

## **Laboratory Standard Operating Procedures**

*Approved by The CKD Biomarkers Consortium  
Quality Control Committee*

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## Laboratory Protocol

### Quantitation of Albumin in Urine and CSF

**Date:** 23 January 2013

**Version Number:** 1.3

**Brief Summary of Procedure:** This laboratory protocol describes the automated method to quantify albumin in human urine and CSF samples using the automated analyzer from Roche Diagnostics (COBAS c501). Detailed specific information is provided that describes the reagent components of the assay, sample type(s) required for analysis, the nature of the measuring reaction and expected performance parameters. Included are expected values for imprecision, reference range estimation, and effect of interfering substances. Also included are procedures to process and evaluate results from Quality Control materials assayed inclusively with patient / unknown samples.

#### Method Review and Approval

Date of Review and Approval	Signatures	
	T.E. Mifflin, Ph.D.	S.R. Master, M.D., Ph.D.



## SOP Training and Awareness Signoff List

My signature below indicates that I have read and understand the contents of this document. If I locate or find errors and items to be corrected, I will notify my supervisor or the laboratory director as soon as possible.

### Distribution and Signoff List

Name of Laboratory Personnel	Training Date	SOP Version	Signature

Comment: \_\_\_\_\_

**Intended use**

In vitro test for the quantitative determination of albumin in human urine and CSF on Roche **cobas** c501 systems.

**Summary**<sup>1,2,3,4,5,6,7,8,9</sup>

Albumin is a non-glycosylated protein with a molecular weight of 66000 daltons. It is synthesized in liver parenchymal cells at a rate of 14 g/day. Quantitatively, albumin is normally the most important protein component (> 50 %) in plasma, CSF and urine. A small, but abnormal albumin excretion in urine is known as microalbuminuria. Causes of microalbuminuria can be glomerular (e.g. due to diabetic microangiopathy, hypertension, minor glomerular lesion), tubular (inhibition of reabsorption) or postrenal. Albumin is also a marker protein for various forms of proteinuria.

In selective glomerular proteinuria, 100-3000 mg albumin/g creatinine are excreted in the urine.

Non-selective glomerular proteinuria is characterized by elevated excretion of high-molecular weight proteins (IgG more than 10 % of the albumin value). Prerenal proteinuria is recognized by a discrepancy between albumin and total protein (albumin accounting for less than 30 %, with concurrent elevation of total protein). Simultaneous elevation of albumin and microproteins is found in glomerulotubular proteinuria occurring due to overloading of tubular reabsorption in glomerulopathy (e.g. nephrotic syndrome), combined glomerular tubulointerstitial nephropathy or in renal failure following diabetic nephropathy or other causes (overflow proteinuria). Albumin has two main functions in plasma: maintaining the oncotic pressure (80 % due to albumin in plasma) and transport. It is the most important transport protein for substances having low water solubility (such as free fatty acids, bilirubin, metal ions, hormones and pharmaceuticals).

Depressed albumin levels are caused by hyperhydration, hepatocellular synthesis insufficiency, secretion disorders in the intravascular space, abnormal distribution between the intravascular and extravascular space, catabolism and loss of albumin, acute phase reactions and congenital analbuminemia.

Blood brain barrier disorders can be reliably quantified with the aid of the albumin CSF/serum ratio. Elevated albumin ratios are indicative of a blood brain barrier disorder.

By simultaneously determining IgG in CSF and serum while taking into account the individual albumin ratios, it is possible to differentiate between IgG originating from the blood and CNS-synthesized immunoglobulin. IgG predominates in multiple sclerosis, chronic HIV encephalitis, neurosyphilis and herpes simplex encephalitis.

A variety of methods, such as radial immunodiffusion, nephelometry and turbidimetry, are available for the determination of albumin.

**Test principle<sup>1</sup>**

Immunoturbidimetric assay.

Anti-albumin antibodies react with the antigen in the sample to form antigen/antibody complexes which, following agglutination, are measured turbidimetrically.

**Reagents - working solutions**

**R1** TRIS buffer: 50 mmol/L, pH 8.0; PEG: 4.2 %; EDTA: 2.0 mmol/L; preservative

**R2** Polyclonal anti-human albumin antibodies (sheep): dependent on titer; TRIS buffer: 100 mmol/L, pH 7.2; preservative

**R3** Reagent for antigen excess check.

Albumin in diluted serum (human); NaCl: 150 mmol/L; phosphate buffer: 50 mmol/L, pH 7.0; preservative

**Precautions and warnings**

For in vitro diagnostic use.

Exercise the normal precautions required for handling all laboratory reagents.

Safety data sheet available for professional user on request.

Disposal of all waste material should be in accordance with local guidelines.

All human material should be considered potentially infectious. This includes human materials used for operation of the instrument such as calibrators and controls. All unknown samples must be considered to be potentially infectious as well. The infectious agents may include any one of the following: bacteria, molds, or viruses.

All products derived from human blood for use on the instrument are prepared exclusively from the blood of donors tested individually and shown to be free from HBsAg and antibodies to HCV and HIV. The infectious agent testing methods applied were FDA-approved or cleared in compliance with the European Directive 98/79/EC, Annex II, List A. However, as no testing method can rule out the potential risk of infection with absolute certainty, the material should be treated just as carefully as a patient specimen. In the event of exposure the directives of the responsible health authorities should be followed.<sup>10,11</sup>

The practice of Universal Precautions must therefore be followed whenever any human – sourced is handled, either for instrument operation or for eventual analysis. This includes both urines and CSF that are to be analyzed.

**Order information**

• Indicates **cobas c** systems on which reagents can be used

Roche/Hitachi  
**cobas c systems**  
**cobas c 501/502**

Tina-quant  
Albumin Gen.2

100 tests	Cat. No. <b>04469658</b> 190	System-ID 07 6743 3	•
C.f.a.s. PUC (5 x 1 mL)	Cat. No. <b>03121305</b> 122	Code 489	
Precinorm PUC (4 x 3 mL)	Cat. No. <b>03121313</b> 122	Code 240	
Precipath PUC (4 x 3 mL)	Cat. No. <b>03121291</b> 122	Code 241	
Precinorm Protein (3 x 1 mL)	Cat. No. <b>10557897</b> 122	Code 302	
Precinorm Protein (3 x 1 mL, for USA)	Cat. No. <b>10557897</b> 160	Code 302	
Precipath Protein (3 x 1 mL)	Cat. No. <b>11333127</b> 122	Code 303	
Precipath Protein (3 x 1 mL, for USA)	Cat. No. <b>11333127</b> 160	Code 303	
Diluent NaCl 9 % (50 mL)	Cat. No. <b>04489357</b> 190	System-ID 07 6869 3	

For **cobas c** 501 analyzer:

**ALBU2:** ACN 153 (Albumin in urine)

**ALBS2:** ACN 628 (Albumin in serum)

**ALBC2:** ACN 407 (Albumin in CSF)

Reagent handling

Ready for use.

Storage and stability

*ALBT2*

Shelf life at 2-8 °C:

See expiration date on **cobas c** pack label.

On-board in use and refrigerated on the analyzer:

12 weeks

*Diluent NaCl 9 %*

Shelf life at 2-8 °C:

See expiration date on **cobas c** pack label.

On-board in use and refrigerated on the analyzer:

12 weeks

### Specimen collection and preparation

For specimen collection and preparation, only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.

Urine

CSF

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

*CSF*

Stability: <sup>12</sup>	up to 3 days	at 2-8 °C
	6 months	at (-15)-(-25) °C
	indefinitely	at (-60)-(-80) °C

*Urine*

Spontaneous, 24-hour urine or 2nd morning urine.

Stability: <sup>13</sup>	7 days	at 15-25 °C
	1 month	at 2-8 °C
	6 months	at (-15)-(-25) °C

Materials provided

See "Reagents - working solutions" section for reagents.

**Calibrator.** Use Roche CFAS Calibrator. Follow the instructions in the CFAS Product Insert attached as an Appendix for information regarding preparation, use and stability.

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### Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

#### Application for urine

##### cobas c 501/502 test definition

Assay type	2 Point End
Reaction time / Assay points	10 / 10-34
Wavelength (sub/main)	700/340 nm
Reaction direction	Increase
Units	mg/L (μmol/L, mg/dL)
Reagent pipetting	Diluent (H <sub>2</sub> O)
R1	100 μL
R2	20 μL
R3	6 μL

#### Sample volumes

	Sample	Sample	Sample dilution Diluent (NaCl)
Normal	6.0 μL	–	–
Decreased	6.0 μL	15 μL	150 μL
Increased	12 μL	–	–

#### Application for CSF

##### cobas c 501/502 test definition

Assay type	2 Point End
Reaction time / Assay points	10 / 10-34
Wavelength (sub/main)	700/340 nm
Reaction direction	Increase
Units	mg/L (μmol/L, mg/dL)
Reagent pipetting	Diluent (H <sub>2</sub> O)
R1	100 μL
R2	20 μL
R3	6 μL

#### Sample volumes

	Sample	Sample	Sample dilution Diluent (NaCl)
Normal	6.0 μL	10 μL	110 μL
Decreased	3 μL	5 μL	180 μL
Increased	12 μL	10 μL	110 μL

#### Calibration

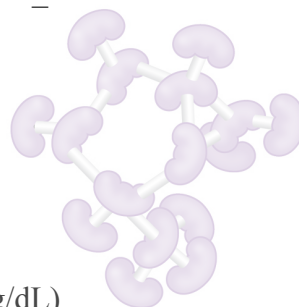
##### Calibrators

S1: H<sub>2</sub>O

S2-6: C.f.a.s. PUC

Multiply the lot-specific C.f.a.s. PUC calibrator value by the factors below to determine the standard concentrations for the six-point calibration curve:

S2:	0.0138	S5:	0.467
S3:	0.0228	S6:	1.00
S4:	0.0450		



Calibration mode RCM

Calibration frequency Full calibration after reagent lot change and as required following quality control procedures

Traceability: This method has been standardized against the reference preparation of the IRMM (Institute for Reference Materials and Measurements) BCR470/CRM470 (RPPHS - Reference Preparation for Proteins in Human Serum).<sup>14</sup>

### Quality control Materials and Sources

For quality control, use Biorad Controls described below. Follow the instructions in the corresponding BioRad Product Inserts attached as Appendices for preparation, use and stability of these control materials.

Other suitable control material can be used in addition (e.g., Roche Diagnostics).

### Urine

- Bio-Rad Lyphochek Quantitative Chemistry Control levels Normal and Abnormal for urine albumin ('Microalbumin'). Store at -80 °C until needed. Then reconstitute and store @ 4-10°C until needed. Can be stored at 4 °C for 30 days past reconstitution.
- Bio-Rad Liquichek Urine Chemistry Control Levels 1 and 2 used for urine albumin ('Microalbumin'). Store at -80 °C until needed. Then thaw and store @ 4-10 °C until needed. Can be stored at 4 °C for 30 days past thawing.

### Quality Control Procedures:

The recommended control requirements for the Roche COBAS c501 Microalbumin assay is a single sample of each Bio-Rad control level to be tested at least once every 8 hours. It is also recommended that a single sample of each level of the assayed control be run during patient testing in the middle of the run and/or at the end of the patient run.

The control values must be within the acceptable ranges specified in the control package insert. If a control is out of its specified limit, the associated test results are invalid and must be retested. The following steps must be followed during the troubleshooting process when an invalid control result is gotten.

### Quality Control Evaluation Procedure:

1. In a stepwise manner, evaluate each assayed control material (serum or urine) to determine whether the albumin values obtained using this method are within  $\pm 2SD$  limit listed by BioRad.
2. If both are within the stated  $\pm 2SD$  limits, accept the run and transfer the unknown results to the reporting worksheet
3. If one control's value is within the  $\pm 2SD$  limits and the other is outside, then follow the Westgard 2 Control evaluation algorithm depicted below before deciding how to determine if the analysis run results can be accepted.
4. If neither of the assayed control albumin results are within limits, then following the stepwise evaluation tree listed below the Westgard diagram on the next page.



Rule	Quality-Control Rule Explanation
1 <sub>2s</sub>	The 1 <sub>2s</sub> rule describes a situation where one control value for any of the two levels of control is outside the mean $\pm$ 2 SD.
1 <sub>3s</sub>	The 1 <sub>3s</sub> rule describes a situation where one control value for any of the two levels of control is outside the mean $\pm$ 3 SD.
2 <sub>2s</sub>	The 2 <sub>2s</sub> rule describes a situation where both control fluids or two consecutive control values of one fluid are outside the mean by either +2 SD or -2 SD.

Table 1. Quality-Control Rules

The three quality-control rules just described are used together when the mean value for the quality-control fluids has been established. The diagram below should help you better understand the three rules.

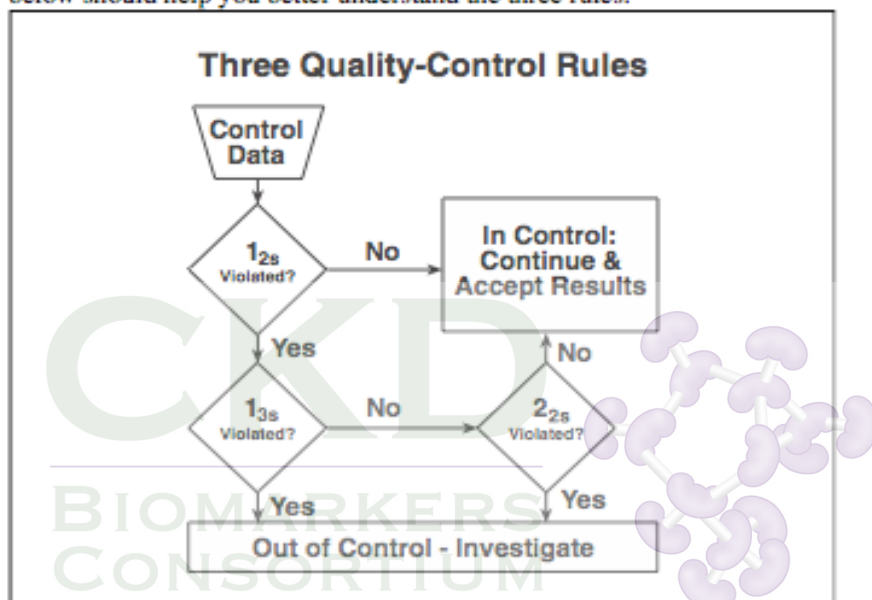


Figure 11. Three Quality-Control Rules

## Corrective Actions to be Taken when QC Materials Exceed Acceptable Limits

- Rerun controls
- If QC is within specified range document correction and proceed with sample testing.
- If QC is **Not** within specified range, document it, make up fresh QC fluid and rerun controls.
- If controls are within specified range, proceed with sample testing.
- If QC is **Not** within specified range, recalibrate the assay.
- If after calibration the QC is within specified range, proceed with testing and document correction.
- If QC is **Not** within specified range after calibration, place a fresh reagent on analyzer and recalibrate.
- If QC is within range after calibrating with fresh reagent, proceed with sample testing and document correction.
- If QC is **Not** within specified range after using fresh reagent and control fluid, do not proceed with sample testing. Check instrument maintenance to see if you can resolve the problem. If not, contact the Laboratory Director.

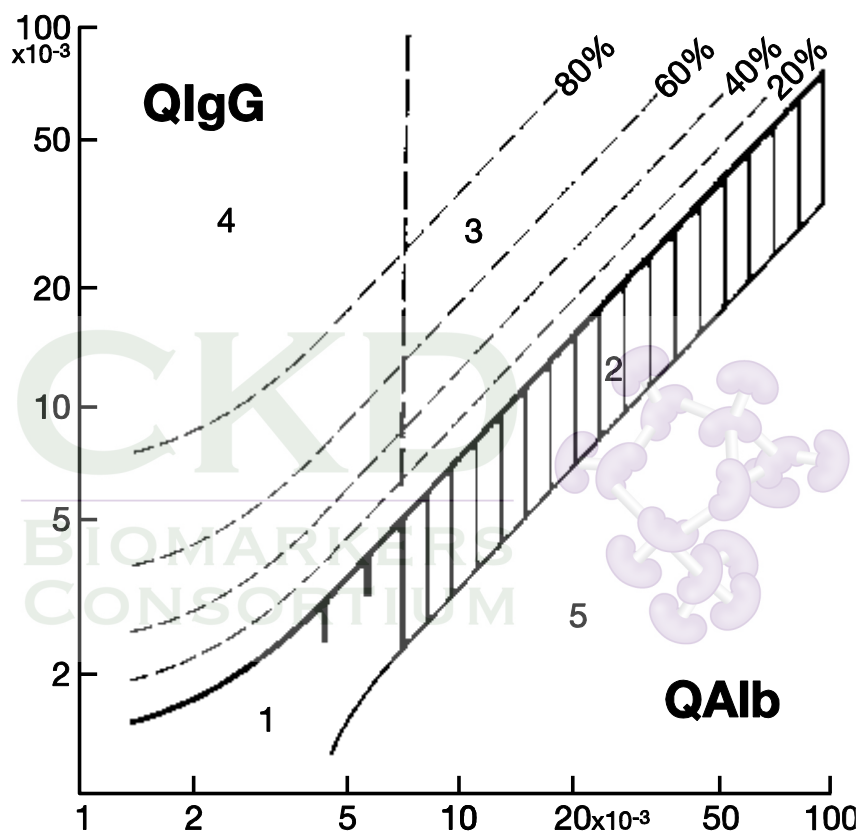
### Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors:

- $\text{g/L} \times 100 = \text{mg/dL}$
- $\text{g/L} \times 15.2 = \mu\text{mol/L}$
- $\text{mg/L} \times 0.1 = \text{mg/dL}$
- $\text{mg/L} \times 0.0152 = \mu\text{mol/L}$

The calculation employs a ratio diagram including hyperbolic functions as differential lines according to Reiber. Results from the determination of IgG and albumin in CSF and serum (IgG and albumin ratios)<sup>4</sup> are plotted.



1. Reference range. 2. Blood brain barrier functional disorder without local IgG synthesis. 3. Blood brain barrier functional disorder with concomitant IgG-synthesis in the CNS. 4. IgG synthesis in the CNS without blood brain barrier functional disorder. 5. As confirmed empirically, there are no values in this region (i.e. values here are due to errors introduced by blood sampling or analytical errors). Generally speaking, cases not associated with local IgG synthesis in the CNS lie below the bold line (hyperbolic function). The percentage values indicate what percentage of the total IgG in CSF (minimum) originates in the CNS relative to the statistically-defined 0 % differential lines.



**Limitations - interference<sup>16</sup>*****Urine***

Criterion: Recovery within  $\pm 10\%$  of initial value.

Icterus: No significant interference up to an I index of 50 (approximate conjugated bilirubin concentration: 855  $\mu\text{mol/L}$  (50 mg/dL)).

Hemolysis: No significant interference up to an H index of 400 (approximate hemoglobin concentration: 248  $\mu\text{mol/L}$  (400 mg/dL)).

No interference by acetone  $\leq 60$  mmol/L, ammonia chloride  $\leq 0.11$  mol/L, calcium  $\leq 40$  mmol/L, creatinine  $\leq 0.18$  mol/L,  $\gamma$ -globulin  $\leq 500$  mg/L, glucose  $\leq 0.19$  mol/L, urea  $\leq 0.8$  mol/L, uric acid  $\leq 5.95$  mmol/L and urobilinogen  $\leq 378$   $\mu\text{mol/L}$ .

Drugs: No interference was found at therapeutic concentrations using common drug panels.<sup>17</sup>

High dose hook-effect: Using the prozone check, no false result without a flag was observed up to an albumin concentration of 40000 mg/L (608  $\mu\text{mol/L}$ , 4000 mg/dL).

***CSF***

Criterion: Recovery within  $\pm 10\%$  of initial value.

Hemolysis: No significant interference up to an H index of 1000 (approximate hemoglobin concentration: 620  $\mu\text{mol/L}$  (1000 mg/dL)).

Icterus: No significant interference up to an I index of 60 (approximate conjugated and unconjugated bilirubin concentration: 600 mg/L (1026  $\mu\text{mol/L}$ , 60 mg/dL)).

High dose hook-effect: Using the prozone check, no false result without a flag was observed up to an albumin concentration of 30000 mg/L (456  $\mu\text{mol/L}$ , 3000 mg/dL).

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

**ACTION REQUIRED**

***Special Wash Programming:*** The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. Refer to the latest version of the Carry over evasion list found with the NaOHD/SMS/Multiclean/SCCS or the NaOHD/SMS/SmpCln1 + 2/SCCS Method Sheets. For further instructions refer to the operator manual.

**cobas c 502 analyzer:** All special wash programming necessary for avoiding carry over is available via the **cobas** link, manual input is not required.

**Where required, special wash/carry over evasion programming must be implemented prior to reporting results with this test.**

**Limits and ranges*****Measuring range******Urine***

12–400 mg/L (0.182–6.08  $\mu\text{mol/L}$ , 1.2–40.0 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:11 dilution. Results from samples diluted by the rerun function are automatically multiplied by a factor of 11.

***CSF***

95–3000 mg/L (1.44–45.6  $\mu\text{mol/L}$ , 9.50–300 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:6.2 dilution. Results from samples diluted by the rerun function are automatically multiplied by a factor of 6.2.

### Lower limits of measurement

*Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantitation (LoQ)*

#### *Urine*

LoB = 2 mg/L (0.030  $\mu$ mol/L, 0.200 mg/dL)

LoD = 3 mg/L (0.046  $\mu$ mol/L, 0.300 mg/dL)

LoQ = 12 mg/L (0.182  $\mu$ mol/L, 1.20 mg/dL)

#### *Serum, plasma*

LoB = 1 g/L (15.2  $\mu$ mol/L, 100 mg/dL)

LoD = 2 g/L (30.4  $\mu$ mol/L, 200 mg/dL)

LoQ = 3 g/L (45.6  $\mu$ mol/L, 300 mg/dL)

#### *CSF*

LoB = 20 mg/L (0.304  $\mu$ mol/L, 2.00 mg/dL)

LoD = 36 mg/L (0.547  $\mu$ mol/L, 3.60 mg/dL)

LoQ = 95 mg/L (1.44  $\mu$ mol/L, 9.50 mg/dL)

The limit of blank, limit of detection and limit of quantitation were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP 17–A requirements.

The limit of blank is the 95<sup>th</sup> percentile value from  $n \geq 60$  measurements of analyte-free samples over several independent series. The limit of blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.

The limit of detection is determined based on the limit of blank and the standard deviation of low concentration samples. The limit of detection corresponds to the lowest analyte concentration which can be detected (value above the limit of blank with a probability of 95 %).

The limit of quantitation is the lowest analyte concentration that can be reproducibly measured with a total error of 30 %. It has been determined using low concentration albumin samples.

Values below the limit of quantitation (12 mg/L (urine); 3 g/L (serum, plasma); 95 mg/L (CSF)) will be flagged by the instrument.

### Expected values

#### *Urine*

2nd morning urine:<sup>4</sup>

Adults: < 20 mg albumin/g creatinine or  
< 2.26 g (34.35  $\mu$ mol) albumin/mol creatinine

Children (3-5 years):<sup>16</sup> < 20 mg/L (0.304  $\mu$ mol/L, 2 mg/dL) albumin  
< 37 mg albumin/g creatinine

24-hour urine:<sup>17</sup>

< 20 mg/L (0.304  $\mu$ mol/L, 2 mg/dL)  
< 30 mg/24 h (0.456  $\mu$ mol/24 h)

*CSF*

Albumin in CSF: <sup>12</sup>	3 months to 4 years:	< 45 mg/dL (< 6.84 $\mu$ mol/L; < 450 mg/L)
	> 4 years	10–30 mg/dL (1.52–4.56 $\mu$ mol/L; 100–300 mg/L)

For information on CSF/serum ratios, see Silverman LM, Christenson RH. Amino acids and proteins. In Tietz NW. ed. Fundamentals of Clinical Chemistry. 4th ed. Philadelphia: WB Saunders 1996: 240–282.

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Roche has not established performance of this assay in a pediatric population.

**Specific performance data**

Representative performance data on the analyzer are given below. Results obtained in individual laboratories may differ.

**Precision**

Precision was determined using human samples and controls in an internal protocol. Repeatability\* (n = 21), intermediate precision\*\* (3 aliquots per run, 1 run per day, 21 days).

The following results were obtained (on the **cobas c 501** analyzer):

*Urine*

<i>Repeatability*</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	<i>mg/L (<math>\mu</math>mol/L, mg/dL)</i>	<i>mg/L (<math>\mu</math>mol/L, mg/dL)</i>	<i>%</i>
Precinorm PUC	30.7 (0.467, 3.07)	0.2 (0.003, 0.02)	0.8
Precipath PUC	108 (1.64, 10.8)	1 (0.01, 0.1)	0.7
Human urine 1	14.3 (0.217, 1.43)	0.2 (0.003, 0.02)	1.6
Human urine 2	252 (3.83, 25.2)	4 (0.06, 0.4)	1.6
<i>Intermediate precision**</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	<i>mg/L (<math>\mu</math>mol/L, mg/dL)</i>	<i>mg/L (<math>\mu</math>mol/L, mg/dL)</i>	<i>%</i>
Precinorm PUC	31.2 (0.474, 3.12)	0.5 (0.008, 0.05)	1.7
Precipath PUC	105 (1.60, 10.5)	1 (0.02, 0.1)	1.2
Human urine 3	13.6 (0.207, 1.36)	0.4 (0.006, 0.04)	2.8
Human urine 4	60.6 (0.921, 6.06)	1.4 (0.021, 0.14)	2.3

*CSF*

<i>Repeatability*</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	<i>mg/L (<math>\mu</math>mol/L, mg/dL)</i>	<i>mg/L (<math>\mu</math>mol/L, mg/dL)</i>	<i>%</i>
CSF Control Low	99.2 (1.51, 9.92)	1.4 (0.02, 0.14)	1.4
Human CSF 1	174 (2.64, 17.4)	3 (0.05, 0.3)	1.7
Human CSF 2	383 (5.82, 38.3)	4 (0.06, 0.4)	1.0
C.f.a.s. PUC	454 (6.90, 45.4)	4 (0.06, 0.4)	0.8

<i>Intermediate precision**</i>	<i>Mean</i> mg/L (μmol/L, mg/dL)	<i>SD</i> mg/L (μmol/L, mg/dL)	<i>CV</i> %
CSF Control Low	91.0 (1.38, 9.1)	2.9 (0.04, 0.29)	3.2
CSF Control High	389 (5.91, 38.9)	7 (0.11, 0.7)	1.7
Human CSF 3	166 (2.53, 16.6)	4 (0.06, 0.4)	2.3
Human CSF 4	366 (5.56, 36.6)	5 (0.07, 0.5)	1.3

\* repeatability = within-run precision

\*\* intermediate precision = total precision / between run precision / between day precision

## Method comparison

### Urine

Albumin values for human urine samples obtained on a Roche/Hitachi **cobas c 501** analyzer (y) were compared with those determined using the same reagent on a Roche/Hitachi 917 analyzer (x).

Sample size (n) = 125

Passing/Bablok<sup>21</sup>

$y = 1.023x - 3.64 \text{ mg/L}$

$\tau = 0.984$

Linear regression

$y = 1.028x - 4.13 \text{ mg/L}$

$r = 0.999$

The sample concentrations were between 12.3 and 386 mg/L (0.187 and 5.87 μmol/L, 1.23 and 38.6 mg/dL).

### CSF

Albumin values for human CSF samples obtained on a Roche/Hitachi **cobas c 501** analyzer (y) were compared with those determined with a nephelometric Albumin test (x).

Sample size (n) = 85

Passing/Bablok<sup>21</sup>

$y = 1.000x - 8.75 \text{ mg/L}$

$\tau = 0.936$

Linear regression

$y = 0.991x + 0.301 \text{ mg/L}$

$r = 0.992$

The sample concentrations were between 115 and 2640 mg/L (1.75 and 40.1 μmol/L, 11.5 and 264 mg/dL).

## References

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Version 3

2010–11

Reagent manufacturer

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim

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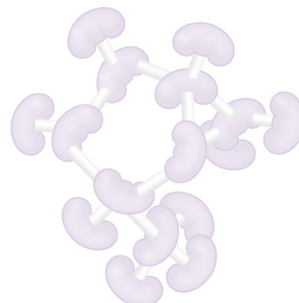
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*Reviewed By: T.E. Mifflin, Ph.D.*

*Date Reviewed: 23 January 2013*



**Entity: University of Minnesota**

**Department: Biochemistry, Molecular Biology and Biophysics**

**Procedure: Analysis of urinary albumin, uromodulin and transferrin by multiple reaction monitoring.**

**Developers: Yun Jiang, Stephen Harvey and Gary Nelsestuen**

## **INTRODUCTION:**

Chronic kidney disease is characterized by slow progression. Early detection of individuals who will advance to disease will offer improved opportunity to alter medication or lifestyle to prevent or delay onset of disease. The objective of this study is to determine the utility of combined protein analyses for purposes of early disease prognosis. In way of background, prior to release of funding for this consortium, findings by broad proteomic screening methods had suggested that analysis of multiple proteins provided an improved prognosis of future kidney disease when compared with current methods (materials in the original application, University of Minnesota, PI Mauer). A common approach is to convert from proteomics methods to ELISA assay. Over 2 years of effort were expended on this objective, including some time after release of funding from this consortium. These extended efforts resulted in the conclusion that uromodulin presented unique problems that were sample-specific and could not be overcome for all, and especially for target, samples. While many typical control samples appeared amenable to ELISA, many experimental samples provided wildly variable values. Freeze-thaw also had a major effect. These findings applied to a commercial uromodulin ELISA kit as well as our own assays. The problem appeared to be related to the tendency for uromodulin to aggregate. Indeed, desk top centrifugation removed 0 to 80% of the ELISA-detected uromodulin from different samples. There was a strong correlation between those samples with wildly variable and high values by ELISA to those with greatest loss during centrifugation. Samples that lost virtually no uromodulin from centrifugation gave the best result by ELISA.

As an attempted explanation for this correlation, we point out that there are a number of features of the ELISA assay that are poorly understood. In this case, we felt that our correlation made sense in light of diffusional theory that we summarize as follows: Without mixing, analytes (proteins in this case) in an ELISA assay should reach the immobilized antibody by diffusion. Application of diffusional theory shows that typical ELISA assay times are insufficient to reach diffusional equilibrium in an ELISA plate well. Applying the relationship  $t = X^2/2D$ , where  $X$ =average diffusion distance for a molecule under standard conditions,  $D$  is the diffusion constant and  $t$  is time, the average albumin molecule ( $D=6 \times 10^{-7} \text{ cm}^2/\text{s}$ ) will require over 9 hours to diffuse the 0.25 cm from the center of the well to the edge for the smallest typical 96 well dimension and 27 hours for larger standard wells (well sizes from

[http://catalog2.corning.com/lifesciences/media/equipment\\_compatibility/MD\\_Microplate\\_Dimension\\_Sheets.htm](http://catalog2.corning.com/lifesciences/media/equipment_compatibility/MD_Microplate_Dimension_Sheets.htm), and time vs. distance from a diffusion calculator at:

[http://www.physiologyweb.com/calculators/diffusion\\_time\\_calculator.html](http://www.physiologyweb.com/calculators/diffusion_time_calculator.html)). Larger proteins with lower diffusion constants such as uromodulin will take longer. Since this is an average, some molecules will not sample the immobilized antibody at all. Average Diffusion from top to bottom of the tube (typically 1 cm) would require 231 hours. Thus, analytes near the bottom of the well, with potential for diffusion to both side and bottom surfaces, will involve geometry quite different from events near the top of the well. Furthermore, antibodies at different places in the tube will experience different exposure to analyte. Despite these issues, ELISA assays can be very accurate if all analytes sample the antibody by the same process and all wells have the same distribution of antibodies over the surface. This suggests that the assay provides some intermediate stage that is constant for all samples but does not represent diffusional equilibrium. However, if some samples contain analyte that reaches the immobilized antibody by other methods (e.g. sedimentation) or remains soluble but is in complex with other molecules that alter its diffusion constant, there can be major deviation from those samples

where analyte is delivered exclusively by diffusion of monodisperse molecules. This would explain the correlation we observed between high and variable values by ELISA and removal of uromodulin by centrifugation. In short, all of the sedimenting protein could reach the surface while not all diffusing protein molecules would. Attempts to address this problem included extensive application of chaotropic agents (urea and guanidine hydrochloride) to solubilize the uromodulin aggregates and/or protein complexes before ELISA assay. After extensive efforts with substantial improvements, these proved only partially successful. We then converted to the following MRM assay where protein aggregates are removed by protease digestion. Even then, substantial modification of standard sample preparation methods for MRM was needed to generate an accurate assay.

A second unknown quantity related to ELISA concerns dissociation of the analyte from the immobilized antibody. Typical high affinity Antibodies used in ELISA assay are reported to have  $K_D=10^{-9}$  M. Association rate constants measured for antibodies in solution are often in the range of  $10^7 \text{ M}^{-1}\text{s}^{-1}$ . These values dictate a typical dissociation rate constant of  $10^{-2} \text{ s}^{-1}$ . Upon removal of associating analyte from the surrounding liquid during the washing procedure, the half-time for dissociation of the analyte should be 69 seconds. This clearly does not occur as typical washing times to remove unbound analyte are far greater. In fact, there is little evidence for dissociation in times of hours. The explanations for these issues are not known. Similar findings often apply to study of rate constants by Surface Plasmon Resonance. Debate of molecular events in these situations has been lively with no clear resolution. Unfortunately, since many of the  $K_D$  values for antibodies are measured in a 96-well plate, they are subject to the same issues outlined above. Again, washing steps that occur over many minutes should result in nearly complete loss of analyte from the surface. Clearly, this does not happen. Given such slow dissociation, it is likely that the dissociation rate has not reached equilibrium, even during the extended initial incubation step. Since no resolution to these issues exists and few are aware, they are most often ignored. This clearly works in many cases but is problematic in others.

The MRM assay also provides for multiplexing. Time and cost for analysis of 50 analytes can be little more than analysis of 1 by the same method. Consequently, new proteins can be added to a single assay as more markers of chronic kidney disease are identified.

### ASSAY PRINCIPLE:

Mass spectrometry offers the ability of multiplex analysis of several analytes in a single sample with a single run. The method may also overcome problems associated with sample storage and freeze/thaw. The procedure involves protein digestion to peptides, addition of known amounts of synthetic target peptides that contain heavy atom amino acids, chromatography of the peptide mixture on ultra-performance liquid chromatography (UPLC) and analysis by mass spectrometry. The target peptides were selected from among the total protein peptides by virtue of signal intensity, lack of interference by contaminants and resistance to degradation by oxidation. The target peptide and heavy atom peptides elute simultaneously and are analyzed by fragmentation. The intensities of the fragment ions (referred to as "transitions") of the normal peptide are compared with those of the heavy atom peptide to obtain the concentration of the normal peptide, from which the concentration of the parent protein is obtained. This assumes that the peptides represent intact albumin and that small peptides or incomplete albumin concentrations do not contribute significantly to the result. The existence of partial protein molecules would not invalidate the test as an approach to diagnosis but only change the interpretation of protein concentration.

A confounding feature of urine is its heterogeneity both with respect to concentration and protein composition. The goal was to develop an assay that could be applied to urine samples without concentration. To achieve this objective, the first reagent added to the urine is highly buffered to eliminate differences of pH and virtually eliminate differences of ionic strength. The chemical



reactions used are not sensitive to pH but not to other remaining minor differences. Later in the procedure, these heterogeneities can lead to different protease digestion results, which appeared especially problematic for uromodulin. Dialysis was used to eliminate heterogeneity of ionic strength and pH and a carrier protein was added to effectively homogenize the protein concentration/composition. Standard plasma proteins such as albumin evidenced much less of this problem. To overcome this issue, every urine sample is effectively made identical by addition of a large amount of a carrier protein, ovalbumin in this case. With a constant level of total protein and similar total protein composition, the protease digestion proceeds at the same level for all samples. As a result, uromodulin became the most accurate protein detected in the sample.

While MRM analysis is commonly used in clinical assays of small molecules, we are unaware of any clinical assays that use peptide MRM to evaluate protein concentration in biological samples. Comparison of MRM vs. antibody-based clinical assays is an important development of this study.

### SPECIMEN:

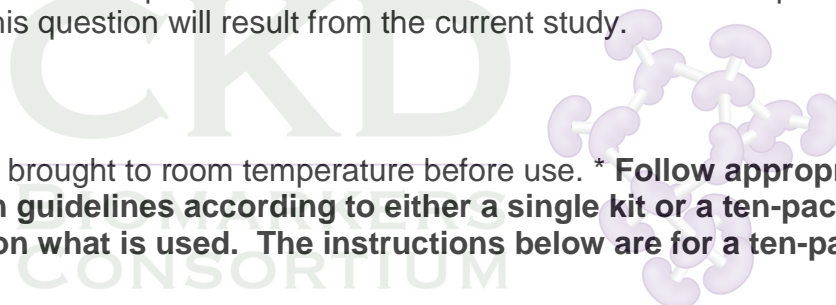
Urine is obtained and stored frozen at -80 degrees C. until the assay is performed. It is then thawed and mixed gently. The procedure has been developed so that samples with normal or slightly elevated albumin need no concentration or dilution.

Centrifugation of the urine sample is to be avoided since it often removes a portion of the uromodulin. Further analysis of this question will result from the current study.

### REAGENTS:

All reagents must be brought to room temperature before use. \* **Follow appropriate reagent preparation guidelines according to either a single kit or a ten-pack of kits, depending upon what is used. The instructions below are for a ten-pack of kits.**

1. MilliQ Water:  
MilliQ is the trade name of the water system purchased from the Millipore Corporation (Continental Water System). MilliQ water is deionized water treated with activated carbon and deionization cartridges and filtered to remove microorganisms larger than 0.22 micrometers.
2. Ovalbumin  
Ovalbumin (purchased from Sigma catalog number A5378-10G),
3. Dithiothreitol  
Dithiothreitol (purchased from Sigma catalog number D-0632)
- 4 Iodoacetamide  
Iodoacetamide (purchased from Sigma, catalog number I1149-25G)
5. 1 M ammonium bicarbonate buffer stock solution pH7.8.  
The stock solution of 1M pH7.8 ammonium bicarbonate buffer is made from ammonium hydroxide solution (purchased from Sigma-Aldrich, catalog number 30501) that was adjusted to pH by bubbling in CO<sub>2</sub> produced by sublimation of dry ice. The solution is kept at room temperature.
6. Hydrochloric acid (purchased from Fisher Scientific, Catalog number A144S-500).
7. Calcium Chloride 1M stock solution. Calcium Chloride is purchased from Sigma, Catalog number C-3881. Solution is kept at room temperature.
7. Sequencing grade modified trypsin. (Promega V5113) Trypsin is kept at -70 degree C. A new vial is used for each batch of sample prep.
8. Heavy atom peptides



Peptides containing one heavy atom amino acid (C13 and N15) were selected on the basis of elution position (minimal overlap with peptides of similar mass), chemical stability and signal intensity in the mass spectrometer. Heavy atom peptides were ordered from the Microchemical Facility at the Mayo Clinic (200 First Street SW Rochester, MN 55905), a facility with extended experience in peptide synthesis. The peptides chosen included:

Uromodulin peptides DLNIK, SLGFDK, FVGQGGAR, Human serum albumin peptides YLYEIAR, AEFAEVSK, LVTDLTK, and transferrin peptide NPDPWAK.

Each labeled peptide was weighed and dissolved in MilliQ water at 2 mg/ml to make stock solutions that are stored at -70 degrees C. A proper amount of each stock solution is mixed and diluted in 5% acetonitrile and 0.1% formic acid to get a mixture of 0.5 µg/ml for each Human serum albumin peptide, 2 µg/ml for each uromodulin peptide and 0.05 µg/ml for the transferrin peptide.

9. Acetonitrile (Fisher Scientific, catalog number A998-1)

10. 10% Formic acid

Add 1 ml 88% Formic acid (Fisher Scientific, catalog number A118<sup>p</sup>-500) into 7.8 ml MilliQ water, mix the solution to get 10% Formic acid.

11. Creatinine assay kit (R&D, catalog number KGE005)

12. Human serum albumin (Sigma, A3782)

13. Uromodulin

Uromodulin was purified from normal human urine and its concentration confirmed by comparison to standard uromodulin purchased from BioVendor (catalog number RD172163100)

14. Human serum transferrin (Sigma, 90190)

## EQUIPMENT AND SUPPLIES REQUIRED:

1. 10µL and 200µL pipets

2. Pipette tips appropriate for above pipettes (Corning catalog number 4153 and 4154)

3. 1L graduated cylinder

4. 0.65 mL microcentrifuge tubes (Dot scientific Inc catalog number RN0650-GNB)

5. 4L plastic beaker.

6. Precision Circulating Water Bath mode 260

6. Magnetic stir bar 7.5cm×1.5cm

7. PH meter (Fisher scientific accumet\* AB15+ Basic)

8. Corning Stirrer

9. PH papers 1~12 (Micro Essential laboratory)

10. Slide-A-Lyzer MINI Dialysis Units, 10K MWCO (Thermo Scientific, catalog number 69572)

11. 12mm×32 mm Screw Top Wide Mouth Vials (Chrom Tech. Inc, Part number CTV-1209GS)

12. 250 µL Vial inserts (Chrom Tech. Inc, Part number CTI-9525)

13. Vial caps (Chrom Tech. Inc Part number CTC-0952-bond)

14. ACQUITY UPLC® BEH C18 column (Waters, 1.7 µl, 2.1×50 mm)

15. AB SCIEX QTRAP® 5500 LC/MS/MS System

## REAGENT PREPARATION:

All reagents must be brought to room temperature before use.

1. 10 mg/ml Ovalbumin. Ovalbumin is weighted and dissolved in MilliQ water at 10 mg/ml. Protein solution is aliquoted and stored at -20 degree C. Each aliquot is frozen and thawed up to 4 times before it is discarded or used up.



CKD  
BIOMARKERS  
CONSORTIUM

2. 70 mM dithiothreitol. Dithiothreitol 5.4 mg is dissolved in 500  $\mu$ l 1M pH 7.8  $\text{NH}_4\text{HCO}_3$  buffer to make a final concentration of 70 mM. Solution is freshly made for each sample batch.
3. Iodoacetimide 300 mM. Iodoacetimide 27.7 mg is dissolved in 0.5 ml MilliQ Water. Solution is freshly made for each sample batch.
4. Dialysis buffer. The dialysis buffer is 0.025 M pH 8.0 ammonium bicarbonate buffer, which is diluted from 1 M pH 7.8 ammonium bicarbonate buffer with MilliQ Water and pH adjusted by adding hydrochloric acid. 1M  $\text{CaCl}_2$  is added at 1 ml per one liter dialysis buffer.

## URINE SAMPLE PREPARATION PROCEDURE:

Thaw frozen urine samples by placing them in a rack that is exposed on all sides to room air. Allow them to thaw without agitation. When thawed, mix by gentle agitation in a vortex mixer. Mix again by drawing 50 microliters (of 100 microliters) into a pipetman pipetter and expelling 4 times. The objective is to keep all particulate matter in suspension and to pipette a homogeneous sample. Immediately transfer the final volume in the pipette (50  $\mu$ l) to a 0.65 ml microcentrifuge tube.

Carrier protein ovalbumin (2.5  $\mu$ l of 10 mg/mL) is added to each sample. Dithiothreitol (7.5  $\mu$ l of 70 mM) is added to each sample. The mixtures are incubated at 65 °C for 50 minutes. After reduction, samples are cooled to room temperature before alkylation by addition of 4  $\mu$ l of 300 mM iodoacetamide for 50 minutes in the dark. Additional dithiothreitol (2  $\mu$ l 70 mM) is added to remove excess iodoacetamide.

Slide-A-Lyzer® MINI Dialysis Units of 10K MWCO are soaked in MilliQ water overnight to remove glue polymers or preservatives. Samples are transferred to the dialysis units and dialysis against 25 mM pH 7.8  $\text{NH}_4\text{HCO}_3$  buffer containing 1 mM  $\text{CaCl}_2$  is conducted for 3 hours. Up to 20 samples can be dialyzed against 4 L of buffer. The sample is then transferred to a new 0.65 ml microcentrifuge tube. Trypsin solution corresponding to 0.8 micrograms of enzyme is added to each sample to produce a protease:protein ratio of 1:32.5~1:40 corresponding to the amount of ovalbumin plus the dynamic range of protein in normal urine samples (0~300  $\mu$ g/ml). Samples are digested at 37 degrees for 18 hours followed by addition of 4  $\mu$ l of acetonitrile, 4  $\mu$ l of 10% formic acid and 4  $\mu$ l of the heavy isotope-labeled peptide mix. The final volume of each sample is 70 ~100  $\mu$ l.

## MRM ASSAY

Each prepared sample (4  $\mu$ l loading volume) is analyzed in duplicate. One of the uromodulin peptides Uro\_DLNIK, gave low intensity and inconsistent results. Therefore, uromodulin concentrations were obtained from the Uro\_FVGQGGAR\_y6 and y7 transitions and the URO\_SLGFDK\_y4 transition.

Acquisition method and LC system settings are described as below.

### 1 Acquisition method

Synchronization Mode: LC SynC

Schedule MRM setting are shown in the table below

	Q1 Mass (Da)	Q3 Mass	Time(min)	ID	DP	CE
--	--------------	---------	-----------	----	----	----

		(Da)	)		(volts)	(volts)
1	467.700	658.300	3.9	HSA_YLYEIR_y5	80.000	23.000
2	443.600	686.300	3.0	HSA_AEFAEVSK_y6	81.000	24.000
3	305.000	381.300	3.0	URO_DLNIK_y3	91.000	20.000
4	399.200	545.500	2.8	URO_FVGQGGAR_y6	86.000	21.000
5	399.200	650.000	2.8	URO_FVGQGGAR_y7	86.000	21.000
6	464.250	651.346	3.9	HSA_YLYEIR_ny5	80.000	25.400
7	440.724	680.361	3.0	HSA_AEFAEVSK_ny6	81.000	24.000
8	396.200	644.350	2.8	URO_FVGQGGAR_ny7	86.000	21.000
9	396.200	545.280	2.8	URO_FVGQGGAR_ny6	86.000	21.000
10	301.860	374.240	3.0	URO_DLNIK_ny3	91.000	20.000
11	395.240	577.300	3.3	HSA_LVTDLTN_ny5	90.000	22.400
12	333.700	466.230	3.1	URO_SLGFDK_ny4	91.000	20.000
13	338.700	476.200	3.1	URO_SLGFDK_y4	91.000	20.000
14	398.200	577.300	3.3	HSA_LVTDLTN_y5	90.000	22.400
15	414.200	501.300	3.1	Trans_NPDPWAK_n2y4	80.000	23.500
16	417.100	507.100	3.1	Trans_NPDPWAK_2y4	80.000	25.000

Polarity: Positive

MRM detection window: 25 sec

Target Scan Time: 0.42 sec

Duration of run (min): 10.996

Delay Time (sec): 0

Cycles: 1571

Cycle (sec): 0.42

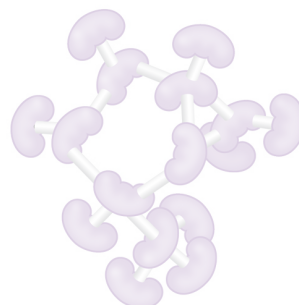
Resolution Q1:Unit

Resolution Q3: Unit

Intensity threshold (total count): 0

Setting time (ms):0

Pause between mass ranges (ms): 3



## 2 Settings for the Shimadzu LC system

### 2.1 Pumps

Pump Mode: Binary Flow

Total Flow: 0.45 ml/min

Pump B Conc: 0%

Pump B Curve:0

## Pressure Limits (Pump A, B)

Minimum: 0 psi

Maximum: 6200 psi

## 2.2 Autosampler settings

Rinsing Volume: 500 µl

Needle Stroke: 52 mm

Rinsing Speed: 35 µl/sec

Sampling Speed: 5 µl

Purge Time: 25 min

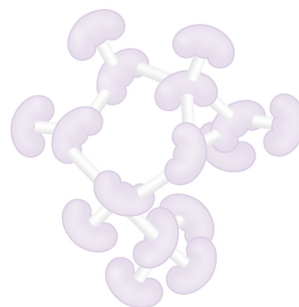
Rinse Dip Time: 0 sec

Rinse Mode: Before and after aspiration

Enable Cooler Unit

Cooler Temperature: 15 degree C

Control Vial Needle Stroke: 50 mm



## 2.3 Oven

Mode CTO-20A

Oven temperature: 56 degree C

Maximum temperature: 90 degree C

## 2.4 Controller

Model: CBM-20A

Power On

## 2.5 Timed Program-table of changes in elution parameters.

	Time (min)	Module	Event	Parameter
1	0.01	Pumps	Pump B Conc	0
2	1.00	Controller	Event	1
3	1.01	Pumps	Pump B Conc	0
4	1.10	Controller	Event	0
5	1.50	Pumps	Pump B Conc	10
6	8.30	Pumps	Pump B Conc	40
7	9.00	Pumps	Pump B Conc	90
8	9.50	Pumps	Pump B Conc	90
9	10.00	Pumps	Pump B Conc	0
10	11.00	Controller	Stop	

2.6 Injection volume (μL): 4

## Data analysis was conducted with MultiQuant (2.0.2) software

### Processing method settings

Gaussian Smooth Width: 0 points

Expected retention times: 3.9 min for HSA\_YLYEIAR y5 / ny5 transition pairs; 3.0 min for HSA\_AEFAEVSK y6 and ny6 transition pairs; 3.3 min for HSA\_LVTDLTK y5/ny5 transition pairs; 2.8 min for Uro\_FVGQGGAR y6/ny6 and y7/ny7 transition pairs; 3.1 min for Uro\_SLGFDK y4/ny4 transition pair; 3.1 min for Trans\_NPDPWAK 2y4/n2y4 transition pairs.

Retention Time Half Window: 30 seconds

Update Expected retention time: NO

Report largest Peak: selected

Min. Peak Width: 3 points

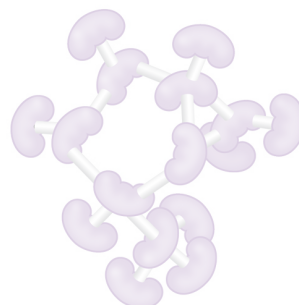
Min. Peak height: 0.00

### Integration Parameters

Noise percentage: 40%

Baseline Sub. Window: 2.00 min

Peak Splitting: 2 points



### Processing of data

Click "File" tab, open "new results table", find interested qtrao5500 data file, select all interested runs, click "next".

Select method, click finish.

Click "display peak review", check each individual peak area; manually change the area if there are errors because of an incorrect retention time, peak splitting or incomplete coverage of the peak (truncated early or late in its elution) because of altered elution time. Split peaks occur in low intensity signals and arise when the instrument fails to detect the signal in one scan but not others. This happens more frequently in Uro\_DLINIK and Trans\_NPDPWAK, because of their low intensity. In the case of incomplete peaks, the sample must be rerun with some modification, such as changing scheduled time, to get the full peak.

Save the result table when finished.



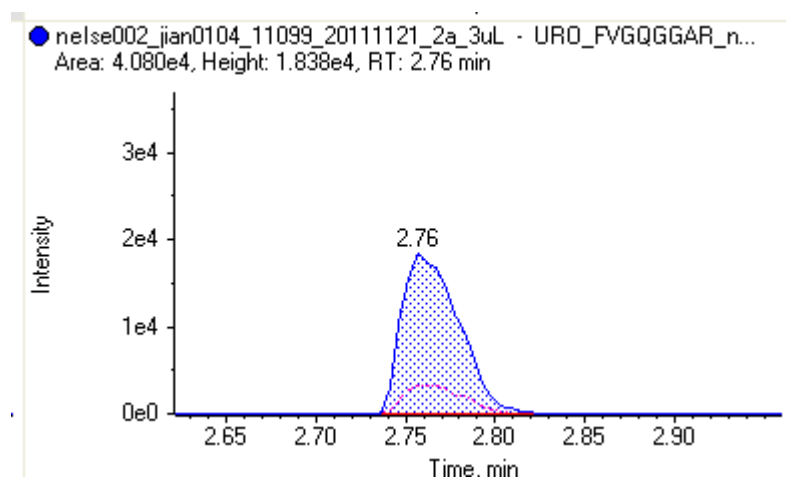


Fig 1. Example of MRM peak view for URO\_FVGQGGAR y7/ny7 pairs (y7 is the y7 transition from the heavy atom peptide while ny7 is transition area for the normal peptide). The blue line (upper line) shows native transition area and the red (lower) line shows the labeled peptide transition area.

### Stable isotope-labeled peptide standardization

The relationship between peak area and protein concentration was determined experimentally. The procedure consists of spiking a sample with known amounts of standard human serum albumin, uromodulin and transferrin. This is carried out in several urine samples as well as in MilliQ water. The MRM assay and concentration calculation are conducted as described. Plots are made of standard protein added vs. added protein for each of the peptides. Slopes are calculated and averaged. For these spike-in experiments, standard Human serum albumin and uromodulin were mixed and diluted in 25 mM pH7.8  $\text{NH}_4\text{HCO}_3$  buffer at a concentration of 25 and 100  $\mu\text{g/mL}$ . Fig 2 gives an example of Human serum albumin peptide YLYEIAR standardization. The sample prepared with water presents an intercept of zero while the intercept of the different urine samples represents the albumin concentration in that sample. The critical measurement is the slope for all samples. Ideally, slopes should be 1.0. However, heavy atom peptides may not be pure and/or the protease digestion may not be complete. This requires that the relationship be determined experimentally as shown.

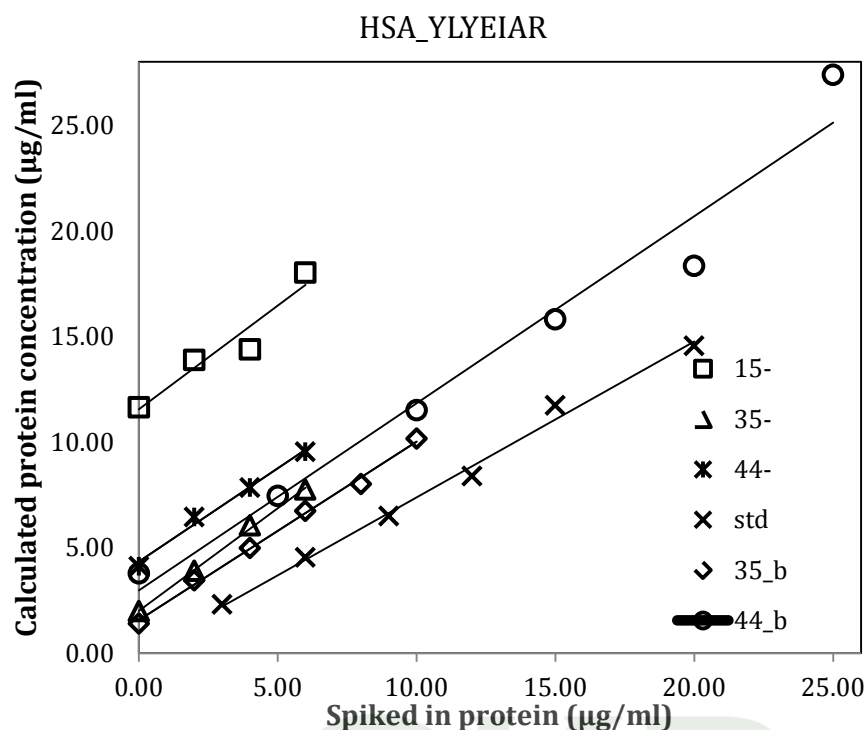


Fig 2. Denominator calculation for Human serum albumin peptide YLYEIAR. Series 15-, 35-, 44- are 3 different urine samples with spiked in standard proteins, std is MilliQ water. Samples 35\_b and 44\_b are urines 35- and 44- with an altered spike-in. The slopes for series std, 35\_b, 44\_b, 44-, 15-, 35- are 0.74, 0.85, 0.88, 0.88, 0.97 respectively to give an average slope of  $0.86 \pm 0.07$ . Average of multiple peptides further enhances the result.

#### EXPORT of DATA AND CALCULATION OF RESULTS:

When the areas of both native and heavy labeled peptides are integrated in MultiQuant, the areas are copied and pasted into an excel spreadsheet in corresponding peptide areas. Retention times are also copied to indicate that the transitions represent the correct peaks. Table 1 shows the raw data and calculation of urine sample ac02, ac03, ac04 albumin concentration based on peptide YLYEIAR and AEFAEVSK.

Uro_FVGQGGAR_y6 and y7									
Sample Name	RT	y6 Area	y7 Area	ny6 Area	ny7 Area	Uncorrected concentration ny6/y6	Uncorrected concentration ny7/y7	Corrected Conc from y6	Corrected Conc from y7
nelse002_jian0104_ac02	2.72	7.89E+04	4.79E+03	5.52E+04	4.04E+03	11.87	14.32	7.59	6.75
nelse002_jian0104_ac02	2.72	7.75E+04	4.51E+03	5.67E+04	3.72E+03	12.42	14.01		
nelse002_jian0104_ac03	2.72	7.63E+04	4.45E+03	1.14E+05	7.68E+03	25.34	29.34	15.63	13.80
nelse002_jian0104_ac03	2.73	7.49E+04	4.42E+03	1.09E+05	7.46E+03	24.68	28.62		
nelse002_jian0104_ac04	2.72	8.09E+04	4.51E+03	2.51E+05	1.73E+04	52.60	64.93	32.90	30.28
nelse002_jian0104_ac04	2.73	8.26E+04	4.79E+03	2.56E+05	1.76E+04	52.68	62.22		

Table 1. Spreadsheet of uromodulin calculation based on peptide FVGQGGAR y6 and y7 fragment ions. Columns of "Retention Time (RT)", "y6area", "y7area", "ny6area" and "ny7area" are all raw data copied from MultiQuant Result file. The "y6area" and "y7area" indicate the abundance of the added heavy labeled peptide, while "ny6area" and "ny7area" indicate the corresponding abundance of the native peptide from the urine sample. "ny6/y6" is the initial calculation of uromodulin concentration



based on peptide ratio, before correction for experimentally determined protein concentration, as illustrated in Figure 2. This initial calculation is:

$$\text{romodulin concentration} = \frac{ny6area}{y6area} \times \frac{84532 \times 4 \times 2}{796.422 \times 50}$$

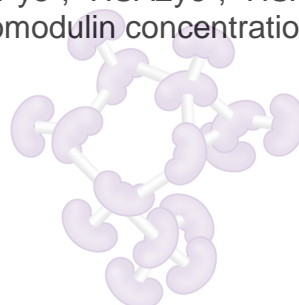
where 84532 is uromodulin MW; 4 is the volume of heavy atom peptide mix added; 2 is the uromodulin peptide concentration in the mixture (2 ug/mL), 796.422 is the MW of the corresponding heavy peptide, 50 is the urine used for sample prep (50 µL). Then primary concentrations from 2 runs are averaged and corrected for experimentally determined protein concentrations (Figure 2) to get the absolute concentration as shown in “corrected Conc from y6” and “y7” columns. Each assay includes multiple peptide measurements, which are considered independent assays. Median values of correlation coefficients for replicates and multiple peptides are typically 5% or less. Any sample presenting a CV greater than 20% is submitted for reanalysis.

HSA1 y5	HSA2y6	HSA3y5	aveHSA	CV HSA	U2-y6	U2-Y7	U3	ave URO	cv Uro
8.75	10.96	8.44	9.38	14.66%	7.59	6.75	7.47	7.27	6.28%
3.02	3.25	2.77	3.01	7.99%	15.63	13.80	14.95	14.79	6.25%
7.54	8.86	6.99	7.79	12.31%	32.90	30.28	30.80	31.33	4.43%

Table 2. Spreadsheet for protein concentration. Columns “HSA1 y5”, “HSA2y6”, “HSA3y5” correspond to the “corrected concentration” columns for each HSA peptide. Human serum albumin concentration (“aveHSA”) is the average of columns “HSA1 y5”, “HSA2y6”, “HSA3y5”. The corresponding CV for HSA is also from these columns. Uromodulin concentration and CV are calculated in the same way.

## Result Entry

Enter the results into the Excel spreadsheet.



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**University of Minnesota  
Advanced Research Diagnostic Laboratory**

<b>Entity:</b>	<b>University of Minnesota</b>
<b>Department:</b>	<b>Advanced Research and Diagnostic Laboratory</b>
<b>Manual:</b>	<b>ARDL Procedure Manual</b>
<b>Procedure:</b>	<b>Serum Beta 2-Microglobulin using ROCHE/HITACHI MODULAR P CHEMICAL ANALYZER</b>

## **INTRODUCTION**

Beta 2-Microglobulin (B2M) is a small globular peptide with a molecular weight of 11,800 Daltons. It is expressed on the extraplasma surface of nearly all nucleated cells. B2M is normally cleared exclusively by the kidneys. It passes freely through the glomerular membrane, and then is reabsorbed to an amount of up to 99.9% by the proximal tubules.

Elevated serum B2M levels occur in renal diseases such as glomerulopathies, tubulopathies, renal failure and amyloidosis, and in other diseases such as rheumatoid arthritis, and autoimmune disease.

Various methods are used to quantitate B2M, including RIA, ELISA, nephelometry and turbidimetry. This procedure is based upon the principle of immunological agglutination with latex reaction enhancement.

Modular P application code: 222

Manual worksheet code: NA

### **Principle:**

The specimen, containing B2M, is mixed with a reagent solution containing a high concentration of latex-bound anti-B2M. Antigen-antibody complexes form, and the latex particles precipitate out of solution, causing turbidity. The amount of turbidity is directly proportional to the concentration of B2M in the sample. The absorbance of this solution is read spectrophotometrically (700 nm), and concentration of B2M is determined by comparing the absorbance data to that of a calibration curve.

### **Specimen:**

Use serum for the procedure. Centrifuge specimens at 1000g for 10 minutes.

Serum is stable for three days at 4°C, six months at -20°C, and longer at -70°C. When samples are stored at -70°C, a single freeze-thaw cycle ranging from 4 to 13 months was shown to have a negligible impact on results [3].

Bilirubin does not interfere up to an I index of 54. Hemolysis does not interfere up to an H index of 1000. Lipemia does not interfere up to an L index 750.

Minimum volume: 100 uL (includes dead volume)

## **Equipment and Supplies:**

1. Roche Modular P chemistry analyzer. Roche Diagnostics, 9115 Hague Road, Indianapolis, IN 46250.
2. Cell Wash Solution II/Acid Wash. Roche product #4880307 (2L bottle). No preparation required. Solution of formic acid, citric acid and nikkol BT-9. Store at room temperature. Stable until expiration date on bottle. No stability time window after opening. This solution is automatically drawn by the Mod P while cleaning reaction cuvettes during analysis.
3. Cell Wash Solution I/NaOH-D. Roche product #1551540 (1800 mL bottle). No preparation required. Solution of sodium hydroxide (1N). Store at room temperature. Stable until expiration date on bottle. After opening a bottle it is stable for 14 days on the instrument. This solution is automatically drawn by the Mod P while cleaning reaction cuvettes during analysis.
4. Reaction cell cuvette segments. Roche product #714-0650 (Four sets of eight segments. Eight segments complete the entire rotor). Soak cuvettes overnight in a solution of 2% Hitergent before installing on the instrument. Perform cell wash and cell blank functions after installation. Change cuvettes quarterly.
5. Hitergent. Roche product #409149 (1L bottle). No preparation required. Solution of ethanolamine, hexahydro-1,3,5-tris (Betahydroxyethyl) triazine and nonidet P-40. Store at room temperature. Stable until expiration date on bottle. Hitergent is an on-board reagent automatically drawn by the Mod P during the daily incubator bath exchange. Hitergent is transferred, as needed, from the 1L bottle to the 66 mL bottle located in position 2D3.
6. Sample cups (micro). Roche product #11406680001.
7. Sample cups (standard). Roche product #729177.

## **Reagents:**

1. Roche product #11660551216, B2-Microglobulin reagent kit (Tina-quant):
  - R1 reagent (2 x 12 mL). Tris/HCl buffer (pH 8.7), NaCl, EDTA, preservatives. See package insert for component concentrations. No preparation required. There are approximately 60 tests per bottle.
  - R2 reagent (2 x 12 mL). Latex particles coated with polyclonal anti-human B2-Microglobulin antibody (rabbit), preservatives. See package insert for concentrations. No preparation required. There are approximately 60 tests per bottle. Because the latex particles may settle during storage, mix the R2 reagent bottle daily when it is on the instrument.
  - Bottle 3. B2M calibrator (human). Open the bottle, being careful to avoid the loss of lyophilizate, and pipette in exactly 1.0 mL water. Then re-cap the bottle and dissolve the contents by gentle swirling. The concentration of the standard is lot-dependant, and the value is provided in the package insert. This concentration value must also be installed on the Modular P.
  - Storage and stability. Keep reagents stored in the refrigerator until use. R1 is stable for 90 days refrigerated on the analyzer. R2 is stable for 90 days refrigerated on the analyzer. Calibrator is stable for 90 days when securely capped and refrigerated.
  - Always change the R1/R2 reagents as a pair. When loading the reagents onto the Mod P, make sure R1 is placed in the R1 rotor, and R2 is placed in the R2 rotor. Remove any bubbles in the reagents prior to loading. Place the reagents in like-numbered locations in the two rotors. This makes it easier to track the chronology of the reagents on the instrument.
2. Milli-Q water. Milli-Q is the trade name of the water system purchase from the Millipore Corporation. Milli-Q is deionized water treated with activated carbon and deionization cartridges and filtered to remove microorganisms larger than 0.22 micrometers. This meets CAP class I water requirements.

3. Roche Precipath U plus control kit. Roche Diagnostics, Gmbh, Sandhofer Strasse 116, D-68305 Mannheim, Germany. Store at 2-8°C until use; stable until expiration date on package. Dilute vial contents with 3.0 mL of kit diluent. Mix by gentle swirling; allow 30 minutes for material dissolution. Control is stable for 5 days following reconstitution, when stored at 2-8°C.
4. Pooled Normal Serum control. Solomon Park Research Laboratories, 12815 NE 124th St. Suite 1, Kirkland, WA 98034. Store frozen at -70°C. Once thawed, product is stable for 7 days at 2-8 °C. Allow the control to reach room temperature (18-25° C) and mix thoroughly by inversion before use.

### **Calibrator:**

See reagent section above for calibrator details.

The B2M calibrator setpoint value is traceable to the WHO standard NIBSC code B2M.

Calibration frequency: Calibration must be performed with each new reagent lot or shipment, every six months and as needed if control values deviate or after major maintenance or repair. The Mod P will automatically perform a two-point calibration (saline + B2M calibrator) when there is a reagent lot number change and when a new reagent bottle set is registered on the instrument. The Mod P will not allow testing to proceed until a successful calibration has been completed.

**Caution: This product is of human and animal origin. Handle as though capable of transmitting infectious disease.**

### **Instrument Setup:**

1. Log into the Mod P using assigned username and password.
2. Reagents. All reagents used on the Mod P are stored in a refrigerated reagent compartment. B2M is a two-reagent system. Reagent 1 must be placed in the outer (R1) rotor; reagent 2 must be placed in the inner (R2) rotor. All reagents have a unique barcoded identifier. Before starting the analysis sequence check the reagent status on the Mod P to confirm there is adequate reagent to complete the anticipated test volume for the day. Discard any bottles that have gone empty. Check the volume of the two wash reagents.
3. Maintenance. Complete the scheduled daily maintenance as described in the Mod P general operations protocol.
4. Order calibration, if indicated (see Mod P general operations protocol).
5. Order controls. If a calibration was requested, the controls should not be ordered until the calibration report has printed. If the controls are ordered and executed before the calibration prints out, the controls will be measured on the previously stored calibration line.

### **Quality Control:**

Two levels of control are assayed each time the B2M method is performed. It is acceptable to run each control at the start of the day, and again at the end of the day. Review Levey-Jennings plots and acceptability criteria for each quality control result daily before reporting results. The operator may run them more frequently, if desired. One control is prepared from

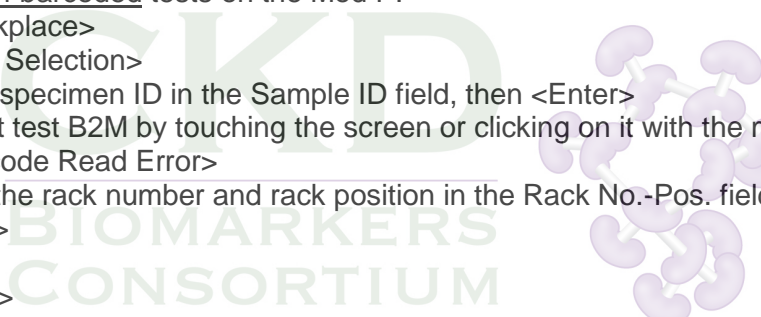
pooled, normal human serum. The other is an abnormal commercial control. Consult quality control charts for current ranges and lots in use. If QC values violate the Westgard Rules 1-3s or 2-2s, perform and document the following corrective actions steps until QC is acceptable:

1. Check reagent and quality control for appropriate lot numbers, expiration dates, preparation and storage conditions.
2. Repeat the analysis using fresh QC material.
3. Perform a calibration.
4. Perform maintenance procedures.
5. If quality control continues to fall outside of the established acceptability limits discontinue testing and notify the supervisor.

### **Procedure:**

After calibration and controls have been measured and evaluated, the test specimens may be loaded onto the Mod P. An abbreviated description of the measurement procedure follows. A more thorough description may be found in the Mod P general operations protocol.

1. If specimens have been frozen, allow them to thaw completely, then mix well. Serum specimens should not require centrifugation unless they have large amounts of suspended material.
2. To order non-barcoded tests on the Mod P:
  - a. <Workplace>
  - b. <Test Selection>
  - c. Enter specimen ID in the Sample ID field, then <Enter>
  - d. Select test B2M by touching the screen or clicking on it with the mouse.
  - e. <Barcode Read Error>
  - f. Enter the rack number and rack position in the Rack No.-Pos. fields.
  - g. <Add>
  - h. <OK>
  - i. <Save>
3. Note the order of positions 1-5 in the sample rack: position 1 is on the right and position 5 is on the left. Place the specimen in the rack so that ¼ to 1 inch of the vial is above the sample rack. This allows the Mod P to detect the presence of the vial in the rack. Orient the vial in the rack so that any barcodes are turned inward, and therefore unreadable. If the testing vials are to be re-capped, arrange the caps so they can be matched up following analysis.
4. To order barcoded tests on the Mod P: Follow instructions as in step 3 above, except that the Container ID (CID) barcode now must face outward so the Mod P can read it. The CID barcode must be oriented vertically. No test ordering is required on the instrument. In this case test ordering has occurred in Misys, and the CID label has been generated for that purpose.
5. After the specimens are in place, put the racks onto the loading platform. The racks will only load in one orientation, as the center track is offset. Do not prepare more than three racks at a time, as evaporation could occur while the instrument goes through the sampling process.
6. Close the cover on the loading platform.
7. On the Mod P computer terminal, press or click <Start>, then <Start> again.
8. Only calibration and control data automatically print out. Patient data hard copies must be requested in <Workplace>, <Data Review>. Highlight the desired records, then <Print>, and <Print> again.
9. Non-barcoded records must be manually entered into Misys, a designated spreadsheet, or website.





10. Barcoded records are accepted using the OEM program in Misys. The method code for the Mod P is UR9.

### **Instrument shutdown:**

After the patient specimens and final controls have been evaluated and accepted, load the green rack (W999) and run it through the instrument. Place three standard sample cups in positions 1, 2 and 3. Fill cup 1 with 1N sodium hydroxide, fill cup 2 with 4N sodium hydroxide, and fill cup 3 with leftover serum. Place it onto the loading platform and press <Start>, and <Start> again. After 18 minutes, the Mod P comes to Stand-by status. If the green rack is not run, the Mod P will take at least one hour to come to Stand-by status.

After coming to Stand-by status the data from each day's run is downloaded from the Mod P computer to a diskette, then to the network folder. Consult the procedure describing this process for details.

Print all Mod P test results, and file in chronological order with the other daily printouts.

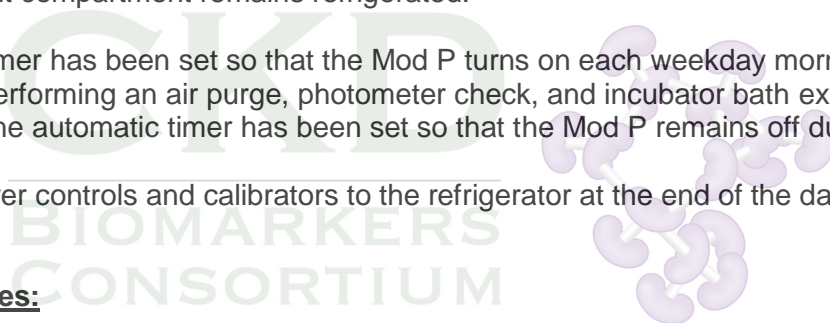
The Mod P is turned off each day after all work is complete. The steps are as follows: <Utility>, <Maintenance>, <Nightly Pipe>, <Select>, <Execute>. This shutdown process requires approximately five minutes. The instrument and its computer are automatically turned off. The reagent compartment remains refrigerated.

An automatic timer has been set so that the Mod P turns on each weekday morning at 0530, automatically performing an air purge, photometer check, and incubator bath exchange during the process. The automatic timer has been set so that the Mod P remains off during weekends.

Return all leftover controls and calibrators to the refrigerator at the end of the day.

### **Expected Values:**

- Roche ranges:  
Serum, adult: 0.8-2.2 mg/dL  
The manufacturer serum reference range (see appendix B, B2M package insert) was compared to results from 13,091 study participant samples in 2012 and 2013. A range of 1.29 – 3.19 mg/L representing the central 95% of the second was determined nonparametrically. Study participant age ranged from 48 – 67 years old and the health status is unknown to this laboratory, thus it is not surprising the central 95% is substantially higher than the manufacturer reported “healthy subjects”.
- Advanced Research and Diagnostic Laboratory ranges: 0.8-2.2  
Misys test code: N/A
- Linear range of the method: 0.2-8.0 mg/L (serum). Specimens exceeding the high limit are automatically diluted by the instrument, and reported accordingly. If a manual dilution is required, dilute the specimen in normal saline, and multiply the result by the dilution factor. Specimens reading below the linear range of the assay should be reported as <0.2 mg/L.



### ASSAY PERFORMANCE

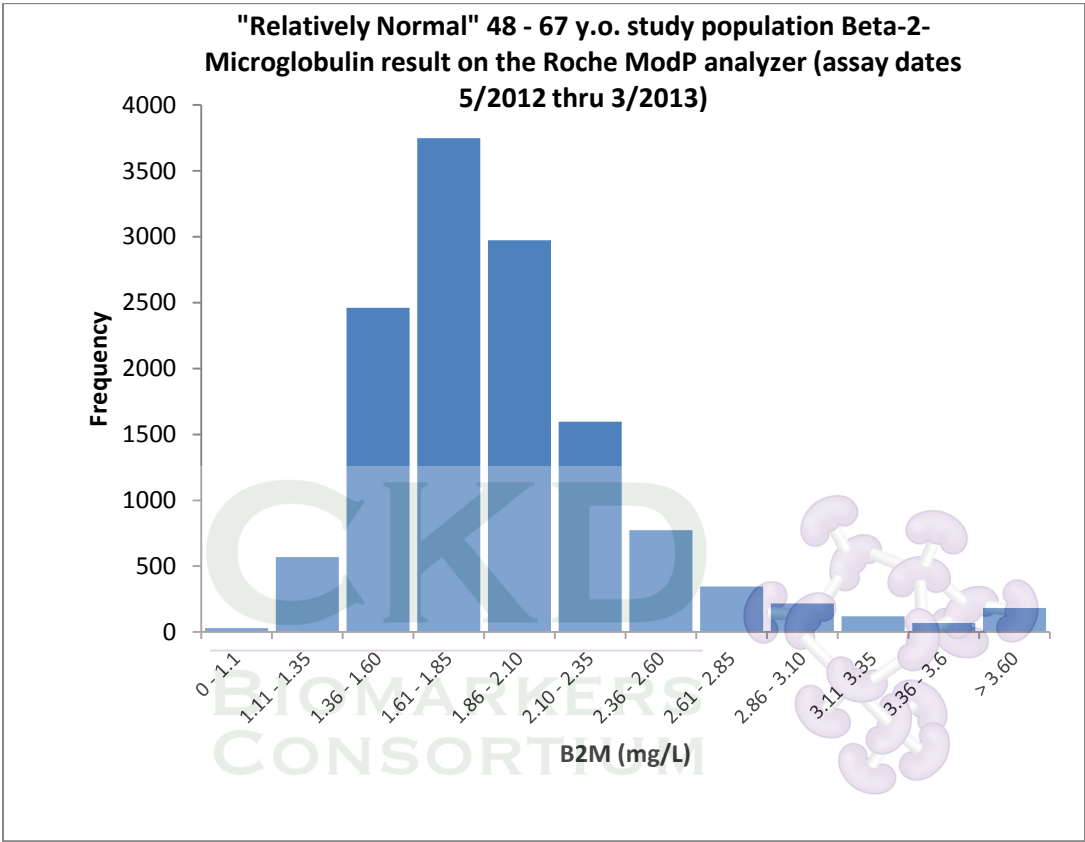
Analytical Range	0.20 - 8.0 mg/L
Limit of Detection (as determined by the manufacture, the value 3SD above the lowest standard, see Ref 4 and appendix B)	0.03 mg/L
Reportable Range	0.20 – 50.00 mg/L
Recovery, normal samples (Performed on 5 normal serum samples and spiked with one part calibrator at a concentration of 17.2 mg/L to four parts normal sample )	88 – 94%
Recovery, uremic samples (Performed on 6 uremic serum samples and spiked with one part calibrator at a concentration of 17.2 mg/L to four parts uremic sample )	96 – 99%
Inter-assay %CV (10 between day replicates at a concentration of 1.67 mg/L)	3.2 %
Within day duplicate range, serum	6.0 %
Reference range (as determined by the manufacturer and validated by the laboratory)	0.8 - 2.2 mg/L

### References:

1. Roche/Hitachi System Application Sheet for B2-Microglobulin, 2005.
2. Roche/Hitachi Modular Analytics Operator's Manual, version 2.0, October 2006.
3. Stephen P. Juraschek , Josef Coresh, Lesley A. Inker, Gregory P. Rynders, John H. Eckfeldt, Elizabeth Selvin. The effects of freeze–thaw on  $\beta$ -trace protein and  $\beta$ 2-microglobulin assays after long-term sample storage. Clin Biochem 2012;45:694 – 696
4. Armbruster DA, Pry T. Limit of the Blank, Limit of Detection and Limit of Quantitation. Clin Biochem Rev Vol 29 Suppl (i) August 2008.

<b>External Ref:</b>	See above
<b>Appendix:</b>	Appendix A: Reference Range evaluation Appendix B: B2M reagent package insert
<b>Written by and Date:</b>	G. Rynders 5/2013
<b>Date Effective:</b>	5/2013
<b>Approved by and Date:</b>	Anthony Killeen, MD, PhD
<b>Date Retired:</b>	

Appendix A: Reference Range Evaluation



Study population B2-Microglobulin		manufacture reference range	0.8 – 2.2 mg/L
Mean =	1.98 mg/L		
Standard deviation =	1.45 mg/L		
N =	13,086		
2.5 %ile =	1.29 mg/L		
97.5 %ile =	3.19 mg/L		
Central 95% reference range for this study population is	1.29 – 3.19 mg/L		



## Appendix B: B2M reagent package insert

11896361001V12

# β2-Microglobulin

Tina-quant β2-Microglobulin

**cobas®**

● Indicates Roche/Hitachi analyzer(s) on which kit(s) can be used

Cat. No.	Bottle	Contents	902	MODULAR	
				P	D
11660551 216	1	REAGENT 2 x 12 mL			
	2	REAGENT 2 x 12 mL	●	●	
	3	CALIBRATOR → 1 x 1 mL			

Some analyzers and kits shown may not be available in all countries. For additional system applications, contact your local Roche Diagnostics representative.

### English

#### System information

For Roche/Hitachi MODULAR P analyzers: ACN 222.

#### Intended use

In vitro test for the quantitative determination of β2-microglobulin in human serum and plasma on Roche automated clinical chemistry analyzers. To be used as an aid in the diagnosis of active rheumatoid factor arthritis and kidney disease.

#### Summary<sup>1,2,3,4,5</sup>

Beta 2-microglobulin (β2-M) was discovered in 1968 by Berggård et al. in the urine of patients with Wilson's disease and in patients with chronic cadmium poisoning. β2-M is a small globular peptide with a molecular weight of 11800 D. It is identical to the light chain of the major histocompatibility complex (MHC) antigen (HLA) 5. Its tertiary structure is homologous to the CH3-IgG immunoglobulin domain. Thus β2-M is expressed on the extraplasma surface of nearly all nucleated cells (exception: trophoblasts). β2-M consists of 100 amino acids with a disulfide-linked loop between amino acid 25 and 81. β2-M is non-covalently associated with the class 1 MHC antigen, and is identical to BDGF (bone-derived growth factor 2), CRG-8, and thymotaxin. β2-M is normally cleared exclusively by the kidneys. It passes freely through the glomerular membrane, and then is reabsorbed to an amount of up to 99.9 % by the proximal tubules. It has been published that elevated serum levels of β2-M occur in renal diseases such as glomerulopathies, tubulopathies, renal failure, and amyloidosis. In addition, it has been reported that other increased serum levels are found in rheumatoid arthritis and autoimmune diseases. Various assay methods are available for β2-microglobulin determination, such as radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), nephelometric immunoassays, and turbidimetric methods. The Roche β2-microglobulin assay is based on the principle of immunological agglutination with latex reaction enhancement.

#### Test principle<sup>1</sup>

Immunoturbidimetric assay.

- Sample and addition of R1
- Addition of R2 and start of reaction:

Latex-bound anti-β2-microglobulin antibodies react with antigen from the sample to form antigen/antibody complexes which are determined turbidimetrically after agglutination.

#### Reagents – working solutions

**R1** TRIS<sup>a</sup>/HCl buffer: 23 g/L, pH 8.7; NaCl: 19 g/L; EDTA: 2 g/L; preservative

**R2** Latex particles coated with polyclonal anti-human β2-microglobulin antibody (rabbit): 0.5 g/L; preservative

**3** β2-microglobulin (human)

a) TRIS = Tris(hydroxymethyl)-aminomethane

#### Precautions and warnings

For in vitro diagnostic use.

Exercise the normal precautions required for handling all laboratory reagents. All human material should be considered potentially infectious. All products derived from human blood are prepared exclusively from the blood of donors tested individually and shown to be free from HBsAg and antibodies to HCV and HIV. The testing methods applied were FDA-approved or cleared in compliance with the European Directive 98/79/EC, Annex II, List A. However, as no testing method can rule out the potential risk of infection with absolute certainty, the material should be handled with the same level of care as a patient specimen. In the event of exposure the directives of the responsible health authorities should be followed.<sup>6,7</sup>

Safety data sheet available for professional user on request.

Disposal of all waste material should be in accordance with local guidelines.

#### Reagent handling

R1: Ready for use

R2: Ready for use

Calibrator 3:

Open the bottle, being careful to avoid the loss of lyophilizate, and pipette in exactly 1.0 mL of distilled/deionized water. Then close the bottle carefully and dissolve the contents by gentle swirling, avoiding the formation of foam.

Store calibrator tightly capped when not in use.

#### Storage and stability

Unopened kit components: Up to the expiration date at 2–8 °C

R1: 90 days opened and refrigerated on the analyzer

R2: 90 days opened and refrigerated on the analyzer

Calibrator: 90 days at 2–8 °C

#### Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.

Serum

Plasma: Li-heparin or EDTA-plasma

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer. Centrifuge samples containing precipitates before performing the assay.

Stability:<sup>8</sup> 3 days at 2–8 °C

6 months at (–15)–(–25) °C

#### Materials provided

See "Reagents – working solutions" section for reagents.

#### Materials required (but not provided)

- Controls: β2-Microglobulin Control Set Serum, Cat. No. 11729683 216
- 0.9 % NaCl
- General laboratory equipment

#### Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

#### Calibration

Traceability: This method has been standardized against the WHO standard.

S1: 0.9 % NaCl solution

S2: Calibrator (bottle 3)

Calibration frequency

2-point calibration is recommended

- after reagent lot change
- as required following quality control procedures

#### Quality control

For quality control use the control material as listed in the "Materials required" section. In addition, other suitable control material can be used. The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within

## Appendix B: B2M reagent package insert

# β2-Microglobulin

### Tina-quant β2-Microglobulin

the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

### Calculation

The analyzer automatically calculates the analyte concentration of each sample.

Conversion factors:  $\text{mg/L} \times 84.7 = \text{nmol/L}$   
 $\text{mg/L} = \mu\text{g/mL}$

### Limitations – interference

Criterion: Recovery within  $\pm 10\%$  of initial value.

Icterus:<sup>9</sup> No significant interference up to an I index of 54 (approximate conjugated and unconjugated bilirubin concentration: 54 mg/dL or 923  $\mu\text{mol/L}$ ).

Hemolysis:<sup>9</sup> No significant interference up to an H index of 1000 (approximate hemoglobin concentration: 1000 mg/dL or 621  $\mu\text{mol/L}$ ).

Lipemia (Intralipid):<sup>9</sup> No significant interference up to an L index of 750. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Rheumatoid factors < 200 IU/mL do not interfere.

No high dose hook-effect up to β2-M concentrations of 5082 nmol/L (60 mg/L).

31 commonly used, in vitro tested pharmaceuticals did not interfere with the assay.

In very rare cases gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

### ACTION REQUIRED

**Special Wash Programming:** The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi analyzers. Refer to the latest version of the carry-over evasion lists and the operator's manual for further instructions. US users refer to the Special Wash Programming document (located on MyLabOnline website) and the operator's manual for special wash instructions. **Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.**

### Limits and ranges

#### Measuring range

Roche/Hitachi 902 analyzer

Measuring range: 16.9–491 nmol/L (0.2–5.80 mg/L or  $\mu\text{g/mL}$ )

On instruments without rerun function, manually dilute samples having higher concentrations with 0.9 % NaCl solution or distilled/deionized water (e.g. 1 + 10). Multiply the result by the appropriate factor (e.g. 11).

Roche/Hitachi MODULAR analyzers

Measuring range: 16.9–678 nmol/L (0.20–8.00 mg/L or  $\mu\text{g/mL}$ )

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:11 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 11.

#### Lower limits of measurement

##### Lower detection limit of the test

Detection limit: 2.54 nmol/L (0.03 mg/L or  $\mu\text{g/mL}$ )

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying three standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability,  $n = 21$ ).

#### Expected values<sup>1</sup>

68–186 nmol/L (0.8–2.2 mg/L or  $\mu\text{g/mL}$ )

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

### Specific performance data

Representative performance data on the analyzers are given below.

Results obtained in individual laboratories may differ.

### Precision

Precision was determined using human samples and controls in an internal protocol with repeatability\* ( $n = 21$ ) and intermediate precision\*\* (1 aliquot per run, 1 run per day, 10 days). The following results were obtained:

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Sample	Repeatability*			Intermediate precision**		
	Mean nmol/L/ mg/L	SD nmol/L/ mg/L	CV %	Mean nmol/L/ mg/L	SD nmol/L/ mg/L	CV %
Human serum	316/ 3.73	2/ 0.02	0.7	108/ 1.27	2/ 0.03	2.0
Control Set low	156/ 1.84	2/ 0.02	1.0	156/ 1.84	3/ 0.03	1.8
Control Set high	490/ 5.78	5/ 0.06	1.0	487/ 5.75	7/ 0.09	1.5

\* repeatability = within-run precision

\*\* intermediate precision = total precision / between run precision / between day precision

### Method comparison

A comparison of the β2-microglobulin determination using the Roche Tina-quant β2-Microglobulin assay (y) with a microparticle enzyme immunoassay for β2-microglobulin (x) in human sera gave the following correlations (mg/L):

Passing/Bablok<sup>10</sup>                      Linear regression  
 $y = 1.226x + 0.143$                        $y = 1.173x + 0.317$   
 $r = 0.89$                                        $r = 0.977$

Number of samples measured: 87

The sample concentrations were between 101 and 809 nmol/L (1.19 and 9.55 mg/L).

### References

1. Junge W, Schlottmann A, Thormeyer I, et al. Evaluation of a Homogeneous Immunoassay for the Determination of Beta-2-Microglobulin in Serum/Plasma. Poster No. 287, 48<sup>th</sup> Annual Meeting of the American Association for Clinical Chemistry, July 28-August 1 (Chicago, Illinois) 1996.
2. Berggard I, Bearn AG. Isolation and Properties of a Low Molecular Weight β2-Globulin Occurring in Human Biological Fluids. J Biol Chem 1968;243:4095.
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4. Steinhoff J, Feddersen A, Wood WG, et al. β2-Microglobulinurie bei Cytomegalievirusinfektionen nach Nierentransplantation. Dtsch med Wschr 1991;116:1008–1012.
5. Niederau CM. β2-Microglobulin - a protein with multiple diagnostic relevance in the past and in the future. Institute of Clinical Chemistry and Laboratory Diagnostics, Heinrich-Heine University, Düsseldorf, Germany 1996.
6. Occupational Safety and Health Standards: bloodborne pathogens (29 CFR Part 1910.1030). Fed. Register.
7. Directive 2000/54/EC of the European Parliament and Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work.
8. Tietz NW. Clinical Guide to Laboratory Tests, 3<sup>rd</sup> ed. Philadelphia, PA: WB Saunders, 1995:436–437.
9. Glick MR, Ryder KW, Jackson SA. Graphical Comparisons of Interferences in Clinical Chemistry Instrumentation. Clin Chem 1986;32:470–475.
10. Passing H, Bablok W, Bender R, et al. A general regression procedure for method transformation. J Clin Chem Clin Biochem 1988 Nov;26(11):783–790.

# β2-Microglobulin

Tina-quant β2-Microglobulin

## Instrument settings

### US users:

Refer to the application sheet and Special Wash Programming document (located on MyLabOnline website) for additional operating information.

### Users of Roche/Hitachi MODULAR analyzers:

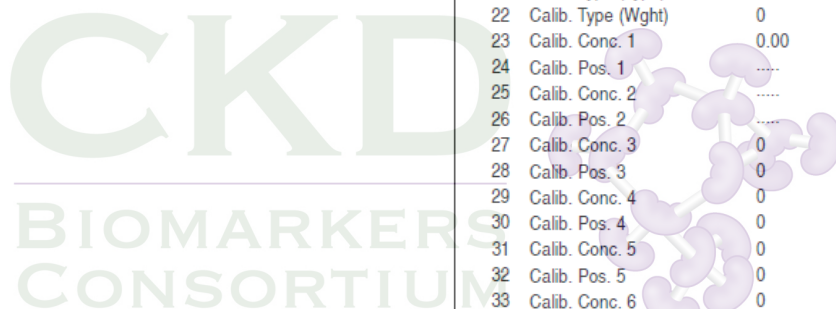
Enter the application parameters via the barcode sheet.

cobas®

## Roche/Hitachi 902 analyzer

No.	<Chemistry>	
1	Test Name	MICGL
2	Assay Code (Mthd)	2 Point End
3	Assay Code (2. Test)	0
4	Reaction Time	10
5	Assay Point 1	23
6	Assay Point 2	33
7	Assay Point 3	0
8	Assay Point 4	0
9	Wavelength (SUB)	0
10	Wavelength (MAIN)	700
11	Sample Volume	3.0
12	R1 Volume	200
13	R1 Pos.	.....
14	R1 Bottle Size	Small
15	R2 Volume	0
16	R2 Pos.	0
17	R2 Bottle Size	Small
18	R3 Volume	200
19	R3 Pos.	.....
20	R3 Bottle Size	Small
21	Calib. Type (Type)	Linear
22	Calib. Type (Wght)	0
23	Calib. Conc. 1	0.00
24	Calib. Pos. 1	.....
25	Calib. Conc. 2	.....
26	Calib. Pos. 2	.....
27	Calib. Conc. 3	0
28	Calib. Pos. 3	0
29	Calib. Conc. 4	0
30	Calib. Pos. 4	0
31	Calib. Conc. 5	0
32	Calib. Pos. 5	0
33	Calib. Conc. 6	0
34	Calib. Pos. 6	0
35	S1 ABS	0
36	K Factor	10000
37	K2 Factor	10000
38	K3 Factor	10000
39	K4 Factor	10000
40	K5 Factor	10000
41	A Factor	0
42	B Factor	0
43	C Factor	0
44	SD Limit	0.1
45	Duplicate Limit	200
46	Sens. Limit	0
47	S1 Abs. Limit (L)	-32000
48	S1 Abs. Limit (H)	32000
49	Abs. Limit	32000
50	Abs. Limit (D/I)	Increase
51	Prozone Limit	-32000
52	Proz. Limit (Upp/Low)	Lower
53	Prozone (Endpoint)	35
54	Expect. Value (L)	.....
55	Expect. Value (H)	.....
56	Instr. Fact. (a)	1.0
57	Instr. Fact. (b)	0.0
58	Key setting	.....

..... Data entered by the operator



## Appendix B: B2M reagent package insert

# $\beta$ 2-Microglobulin

### Tina-quant $\beta$ 2-Microglobulin

For further information, please refer to the appropriate operator's manual for the analyzer concerned, the respective application sheets and method sheets of all necessary components.

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

#### FOR US CUSTOMERS ONLY: LIMITED WARRANTY

Roche Diagnostics warrants that this product will meet the specifications stated in the labeling when used in accordance with such labeling and will be free from defects in material and workmanship until the expiration date printed on the label. THIS LIMITED WARRANTY IS IN LIEU OF ANY OTHER WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR PARTICULAR PURPOSE. IN NO EVENT SHALL ROCHE DIAGNOSTICS BE LIABLE FOR INCIDENTAL, INDIRECT, SPECIAL OR CONSEQUENTIAL DAMAGES.

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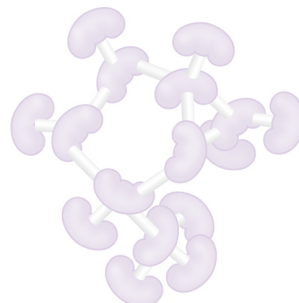


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**University of Minnesota**  
**Advanced Research Diagnostic Laboratory**

<b>Entity:</b>	<b>University of Minnesota</b>
<b>Department:</b>	<b>Advanced Research and Diagnostic Laboratory</b>
<b>Manual:</b>	<b>ARDL Procedure Manual</b>
<b>Procedure:</b>	<b>Beta Trace Protein Measurement on Siemens ProSpec Nephelometer</b>

It is known that the estimation of glomerular filtration rate (GFR) based upon the serum creatinine concentration has its limitations. In particular, individuals with higher muscle mass will generally have higher “normal” levels of circulating creatinine. The calculations devised to estimate GFR do not account for this variable. It is theorized that beta trace protein (BTP), a low molecular weight glycoprotein that is freely filtered through the kidney’s glomerular basement membrane, and has minimal non-renal elimination, may be a better marker for estimation of GFR. BTP (also known as prostaglandin D synthase) is found in high concentrations in nerve tissue throughout the human body. The concentration of BTP in CSF is ~35 times greater than its concentration in blood. The BTP assay described below is not yet approved for clinical use in the United States, but it is available for research applications only.

**Principal:**

A solution of polystyrene particles coated with rabbit antibodies specific to human BTP is incubated with diluted specimen. A reaction occurs between the bound antibody and the BTP in the specimen, resulting in particle aggregation and an increase in light scatter. The BTP concentration of the test specimen is determined by comparing its light scatter intensity to that observed in a calibration curve.

**Specimen:**

Serum is the preferred sample type. EDTA- and heparinized plasma are reported by the manufacture as an acceptable sample types, but only EDTA-plasma has been evaluated by the Advanced Research and Diagnostic Laboratory (see Appendix A). CSF, nasal or oral secretions are also acceptable. Specimens are stable two weeks at 2-8°C. Long term storage should be at –20°C or –70°C. Avoid repeated freeze/thaw cycles. Prior to analysis specimens must be free of any particulate matter. Lipemic samples, or frozen specimens that become turbid after thawing, must be clarified by centrifugation for 10 minutes at 15,000 g.

The ProSpec samples 30 uL. The sample dead volume when sampling from 2 mL Sarstedt vials in the Eppendorf rack is approximately 75 uL. The sample dead volume when sampling from a 10 x 75 mm glass test tube is approximately 250 uL.

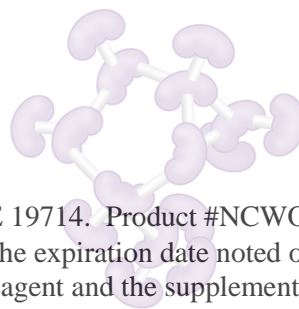
**Equipment and Supplies:**

1. **ProSpec nephelometer**  
Siemens Healthcare Diagnostics Inc., Newark, DE 19714.

2. **ProSpec parts and supplies:**
  - a. N Reaction Buffer. Siemens product #OUMS 65 (5 L bottle). A solution of polyethylene glycol and sodium chloride (11.6 g/L) in phosphate buffer (0.05 mol/L). Contains sodium azide (<1 g/L). Store at room temperature. Stable until expiration date on bottle; stable for six weeks after opening.
  - b. N Diluent. Siemens product #OUMT 65 (5 L bottle). Phosphate buffered saline with sodium azide (<1 g/L). Store at room temperature. Stable until expiration date on bottle.
  - c. N Cuvette segments for BN ProSpec. Siemens product #OVLK 31 (300 segments of 9 cuvettes each). Cuvettes are a single-use item, and must be manually changed by the operator.
  - d. N Predilution wells. Siemens product #OVIC 11 (1100 segments of 6 wells each). Used for automatic dilutions prepared by the ProSpec.
  - e. N Evaporation caps for reagent vials (5 mL). Siemens product #OVLC 31 (100 pieces). The caps are placed directly on reagent bottles stored in the refrigerated reagent chamber. The design allows for sampling by the instrument, but prevents evaporation of the reagent.
  - f. Disposable glass culture tubes, 10 x 75 mm. Cardinal Health cat. no. T1290-2. If necessary, specimens are transferred into these tubes prior to instrument sampling.
3. **Pipets:**  
Adjustable, 20-200  $\mu$ L and 100-1000  $\mu$ L.

### **Reagents:**

1. **N Latex BTP reagent kit:**  
Siemens Healthcare Diagnostics Inc., Newark, DE 19714. Product #NCWO 11. Store at 2 to 8°C until put into use. The kit is stable until the expiration date noted on the box. The reconstituted antibody-coated latex particle reagent and the supplemental reagent are stable for four weeks if stored, with their evaporation caps in place, in the ProSpec's refrigerated reagent chamber. The following components are included in the kit:
  - a. N BTP Reagent, 3 x 36 tests, 2 mL bottles. Lyophilized polystyrene particles coated with rabbit antibodies to human BTP. Resuspend the particles in 2.0 mL of distilled water. The prepared reagent can be used after 30 minutes. Gently mix the reagent daily. It is stable for four weeks if stored, with evaporation cap in place, in the ProSpec's refrigerated reagent chamber.
  - b. N BTP Supplementary Reagent, 3 x 0.6 mL bottles. Rabbit immunoglobulin in buffered solution. It is used to suppress interference from rheumatoid factors. No preparation required.
  - c. N/T Protein Control LC. 3 x 1.0 mL. Siemens catalog no. OQLW15. Lyophilized human urine and serum proteins with polygeline and rabbit albumin. Reconstitute with 1.0 mL distilled water. Allow to stand for 30 minutes, then mix well prior to first use. Avoid foam formation and vigorous shaking. The control is stable for two weeks following preparation when stored at 2 to 8°C. Do not freeze. Although Siemens provides a target range of values for each lot number of control, the laboratory determines its own mean and acceptable ranges.





2. **N Protein Standard UY:**

Siemens product #OQLV09 (3 x 0.5 mL). Lyophilized human urine proteins. Store at 2 to 8°C until put into use. The BTP concentration in the standard will vary with lot. Siemens provides periodic calibrator lot and concentration updates on compact disk. When these parameters are read into the ProSpec system, it is only necessary for the instrument to read the calibrator's barcode to determine its BTP concentration. The reference line is valid until controls demonstrate drift, the reagent lot changes, or the calibrator lot changes. At these junctures, re-calibration must be performed.

Reconstitute with 0.5 mL Milli-Q water. Allow to stand for 30 minutes, then mix well prior to first use. The standard is stable for two weeks following preparation when stored at 2 to 8°C.

3. **Milli-Q water.**

Milli-Q is the trade name of the water system purchased from the Millipore Corporation. Milli-Q water is deionized water treated with activated carbon and deionization cartridges and filtered to remove microorganisms larger than 0.22 micrometers. This meets CAP Class I water requirements.

**Instrument Setup:**

All reagents used on the BN ProSpec are stored on-board in a refrigerated reagent compartment. The instrument features a heated sample/reagent probe that warms the refrigerated reagent to 37°C before dispensing it into the 37°C reaction cuvette. Each bottle has a barcoded lot number affixed to it.

Before beginning any testing check the reagent volume to make sure enough is available for the scheduled work. The ProSpec uses 50 uL of antibody reagent per sample, so a 2 mL bottle equates to nearly 40 measurements. The ProSpec uses 15 uL of supplementary reagent per sample, so a 0.6 mL bottle equates to nearly 40 tests. To check available reagents, <Loading>, <Reagents>. The BTP antibody appears under the Reagent column as "bTP". The supplementary reagent appears as "SR bTP". The other category headings are as follows: Lot = lot number, Required = reagent volume required for the tests currently ordered, Available = reagent volume currently available in that bottle, Seq/Pos = reagent segment number and position within that segment, Status = reagent status.

Note: Because BTP is not a FDA-approved assay, there are certain features of the test that are not fully loaded on the ProSpec. One element that is missing is a value in the "Required" column. The ProSpec does not tally the required reagent volumes as the BTP tests are ordered. The value remains "0". However, the ProSpec will produce a yellow flag in the Reagents & Controls box when the total volume of reagent required for the ordered BTP tests exceeds the amount detected by the instrument.

Check the availability of dilution cups and reaction cuvettes prior to starting:

- a. <System>
- b. <Cuvettes and dilution cups>
- c. <Cuvettes and dilution cups [F12]>
- d. <OK> at prompt to access the right cover.
- e. Lift right cover
- f. <Select all [F9]>

- g. Replace the dilution strips and reaction cuvettes that have an “X” in them. Replace cuvette dome lid.
- h. <Save>
- i. Close right cover.
- j. Check display to make sure the updates were registered by the instrument.

The BN ProSpec and its external computer are not turned off after each day’s use. Therefore there is no need for a routine boot-up procedure.

See the separate maintenance procedure for scheduled maintenance tasks.

### **Quality Control:**

There are two levels of controls routinely assayed. The first is a pooled human serum collected from 12 normal donors. It is stored at -70°C. The other control is the LC control described in the reagents section above. Consult posted information for current lot numbers and acceptable ranges. The controls are assayed on the BN ProSpec as specimen unknowns, ordered like a routine test specimen. All values are plotted on the monthly spreadsheet located in the ProSpec folder within the Daily QC Tally folder in the Q: drive. Review Levey-Jennings plots and acceptability criteria for each quality control result daily.

### **Procedure:**

1. If specimens have been previously frozen, allow them to thaw completely, and then mix well. Remove any particulates as described in the Specimen section above.
2. Calibration is performed at least every 6 months, with reagent lot changes, if control values deviate or after major repair or maintenance. To calibrate:
  - a. Load the ProSpec with reagents: <Loading [F4]> <Reagents [F8]> Click on an existing reagent position <Access [Shift+F6]>. Answer OK to the prompt, and the ProSpec positions the selected reagent under the access door. Open the instrument’s right cover and the reagent door. **Note: Never open either of the ProSpec’s covers without requesting, then being granted access by the computer system. If this step is not followed the instrument may have to be reset.** The reagent rotor may be manually rotated. Load the reagent into position 1 or 3 in any of the white reagent wedges. Both reagents (polystyrene particle/antibody and supplemental) are required to perform the test. The bottle barcodes must face out through the window of the wedge in order for the ProSpec to register it. The yellow wedges are for controls only.
  - b. Close the covers after loading all the reagents, and answer the prompts on the computer to commence registration.
  - c. Load the ProSpec with calibrator. Remove the cap, and then place a bottle of N Protein Standard UY in any position on the calibrator segment (#2001). Turn the bottle so its barcode faces out through the gap in the holder.
  - d. Click on <System> <Replace Sample>. Accept the prompt, and then open the left cover of the instrument. Place the calibrator segment in any of the three locations on the sample rotor.
  - e. Close the left door and answer the computer prompts. The ProSpec is now ready for calibration.
  - f. Click on <Calibration [F5]> at the top of the screen.

- g. In the Calibration window, select test <bTP> in the Assays box.
  - h. In the Reagents box, select the appropriate reagent lot number requiring calibration.
  - i. If the reagent lot has never been calibrated, highlight the current lot number and click on the <Measure> icon on the left of the screen.
  - j. If the reagent lot has been previously calibrated, click on the <Show Curve> icon on the left of the screen. When the existing curve is displayed, click on the <Repeat> icon on the left of the screen. The ProSpec will store up to two calibrations for three reagent lot numbers. If a prompt appears asking whether a new dilution series is needed, answer "Yes". The calibration process begins automatically, and requires approximately 12 minutes.
  - k. The ProSpec prepares the serial standard dilutions required to construct a complete standard line. The instrument will automatically accept the line if it meets established limits. Print a copy of the calibration curve by following steps e, f, g, and i above. Click on the printer icon to create a hard copy. Keep this copy in the logbook along with a printout of the day's results. Also place a copy in the ProSpec Calibrations logbook.
3. If calibration is not required, or if the calibration has been successfully completed, the next step is to assay controls. Controls on the ProSpec are assayed like specimen samples:
    - a. Click on <Lab Journal [F3]> at the top of the screen.
    - b. Select <New [F7]> from left-side menu.
    - c. Key in the control (or specimen) ID in the Sample Data field. Use the <TAB> key to move between fields.
    - d. In the Assays field select <bTP> from the dropdown menu.
    - e. Select <Apply> to accept.
  4. After the controls are ordered, load the specimens onto the instrument. Before loading, clear any existing specimen assignments from all sample segments:
    - a. At top of screen select <Loading [F4]>.
    - b. In the Segment ID dropdown field select the sample segment to be cleared (010108, 010207, 010306, 610100, 610209, or 610308). Note: the labels on the sample segments do not include the final two digits.
    - c. <Double right-click> on any assigned cell.
    - d. Answer <YES> to the dialog box that appears. This will clear all specimens from this segment.
    - e. Click on <Apply [Shift+F10]> in the lower left screen corner.
    - f. Exit the Loading window by choosing <System> at the top of the screen. This step clears the IDs that were just removed from the segment from the pending test list.
    - g. Again select <Loading [F4]>.
    - h. Select the Segment ID from the drop down menu in the center of the screen.
    - i. From the ID box at screen right, left-click-hold on the desired ID. Drag the ID to the segment cell that will hold the specimen. Continue for all IDs. After all specimens are transferred or if additional segments are needed, click on <Apply>. If additional specimens are to be loaded, then select the next segment to load. Continue this process for the remaining IDs.
  5. With the loading window still on the screen, or after printing out the loading window, begin transferring the specimens to the sample segment.

6. If using sample segments 010108, 010207 or 010306 transfer 300 uL of control or specimen to a 10x75 mm disposable glass tube, then place the tube in the correct location on the sample segment. This option should be chosen if the original specimen container is too large to fit in the white tube bracket. If the specimen is in a Nunc-style tube, the ProSpec should successfully sample it if the vial is placed directly into the white bracket. Turn all sample barcodes inward so they are not detected by the instrument. These sample segments have their background barcode blocked by a piece of paper so that the instrument will not detect the barcode through the glass tube, and interpret that as a missing specimen. Do not remove this paper.
7. If using sample segments 610100, 610209 or 610308 place a 2 mL Sarstedt vial directly into the rack. If the specimen was originally provided in this type of vial place it directly into the rack and turn any barcode labels inward so they are not read by the instrument. Keep the screw caps in order so they can be re-used on the correct vials at the end of the run. If the specimen was provided in a different type of container, the specimen may be transferred to an empty Sarstedt vial for testing as with the 10 x 75 mm glass tubes in step 6. In either case, the volume of specimen in the Sarstedt vial must not exceed 1 mL as this causes sampling interruption on the ProSpec.
8. After all of the specimens and controls have been loaded onto sample segments, place the segments onto the sample rotor. Access must be requested by selecting <System>, then <Replace Samples>. Answer <OK> to the prompt, and then wait for access clearance.
9. Lift the left door, and place the sample segments into the rotor brackets. The segments may be loaded in any sequence, but it is preferable to place the first ordered sample rack in position 1, the second in position 2, etc. Close the left door. When prompted, click on <Close [Shift+F12]>. Answer <OK> at the next prompt.
10. The ProSpec now automatically begins sampling, diluting and measuring. There is no "Start" button on the instrument. The System screen will show the amount of time remaining in the programmed run, and it will alert the operator to any error messages. Common problems and their solutions are found in the Instruction Manual, Chapter 14.
11. The run is complete when the instrument functions have ceased, and the Walk Away Time on the System screen reads 0 minutes. Remove specimens from the instrument by requesting access to the sample rotor: <System>, <Replace Samples>. Answer the prompts as above when loading, then remove the segments and close the door. Before discarding the secondary tubes into a biohazard waste container, review results as described in the next section. Re-cap all primary vials, and discard/store/inventory as defined in specific study protocols.
12. Before preparing another batch of specimens, check reagent volume, detergent level, and dilution cup and cuvette availability on the instrument. Replenish or replace, as necessary.
13. There is no shutdown procedure for the ProSpec. Remove all completed specimens, exhausted reagent bottles and used dilution cups and reaction cuvettes at the end of the day.

### **Results/Expected Values:**

1. Results will not print out automatically. At the conclusion of the run, review the results by selecting <Lab Journal [F3]>. Results may be printed from this screen by highlighting the results to print, then selecting the Print icon.
2. The measurable reporting on a routinely (1:100 in N Diluent) diluted specimen is 0.25 – 16.00 mg/L. This range will vary slightly depending upon calibrator lot number. Results exceeding the upper concentration limit for a given dilution will be automatically diluted and repeated by the ProSpec. Results reading less than the lowest limit will print out as such.
3. Results are reported to three decimal places in mg/L. A serum or plasma result should not typically read less than the lowest value of the technical range. If this occurs investigate the sample for excessive lipemia, clots, incorrect specimen type, dilution problem or anything other type of situation that could yield a sub-optimal reaction. If the specimen is very lipemic, remove the lipids by high-speed centrifugation, and re-assay the clear infranate.
4. The BTP value typically mirrors creatinine level. Extreme results may be confirmed by checking a creatinine value on the Roche ModP.
5. Since it is not an FDA-approved test, Siemens does not provide a reference range for BTP. An alternative source (see reference 3) provides the following reference range based upon a survey of 100 healthy males and 100 healthy females: 0.402 – 0.738 mg/L. The manufacturer's serum reference range was compared to results from 8,766 NHANES participant samples in 2009 and 2010 (see Appendix B). A range of 0.318 – 1.370 mg/L representing the central 95% of the second examination of the NHANES III samples was determined nonparametrically. NHANES is a population-based sample of the US population and no effort was made to exclude those with renal or other disease conditions. Hence, it is not surprising the 97.5 percentile of the NHANES population is substantially higher than reported by the manufacturer for "health subjects". Participant age, gender and ethnicity of the NHANES samples are not available to us.
6. BTP does not have a test code in Misys. All reporting is done through study-specific spreadsheets or websites.
7. Analytical Measurement Range: 0.25-16.00 mg/L
8. Clinically Reportable Range: 0.10-80.00 mg/L

### **References:**

1. Siemens BN ProSpec Nephelometer Instruction Manual. Siemens Diagnostics GmbH, Postbox 1149, D35001 Marburg 1, Germany.
2. Siemens N Latex BTP reagent package insert. Siemens Healthcare Diagnostics, Newark, DE 19714, U.S.A. March 2000

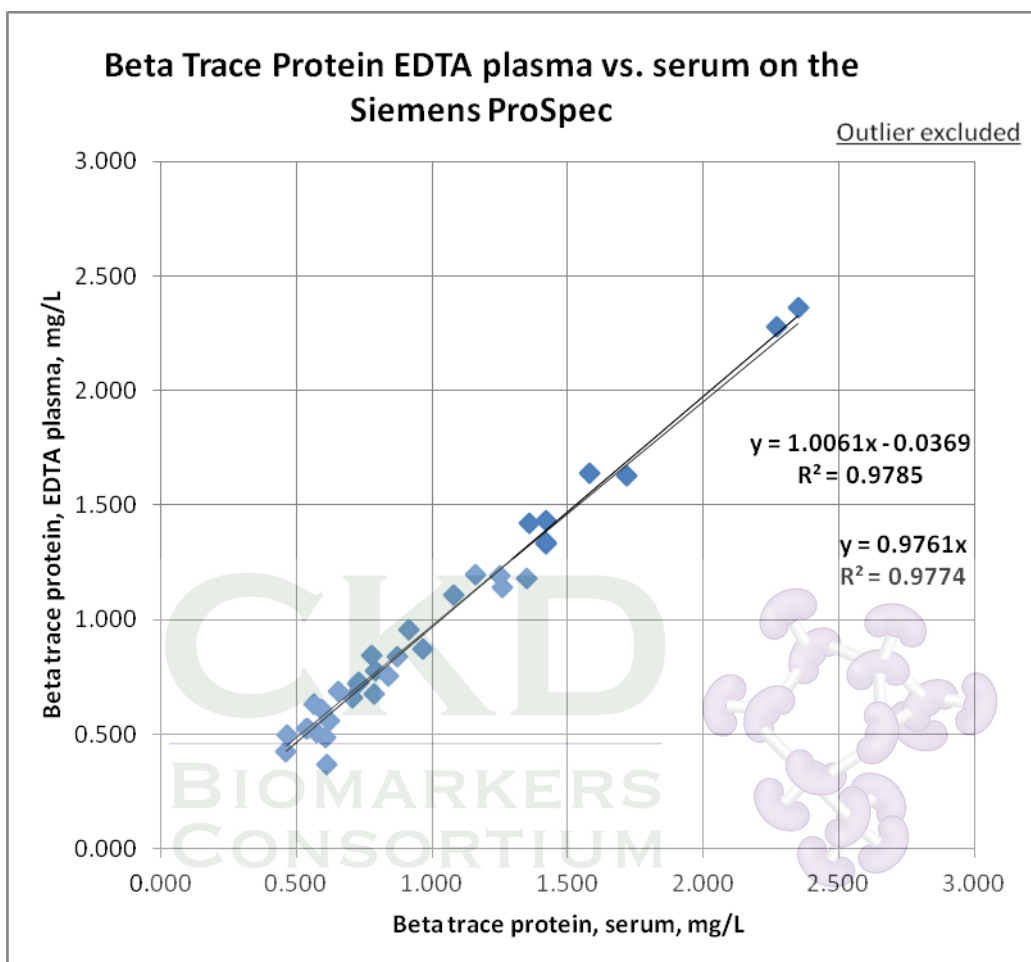


3. “Serum levels of beta-trace protein and its association to diuresis in haemodialysis patients”. Gerhard T, Poge U, Stoffel-Wagner B, Klein B, Klehr HU, Sauerbruch T, Woitas RP. Nephrol Dial Transplant (2007) 1 of 6.

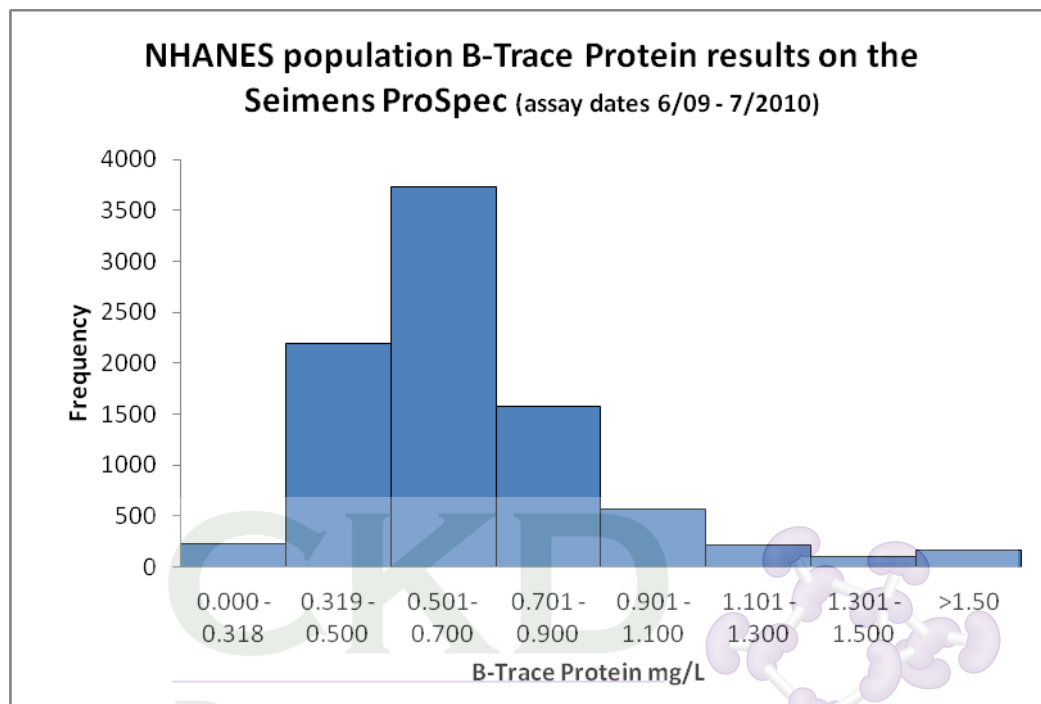
<b>External Ref:</b>	Siemens BN ProSpec Nephelometer Instruction Manual. Siemens Diagnostics GmbH, Postbox 1149, D35001 Marburg 1, Germany. Siemens N Latex BTP reagent package insert. Siemens Healthcare Diagnostics, Newark, DE 19714, U.S.A. March 2000 “Serum levels of beta-trace protein and its association to diuresis in haemodialysis patients”. Gerhard T, Poge U, Stoffel-Wagner B, Klein B, Klehr HU, Sauerbruch T, Woitas RP. Nephrol Dial Transplant (2007) 1 of 6.
<b>Internal Ref:</b>	
<b>Source:</b>	
<b>Date Written:</b>	2/10
<b>Approved by:</b>	6/3/10 G Rynders, Medical Director 6/3/10
<b>Approved by:</b>	Anthony Killeen, MD, PhD; Medical Director
<b>Date Effective:</b>	
<b>Date Revised:</b>	5/11L Barth, 11/2012 J. Peters
<b>Date Reviewed:</b>	11/2012 J. Peters



## Appendix A. Comparison of beta trace protein measurements from EDTA plasma vs. serum on the Siemens ProSpec 6/2011



## Appendix B. Reference Range Validation



### NHANES BTP

Mean =	0.6722
Standard deviation =	0.5975
N =	8766
2.5 %ile =	0.318
97.5 %ile =	1.370

Manufacturer's suggested reference range = 0.40 – 0.74 mg/L

UL: Core Proteomics Laboratory

**Title: human plasma bradykinin LCMS-based MRM assay**

**Reference: human plasma BK-MRM SOP-02**

**Version Number: 1.1**

**Effective Date: January 31, 2013**

**Review Date:**

Author	Michael L. Merchant, PhD	Reviewed by		Approved by	
Title	Associate Professor, Medicine	Title		Title	
Signature		Signature		Signature	
Date		Date		Date	

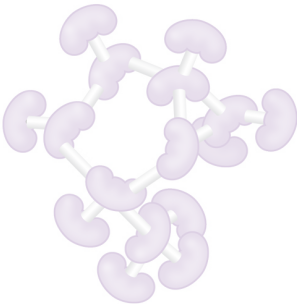
Document History			
Date	Comment	Reviewer	Signature

Reference: **human plasma BK SOP-05**

Version: 1.5

Author: Michael L. Merchant, PhD

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## Purpose

This Standard Operating Procedure (SOP) describes the process for the analysis of bradykinin, hydroxylated bradykinin, des-Arg9-bradykinin and des-Arg9-hydroxylated bradykinin analogues in human EDTA-plasma. This SOP for quantification of these kinins has not been validated for citrated-, heparinized-, guanidine thiocyanate-trifluoroacetic acid- (GTC-TFA), or sodium fluoride-plasma or for serum.

## Introduction

Bradykinin (BK) is a peptide hormone with the sequence, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (RPPGFSPFR). This peptide released by hydrolysis from the parent protein kininogen by the action of proteases, primarily kallikrein(s), and degraded by numerous proteases including carboxylprolylpeptidases such as the angiotensin converting enzyme I (ACE). Circulating BK levels have been documented to be altered in chronic inflammatory states. In addition to the unmodified peptide, other endogenous modified forms of BK include proteolytic fragments such as des-Arg-BK (or BK1-8), prolyl hydroxylated forms primarily hydroxylation of the third proline (BK3), and combinations of the two.

## Procedure

**Background:** Plasma concentrations of BK and modified BKs will be determined using a LCMS approach incorporating a selective reaction ion monitoring-mass spectrometry (SRM) step.

### Sample requirements:

- A. **All Samples are maintained is storage at -80 C until use for analysis. Samples are thawed on ice until processed for analysis.**
- B. **EDTA plasma.** The current kinin workflow has been used EDTA plasma. This kinin specific workflow has not been validated for citrated-, heparinized-, guanidine thiocyanate-trifluoroacetic acid- (GTC-TFA)-, or sodium fluoride-plasma or for serum.
  - a. Work to evaluate the adaptation of this SOP to serum bradykinin measurements is on-going
  - b. The effects of sample collection, sample processing and storage conditions have not been established. These effects are expected to be highly dependent on the methods incorporated into the various protocols that have been approved and adopted by the various NIH studies and tertiary collection sites. It is impossible to predict all the methods that have been applied for plasma or serum collection. However, the effects of sample collection/processing/storage conditions will be determined by spike in study protocols per current research design for EDTA-, citrate- and serum samples.

**Method summary for peptide isolation:** Peptides from patient samples will be isolated, yield estimated using a peptide compatible protein assay, and analyzed by LC-MS<sup>n</sup> methods. Prior to peptide isolation standard peptide(s) will be spiked into the samples. Samples will be assayed using a Bicinchoninic Acid (BCA) Protein Assay (μBCA Protein Assay, Thermo Scientific #23235) or equivalent protein assay to estimate yield of recovered peptide. Samples will be diluted using 2% acetonitrile/0.5% formic acid and analyzed by LC-MS<sup>n</sup> methods.

Reference: **human plasma BK SOP-05**

Version: 1.5

Author: Michael L. Merchant, PhD

### Detailed procedure for peptide isolation:

#### A. Isolation of plasma peptides by ultrafiltration:

- a. Add 380µL 18MΩ water to 1.5mL vial(s).
- b. Add 10µL internal standard (IS) at concentration of 5pmol/µL. (IS = a synthetic nonapeptide based on a scrambled BK sequence).
- c. Add 100µL EDTA-plasma, mix, transfer to vial with 0.45µm filter, centrifuge at 8K rpm for 1min.
- d. Add 2.5mL 0.1% formic acid solution to 15mL Falcon tube. Transfer filtrate from previous step into the 2.5mL 0.1% formic acid solution in the 15mL Falcon tube.
- e. Transfer maximally volume of diluted, filtered EDTA-plasma sample into an Amicon Ultra-4 (5,000 NMWCO) (Catalogue# UFC801024) device.
- f. Centrifuge at 3200g, 4° C for 20 minutes in a clinical centrifuge to concentrate liquid a final volume of 0.15-0.2mL.
- g. The non-retained fraction (also referred to as the ultrafiltrate) is removed from the lower chamber, 0.5mL aliquots placed into 1.5mL low protein binding Eppendorf tubes.
- h. Keep tubes for immediate use on ice. All tubes not for immediate use label appropriately and store the peptide samples at -80°.
- i. Determine peptide content using µBCA Protein Assay (Thermo Scientific #23235); see method at end of this document.

#### B. Isolation of plasma peptides by precipitation:

- a. Transfer 10µL EDTA-plasma into a 0.5µL low protein binding Eppendorf tube and place tube on ice.
- b. While tube is on ice add 10µL of 10% acetic acid to the EDTA-plasma.
- c. While tube is on ice add 10µL of IS solution.
- d. While tube is on ice add 88µL of 99.9% acetonitrile/0.1% trifluoroacetic acid.
- e. Lightly vortex for 30 sec then centrifuge at 15,000 rcg for 15 min.
- f. Draw off supernatant (approx. 100µl), lyophilize supernatant for 1.5 hrs.
- g. Re-suspend in sample in 0.1% formic acid solution.
- h. Aliquot 0.5mL volumes of sample into 1.5mL low protein binding Eppendorf tubes.
- i. Keep tubes for immediate use on ice. All tubes not for immediate use label appropriately and store the peptide samples at -80°.
- j. Determine peptide content using µBCA Protein Assay (Thermo Scientific #23235); see method at end of this document.



### C. LCMS analysis of sample for kinin peptides.

**LCMS data collection and analysis method summary:** 0.5µg total peptide per 10uL injection will be separated using a Waters nanoAcuity UHPLC fitted with a trap column and nanoLC column. The bound peptide fraction will be eluted over a 22min 2.5% to 80% acetonitrile gradient in 0.1% formic acid/18MΩ water into a Thermo LTQ-Orbi-XL mass spectrometer.

An extracted ion mass chromatogram (XIC) for bradykinin and other kinins including novel kinins such as 3,7-bis hydroxyprolyl-bradykinin (BK3,7) will be generated using the Xcaliber software package (Thermo-Fisher Scientific) and the doubly-charged peptide ions (bradykinin, MH+2 = 530.9; 3,7-bis hydroxyprolyl-bradykinin, MH+2 = 546.9) will be selected for use in subsequent quantitative analysis experiments. The dominant SRM transition for BK has been identified as a des-Arg fragment ion of the parent peptides that for BK and BK3,7 will be followed as:

- a. BK, m/z 530.9 → 904.20
- b. 3,7-bis hydroxyprolyl-bradykinin, m/z 546.9 → m/z 936.2

For construction of the external standard curve, kinin concentrations ranging between 1 fmol and 1 nmol (n=5 per concentration) of the natural abundance peptides will be prepared in PBS and processed as described previously. Three standard curves will be prepared and analyzed by RPLC-nanosprayESI-MS/MS. The SRM peak area for the natural abundance isotope will be plotted versus the peptide concentration and data will be fit to a linear equation. A regression analysis will be performed for BK and 3,7-bis hydroxyprolyl-BK to determine the lower limits of detection and quantification. Standard curves will be run monthly. Replicate data point CV should be less than 20%.

**Note:** At this time the methods are being used that incorporate an internal standard (IS) peptide to facilitate normalization of run to run bradykinin data. That peptide is added to the sample as early as possible in the sample handling at a level of 5pmol. At this time that peptide consists of a 9-amino acid peptide containing the same number and type of amino acids as bradykinin but present in a different order. That peptide is referred to as scrambled BK.

**Results Reporting Summary:** Results will be reported as a normalized, absolute value representing the ratio of area of the target to the IS peptide. An calculated concentration will be based on external calibration curves of known and compared to reference ranges published literature values (Duncan AM et al. Kinins in humans, *Am J Physiol Regul Integr Comp Physiol* **278**:R897-R904, 2000) for bradykinin and other analogues.

## Location

Baxter I, Room 207A

University of Louisville, School of Medicine, Department of Medicine, Division of Nephrology

## Required equipment / reagents (as specific or equivalent)

- a. Centrifuges- clinical and microcentrifuge.  
Note- centrifuges are maintained on an as need basis and are not routinely calibrated
- b. Centrifugal filtration devices (3,000 Da nominal molecular weight cut-off, NMWCO)
- c. Vortex  
Note- vortexers are maintained on an as need basis and are not routinely calibrated
- d. Standard and multichannel pipettes  
Note- pipettes are serviced every 6-months on site using an commercial provider of calibration services.
- e. LCMS system- calibrated onsite per manufacturer's guidelines for flow calibration (UHPLC) and mass calibration (TSQ).

### Configuration 1

- i. HPLC- Waters nanoAcuity nanoLC
- ii. HPLC column- **Trap Column:** u-precolumn 300 um i.d., 5 mm, packed with C18 PepMap 100, 5 um, 100A (Thermo P/N 160454); **nanoLC separating column:** 100 um i.d., 13 cm in length, packed with Aries XB-C18, 3.6 um, 200A (phenomenex).
- iii. Source- EASY-Spray nano-ESI source
- iv. Mass Spectrometer- (1) Thermo Finnigan LTQ-Orbitrap XL

### Alternate Configuration 2

- v. HPLC- autosampler-Proxeon EASY-nLC 1000
- vi. UHPLC column- **Dionex Trap Column:** u-precolumn 300 um i.d., 5 mm, packed with C18 PepMap 100, 5 um, 100A (Thermo P/N 160454); **Dionex Separating column:** Acclaim® PepMap™ RSLC column 50µm I.D. C18
- vii. Source- EASY-Spray nano-ESI source
- viii. Mass Spectrometer- Quantum Discovery Max triple quadrupole mass spectrometer

## Standards

BK (light and heavy) standard synthetic peptides are used where the internal standard heavy isotope labeled amino acid is either the first or last arginine. These peptides will be sequence validated by MALDI-TOF MS/MS methods and purity/composition analysis by elemental analysis.

**Note:** At this time the methods are being used that incorporate an internal standard (IS) peptide to facilitate normalization of run to run bradykinin data. That peptide is added to the sample as early as possible in the sample handling at a level of 5pmol. At this time that peptide consists of a 9-amino acid peptide containing the same number and type of amino acids as bradykinin but present in a different order. That peptide is referred to as scrambled BK.

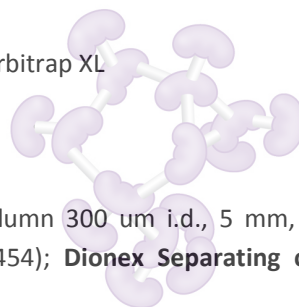
**Note:** Analytic sensitivity is dependent on LCMS analysis platform. The selected platform for plasma kinin analysis has not been finalized. Once finalized the analytic sensitivity will be reported for the lowest limit of replicate detection while maintaining a CV of less than 20% for that concentration.

Reference: **human plasma BK SOP-05**

Version: 1.5

Author: Michael L. Merchant, PhD

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### Buffers-

LC solvent A- 5% acetonitrile/0.05% formic acid

LC solvent B- 80% acetonitrile/0.05% formic acid

Buffer preparation (prepared weekly, filtered and stored at RT)-

LC Solvents	
Buffer	Components
LC buffer "A".	5% acetonitrile/0.05% formic acid
	50mL LCMS grade acetonitrile 500uL formic acid Queue to 1000mL with LCMS grade water
LC buffer "B".	800mL LCMS grade acetonitrile 500uL formic acid Queue to 1000mL with LCMS grade water

### Experimental Procedure Data

#### Sample handling

Clinical samples are thawed on ice. Vortex the tubes for 5 sec, centrifuge at 4°C, and kept on ice and proceed for the analysis.

#### Prepare Kinin standards for configuration 1

Prepare appropriate QC standards:

Example: 1pmol/μL combined bradykinin (scrambled BK + BK + BK3+BK7+BK3,7) in buffer A.

Note: Target concentration for infusion- 0.01-10 nM or 0.01 - 1E-9 mol/L.

Target volume for sample preparation = 10mL or 0.01L or 1E-2 L

Mass needed = (1E-2 L) (1E-8 mol/L) (x) where x = molecular mass

BK	x=1060.56	(1E-2)(1E-8)(1.06E+3)= 1.06E-7 grams or 0.106 ug
BK H3	x=1076.56	(1E-2)(1E-8)(1.08E+3)= 1.08E-7 grams or 0.108 ug
BK H7	x=1076.56	(1E-2)(1E-8)(1.08E+3)= 1.08E-7 grams or 0.108 ug
BK H3,7	x=1092.55	(1E-2)(1E-8)(1.09E+3)= 1.09E-7 grams or 0.109 ug
Scrambled BK	x=1060.56	(1E-2)(1E-8)(1.06E+3)= 1.06E-7 grams or 0.106 ug

Stock solutions are adjusted to:

[BK]	0.106μg/μL and 230μL
[BK H3]	0.108μg/μL and 230μL
[BK H7]	5.0μg/μL and 40μL
[BK H3,7]	5.0μg/μL and 40μL

Therefore a 10nM concentration of BK or BK H3 requires 1ul of stock dissolved into 10mL.

For BK H7 and BK H3,7 requires a dilution from 5 ug/ul down to the appropriate concentration.

Reference: **human plasma BK SOP-05**

Version: 1.5

Author: Michael L. Merchant, PhD

### Methodology for Configuration 1

**Chromatography:** Chromatographic separation of kinin peptides using 13 cm in length, packed with Aries XB-C18, 3.6  $\mu$ m, 200A (phenomenex) and a Waters nanoAcuity HPLC.

**Solvent A:** 0.1% formic acid in water (LC-MS Grade from Sigma); **Solvent B:** 0.1% formic acid in acetonitrile (LC-MS Grade from Sigma)

**Trapping:** 100% Solvent A at 4  $\mu$ L/min for 4 min.

LC Gradient as in table below			
Time (min)	Flow ( $\mu$ L/min)	A (%)	B (%)
0	0.2	97.5	2.5
15	0.2	50	50
17	0.3	20	80
22	0.3	20	80
24	0.3	97.5	2.5
35	0.2	97.5	2.5

### MRM parameters for targeted peptide LCMS analysis:

- Isolation width: 3Da
- Collision Energy: 35
- Activation Q: 0.25
- Activation Time: 30msec

Transition specific parameters for detection and quantification of kinin peptides using LCMS configuration 1.						
Duration (min)	Peptide	Precursor (m/Z)	Z	Fragment (m/Z)	Z	Rt (min)
5-16.05	BK-3,7	546.78	2	936.42	1	14.1
	BK-3	538.79	2	920.42	1	15.0
	BK-S (I.S.)	354.19	3	369.22	1	15.2
	BK	530.77	2	904.47	1	15.4
	Des-Arg9-BK-3,7	468.73	2	658.33	1	15.5
16.05-25	Des-Arg9-BK3	460.74	2	658.33	1	16.4
	Des-Arg9-BK	452.74	2	642.34	1	16.7

Reference: **human plasma BK SOP-05**

Version: 1.5

Author: Michael L. Merchant, PhD

### 3.5 Quality Control

#### Quality controls:

- a. Samples: Prior to shipment and analysis samples are de-identified and blinded replicates are interwoven to aid in determining technical precision.
- b. Preparation: Prior to analysis, samples are randomized during sample handling and in ordering for sample analysis to minimize the effects of systematic changes in instrumentation or reagent preparation.
- c. Accuracy and acceptance of results: Calibration curves will be established for internal standards prior to the initiation of sample analysis. All measurements will be conducted with technical replicates (n=5). Full external calibration curves for standards will be repeated every two weeks. Single external calibration points (concentrations selected from the mid-point value of the external calibration curve) will be interwoven into each assembled series of samples at the 25%, 50%, and 75% as well as after completion of the last study sample. The estimated standards concentration in comparison with the known amount will be used to measure the accuracy of the method at estimating analyte concentrations. Acceptable CV for the difference between the estimated and known analyte concentrations are 80%-120% of the known concentration.
- d. All the samples and standards are run in with a technical replicate.
- e. Samples with an internal standard variation of more than 20% CV are repeated.

#### Additional quality control:

- a. Two external specimen control pools will be created (one high and one low concentration QC sample) to monitor the performance of the entire analytical process including:
  - a. isolation of peptide
  - b. BCA assay
  - c. sample injection into the mass spectrometer,
  - d. measurement of bradykinin derivative by LC MS/MS.
- b. These QC sample(s) will be obtained from a large volume of EDTA plasma with bradykinin levels level determined by spiking plasma with a known concentration of bradykinin.
- c. The specimens will be aliquoted (n>1000) and stored at -80C.
- d. These aliquots will be used to
  - a. evaluate total test precision
  - b. establish acceptable performance range for QC samples
  - c. run routine QC on the day of each experimental assay.

### μBCA Protein Assay (Thermo Scientific #23235)

Dissolve a BSA standard (BioRad #500-0007) with 18MΩ·cm H<sub>2</sub>O to give a 9.8mg/mL stock and aliquot into 60μL volumes. Store the aliquots at –70°C. Create the standard curve concentrations as follows:

	<u>Volume 9.8mg/mL stock</u>	<u>18MΩ·cm H<sub>2</sub>O</u>
9.8mg/mL BSA	20μL	0μL
7.0mg/mL BSA	14.29μL	5.71μL
5.6mg/mL BSA	11.43μL	8.57μL
2.8mg/mL BSA	5.71μL	14.29μL
1.4mg/mL BSA	2.86μL	17.14μL
1.0mg/mL BSA	2.04μL	17.96μL
0.5mg/mL BSA	2.04μL	37.96μL
0mg/mL BSA	0μL	20μL

In the protocol we follow for the μBCA assay, we dilute 1μL sample or standard into 149μL 18MΩ·cm H<sub>2</sub>O. We make duplicates of each standard and triplicates of each sample. For a broader range standard curve, 19.6mg/mL and 29.4mg/mL standard points may be added. To get these concentrations, use 2μL of the 9.8mg/mL standard (dilute into 148μL 18MΩ·cm H<sub>2</sub>O) to get a final concentration of 19.6mg/mL and 3μL of the 9.8mg/mL standard (dilute into 147μL 18MΩ·cm H<sub>2</sub>O) to get a final concentration of 29.4mg/mL.

Prepare the μBCA working reagent; the working reagent is composed of 50 parts reagent A, 48 parts reagent B, and 2 parts reagent C. Add 150μL of the μBCA working reagent to each standard and sample and mix with a multichannel pipettor (draw and expel working reagent several times into diluted sample). After adding the reagent to all wells, cover plate with plastic adhesive film. Incubate for 2 hours at 37°C. Allow plate to cool to room temperature for 10min while setting up spectrophotometer. Invert the plate immediately before reading to wet the film. Read absorbance at 562nm on the spectrophotometer. A quadratic equation fits well through all standard points.

**Note:** Careful pipetting is critical. Use the same pipettor for the standard curve dilution series if possible, and standardize the way the volumes of liquid are drawn and dispensed (for example, always draw from the surface of the standard or sample, and always wash the tip by pipetting up and down when adding the standard or sample to the water).



## **Bottinger Lab: Development of modified SOP for detection of iC3b in human urine adapting Quidel iC3b Sandwich ELISA kit.**

### **Sandwich ELISA kit for detection of iC3b in human urine. (Edited by Dr. Bonventre's lab)**

#### **1. Introduction**

##### **1.1 Background of the method**

As described in the kit instructions the activities of Complement components are limited by cleavage by specific proteases in order to prevent collateral damage to self cells. In the case of complement component C3 cleavage produces two active molecules iC3b and C3b with altered activity from their precursor. Cleavage of C3b gives rise to "inactive" C3b or iC3b which in turn has its own contribution to innate immunity. This ELISA kit uses immobilized anti-human iC3b antibody coated wells of a 96-well plate to capture iC3b from human bodily fluids. The trapped iC3b is subsequently detected with horseradish peroxidase-conjugated (Goat) antibody to another iC3b epitope. NOTE: The manufacturer's manual is only validated for detection of iC3b in human plasma or serum! Significant modifications were developed by the Bottinger Lab to achieve satisfactory assay performance for iC3b detection in human urine

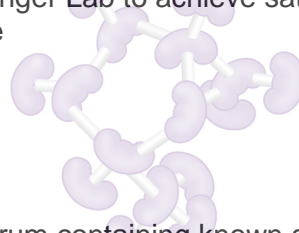
##### **1.2 Reagents and consumables.**

###### **1.2.1 Supplied by Quidel in the kit**

- iC3b standards are supplied as diluted human serum containing known amounts of iC3b in PBS, protein stabilizers, 0.01%Thimerosal. Standard concentrations are listed below BUT can vary therefore concentrations must be checked against the Certificate of Analysis found in each box
  - C = 1.29 µg/ml
  - B = 0.59
  - A = 0.14
- ELISA plate coated with a mouse anti-human iC3b monoclonal antibody.
- Hydrating reagent for solubilization of standards: 0.035% ProClin® 300.
- Peroxidase-conjugated (Goat) anti-human iC3b in PBS, stabilizers and 0.01% Thimerosal.
- Substrate Diluent: 0.1 M citrate buffer and 0.05% peroxide.
- Substrate contains 0.7% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).
- 20 x Wash Solution concentrate (1 x PBS 0.05% Tween-20, 0.01% Thimerosal)
- Stop Solution (250mM oxalic acid)

###### **1.2.2 User supplied**

- Spectrophotometer (Molecular Devices SpectraMax Plus 384)
- Refrigerated Centrifuge (Sorvall Benchtop swing bucket centrifuge, Rotor RTH-250)



CKD  
BIOMARKERS  
CONSORTIUM

- Vortexer
- Electronic Multichannel pipettes
  - E4 XLS multichannel pipette (dispenses 2 µl-20 µl)(Rainin, catalog # E8-20XLS)
  - EDO 3-Pls multichannel pipette (dispenses 100 µl-1200 µl) (Rainin, catalog # E8-1200)
- Single Channel Manual Pipettes
  - Rainin Classic 0.5 µl-10 µl (catalog # PR-10)
  - Rainin Classic 10 µl-100 µl (catalog # PR-100)
  - Rainin Classic 100 µl-1000 µl (catalog # PR-1000)
- 50 ml reagent reservoir (Fisher Scientific)

**1.3 Plate washing**

- Add about 200 µl of PBS or Wash Solution (as required) to each well with a 10 ml pipette by slowly dispensing in a constant stream and moving the pipette from well to well to fill each well of the plate.
- For the first wash of a set let the plate stand for two minutes before proceeding.
- Immediately remove the solution by tipping the plate upside down with a throwing motion to expel the solution into a basin
- Tap the plate upside down on paper towel to remove remaining solution.

**2. ELISA Assay**

**2.1 Sample handling**

- Sample tubes will be arranged in open tube racks with an empty space between each tube for better air circulation. Place these racks in 30°C incubator until all samples are thawed (The tubes will be still cold after the samples are thawed). Vortex the tubes for 5 sec, centrifuge at 4°C 3000 RPM using RTH-250 rotor on Sorvall Bench top Centrifuge, and keep on ice and proceed for the analysis.

**2.1.1: Stability of iC3b:** Stability of iC3b was determined in samples with low, intermediate and high concentrations of iC3b. The freeze thaw stability of the analyte was evaluated by freeze thawing one time ( Comparison against “ sample conc” and “ 0 hr conc). Further, the stability of the analyte was evaluated by incubating the same at room temperature for 8 hr and 24 hr. As outlined in the table below, iC3b is stable with single freeze thaw cycle and incubation at room temperature for **24 hrs**


**2.2 Standard curve with protein standards and variation from manufacturer’s instructions**

- The standards are supplied by the manufacturer in lyophilized form and are solubilized in hydrating solution as per manufacturer.
- To extend the range of standards to detect lower concentrations of iC3b the lowest concentration of protein standard (0.14 µg/ml) was further diluted 1:1 in hydrating solution to give: 0.07, 0.035, 0.0175 and 0.00875 µg/ml.

### 2.3 Loading the urine analyte and standards

- Setup a template plate by adding 120 µl aliquots of undiluted urine analyte and protein standards into the wells of a Polypropylene template plate following the Template Map shown in Figure 1 below.
- As quickly and carefully as possible (following the guidelines for pipetting described above) load the ELISA plate with the multichannel pipette set at 100ul.
- Cover the fully loaded ELISA plate with an adhesive plate sealer. Secure the plate(s) on the IKA orbital shaker with clear tape on each corner and incubate at setting 7 (about 80 revolutions of the table per minute) for 1 h at room temperature (18-25°C).

**Figure 1. Template Map**

A	S1	S1	U01	U01	U09	U09	U17	U17	U25	U25	U33	U33
B	S2	S2	U02	U02	U10	U10	U18	U18	U26	U26	U34	U34
C	S3	S3	U03	U03	U11	U11	U19	U19	U27	U27	UC1	UC1
D	S4	S4	U04	U04	U12	U12	U20	U20	U28	U28	UC2	UC2
E	S5	S5	U05	U05	U13	U13	U21	U21	U29	U29	UC3	UC3
F	BL	BL	U06	U06	U14	U14	U22	U22	U30	U30	PS1	PS1
G	KC1	KC1	U07	U07	U15	U15	U23	U23	U31	U31	PS2	PS2
H	KC2	KC2	U08	U08	U16	U16	U24	U24	U32	U32	PS3	PS3

S1-S5: Standards

Un01-Un34: Unknown Samples

KC1-KC2: Controls provided by the kit

UC1-UC3: Urine Controls developed in house with low, intermediated and high levels of the analyte

PS1-PS3: Proficiency samples

### 2.3 Modified wash procedure to remove urine and unbound proteins and prevent cross-well contamination

- Aspirate and discard the urine and standards with the multichannel pipette using techniques in section 1.3 and being careful to tilt the plate to remove as much of the solution without touching below the halfway point of the well.
- Add 200 µl of Wash Solution from a pipette basin with the multichannel pipette to each well.
- Incubate the first wash for 1 min.
- Aspirate the solution with the multichannel pipette.

- Perform three further washes by the plate washing procedure in section 1.6.

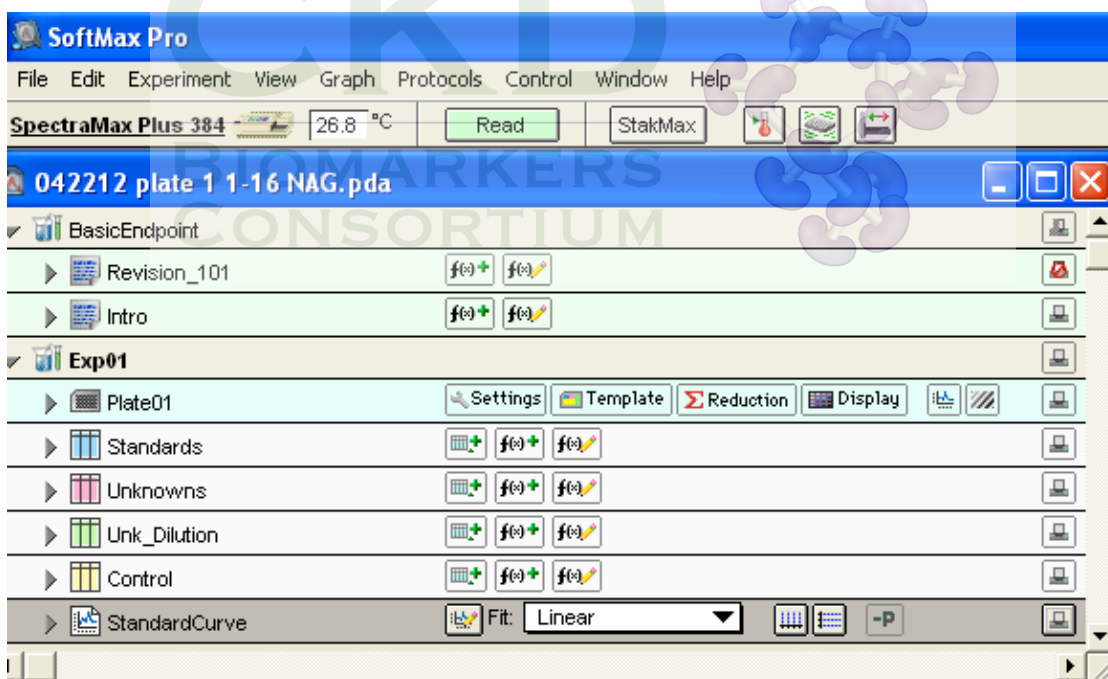
#### **2.4 Binding labeled Detection antibody to the captured protein**

- Immediately after the last wash add the contents of the tube of iC3b Conjugate (7 ml) to a pipette basin,
- Dispense 50  $\mu$ l of the iC3b conjugate in each well with the multichannel pipette.
- Incubate with shaking for 30 min at room temp with shaking.
- Wash the plate five times as per section 1.4.
- Dry the bottom of the plate with a KimWipe tissue to remove liquid, clean and polish the surface to allow light transmission in the plate reader.

#### **2.5 Prepare and add substrate and develop**

- Add 500 $\mu$ l of substrate concentrate to 10 ml of substrate diluent in a pipette basin.
- Dispense 100  $\mu$ l of the substrate into each well with the multichannel pipette.
- Incubate in a closed, empty drawer for 15 min with occasional very gentle tapping to mix the reaction components and disperse the color.
- Stop development with 100  $\mu$ l/well of Stop Solution
- Tap gently to mix the solution, disperse color and stop the HRP activity.

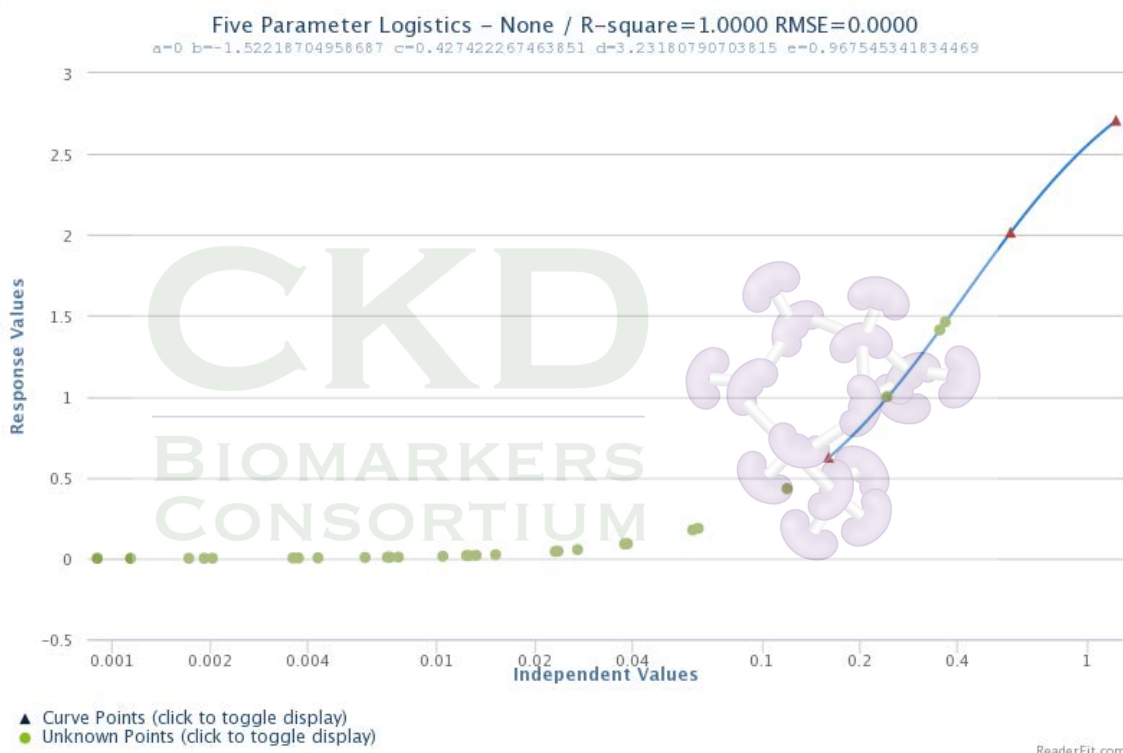
### **3. Data Generation & Processing**



#### **3.1 Data Output**

- As indicated above, the 96 well plate is placed in a Spectrophotometer and absorbance read by pressing “Read” icon in SoftMax Pro software.

- Data will be automatically generated by SoftMax Pro 5.4 software that is integrated into the instrument operating software, once the machine is done with the reading (shown below).
- The SoftMax Pro software uses serially diluted standards to generate a standard curve. The software has user-selected options to choose the type of regression analysis. We use linear regression analysis to quantitate the concentrations of unknown samples.
- For each analyte standard, a concentration is back-calculated by plotting the absorbance of the standard on the standard curve. (Below is the typical standard curve)



**Figure 2. Exemplary Standard Curve**

### 3.2 Data Processing and Data Calculations

- The SoftMax automatically generates the values of unknowns based on the standard curve. The data generated includes the absorbance, predicted concentration of unknowns, standard error, % CV, and errors in the run of all samples (below is the typical data output page).
- Data will be exported from Softmax pro to Microsoft Excel using the export file option. The program will export information from the run including Sample ID, information of the wells, absorbance of each replicate, concentration of each replicate, mean concentration of the sample, standard deviation, and % CV. Two control urine specimens are



evaluated on every plate. Three proficiency samples will be run every week.

- In addition to sample ID (Biocon ID), each tube will be numerically labeled for cross verification in the order we aliquot the samples on the plate. Once the data are transferred and compiled, we will again cross check the numerical number on the tube with the barcode scan.
- Another member in the facility will perform a secondary check to further validate the sample order.

### 3.3 Performance of the ELISA assay on urine samples.

- As the ELISA was specifically designed for measuring serum samples, we have performed additional recovery and interference studies in normal and nephrotic urine samples. The samples were spiked with various concentrations of the analyte and the assay stability was measured at different pH and dilution. As outlined in the table below, the urine pH adjustments or sample dilution didn't significantly alter measurements of high, medium or low levels of C3a.

Type of assay	Spiked Concentration	% Recovery		
		Neat Samples Average pH 6.1	Undiluted urine samples. Average adjusted pH 7.2	Diluted Urine Samples (1 in 5 with sample diluent). Average pH 7.1
iC3b Assay	4.808%	92.6±1.6%	92.3±1.5%	93.7±1.3%
	3.075%	95.1±0.9%	89.9±0.7%	92.6±1.3%
	1.242%	95.1±0.7%	91.5±1.4%	93.1±1.3%
	0.152%	96.1±0.7%	92.8±1.0%	91.1±2.0%
	0.081%	94.0±1.0%	92.5±0.9%	92.6±1.4%

- The assay was further evaluated whether recovery of spiked analytes were altered by nephrotic range proteinuria with or without uremia. As shown below, the assay was quite stable in samples collected from patients with high-grade proteinuria or advanced CKD stage V.

			% Recovery in sample		
			iC3b		
Sample #	URINE PROT.	eGFR	1:1	1:2	1:4
9038	Neg	15.2	90	88	89
50073	Trace	10.5	99	94	93
40036	nephrotic	12.1	94	94	96
70056	nephrotic	8.9	91	92	88
70067	nephrotic	106.3	87	89	88
70083	nephrotic	119.1	87	93	86

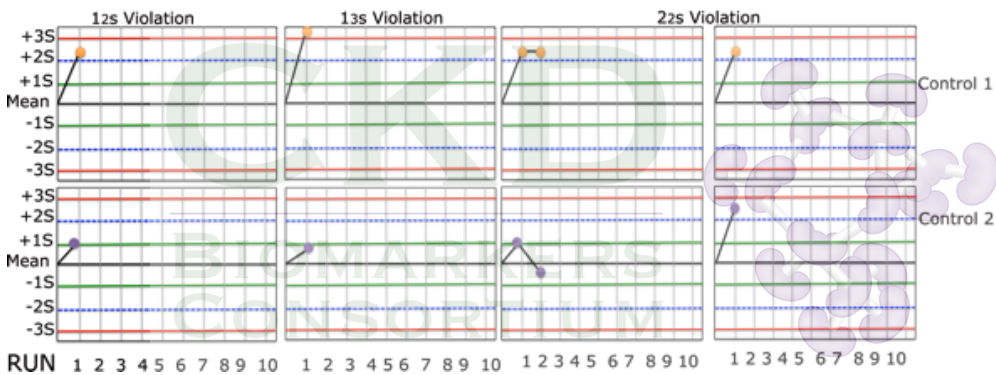
## 4. Quality Control

### 4.1. Quality control of the Assay.

- We will validate that Complement IC3b levels obtained using the assay on control specimens are within mean  $\pm$  2 SD limit established from the Validation Protocol.
- If both samples are within mean  $\pm$  2 SD limit, we will accept the data. If one of the two control values is outside mean  $\pm$  2 SD, we will employ the Westgard 2 rules to determine if the analysis run results can be accepted.
- If neither of the assayed control results is within mean  $\pm$  2 SD limits, then we will follow the following Westgard three-quality control rule.

RULE	QUALITY CONTROL RULE EXPLANATION
12s	One value of any of the two controls used in the assay is outside of the mean+2 SD or mean-2 SD of that control
13s	One value of any of the two controls used in the assay is outside of the mean+3 SD or mean-3 SD of that control
22s	Both controls used in the assay are outside of their mean $\pm$ SD on the same assay or two consecutive values of one control is outside of mean $\pm$ SD

Levey-Jenning's plots of controls:



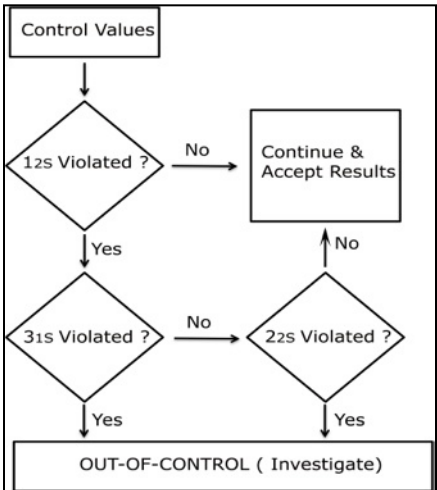
#### 4.2 Quality Control for each sample

- Sample measurements will be repeated if the %CV of the sample is  $> 15\%$
- Sample measurements will be repeated if the absorbance of the sample is below 0.020 to make sure that the low absorbance values are not due to pipetting error.

#### 4.3 Quality Control troubleshooting procedure

If the controls fail the above quality control procedures, then we will employ the following procedure to resolve the issue:

- Rerun Control
- If the quality control values pass the above described westgard rules and proceed with unknown testing
- If the quality control failed again, make up fresh reagents and rerun controls and document the problem



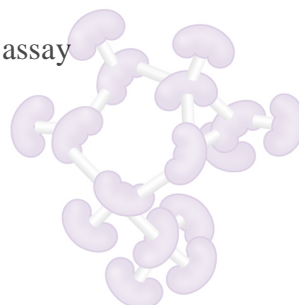
- If the quality control indicates that the westgard rules are passed, proceed with testing
- If QC fails again, recalibrate the assay (use new standard lot)
- If after calibration the QC determinations have passed westgard criteria, proceed with testing and document correction
- If Westgard criteria were not met after preparation of fresh reagents, do not proceed with the analysis and contact the Laboratory Director.

#### **4.4 Data storage and reporting**

- After the analysis has passed the quality control test, Complement IC3b values of unknown samples and their corresponding %CV and SD will be copied and pasted into a master excel data sheet in the sequential order on sheet 1 in excel workbook. COMPLEMENT IC3B values, %CV, SD of quality control samples and proficiency samples will be pasted on sheet 2. Both sheet 1 & 2 will be updated with addition of data from each run.
- The master data sheet that will go to coordinating center will contain the following information
  1. Sample ID (Bio Con ID)
  2. Date of the assay
  3. Platform and Instrument# used for the assay
  4. Reagent lot numbers
  5. % CV across the sample replicates

BIOMARKERS  
CONSOR

#### **APPENDIX:**



#### **Data processing steps used in Dr. Erwin Bottinger's laboratory**

##### **3.1 Reading the plate**

- Use the Perkin Elmer 1420 Multilabel Counter and plate reader to read the color intensity of urine and standards to determine concentrations.
- The menu options for the reader are shown in screen shots below

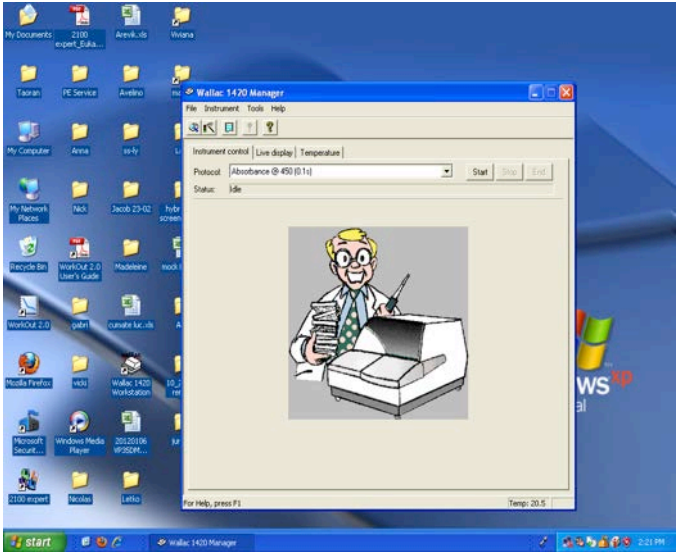


Figure 2. Find the Wallace 1420 operating software Manager. Go to Tools > Start Wizard

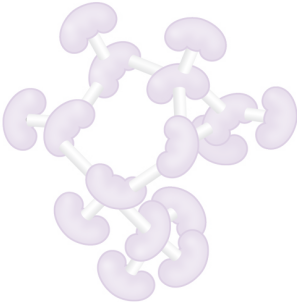
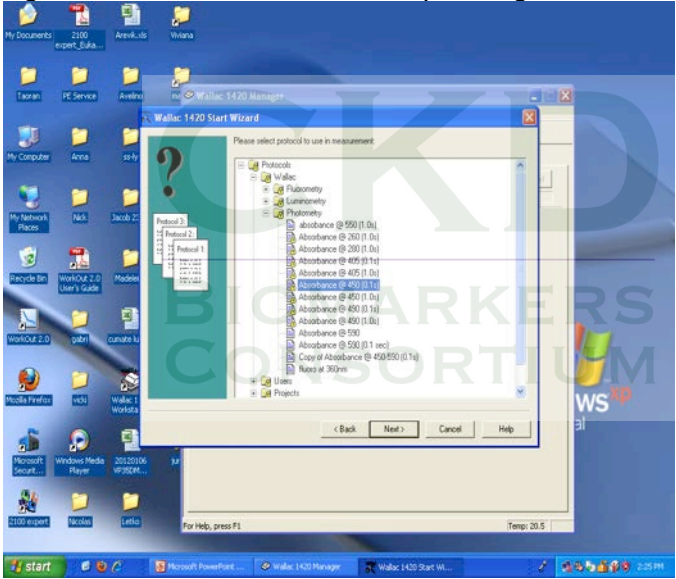


Figure 3. Find Photometry absorbance @ 405nm (0.1s). Reading time can be changed if warranted.

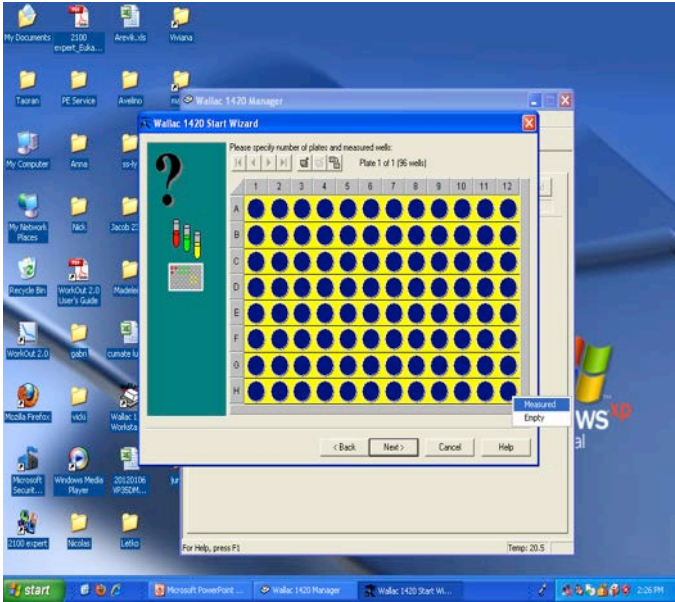


Figure 4. Highlight all wells to ensure all are read.

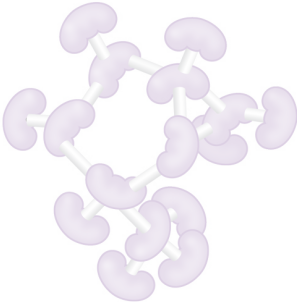
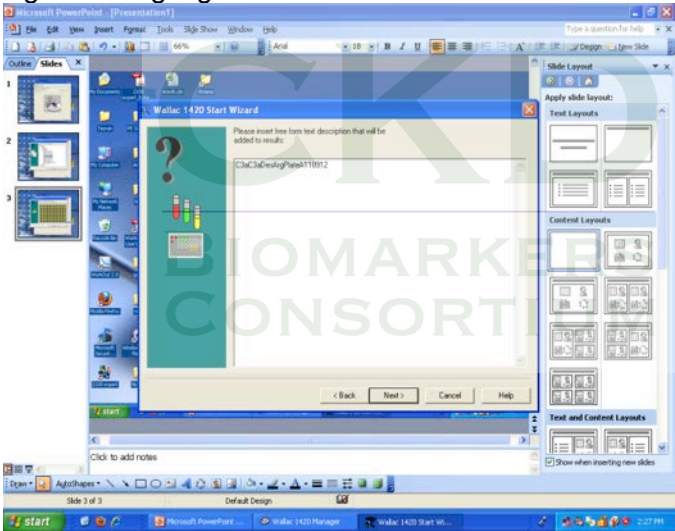


Figure 5. Can be left blank



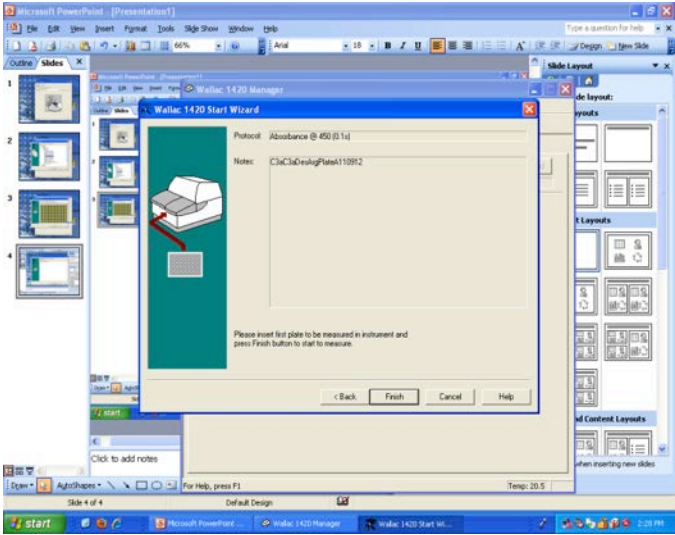


Figure 6. Click Finish to start

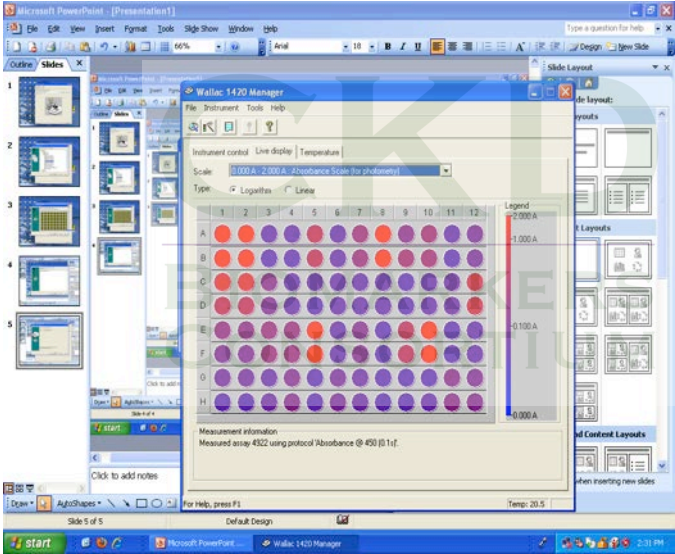
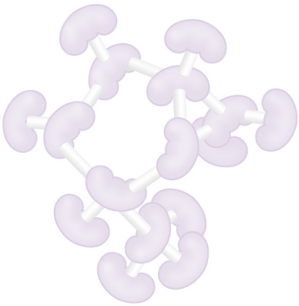


Figure 7. Results seen on the Live display tab.





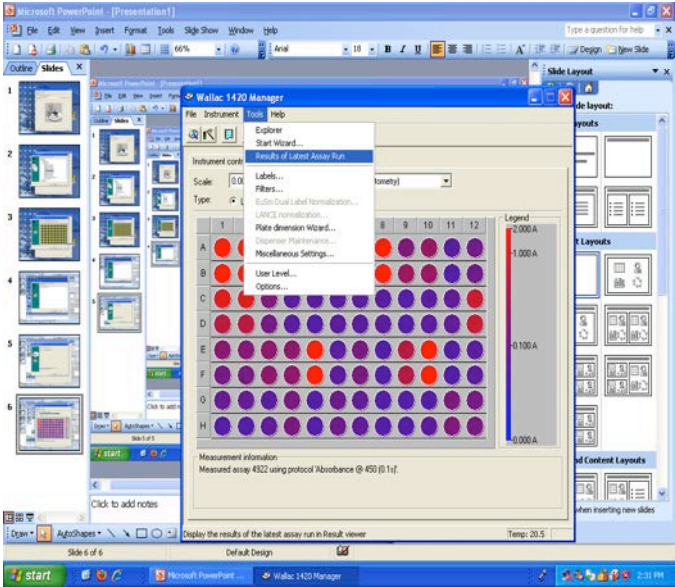


Figure 8. To retrieve the data use Tools tab and scroll to Results of Latest Assay Run

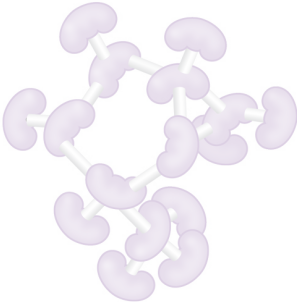
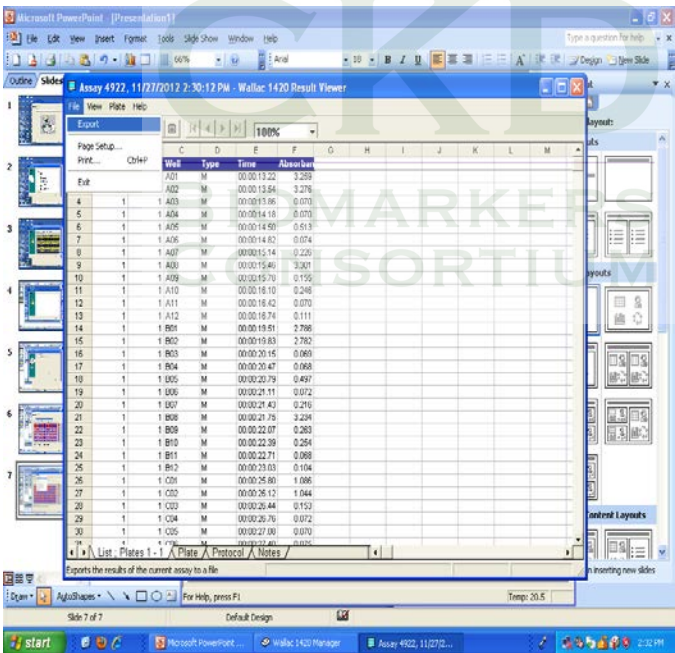


Figure 9. The data file in Excel spreadsheet format must be exported to a file for retrieval and analysis.

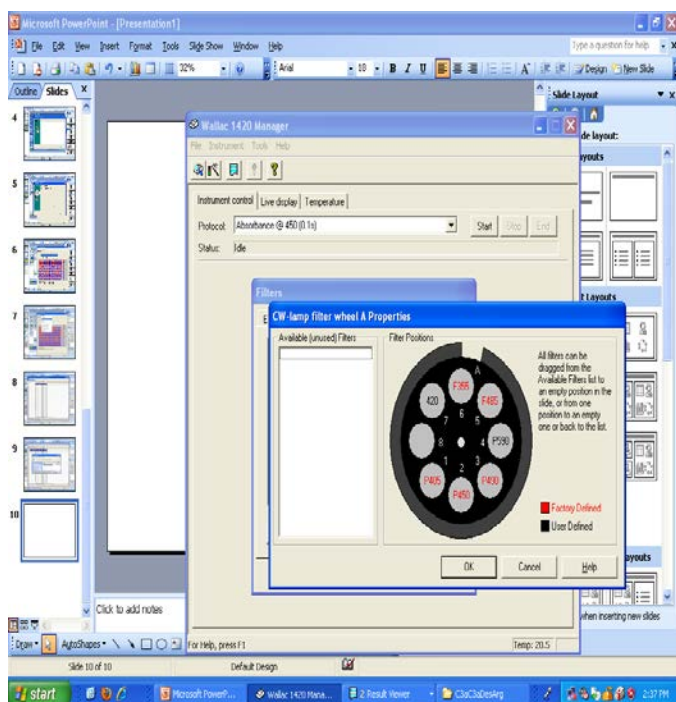


Figure 10. Confirmation that the filter for 405nm is present. For physical confirmation the wheel can be removed.

### 3.2 Preparing the Raw data for plotting and analysis

- The data is saved in Excel in the form of a table reflecting the ELISA plate.
- The data has to be prepared for plotting before concentrations can be determined.
- The process has been expedited by recording a macro of all the steps required to prepare the data.
- The macro accomplishes the following very quickly and reproducibly:
  - Absorbance values of the standards are copied from the Raw data and pasted into an unused part of the sheet (from A2 – B9 to A15 – B22) and labeled as Std 1 and Std2 respectively (Arrow 1, red).
  - The average is determined of the duplicate values and recorded in the appropriate row (C15 – C22, Arrow 3, green).
  - The average of the Blank readings is copied to the cells D15-D22 (Arrow 4, light blue) for subtraction from the average concentration absorbance values (Oval 5, purple)
  - The corrected absorbance values for the standards is copied and pasted in an unused area (G15-G22, Circle blue/black)
  - Concentration values are left open to be inserted for the particular assay
  - The urine A450 values are duplicated vertically as shown in Figure 1 and run consecutively from left to right starting at C3 and C4 and ending at L8 and L9.
  - These data are copied one double row at a time (C3 and C4 to L3 and L4) and using the Paste Special, Transpose function are transferred to B30-B40 to transpose from row to column presentation.

- Consecutive rows are added below until all the urine A450 data is in the columns B30-B70 (Arrow 2, dark blue).
- The rows of duplicate values are labeled consecutively 1 – 40 titled Urine#
- The data columns are labeled A405 1 and A405 2 respectively.
- The average is determined and recorded alongside then highlighted and Paste Special, value back in the same column and labeled “Average”.
- The Blank or background average from above is recorded and repeated alongside the urine A405 data and labeled “Background” in preparation for subtraction.
- Subtraction is done and reported in the next column as “Ave-Bckgrnd” and copied, Paste Special values i.e. only values are recorded.
- To remove subzero data the following formula is placed into the top cell of cell G30 “=IF(F30<0,0,F30)” and dragged down to G70. This leaves unchanged all positive numbers and zero but changes negative numbers to 0.
- This final column G30-G70 provides the data for plotting and concentration determination along with the Std A405 average corrected values and the concentrations which have to be entered retrospectively into the worksheet.

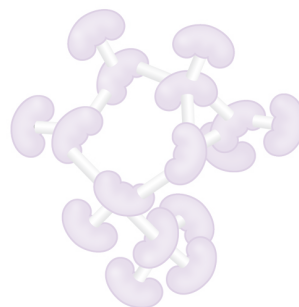
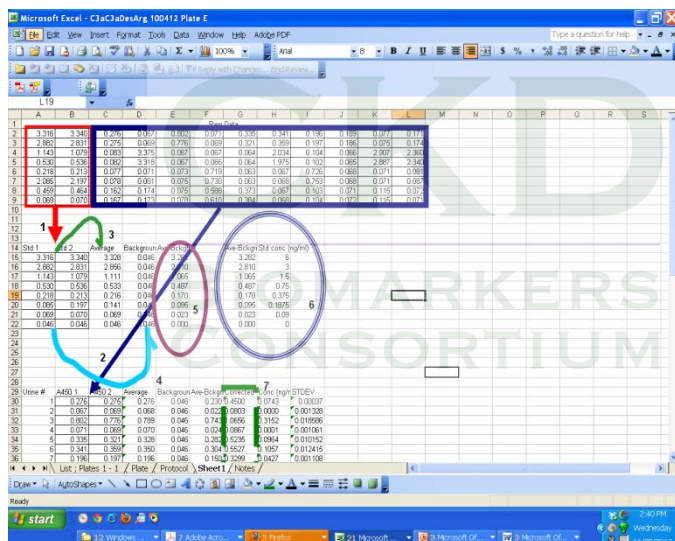
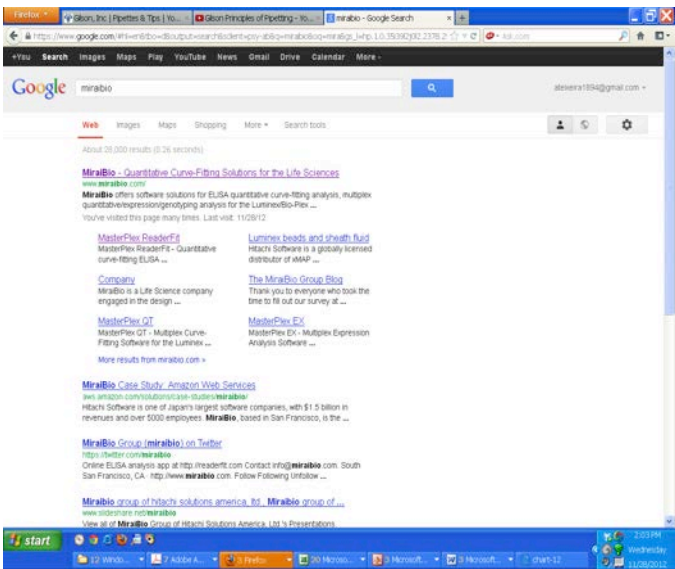


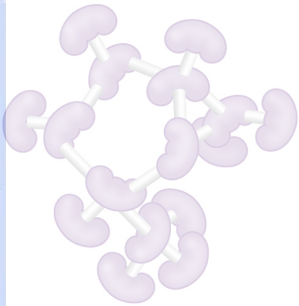
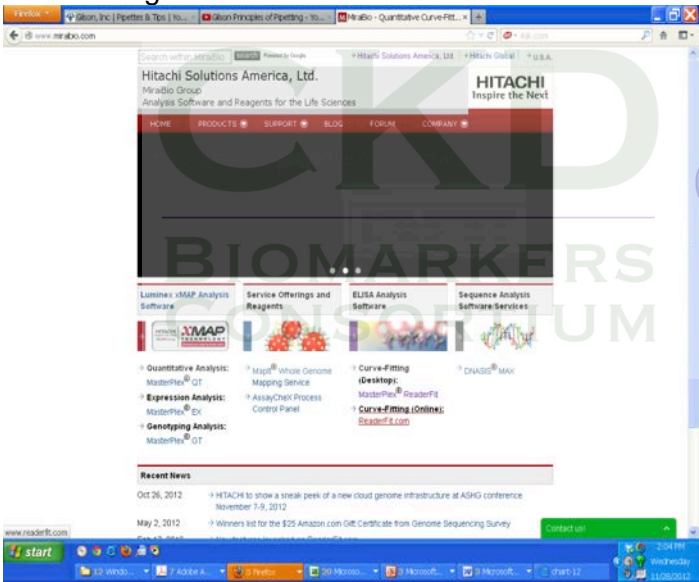
Figure 11. The Excel spreadsheet with the data showing the steps recorded in a macro for the purpose of preparing data for plotting.

### 3.3 Plotting the data

- The data is plotted using 4-parameter logistic curve fitting through the MasterPlex ReaderFit curve-fitting software for ELISA by Miraibio Group of Hitachi Software Engineering.
- The menu options and responses with the software are recorded for an example ELISA.



**Figure 12.** Locate the website through Google using MiraiBio as search term. Enter through the MiraiBio link.



**Figure 13.** Enter the ELISA Analysis Software, Curve-fitting (Online) ReaderFit.com tab



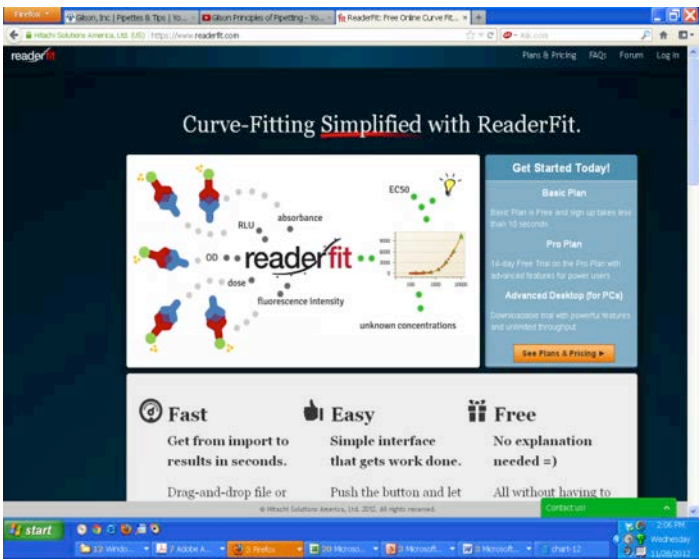


Figure 14. Log in at the ReaderFit page

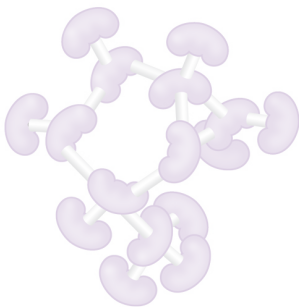
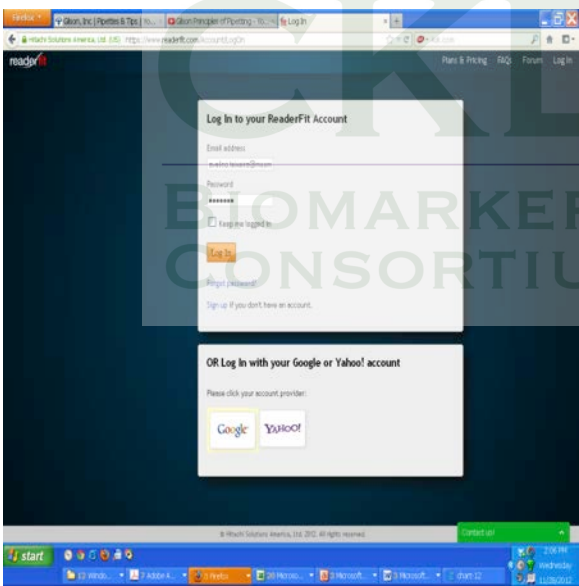


Figure 15. Login page

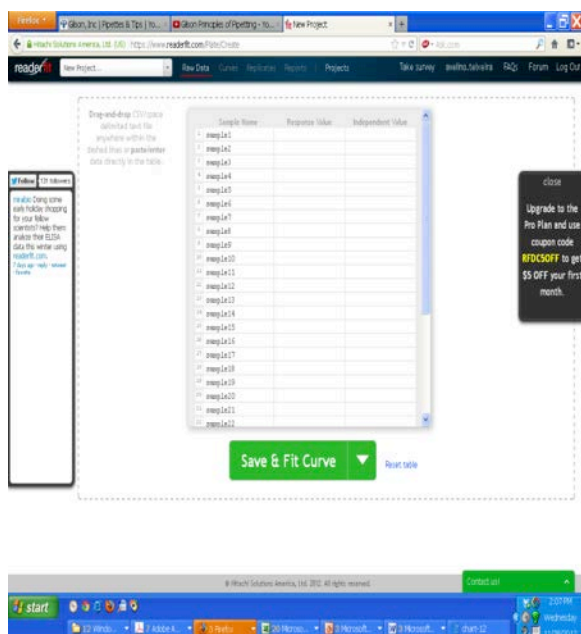


Figure 16. Opens to the data entry page.

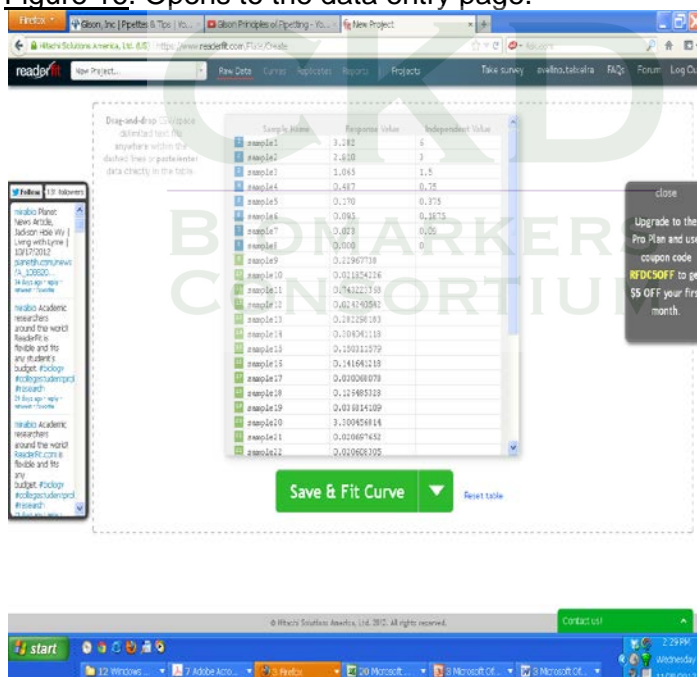
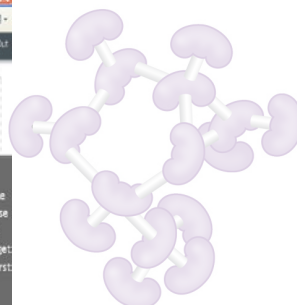


Figure 17.

- Copy from the prepared Excel file as shown in section 3.2 and paste the Standards A405 corrected averages (Response Values) and the Concentrations (Independent Values) into the top rows of the Raw Data table.
- Copy and paste the concentrations into the Response Value column below.
- Only 32 entries are available for entry on the Free Software version of Readerfit
- The remaining entries are submitted in a second file.
- Click the green "Save and Fit Curve" button.





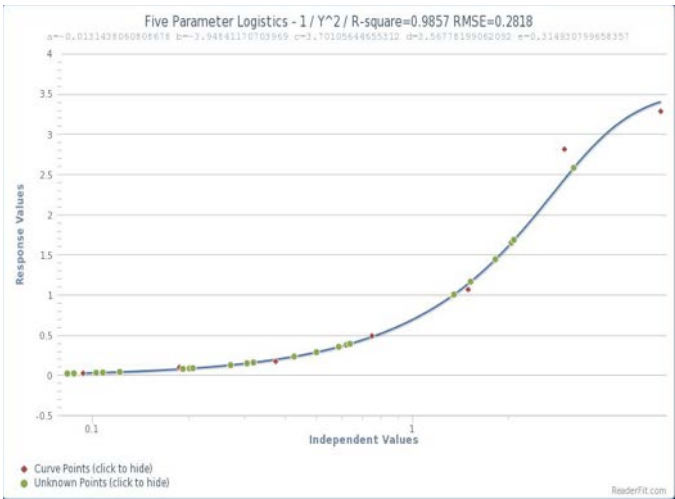


Figure 18 Resulant fitted curve showing the Standards in red and the unknowns in green

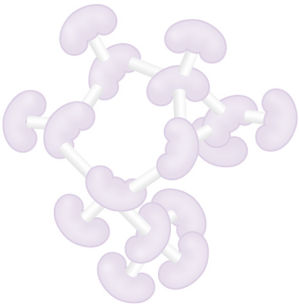
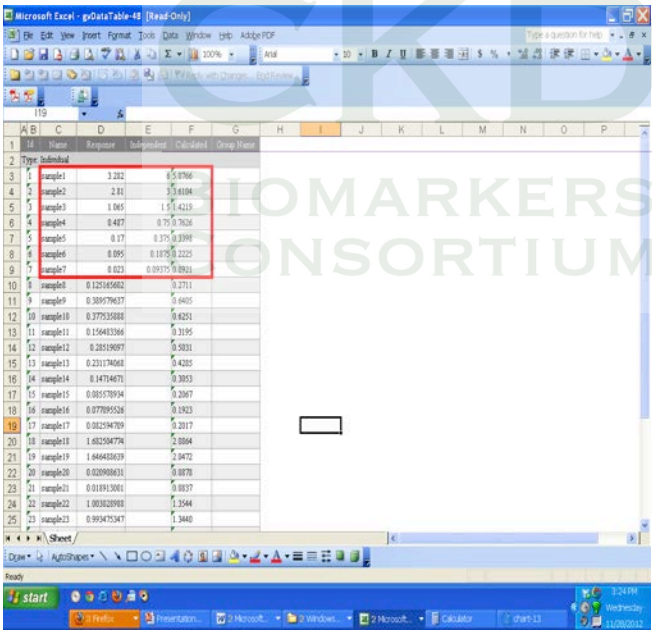


Figure 19

- Click on reports tab and Export to xls for the table shown above.
- The data highlighted in red outline above shows the response to the standards and closeness of fit of calculated data to the concentrations made on the bench.
- R-squared value and RMSE value of 1 and 0 respectively show ideal fit and large deviations from this indicate compromised standard curve and unreliable data.

### 3.4 Quality control of the data

- Coefficient of Variation (CV) is derived from the ratio of the standard deviation to the non-zero mean of duplicate concentration values, i.e. Coefficient of Variation **CV = Standard Deviation / Mean**. CV is expressed as percentage by multiplying CV by 100.
- For any sampled assay, a %CV value of more than 20% is a threshold for removal of the value and scheduling for repeat measurement of this sample in a subsequent assay.



# **Development of modified SOP for detection of C3a in human urine adapting Quidel C3a Sandwich ELISA kit. (Edited by Dr. Bonventre's lab)**

## **1. Introduction**

### **1.1 Background of the method**

As the kit instructions indicate C3 convertase cleaves the Complement component C3 to C3a and C3b. C3a, the subject of this ELISA kit is a 77 amino acid protein which is rapidly stabilized by the serum enzyme carboxypeptidase N to a more stable, 76 amino acid form C3a des-Arg. For the purposes of this ELISA both forms will be referred to as C3a. NOTE: The manufacturer's manual is only validated for detection of C3a in human plasma or serum! Significant modifications were developed by the Bottinger Lab to achieve satisfactory assay performance for C3a detection in human urine.

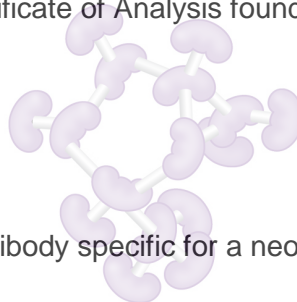
### **1.2 Reagents and consumables.**

#### **1.2.1 Supplied by Quidel in the kit**

- C3a Plus Standards concentrations are listed below BUT can vary therefore concentrations must be checked against the Certificate of Analysis found in each box
  - E = 5.4 ng/ml
  - D = 2.46
  - C = 0.52
  - B = 0.21
  - A = 0.05
- ELISA plate coated with a mouse monoclonal antibody specific for a neo-epitope on human C3a.
- Horseradish Peroxidase-conjugated polyclonal antibody to the C3a region of C3
- TMB Substrate. Ready to use. Contains 3,3',5,5'-tetramethylbenzidine (TMB) and
- Hydrogen Peroxide
- 20 x Wash Solution concentrate (1 x PBS 0.05% Tween-20, Proclin 300)
- Stop Solution (1N HCl)

#### **1.2.2 User supplied**

- Spectrophotometer (Molecular Devices SpectraMax Plus 384)
- Refrigerated Centrifuge (Sorvall Benchtop swing bucket centrifuge, Rotor RTH-250)
- Vortexer
- Electronic Multichannel pipettes
  - E4 XLS multichannel pipette (dispenses 2 µl-20 µl)(Rainin, catalog # E8-20XLS)
  - EDO 3-Pls multichannel pipette (dispenses 100 µl-1200 µl) (Rainin, catalog # E8-1200)
- Single Channel Manual Pipettes
  - Rainin Classic 0.5 µl-10 µl (catalog # PR-10)



CKD  
BIOMARKERS  
CONSORTIUM



- Setup a template plate by adding 120 µl aliquots of undiluted urine analyte and protein standards into the wells of a Polypropylene template plate following the Template Map shown in Figure 1 below.
- As quickly and carefully as possible (following the guidelines for pipetting described above) load the ELISA plate with the 12 tip multichannel pipette set at 100ul.
- Cover the fully loaded ELISA plate with an adhesive plate sealer. Secure the plate(s) on the IKA orbital shaker with clear tape on each corner and incubate at setting 7 (about 80 revolutions of the table per minute) for 1 h at room temperature (18-25°C).

**Figure 1. Template Map**

A	S1	S1	U01	U01	U09	U09	U17	U17	U25	U25	U33	U33
B	S2	S2	U02	U02	U10	U10	U18	U18	U26	U26	U34	U34
C	S3	S3	U03	U03	U11	U11	U19	U19	U27	U27	UC1	UC1
D	S4	S4	U04	U04	U12	U12	U20	U20	U28	U28	UC2	UC2
E	S5	S5	U05	U05	U13	U13	U21	U21	U29	U29	UC3	UC3
F	BL	BL	U06	U06	U14	U14	U22	U22	U30	U30	PS1	PS1
G	KC1	KC1	U07	U07	U15	U15	U23	U23	U31	U31	PS2	PS2
H	KC2	KC2	U08	U08	U16	U16	U24	U24	U32	U32	PS3	PS3

S1-S5: Standards

Un01-Un34: Unknown Samples

KC1-KC2: Controls provided by the kit

UC1-UC3: Urine Controls developed in house with low, intermediated and high levels of the analyte

PS1-PS3: Proficiency samples

#### **2.4 Modified wash procedure to remove urine and unbound proteins and prevent cross-well contamination**

- Aspirate and discard the urine and standards with the multichannel pipette using techniques in section 1.3 and being careful to tilt the plate to remove as much of the solution without touching below the halfway point of the well.
- Add 200 µl of Wash Solution from a pipette basin with the multichannel pipette to each well.
- Incubate the first wash for 1 min.
- Aspirate the solution with the multichannel pipette.
- Perform three further washes by the plate washing procedure in section 1.6.

#### **2.5 Binding labeled Detection antibody to the captured protein**

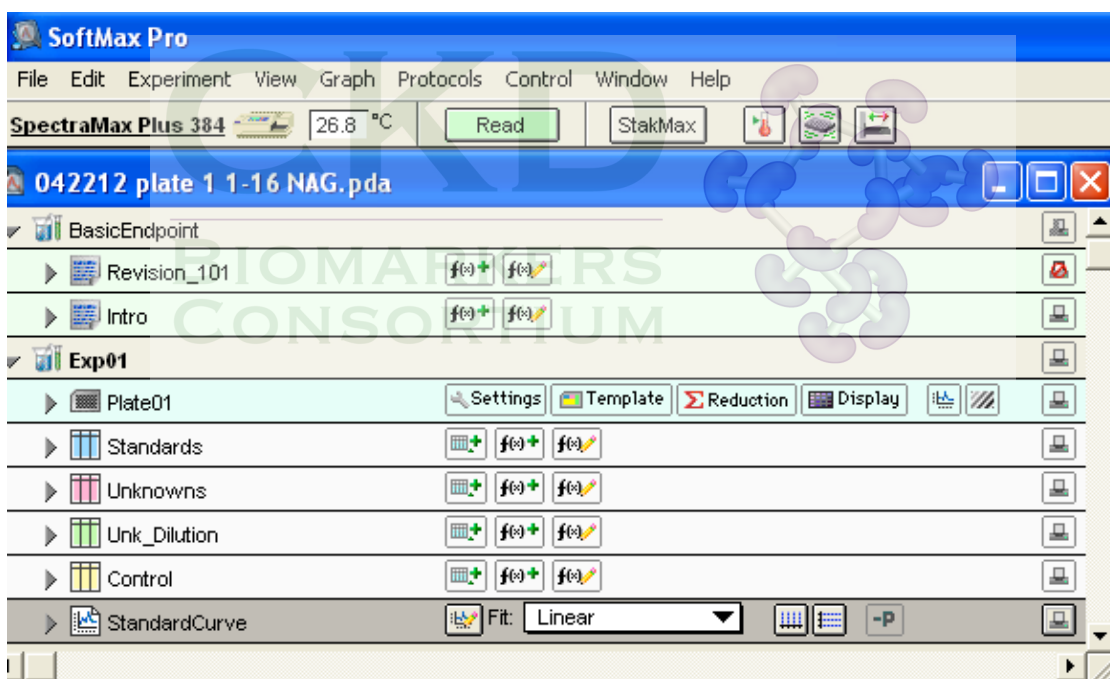
- Immediately after the last wash add the contents of the tube of C3a Plus Conjugate (7 ml) to a pipette basin,

- Dispense 50  $\mu$ l of the C3a conjugate in each well with the multichannel pipette.
- Incubate with shaking for 30 min at room temp with shaking.
- Wash the plate five times as per section 1.4.
- Dry the bottom of the plate with a KimWipe tissue to remove liquid, clean and polish the surface to allow light transmission in the plate reader.

## 2.6 Add TMB substrate and develop

- Add the contents of the TMB Substrate bottle to a pipette basin.
- Dispense 100  $\mu$ l of the substrate into each well with the multichannel pipette.
- Incubate in a closed, empty drawer for 15 min with occasional very gentle tapping to mix the reaction components and disperse the color.
- Stop development with 100  $\mu$ l/well of Stop Solution
- Tap gently to mix the solution, disperse color and stop the HRP activity.

## 3. Data Generation & Processing

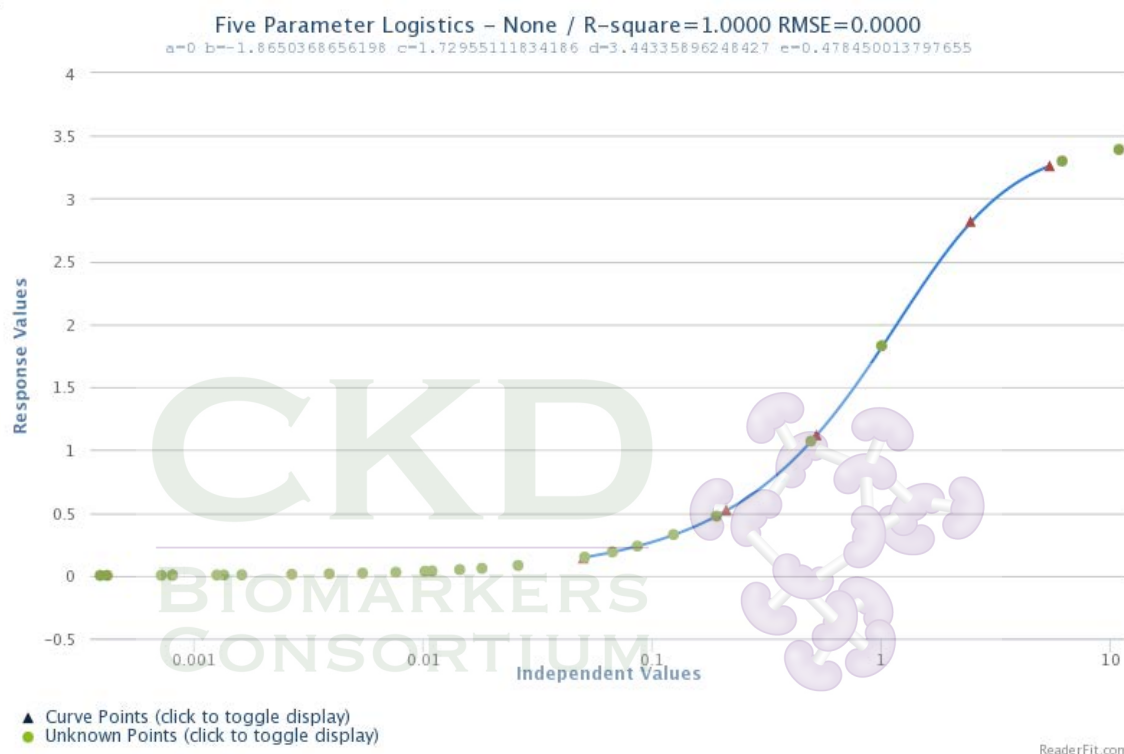


### 3.1 Data Output

- As indicated above, the 96 well plate is placed in a Spectrophotometer and absorbance read by pressing “Read” icon in SoftMax Pro software.
- Data will be automatically generated by SoftMax Pro 5.4 software that is integrated into the instrument operating software, once the machine is done with the reading (shown below).



- The SoftMax Pro software uses serially diluted standards to generate a standard curve. The software has user-selected options to choose the type of regression analysis. We use linear regression analysis to quantitate the concentrations of unknown samples.
- For each analyte standard, a concentration is back-calculated by plotting the absorbance of the standard on the standard curve. (Below is the typical standard curve)



**Figure 2. C3a Standard Curve**

### 3.2 Data Processing and Data Calculations

- The SoftMax automatically generates the values of unknowns based on the standard curve. The data generated includes the absorbance, predicted concentration of unknowns, standard error, % CV, and errors in the run of all samples (below is the typical data output page).
- Data will be exported from Softmax pro to Microsoft Excel using the export file option. The program will export information from the run including Sample ID, information of the wells, absorbance of each replicate, concentration of each replicate, mean concentration of the sample, standard deviation, and % CV. Two control urine specimens are evaluated on every plate. Three proficiency samples will be run every week.

- In addition to sample ID (Biocon ID), each tube will be numerically labeled for cross verification in the order we aliquot the samples on the plate. Once the data are transferred and compiled, we will again cross check the numerical number on the tube with the barcode scan.
- Another member in the facility will perform a secondary check to further validate the sample order.

**3.3 Performance of the ELISA assay on urine samples.**

- As the ELISA was specifically designed for measuring serum samples, we have performed additional recovery and interference studies in normal and nephrotic urine samples. The samples with were spiked with various concentrations of the analyte and the assay stability was measured at different pH and dilution. As outlined in the table below, the urine pH adjustments or sample dilution didn't significantly alter measurements of high, medium or low levels of C3a.

Type of assay	Spiked Concentration	% Recovery		
		Neat Samples Average pH 6.1	Undiluted urine samples. Average adjusted pH 7.2	Diluted Urine Samples (1 in 5 with sample diluent). Average pH 7.1
C3a Assay	4.808	93.9±0.9	90.9±0.7	92.5±1.6
	3.075	93.7±0.9	94.4±1.3	93.0±1.4
	1.242	92.8±1.1	90.9±0.7	93.3±1.3
	0.152	91.9±0.9	93.8±1.2	91.9±1.6
	0.081	92.7±1.3	93.8±1.3	94.9±1.2

- The assay was further evaluated whether recovery of spiked analytes were altered by nephrotic range proteinuria with or without uremia. As shown below, the assay was quite stable in samples collected from patients with high-grade proteinuria or advanced CKD stage V.

			% Recovery in sample		
			C3a		
Sample #	URINE PROT.	eGFR	1:1	1:2	1:4
9038	Neg	15.2	96	98	96
50073	Trace	10.5	98	95	92
40036	nephrotic	12.1	89	94	97
70056	nephrotic	8.9	90	89	95
70067	nephrotic	106.3	99	93	95
70083	nephrotic	119.1	92	93	95

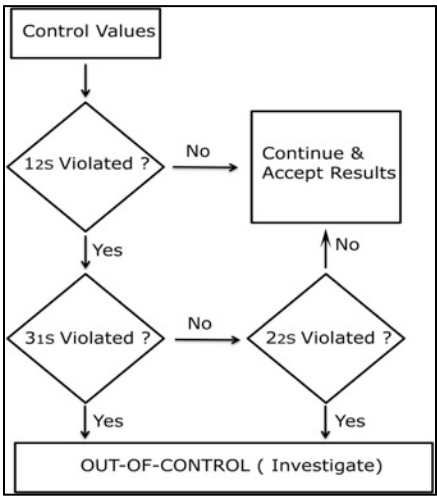
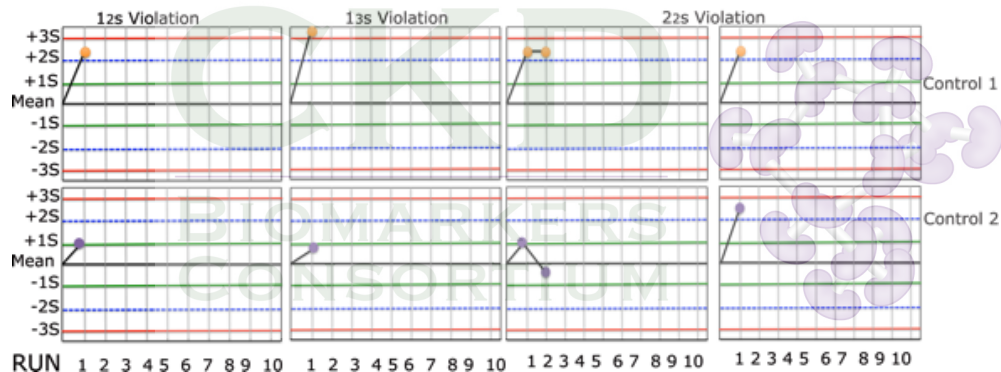
**4. Quality Control**

4.1. Quality control of the Assay.

- We will validate that Complement C3a levels obtained using the assay on control specimens are within mean  $\pm$  2 SD limit established from the Validation Protocol.
- If both samples are within mean  $\pm$  2 SD limit, we will accept the data. If one of the two control values is outside mean  $\pm$  2 SD, we will employ the Westgard 2 rules to determine if the analysis run results can be accepted.
- If neither of the assayed control results is within mean $\pm$  2 SD limits, then we will follow the following Westgard three-quality control rule.

RULE	QUALITY CONTROL RULE EXPLANATION
12S	One value of any of the two controls used in the assay is outside of the mean+2 SD or mean-2 SD of that control
13S	One value of any of the two controls used in the assay is outside of the mean+3 SD or mean-3 SD of that control
22s	Both controls used in the assay are outside of their mean $\pm$ SD on the same assay or two consecutive values of one control is outside of mean $\pm$ SD

Levey-Jenning's plots of controls:



4.2 Quality Control for each sample

- Sample measurements will be repeated if the %CV of the sample is > 15%

- Sample measurements will be repeated if the absorbance of the sample is below 0.020 to make sure that the low absorbance values are not due to pipetting error.

#### **4.3 Quality Control troubleshooting procedure**

If the controls fail the above quality control procedures, then we will employ the following procedure to resolve the issue:

- Rerun Control
- If the quality control values pass the above described westgard rules and proceed with unknown testing
- If the quality control failed again, make up fresh reagents and rerun controls and document the problem
- If the quality control indicates that the westgard rules are passed, proceed with testing
- If QC fails again, recalibrate the assay (use new standard lot)
- If after calibration the QC determinations have passed westgard criteria, proceed with testing and document correction
- If Westgard criteria were not met after preparation of fresh reagents, do not proceed with the analysis and contact the Laboratory Director.

#### **4.4 Data storage and reporting**

- After the analysis has passed the quality control test, Complement C3a values of unknown samples and their corresponding %CV and SD will be copied and pasted into a master excel data sheet in the sequential order on sheet 1 in excel workbook. COMPLEMENT C3A values, %CV, SD of quality control samples and proficiency samples will be pasted on sheet 2. Both sheet 1 & 2 will be updated with addition of data from each run.
- The master data sheet that will go to coordinating center will contain the following information
  1. Sample ID (Bio Con ID)
  2. Date of the assay
  3. Platform and Instrument# used for the assay
  4. Reagent lot numbers
  5. % CV across the sample replicates

### **APPENDIX:**

## Data processing steps used in Dr. Erwin Bottinger's laboratory

### 3.1 Reading the plate

- Use the Perkin Elmer 1420 Multilabel Counter and plate reader to read the color intensity of urine and standards to determine concentrations.
- The menu options for the reader are shown in screen shots below

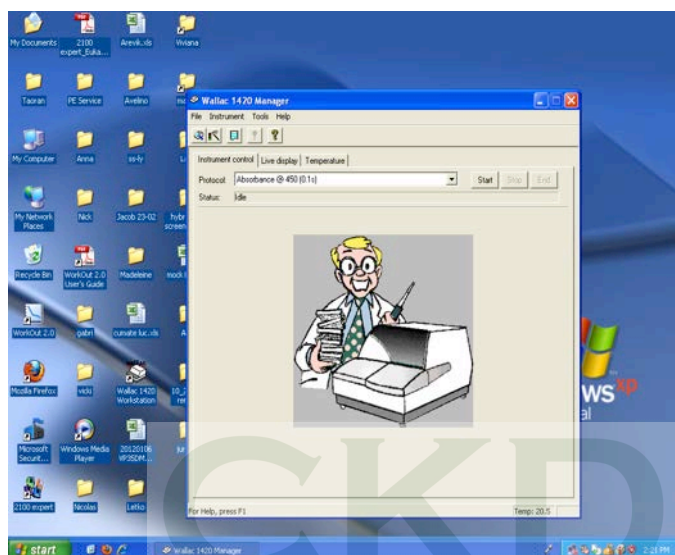


Figure 2. Find the Wallac 1420 operating software Manager. Go to Tools > Start Wizard

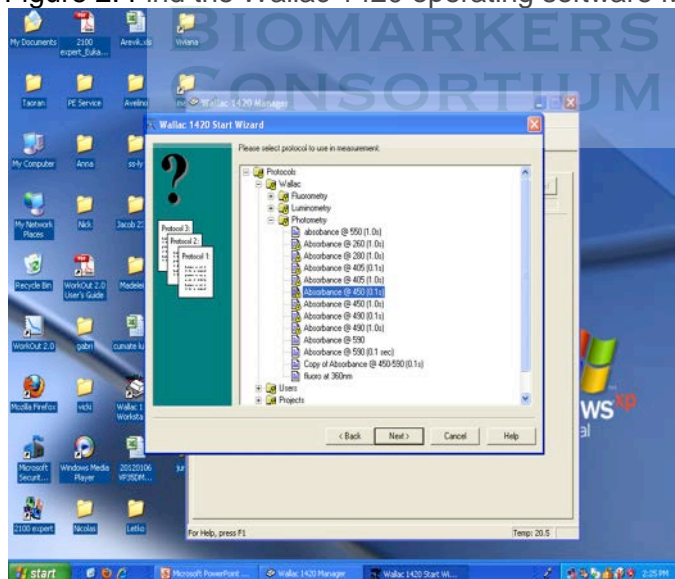


Figure 3. Find Photometry absorbance @ 450nm (0.1s). Reading time can be changed if warranted.





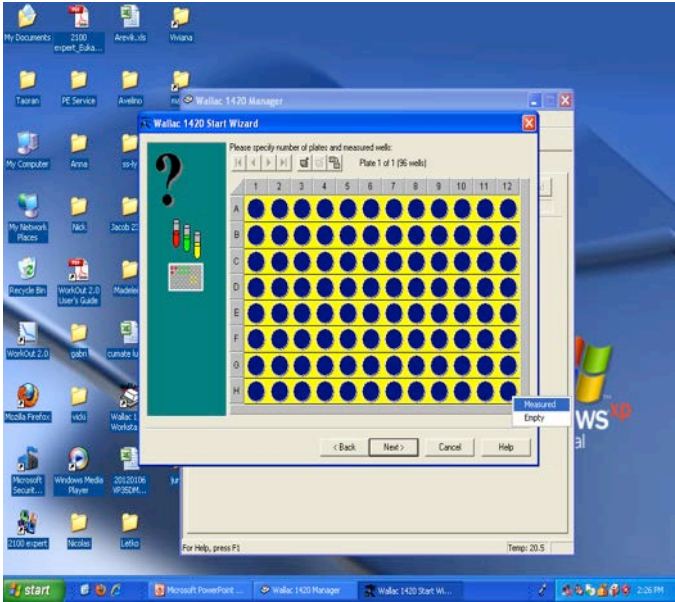


Figure 4. Highlight all wells to ensure all are read.

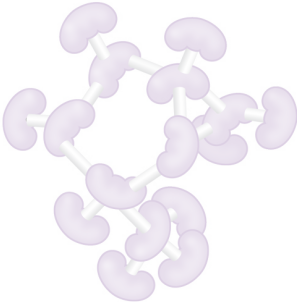
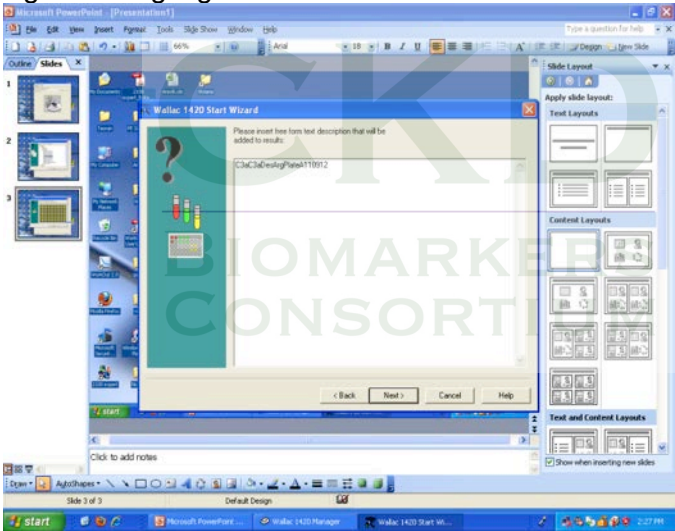


Figure 5. Can be left blank



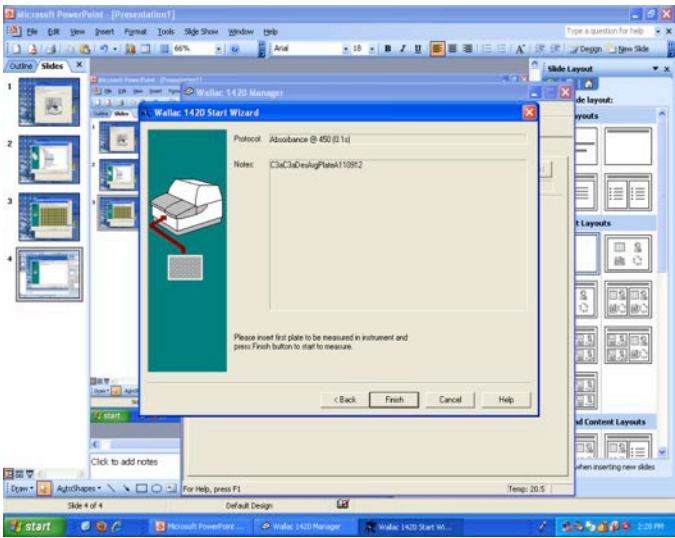


Figure 6. Click Finish to start

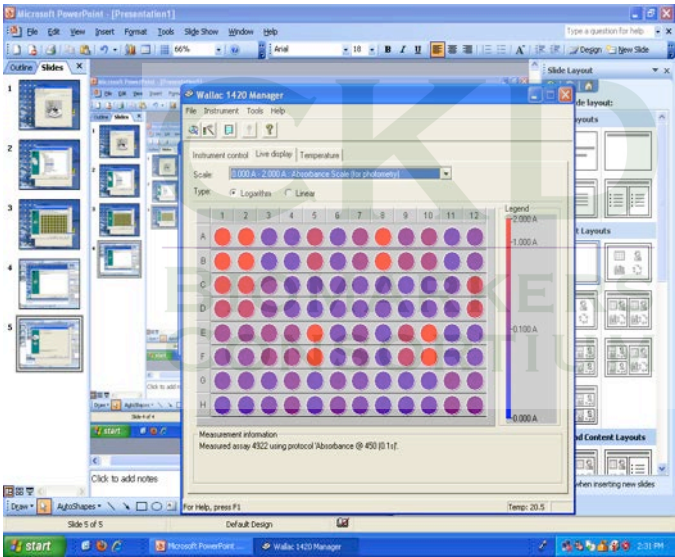
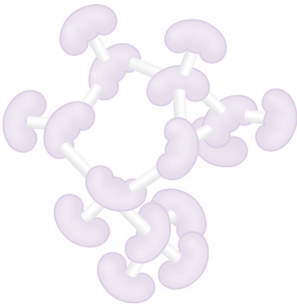


Figure 7. Results seen on the Live display tab.



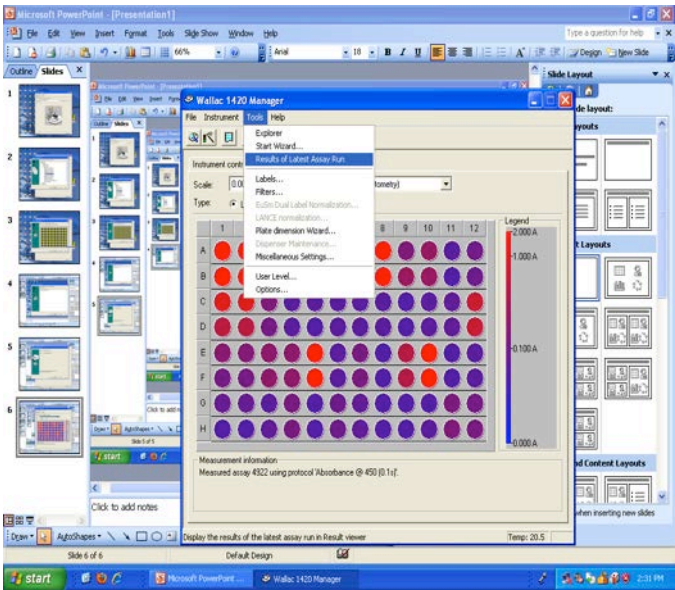


Figure 8. To retrieve the data use Tools tab and scroll to Results of Latest Assay Run

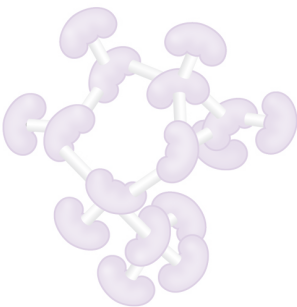
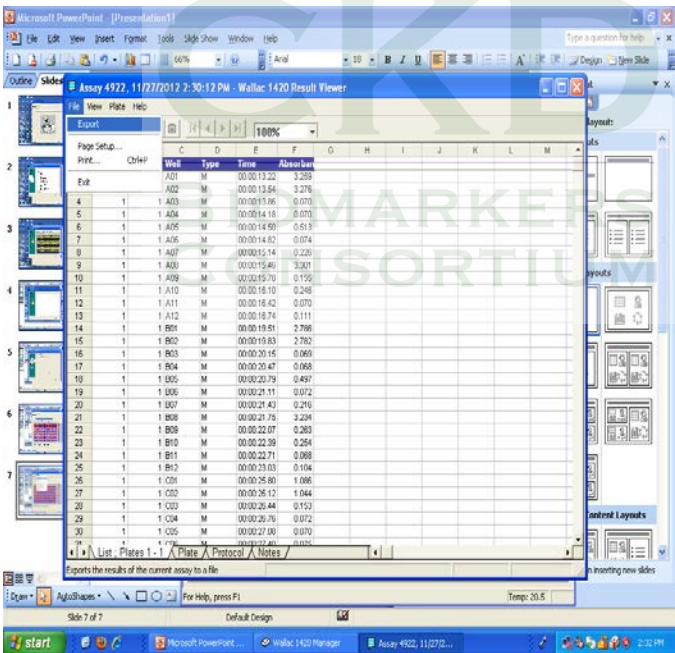


Figure 9. The data file in Excel spreadsheet format must be exported to a file for retrieval and analysis.

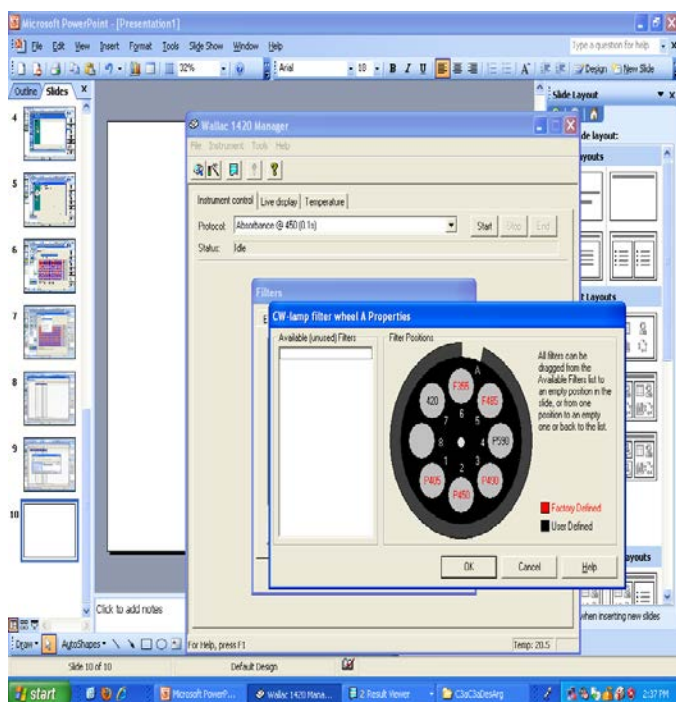


Figure 10. Confirmation that the filter for 450nm is present. For physical confirmation the wheel can be removed.

### 3.2 Preparing the Raw data for plotting and analysis

- The data is saved in Excel in the form of a table reflecting the ELISA plate.
- The data has to be prepared for plotting before concentrations can be determined.
- The process has been expedited by recording a macro of all the steps required to prepare the data.
- The macro accomplishes the following very quickly and reproducibly:
  - Absorbance values of the standards are copied from the Raw data and pasted into an unused part of the sheet (from A2 – B9 to A15 – B22) and labeled as Std 1 and Std2 respectively (Arrow 1, red).
  - The average is determined of the duplicate values and recorded in the appropriate row (C15 – C22, Arrow 3, green).
  - The average of the Blank readings is copied to the cells D15-D22 (Arrow 4, light blue) for subtraction from the average concentration absorbance values (Oval 5, purple)
  - The corrected absorbance values for the standards is copied and pasted in an unused area (G15-G22, Circle blue/black)
  - Concentration values are left open to be inserted for the particular assay
  - The urine A450 values are duplicated vertically as shown in Figure 1 and run consecutively from left to right starting at C3 and C4 and ending at L8 and L9.
  - These data are copied one double row at a time (C3 and C4 to L3 and L4) and using the Paste Special, Transpose function are transferred to B30-B40 to transpose from row to column presentation.

- Consecutive rows are added below until all the urine A450 data is in the columns B30-B70 (Arrow 2, dark blue).
- The rows of duplicate values are labeled consecutively 1 – 40 titled Urine#
- The data columns are labeled A450 1 and A450 2 respectively.
- The average is determined and recorded alongside then highlighted and Paste Special, value back in the same column and labeled “Average”.
- The Blank or background average from above is recorded and repeated alongside the urine A450 data and labeled “Background” in preparation for subtraction.
- Subtraction is done and reported in the next column as “Ave-Bckgrnd” and copied, Paste Special values i.e. only values are recorded.
- To remove subzero data the following formula is placed into the top cell of cell G30 “=IF(F30<0,0,F30)” and dragged down to G70. This leaves unchanged all positive numbers and zero but changes negative numbers to 0.
- This final column G30-G70 provides the data for plotting and concentration determination along with the Std A450 average corrected values and the concentrations which have to be entered retrospectively into the worksheet.
- Standard deviations greater than 20% are disregarded and those samples are put aside for reanalysis

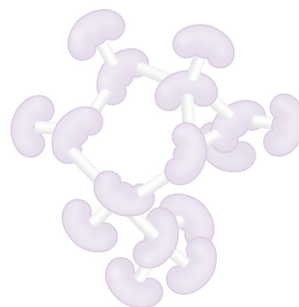
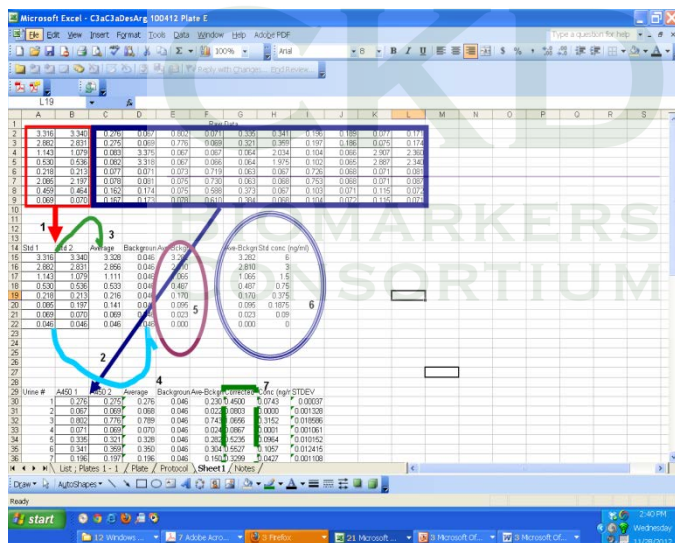
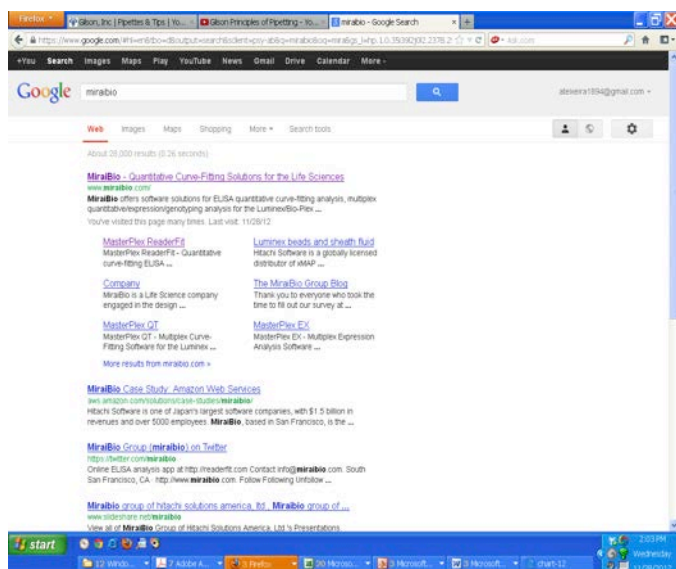


Figure 11. The Excel spreadsheet with the data showing the steps recorded in a macro for the purpose of preparing data for plotting.

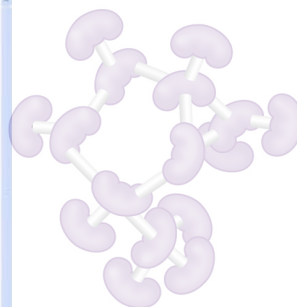
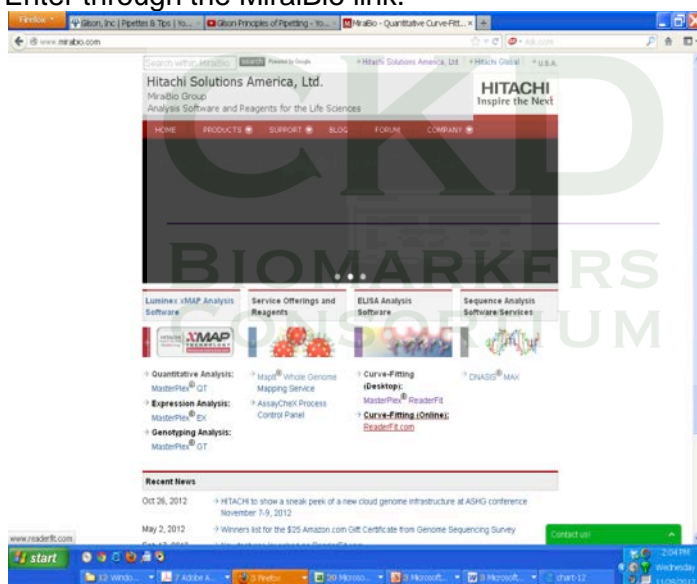
### 3.3 Plotting the data

- The data is plotted using 4-parameter logistic curve fitting through the MasterPlex ReaderFit curve-fitting software for ELISA by Miraibio Group of Hitachi Software Engineering.
- The menu options and responses with the software are recorded for an example ELISA.





**Figure 12.** Locate the website through Google using MiraiBio as search term. Enter through the MiraiBio link.



**Figure 13.** Enter the ELISA Analysis Software, Curve-fitting (Online) ReaderFit.com tab

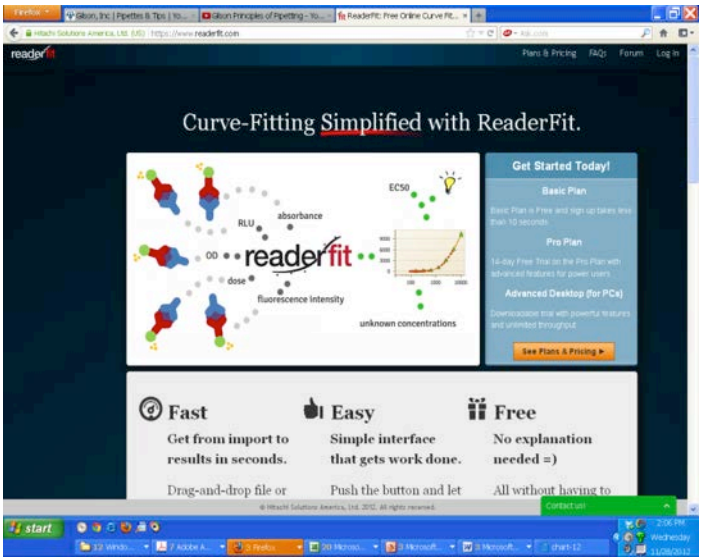


Figure 14. Log in at the ReaderFit page

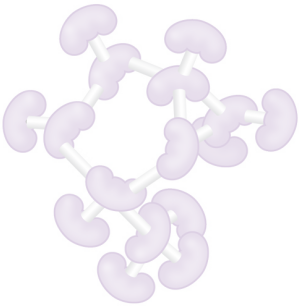
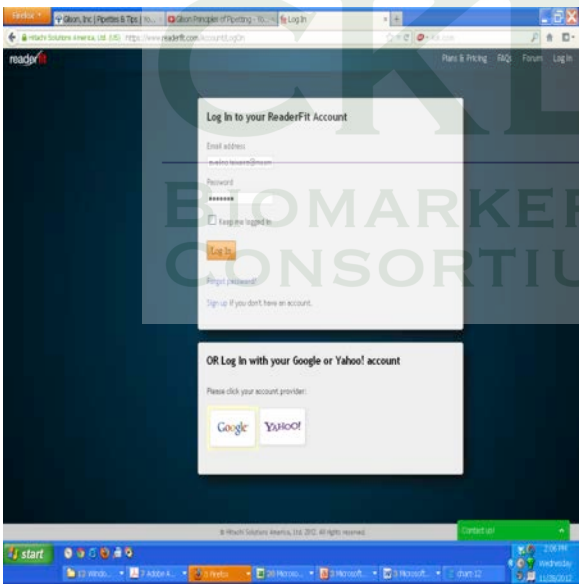


Figure 15. Login page



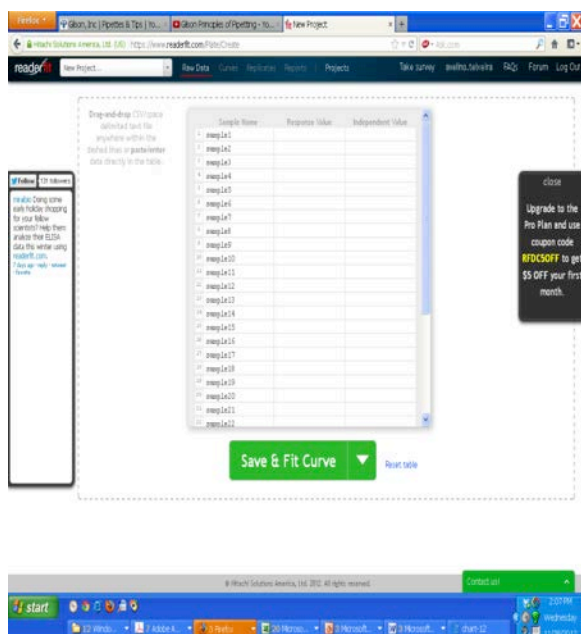


Figure 16. Opens to the data entry page.

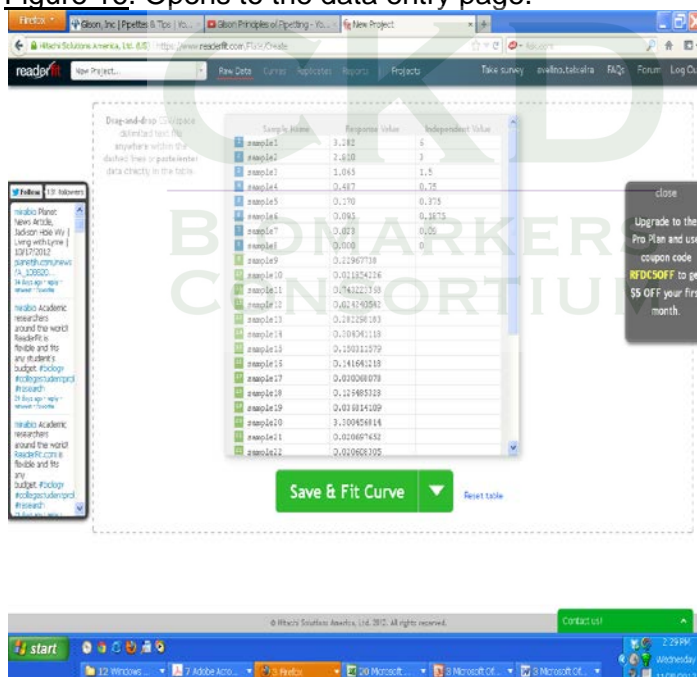
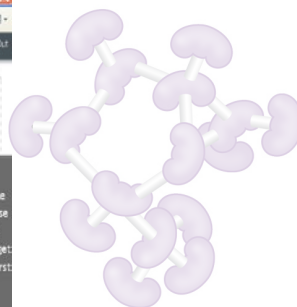


Figure 17.

- Copy from the prepared Excel file as shown in section 3.2 and paste the Standards A450 corrected averages (Response Values) and the Concentrations (Independent Values) into the top rows of the Raw Data table.
- Copy and paste the concentrations into the Response Value column below.
- Only 32 entries are available for entry on the Free Software version of Readerfit
- The remaining entries are submitted in a second file.
- Click the green "Save and Fit Curve" button.



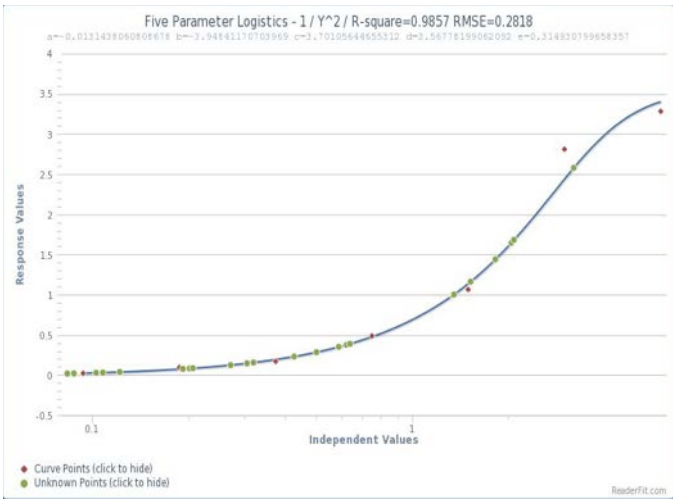


Figure 18 Resulant fitted curve showing the Standards in red and the unknowns in green

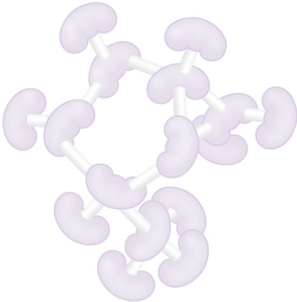
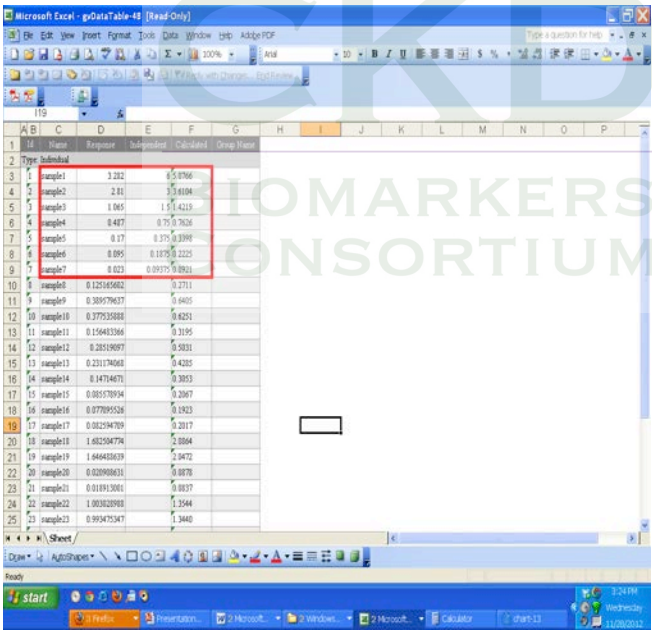
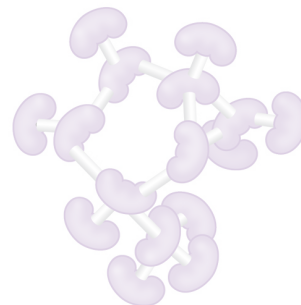


Figure 19

- Click on reports tab and Export to xls for the table shown above.
- The data highlighted in red outline above shows the response to the standards and closeness of fit of calculated data to the concentrations made on the bench.
- R-squared value and RMSE value of 1 and 0 respectively show ideal fit and large deviations from this indicate compromised standard curve and unreliable data.

3.4 Quality control of the data

- Coefficient of Variation (CV) is derived from the ratio of the standard deviation to the non-zero mean of duplicate concentration values, i.e. Coefficient of Variation **CV = Standard Deviation / Mean**. CV is expressed as percentage by multiplying CV by 100.
- For any sampled assay, a %CV value of more than 20% is a threshold for removal of the value and scheduling for repeat measurement of this sample in a subsequent assay.



# **Development of modified SOP for detection of C5b-9 in human urine adapting Quidel C5b-9 Sandwich ELISA kit. (Edited by Dr. Bonventre's lab)**

## **1. Introduction**

### **1.1 Background of the method**

As the kit instructions indicates the Terminal Complement Complex (TCC, SC5b-9) is generated by the assembly of C5 through C9 as a consequence of activation of the complement system by either the classical, lectin or alternative pathway. The membrane attack complex (MAC), a form of TCC, is a stable complex that mediates the irreversible target cell membrane damage associated with complement activation. This ELISA kit uses a mouse monoclonal antibody specific for the C9 ring of human SC5b-9 coated to wells of a 96-well plate to capture the complex. The trapped SC5b-9 is subsequently detected with horseradish peroxidase-conjugated (Goat) antibodies to antigens of SC5b-9. NOTE: The manufacturer's manual is only validated for detection of SC5b-9 in human plasma or serum! Significant modifications were developed by the Bottinger Lab to achieve satisfactory assay performance for SC5b-9 detection in human urine

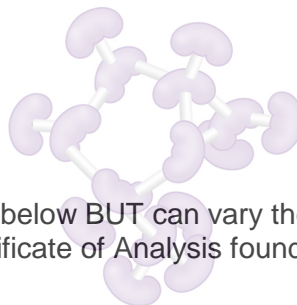
### **1.2 Reagents and consumables.**

#### **1.2.1 Supplied by Quidel in the kit**

- SC5b-9 Plus Standards concentrations are listed below BUT can vary therefore concentrations must be checked against the Certificate of Analysis found in each box
  - E = 198 ng/ml
  - D = 128
  - C = 49
  - B = 13
  - A = 0
- ELISA plate coated with a mouse monoclonal antibody specific for the C9 ring of SC5b-9.
- Horseradish Peroxidase-conjugated (Goat) antibodies to antigens of SC5b-9
- TMB Substrate. Ready to use.
- 20 x Wash Solution concentrate (1 x PBS 0.05% Tween-20, Proclin 300)
- Stop Solution (2N H<sub>2</sub>SO<sub>4</sub>)

#### **1.2.2 User supplied**

- Spectrophotometer (Molecular Devices SpectraMax Plus 384)
- Refrigerated Centrifuge (Sorvall Benchtop swing bucket centrifuge, Rotor RTH-250)
- Vortexer
- Electronic Multichannel pipettes
  - E4 XLS multichannel pipette (dispenses 2 µl-20 µl)(Rainin, catalog # E8-20XLS)



CKD  
BIOMARKERS  
CONSORTIUM

- EDO 3-Pls multichannel pipette (dispenses 100 µl-1200 µl) (Rainin, catalog # E8-1200)
- Single Channel Manual Pipettes
  - Rainin Classic 0.5 µl-10 µl (catalog # PR-10)
  - Rainin Classic 10 µl-100 µl (catalog # PR-100)
  - Rainin Classic 100 µl-1000 µl (catalog # PR-1000)
- 50 ml reagent reservoir (Fisher Scientific).

**1.3 Plate washing**

- Add about 200 µl of PBS or Wash Solution (as required) to each well with a 10 ml pipette by slowly dispensing in a constant stream and moving the pipette from well to well to fill each well of the plate.
- For the first wash of a set let the plate stand for two minutes before proceeding.
- Immediately remove the solution by tipping the plate upside down with a throwing motion to expel the solution into a basin
- Tap the plate upside down on paper towel to remove remaining solution.

**2. ELISA Assay**

**2.1 Sample handling**

- Sample tubes will be arranged in open tube racks with an empty space between each tube for better air circulation. Place these racks in 30°C incubator until all samples are thawed (The tubes will be still cold after the samples are thawed). Vortex the tubes for 5 sec, centrifuge at 4°C 3000 RPM using RTH-250 rotor on Sorvall Bench top Centrifuge, and keep on ice and proceed for the analysis.
- **2.1.1: Stability of C5b-9:** Stability of C5b-9 was determined in samples with low, intermediate and high concentrations of C5b-9. The freeze thaw stability of the analyte was evaluated by freeze thawing one time ( Comparison against “ sample conc” and “ 0 hr conc). Further, the stability of the analyte was evaluated by incubating the same at room temperature for 8 hr and 24 hr. As outlined in the table below, **C5b-9** is stable with single freeze thaw cycle and incubation at room temperature for 24h.


**2.2 Standard curve with protein standards and variation from manufacturer’s instructions**

- The standards are supplied by the manufacturer ready for use.

- To extend the range of standards to detect lower concentrations of SC5b-9 the lowest concentration of protein standard (0.14 µg/ml) was further diluted 1:1 in hydrating solution to give: 6.5, 3.25, 1.625 ng/ml

### 2.3 Loading the urine analyte

- Add about 200 µl of PBS or Wash Solution (as required) to each well with a 10 ml pipette by slowly dispensing in a constant stream and moving the pipette from well to well to fill each well of the plate.
- Setup a template plate by adding 120 µl aliquots of undiluted urine analyte and protein standards into the wells of a Polypropylene template plate following the Template Map shown in Figure 1 below.
- As quickly and carefully as possible (following the guidelines for pipetting described above) load the ELISA plate with the 12 tip multichannel pipette set at 100ul.
- Cover the fully loaded ELISA plate with an adhesive plate sealer. Secure the plate(s) on the IKA orbital shaker with clear tape on each corner and incubate at setting 7 (about 80 revolutions of the table per minute) for 1 h at room temperature (18-25°C).

**Figure 1. Template Map**

A	S1	S1	U01	U01	U09	U09	U17	U17	U25	U25	U33	U33
B	S2	S2	U02	U02	U10	U10	U18	U18	U26	U26	U34	U34
C	S3	S3	U03	U03	U11	U11	U19	U19	U27	U27	UC1	UC1
D	S4	S4	U04	U04	U12	U12	U20	U20	U28	U28	UC2	UC2
E	S5	S5	U05	U05	U13	U13	U21	U21	U29	U29	UC3	UC3
F	BL	BL	U06	U06	U14	U14	U22	U22	U30	U30	PS1	PS1
G	KC1	KC1	U07	U07	U15	U15	U23	U23	U31	U31	PS2	PS2
H	KC2	KC2	U08	U08	U16	U16	U24	U24	U32	U32	PS3	PS3

S1-S5: Standards

Un01-Un34: Unknown Samples

KC1-KC2: Controls provided by the kit

UC1-UC3: Urine Controls developed in house with low, intermediated and high levels of the analyte

PS1-PS3: Proficiency samples

### 2.4 Modified wash procedure to remove urine and unbound proteins and prevent cross-well contamination

- Aspirate and discard the urine and standards with the multichannel pipette using techniques in section 1.3 and being careful to tilt the plate to remove as much of the solution without touching below the halfway point of the well.
- Add 200 µl of Wash Solution from a pipette basin with the multichannel pipette to each well.
- Incubate the first wash for 1 min.



- Aspirate the solution with the multichannel pipette.
- Perform three further washes by the plate washing procedure in section 1.6.

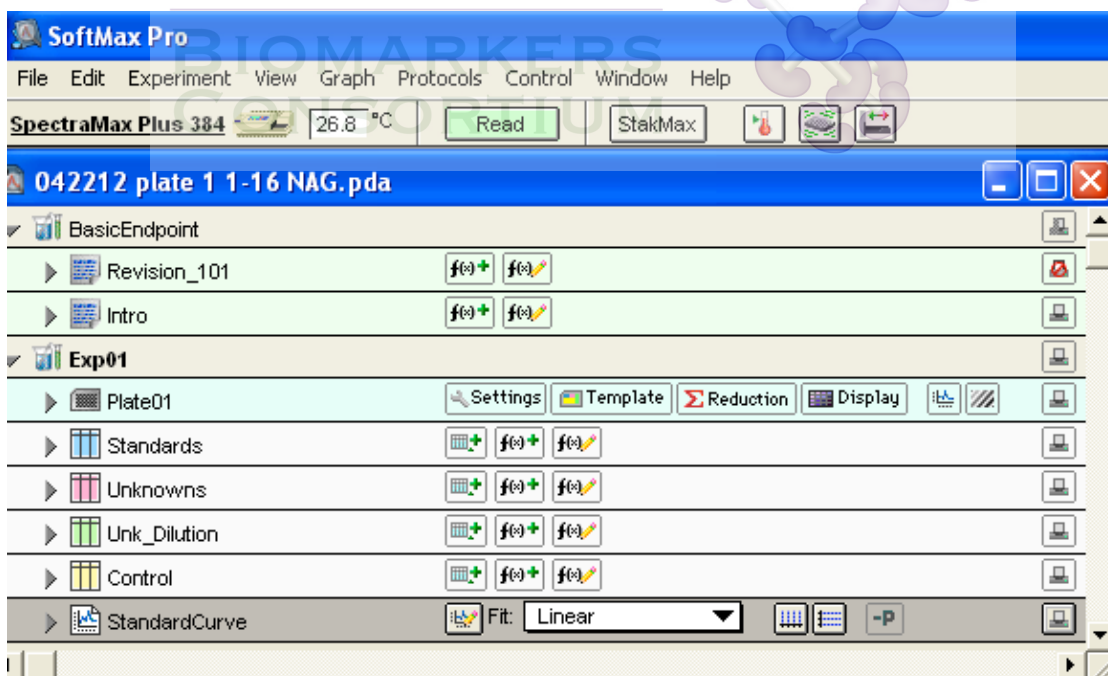
## 2.5 Binding labeled Detection antibody to the captured protein

- Immediately after the last wash add the contents of the tube of SC5b-9 Plus Conjugate (7 ml) to a pipette basin,
- Dispense 50  $\mu$ l of the SC5b-9 conjugate in each well with the multichannel pipette.
- Incubate with shaking for 30 min at room temp with shaking.
- Wash the plate five times as per section 1.4.
- Dry the bottom of the plate with a KimWipe tissue to remove liquid, clean and polish the surface to allow light transmission in the plate reader.

## 2.6 Add TMB substrate and develop

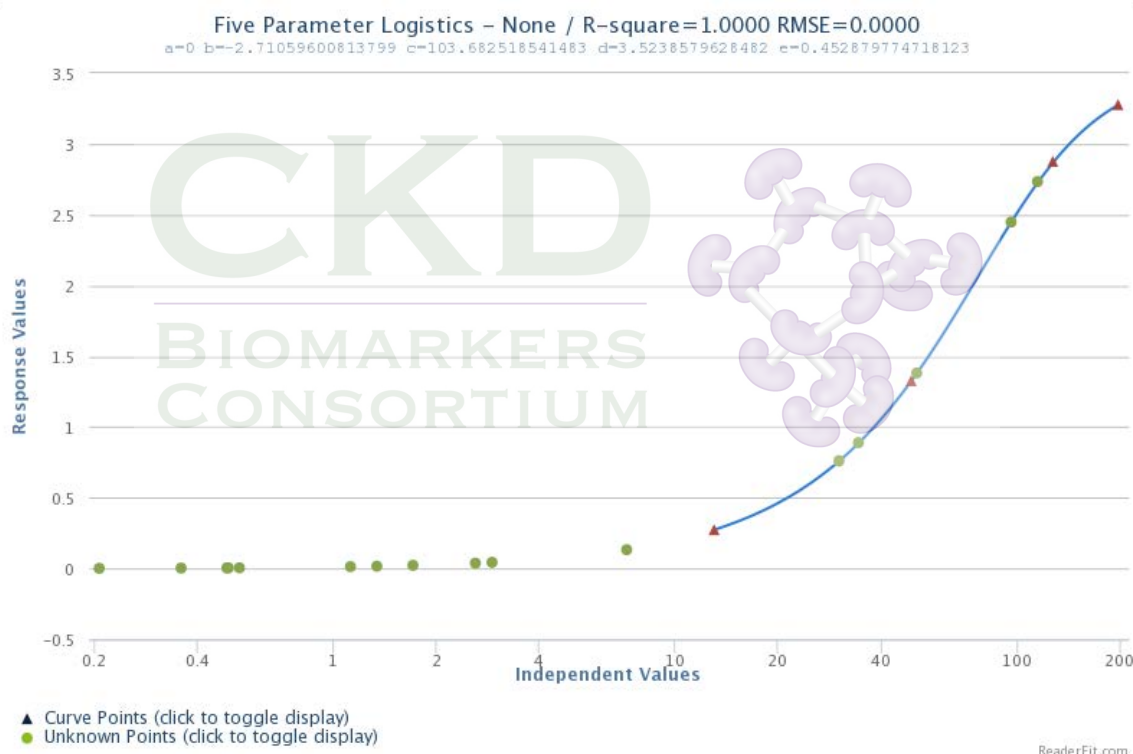
- Add the contents of the TMB Substrate bottle to a pipette basin.
- Dispense 100  $\mu$ l of the substrate into each well with the multichannel pipette.
- Incubate in a closed, empty drawer for 15 min with occasional very gentle tapping to mix the reaction components and disperse the color.
- Stop development with 100  $\mu$ l/well of Stop Solution
- Tap gently to mix the solution, disperse color and stop the HRP activity.

## 3. Data Generation & Processing



### 3.1 Data Output

- As indicated above, the 96 well plate is placed in a Spectrophotometer and absorbance read by pressing “Read” icon in SoftMax Pro software.
- Data will be automatically generated by SoftMax Pro 5.4 software that is integrated into the instrument operating software, once the machine is done with the reading (shown below).
- The SoftMax Pro software uses serially diluted standards to generate a standard curve. The software has user-selected options to choose the type of regression analysis. We use linear regression analysis to quantitate the concentrations of unknown samples.
- For each analyte standard, a concentration is back-calculated by plotting the absorbance of the standard on the standard curve. (Below is the typical standard curve)



**Figure 2. Standard Curve**

### 3.2 Data Processing and Data Calculations

- The SoftMax automatically generates the values of unknowns based on the standard curve. The data generated includes the absorbance, predicted concentration of unknowns, standard error, % CV, and errors in the run of all samples (below is the typical data output page).
- Data will be exported from Softmax pro to Microsoft Excel using the export file option. The program will export information from the run

including Sample ID, information of the wells, absorbance of each replicate, concentration of each replicate, mean concentration of the sample, standard deviation, and % CV. Two control urine specimens are evaluated on every plate. Three proficiency samples will be run every week.

- In addition to sample ID (Biocon ID), each tube will be numerically labeled for cross verification in the order we aliquot the samples on the plate. Once the data are transferred and compiled, we will again cross check the numerical number on the tube with the barcode scan.
- Another member in the facility will perform a secondary check to further validate the sample order.

**3.3 Performance of the ELISA assay on urine samples.**

- As the ELISA was specifically designed for measuring serum samples, we have performed additional recovery and interference studies in normal and nephrotic urine samples. The samples with were spiked with various concentrations of the analyte and the assay stability was measured at different pH and dilution. As outlined in the table below, the urine pH adjustments or sample dilution didn't significantly alter measurements of high, medium or low levels of C3a.

Type of assay	Spiked Concentration	% Recovery		
		Neat Samples Average pH 6.1	Undiluted urine samples. Average adjusted pH 7.2	Diluted Urine Samples (1 in 5 with sample diluent). Average pH 7.1
SC5B-9 Assay	4.808%	92.3±1.3%	94.9±1.3%	91.6±1.5%
	3.075%	92.9±1.3%	93.3±1.1%	89.0±1.6%
	1.242%	94.9±0.9%	94.2±1.3%	92.6±1.6%
	0.152%	94.8±0.6%	92.8±1.3%	94.2±1.0%
	0.081%	96.4±1.0%	91.7±1.4%	90.9±1.1%

- The assay was further evaluated whether recovery of spiked analytes were altered by nephrotic range proteinuria with or without uremia. As shown below, the assay was quite stable in samples collected from patients with high-grade proteinuria or advanced CKD stage V.

			% Recovery in sample		
			C5b-9		
Sample #	URINE PROT.	eGFR	1:1	1:2	1:4
9038	Neg	15.2	91	91	95
50073	Trace	10.5	96	90	89
40036	nephrotic	12.1	99	90	96
70056	nephrotic	8.9	97	98	89
70067	nephrotic	106.3	87	88	91
70083	nephrotic	119.1	95	96	93

4. Quality Control

4.1. Quality control of the Assay.

- We will validate that SC5b-9 levels obtained using the assay on control specimens are within mean  $\pm$  2 SD limit established from the Validation Protocol.
- If both samples are within mean  $\pm$  2 SD limit, we will accept the data. If one of the two control values is outside mean  $\pm$  2 SD, we will employ the Westgard 2 rules to determine if the analysis run results can be accepted.
- If neither of the assayed control results is within mean $\pm$  2 SD limits, then we will follow the following Westgard three-quality control rule.

RULE	QUALITY CONTROL RULE EXPLANATION
12s	One value of any of the two controls used in the assay is outside of the mean+2 SD or mean-2 SD of that control
13s	One value of any of the two controls used in the assay is outside of the mean+3 SD or mean-3 SD of that control
22s	Both controls used in the assay are outside of their mean $\pm$ SD on the same assay or two consecutive values of one control is outside of mean $\pm$ SD

Levey-Jenning's plots of controls:



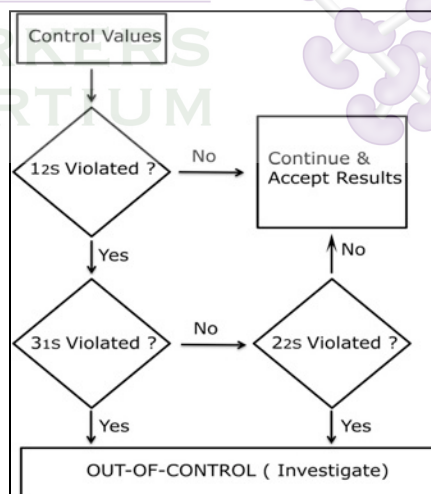
#### 4.2 Quality Control for each sample

- Sample measurements will be repeated if the %CV of the sample is > 15%
- Sample measurements will be repeated if the absorbance of the sample is below 0.020 to make sure that the low absorbance values are not due to pipetting error.

#### 4.3 Quality Control troubleshooting procedure

If the controls fail the above quality control procedures, then we will employ the following procedure to resolve the issue:

- Rerun Control
- If the quality control values pass the above described westgard rules and proceed with unknown testing
- If the quality control failed again, make up fresh reagents and rerun controls and document the problem
- If the quality control indicates that the westgard rules are passed, proceed with testing
- If QC fails again, recalibrate the assay (use new standard lot)
- If after calibration the QC determinations have passed westgard criteria, proceed with testing and document correction
- If Westgard criteria were not met after preparation of fresh reagents, do not



proceed with the analysis and contact the Laboratory Director.

#### 4.4 Data storage and reporting

- After the analysis has passed the quality control test, SC5b-9 values of unknown samples and their corresponding %CV and SD will be copied and pasted into a master excel data sheet in the sequential order on sheet 1 in excel workbook. SC5b-9 values, %CV, SD of quality control samples and proficiency samples will be pasted on sheet 2. Both sheet 1 & 2 will be updated with addition of data from each run.

- The master data sheet that will go to coordinating center will contain the following information
  1. Sample ID (Bio Con ID)
  2. Date of the assay
  3. Platform and Instrument# used for the assay
  4. Reagent lot numbers
  5. % CV across the sample replicates

## APPENDIX (Data processing steps used in Dr. Erwin Bottinger's laboratory)

### 3. Data Capture and analysis

#### 3.1 Reading the plate

- Use the Perkin Elmer 1420 Multilabel Counter and plate reader to read the color intensity of urine and standards to determine concentrations.
- The menu options for the reader are shown in screen shots below

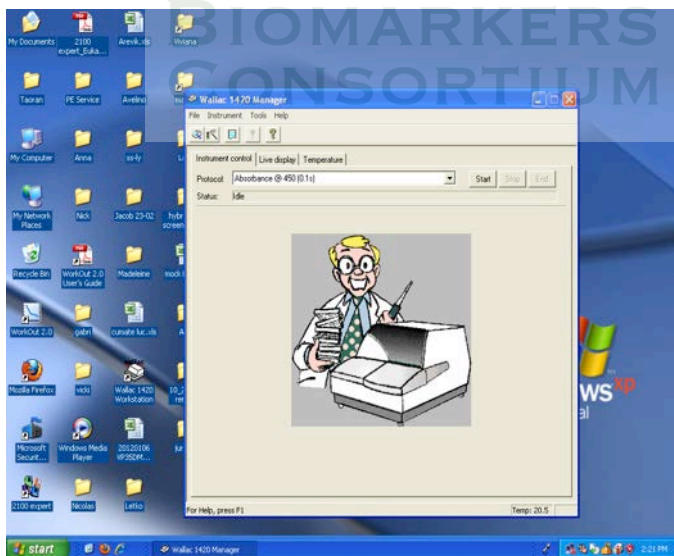


Figure 2. Find the Wallace 1420 operating software Manager. Go to Tools > Start Wizard





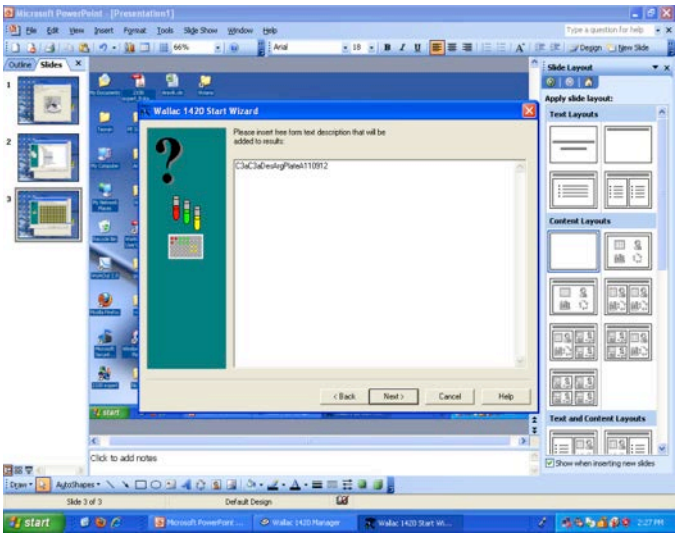
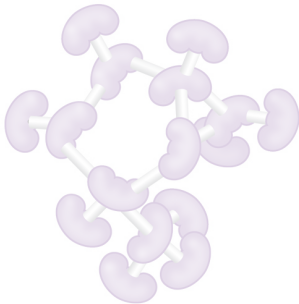


Figure 5. Can be left blank



Figure 6. Click Finish to start



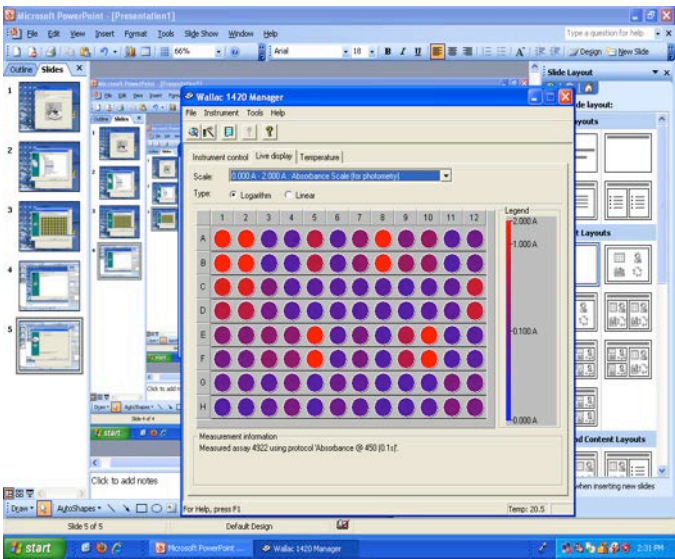


Figure 7. Results seen on the Live display tab.

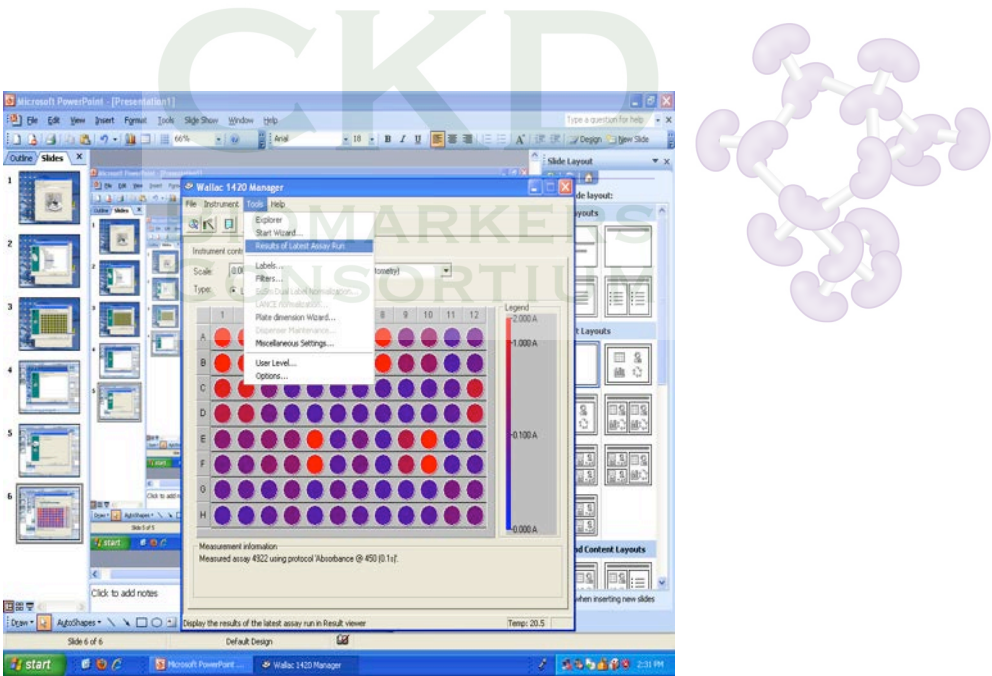


Figure 8. To retrieve the data use Tools tab and scroll to Results of Latest Assay Run

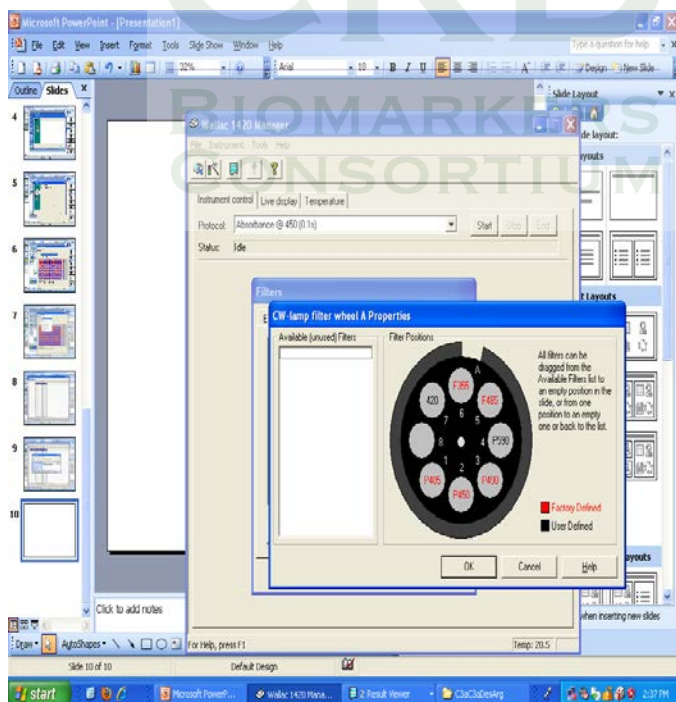


Figure 10. Confirmation that the filter for 450nm is present. For physical confirmation the wheel can be removed.



### **3.2 Preparing the Raw data for plotting and analysis**

- The data is saved in Excel in the form of a table reflecting the ELISA plate.
- The data has to be prepared for plotting before concentrations can be determined.
- The process has been expedited by recording a macro of all the steps required to prepare the data.
- The macro accomplishes the following very quickly and reproducibly:
  - Absorbance values of the standards are copied from the Raw data and pasted into an unused part of the sheet (from A2 – B9 to A15 – B22) and labeled as Std 1 and Std2 respectively (Arrow 1, red).
  - The average is determined of the duplicate values and recorded in the appropriate row (C15 – C22, Arrow 3, green).
  - The average of the Blank readings is copied to the cells D15-D22 (Arrow 4, light blue) for subtraction from the average concentration absorbance values (Oval 5, purple)
  - The corrected absorbance values for the standards is copied and pasted in an unused area (G15-G22, Circle blue/black)
  - Concentration values are left open to be inserted for the particular assay
  - The urine A450 values are duplicated vertically as shown in Figure 1 and run consecutively from left to right starting at C3 and C4 and ending at L8 and L9.
  - These data are copied one double row at a time (C3 and C4 to L3 and L4) and using the Paste Special, Transpose function are transferred to B30-B40 to transpose from row to column presentation.
  - Consecutive rows are added below until all the urine A450 data is in the columns B30-B70 (Arrow 2, dark blue).
  - The rows of duplicate values are labeled consecutively 1 – 40 titled Urine#
  - The data columns are labeled A450 1 and A450 2 respectively.
  - The average is determined and recorded alongside then highlighted and Paste Special, value back in the same column and labeled "Average".
  - The Blank or background average from above is recorded and repeated alongside the urine A450 data and labeled "Background" in preparation for subtraction.
  - Subtraction is done and reported in the next column as "Ave-Bckgrnd" and copied, Paste Special values i.e. only values are recorded.
  - To remove subzero data the following formula is placed into the top cell of cell G30 " $=IF(F30<0,0,F30)$ " and dragged down to G70. This leaves unchanged all positive numbers and zero but changes negative numbers to 0.
  - This final column G30-G70 provides the data for plotting and concentration determination along with the Std A450 average corrected values and the concentrations which have to be entered retrospectively into the worksheet.
  - Standard deviations greater than 20% are disregarded and those samples are put aside for reanalysis

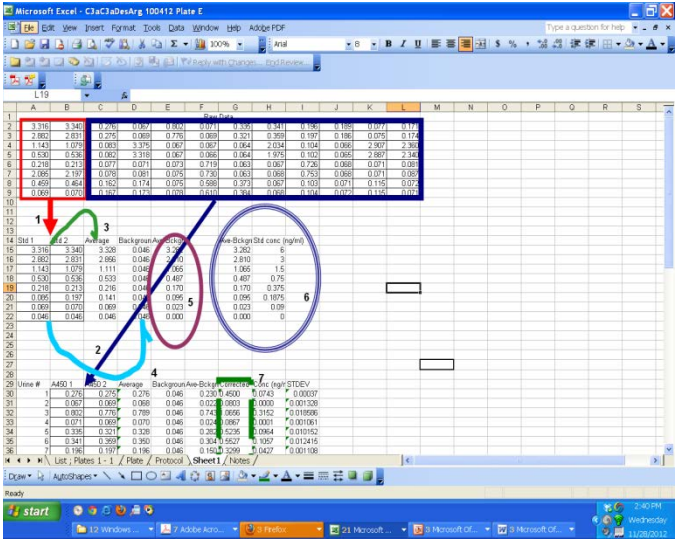


Figure 11. The Excel spreadsheet with the data showing the steps recorded in a macro for the purpose of preparing data for plotting.

### 3.3 Plotting the data

- The data is plotted using 4-parameter logistic curve fitting through the MasterPlex ReaderFit curve-fitting software for ELISA by Miraibio Group of Hitachi Software Engineering.
- The menu options and responses with the software are recorded for an example ELISA.

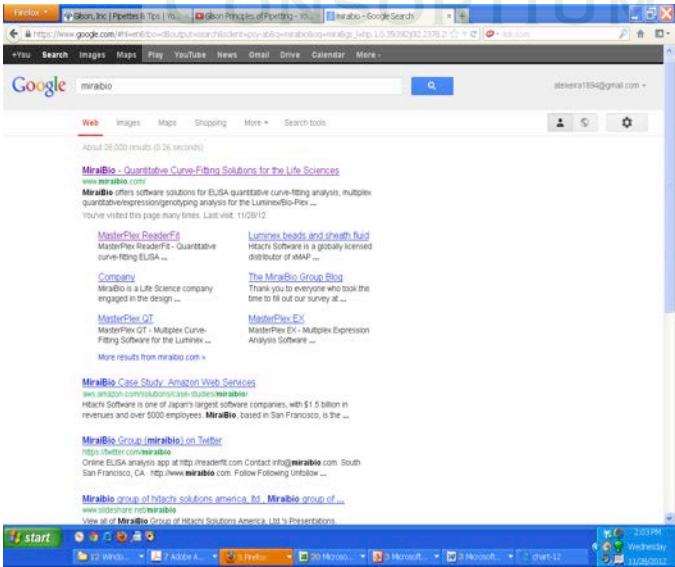


Figure 12. Locate the website through Google using MiraiBio as search term. Enter through the MiraiBio link.





Figure 13. Enter the ELISA Analysis Software, Curve-fitting (Online) ReaderFit.com tab

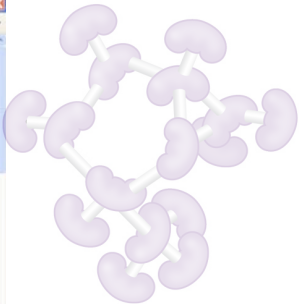
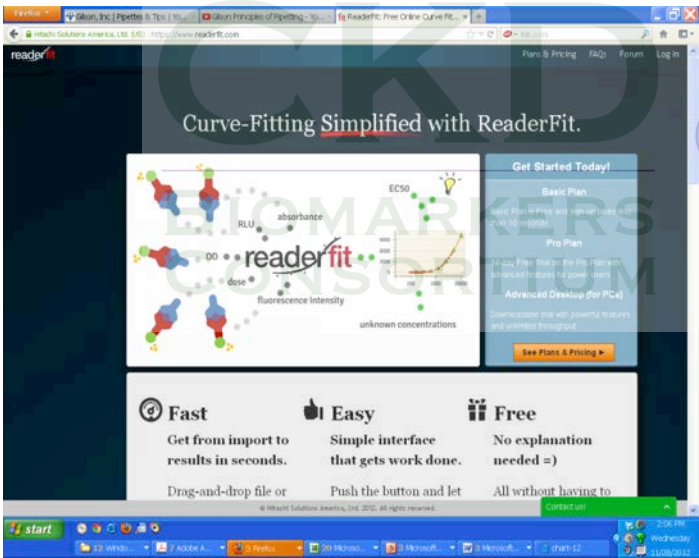


Figure 14. Log in at the ReaderFit page

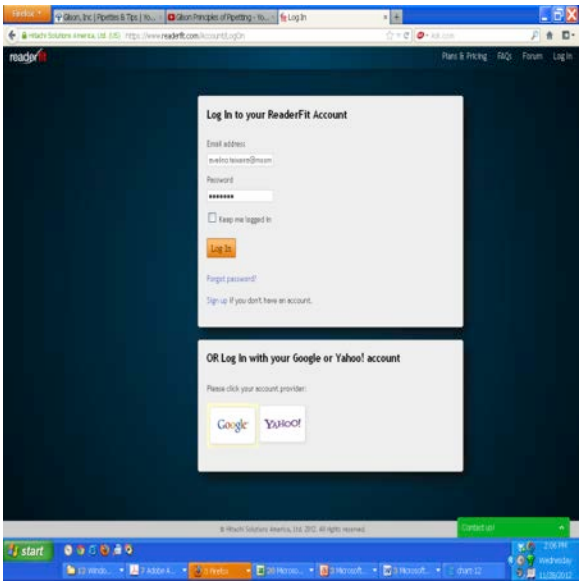


Figure 15. Login page

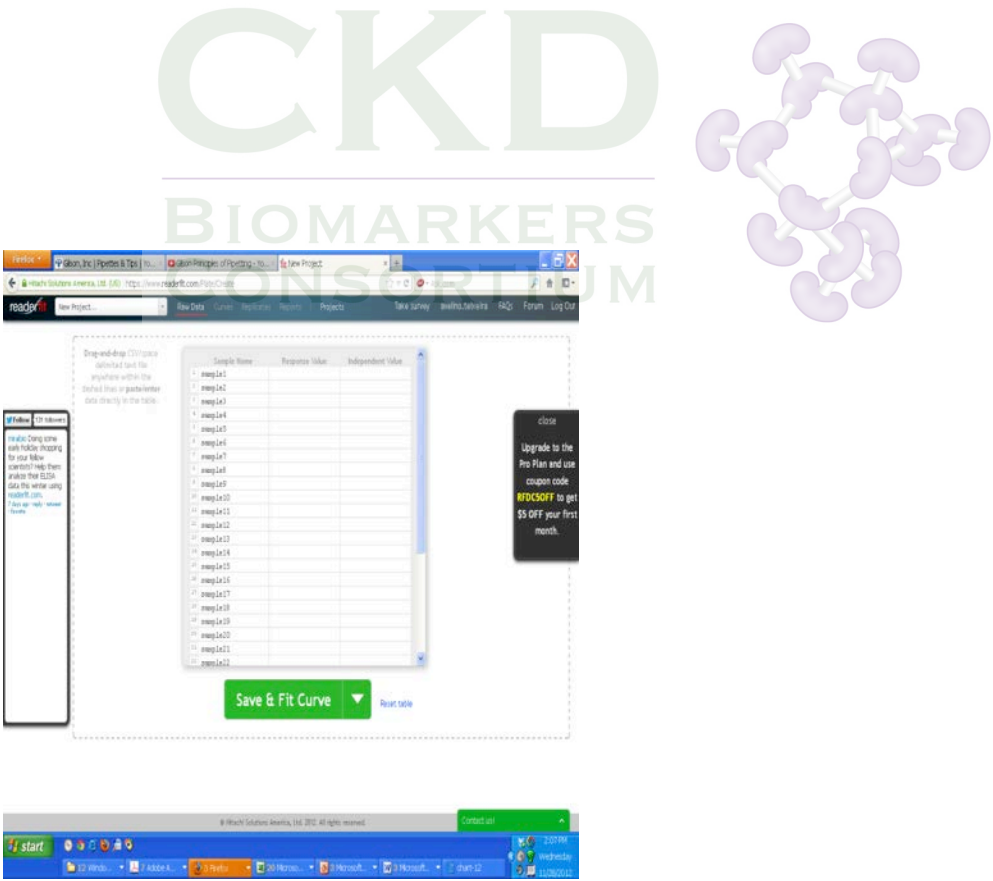


Figure 16. Opens to the data entry page.

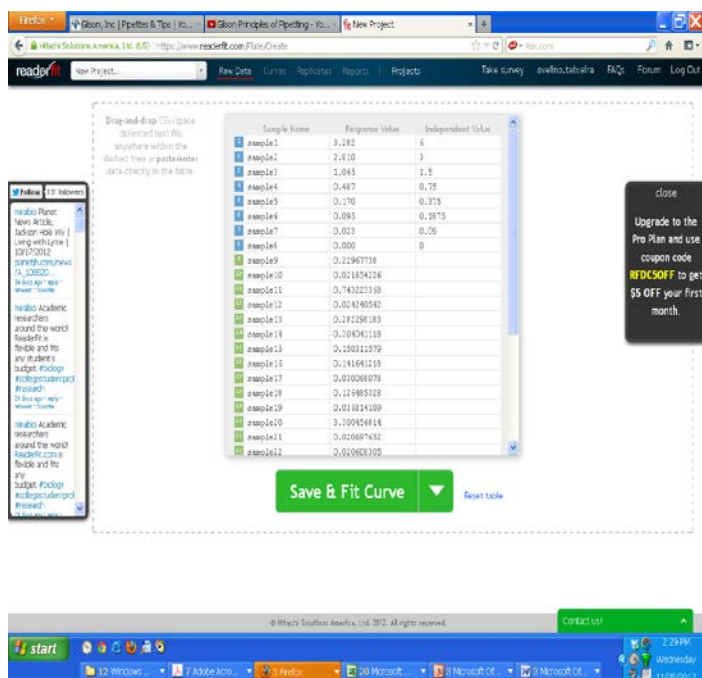


Figure 17.

- Copy from the prepared Excel file as shown in section 3.2 and paste the Standards A450 corrected averages (Response Values) and the Concentrations (Independent Values) into the top rows of the Raw Data table.
- Copy and paste and the concentrations into the Response Value column below.
- Only 32 entries are available for entry on the Free Software version of Readerfit
- The remaining entries are submitted in a second file.
- Click the green “Save and Fit Curve” button.

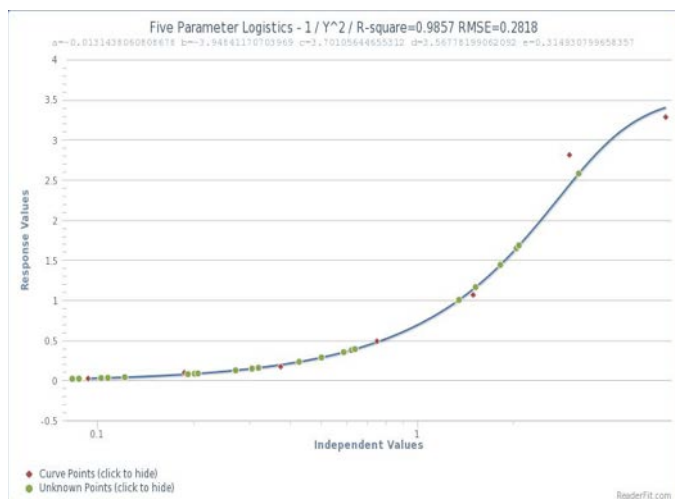


Figure 18 Resulant fitted curve showing the Standards in red and the unknowns in green

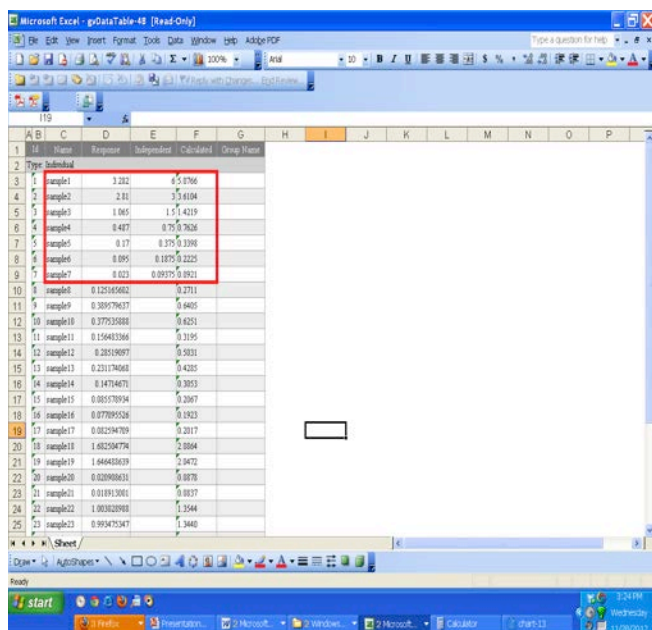


Figure 19

- Click on reports tab and Export to xls for the table shown above.
- The data highlighted in red outline above shows the response to the standards and closeness of fit of calculated data to the concentrations made on the bench.
- R-squared value and RMSE value of 1 and 0 respectively show ideal fit and large deviations from this indicate compromised standard curve and unreliable data.

### 3.4 Quality control of the data

- Coefficient of Variation (CV) is derived from the ratio of the standard deviation to the non-zero mean of duplicate concentration values, i.e. Coefficient of Variation **CV = Standard Deviation / Mean**. CV is expressed as percentage by multiplying CV by 100.
- For any sampled assay, a %CV value of more than 20% is a threshold for removal of the value and scheduling for repeat measurement of this sample in a subsequent assay.



**Translational Core Laboratory (TCL)**  
Institute for Translational Medicine and Therapeutics (ITMAT)  
Smilow Center for Translational Research  
Philadelphia, PA 19104

## Laboratory Protocol

### Quantitation of Creatinine in blood and urine

**Date:** 6 December 2012

**Version Number:** 1.2

**Brief Summary of Procedure:** This laboratory protocol describes the automated method to quantify creatinine in blood and urine samples using the automated analyzer from Roche Diagnostics (COBAS c501). Detailed specific information is provided that describes the reagent components of the assay, sample type(s) required for analysis, the nature of the measuring reaction and expected performance parameters. Included are expected values for imprecision, reference range estimation, and effect of interfering substances.

#### Method Review and Approval

Date of Review and Approval	Signatures	
	T.E. Mifflin, Ph.D.	S.R. Master, M.D., Ph.D.

## SOP Training and Awareness Signoff List

My signature below indicates that I have read and understand the contents of this document. If I locate or find errors and items to be corrected, I will notify my supervisor or the laboratory director as soon as possible.

### Distribution and Signoff List

Name of Laboratory Personnel	Training Date	SOP Version	Signature

Comment: \_\_\_\_\_



## Intended use

In vitro test for the quantitative determination of creatinine in human serum, plasma and urine on Roche **cobas c501** system.

## Summary<sup>1,2,3,4,5</sup>

Chronic kidney disease is a worldwide problem that carries a substantial risk for cardiovascular morbidity and death. Current guidelines define chronic kidney disease as kidney damage or glomerular filtration rate (GFR) less than 60 mL/min per 1.73 m<sup>2</sup> for three months or more, regardless of cause.

The assay of creatinine in serum or plasma is the most commonly used test to assess renal function. Creatinine is a break-down product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). It is freely filtered by the glomeruli and, under normal conditions, is not re-absorbed by the tubules to any appreciable extent. A small but significant amount is also actively secreted.

Since a rise in blood creatinine is observed only with marked damage of the nephrons, it is not suited to detect early stage kidney disease. A considerably more sensitive test and better estimation of glomerular filtration rate (GFR) is given by the creatinine clearance test based on creatinine's concentration in urine and serum or plasma, and urine flow rate. For this test a precisely timed urine collection (usually 24 hours) and a blood sample are needed. However, since this test is prone to error due to the inconvenient collection of timed urine, mathematical attempts to estimate GFR based only on the creatinine concentration in serum or plasma have been made. Among the various approaches suggested, two have found wide recognition: that of Cockcroft and Gault and that based on the results of the MDRD trial. While the first equation was derived from data obtained with the conventional Jaffé method, a newer version of the second is usable for IDMS-traceable creatinine methods. Both are applicable for adults. In children, the Schwartz formula is used.

In addition to the diagnosis and treatment of renal disease, the monitoring of renal dialysis, creatinine measurements are used for the calculation of the fractional excretion of other urine analytes (e. g., albumin,  $\alpha$ -amylase). Numerous methods were described for determining creatinine. Automated assays established in the routine laboratory include the Jaffé alkaline picrate method in various modifications, as well as enzymatic tests.

## Test principle<sup>6,7,8</sup>

This kinetic colorimetric assay is based on the Jaffé method. In alkaline solution, creatinine forms a yellow-orange complex with picrate. The rate of dye formation is proportional to the creatinine concentration in the specimen. The assay uses "rate-blanking" to minimize interference by bilirubin. To correct for non-specific reaction caused by serum/plasma pseudo-creatinine chromogens, including proteins and ketones, the results for serum or plasma are corrected by -26  $\mu\text{mol/L}$  (-0.3 mg/dL).



## Reagents - working solutions

**R1** Potassium hydroxide: 900 mmol/L; phosphate: 135 mmol/L; pH  $\geq$  13.5; preservative; stabilizer

**R2/R3** Picric acid: 38 mmol/L; pH 6.5; non reactive buffer

• Indicates **cobas c** systems on which reagents can be used

Order information			Roche/Hitachi <b>cobas c</b> systems	
			<b>cobas c</b> 311	<b>cobas c</b> 501/502
Creatinine Jaffé Gen.2				
700 tests	Cat. No. <b>04810716</b> 190	System-ID 07 6928 2	•	•
Calibrator f.a.s. (12 x 3 mL)	Cat. No. <b>10759350</b> 190	Code 401		
Calibrator f.a.s. (12 x 3 mL, for USA)	Cat. No. <b>10759350</b> 360	Code 401		
Precinorm U plus (10 x 3 mL)	Cat. No. <b>12149435</b> 122	Code 300		
Precinorm U plus (10 x 3 mL, for USA)	Cat. No. <b>12149435</b> 160	Code 300		
Precipath U plus (10 x 3 mL)	Cat. No. <b>12149443</b> 122	Code 301		
Precipath U plus (10 x 3 mL, for USA)	Cat. No. <b>12149443</b> 160	Code 301		
Precinorm U (20 x 5 mL)	Cat. No. <b>10171743</b> 122	Code 300		
Precipath U (20 x 5 mL)	Cat. No. <b>10171778</b> 122	Code 301		
Precinorm PUC (4 x 3 mL)	Cat. No. <b>03121313</b> 122	Code 240		
Precipath PUC (4 x 3 mL)	Cat. No. <b>03121291</b> 122	Code 241		
Diluent NaCl 9 % (50 mL)	Cat. No. <b>04489357</b> 190	System-ID 07 6869 3		

## Precautions and warnings

For in vitro diagnostic use.

Exercise the normal precautions required for handling all laboratory reagents.

Safety data sheet available for professional user on request.

Disposal of all waste material should be in accordance with local guidelines.

All human material should be considered potentially infectious. This includes human materials used for operation of the instrument such as calibrators and controls. All unknown samples must be considered to be potentially infectious as well. The infectious agents may include any one of the following: bacteria, molds, or viruses.

All products derived from human blood for use on the instrument are prepared exclusively from the blood of donors tested individually and shown to be free from HBsAg and antibodies to HCV and HIV. The infectious agent testing methods applied were FDA-approved or cleared in compliance with the European Directive 98/79/EC, Annex II, List A. However, as no testing

method can rule out the potential risk of infection with absolute certainty, the material should be treated just as carefully as a patient specimen. In the event of exposure the directives of the responsible health authorities should be followed.<sup>10,11</sup>

The practice of Universal Precautions must therefore be followed whenever any human – sourced material is handled, either for instrument operation or for eventual analysis. This includes all serum, plasma, urine and CSF samples that are to be analyzed.

This kit contains components classified as follows according to the European directive 1999/45/EC.



**C** – Corrosive. R1 contains potassium hydroxide.

R 1: Explosive when dry. R 4: Forms very sensitive, explosive metallic compounds. R 34: Causes burns.

S 24-25: Avoid contact with skin and eyes. S 26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S 35: This material and its container must be disposed of in a safe way.

S 36/37/39: Wear suitable protective clothing, gloves and eye/face protection.

S 45: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Contact phone: all countries: +49-621-7590, USA: +1-800-428-2336

Reagent handling

Ready for use.

## Storage and stability

*CREJ2*

Shelf life at 15-25 °C: See expiration date on **cobas c** pack label.

On-board in use and refrigerated on the analyzer: 8 weeks

*Diluent NaCl 9 %*

Shelf life at 2-8 °C: See expiration date on **cobas c** pack label.

On-board in use and refrigerated on the analyzer: 12 weeks

## Specimen collection and preparation<sup>9</sup>

For specimen collection and preparation, only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.

### Serum.

### Plasma: Li-heparin and K<sub>2</sub>-EDTA plasma.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

**Urine.**

Collect urine without using additives. If urine must be collected with a preservative for other analytes, only hydrochloric acid (14 to 47 mmol/L urine, e.g. 5 mL 10 % HCl or 5 mL 30 % HCl per liter urine) or boric acid (81 mmol/L, e.g. 5 g per liter urine) may be used.

**Stability in SAMPLES**

Stability in *serum/plasma*:<sup>10</sup>

7 days at 15-25 °C

7 days at 2-8 °C

3 months at (-15)-(-25) °C

Stability in *urine* (without preservative):<sup>10</sup>

2 days at 15-25 °C

6 days at 2-8 °C

6 months at (-15)-(-25) °C

Stability in *urine* (with preservative):<sup>11</sup>

3 days at 15-25 °C

8 days at 2-8 °C

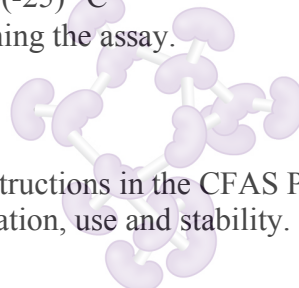
3 weeks at (-15)-(-25) °C

Centrifuge samples containing precipitates before performing the assay.

Materials provided

See "Reagents - working solutions" section for reagents.

**Calibrator.** Use Roche CFAS Calibrator. Follow the instructions in the CFAS Product Insert attached as an Appendix for information regarding preparation, use and stability.



**Assay**

For optimum performance of the assay, follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

**Application for serum and plasma****cobas c 501/502 test definition**

Assay type	Rate A		
Reaction time / Assay points	10 / 42-52 - 24-34 (STAT 4 / 17-27)		
Wavelength (sub/main)	570/505 nm		
Reaction direction	Increase		
Units	μmol/L (mg/dL, mmol/L)		
Reagent pipetting		Diluent (H <sub>2</sub> O)	
R1	13 μL	77 μL	
R3	17 μL	30 μL	
<i>Sample volumes</i>	<i>Sample</i>	<i>Sample dilution</i>	
		<i>Sample</i>	<i>Diluent (NaCl)</i>
Normal	10 μL	—	—
Decreased	10 μL	20 μL	80 μL
Increased	10 μL	—	—

Enter the correction value for the non-specific protein reaction as the instrument factor  $y = ax + b$  for mg/dL or for μmol/L, where **a = 1.0** and **b = -0.3** (mg/dL) or **a = 1.0** and **b = -26** (μmol/L).

**Application for urine****cobas c 501/502 test definition**

Assay type	Rate A	
Reaction time / Assay points	10 / 42-52 - 24-34 (STAT 4 / 17-27)	
Wavelength (sub/main)	570/505 nm	
Reaction direction	Increase	
Units	μmol/L (mg/dL, mmol/L)	
Reagent pipetting	Diluent (H <sub>2</sub> O)	
R1	13 μL	77 μL
R3	17 μL	30 μL

<i>Sample volumes</i>	<i>Sample</i>	<i>Sample dilution</i>	
		<i>Sample</i>	<i>Diluent (NaCl)</i>
Normal	10 μL	6 μL	144 μL
Decreased	10 μL	2 μL	180 μL
Increased	10 μL	10 μL	115 μL
Calibration	S1: H <sub>2</sub> O S2: C.f.a.s.		
Calibrators			
Calibration mode	Linear		
Calibration frequency	2-point calibration after reagent lot change and as required following quality control procedures		

Traceability: This method has been standardized against ID/MS.

For the USA, this method has been standardized against a primary reference material (SRM 914).

**Quality control**

For quality control, use Biorad Controls.

Other suitable control material can be used in addition.

*Serum/plasma*

For quality control, use the BioRad Controls. Follow the corresponding BioRad Product Inserts attached as Appendices for preparation, use and stability. Other suitable control material can be used in addition.

*Urine*

For quality control, use the BioRad Controls. Follow the corresponding BioRad Product Inserts attached as Appendices for preparation, use and stability.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the limits.

Follow the applicable government regulations and local guidelines for quality control.



**Quality Control Procedures:**

The recommended control requirements for the Roche COBAS c501 Creatinine assay is a single sample of each Bio-Rad control level to be tested at least once every 8 hours. It is also recommended that a single sample of each level of the assayed control be run during sample testing in the middle of the run and/or at the end of the sample run. Randox urine Chemistry control is tested at least once every 8 hours.

**List of Normal and Abnormal Controls:**

- Bio-Rad Lyphochek Assayed Chemistry Control levels 1 and 2 for serum Creatinine
- Bio-Rad Liquichek Unassayed Chemistry Control Levels 1 and 2 used for serum Creatinine run once a week.
- Bio-Rad Liquichek Urine Chemistry Control Levels 1 and 2 used for urine Creatinine
- Randox Assayed Urine Control Levels 1 and 2 used for urine Creatinine

The control values must be within the acceptable ranges specified in the control package insert. If a control is out of its specified limit, the associated test results are invalid and must be retested. The following steps must be followed during the troubleshooting process when an invalid control result is gotten.

**Quality Control Troubleshooting Procedure:**

1. In a stepwise manner, evaluate each assayed control material (serum or urine) to determine whether the creatinine values obtained using this Roche c501 method are within  $\pm 2SD$  limit listed by BioRad or RANDOX.
2. If both are within the stated  $\pm 2SD$  limits, accept the run and transfer the unknown results to the reporting worksheet
3. If one control's value is within the  $\pm 2SD$  limits and the other is outside, then follow the Westgard 2 Control evaluation algorithm depicted on the next page before deciding how to determine if the analysis run results can be accepted.
4. If neither of the assayed control creatinine results are within limits, then following the stepwise evaluation tree listed below the Westgard diagram on the next page.

Rule	Quality-Control Rule Explanation
1 <sub>2s</sub>	The 1 <sub>2s</sub> rule describes a situation where one control value for any of the two levels of control is outside the mean $\pm$ 2 SD.
1 <sub>3s</sub>	The 1 <sub>3s</sub> rule describes a situation where one control value for any of the two levels of control is outside the mean $\pm$ 3 SD.
2 <sub>2s</sub>	The 2 <sub>2s</sub> rule describes a situation where both control fluids or two consecutive control values of one fluid are outside the mean by either +2 SD or -2 SD.

Table 1. Quality-Control Rules

The three quality-control rules just described are used together when the mean value for the quality-control fluids has been established. The diagram below should help you better understand the three rules.

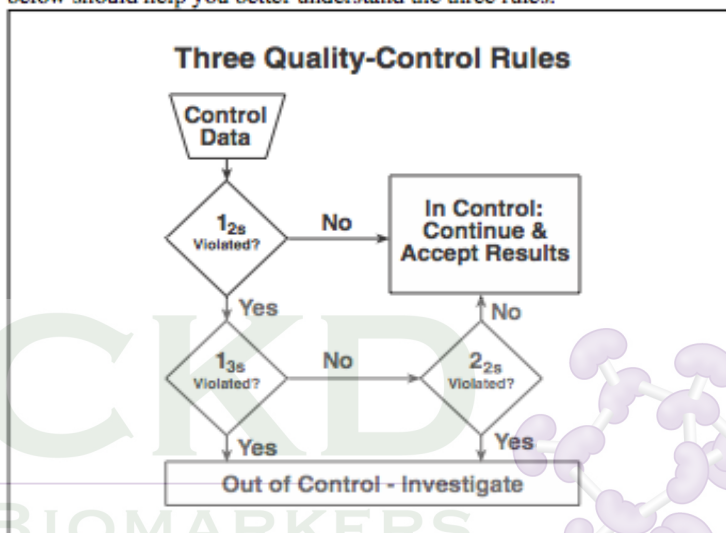


Figure 11. Three Quality-Control Rules

### STEPS to Follow if QC Values are Not Within Acceptable Limits

- Rerun controls
- If QC is within specified range document correction and proceed with sample testing.
- If QC is **Not** within specified range, document it, make up fresh QC fluid and rerun controls.
- If controls are within specified range, proceed with sample testing.
- If QC is **Not** within specified range, recalibrate the assay.
- If after calibration the QC is within specified range, proceed with testing and document correction.
- If QC is **Not** within specified range after calibration, place a fresh reagent on analyzer and recalibrate.
- If QC is within range after calibrating with fresh reagent, proceed with sample testing and document correction.
- If QC is **Not** within specified range after using fresh reagent and control fluid, do not proceed with sample testing. Check instrument maintenance to see if you can resolve the problem. If not, contact the Laboratory Director.

## Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors:  $\mu\text{mol/L} \times 0.0113 = \text{mg/dL}$

$\mu\text{mol/L} \times 0.001 = \text{mmol/L}$

## Limitations – interference<sup>12</sup>

Criterion: Recovery within  $\pm 10\%$  of initial value at a creatinine concentration of  $80 \mu\text{mol/L}$  ( $0.90 \text{ mg/dL}$ ) in serum/plasma and  $2500 \mu\text{mol/L}$  ( $28.3 \text{ mg/dL}$ ) in urine.

### Serum/plasma

Icterus (*CREJ2*): No significant interference up to an I index of 5 for conjugated bilirubin and 10 for unconjugated bilirubin (approximate conjugated bilirubin concentration:  $86 \mu\text{mol/L}$  ( $5 \text{ mg/dL}$ ) and approximate unconjugated bilirubin concentration:  $171 \mu\text{mol/L}$  ( $10 \text{ mg/dL}$ )).

Icterus (*SCRE2*): No significant interference up to an I index of 2 for conjugated bilirubin and 3 for unconjugated bilirubin (approximate conjugated bilirubin concentration:  $34 \mu\text{mol/L}$  ( $2 \text{ mg/dL}$ ) and approximate unconjugated bilirubin concentration:  $51 \mu\text{mol/L}$  ( $3 \text{ mg/dL}$ )).

Hemolysis: No significant interference up to an H index of 1000 (approximate hemoglobin concentration:  $621 \mu\text{mol/L}$  ( $1000 \text{ mg/dL}$ )).

Lipemia (Intralipid): No significant interference up to an L index of 800. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Drugs: No interference was found at therapeutic levels using common drug panels.<sup>13,14</sup>

Exception: Cefoxitin causes artificially high creatinine results.

Exception: Cyanokit (Hydroxocobalamin) may cause interference with results.

Values  $< 15 \mu\text{mol/L}$  ( $< 0.17 \text{ mg/dL}$ ) or negative results are reported in rare cases in children  $< 3$  years and in elderly patients. In such cases use the Creatinine plus test to assay the sample.

Do not use Creatinine Jaffé for the testing of creatinine in hemolyzed samples from neonates, infants or adults with HbF levels  $\geq 60 \text{ mg/dL}$  for *CREJ2* applications ( $\geq 30 \text{ mg/dL}$  for *SCRE2* applications).<sup>15</sup> In such cases, use the Creatinine plus test ( $\leq 600 \text{ mg/dL}$  HbF) to assay the sample.

Estimation of the Glomerular Filtration Rate (GFR) on the basis of the Schwartz Formula can lead to an overestimation.<sup>16</sup>

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.

### Urine

Icterus: No significant interference up to a conjugated bilirubin concentration of  $855 \mu\text{mol/L}$  ( $50 \text{ mg/dL}$ ).

Hemolysis: No significant interference up to a hemoglobin concentration of  $621 \mu\text{mol/L}$  ( $1000 \text{ mg/dL}$ ).

Glucose  $< 120 \text{ mmol/L}$  ( $< 2162 \text{ mg/dL}$ ) and urobilinogen  $< 676 \mu\text{mol/L}$  ( $< 40 \text{ mg/dL}$ ) do not interfere.

Drugs: No interference was found at therapeutic levels using common drug panels.<sup>14</sup>

Exception: Cyanokit (Hydroxocobalamin) may cause interference with results.

High homogentisic acid concentrations in urine samples lead to false results.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

**ACTION REQUIRED**

**Special Wash Programming:** The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. The latest version of the Carry over evasion list can be found with the NaOHD/SMS/Multiclean/SCCS or the NaOHD/SMS/SmpCln1 + 2/SCCS Method Sheets. For further instructions refer to the operator manual.

**cobas c 502 analyzer:** All special wash programming necessary for avoiding carry over is available via the **cobas** link, manual input is not required.

**Where required, special wash/carry over evasion programming must be implemented prior to reporting results with this test.**

Limits and ranges

**Measuring range***Serum/plasma*

15-2200  $\mu\text{mol/L}$  (0.17-24.9 mg/dL)

The technical limit in the instrument setting is defined as 0.47-25.2 mg/dL due to the compensation factor of 0.3

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:5 dilution. Results from samples diluted by the rerun function are automatically multiplied by a factor of 5.

*Urine*

375-55000  $\mu\text{mol/L}$  (4.2-622 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:3.6 dilution. Results from samples diluted by the rerun function are automatically multiplied by a factor of 3.6.

**Lower limits of measurement***Lower detection limit of the test**Serum/plasma*

15  $\mu\text{mol/L}$  (0.17 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying three standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

*Urine*

375  $\mu\text{mol/L}$  (4.2 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying three standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

**Expected values***Serum/plasma**Adults*<sup>17</sup>

Females	44-80 $\mu\text{mol/L}$	(0.50-0.90 mg/dL)
Males	62-106 $\mu\text{mol/L}$	(0.70-1.20 mg/dL)

*Children*<sup>18</sup>

Neonates (premature)	25-91 $\mu\text{mol/L}$	(0.29-1.04 mg/dL)
Neonates (full term)	21-75 $\mu\text{mol/L}$	(0.24-0.85 mg/dL)
2-12 m	15-37 $\mu\text{mol/L}$	(0.17-0.42 mg/dL)
1- < 3 y	21-36 $\mu\text{mol/L}$	(0.24-0.41 mg/dL)
3- < 5 y	27-42 $\mu\text{mol/L}$	(0.31-0.47 mg/dL)
5- < 7 y	28-52 $\mu\text{mol/L}$	(0.32-0.59 mg/dL)
7- < 9 y	35-53 $\mu\text{mol/L}$	(0.40-0.60 mg/dL)
9- < 11 y	34-65 $\mu\text{mol/L}$	(0.39-0.73 mg/dL)
11- < 13 y	46-70 $\mu\text{mol/L}$	(0.53-0.79 mg/dL)
13- < 15 y	50-77 $\mu\text{mol/L}$	(0.57-0.87 mg/dL)

*Urine**1st morning urine*<sup>17</sup>

Females	2470-19200 $\mu\text{mol/L}$	(28-217 mg/dL)
Males	3450-22900 $\mu\text{mol/L}$	(39-259 mg/dL)

*24-hour urine*<sup>19</sup>

Females	7000-14000 $\mu\text{mol/24 h}$	(740-1570 mg/24 h)
Males	9000-21000 $\mu\text{mol/24 h}$	(1040-2350 mg/24 h)

Creatinine clearance<sup>19,20</sup>

71-151 mL/min

Refer to reference 21 for a prospective study on creatinine clearance in children.<sup>21</sup>

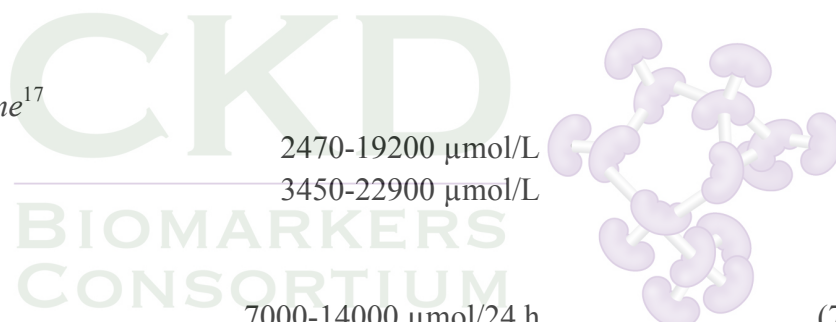
Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

## Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

**Precision**

Precision was determined using human samples and controls in an internal protocol. *Serum/plasma*: repeatability\* (n = 21), intermediate precision\*\* (3 aliquots per run, 1 run per day, 21 days); *Urine*: repeatability\* (n = 21), intermediate precision\*\* (3 aliquots per run, 1 run per day, 10 days). The following results were obtained:



*Serum/plasma (CREJ2)*

<i>Repeatability*</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	$\mu\text{mol/L (mg/dL)}$	$\mu\text{mol/L (mg/dL)}$	%
Precinorm U	105 (1.19)	2 (0.03)	2.1
Precipath U	360 (4.07)	4 (0.05)	1.1
Human serum 1	206 (2.33)	3 (0.03)	1.2
Human serum 2	422 (4.77)	5 (0.06)	1.3

<i>Intermediate precision**</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	$\mu\text{mol/L (mg/dL)}$	$\mu\text{mol/L (mg/dL)}$	%
Precinorm U	101 (1.14)	4 (0.05)	3.5
Precipath U	351 (3.97)	8 (0.09)	2.2
Human serum 3	201 (2.27)	5 (0.06)	2.5
Human serum 4	411 (4.64)	9 (0.10)	2.2

*Urine (CRJ2U)*

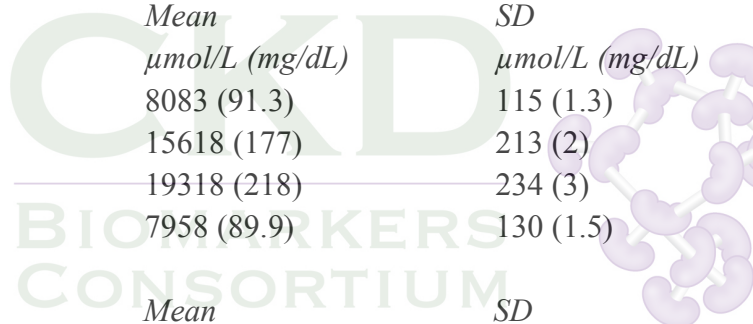
<i>Repeatability*</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	$\mu\text{mol/L (mg/dL)}$	$\mu\text{mol/L (mg/dL)}$	%
Control Level 1	8083 (91.3)	115 (1.3)	1.4
Control Level 2	15618 (177)	213 (2)	1.4
Human urine 1	19318 (218)	234 (3)	1.2
Human urine 2	7958 (89.9)	130 (1.5)	1.6

<i>Intermediate precision**</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	$\mu\text{mol/L (mg/dL)}$	$\mu\text{mol/L (mg/dL)}$	%
Control Level 1	8130 (91.9)	164 (1.9)	2.0
Control Level 2	15533 (176)	251 (3)	1.6
Human urine 3	19353 (219)	385 (4)	2.0
Human urine 4	7932 (89.6)	166 (1.9)	2.1

*Serum/plasma (SCRE2)*

<i>Repeatability*</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	$\mu\text{mol/L (mg/dL)}$	$\mu\text{mol/L (mg/dL)}$	%
Precinorm U	106 (1.20)	2 (0.02)	2.2
Precipath U	346 (3.91)	5 (0.06)	1.5
Human serum 1	543 (6.14)	6 (0.07)	1.1
Human serum 2	69 (0.78)	2 (0.02)	3.1

<i>Intermediate precision**</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	$\mu\text{mol/L (mg/dL)}$	$\mu\text{mol/L (mg/dL)}$	%
Precinorm U	100 (1.13)	4 (0.05)	4.0
Precipath U	334 (3.77)	10 (0.11)	3.0





Human serum 3	522 (5.90)	12 (0.14)	2.4
Human serum 4	64 (0.72)	3 (0.03)	5.0

*Urine (SCR2U)*

<i>Repeatability*</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	$\mu\text{mol/L (mg/dL)}$	$\mu\text{mol/L (mg/dL)}$	%
Control Level 1	6287 (71.0)	82 (0.9)	1.2
Control Level 2	15252 (172)	182 (2)	1.2
Human urine 1	24174 (273)	212 (2)	0.9
Human urine 2	2146 (24.2)	48 (0.5)	2.2

<i>Intermediate precision**</i>	<i>Mean</i>	<i>SD</i>	<i>CV</i>
	$\mu\text{mol/L (mg/dL)}$	$\mu\text{mol/L (mg/dL)}$	%
Control Level 1	6943 (78.5)	114 (1.3)	1.6
Control Level 2	15394 (174)	229 (3)	1.5
Human urine 3	24230 (274)	354 (4)	1.5
Human urine 4	2184 (24.7)	54 (0.6)	2.5

\* repeatability = within-run precision

\*\* intermediate precision = total precision / between run precision / between day precision

**Method comparison**

Creatinine values for human serum, plasma and urine samples obtained on a Roche/Hitachi **cobas c** 501 analyzer (y) were compared with those determined on Roche/Hitachi 917/MODULAR P analyzers (x), using the corresponding Roche/Hitachi reagent.

*Serum/plasma (CREJ2)*

Sample size (n) = 273

Passing/Bablok<sup>22</sup>

$$y = 1.000x - 0.653 \mu\text{mol/L}$$

$$\tau = 0.973$$

Linear regression

$$y = 1.002x - 0.978 \mu\text{mol/L}$$

$$r = 0.999$$

The sample concentrations were between 38 and 2178  $\mu\text{mol/L}$  (0.429 and 24.6 mg/dL).

*Urine (CRJ2U)*

Sample size (n) = 223

Passing/Bablok<sup>22</sup>

$$y = 0.999x + 20.7 \mu\text{mol/L}$$

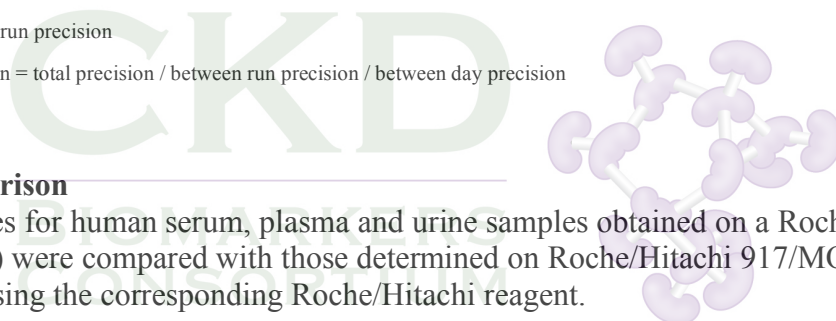
$$\tau = 0.969$$

Linear regression

$$y = 0.999x + 41.5 \mu\text{mol/L}$$

$$r = 0.999$$

The sample concentrations were between 934 and 50228  $\mu\text{mol/L}$  (10.6 and 568 mg/dL).



*Serum/plasma (SCRE2)*

Sample size (n) = 224

Passing/Bablok<sup>22</sup>

$$y = 1.000x - 14.4 \mu\text{mol/L}$$

$$\tau = 0.964$$

Linear regression

$$y = 0.996x - 12.2 \mu\text{mol/L}$$

$$r = 0.999$$

The sample concentrations were between 66 and 1775  $\mu\text{mol/L}$  (0.746 and 20.1 mg/dL).

*Urine (SCR2U)*

Sample size (n) = 223

Passing/Bablok<sup>22</sup>

$$y = 0.999x + 67.8 \mu\text{mol/L}$$

$$\tau = 0.973$$

Linear regression

$$y = 0.998x + 113 \mu\text{mol/L}$$

$$r = 0.999$$

The sample concentrations were between 931 and 48729  $\mu\text{mol/L}$  (10.5 and 551 mg/dL).

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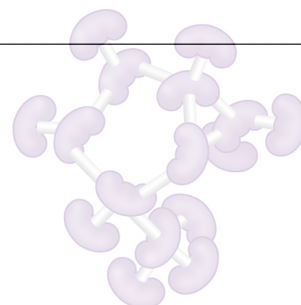
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Alternative method

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CKD  
BIOMARKERS  
CONSORTIUM



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Version 11

2010-07

Reagent manufacturer

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim

[www.roche.com](http://www.roche.com)

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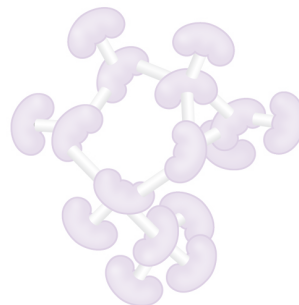
Roche Diagnostics, Indianapolis, IN

US Customer Technical Support: 1-800-428-2336

Source document

Reagent Name: CREJ2

Package Insert Version: 2010-04 V11 English



*Reviewed By: T.E. Mifflin, Ph.D.*

*Date Reviewed: 6 December 2012*

**University of Minnesota  
Advanced Research Diagnostic Laboratory**

<b>Entity:</b>	<b>University of Minnesota</b>
<b>Department:</b>	<b>Advanced Research and Diagnostic Laboratory</b>
<b>Manual:</b>	<b>ARDL Procedure Manual</b>
<b>Procedure:</b>	<b>CYSTATIN C using ROCHE/HITACHI MODULAR P CHEMICAL ANALYZER</b>

### **INTRODUCTION**

Cystatin C is a 120 amino acid, nonglycated basic protein of the cystatin superfamily of cysteine proteinase inhibitors. All investigated human nucleated cells produce it, its rate of production does not change during inflammatory states, and it is present in all investigated body fluids. Cystatin C is primarily catabolized by renal glomerular filtration followed by renal tubular reabsorption and degradation of the protein. The low molecular mass of cystatin C, coupled with its steady cellular production, indicates that its plasma concentration is directly determined by the glomerular filtration rate (GFR), making cystatin C an excellent indicator of GFR. Diminished GFR is indicated by an increase in the plasma cystatin C concentration. Serum creatinine concentration is also a GFR indicator, but studies have shown cystatin C to have greater diagnostic accuracy.

Modular P application code: 313

Manual worksheet code: N/A

### **PRINCIPLE**

Serum is mixed with cystatin C immunoparticles. Cystatin C present in the sample binds to the antibody bound to the particles, and aggregation occurs. The formed complexes absorb light, and by turbidimetry the absorption is related to cystatin C concentration via interpolation on an established standard calibration curve. Light absorption is measured at 546 nm (secondary wavelength = 700 nm). Rheumatoid factor does not interfere in this assay because the cystatin C antibody is from avian source.

### **SPECIMEN**

Use human serum for the procedure. The manufacturer states EDTA plasma and heparin plasma are acceptable but have not been validated by this laboratory. Centrifuge samples for 10 minutes at 1000g. Serum is stable for 14 days at room temperature, 21 days at 2°C-8°C, and for at least ten years at -20°C. Samples can be shipped without special cooling, but must be analyzed within 14 days of receipt.

Bilirubin does not interfere up to 80 mg/dL. Hemolysis does not interfere up to 700 mg/dL. Lipemia does not interfere up to a triglyceride level of 1100 mg/dL.

Minimum volume: 100 uL (includes dead volume)

## **EQUIPMENT AND SUPPLIES**

1. Roche Modular P chemistry analyzer. Roche Diagnostics, 9115 Hague Road, Indianapolis, IN 46250.
2. Cell Wash Solution II/Acid Wash. Roche product #4880307 (2L bottle). No preparation required. Solution of formic acid, citric acid and nikkol BT-9. Store at room temperature. Stable until expiration date on bottle. No stability time window after opening. This solution is automatically drawn by the Mod P while cleaning reaction cuvettes during analysis.
3. Cell Wash Solution I/NaOH-D. Roche product #1551540 (1800 mL bottle). No preparation required. Solution of sodium hydroxide (1N). Store at room temperature. Stable until expiration date on bottle. After opening a bottle it is stable for 14 days on the instrument. This solution is automatically drawn by the Mod P while cleaning reaction cuvettes during analysis.
4. Reaction cell cuvette segments. Roche product #714-0650 (Four sets of eight segments. Eight segments complete the entire rotor). Soak cuvettes overnight in a solution of 2% Hitergent before installing on the instrument. Perform cell wash and cell blank functions after installation. Change cuvettes quarterly.
5. Hitergent. Roche product #409149 (1L bottle). No preparation required. Solution of ethanolamine, hexahydro-1,3,5-tris (Betahydroxyethyl) triazine and nonidet P-40. Store at room temperature. Stable until expiration date on bottle. Hitergent is an on-board reagent automatically drawn by the Mod P during the daily incubator bath exchange. Hitergent is transferred, as needed, from the 1L bottle to the 66 mL bottle located in position 2D3.
6. Sample cups (micro). Roche product #11406680001.
7. Sample cups (standard). Roche product #729177.

## **REAGENTS**

1. Gentian Cystatin C Reagent Kit (Gentian AS, PO Box 733, N-1509, Moss, Norway), reference #1101:
  - R1 reagent (1 x 58 mL). MOPS buffered saline, sodium azide. See insert for concentrations. No preparation required. There are approximately 250 tests per bottle.
  - R2 reagent (1 x 10 mL). Purified immunoglobulin fraction that is directed against cystatin C. The antibody is covalently bound to uniform polystyrene particles. Human cystatin C was used as the immunogen in the process of generating the immunoparticles. The particle suspension is preserved in a solution of 15 mmol/L sodium azide and antibiotics. No preparation required. There are approximately 250 tests per bottle.
  - Storage and stability. Keep reagents stored in refrigerator until use. R1 is stable for 63 days refrigerated on the analyzer. R2 is stable for 63 days refrigerated on the analyzer.
  - Since these reagent bottles use "generic" barcodes, the location of the reagents must be manually assigned on the Mod P. Roche reagents have a unique barcode that codes for the test parameters, so they do not require manual assignment. The steps for assigning the reagent positions are as follows:
    1. With Mod P in Standby mode, click on the Reagent tab in the home screen.



2. Click on the Setting tab.
  3. Click on an open reagent position. Select a position number that has an opening in the R1 rotor and in the R2 rotor.
  4. Click on Manual Registration at the bottom of the screen.
  5. Click on the "Test" radio button.
  6. Select CYSC in the Test dropdown menu.
  7. Under Reagent Type, choose R1 for reagent 1, and choose R3 for reagent 2.
  8. Under Bottle Size, choose C(70 mL) for reagent 1, and choose B(20 mL) for reagent 2.
  9. A third reagent bottle, containing saline, must also be assigned to an open position as described above. The saline is used as a diluent for the reagent and for elevated specimens.
  10. Select OK when completed.
- As the reagent is consumed, periodically add additional reagent from the kit to the assigned generic bottles. The bottles do not have to be exchanged when reagent is added. However, if a new reagent lot is started, new bottles should be installed at that time.
2. Milli-Q water. Milli-Q is the trade name of the water system purchase from the Millipore Corporation. Milli-Q is deionized water treated with activated carbon and deionization cartridges and filtered to remove microorganisms larger than 0.22 micrometers. This meets CAP class I water requirements.
  3. Gentian cystatin C control kit. Gentian AS, PO Box 733, N-1509 Moss, Norway. Two levels, low and high. Store at 2-8°C until use; stable until expiration date on package. Controls require no preparation prior to use.
  4. Pooled Normal Serum control. Solomon Park Research Laboratories, 12815 NE 124th St. Suite 1, Kirkland, WA 98034. Store frozen at -70°C. Once thawed, product is stable for 7 days at 2-8 °C. Allow the control to reach room temperature (18-25° C) and mix thoroughly by inversion or gentle vortex before use.

**CALIBRATION**

Gentian Cystatin C Calibrator, reference #1012, 1 x 1 mL. The calibrator is stable until the expiration date on the bottle when stored at 4°C. The calibrator requires no preparation, and is ready for immediate use.

The calibrator is standardized against the international calibrator standard ERM-DA47/IFCC.

Calibration frequency: Calibration must be performed with each new reagent lot or shipment, every six months and as needed if control values deviate or after major maintenance or repair. Monitor control values to determine stability of the current calibration.

**Caution: This product is of human and animal origin. Handle as though capable of transmitting infectious disease.**

## **INSTRUMENT SET-UP**

1. Log into the Mod P using assigned username and password.
2. Reagents. All reagents used on the Mod P are stored in a refrigerated reagent compartment. Cystatin C is a three-reagent system. Reagent 1 must be placed in the outer (R1) rotor; reagent 2 must be placed in the inner (R2) rotor. Reagent 3 (saline) can be assigned in either rotor. Before starting the analysis sequence check the reagent status on the Mod P to confirm there is adequate reagent to complete the anticipated test volume for the day. Replenish any bottles that have low volume.
3. Maintenance. Complete the scheduled daily maintenance as described in the Mod P general operations protocol.
4. Order calibration, if indicated (see Mod P general operations protocol).
5. Order controls. If a calibration was requested, the controls should not be ordered until the calibration report has printed. If the controls are ordered and executed before the calibration prints out, the controls will be measured on the previously stored calibration line.

## **QUALITY CONTROL**

Three levels of control are assayed each time the cystatin C method is performed. It is acceptable to run each control at the start of the day, and again at the end of the day. Review Levey-Jennings plots and acceptability criteria for each quality control result daily before reporting results. The operator may run them more frequently, if desired. One control is prepared from pooled, normal human serum. The others are commercial controls of varying concentrations. Consult quality control charts for current ranges and lots in use. If QC values violate the Westgard Rules 1-3s or 2-2s, perform and document the following corrective actions steps until QC is acceptable:

1. Check reagent and quality control for appropriate lot numbers, expiration dates, preparation and storage conditions.
2. Repeat the analysis using fresh QC material.
3. Perform a calibration.
4. Perform maintenance procedures.
5. If quality control continues to fall outside of the established acceptability limits discontinue testing and notify the supervisor.

In January 2012 this method, calibrated with the Gentian calibrator, was evaluated by assaying the ERM-DA47/IFCC reference material. This standard has an assigned value of 5.48 mg/L. Eight replicates assayed over four days yielded an average value of 5.62 mg/L. This reference material should be measured periodically to assure method accuracy.

## **PROCEDURE**

After calibration and controls have been measured and evaluated, the test specimens may be loaded onto the Mod P. An abbreviated description of the measurement procedure follows. A more thorough description may be found in the Mod P general operations protocol.

1. If specimens have been frozen, allow them to thaw completely, then mix well. Serum specimens should not require centrifugation unless they have visible suspended particles or clots.
2. To order non-barcoded tests on the Mod P:
  - a. <Workplace>
  - b. <Test Selection>
  - c. Enter specimen ID in the Sample ID field, then <Enter>
  - d. Select test CYSC by touching the screen or clicking on it with the mouse.
  - e. <Barcode Read Error>
  - f. Enter the rack number and rack position in the Rack No.-Pos. fields.
  - g. <Add>
  - h. <OK>
  - i. <Save>
3. Note the order of positions 1-5 in the sample rack: position 1 is on the right and position 5 is on the left. Place the specimen in the rack so that ¼ to 1 inch of the vial is above the sample rack. This allows the Mod P to detect the presence of the vial in the rack. Orient the vial in the rack so that any barcodes are turned inward, and therefore unreadable. If the testing vials are to be re-capped, arrange the caps so they can be matched up following analysis.
4. To order barcoded tests on the Mod P: Follow instructions as in step 3 above, except that the barcode now must face outward so the Mod P can read it. The barcode must be oriented vertically. No test ordering is required on the instrument. In this case test ordering has occurred in Misys, and a label has been generated for that purpose, or the specimen has a non-Misys barcode label and a user-defined default battery has been installed on the Mod P.
5. After the specimens are in place, put the racks onto the loading platform. The racks will only load in one orientation, as the center track is offset. Do not prepare more than three racks at a time, as evaporation could occur while the instrument goes through the sampling process.
6. Close the cover on the loading platform.
7. On the Mod P computer terminal, press or click <Start>, then <Start> again.
8. Only calibration and control data automatically print out. Patient data hard copies must be requested in <Workplace>, <Data Review>. Highlight the desired records, then <Print>, and <Print> again.
9. Non-barcoded records must be manually entered into Misys, a designated spreadsheet, or website.
10. Barcoded records are accepted using the OEM program in Misys. The method code for the Mod P is UR9.

### **INSTRUMENT SHUT DOWN**

After the patient specimens and final controls have been evaluated and accepted, load the green rack (W999) and run it through the instrument. Place three standard sample cups in positions 1, 2 and 3. Fill cup 1 with 1N sodium hydroxide, fill cup 2 with 4N sodium hydroxide, and fill cup 3 with leftover serum. Place it onto the loading platform and press <Start>, and <Start> again. After 18 minutes, the Mod P comes to Stand-by status. If the green rack is not run, the Mod P will take at least one hour to come to Stand-by status.

After coming to Stand-by status the data from each day's run is downloaded from the Mod P computer to a diskette, then to the network folder. Consult the procedure describing this process for details.

Print all Mod P test results, and file in chronological order with the other daily printouts.

The Mod P is turned off each day after all work is complete. The steps are as follows: <Utility>, <Maintenance>, <Nightly Pipe>, <Select>, <Execute>. This shutdown process requires approximately five minutes. The instrument and its computer are automatically turned off. The reagent compartment remains refrigerated.

An automatic timer has been set so that the Mod P turns on each weekday morning at 0530, automatically performing an air purge, photometer check, and incubator bath exchange during the process. The automatic timer has been set so that the Mod P remains off during weekends.

### **EXPECTED VALUES**

- Gentian ranges:  
Serum, adult: 0.51-1.05 mg/L  
Serum, 5-15 years: 0.51-1.05 mg/L
- Advanced Research and Diagnostic Laboratory ranges:  
Misys test code: N/A  
Serum, adult: 0.51-1.05 mg/dL  
The manufacture serum reference range was compared to results from 13,091 study participant samples in 2012 and 2013. A range of 0.60 – 1.34 mg/L representing the central 95% of the second was determined nonparametrically. Study participant age ranged from 48 – 67 years old and the health status is unknown to this laboratory, thus it is not surprising the central 95% is substantially higher than the manufacture reported “healthy subjects”.
- Linear range of the method: 0.32-8.00 mg/L (serum). Specimens exceeding the high limit are automatically diluted (2:3) by the instrument, and reported accordingly. If a manual dilution is required, dilute the specimen in normal saline, and multiply the result by the dilution factor.

### **ASSAY PERFORMANCE**

Analytical Range	0.32 - 8.00 mg/L
Reportable Range	0.32 – 20.00 mg/L
Limit of Detection (as reported by the manufacturer)	0.03 mg/L
Limit of Quantitation (as reported by the manufacturer)	0.32 mg/L
Recovery, normal samples (Performed on 5 normal serum samples and spiked with one part calibrator at a concentration of 7.2 mg/L to four parts normal sample )	102 – 105%
Recovery, uremic samples (Performed on 6 uremic serum samples and spiked with one part calibrator at a concentration of 7.2 mg/L to four parts uremic sample )	100 - 105%
Intra-assay %CV (10 within day replicates at a concentration of 0.97 mg/L)	0.8%
Inter-assay %CV (10 between day replicates at a concentration of 1.54 mg/L)	0.7%

Inter-assay %CV (10 between day replicates at a concentration of 0.94 mg/L)	4.1%
Inter-assay %CV (10 between day replicates at a concentration of 3.29 mg/L)	2.8%
Within day duplicate range, serum	6.2%
Manufacture Reference range (see Appendix A)	0.51 – 1.05 mg/L

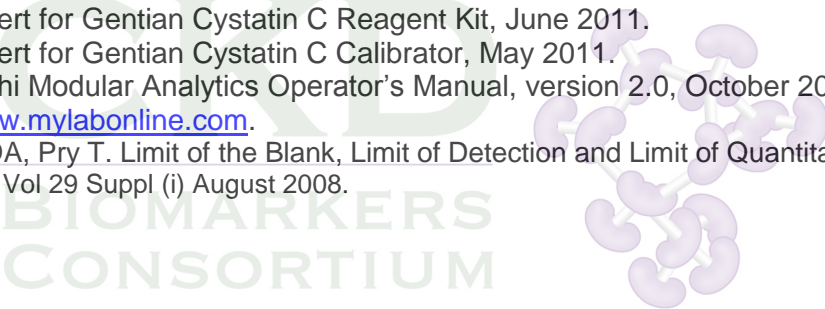
**NOTES**

1. There are several equations available for estimating glomerular filtration rate (GFR) based upon the cystatin C concentration in serum. Gentian offers the following:

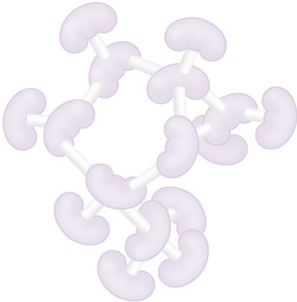
$$\text{GFR (mL/min/1.73 m}^2\text{)} = 79.901/\text{cystatin c (mg/L)}^{1.4389}$$

**REFERENCES**

1. Package insert for Gentian Cystatin C Reagent Kit, June 2011.
2. Package insert for Gentian Cystatin C Calibrator, May 2011.
3. Roche/Hitachi Modular Analytics Operator’s Manual, version 2.0, October 2006 available online at [www.mylabonline.com](http://www.mylabonline.com).
4. Armbruster DA, Pry T. Limit of the Blank, Limit of Detection and Limit of Quantitation. Clin Biochem Rev Vol 29 Suppl (i) August 2008.

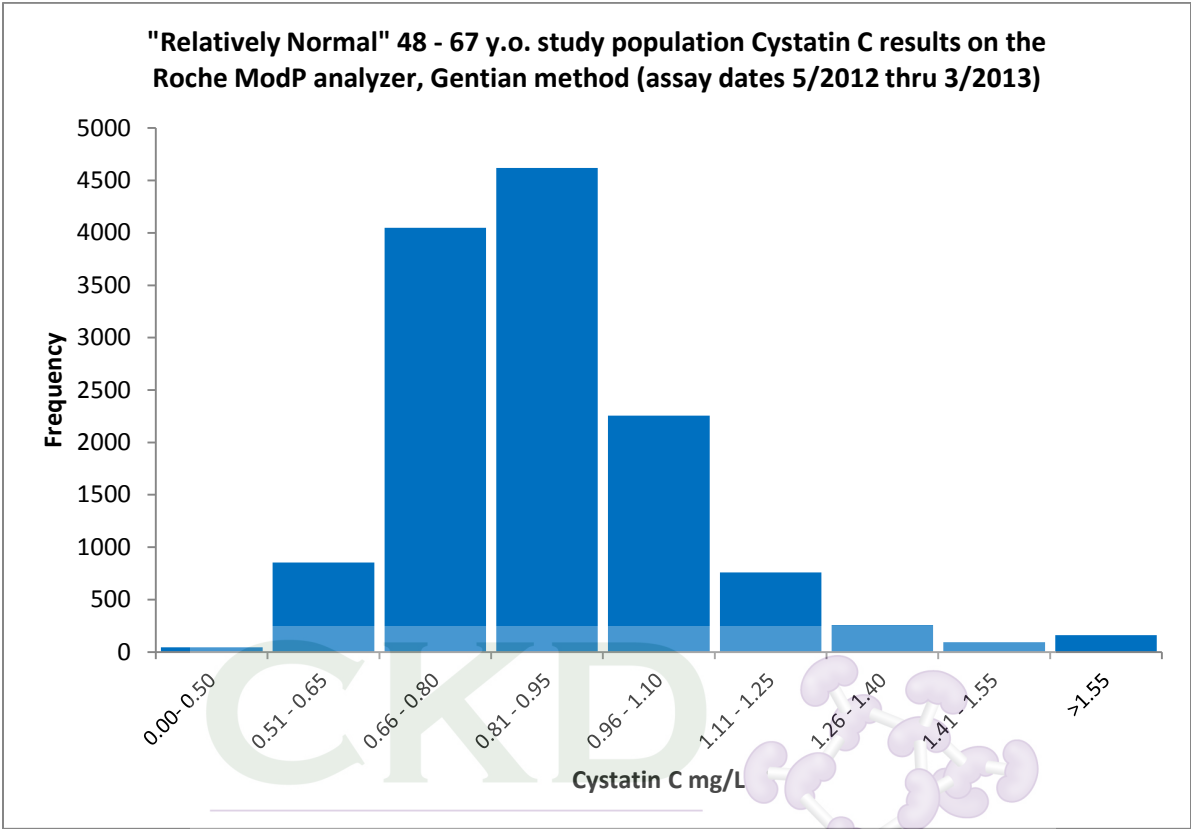


<b>External Ref:</b>	See above
<b>Appendix</b>	A. Reference Range comparison B. Gentian Cystatin C Reagent package insert C. Gentian Cystatin C Calibrator package insert
<b>Source:</b>	
<b>Written by and Date:</b>	Greg Rynders, July 2012
<b>Date Effective:</b>	9/2012
<b>Approved by and Date:</b>	Anthony Killeen, MD, PhD
<b>Date Retired:</b>	





Appendix A



CKD  
BIOMARKERS  
CONSORTIUM

manufacture  
reference range 0.51 – 1.05

Study Population Cystatin C	
Mean =	0.89 mg/L
Standard deviation =	0.31 mg/L
N =	13,091
2.5 %ile =	0.60 mg/L
97.5 %ile =	1.34 mg/L
Central 95% reference range for this study population is	0.60 – 1.34 mg/L

**gentian****Gentian Cystatin C Reagent Kit**

REF1101 (Contains: Assay Buffer + Immunoparticles)

**CE**

IVD

For in vitro diagnostic use only

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**Intended Use**

The Gentian Cystatin C Immunoassay is an in vitro diagnostic test for quantitative determination of cystatin C in human serum and plasma. The measurement of cystatin C is used in the diagnosis and treatment of renal diseases.

**Summary and Explanation of Test**

The non-glycosylated basic protein, cystatin C (molecular weight 13.2 kD), is produced at a constant rate in nearly every nucleated cell in the human body [1]. It is freely filtered through a normal Glomerular membrane, and is then reabsorbed and almost entirely catabolized in the proximal tubules. Hence, the cystatin C concentration in human blood is closely related to glomerular filtration rate (GFR) [2]. A reduction in the GFR causes a rise in the cystatin C concentration. The cystatin C concentration has not been shown to be significantly influenced by other factors such as muscular mass, inflammatory diseases, sex, age or diet [2, 3 and 4].

**Calibrator Standardisation**

Gentian Cystatin C Calibrator is standardised against the international calibrator standard ERM-DA471/IFCC.

**GFR prediction calculation**

Several cystatin C based prediction equations for calculation of GFR for adults and children have been published. It should be noted that these formulas were evaluated with different Cystatin C assays (particle-enhanced nephelometric immunoassay PENIA or particle enhanced turbidimetric immunoassay PETIA) and may reveal inaccurate GFR results if an inappropriate combination of formula and assay is used. For calculation of GFR from cystatin C values measured with the Gentian assay the following prediction equation is recommended using mg/L as the unit factor [7]. The equation is valid for persons above 14 years.

$$\text{GFR [mL/min/1.73 m}^2\text{]} = \frac{79.901}{\text{Cystatin C (mg/L)}^{1.438}}$$

**Principle of the Method**

Serum or plasma sample from human is mixed with Gentian Cystatin C immunoparticles. Cystatin C from the sample and anti-cystatin C from the immunoparticles aggregates. The complex particles created absorb light, and by turbidimetry the absorption is related to cystatin C concentration via interpolation on an established standard calibration curve. The instrument platform used for Gentian Cystatin C measurement will automatically calculate the results.

**Assay Reagents****Materials Provided in Cystatin C Reagent Kit, REF 1101:**

Cystatin C Assay Buffer, bottle of 58 mL      Reagent 1  
Cystatin C Immunoparticles, bottle of 10 mL,      Reagent 2

**Material required, but not provided:**

Gentian Cystatin C Calibrator, 1 level, vial of 1 mL      REF 1012  
Gentian Cystatin C Calibrator Kit, 8 levels, vials of 1 mL      REF 1051  
Gentian Cystatin C Control Kit, 2 vials of 1 mL      REF 1019  
Gentian Cystatin C Control Kit, 2 vials of 5 mL      REF 1028

All materials are ready for use.

**Composition**

**Cystatin C Immunoparticles** is a purified immunoglobulin fraction that is directed against cystatin C which is covalently attached to uniform polystyrene particles. Human cystatin C was used as immunogen in the process of generating the immunoparticles. It is provided as a ready to use suspension, preserved with 15 mmol/L sodium azide and antibiotics. The cystatin C immunoparticles are ready to use.

**Cystatin C Assay Buffer** is a MOPS [3-(N-Morpholino)-propanesulfonic acid] buffered saline, preserved with sodium azides. The buffer is ready to use.

**Cystatin C Calibrator** is a delipidated human serum pool spiked with human cystatin C. Antibiotics are used as preservation. The calibrator is ready to use.

**Cystatin C Control Kit** consists of cystatin C control Low and High. It is made from a delipidated human serum pool spiked with human cystatin C. Antibiotics are used as preservation. The controls are ready to use.

**Warnings and Precautions**

1. The Gentian Cystatin C Immunoassay reagents are for in vitro use only, and must be handled by qualified personnel.
2. Reagents containing antibiotics must be handled with due caution.
3. Reagents containing sodium azide must be handled with due caution: Do not ingest or allow contact to skin or mucous membranes. The sodium azide concentration of this product is not characterised as dangerous. Although, accumulated Na<sub>2</sub> in lead and copper pipes may cause generation of explosive metal azides. To prevent this, rinse thoroughly if discarded into the drain.
4. Serum used in the manufacture of calibrators and controls are tested for hepatitis HBsAg, anti-HCV, anti-HIV1 and anti-HIV2 and found to be negative. Nevertheless, the materials contain substances of human and animal origin and must be handled with due care. Disposal of any discarded materials should be in accordance to local requirements.

**Storage and stability**

Shelf life at 2-8 °C: See expiry date on the label  
Stability after opening: 9 weeks at 2-8 °C  
On-board stability: 9 weeks at correct temperature (2-8 °C)

**Specimen**

Required sample material is human serum, heparinized plasma and EDTA plasma. It is recommended to analyse the samples as fresh as possible. Sample stability testing showed that cystatin C in serum and plasma samples are stable for 14 days at room temperature (8-25°C), 21 days if stored at 2-8°C and stored at below -20°C for at least 10 years [9]. Mix samples well before analysing. The samples can be shipped without special cooling and must then be analysed within 14 days after shipment.

**Procedure****Application Notes**

Methods for the Gentian cystatin C Immunoassay are established on several clinical chemistry analysers. Detailed and validated Application Notes describing the procedures for installation and analysing are available from Gentian AS. The Application Notes are also available at the following link: [www.gentian.no](http://www.gentian.no)  
Maintenance, operation and precautions must be handled in accordance with the specific instrument manual.

## Appendix B Gentian Cystatin C Reagent package insert page 2

### Instrument installation

A defined application for the Gentian Cystatin C immunoassay must be installed in accordance with the specific instrument's Settings for the instrument in question. For instrument setup information, consult the specific Gentian Cystatin C immunoassay Application Note and the specific instrument manual.

### Loading of Reagents

Load the Gentian Cystatin C immunoassay reagents to the instrument as described in the specific instrument's Application Note. Reagents shall be stored at 2-8°C when not in use.

The reagent tubes provided (REF: 1101) fits directly into several instruments:

Abbott<sup>1</sup>, Architect<sup>1</sup>  
Roche<sup>2</sup>, Hitachi<sup>3</sup> 917 and Modular<sup>2</sup> P  
Beckman Coulter AU<sup>4</sup> series (Former Olympus),  
Siemens<sup>1</sup>, Advia<sup>1</sup> series

The reagent tubes may fit directly into other instruments as well, but are not tested by Gentian.

### Establishment of the Calibration Curve

The calibration curve is established from a dilution series automatically prepared by the instrument, as defined in the Application Note (for some instruments it is required to use pre-diluted calibrators, REF 1051). A multi-point standard calibration curve is established over the points achieved from the dilution series. Instructions are given in the specific Gentian Cystatin C immunoassay instrument Application Note and in the instrument manual. Calibrator values are lot dependent and a new calibration must be performed whenever a new calibration lot is used. The calibrator's assigned value is given on the analytical value sheet provided with the calibrator. The stability of a calibration curve is normally 4 weeks.

### QC Controls

The Gentian Cystatin C controls low and high must be assayed each day before any samples are assayed in order to validate the calibration curve. The controls have an assigned value range that must be met before measuring samples. The assigned values are given in the analytical value sheet included with the control kit (REF 1019, 1025). If the control values are not valid, repeat the control measurements. If the calibration cannot be performed without error, or valid control values cannot be reproduced, contact Gentian for support.

### Measuring Patient Samples

When a valid calibration has been performed and the control values are within the valid range, serum or plasma samples may be measured. Check that minimum volume of sample is present and assay the samples according to the instructions given in the Application Note and the instrument manual.

### Results

The results are evaluated automatically by the analyser for all applications established for Gentian Cystatin C immunoassay. The results are presented in mg/L.

### Limitations

The materials shall not be used past expiration date. No interference is detected for this product with triglycerides, haemoglobin, intralipid or bilirubin at tested concentrations. There is no interference detected with the drugs tested on recommendation from Sonntag and Scholer [5]. There is no RF interference present in the Gentian Cystatin C immunoassay because the antibodies are made from avian (chicken) [6]. For details see Application Notes.

### Reference Intervals

Gentian followed the CLSI Guideline, C28-A2 to determine the transferability of the reference interval. The reference interval was determined from a population of ostensibly healthy subjects with no history of renal disease. A total of 136 samples obtained from 58 males and 78 females ranging in age from 20 to 80 years were tested. The reference interval was calculated non-parametrically and was determined to be 0.51-1.05 mg/L. This represents the central 95 % of the population. It is recommended that every laboratory should determine a local reference interval since values may vary depending on the population tested.

Package insert Gentian Cystatin C Reagent Kit

In a separate study involving 250 healthy children (48% boys, 54 % girls) in the age from 5 to 15 years, the reference range 0.51 - 1.05 mg/L was confirmed in all ages down to 5 years of age [8].

### Symbols Key

	Lot number
	Temperature limitations
	In Vitro Diagnostic Medical Device
	Expiration date
	Consult instructions for use
	Manufacturer
	Catalogue Number

### References

- [1] Abrahamson MJ, et al: Biochem. J. 1990;268:287-94
- [2] Laterza OF, et al: Clin Chem 2002;48:563-96.
- [3] Grubb AO. Adv Clin Chem 2000;36:93-99.
- [4] Filler G et al: Clin. Biochem. 2005; 38: 1-8.
- [5] Sonntag G, Scholer A. Ann Clin Biochem 2001; 38:375-85.
- [6] Larsson A et al: Poultry Science 1999;72:1807-1812.
- [7] Hladik M et al: Scand J Clin Lab Invest 2007; 1-8
- [8] Nitsch D, et al: The Uppsala Family Stud. Am J Kidney Dis. 2011;57(6):863-872
- [9] Shlipak MG, et al: Clinical Chemistry 57: 737-745, 2011

### Manufacturer:

Gentian AS, PO BOX 733, N-1509 Moss, Norway

EC REP Authorized European Representative in EU:

Gentian AS, PO BOX 733, N-1509 Moss, Norway

TEL: +47 69 33 99 05

FAX: +47 69 34 09 82

<http://www.gentian.no>

- 1 - Registered Trademark of Abbott Diagnostics
- 2 - Registered Trademark of Roche Diagnostics
- 3 - Registered Trademark of Beckman Coulter Inc
- 4 - Registered Trademark of Siemens Medical Diagnostics

r04en-June 2015

## Appendix C Gentian Cystatin C Calibrator package insert

Page 1 of 1

**gentian**

### Gentian Cystatin C Calibrator

REF1012

CE

IVD

For in vitro diagnostic use only

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#### Intended Use

The Gentian Cystatin C immunoassay is an in vitro diagnostic test for quantitative determination of cystatin C in human serum and plasma. The measurement of cystatin C is used in the diagnosis and treatment of renal diseases.

#### Gentian Cystatin C Calibrator Indication for use

The Gentian Cystatin C Calibrator is intended to be used to establish a calibration curve for measuring Cystatin C concentration in human serum or plasma with the Gentian Cystatin C immunoassay.

#### Calibrator Standardisation

Gentian Cystatin C Calibrator is standardised against the International calibrator standard ERM-DA471/IFCC.

#### Reagents

##### Composition

The Gentian Cystatin C Calibrator is a delipidated human serum pool spiked with human cystatin C. Antibiotics are used as preservative. The calibrator is ready to use.

##### Calibrator Value Assignment

The calibrator value, given in the enclosed Analytical Value Sheet, is assigned according to Gentian's value transfer protocol as recommended in ISO 17511 [1] for calibrators and controls. The calibrator comprises human cystatin C isolated from human urine.

#### Warnings and Precautions

1. The Gentian Cystatin C Calibrator is for in vitro use only, and must be handled by qualified personnel.
2. The Gentian Cystatin C Calibrator contains antibiotics and must be handled with due cautions.
3. Serum used in the manufacturing of the Gentian Cystatin C Calibrator are tested for hepatitis HBsAg, anti-HCV, anti-HIV1 and anti-HIV2 and found to be negative. The testing was done with FDA approved methods. Nevertheless, the materials contain substances of human origin and must be handled with due care. Disposal of any discarded materials should be in accordance to local requirements.

#### Storage

Store at 2-8°C. Do not use past the expiration date stated on the label.

#### Assay reagents

##### Materials Provided:

Cystatin C Calibrator, 1 level, vial of 1ml REF 1012

##### Material required, but not provided:

Gentian Cystatin C Reagent Kit REF 1101

Gentian Cystatin C Control Kit, 2 vials of 1 ml REF 1019

Gentian Cystatin C Control Kit, 2 vials of 5 ml REF 1026

All materials are ready for use.

#### Procedure

Methods for the Gentian Cystatin C immunoassay are established on several clinical chemistry analyzers. Detailed and validated Application Notes describing the procedures for installation and analysing are available from Gentian AS. The Application Notes are also available at [www.gentian.no](http://www.gentian.no)

#### Internal quality control

It is necessary to evaluate the calibration curve by assaying the Gentian Cystatin C Control Low (REF 1020) and the Control High (REF 1021) each day before any samples are assayed in order to validate the calibration curve. See the Analytical Value Sheet provided with the controls for assigned values and confidence intervals. If the results fall outside the given intervals a recalibration is recommended. Also when the reagent lots are changed a recalibration is recommended. Otherwise recalibrate within the interval stated in the instrument specific Application Note.

#### Calibration curve

A 6 point calibration curve is established from a dilution series automatically prepared by the instrument, as defined in the instrument specific Application Note. The established calibration curve is usually stable for 4 weeks. When reagent lots are changed or measured control values are outside the assigned range given in the Analytical Value Sheet, a recalibration is recommended.

#### Symbols Key

Lot number	Lot number
Temperature limitations	Temperature limitations
IVD	In Vitro Diagnostic Medical Device
Expiration date	Expiration date
Consult instructions for Use	Consult instructions for Use
Manufacturer	Manufacturer
REF	Catalogue Number

#### References

- [1] ISO 17511; In Vitro Diagnostic Medical Devices-Measurement of Quantities in Biological Samples- Metrological Traceability of Values Assigned to Calibrators and Control Materials. First edition 2003-08-15.



#### Manufacturer:

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**University of Minnesota  
Advanced Research Diagnostic Laboratory**

<b>Entity:</b>	<b>University of Minnesota</b>
<b>Department:</b>	<b>Advanced Research and Diagnostic Laboratory</b>
<b>Manual:</b>	<b>ARDL Procedure Manual</b>
<b>Procedure:</b>	<b>FGF-23 Immunoassay from Kainos Laboratories, Inc.</b>

### **Principle**

FGF-23 is a 2-step enzyme-linked immunosorbent assay. Refer to appended manufacturer's package insert FGF-23 ELISA Kit, Kainos Laboratories, Tokyo, Japan.

Two specific murine monoclonal antibodies bind to full length FGF-23. One antibody is conjugated to horseradish peroxidase (HRP) for detection. In the first reaction a sample containing FGF-23 is incubated with the immobilized antibody in a microtiter well, FGF-23 in the sample is captured by the antibody. At the end of this reaction, the well is washed to remove unbound FGF-23 and other components. In the second reaction, this immobilized FGF-23 is incubated with horseradish peroxidase labeled antibody to form a sandwich complex: anti FGF-23 antibody (N-terminal) – FGF-23 – HRP labeled anti FGF-23 antibody (G-terminal). At the end of this reaction the well is washed to remove unbound components. In the enzyme reaction the sandwich complex is incubated with a substrate solution and then measured by spectrophotometric plate reader. The enzymatic activity of the complex bound to the well is directly proportional to the amount of FGF-23 in the sample.

### **Specimen Type and Handling**

Serum and EDTA plasma are acceptable. See appendix A for laboratory performed comparisons between serum and EDTA plasma. Minimum volume: 100 µL in a 2mL microcentrifuge tube (50µL for test and additional 50µL for dead space).

Intact FGF-23 is highly unstable. Sample processing should be completed as soon as possible after collection. Samples should be stored frozen at -80°C. It is preferable to assay FGF-23 on a never-thawed specimen, however, up to 3 freeze-thaws cycles are acceptable provided that the sample has been returned to frozen storage immediately after previous use.

### **Specimen Collection**

**Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store. Samples should be stored frozen at -80°C. It is preferable to assay FGF-23 on a never-thawed specimen, however, up to 3 freeze-thaws cycles are acceptable provided that the sample has been returned to frozen storage immediately after previous use.

**Plasma** - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Remove plasma and assay immediately or aliquot and store. Samples should be stored frozen at -80°C. It is preferable to assay FGF-23 on a never-thawed specimen, however, up to 3 freeze-thaws cycles are acceptable provided that the sample has been returned to frozen storage immediately after previous use.

## Interferences or limitations

The manufacturer reports ascorbic acid (100 mg/dL and lower), bilirubin (50 mg/dL and lower), chylomicrons (3000 formazine turbidity units and lower), and rheumatoid factor (500 IU/mL and lower), each have almost no effect on measurements.

## Procedure

Refer to appended manufacturer's procedure for complete instructions. Notes:

- Prepare daily worksheet. Refer to *the ARDL General ELISA Procedure* and see appended example.
- Use MilliQ water for preparation of the wash buffer.
- Standards 1 and 2 are assayed in triplicate; standards 3 and 4 are assayed in duplicate; standard 5 is assayed in singleton; standards 6 and 7 are not assayed. ARDL has verified that a standard curve produces equivalent results whether standards 6 and 7 are included or excluded from the standard curve calculation.
- Use the Biomek NXp method "FGF-23 (Kainos) (revision 12)" to pipette the standards, controls, samples and Assay Diluent into the ELISA plate. Refer to the *ARDL Biomek NXp General Operation Procedure* for instrument maintenance and operating instructions.

## Manufacture Kit Instructions:

1. Add 50 uL of Assay Diluent into the well
2. Add 50 uL of FGF23 Standards or sample into each well,
3. Cover plate with plate sealer.
4. Incubate plate at room temperature for 2 hours on an orbital shaker set at 450 RPM.
5. Remove the plate sealer.
6. Using a multi-channel pipette aspirate the reaction solution.
7. Add 300 uL of Wash Buffer solution to the well and then aspirate and discard.
8. Invert plate and blot on clean paper towel after all plate wells have been washed.
9. Repeat steps 7 and 8 three more times for a total of four washes. Note: work quickly with a multi-channel pipette to avoid drying out of the wells and make sure the pipette tips do not scratch the well bottoms.
10. Add 100 uL of FGF23 Conjugate into each well
11. Cover plate with plate sealer.
12. Incubate plate at room temperature for 1 hour on an orbital shaker set at 450 RPM.
13. Remove the plate sealer.
14. Remove the reaction solution.
15. Add 300 uL of Wash Buffer solution to the well and then aspirate from well.
16. Invert plate and blot on clean paper towel after all wells on the plate have been washed..
17. Repeat steps 15 and 16 three more times for a total of four times. Note: work quickly with a multichannel pipette to avoid drying out of the wells and make sure the pipette tips do not scratch the well bottoms
18. Add 100 uL of Substrate into each well.
19. Cover plate with plate sealer and aluminum foil.
20. Incubate plate at room temperature for 30 minutes.
21. Remove the plate sealer and aluminum foil.
22. Immediately add 100 uL of Stop Solution into each well.
23. Place the plate on the orbital shaker set at 450 RPM for 1 minute.
24. Determine the optical density of each well by placing the plate on the SpectraMax 190 plate reader with the absorbance at 450 nm (secondary wavelength 600-650 nm) within 10 minutes.

## Dilutions



No maximum dilution is indicated in manufacturer's insert. ARDL will dilute samples greater than 800 pg/mL (the highest standard) using Standard 1 (0 ng/mL) as the diluent. Dilute sample 1:20 by pipetting 10uL sample and 190uL diluent into a 2mL tube; vortex briefly to mix; assay as usual, multiplying by the dilution factor to determine the final sample concentration. The linearity of the assay has been verified up to a 1:1000 dilution.

### Calibration

Calibrators included with the FGF-23 ELISA kit are assayed on each ELISA plate, with the exceptions noted in the Procedure section.

### Quality Control

Two levels of manufacture quality controls and a pooled serum quality control (see reagent section) are analyzed in duplicate on each ELISA plate. Review Levey-Jennings plots and acceptability criteria for each quality control result daily before reporting results. Control values from each plate are compared to established control ranges. Violation of Westgard Rules 1-3s or 2-2s will result in corrective action.

If quality control fails the 1-3s or 2-2s Westgard rule take the following corrective action steps:

- With a new assay kit that includes controls and additional samples, reanalyze six samples from the failed plate that represent a range of results. If the controls from the new batch pass the Westgard rules and the repeat sample results are within the acceptable duplicate range, accept the batch, document the corrective action and proceed with testing.

If the quality control fails after using a new kit, document corrective action, discontinue testing and notify the laboratory supervisor.

### New Lot Verification

A single lot is purchased to complete each study. If this is impossible because of reagent expiration dates, correlation of 20 samples between the two lots is performed, and a factor to harmonize results is used if kits are >3% different.

### Maintenance

Refer to *ARDL Biomek NXp General Operation Procedure*, *ARDL SpectraMax 190 plate reader procedure* and *ARDL General ELISA procedure* for maintenance procedures.

### Reagents and Supplies

- Refer to appended manufacturer's procedure for reagents included in FGF-23 ELISA kit.
- Pooled Normal Serum control. Solomon Park Research Laboratories, 12815 NE 124th St. Suite 1, Kirkland, WA 98034. Store at -70°C for up to 4 years, at refrigerated temperature for 1 day and at room temperature for up to 4 hours. Allow the control to reach room temperature (18-25°C) and mix thoroughly by inversion before use.
- Low and high controls. Kainos Laboratories Inc, Tokyo, Japan. Prepared from FGF-23 ELISA kit standards. Prepare low control by diluting the 800 pg/mL standard from ELISA kit 1:40 with the 0 pg/mL standard. Aliquot 125uL into 2mL microcentrifuge tube. Prepare high control by diluting 800 pg/mL standard from ELISA kit 1:10 with 0 pg/mL standard from ELISA kit. Aliquot 125uL into 2mL microcentrifuge tube. Stable at -80°C for up to 1 year, at refrigerated temperature for 1 day and at room temperature for up to 4 hours.
- MilliQ water
- Biomek NXp (Beckman Instruments, Inc. 2500 N. Harbor Blvd., Fullerton, CA 92834-3100)
- Biomek pipet tips, reagent reservoirs and labware; refer to appended *ARDL Biomek NXp General Operation Procedure*
- Orbital microplate shaker (Labnet International, Woodbridge NJ)

- Rainin 12-channel pipet and pipet tips
- Reagent reservoirs

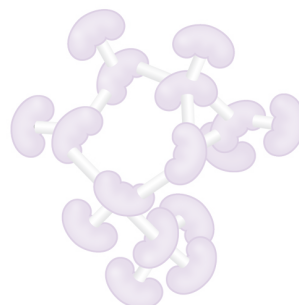
## Results

1. Quality control results must be in acceptable ranges.
2. Review plate reader printout for result flags and take appropriate action. The plate reader will flag samples with an “R” in the “R” column that have an OD that is less than the lowest standard or greater than the highest standard. Note in the results sheet that the sample requires dilution (if > highest standard).
3. Report all values calculated by the plate reader software. For values <3 pg/mL, indicate that result is “below the sensitivity of the assay stated by the manufacturer.”
4. Results are downloaded from the plate reader onto a spreadsheet, see *ARDL General ELISA procedure*.
5. Results must be reviewed for accuracy by a second technologist before reporting, see *ARDL General ELISA procedure*.

## Reference Range

No reference range is indicated by the manufacturer.

Results were obtained from 476 study participant samples in 2012. A range of 23.7 – 97.1 mg/L representing the central 95% was determined nonparametrically. Study participants age ranged from 48 – 67 years old and the health status is unknown to this laboratory.



## Critical Values

None

## Assay performance

Analytical Range	3 – 800 pg/mL
Limit of Detection (as determined by the kit manufacture, method not stated. Lab measured LOD =2.2 pg/mL as calculated by analyzing the blank 30 times and calculating 2SD above the blank.)	3 pg/mL
Recovery, normal samples (Performed on 5 normal serum samples and spiked with one part standard at a concentration of 250 pg/mL to four parts normal sample )	96 – 110%
Recovery, uremic samples (Performed on 6 uremic serum samples and spiked with one part standard at a concentration of 250 pg/mL to four parts uremic sample )	97 - 110%
Intra-assay %CV (10 within day replicates at a concentration of 31.14 pg/mL)	8.0%
Intra-assay %CV (10 within day replicates at a concentration of 52.84 pg/mL)	9.1%
Inter-assay %CV (43 between day replicates at a concentration of 42.12 pg/mL)	9.7%
Within day duplicate range	14.5%
Reference range* (See appendix C)	Serum 23.7 -97.1 pg/mL

## Computer Entry

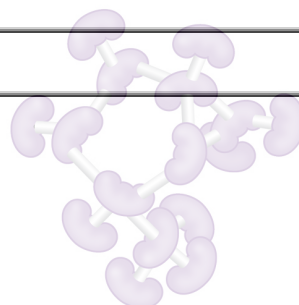
1. Results are downloaded from the plate reader onto a spreadsheet, see *ARDL General ELISA procedure*.
2. Report all values calculated by the plate reader software. For values <3 pg/mL, indicate that result is “below the sensitivity of the assay stated by the manufacturer.”

## References

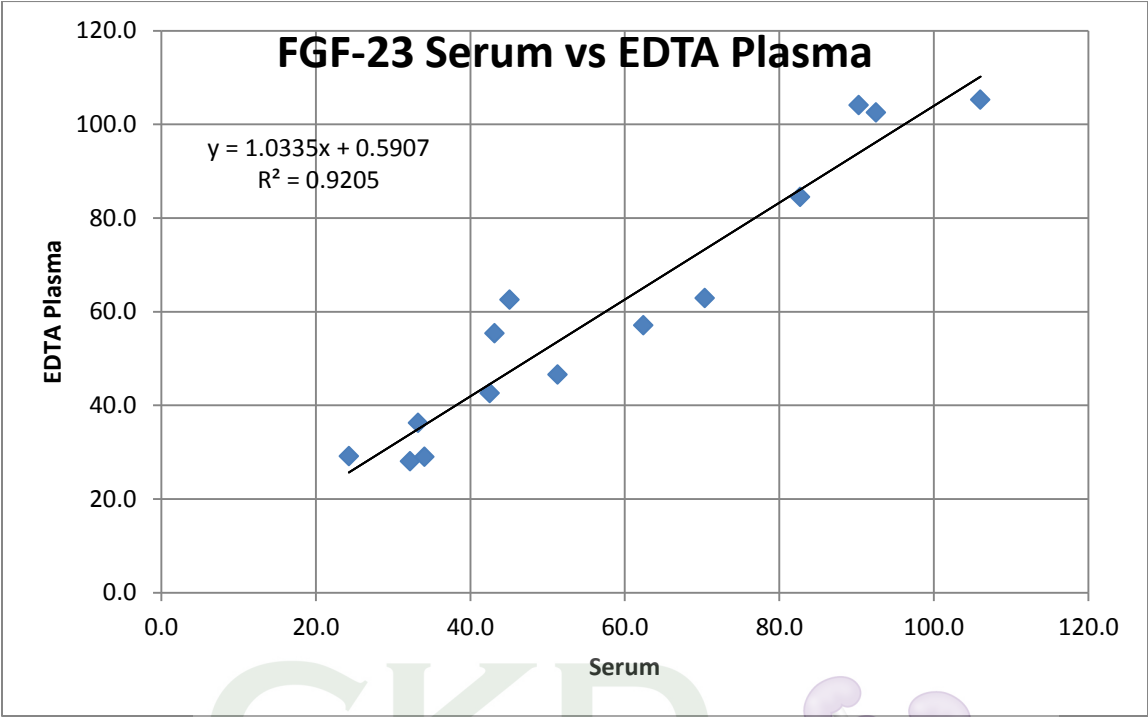
1. FGF-23 ELISA Kit package insert, June 2011. Kainos Laboratories, Inc., 38-18, Hongo 2-Chrome Bunkyo-ku, Tokyo, Japan 113-0033
2. Armbruster DA, Pry T. Limit of the Blank, Limit of Detection and Limit of Quantitation. Clin Biochem Rev Vol 29 Suppl (i) August 2008.

<b>External Ref:</b>	
<b>Internal Ref:</b>	ARDL Biomek NXp General Operation Procedure ARDL SpectraMax 190 plate reader procedure ARDL General ELISA procedure
<b>Appendixes</b>	A. Comparison of Serum and EDTA plasma B. Example Worksheet C. Reference Range Validation D. Package insert Kainos Laboratories Inc FGF-23 ELISA Kit
<b>Written By and Signature:</b>	Scott Stahowiak, Naomi Hanson, Valerie Arends
<b>Date Written:</b>	3/2013
<b>Approved by:</b>	Anthony Killeen, MD, PhD
<b>Date Effective:</b>	5/2013
<b>Date Retired:</b>	

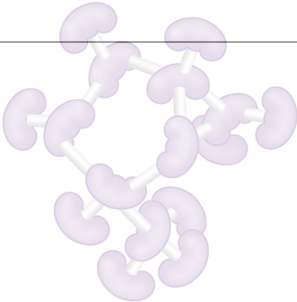
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CONSORTIUM



Comparison of Serum vs EDTA Plasma 3/2013



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CONSORTIUM



Appendix B

Intact FGF-23 (Kainos) Immunoassay Worksheet

using Biomek NXp method "FGF-23 (Kainos) (revision 12)"

Reagent	Lot	Date	Reagent	Lot	Date
FGF-23 kit	245	10/2013	Low control (~20 pg/mL)	150-1	2/6/2012
Microplate	245	10/2013	High control (~80 pg/mL)	150-2	2/6/2012
Conjugate	245	10/2013			
Standards	245	10/2013			
Assay diluent	245	10/2013			
Substrate	245	10/2013			
Wash buffer	245	10/2013			
Stop solution	245	10/2013			

samples not diluted  
need 50uL sample per well

Date:    
Tech:   
Results entered/checked:   
QC entered/checked:   
plate shaker used:

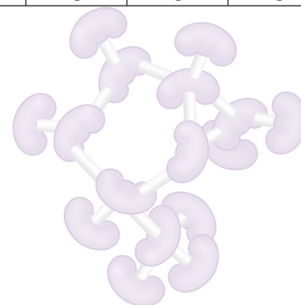
Spectrophotometer
Wavelength 450 nm

Incubation	1 (2h)	2 (1h)	3 (30m)
Temp	RT on shaker (450 RPM)	RT on shaker (450 RPM)	RT on bench (dark)
In/Out			

BIOMEK: ELISA PLATE in P11

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1 (0)	Std 4 (100)	0	0	0	0	0	0	0	0	0	0
B	Std 1 (0)	Std 4 (100)	0	0	0	0	0	0	0	0	0	0
C	Std 1 (0)	Std 5 (250)	0	0	0	0	0	0	0	0	0	0
D	Std 2 (10)	Pool CP	0	0	0	0	0	0	0	0	0	0
E	Std 2 (10)	Pool CP	0	0	0	0	0	0	0	0	0	0
F	Std 2 (10)	low control	0	0	0	0	0	0	0	0	0	0
G	Std 3 (50)	high control	0	0	0	0	0	0	0	0	0	pool CQ
H	Std 3 (50)	0	0	0	0	0	0	0	0	0	0	pool CQ

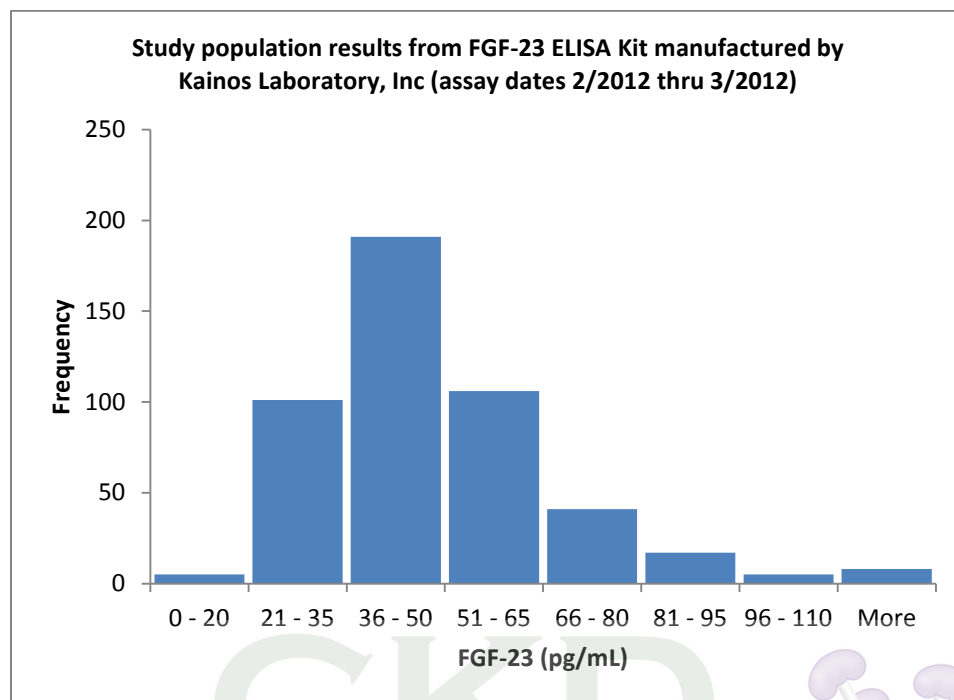
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## Appendix C

FGF-23 reference range from an apparently “normal” population.



### Study population statistics

		Manufacture stated reference range: None reported.
Mean	49.0 pg/mL	
SD	19.6 pg/mL	
N	476	
2.5%ile	23.7 pg/mL	
97.5%ile	97.1 pg/mL	
Study population central 95%ile	23.7 – 97.1 pg/mL	

Read carefully before using the product

Research use only

## FGF-23 ELISA Kit

### 【INTRODUCTION】

FGF-23 was discovered as 22th protein of fibroblast growth factor family. FGF-23 is produced as a 226 amino acid polypeptide which has a cleavage site and the fragmented peptides are recognized in serum with full length peptide.

It is suggested that serum full length FGF-23 level may be useful as an indicator for severity of osteomalacia such as tumor-induced osteomalacia, X-linked hypophosphatemic rickets and autosomal dominant hypophosphatemic rickets.

### 【CAUTION】

- 1) The kit is intended only for research purposes, not for use in diagnostic procedures.
- 2) The reliability of results cannot be guaranteed if the kit is used with a method or for a purpose other than those stipulated.
- 3) This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions.

### 【COMPONENTS】

- 1) **FGF-23 Antibody coated Microplate** (FGF-23 Microplate)  
96 well microplate (12 strips of 8 well) coated with a murine monoclonal antibody against FGF-23.
- 2) **HRP labeled FGF-23 Antibody** (FGF-23 Conjugate)  
12 mL of murine monoclonal antibody against FGF-23 conjugated to horseradish peroxidase, with preservatives.
- 3) **FGF-23 Standards** (Std1-Std7)  
7 vials of FGF-23 (Std1:12 mL, Std2-7:0.5 mL) in buffered base with preservative.
- 4) **Assay Diluent** (Assay Diluent)  
1 bottle containing 12 mL of buffer.
- 5) **Color Reagent** (Substrate)  
1 bottle containing 12 mL of tetramethylbenzidine (TMBZ) with urea hydrogen peroxide.
- 6) **Wash Buffer Concentrate** (Wash Buffer (×5))  
1 bottle containing 90 mL of a 5-fold concentrated solution of buffered surfactant with preservative.
- 7) **Stop Solution** (Stop Solution)  
1 bottle containing 12 mL of 0.5 mol/L Sulfuric acid.
- 8) **Plate Sealer**  
3 included in kit.

### 【INTENDED USE】

Measurement of FGF-23 in serum

### 【PRINCIPLE OF THE EXAMINATION METHOD】

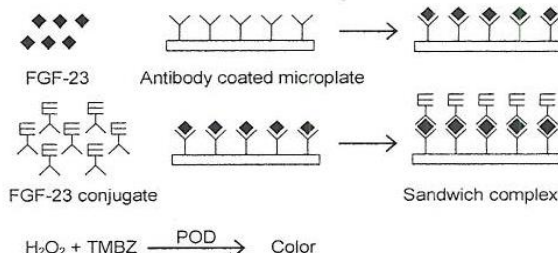
#### 1. Principle

The assay principle is a two-step enzyme-linked immunosorbent assay.

In first reaction, a sample containing FGF-23 is incubated with the immobilized antibody in a microtiter well. FGF-23 in sample is captured by the antibody. In second reaction, this immobilized FGF-23 is incubated with HRP labeled antibody to form a "sandwich" complex.

Because Peroxidase bound to the solid phase depends on the amount of FGF-23, the sample amount of FGF-23 can be

determined by colorimetrically measuring the amount of detached oxidized (colored) TMBZ using urea hydrogen peroxide ( $H_2O_2$ ) and 3,3',5,5'-Tetramethylbenzidine (TMBZ) as the substrates.



#### 2. Feature

- 1) High specificity:  
Measurable without the influence of serum components.
- 2) High sensitivity:  
The minimal detectable concentration is 3 pg/mL.
- 3) Wide range:  
The maximal detectable concentration is 800 pg/mL.

### 【LIMITATIONS OF THE EXAMINATION PROCEDURE】

#### 1. Samples

- 1) Serum sample is used for measurement.
- 2) After sampling, measure as soon as possible. If samples must be stored, freeze at  $-20^{\circ}C$  or lower.
- 3) The intact FGF-23 molecule appears to be highly unstable resulting in decreased immunoreactivity over time.
- 4) Besides human, analytic species can be measured mouse, rat and monkey.

#### 2. Interference<sup>2)</sup>

- 1) Ascorbic acid (100 mg/dL or lower), bilirubin (50 mg/dL or lower), and chylomicrons (3000 turbidity units or lower), rheumatoid factor (500 IU/mL or lower) each have almost no effect on measurements.
- 2) The sample and all reagents should be pipetted carefully to minimize air bubbles in the well.

#### 3. Others

- 1) A calibration curve must be prepared for each new assay.
- 2) If multiple samples assayed, make sure to keep the intervals to add each reagent constant so that each reaction completes in the defined time period.
- 3) The washing step is also an important part of the total assay procedure. If you using an automated microtiter plate washer, please completely removal of Wash Buffer Solution.
- 4) Avoid the well from drying out during the wash process.
- 5) Make sure the well bottoms do not get scratched or soiled.

### 【EXAMINATION PROCEDURE】

#### 1. Preparation of Reagents

- 1) Wash Buffer Solution  
Before using, dilute the contents to 5-fold with deionized water and mix well.

- 2) Reagents other than the above  
Ready to use.

## 2. Material Required but not Provided

- 1) Automatic pipettes, Multi-channel pipettes, Disposable pipette tips
- 2) Plate mixer
- 3) Microtiter plate reader ( $\lambda=450, 600-650$  nm)

## 3. Assay Method

- 1) Add 50  $\mu$ L of Assay Diluent into the well.
- 2) Add 50  $\mu$ L of FGF-23 Standards or sample into each well, and cover the plate with a plate sealer.
- 3) Incubate plate at room temperature for 2 hours on a plate mixer.
- 4) Remove the plate sealer. After removing the reaction solution, add 300  $\mu$ L of Wash Buffer Solution into each well and then discard the Wash Buffer Solution. Repeat this process 4 times. Remove any droplets remaining in the wells by tapping the plate on paper towel or similar absorbent material.

Note: In order to avoid wells from drying out when adding Wash Buffer Solution, work quickly with a multi-channel pipette or a similar device.

Note: Make sure the pipette tips do not scratch the well bottoms trying to remove the solution from the well.

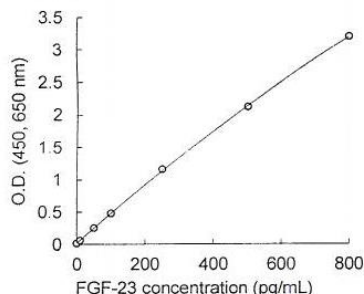
- 5) Add 100  $\mu$ L of FGF-23 Conjugate into each well and cover the plate with a plate sealer.
- 6) Incubate plate at room temperature for 1 hour on a plate mixer.
- 7) Repeat in the same manner 4).
- 8) Add 100  $\mu$ L of Substrate into each well and cover the plate with a plate sealer and an aluminum foil.
- 9) Incubate plate at room temperature for 30 minutes.
- 10) Remove plate sealer and aluminum foil. Immediately add 100  $\mu$ L of Stop Solution into each well. Shake the plate on the plate mixer for 1 minute.
- 11) Read the absorbance at 450 nm (secondary wavelength 600-650 nm) within 10 minutes by microtiter plate reader.

## 【RESULTS】

### 1. Calculation Results

The standard curve is generated by plotting the corrected absorbance of the Std1-Std7 levels on the ordinate against the standard concentration on the abscissa using linear-linear or log-log graph paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results.

### 2. Calibration Curve



### 3. Notes

- 1) In case FGF-23 concentration in the sample exceeds the measurable range, dilute the sample with Std1 and repeat the assay.

- 2) Some samples may cause reaction with other substances and interfere with the assay. When measured value and result have question, confirm the results in retest or other method.

## 【WARNINGS AND PRECAUTIONS】

### 1. Warnings

- 1) Serum samples should be handled as potentially infectious as they may contain infectious agents including HBV, HCV, HIV, etc.
- 2) Any instruments that have come to contact samples should also be treated potentially infectious.
- 3) Wear protective gloves to avoid infection.
- 4) In case any of the reagents contact eyes, mouth and/or skin, immediately flush with copious amount of water then consult a physician as necessary.

### 2. Handling Procedures

- 1) Do not freeze.
- 2) Use any opened reagents as soon as possible. Store any unused Antibody coated Strips in the resealable aluminum pouch with desiccant to protect from moisture.
- 3) The kit should be used before the expiration date printed on the product label.
- 4) Do not pool reagents even if the Lot No. of kits are the same. Do not combine reagents from different kits.

### 3. Disposal Procedures

- 1) Dispose of used reagent bottles as medical waste or industrial waste according to the rules stipulated for waste materials.
- 2) Wipe off if the released amount is small. Flush with copious amount of water if a large volume is released.

## 【STORAGE】

Store at 2-10°C in dark.

## 【WARRANTY】

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions.

KAINOS LABORATORIES, INC. disclaims any implied warranty of merchantability or fitness for a particular purpose, and in no event shall KAINOS LABORATORIES, INC. be liable for consequential.

Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser.

This warranty gives you specific legal right and you may have other rights which vary from state to state.

## 【LITERATURE REFERENCES】

- 1) Yuji Yamazaki : J Clin Endocrinol Metab, 87, 11 : 4957-4960 (2002)
- 2) In-house data

## 【CUSTOMER SERVICE】

KAINOS LABORATORIES, Inc.

38-18, Hongo 2-chome, Bunkyo-ku, Tokyo 113-0033, Japan

e-mail : fgf-23@kainos.co.jp

MANUFACTURER



KAINOS LABORATORIES, INC.

38-18, Hongo 2-chome, Bunkyo-ku, Tokyo 113-0033, Japan



## Nephrology Research laboratory, The Ohio State University

**Title:** Hepcidin Urine ELA-SOP2

**Reference:**

**Version:** 1.3

**Total pages:**

**Origination Date:** 12/13/12

**Supersedes Date:** N/A

**Effective Date:** 4/30/2014

**Written by:** Cassie Moyer, Research Assistant;

**Signature:**

**Date:**

**Modified by:** Anthony Alvarado, Research Fellow;

**Signature:**

**Date:**

**Reviewed by:** Xiaolan Zhang, Research Scientist;

**Signature:**

**Date:** 4/30/14

**Approved by:** Brad H Rovin, Division Director;

**Signature:**

**Date:** 4/30/14

**Distributed to:** Lab310 and Nephrology Clinical Trial Unit

**# of Copies:** 2

### Document History:

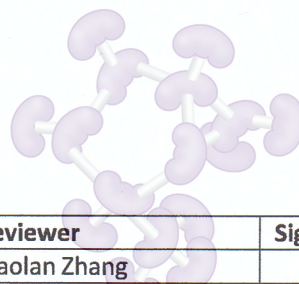
Date	Version	Comment	Reviewer	Signature
	1.0	New SOP	Xiaolan Zhang	
6/04/13	1.1	Modified by Cassie	Xiaolan Zhang	
9/05/13	1.2	Modified by Anthony	Xiaolan Zhang	
4/30/14	1.3	Modified by Xiaolan	Xiaolan Zhang	

Effective Date:

Page 1 of 6

DATE PRINTED: 4/30/14

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9/05/13	1.2	Modified by Anthony	Xiaolan Zhang	
4/30/14	1.3	Modified by Xiaolan	Xiaolan Zhang	Signed



### **Purpose:**

- 1.1. Hepcidin is an antimicrobial peptide originally found in human urine and blood, and it plays a central role in iron homeostasis and inflammation regulation. Increased hepcidin contributes to anemia of chronic disease (ACD) associated with inflammation, chronic kidney disease and some cancers. Decreased hepcidin results in iron overload in hereditary hemochromatosis and ineffective erythropoiesis.
- 1.2. Principle of the assay: The Hepcidin-25 EIA kit (S-1337) from Bachem employs the competitive ELISA technique. The plate is incubated with antiserum which is captured by the pre-coated antibody on the immunoplate. A known concentration of biotinylated tracer competes with varying concentrations of unlabeled standard or sample peptide for binding to the antiserum. After washing away any unbound substances, captured Bt-tracer is bound by streptavidin-conjugated horseradish peroxidase which produces a soluble colored product after TMB substrate is added. The color development is stopped and the intensity of the color is measured.
- 1.3. This immunoassay must be validated for measurement of hepcidin in urine samples before use.

### **2. Specimen Maintenance/Preparation:**

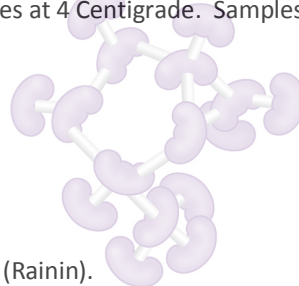
- 2.1. Specimen type: Urine
  - 2.1.1. Samples will arrive frozen and should be stored at -80 Centigrade until thawed once immediately before testing. The information on the number of freeze-thaw cycles prior to the shipment should be recorded.
  - 2.1.2. Minimum sample volume: 25 uL
  - 2.1.3. Immediately before use sample tubes will be arranged in open tube racks with an empty space between each tube for better circulation. Keep the rack in room temperature water bath until all samples are thawed. After which sample tube will be vortex for 5 seconds to resuspend particles. Then samples will be centrifuge at 16.1 g for 5 minutes at 4 Centigrade. Samples will be kept on ice for further analysis.

### **3. Equipment:**

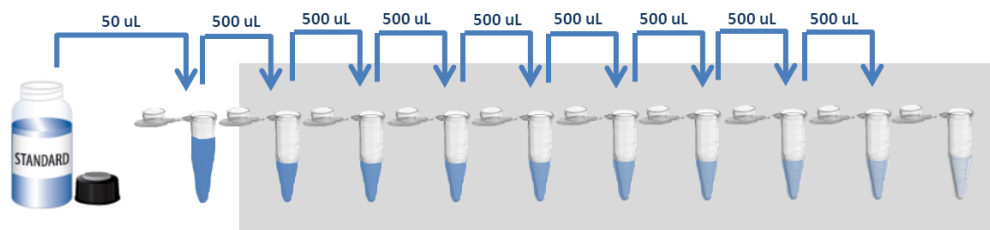
- 3.1. Epoch (BioTek) 96- well microtiter Plate Reader
- 3.2. Gen5 2.01 Computer Software
- 3.3. 96-well plate washer and shaker
- 3.4. Sterile deionized water
- 3.5. Automated Pipettes; Finnpiptette (LabSystems), EDP3-plus (Rainin).

### **4. Reagents/Supplies:**

- 4.1. For all kit components, follow expiration date guidelines in kit. DO NOT mix reagents from different kits.
- 4.2. Bring all reagents to ambient temperature before use (one hour at room temperature).
- 4.3. Hepcidin-25 (human) Enzyme Immunoassay Kit. (Bacham Cat. No. S-1337)
  - 4.3.1. Hepcidin 96-well Immunoplate
    - a. Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 year at 2-4°C.
  - 4.3.2. Hepcidin-25 Standard
    - a. Original lyophilized aliquot can be store for up to 1 year at -20°C (Bachem S-1337 kit protocol). Avoid repeated freeze-thaw cycles.
    - b. Reconstitute the Hepcidin-25 Standard with 1.0 mL of EIA Buffer (1X). This reconstitution produces a stock solution of 1.0ug/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Use microcentrifuge tubes.
      - 4.3.2.b.1. Pipette 950 µL of EIA Buffer into one tube. Pipette 500 µL of EIA Buffer into the remaining eleven tubes.
      - 4.3.2.b.2. Use the stock solution to produce a dilution series (fig 1 below).
      - 4.3.2.b.3. Mix each tube thoroughly before the next transfer.
      - 4.3.2.b.4. The second dilution standard serves as the high standard (25.0ng/mL). The EIA Buffer only serves as the zero standard (0 ng/mL). Diluted standards are made freshly for each run.

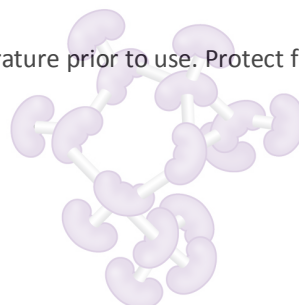






**FIGURE 1. Dilutions of Hepcidin-25 Standard**

- 4.3.3. Anti-Hepcidin-25 (human) Rabbit Antiserum
  - a. May be stored for up to 1 year at -20 °C.
  - b. Reconstitute Antiserum in 5 mL of EIA Buffer approximately 10 minutes before use.
- 4.3.4. EIA Buffer concentrate
  - a. May be stored for up to 1 year at 2-4 °C.
  - b. Dilute 50 mL of EIA Buffer Concentrate into sterile deionized or USP pure water to yield 1000 mL of EIA Buffer (1X).
- 4.3.5. Biotinylated Hepcidin-25 (human) Tracer
  - a. May be stored for up to 1 year at -20 °C.
  - b. Reconstitute Bt-tracer in 5 mL of EIA Buffer approximately 10 minutes before use.
- 4.3.6. HRP Streptavidin
  - a. May be stored for up to 1 year at 2-4 °C.
- 4.3.7. TMB Substrate
  - a. May be stored for up to 1 year at 2-4 °C.
  - b. TMB substrate should be brought to room temperature prior to use. Protect from light.
- 4.3.8. Stop solution (2N HCl)
  - a. May be stored for up to 1 year at 2-4 °C.
- 4.3.9. Plate Sealers (adhesive films)
- 4.4. Items not included in kit:
  - 4.4.1. Room temperature water bath
  - 4.4.2. Eppendorf centrifuge 5415R
  - 4.4.3. SARSTEDT 96-well flat bottom microtest plate
  - 4.4.4. SARSTEDT 1.5 mL microcentrifuge tubes
  - 4.4.5. VWR mini plate shaker
  - 4.4.6. Beckman Coulter MW96 plate washer
  - 4.4.7. opaque plate cover
  - 4.4.8. Pipettes and pipette tips
  - 4.4.9. Mechanical stepper pipette
  - 4.4.10. Deionized, distilled water
  - 4.4.11. Control samples (see section 7.)



## 5. SPECIAL SAFETY PRECAUTIONS:

- 5.1. This assay uses human source materials and should be performed in a BSLII laboratory facility.
- 5.2. Personal protective equipment:
  - 5.2.1. gloves
  - 5.2.2. gown
  - 5.2.3. optional face shield is available
- 5.3. The stop solution provided with this kit is an acid. Use care when working with acids and wash hands thoroughly after handling.

## 6. CALIBRATION/PROGRAMMING/MAINTENANCE:

- 6.1. Calibration

- 6.1.1. A standard curve is plotted with each run of the assay. Controls with known values are analyzed, along with the standards and patients, to verify an acceptability of the assay performance.
- 6.1.2. The Epoch (BioTek) plate reader automatically performs an internal calibration before each reading.
- 6.2. Maintenance procedure
  - 6.2.1. Daily Maintenance
    - a. Check for spills or excessive dust and wipe outside surfaces of reader as needed.
  - 6.2.2. Weekly Maintenance
    - a. Clean the outside surfaces of the Epoch plate reader using a paper towel that has been dampened with water. Do not use abrasive cleaners. If required, clean the surfaces using a mild dish soap solution diluted with water, or glass cleaner, and then wipe with a clean damp cloth to remove any residue. Do not spray cleaner directly onto the instrument.

## 7. Quality Control:

- 7.1. Control materials are individual urine donor from in-house sample pool that has been tested multiple times to establish an acceptable range. These control urine samples are stored at -80°C freezer in box label Urine biopsy. Aliquot the urine into 10-12, 1.5mL tubes. Stored samples are recorded in Excel spreadsheet.
  - 7.1.1. Current acceptable range for low control (Bio5): **3.71 – 13.18** ng/ml
  - 7.1.2. Current acceptable range for medium control (Bio8): **81.19 – 177.30** ng/ml
  - 7.1.3. Current acceptable range for high control (Bio12): **193.32 – 275.64** ng/ml
- 7.2. Control samples are prepared in the same way as patient samples. Pull samples from -80°C freezer. Sample tubes will be arranged in open tube racks with an empty space between each tube for better circulation. Keep the rack in room temperature water bath until all samples are thawed. After which sample tube will be taken out of water and vortex for 5 seconds to resuspend particles. The tubes will be kept on ice for further analysis. To the degree possible, measure the analytes of interest without refreezing the aliquot tube. If the aliquot tubes are to be refrozen, make marks that how many times it has been freeze and thawed.
- 7.3. Three levels of control, a low, medium and high control, are included in duplicate with each run.
- 7.4. For a run to be considered valid, all levels of control must fall into the pre-established posted range.
  - 7.4.1. If the duplicates of each level are within the established  $\pm 2SD$  range, AND their CV's are  $\leq 15\%$ , then the QC is acceptable.
  - 7.4.2. If one replicate of a control value is outside the established  $\pm 2SD$  range, the Westgard 2 rules will be applied to determine the validity of the run.
    - a. ONE replicate of any two controls MAY be outside the established  $\pm 2SD$  range, CV of the replicates must still be  $\leq 15\%$ .
    - b. If one or both replicate values are outside of the  $\pm 3SD$  range, the run must be rejected and repeated.
    - c. If both replicate values are out of the established  $\pm 2SD$  range, the run must be rejected and repeated.
- 7.5. Control values will be recorded in a log and reviewed periodically to monitor for statistical drift and shifts. A recent QC levey-jennings plot is presented in Figure 2 for three in house urine controls run between Oct 2013 to Nov 2014. The acceptable range is Mean $\pm 2SD$ .
- 7.6. New lots of controls will be tested in parallel with existing lots and an acceptable range determined before placing the new lot into service and discontinuing the current lot.

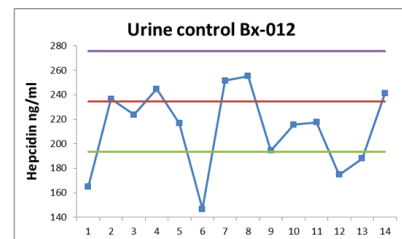
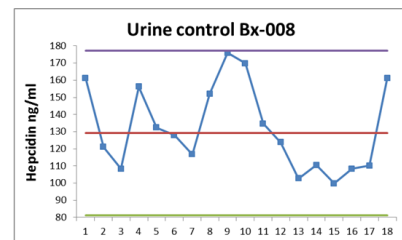
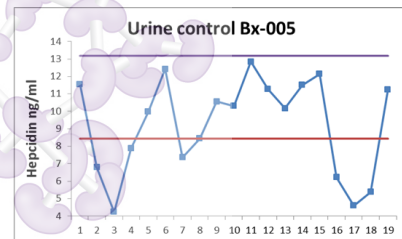


Figure 2. QC Levey-Jennings Plot Oct-Nov 2013

## 8. Test Procedure:

- 8.1. Bring all reagents and samples to room temperature before use. All standards, samples, and controls will be assayed in duplicate and plotted on a plate template.
- 8.2. Prepare all reagents, working standards, and samples as directed in the previous sections.
- 8.3. Remove excess immunoplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
- 8.4. Add 25  $\mu$ L of Antiserum to each well excluding blank wells. Add 25  $\mu$ L of EIA Buffer (1X) to blank wells. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature.
- 8.5. Add 50  $\mu$ L of Standard, sample\* (see 9.4), or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
- 8.6. Add 25  $\mu$ L of Bt-tracer to each well. Cover with adhesive strip provided. Incubate overnight at 4°C.
- 8.7. The following day, bring all reagents and immunoplate to room temperature before use.
- 8.8. Aspirate each well and wash, repeating the process for a total of five washes. Wash by filling each well with EIA 1X Buffer (400  $\mu$ L) using the Beckman Coulter MW96 plate washer. After the last wash, remove any remaining Wash Buffer by inverting the plate and blot it against clean paper towels.
- 8.9. Add 100  $\mu$ L of Streptavidin-HRP to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
- 8.10. Repeat the aspiration/wash as in step 8.8.
- 8.11. Add 100  $\mu$ L of TMB Substrate Solution to each well. Incubate for 10 minutes at room temperature, mixing on shaker at 300 rpm. Protect from light by completely covering the plate with an opaque plate cover.
- 8.12. Add 100  $\mu$ L of Stop Solution to each well.
- 8.13. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. Set wavelength correction to 570 nm.
- 8.14. The following instructions in steps 8.14.1-8.14.11 are for the Epoch (BioTek) plate reader used with PC name: imimm-c3jgbm1. For other plate readers, please see section 8.15.
  - 8.14.1. Turn on the Epoch plate reader and allow it to warm up.
  - 8.14.2. Open the Gen5 2.01 software by clicking the icon on the desktop.
  - 8.14.3. Login with username and password.
  - 8.14.4. Under the "Create a New Item" heading, select "Experiment".
  - 8.14.5. Open the "HepcidinEIA S-1337" protocol, saved in C:\Program Files\BioTek\Gen52.01\Protocols.
  - 8.14.6. In the left-most window of the screen, expand the "Protocol" tab. Double click on "Plate Layout" and adjust the layout and/or dilution factors as appropriate. Press "OK" when complete.
  - 8.14.7. In the toolbar across the top of the screen, press the "Read Plate" button (green arrow overlying a grey rectangle).
  - 8.14.8. A prompt will be displayed instructing the user to place the plate on the carrier. Do so and press "OK" to begin plate reading.
  - 8.14.9. After the plate has been read, a window will be displayed prompting the user to save the experiment file. Select the appropriate location and press "Save".
  - 8.14.10. Once the reading is complete and plate has been saved, click the "Statistics" tab. From the "Data:" drop-down menu, select "Conc x Dil". Click the button with the Excel logo to export the data to a new excel document.
  - 8.14.11. In Gen5, select the "Graphs" tab. Export the "Curve" and "Results" to the excel document in the same manner as step 8.14.10.
- 8.15. For plate readers other than the Epoch, please utilize the following guidelines:
  - 8.15.1. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

## 9. Calculations:

- 9.1. Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. This is done by the Gen5 2.01 software automatically.
- 9.2. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The best fit line can be determined by a 4-parameter non-linear regression analysis. This procedure is performed by the Gen5 2.01 software.
- 9.3. Because urine samples have been diluted prior to the assay, the measured concentrations must be multiplied by the dilution factor. (This is done automatically by the Gen 5 2.01 software, dilution factors are entered in by the user.)
- 9.4. **If measured OD results do not fall within the range of the corresponding standard curve OD values for concentrations 6.25ng/mL to 0.0 ng/mL**, further dilutions or neat samples must be run. Dilute samples as needed and repeat assay procedure.

## 10. REPORTING RESULTS/DATA TRANSFER:

- 10.1. Reference intervals: no literature from the manufacturer available. Local reference range is in the process of being established.
- 10.2. Analytical Measurement Range: 0- 6.25 ng/ml
- 10.3. Hepcidin-25 final values will be reported as an average of two replicates with the corresponding CV. Values will be reported in ng/ml and rounded to three decimal points.
- 10.4. Results from valid runs will be copied from the exported data Excel sheet into a master Excel reporting workbook in sequential order. Patient data, control samples, standard curve data and graph for each experiment will be included on the same sheet. This master workbook will be transferred securely to the coordinating site and contain the following information:
  - 10.4.1. Sample ID
  - 10.4.2. Date of the assay
  - 10.4.3. Performer of the assay
  - 10.4.4. Reagent lot numbers (kit)
  - 10.4.5. Number of freeze/thaw cycles for each sample and control
  - 10.4.6. Concentration of Hepcidin-25 (ng/mL) for patient samples and controls
  - 10.4.7. %CV between replicates for patient samples and controls as needed

## 11. OTHER TECHNICAL CHARACTERISTICS:

- 11.1. Assay precision: Please refer to Reference 12.2
- 11.2. Analytical sensitivity of the assay: Please refer to Reference 12.2
- 11.3. Sample Linearity: Please refer to Reference 12.2
- 11.4. Urine sample stability: Please refer to Reference 12.2
  - 11.4.1 Please refer to Reference 12.2
  - 11.4.2 Sample requirement studies indicated that common conditions for sample storage and processing usually do not cause >10% variation in detection of the analyte. However, investigators should avoid extreme conditions such as storage of urine at room temperature for > 4 hours, or at refrigerated conditions for more than 24 hours, or >3 cycles of freeze-thaw.
- 11.5. Limit of detection: Please refer to Reference 12.2
- 11.6. Recovery rate: Please refer to Reference 12.2

## 12 References:

- 12.1 Package Insert: Peninsula Laboratories, LLC Peptide Enzyme Immunoassay (EIA) Protocols: Protocol V (Ab1hr.Std2hr.BtON)
- 12.2 Validation of Human Urine Hepcidin Measurement on Bachem Hepcidin-25 (human) EIA Kit (attached, Sept 2013)

## Validation of Human Urine Hepcidin Measurement on Bachem Hepcidin-25 (human) EIA Kit

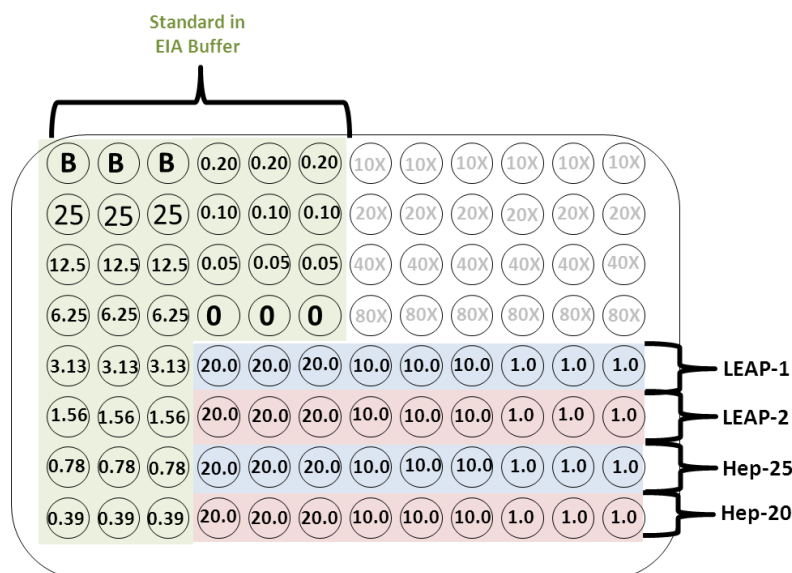
**Intended Use:** urine Hepcidin-25 as a prognostic biomarker for lupus nephritis

The Bachem Hepcidin-25 (human) EIA Kit S-1337 is validated by Peninsula Laboratories, LLC for the use of measuring Hepcidin-25 in human serum/plasma. To measure urine levels of Hepcidin-25, the kit must first be validated for use with urine. Seven validation experiments were conducted to determine:

- Peptide Specificity
- Buffer choices
- Assay precision
- Spike and Recover
- Sample Linearity
- Sensitivity
- Urine sample stability and lifespan
- Urine Internal Control

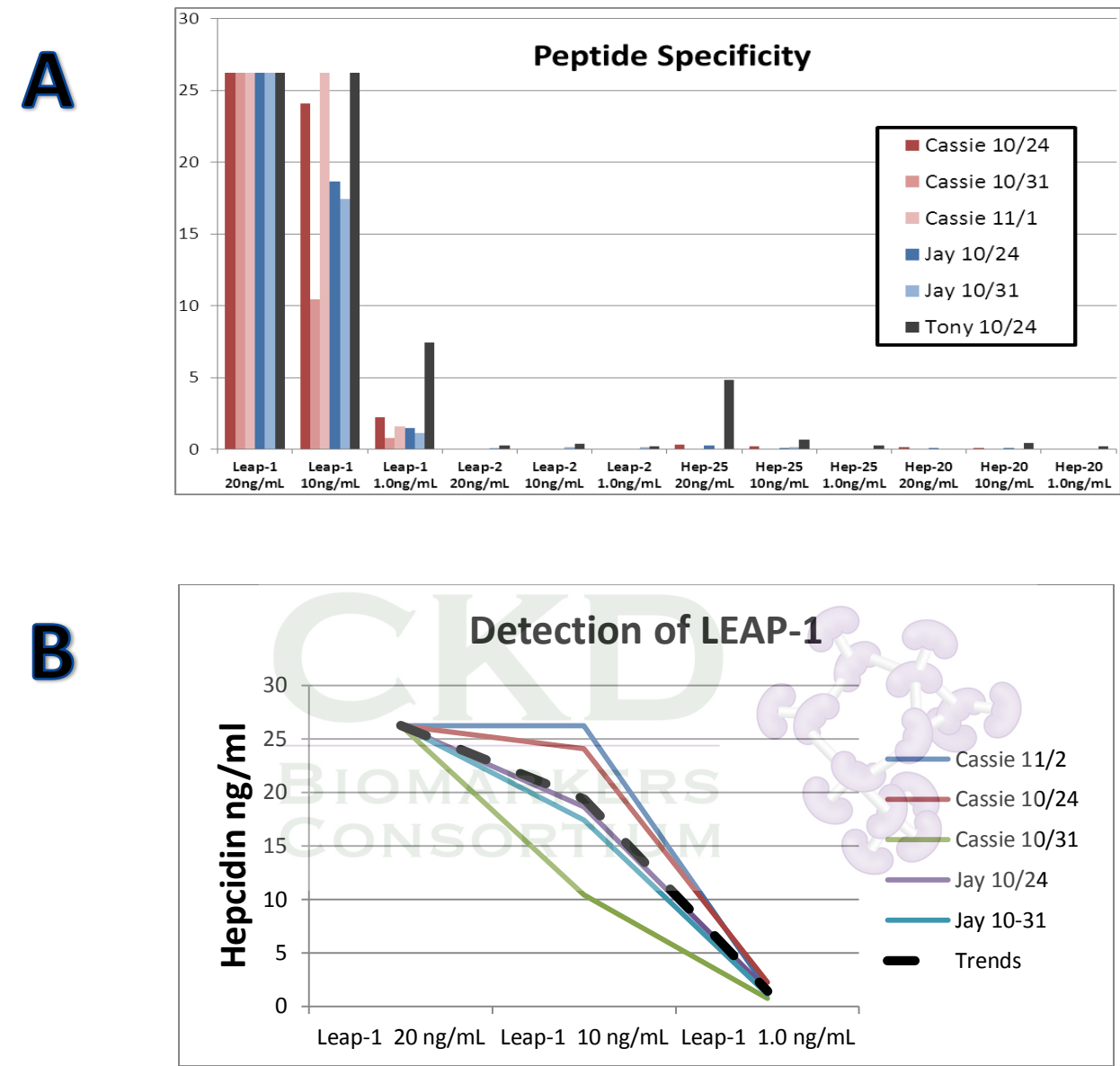
### 1) Peptide Specificity

**Method:** In order to determine whether the Bachem Kit S-1337 can specifically detect hepcidin peptides, four different hepcidin peptides were plated at 1.0, 10.0 and 20.0ng/mL and measured against the prepared standard (using the kit S-1337 standard peptide diluted in EIA Buffer). Two of the peptides were ordered from Peptides International – **LEAP-1** (PLP-4392-s LOT#620510) and **LEAP-2** (PLP-4405-s LOT#560627). The remaining two peptides were ordered from Alpha Diagnostics – **Hep-25** (Cat#HEPC61-P LOT#45662P1) and **Hep-20** (Cat#HEPC71-P LOT#29760P1). All standards, samples and blanks were plated in triplicates (See Figure 8). This method was used on both the S-1337 Kit plate as well as a Goat Anti-Rabbit IgG self-coated plate (all reagents are from the kit S-1337, performed by Jay and Tony). This experiment was completed three times on different days (10-24-12, 10-31-12 and 11-1-12).



**Figure 1.** Plate template for peptide specificity validation ELISA

**Results:** Absorbance of ELISA was read at 450nm. The concentration of all four peptides was calculated from the standard curve generated (Figure 1-A). **Note:** The results from Tony’s plate are incorrect due to contamination with Leap-1 peptide so the data is not included in Figure 2-B



**Figure 2.** Detection of hepcidin peptide Leap-1, Leap-2, Hep-25 and Hep-20 using Bachem S-1337 (Cassie) and self-coated kit (Jay).

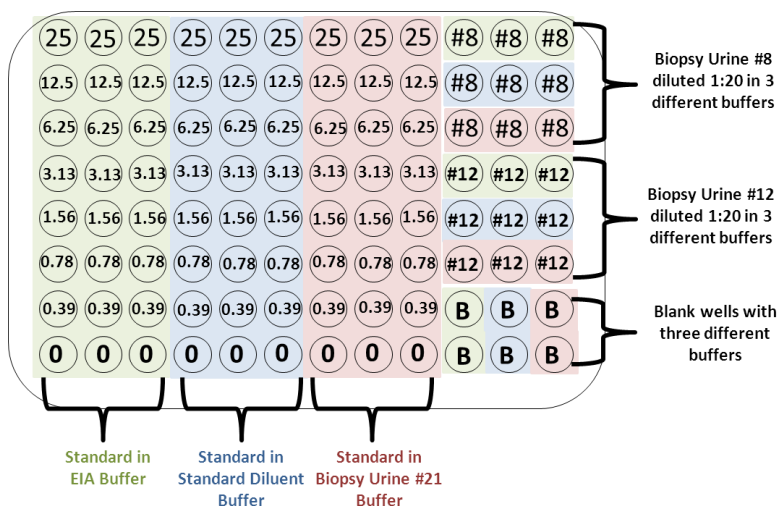
**Conclusion:** The results show that only Leap-1 was specifically detected using the Bachem kit (whether using the kit plate or a self-coated plate with kit reagents). The kit cannot detect Leap-2 (a Hep-20 peptide). It is unclear why the kit could not detect Hep-25 since it should be the same peptide as Leap-1. A previous lab work did not show that hepcidin peptide from ADL is not working in western blot either. From these results, it can be assumed that if Hep-20 peptides were present in urine samples, the Bachem kit S-1337 would not be able to detect them.

2) Buffer choice

**Method:** Three buffers were chosen for comparison – EIA Buffer (1X) provided by the kit, Standard Diluent [1ug/mL] provided by the kit and a 1:20 dilution of Biopsy Urine #21 in EIA Buffer. The hep-25 standard peptide (provided by the kit) was prepared as an 8 Standard serial dilution from 25ng/mL to 0ng/mL in each of the three buffers. Biopsy



Urine #8 and #12 were prepared as a 1:20 dilution in each of the three buffers. All standards and samples were plated in triplicates (See Figure 3). Duplicate blanks containing each of the three buffers were plated. This method was used on both the S-1337 Kit plate as well as a Goat Anti-Rabbit IgG self-coated plate. This experiment was completed three times on different days (9-19-12, 9-26-12 and 9-28-12).



**Figure 3.** Plate template for Dilution Buffer Choice validation ELISA.

**Results:** Absorbance of ELISA was read at 450nm. Each plate was read three different times using each of the three standards. All the raw data were presented in table 1. The hepcidin concentration of Urine Biopsy #8 and #12 was calculated using each standard curve (table 1). **Note:** the results shown are for the Bachem S-1337 kit plate only. The self-coated plates were not performed correctly.

EIA as buffer	9/19/2012	9/26/2012	9/28/2012	Mean	SD	CV%
Diluent Buffer [25]	23.767	23.358	UD	23.563	0.289	1.227
Diluent Buffer [12.5]	18.026	12.622	12.530	14.393	3.147	21.865
Diluent Buffer [6.25]	7.555	6.257	5.464	6.425	1.056	16.429
Diluent Buffer [3.125]	3.845	3.403	2.840	3.363	0.504	14.980
Diluent Buffer [1.5625]	2.055	1.811	1.574	1.813	0.241	13.263
Diluent Buffer [0.78125]	0.959	0.912	0.796	0.889	0.084	9.437
Diluent Buffer [0.390625]	0.459	0.471	0.385	0.438	0.047	10.626
Diluent Buffer [0]	0.020	0.014	0.008	0.014	0.006	42.857
Urine #21 Buffer [25]	UD	21.684	UD	21.684	UD	#VALUE!
Urine #21 Buffer [12.5]	UD	13.211	21.015	17.113	5.518	32.246
Urine #21 Buffer [6.25]	11.960	6.225	6.738	6.482	0.363	5.597
Urine #21 Buffer [3.125]	6.324	3.683	3.258	3.471	0.301	8.659
Urine #21 Buffer [1.5625]	3.290	1.888	1.835	1.862	0.037	2.013
Urine #21 Buffer [0.78125]	1.806	1.053	0.938	0.996	0.081	8.168
Urine #21 Buffer [0.390625]	0.961	0.559	0.486	0.523	0.052	9.879
Urine #21 Buffer [0]	0.057	0.042	0.081	0.062	0.028	44.841
Biopsy #8 in EIA	194.683	145.008	183.494	174.395	26.058	14.942
Biopsy #8 in Diluent	246.085	229.673	173.375	216.378	38.135	17.624
Biopsy #8 in Urine #21	278.912	249.747	184.518	237.726	48.332	20.331
Biopsy #12 in EIA	396.496	359.855	407.469	387.940	24.933	6.427
Biopsy #12 in Diluent	476.584	410.439	391.771	426.265	44.566	10.455
Biopsy #12 in Urine #21	341.373	312.253	408.073	353.900	49.123	13.880

Diluent as Buffer	9/19/2012	9/26/2012	9/28/2012	Mean	SD	CV%
EIA Buffer [25]	23.585	UD	16.453	20.019	5.043	25.191
EIA Buffer [12.5]	9.944	11.656	11.613	11.071	0.976	8.818
EIA Buffer [6.25]	4.364	5.510	5.313	5.062	0.613	12.104
EIA Buffer [3.125]	2.494	2.819	3.052	2.788	0.280	10.051
EIA Buffer [1.5625]	1.351	1.427	1.973	1.584	0.339	21.425
EIA Buffer [0.78125]	0.624	0.677	0.806	0.702	0.094	13.328
EIA Buffer [0.390625]	0.333	0.311	0.365	0.336	0.027	8.074
EIA Buffer [0]	UD	UD	0.012	0.012	UD	#VALUE!
Urine #21 Buffer [25]	UD	22.561	UD	22.561	UD	#VALUE!
Urine #21 Buffer [12.5]	UD	12.976	16.639	14.808	2.590	17.492
Urine #21 Buffer [6.25]	9.396	5.859	6.908	7.388	1.817	24.590
Urine #21 Buffer [3.125]	4.828	3.368	3.512	3.903	0.805	20.616
Urine #21 Buffer [1.5625]	2.585	1.681	1.956	2.074	0.463	22.344
Urine #21 Buffer [0.78125]	1.447	0.907	0.964	1.106	0.297	26.825
Urine #21 Buffer [0.390625]	0.790	0.464	0.477	0.577	0.185	31.989
Urine #21 Buffer [0]	0.036	0.019	0.059	0.038	0.020	52.829
Biopsy #8 in EIA	147.583	138.001	181.180	155.588	22.675	14.574
Biopsy #8 in Diluent	189.043	225.356	156.294	190.231	34.546	18.160
Biopsy #8 in Urine #21	216.968	246.009	181.152	214.710	32.487	15.131
Biopsy #12 in EIA	329.417	365.967	379.650	358.345	25.969	7.247
Biopsy #12 in Diluent	429.048	410.600	354.343	397.997	38.914	9.778
Biopsy #12 in Urine #21	337.503	313.270	310.454	320.409	14.871	4.641

Urine #21 as buffer	9/19/2012	9/26/2012	9/28/2012	Mean	SD	CV%
EIA Buffer [25]		UD	12.084	12.084	UD	#VALUE!
EIA Buffer [12.5]		11.722	9.079	10.401	1.869	17.969
EIA Buffer [6.25]		5.401	4.460	4.931	0.665	13.495
EIA Buffer [3.125]		2.680	2.610	2.645	0.049	1.871
EIA Buffer [1.5625]		1.303	1.689	1.496	0.273	18.245
EIA Buffer [0.78125]		0.586	0.670	0.628	0.059	9.458
EIA Buffer [0.390625]		0.247	0.271	0.259	0.017	6.552
EIA Buffer [0]		UD	UD	UD	UD	#VALUE!
Diluent Buffer [25]		23.939	22.812	23.376	0.797	3.409
Diluent Buffer [12.5]		12.631	8.817	10.724	2.697	25.148
Diluent Buffer [6.25]		5.727	4.414	5.071	0.928	18.310
Diluent Buffer [3.125]		2.948	2.644	2.796	0.215	7.688
Diluent Buffer [1.5625]		1.477	1.444	1.461	0.023	1.598
Diluent Buffer [0.78125]		0.684	0.682	0.683	0.001	0.207
Diluent Buffer [0.390625]		0.315	0.274	0.295	0.029	9.844
Diluent Buffer [0]		UD	UD	UD	UD	#VALUE!
Biopsy #8 in EIA		136.299	145.012	140.656	6.161	4.380
Biopsy #8 in Diluent		226.571	125.157	175.864	71.711	40.776
Biopsy #8 in Urine #21		248.812	142.946	195.879	74.859	38.217
Biopsy #12 in EIA		373.395	275.651	324.523	69.115	21.298
Biopsy #12 in Diluent		423.728	257.773	340.751	117.348	34.438
Biopsy #12 in Urine #21		316.990	309.293	313.142	5.443	1.738

Table 1. Hepcidin raw data in validation of buffer choice on Bachem kit S-1337. Hepcidin value is presented as ng/ml.

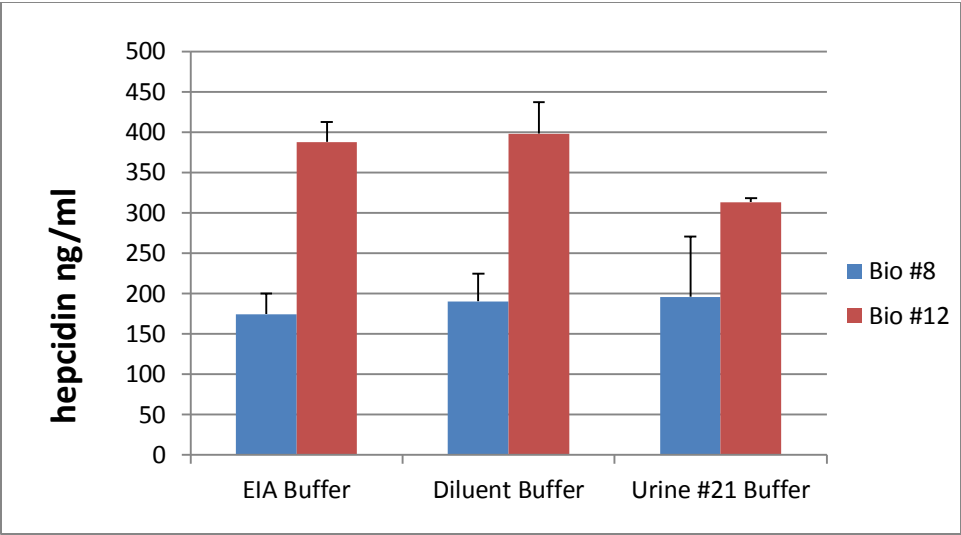


Figure 4. Urine hepcidin measurement using 3 different buffers.

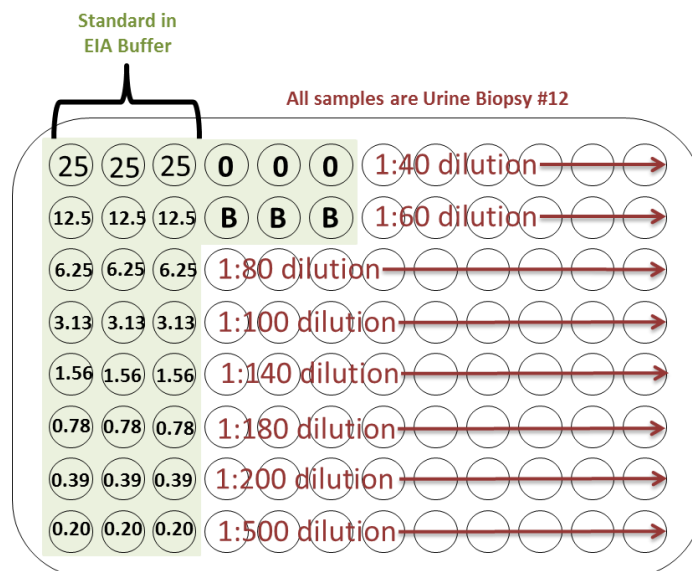
		EIA as Buffer	Diluent as Buffer	Urine #21 as Buffer
Bio #8	Mean	174.40	190.23	195.88
	SD	26.06	34.55	74.86
	CV	0.15	0.18	0.38
Bio #12	Mean	387.94	398.00	313.14
	SD	24.93	38.91	5.44
	CV	0.06	0.10	0.02

Table 2. Urine sample hepcidin measurement using 3 different buffers. The EIA as buffer is chosen as it provide small CV in experiments (highlighted in red).

**Conclusions:** All standard curves using different buffer have very good correlation of 4-parameter fitting and  $R^2$  is around 1.0. The EIA Buffer generated standard provides the most accurate urine sample Bio #8 and Bio #12 measurement as it provide small SD and inter CV between different plate and day of experiments (Figure 4 and Table 2). EIA Buffer was chosen as the appropriate dilution buffer for urine samples.

### 3) Assay Precision

**Method:** To calculate the intra %CV for the Bachem S-1337 kit, Biopsy Urine #12 was plated at eight dilutions of 6 to 9 repeats on a single ELISA kit plate (6/4/13). The standard was plated in triplicates from 25.0ng/mL to 0ng/mL (See Figure 5). To calculate the inter %CV for the Bachem S-1337 kit, Biopsy Urine #8 was plated in duplicates at 1:20 dilution on 22 different plates, performed on different days (11-19-12, 11-20-12, 11-26-12, 11-27-12, 11-28-12, 11-29-12 and 11-30-12) by the same experimenter. The standard and blank were plated in duplicates. More standard precision results can be provide from buffer choice test in this validation from Section 2 buffer choice.



**Figure 5.** Plate template for intra %CV validation ELISA

**Results:** Absorbance of ELISA was read at 450nm. The calculations for inter and intra %CV were as followed.

**Intra-%CV: Standard Deviation** (of all 6-9 measured concentration values)/**Mean** (of all 6-9 measured concentration values) \* 100

**Inter-%CV: Standard Deviation** (of all measured concentration values from all 22 plates)/**Mean** (of all measured concentration values from all 22 plates)\* 100

The measured Intra %CV should fall below 15% and the measured Inter %CV should fall below 20% to validate the Bachem S-1337 kit for use of urine samples. All CV's greater than the allowed percentage are indicated in RED (see Table 3 and Table 4).

	40X	60X	80X	100X	140X	180X	200X	500X
Measured Concentrations (6/4/13)	216.635	166.003	197.885	228.59	245.073	234.426	262.73	354.282
	226.644	207.674	195.707	260.205	211.878	274.522	251.573	390.677
	213.857	171.003	224.73	267.35	225.467	251.709	278.438	376.611
	168.439	197.535	217.746	258.223	213.517	234.023	235.787	335.11
	235.223	193.702	216.602	248.787	223.759	258.63	250.296	369.659
	229.192	228.247	240.485	247.865	291.567	224.379	257.801	447.235
			224.932	254.334	268.306	261.997	346.188	447.915
			234.372	258.771	238.307	230.963	239.27	290.676
			288.381	293.509	263.772	261.13	NA	354.455
Average	214.998	194.027	226.760	257.515	242.405	247.975	265.260	374.069
STDev	24.157	23.174	27.462	17.384	27.316	17.415	35.336	50.444
%CV	11.236	11.944	12.111	6.751	11.269	7.023	13.321	13.485

**Table 3.** Calculated Intra-%CV using Bio #12 human urine sample.

Plate #	Biopsy Urine #8 [ng/mL]
1	119.012
2	151.995
3	116.551
4	147.407
5	137.773
6	144.637
7	136.995
8	170.21
9	90.653
10	165.832
11	163.841
12	167.131
13	128.396
14	180.243
15	138.597
16	221.844
17	178.718
18	152.809
19	159.365
20	119.386
21	167.79
22	178.845
Avg	151.729
StDev	28.214
%CV	18.595

**Table 4.** Calculated Inter-%CV for Bio #8 human urine sample.

Hepcidin spiked	25 ng/ml	12.5 ng/ml	6.25 ng/ml	3.12 ng/ml	1.56 ng/ml	0.78 ng/ml	0.39 ng/ml	0 ng/ml
EIA/Diluent	1.2274	21.8646	16.4289	14.9796	13.2633	9.4374	10.6257	42.8571
EIA/Urine	#DIV/0!	32.2460	5.5966	8.6593	2.0133	8.1685	9.8792	44.8409
Diluent/EIA	25.1915	8.8181	12.1040	10.0512	21.4254	13.3280	8.0735	#DIV/0!
Diluent/Urine	#DIV/0!	17.4920	11.6199	2.9600	10.6931	4.3084	1.9537	72.5238
Urine/EIA	#DIV/0!	17.9692	13.4953	1.8714	18.2449	9.4581	6.5523	#DIV/0!
Urine/Diluent	3.4092	25.1483	18.3104	7.6881	1.5977	0.2071	9.8443	#DIV/0!

**Table 5.** Standard inter CV% in spike / recovery assay using different buffer. EIA/Diluent, standard curve in EIA buffer with standard spiked in diluent buffer; EIA/Urine, standard curve in EIA buffer with standard spiked in urine #21; Diluent/EIA, standard curve in Diluent buffer with standard spiked in EIA buffer; Diluent/Urine, standard curve in Diluent buffer with standard spiked in Urine #21; Urine/EIA, standard curve in Urine #21 with standard spiked in EIA buffer; Urine/Diluent, standard curve in Urine #21 with standard spiked in diluent buffer.

## CV% in choice of buffer

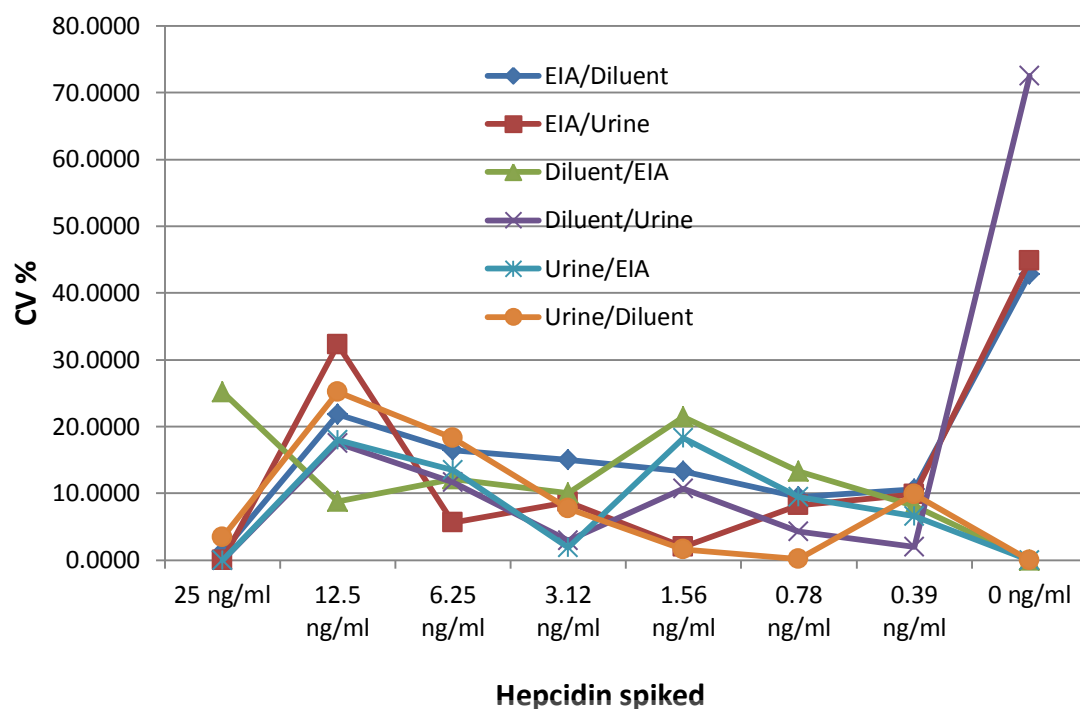


Figure 6. Inter-CV % in buffer choices for Bachem S-1337 EIA kit.

**Conclusions:** It is clearly suggested that inter-CV is below 20% between standard the lowest concentration tested 0.39 to 6.25 ng/ml in spiking and recovery test. Both the Intra and Inter-%CVs for urine sample hepcidin were below the cut-off percentages of 15% and 20% respectively. These results suggest that the kit is valid for the accurate measurement of urine hepcidin levels with standard range from the lowest concentration tested 0.39 to 6.25 ng/ml.

## 4) Standard Spike and Recovery

**Method:** The Spike and Recovery test was recovered from our buffer choice experiment on 9/26/12 and 9/28/12 (see section 2 Buffer choice), according to R&D protocol

([http://www.woongbee.com/0NewHome/RnD/ELISA/RnD\\_%20SPIKEandREC2006.pdf](http://www.woongbee.com/0NewHome/RnD/ELISA/RnD_%20SPIKEandREC2006.pdf)). The low hepcidin urine Bio #21 was used as sample diluent for standard spiking. Standard, samples and blanks were plated in triplicates (see section 2). This method was used on both the S-1337 Kit plate as well as a Goat Anti-Rabbit IgG self-coated plate (performed by Cassie). Only data from S-1337 kit are presented.

**Results:** Absorbance of ELISA was read at 450nm. Recovery and linearity were calculated as percentages using the following formulas.

**Recovery** (%Recovery) = (Observed concentration – Neat concentration)/ Expected concentration \* 100

**Linearity** (%Recovery) = (Observed concentration \* Dilution Factor)/ Expected concentration\* 100

The results for each sample are outlined in the tables below. Values that could not be determined (because the measured concentration was greater than the standard curve values) are indicated by “?????”. Ideal %Recovery should fall within the range of 80-120%. All percent outside this range are indicated in Yellow.



EIA as standard buffer

Standard spiked	9/26/2012	9/28/2012	Average	Recovery
Urine #21 Buffer [25]	21.684	?????	21.684	86.49
Urine #21 Buffer [12.5]	13.211	21.015	17.113	136.412
Urine #21 Buffer [6.25]	6.225	6.738	6.4815	102.72
Urine #21 Buffer [3.125]	3.683	3.258	3.4705	109.088
Urine #21 Buffer [1.5625]	1.888	1.835	1.8615	115.3846154
Urine #21 Buffer [0.78125]	1.053	0.938	0.9955	119.7435897
Urine #21 Buffer [0.390625]	0.559	0.486	0.5225	118.2051282
Urine #21 Buffer [0]	0.042	0.081	0.0615	0

Diluent as standard buffer

Standard spiked	9/26/2012	9/28/2012	Average	Recovery
Urine #21 Buffer [25]	22.561	?????	22.561	90.088
Urine #21 Buffer [12.5]	12.976	16.639	14.8075	118.148
Urine #21 Buffer [6.25]	5.859	6.908	6.3835	101.512
Urine #21 Buffer [3.125]	3.368	3.512	3.44	108.832
Urine #21 Buffer [1.5625]	1.681	1.956	1.8185	114.0705128
Urine #21 Buffer [0.78125]	0.907	0.964	0.9355	114.9358974
Urine #21 Buffer [0.390625]	0.464	0.477	0.4705	110.6410256
Urine #21 Buffer [0]	0.019	0.059	0.039	0

Table 6. Raw data of spike / Recovery of hepcidin standard in different buffer.

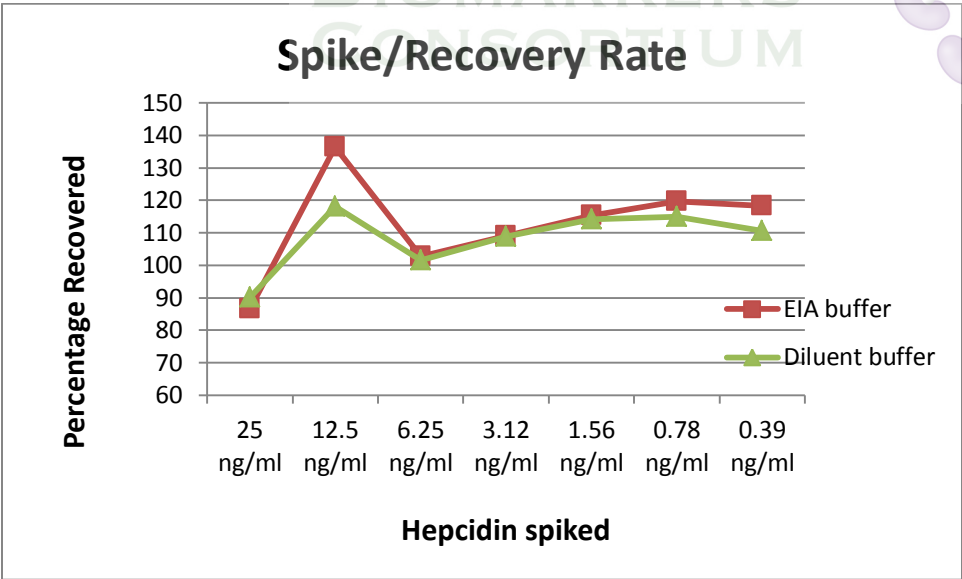


Figure 7. Hepcidin standard spike / recovery rate in both EIA and Diluent buffer.

**Conclusions:** These experiments show that the hepcidin spike/recovery rate are between 80-120% below OD of less than 6.25 ng/ml hepcidin standard, which is consistent to the good inter CV result range (less than 20%) below hepcidin 6.25 ng/ml standard. So we postulate that the most accurate concentration reading are within the OD range

**correspondent the hepcidin standard 6.25 ng/ml below.** This also means that human urine does not contain components interfering hepcidin EIA assay.

Another supportive experiments done in line with this validation (from UO1 urine samples 2012-2013) is that off –OD- range urine measurement were repeated using appropriate dilution to keep OD within the suggested OD range ( OD correspondent to 6.25 ng/ml below). The data summary and graph is presented in figure 8. It is indicated that any measurement with OD lower than standard 6.25 ng/ml is significantly higher than real hepcidin value so all the urine samples with lower OD should be re-measured until adjusted to the suggested OD range.

OD/StandardRange (ng/ml)	<0.39	1.56-6.25	6.25-12.5	12.5-25	>25
Fold	0.922584856	1.015514542	1.616273525	1.745882118	2.192263911
SD	0.88024453	0.672161117	1.381073761	0.902955261	0.485139532
N	31	14	69	44	10
P-value	0.633629267	0.623182	0.000467276	3.57581E-06	7.82141E-05

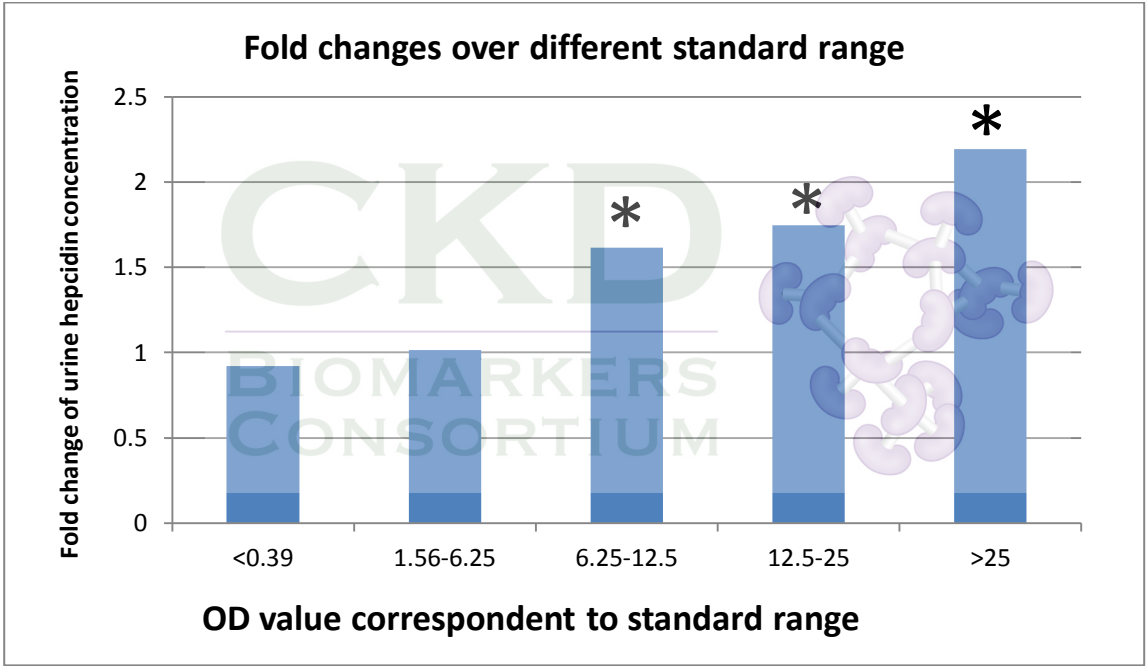


Figure 8. Determination of OD range for accurate urine hepcidin measurement. The raw data summary was presented in the top table. Fold change is calculated using off-range-OD hepcidin concentration normalized to within-range-OD hepcidin concentration. \* indicates where the significance is in the graph presentation.

### 5) Sample Linearity (proper urine dilution)

**Method:** To determine whether the Bachem S-1337 kit provide linearity on measuring urine hepcidin and what proper dilution gives most accurate urine hepcidin value, **Biopsy Urine #12** was plated with a dilution factor of 100, 150, 200, 300 and 500 for natural linearity since it has a high concentration of hepcidin (greater than 60% of the high standard). All standards, blanks and urine dilution were plated in triplicates. This experiment was completed twice on different days by two performers (7-26-13 and 8-2-13).

**Results:** Absorbance of ELISA was read at 450nm. The concentration of hepcidin at all seven dilution factors was calculated from the standard curve generated and the Inter CV was calculated (Table 7). A dilution parallelism was used to compare the percent recovery of concentration values between dilutions (see Figure 9).

RecoveryRate

	T/7-26-13	X/7-26-13	T/8-2-13	X/8-2-13	Trend
1:100	1.0362368	1.1269513	1.0710744	0.8794325	1.0284238
1:150	1.032135	1.1519436	1.1812098	0.8613654	1.0566635
1:200	1.0967325	1.0326166	1.3051309	0.9166705	1.0877876
1:300	1.0135908	1.2389512	1.6125	1.0259562	1.2227496
1:500	1.0638852	1.7020444	2.3189968	1.3537668	1.6096733

**Table 7.** Dilution Parallelism between dilutions of Biopsy Urine #12. The 40X dilution is considered the “NEAT” dilution therefore, percentage of recovery is calculated by dividing the concentration of each dilution by the concentration measured at 40X.

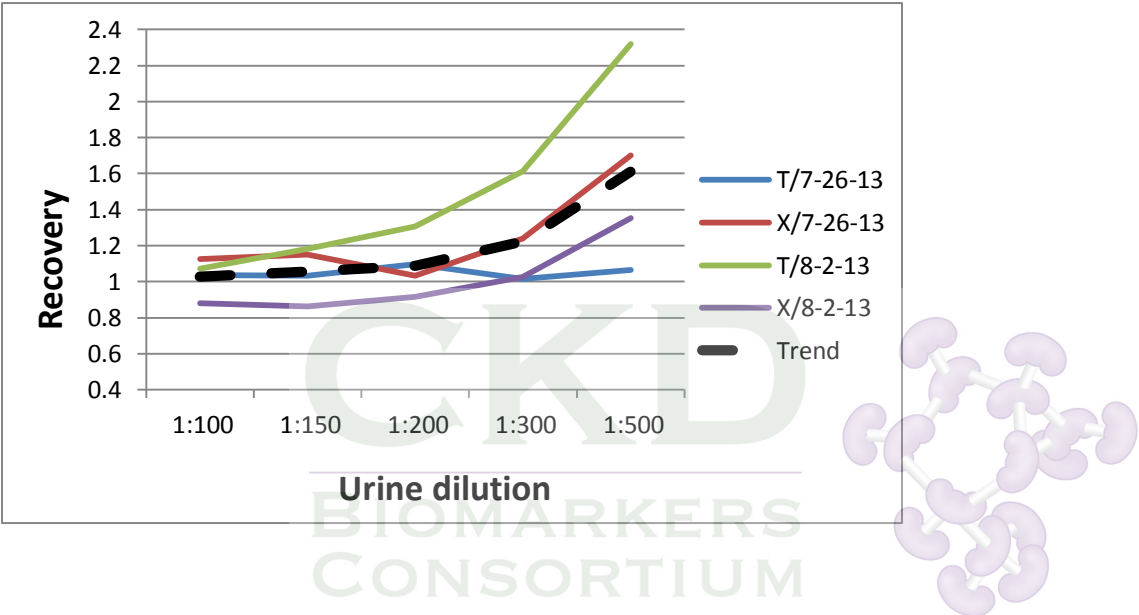
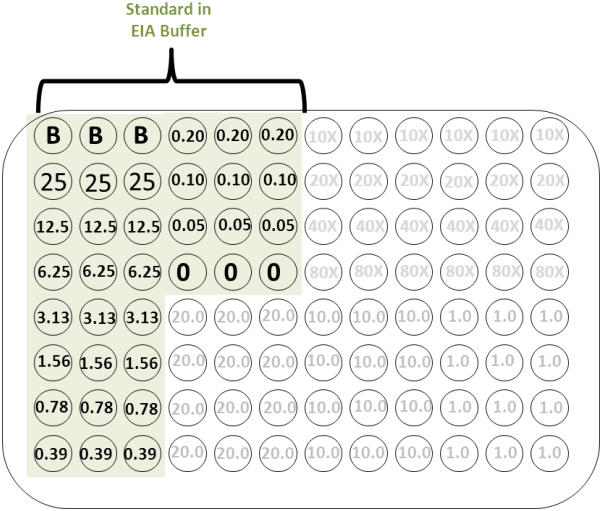


Figure 9. Schematic presentation of acceptable recovery range and its corresponding standard concentration with OD.

**Conclusion:** In table 7, all the ODs are within the standard curve range of 0.39ng/mL to 6.25ng/mL (the most “linear” portion and good CV of the standard curve). The Bio#12 urine presents its linearity between 100 and 200 times of dilutions.

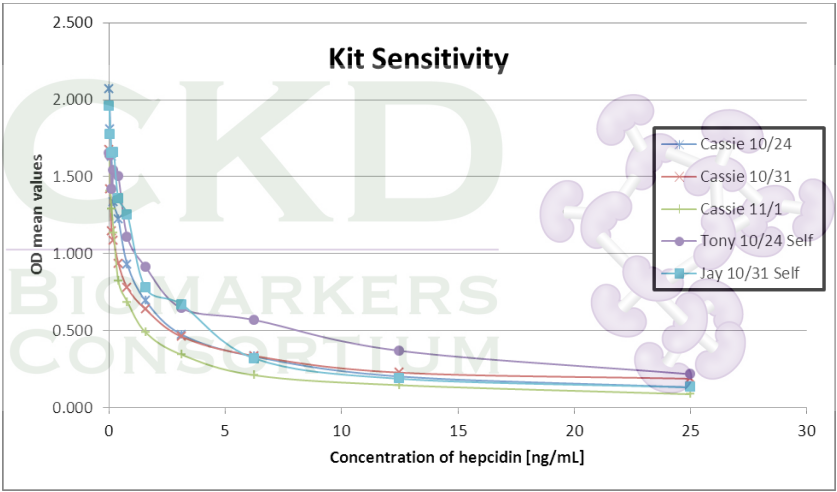
### 6) Sensitivity

**Method:** An 11-standard was prepared to evaluate the sensitivity of the Bachem S-1337 kit. Standard and blanks were plated in triplicates (See Figure 10). This method was used on both the S-1337 Kit plate as well as a Goat Anti-Rabbit IgG self-coated plate (performed by Jay and Tony). This experiment was completed three times on different days (10-24-12, 10-31-12 and 11-1-12).



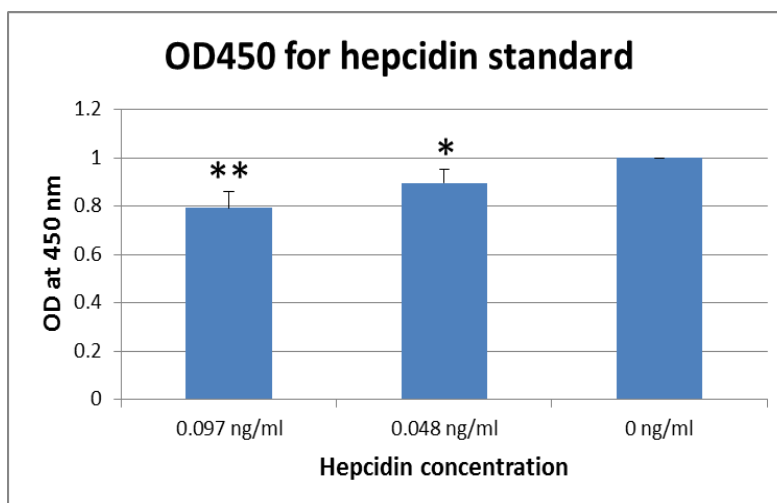
**Figure 10.** Plate template for sensitivity validation ELISA

**Results:** Absorbance of ELISA was read at 450nm. The generated standard curve for each plate was graphed (see Figure 10).



**Figure 10.** Comparison of both kit and self-coated plate generated standard curves.

	0.097 ng/ml	0.048 ng/ml	0 ng/ml
10/24 Cassie	0.786692444	0.874013211	0.999838892
10/31 Cassie	0.685583433	0.849064118	1.000199124
11/1 Cassie	0.792765175	0.864500307	0.999795626
10/24 Tony	0.85887341	0.990308904	0.999798102
10/31 Jay	0.843367347	0.905612245	0.999829932
Average	0.793456362	0.896699757	0.999892335
SD	0.067921451	0.056264427	0.000172556



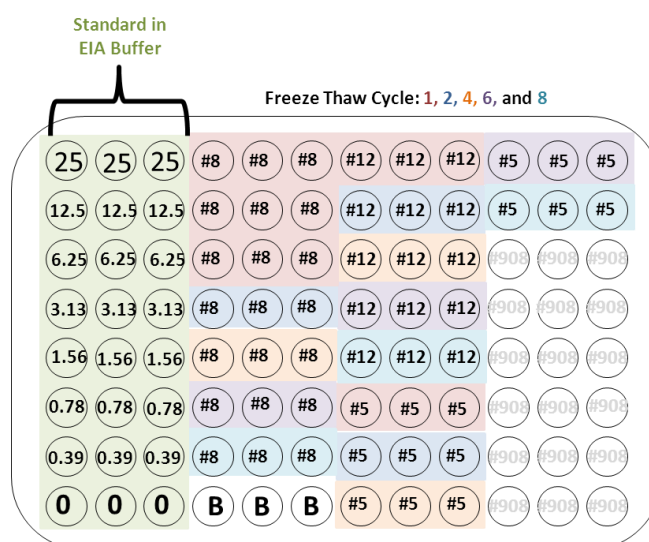
**Figure 11.** OD readings at 450 nm and its p-value in different hepcidin concentrations.

**Conclusion:** The self-coated plate standards are not as well fit to the curve. However, in all five plates, the OD values are significantly different at hepcidin 0.048 ng/ml (\*P < 0.05) and Hepcidin 0.097 ng/ml (\*\*P<0.01). This indicates that the kit is sensitive for lower than even 0.05ng/mL concentration of hepcidin.

## 7) Urine Sample Stability and Lifespan

**Method:** To determine the stability of urine hepcidin, Biopsy Urine #5, #8 and #12 were aliquoted after freeze and thaw cycles 2, 4, 6 and 8. The urine samples were frozen in -80°C freezer for 30 minutes and then thawed in a room temperature water bath for 5 minutes. This freeze/thaw cycle was repeated up to 8 times. The standard, aliquoted samples and blank were plated in triplicates (see Figure 12). This method was used on both the S-1337 Kit plate as well as a Goat Anti-Rabbit IgG self-coated plate (performed by Cassie). This experiment was completed three different times on different days (11-14-12, 11-15-12 and 11-16-12).

To access the urine hepcidin stability over storage lifespan, we compared the hepcidin value measured in 2009/2010, with our current measurement in 2013. Data used are all from best OD range to ensure less variability.



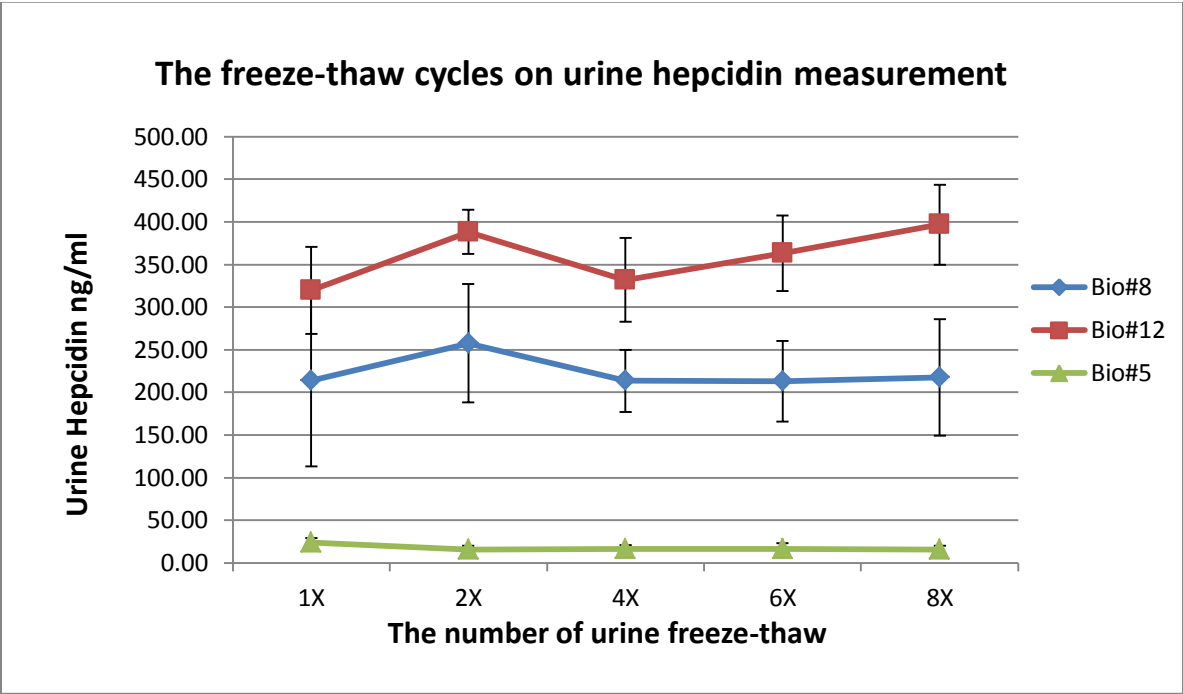
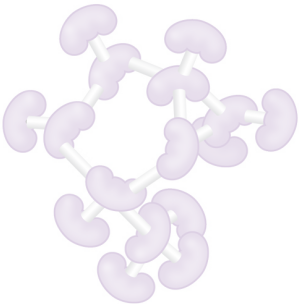
**Figure 12.** Plate template for urine stability validation of ELISA

**Results:** Absorbance of ELISA was read at 450nm. The sample concentration was determined from the standard curve and represented in following table. The single factor ANOVA test was used to determine the p-value between groups of 1, 2, 4, 6 and 8 freeze thaw cycles. A p-value under 0.05 is considered significant different between the freeze-thaw cycles. **Note:** The graph data and p-values provided below are from the kit plate only.

Kit Plate	Average Bio#8 Values				
Date	1X	2X	4X	6X	8X
11/14/2012	256.03	285.28	231.16	255.52	263.29
11/15/2012	228.89	234.14	251.49	216.92	219.49
11/16/2012	157.26	252.70	158.17	167.30	169.70

Kit Plate	Average Bio#12 Values				
Date	1X	2X	4X	6X	8X
11/14/2012	348.28	463.93	353.25	356.52	394.02
11/15/2012	402.32	327.73	352.70	413.43	466.57
11/16/2012	207.36	372.32	290.01	319.25	329.86

Kit Plate	Average Bio#5 Values				
Date	1X	2X	4X	6X	8X
11/14/2012	27.52	17.96	20.70	23.58	19.64
11/15/2012	25.85	18.39	16.89	15.15	16.44
11/16/2012	18.32	11.01	11.98	11.54	11.09





**Figure 13.** The impact of freeze-thaw cycles on urine hepcidin measurement (raw data table and graph). The urine hepcidin value were presented as mean  $\pm$ SD for three internal control urine samples after multiple freeze-thaw cycles on commercial kit plates (both in graph and table).

ANOVA Bio#8						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3782.28	4.00	945.57	0.49	0.74	5.19
Within Groups	9562.37	5.00	1912.47			
Total	13344.65	9.00				

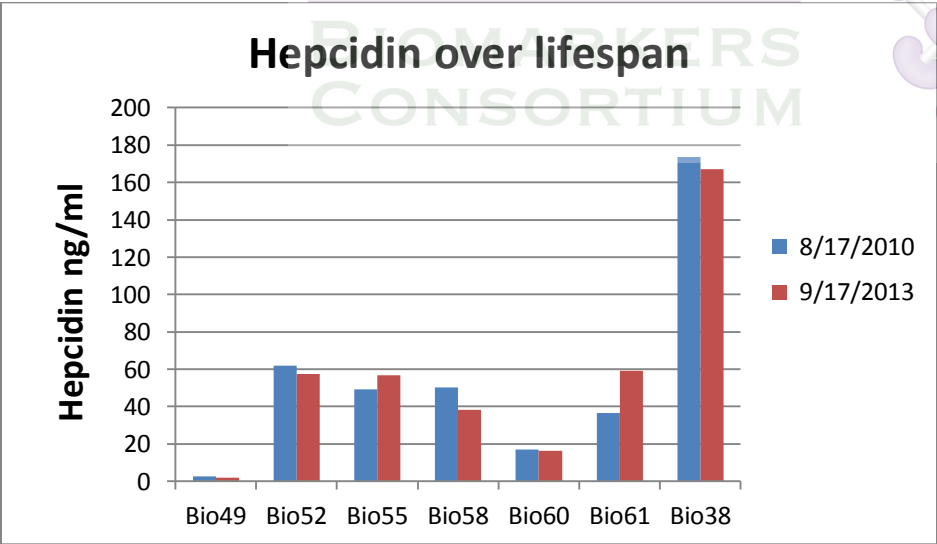
  

ANOVA Bio#12						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	10869.58	4.00	2717.40	0.38	0.82	5.19
Within Groups	35743.06	5.00	7148.61			
Total	46612.64	9.00				

ANOVA Bio#5						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	105.24	4.00	26.31	1.49	0.33	5.19
Within Groups	88.42	5.00	17.68			
Total	193.66	9.00				

Table 8. The single factor ANOVA analysis on freeze-thaw urine hepcidin data.



**Figure 14.** Hepcidin value over storage lifespan. Using the same biopsy urine samples hepcidin value were compared between measurement done in 2010 and 2013. The p-value for this paired analysis is 0.8324, indicating no significant changes over years of storage at -80°C.

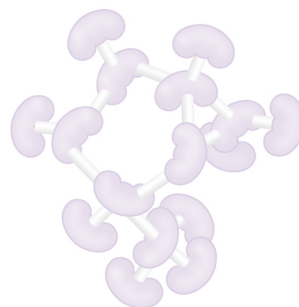
**Conclusions:** These results demonstrated that urine samples with freeze-thaw between 1 and 8 times, and stored up to 3 years do not show significant differences in hepcidin measurement using Bachem S-1337. Although intra and inter CV are high on some measurement these can be improved by using narrower OD range of middle standards (as recommended by this validation). More accurate freeze/thaw results may be obtained by limiting intra-CV under 15%.

## 8) Urine Internal Control

To develop our in-house urine internal control for urine hepcidin QC, we select three urine covering from low, medium and high concentration of hepcidin, Bio5, Bio8 and Bio12. Urine samples were thawed at room temperature water bath for 5 min, vortexed for 10 sec and spun down at 16.1g for 5 min at 4°C. Using recommended OD range from above (correspondent to standard lower than 6.25 ng/ml), we diluted urine Bio5 1:10, Bio8 1:50 and Bio12 1:100. All intra-CV are less than 15% for the duplicates. Please note that these concentration may be different to the concentration presented in validation experiments above due to different dilution factors used to fit into narrow OD range recommended (correspondent to hepcidin 6.25 ng/ml lower). All these experiments are done in Aug and Sept 2013.

New lots of in-house control will be tested in parallel with existing lots, and an acceptable range will be determined using Levey-Jennings graphs for interpretation before placing new lots into service.

	Bio5	Bio8	Bio12
1	9.343	116.843	206.174
2	10.845	162.452	221.823
3	4.248	108.275	236.606
4	5.461	90.393	223.84
5	7.873	156.217	244.87
6	9.976	132.288	270.378
7	10.564	127.877	220.518
8	10.305	152.037	251.636
9	7.371	116.803	
Average	8.4428889	129.242778	234.480625
SD	2.3680111	24.0276713	20.5810004
Mean	6.0748778	105.215106	213.8996246
1SD	10.8109	153.270449	255.0616254
Mean	3.7068668	81.1874352	193.3186242
2SD	13.178911	177.29812	275.6426258



**Table 9.** Urine hepcidin concentration determination for internal control.

Conclusion: Three in-house internal control were determined using Bachem S-1337. The acceptable concentrations ranges are mean  $\pm$  2SD:

**Bio 5:** 3.71 – 13.18 ng/ml

**Bio 8:** 81.19 – 177.30 ng/ml

**Bio 12:** 193.32 – 275.64 ng/ml

## Overall thoughts and conclusions:

The validation results show that the kit is specific for Hepcidin-25 and very sensitive to concentrations lower than 0.05ng/mL. Dilution parallelism and natural linearity show that regardless of dilution, a consistent concentration can be achieved **if the OD measured higher than corresponding OD of 6.25ng/mL**. The inter and intra CV falling below the cut-off percentages of 20% and 15% respectively can be used for accurate and consistent measurements of hepcidin. Human urine samples are stable for up to 8 freeze-thaw cycles in this validation test. From these results, the Bachem S-1337 Hepcidin ELISA is validated for use with urine samples. The following parameters should be used for the most accurate results.

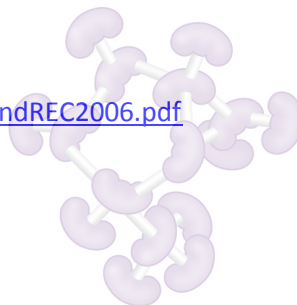
### Parameters:

1. Use a 9-point standard curve from 25ng/mL to 0ng/mL.
2. Dilute all urine samples at 1:20. If the measured OD is lower than the OD value measured for the standard curve at 6.25ng/mL then the sample must be diluted more at 1:50 or 1:100 to achieve an OD higher than the OD of 6.25ng/mL hepcidin standard.
3. Biopsy #5 (1:10), Biopsy #8 (1:50) and Biopsy #12 (1:100) are recommended for each ELA run as sample controls.
4. Any urine hepcidin measurement with CV greater than 15% should be repeated.

### References:

[http://www.woongbee.com/0NewHome/RnD/ELISA/RnD\\_%20SPIKEandREC2006.pdf](http://www.woongbee.com/0NewHome/RnD/ELISA/RnD_%20SPIKEandREC2006.pdf)

CKD  
BIOMARKERS  
CONSORTIUM



UL: Clinical Proteomics Laboratory

**Title: human urine IPPK qLCMS-based SRM assay**

**Reference:**

**Version Number: 1.2**

**Effective Date: January 8, 2015**

**Review Date:**

Author	Michael L. Merchant, PhD	Reviewed by		Approved by	
Title	Associate Professor	Title		Title	
Signature		Signature		Signature	
Date		Date		Date	

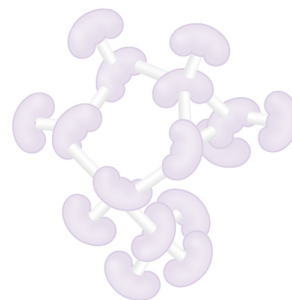
Document History			
Date	Comment	Reviewer	Signature

Reference: **human Urine IPPK-01**

Version: 1.2

Author: Michael L. Merchant, PhD

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## Purpose

This Standard Operating Procedure (SOP) describes the process for the analysis of inositol-1,3,4,5,6-pentakisphosphate-2 kinase (IPPK) in human urine using selective reaction monitoring (SRM) methods with a nanoflow ultra-high pressure liquid chromatography (nUHPLC), nanoelectrospray ionization source and a triple quadrupole (QqQ) mass spectrometer (MS). This SOP for quantification of IPPK has not been validated for serum or plasma.

## Introduction

The IPPK gene has four splice variants with the principle transcript encoding a protein (SwissProt number Q9H8X2) of 491 amino acids and a translated molecular weight of 56,017 Daltons. The observed mass of the translated gene product may increase with protein post-translational modification. The protein acts as a lipid kinase that phosphorylates the inositol position 2 hydroxyl group of inositol-1,3,4,5,6-pentakisphosphate to form inositol-1,2,3,4,5,6-hexakisphosphate (InsP6). InsP6 has a variety of functions; reported to include stimulation of DNA repair, endocytosis, mRNA export and ion channel regulation. It has been reported to protect cells from TNF-alpha-induced apoptosis. Increased abundance IPPK has been reported for renal tissue and urine of T1D patients with microalbuminuria that experience early renal function decline.

## Procedure

**Background:** Urine concentrations of IPPK will be determined using a LCMS approach incorporating a SRM step to quantify specific tryptic peptide(s) against an external calibration curve using stable isotope labeled peptide(s) as an internal standard(s).

### Sample requirements:

- A. **All samples are maintained in storage at -80 C until used for analysis. Samples are thawed on ice until processed for analysis. Sample aliquots as provided by clinical sites are spun at 17,000 xg for 10min at 4 C and then an aliquot is removed for analysis. The remaining sample is returned to - 80 C storage.**
- B. **Urine.** The current workflow has been used in normal human urine. This specific workflow has not been validated for serum or plasma.
  - a. The effects of sample collection, sample processing and storage conditions have not been established. These effects are expected to be highly dependent on the methods incorporated into the various protocols that have been approved and adopted by the various NIH studies and tertiary collection sites. It is impossible to predict all the methods that have been applied for urine collection. However, the effects of sample collection/processing/storage conditions will be determined by spike in study protocols per current research design for samples.

**Method summary for sample analysis:** Aliquots of urine protein samples will be processed for quantitative LCMS analysis using a urine-modified Filter-Aided Sample Preparation (U-FASP) method. Urine protein values provided by the CKD Biocon reference laboratory will be used as the reference protein concentration value to determine volume of urine to be used in digestion. In the absence of urine protein values, urine albumin values provided by the CKD Biocon reference laboratory will be used as the reference protein concentration value to determine volume of urine to be used in digestion. When neither value is provided by the CKD Biocon reference laboratory the protein value of the urine will be estimated using a Bradford protein assay and duplicate urine volumes of 1-10uL of sample. When the protein concentration is below the level of quantification a volume of 100uL will be used for processing. Twenty-five micrograms (25ug) of urine protein will be reduced, alkylated, transferred to a 10,000 Dalton nominal molecular weight cut-off (NMWCO) spin filtration device, buffer exchanged into suitable digestion buffer, internal standard peptide(s) added, trypsin added, incubated overnight for digestion, sample spun through filter to isolate <10,000 Dalton fraction, diluted to 25ng/uL concentration, transferred to an autosampler vial, loaded into auto sampler tray, and the signal area of 3- or more fragment ions derived from the target peptide(s) and internal standard peptide(s) are quantified from a nUHPLC-SRM-MS run. The concentration of the target peptide is determined using the ratio of the target peptide fragment areas to the internal standard fragment areas against an external calibration curve using analytical LC-SRM-MS software.

Reference: **human Urine IPPK-01**

Version: 1.2

Author: Michael L. Merchant, PhD

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## Detailed procedure for sample digestion:

### Urine Filter-Aided Sample Preparation (U-FASP) Method:

- a. Add the volume of protein equivalent to 25µg of urine protein or 100µL urine sample (undetermined concentration) to labeled low protein binding 1.5mL vial(s) (Cat # 72.690.001, Sarstedt, Newton, NC)
  - i. Queue up the urine volume to 100µL using 18MΩ LCMS grade water (Cat. #51140, Thermo Pierce, Rockford, IL) as necessary.
- b. Add 80mM dithiothreitol (DTT) (Cat. #D0632, Sigma-Aldrich, St. Louis, MO) to the sample for a final concentration of 4mM, lightly vortex to mix, spin to recover in bottom of tube and incubate at 56 C for 45min.
- c. Add 150mM iodoacetamide (IAA) (Cat. #I1149, Sigma-Aldrich, St. Louis, MO) to the sample for a final concentration of 15mM, lightly vortex to mix, spin to recover in bottom of tube and incubate in dark at room temperature for 15min.
- d. Transfer reaction mixture to a Microcon-10 spin filtration device (YM-10 membrane, Cat. #MRCPT010, EMD Millipore, Billerica, MA), dilute with 200µL 8M urea (Cat. #U6504, Sigma-Aldrich, St. Louis, MO)/0.05M triethylammonium bicarbonate -HCl pH8.5 (Cat. #17902, Fluka, Sigma-Aldrich, St. Louis, MO) and centrifuge at 14,000xg, 20 C for 20min.
  - i. Repeat addition of 200µL 8M urea/0.05M triethylammonium bicarbonate pH8.5, and centrifuge at 14,000xg, 20 C for 40min.
  - ii. Repeat twice the addition of 200µL 8M urea/0.05M triethylammonium bicarbonate pH8.5, and centrifuge at 14,000xg, 20°C for 40min.

**(Note: the sample has gone through 3 washes and now prepared for addition of digestion buffer, internal standard and the digestion protease- mass spectrometry grade trypsin).**
- e. Transfer Microcon-10 reaction vial to new, labeled low protein binding collection vial.
- f. Add 200µL 0.8M urea/0.05M triethylammonium bicarbonate pH8.5, and 400ng mass spectrometry grade trypsin (Cat. #V115A, Promega, Madison, WI).
- g. Snap close lid to reaction vial. Seal with parafilm. Incubate at 37 C overnight with light agitation (100 RPM).
- h. Recover vial from incubator. Place on ice to cool reaction vial. Centrifuge at 14,000xg, 20°C for 40min.
- i. Add 50µL 0.5M NaCl (Cat. #S9625, Sigma-Aldrich, St. Louis, MO) to the reaction vial. Centrifuge at 14,000xg, 20°C for 40min to collect tryptic peptides that were previously adhered to the YM-10 membrane.
- j. Remove and discard Microcon-10 reaction vial.
- k. Add 1.4µL of 50% formic acid (Cat. #09676, Fluka, Sigma-Aldrich, St. Louis, MO) to the 250µL pooled sample in collection vial to acidify sample.
- l. Add 10µL of internal standard (IS) at concentration of 600fmol/µL. (IS – a synthetic tryptic peptide based from IPPK digestion).
- m. Measure and record volume of sample in collection vial using clean 500µL glass Hamilton syringe (Cat. # 81265, Hamilton Syringe Co., Reno, NV).
- n. Calculate concentration of peptides in sample vial based on the starting amount of urine protein (10µg) and volume measured using Hamilton syringe. Expected concentration is approximately 40ng/µL.
- o. Keep tubes for immediate use on ice. All tubes not for immediate use label appropriately and store the peptide samples at -80°C.
  - i. **NOTE:** For nUHPLC-SRM-MS analysis the desired amount of tryptic digest on column is 100ng. The optimal EASY-nLC 1000 (Thermo Fisher Scientific, West Palm Beach, FL) sample injection is based on the sample loop size of 10µL. Therefore the desired concentration of urine protein digest in sample vial is 10ng/µL.
- p. Each digested sample equivalent to 1/5<sup>th</sup> of total digested urinary protein (5µg) are desalted and concentrated using C18 reversed phased desalting columns (Cat. #SUM SS18V, The NEST Group, Inc, South borough, MA) using the procedure described in Appendix D.

Reference: **human Urine IPPK-01**

Version: 1.2

Author: Michael L. Merchant, PhD

- q. Peptides contained in 300 $\mu$ L of chromatography buffer B (80% acetonitrile/0.1% formic acid) after completion of NEST clean-up procedure are frozen at -80C, brought to dryness using the SpeedVac and diluted using chromatography buffer A (2% acetonitrile/0.1% formic acid) to a final volume of 200 $\mu$ L.
- r. Sample from is transferred from 1.5mL vial to a low protein binding autosampler vial for analysis.



## **uHPLC-SRM-MS analysis of urine protein tryptic digest for IPPK peptides.**

### **Standards**

**External Calibration Curve-** IPPK (light and heavy) synthetic peptides are used where the internal standard heavy isotope labeled amino acid is either leucine based or arginine based. These peptides will be sequence validated by MALDI-TOF MS/MS methods and purity/composition analysis by elemental analysis.

**Note:** At this time the IPPK internal standard (IS) peptide is being after the digestion simultaneously with acidification of the digestion to inhibit further proteolytic activity and to facilitate normalization of run to run variability in the IPPK data.

**Note:** Analytic sensitivity is dependent on LCMS analytical platform. The analytic sensitivity will be reported for the lowest limit of replicate detection while maintaining a CV of less than 20% for that concentration.

**Method summary for unknown sample analysis:** A full loop injection (10 $\mu$ L) of a 25ng/ $\mu$ L urine protein digest that also contains 6pmol/ $\mu$ L internal standard IPPK peptide (IPPK-IS) will be separated with a 30 min 2% to 48% acetonitrile/0.1% formic acid gradient using a Proxeon EASY-nLC 1000 fitted to load onto a Dionex Acclaim PepMap 100 trap column and a RSLC Pepmap 100 C18 reversed phase resolving column prior to introduction by nanoelectrospray using a Nanospray Flex source (ThermoElectron) into a Thermo TSQ Quantum Discovery MAX triple quadrupole mass spectrometer. Controller software (Thermo Xcaliber version 2.5) will be used to control sample injection, gradient separation, and generate peptide collision induced dissociation (CID) data for the target IPPK and IPPK-IS peptides. LCMS (RAW) data files for all unknown sample LCMS runs and for external calibration curves will be imported into the analytic software (Skyline version 2.6) for quantification of IPPK concentration in each unknown.

For construction of the external standard curve, IPPK concentrations ranging between 2.5 fmol and 800 fmol on column of the natural abundance peptides will be prepared in a urine matrix and processed as described previously. Three standard curves will be prepared and analyzed by UHPLC-nanosprayESI-MS/MS. The SRM peak area for the natural abundance isotope will be plotted versus the peptide concentration and data will be fit to a linear equation using Skyline version 2.6 and MS Excel. A regression analysis will be performed for IPPK to determine the lower limits of detection and quantification. Standard curves will be run weekly. Replicate data point CV should be less than 20%.

### **Results Reporting**

Skyline software will be used to quantify IPPK in the patient urine sample based on the ratio of IPPK fragment ions to the IPPK-IS fragment ions and the external calibration curve. The urine IPPK concentration will be reported as fmole of IPPK per unit mass of urine protein or per unit volume ( $\mu$ L) of starting patient urine. These values may be converted to other clinical units based on data accessible to Protocol B members.

## Location

Baxter I, Room 207A

University of Louisville, School of Medicine, Department of Medicine, Division of Nephrology & Hypertension

## Required equipment / reagents (as specific or equivalent)

- Centrifuges- clinical and microcentrifuge.  
Note- centrifuges are maintained on an as need basis and with annual servicing to verify speed settings of RPM's within +/- 10%
- Centrifugal filtration devices (Microcon-10 with YM10 filter having 10,000 Da nominal molecular weight cut-off, NMWCO)
- Vortexer  
Note- vortexers are maintained on an as need basis
- Standard and multichannel pipettes  
Note- pipettes are serviced every 6-12-months on site using a commercial provider of calibration services.
- LCMS system- calibrated onsite per manufacturer's guidelines for flow calibration (UHPLC) and mass calibration (TSQ).
  - HPLC- autosampler-Proxeon EASY-nLC 1000
  - UHPLC column- **Dionex Trap Column**: u-precolumn 300 um i.d., 5 mm, packed with C18 PepMap 100, 5 um, 100A (Thermo P/N 160454); **Dionex Separating column**: Acclaim® PepMap™ RSLC column 50µm I.D. C18
  - Source- EASY-Spray nano-ESI source
  - Mass Spectrometer- Thermo TSQ Quantum Discovery MAX triple quadrupole mass spectrometer
- Analytic software- Skyline version 2.6 (<https://skyline.gs.washington.edu>)

## Buffers-

LC solvent A- 2% acetonitrile/0.1% formic acid (total volume prepared 0.025L)

LC solvent B- 80% acetonitrile/0.1% formic acid (total volume prepared 0.025L)

Buffer preparation (prepared every 15 days, sonicated to degas and then stored at RT)-

LC Solvents	
Buffer	Components
LC buffer "A".	2% acetonitrile/0.1% formic acid
	0.5mL LCMS grade acetonitrile 25uL formic acid Queue to 25mL with LCMS grade water
LC buffer "B".	20mL LCMS grade acetonitrile 25uL formic acid Queue to 25mL with LCMS grade water

Reference: **human Urine IPPK-01**

Version: 1.2

Author: Michael L. Merchant, PhD

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## Experimental Procedure Data

### Sample handling

Samples are maintained in storage at -80°C until used for analysis. Samples are thawed on ice until processed for analysis. Sample aliquots as provided by clinical sites are spun at 17,000 xg for 10min at 4°C and then an aliquot is removed for analysis. The remaining sample is returned to -80°C storage.

### Prepare IPPK standards for external calibration curve:

All IPPK peptide solutions are prepared from lyophilized samples received from the commercial source. Samples are solubilized in LCMS grade, 18MΩ·cm H<sub>2</sub>O to yield working concentration of 6mM. Aliquots of peptides are sent for elemental compositional analysis using services provided by UC Davis (<http://stableisotopefacility.ucdavis.edu/>).

Primary stock freezer solutions of IPPK are prepared in water to a final concentration of 6mM. Two intermediate dilutions are made in urine matrix to achieve a final set of calibration points having concentrations shown below.

Urine matrix-

Working solutions needed to prepared calibration curve.

Solution A- 160fmol/μL IPPK and 6fmol/μL IPPK-IS, 25ng/uL urine matrix

Solution B- 6fmol/μL IPPK-IS, 25ng/μL urine matrix

Vial label	Volume of A	Volume of B	Final Volume (μL)	IPPK (fmol) on column	
8	40μL of Solution A	40μL of Solution B	80μL	800	
7	30μL vial 8	120μL of Solution B	150μL	160	
6	50μL vial 7	50μL of Solution B	100μL	80	
5	50μL vial 6	50μL of Solution B	100μL	40	
4	20μL vial 5	60μL of Solution B	80μL	10	
3	10μL vial 4	30μL of Solution B	40μL	2.5	
2	10μL Buffer A	40μL of Solution B	50μL	0	IPPK-IS/Matrix blank
1	10μL Buffer A	40μL of Buffer B	50μL	0	Buffer A+B blank

### Methodology for Chromatography:

**Solvent A:** 2% acetonitrile/0.1% formic acid in water (LCMS Grade from Sigma)

**Solvent B:** 80% acetonitrile/0.1% formic acid (LCMS Grade from Sigma)

LC Gradient as in table below			
Time (min)	Flow (uL/min)	A (%)	B (%)
0.0	0.3	100	0
30.0	0.3	43	57
30.1	0.3	5	95
35.0	0.3	5	95

Reference: **human Urine IPPK-01**

Version: 1.2

Author: Michael L. Merchant, PhD

**SRM parameters for IPPK and IPPK-IS LCMS analysis:**

- a. Isolation width: 0.002Da
- b. Collision Energy: 15

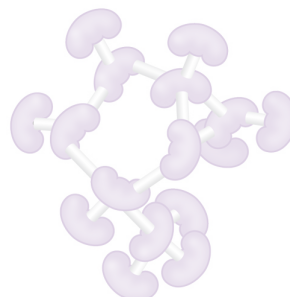
Transition specific parameters for detection and quantification of IPPK peptide using specified LCMS configuration.

Duration (min)	Peptide	Precursor (m/Z)	Z	Fragment (m/Z)	Z	Rt (min)
0.5-29.5	IPPK 260-ELVHVITR-267;	322.86	3			11.5-11.6
				313.19	1	
				389.25	1	
				488.32	1	
0.5-29.5	IPPK-IS 260-EL(13C6,15N)VHVITR-267	325.20	3			11.5-11.6
				313.19	1	
				389.25	1	
				488.32	1	



## Quality Control

- a. **Samples:** Prior to shipment and analysis samples are de-identified and blinded replicates are interwoven to aid in determining technical precision.
- b. **Preparation:** Prior to analysis samples are randomized during sample handling and in ordering for sample analysis to minimize the effects of systematic changes in instrumentation or reagent preparation.
- c. **Accuracy and acceptance of results:** Calibration curves will be established for internal standards prior to the initiation of sample analysis. Each calibration curve will be established with technical replicates (n=3). Full external calibration curves of standards in matrix will be repeated 100 samples. Single external calibration points (concentrations selected from the mid-point value of the external calibration curve and used for quality control) will be interwoven into each assembled flight of samples at the beginning of the weekly sample set. The estimated standards concentration in comparison with the known amount will be used to measure the accuracy of the method at estimating analyte concentrations. Acceptable CV for the difference between the estimated and known analyte concentrations are 80%-120% of the known concentration.
- d. All the samples and standards are run in with a technical replicate.
- e. Samples with an internal standard variation of more than 20% CV are repeated.



## Appendix A. Additional consideration to LCMS performance

### Buffer considerations

Buffers were changed every 15 days or earlier if determined necessary. Buffer A and wash solution (2% acetonitrile and in 0.1% formic acid and water) and Buffer B (80% acetonitrile in 0.1% formic acid and water) were prepared in 25mL Schott Duran bottles and a 50mL autosampler wash bottle and degassed by sonicating for 30 minutes. Solvents remaining in Pumps A, B and S and the volume of the nano-UHPLC system were replaced with new degassed solvents by filling selected pumps and ejecting into the waste container using the Purge Solvent script. Air was removed from pump heads by pressurizing pumps and releasing pressure into flow lines toward the waste container using the Flush Air Script. This script terminates after the first iteration occurs resulting in a flush volume that is below the set threshold (12 $\mu$ L). At this point, the system is ready for use.

### UHPLC column considerations

Columns were characterized before use. Initial pressures of the Acclaim PepMap precolumn by itself and the Acclaim PepMap precolumn and column in place were recorded. New columns were conditioned by injecting seven replicates of apomyoglobin tryptic digests (2ng injected on column). These replicates were kept as a reference for column performance and quality control. Intensity and peak widths of peptides and resulting fragments collected using both full scan (300 and 1500 m/z) and SRM (650>204, 650>351, 650>408) scan events were evaluated and incorporated into sample batches for quality control by comparing previous injections made under identical conditions.

### NanoFlex ion source positioning

The NanoFlex Ion Source is positioned to deliver optimal signal and spray stability by directly infusing IPP2K peptide ELVHVITR (300nM ELVHVITR peptide dissolved in 50% acetonitrile/50% water/0.001% formic acid) using the Direct Infusion script. During infusion, the XYZ manipulator is positioned so the emitter is aligned with the initial point of sample introduction into the mass spectrometer, the ion transfer tube. Signal is optimized during infusion by both visually adjusting the XYZ manipulator to align the emitter with the ion transfer tube opening and also by monitoring signal intensity of the primary transition (322>488) for ELVHVITR while the manipulator is adjusted in all three directions (x, y and z).

## Appendix B

### μBCA Protein Assay (Thermo Scientific #23235)

Dissolve a BSA standard (BioRad #500-0007) with 18MΩ·cm H<sub>2</sub>O to give a 9.8mg/mL stock and aliquot into 60μL volumes. Store the aliquots at –70°C. Create the standard curve concentrations as follows:

	<u>9.8mg/mL stock</u>	<u>18MΩ·cm H<sub>2</sub>O</u>
9.8mg/mL BSA	20μL	0μL
7.0mg/mL BSA	14.29μL	5.71μL
5.6mg/mL BSA	11.43μL	8.57μL
2.8mg/mL BSA	5.71μL	14.29μL
1.4mg/mL BSA	2.86μL	17.14μL
1.0mg/mL BSA	2.04μL	17.96μL
0.5mg/mL BSA	2.04μL	37.96μL
0mg/mL BSA	0μL	20μL

In the protocol we follow for the μBCA assay, we dilute 1μL sample or standard into 149μL 18MΩ·cm H<sub>2</sub>O. We make duplicates of each standard and triplicates of each sample. For a broader range standard curve, 19.6mg/mL and 29.4mg/mL standard points may be added. To get these concentrations, use 2μL of the 9.8mg/mL standard (dilute into 148μL 18MΩ·cm H<sub>2</sub>O) to get a final concentration of 19.6mg/mL and 3μL of the 9.8mg/mL standard (dilute into 147μL 18MΩ·cm H<sub>2</sub>O) to get a final concentration of 29.4mg/mL.

Prepare the μBCA working reagent; the working reagent is composed of 50 parts reagent A, 48 parts reagent B, and 2 parts reagent C. Add 150μL of the μBCA working reagent to each standard and sample and mix with a multichannel pipettor (draw and expel working reagent several times into diluted sample). After adding the reagent to all wells, cover plate with plastic adhesive film. Incubate for 2 hours at 37°C. Allow plate to cool to room temperature for 10min while setting up spectrophotometer. Invert the plate immediately before reading to wet the film. Read absorbance at 562nm on the spectrophotometer. A quadratic equation fits well through all standard points.

**Note:** Careful pipetting is critical. Use the same pipette for the standard curve dilution series if possible, and standardize the way the volumes of liquid are drawn and dispensed (for example, always draw from the surface of the standard or sample, and always wash the tip by pipetting up and down when adding the standard or sample to the water).

## Appendix C.

### Creating a Standard Curve for the Bradford Assay (BioRad Cat. #500-0002)

Dissolve the BSA with 18 MΩ H<sub>2</sub>O to give a 9.8 mg/mL stock and aliquot into 60 µL volumes. Store the aliquots at –20 °C. Create the standard curve concentrations as follows:

	<u>Volume 9.8 mg/mL stock</u>	<u>Volume 18 MΩ H<sub>2</sub>O</u>
9.8 mg/mL BSA	20 µL	0 µL
7.0 mg/mL BSA	14.29 µL	5.71 µL
5.6 mg/mL BSA	11.43 µL	8.57 µL
2.8 mg/mL BSA	5.71 µL	14.29 µL
1.4 mg/mL BSA	2.86 µL	17.14 µL
1.0 mg/mL BSA	2.04 µL	17.96 µL
0.5 mg/mL BSA	2.04 µL	37.96 µL (20µL + 17.96µL)
0 mg/mL BSA	0 µL	20 µL

The BioRad Cat. #500-0002 protocol used is the Bradford microplate assay. 1µL sample should be diluted into 199µL 18 MΩ·cm H<sub>2</sub>O. 1µL of each standard and 1µL of sample buffer should be diluted into 199µL 18 MΩ H<sub>2</sub>O (if sample buffer is unknown, just add 199µL water and 1 µL standard). Depending on the concentration of the samples, it may be necessary to add more or less than 1µL of the sample; the goal is to have the sample absorbance within the readable portion of the standard curve (0.5 – 9.8mg/mL).

The analysis utilizes duplicates of each standard and triplicates of each sample. One of the 0 mg/mL BSA standards serves as the blank for spectrophotometer readings. Add 50 µL Bradford dye to each well for the microplate assay. Cover with microplate film and invert to mix. Incubate for 5 minutes at room temperature. Read absorbance at 595nm on a spectrophotometer or plate reader.

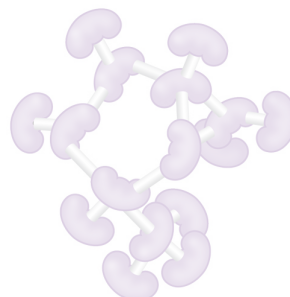
**Note:** Careful pipetting is critical. Use the same pipettor for the standard curve dilution series if possible, and standardize the way the volumes of liquid are drawn and dispensed (for example, always draw from the surface of the standard or sample, and always wash the tip by pipetting up and down when adding the standard or sample to the water).

## Appendix D

### C18 NEST Desalting Procedure

#### Cleanup with C18 PROTO™, 300Å Ultra MicroSpin Column (Cat. #SUM SS18V, The Nest Group, Inc, Southborough, MA)

1. Make solutions A=2% v/v acetonitrile / 0.1% v/v formic acid and B=80% v/v acetonitrile / 0.1% v/v formic acid.
2. Place the spin column with the adapter collar into a 2mL microtube (empty this waste tube as needed).
3. Add 100µL B to the spin column, and centrifuge at 110xg for 2min; repeat twice.
4. Add 100µL A to the spin column and centrifuge as above; repeat twice.
5. Place the column into a clean 2mL microtube, load sample in 100µL of A (note that with the FASP method, higher concentrations of formic acid may be needed to acidify the sample), and centrifuge as above; pass the flow-through liquid through the column a second time.
6. Place the spin column into the waste tube, add 100µL of A, and centrifuge as above; repeat twice.
7. Place the column into a clean 2mL microtube, add 100µL of B, and centrifuge as above; repeat twice.
8. Freeze the eluate at -80°C, and then dry in a SpeedVac. Store the dried residue at -80°C.
9. Dissolve the residue in 2% v/v acetonitrile / 0.1% v/v formic acid prior to MS analysis.



**BWH: Center for Kidney Diseases  
HIM 550, Harvard Institutes of Medicine  
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Phone # 617-525-5988**

**Title: human urinary KIM-1 microbead based assay**

**Reference: human urine KIM-1/sop/001**

**Version Number: 1.1**

**Date: October 3, 2012**

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Date		Date		Date	

Document History			
Date	Comment	Reviewer	Signature
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Reference: human urine KIM-1/SOP/001

Version: 1.1

Author: Venkata S. Sabbiseti

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## 1. Purpose

This Standard Operating Procedure (SOP) describes the process for the quantitative assessment of ectodomain of Kidney injury molecule-1 (KIM-1) in human urine specimens. The concentration of KIM-1 in urine will be measured using microbead-based sandwich ELISA (Luminex) method that was developed and extensively validated in our laboratory. In brief, 30 µl of urine specimen, control urine specimen, and serially diluted human KIM-1 recombinant proteins (standards) will be incubated with KIM-1 antibody coupled polystyrene microbeads (~5000 beads/well) for 1 hr followed by 3X washing with Phosphate buffered saline (PBS)-tween (PBST) buffer. The samples will then be incubated with biotinylated KIM-1 secondary antibody for 45 min followed by washing 3X

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with PBST. Quantification will be achieved by incubating samples with streptavidin coupled to picroerythrin for 15 min, which is excited at 532 nm. The signal from this flurochrome is directly proportional to the amount of antigen bound at the microbead surface that will be detected using the BioRad Bio-Plex system. Values will be interpreted using a 13 point standard five parametric logistic regression analysis.

## 2. Introduction

KIM-1 was originally identified in our laboratory as an early marker of proximal tubular injury and the most highly upregulated protein in the proximal tubule after renal injury of many types. Tubular KIM-1 expression is virtually undetectable in healthy kidney tissues but is strongly induced in acute and chronic kidney disease (CKD), including transplant dysfunction, where it is significantly associated with tubulo-interstitial damage and inflammation. KIM-1 is a member of the immunoglobulin superfamily (IgSF) that contains a single IgV like domain sitting adjacent to a mucin domain. The IgV domain of KIM-1 also contains N-linked glycosylation sites and the mucin domain contains numerous O-linked glycosylation sites. There is a transmembrane domain and a cytoplasmic domain containing at least three tyrosine phosphorylation sites. The cell surface (mature) form of KIM-1 is a 104 kDa protein. The shed, soluble ectodomain KIM-1 protein, that serves as a urinary biomarker, is approximately 90 kDa in size.

## 3. Sample Collection, Handling & Storage

Frozen urines (Spot, timed or 24 hr collection) obtained from investigators will be immediately stored at – 80 °C. KIM-1 is stable for up to at least 5 freeze thaw cycles. Urine specimens that were collected at BWH will be placed on ice immediately, and centrifuged at 1000 rpm for five minutes to pellet the particulate matter. Supernatant will be aliquoted into 1.8 ml screw cap centrifuge tubes (generally a maximum of 1 ml /tube) and stored in an -80 °C freezer ideally within 1 hour but overnight preservation on ice does not appear to alter the level of urinary KIM-1. All the personnel handling the specimens will have undergone Environmental Health & Safety training at Brigham & Women's Hospital. Protective gear including lab coats and gloves must be worn while working in the laboratory. The addition of preservatives to urine specimens is investigators' choice. In our studies, we didn't see any significant difference in KIM-1 levels in samples that were stored at -80 °C with and without preservatives.

## 4. Procedure

### 4.1. Location

Harvard Institutes of Medicine Room 550

Renal Division/ Department of Medicine/ Brigham and Women's Hospital

### 4.2. Required equipment / reagents

- 96 well micro filter plate (Millipore, Catalog # MAVBN 1250)
- Vacuum manifold (\*Maximum suction 3 psi\*) (Millipore, Catalog # MSVMHTS00)

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- Bio-Plex Luminex 200 & 100 readers (Bio-Rad)
- MilliQ water (Type 1, reagent grade water)
- Refrigerated bench top centrifuge for sample centrifugation (Sorvall Bench top swing bucket centrifuge, Rotor RTH-250)
- Refrigerated Centrifuge for bead coupling reactions (Eppendorf 5430R, Rotor FA-45-30-11)
- Vortexer (Denville Scientific)
- Electronic Multichannel pipettes
  - E8 XLS multichannel pipette (dispenses 2 µl-20 µl) (Rainin, catalog # E8-20XLS)
  - EDO 3-Pls multichannel pipette (dispenses 100 µl-1200 µl) (Rainin, catalog # E8-1200)
- Single Channel Manual Pipettes
  - Rainin Classic 0.5 µl-10 µl (catalog # PR-10)
  - Rainin Classic 10 µl-100 µl (catalog # PR-100)
  - Rainin Classic 100 µl-1000 µl (catalog # PR-1000)
- 50 ml reagent reservoir (Fisher Scientific)
- Plate shaker (Denville Scientific)
- Orbital Plate shaker (IKA & Denville Scientific, Max speed 11000 rpm)
- 30°C incubator (Thermo Scientific)
- KIM-1 Standard Protein (RnD Systems, 1750-TM)
  - Lyophilized from 0.2 µm filtered solution in PBS
  - Purity: > 90%, as determined by SDS-PAGE and visualized by silver stain
  - Recombinant protein stock solution will be prepared at a concentration of 100 µg/ml with sterile 1 X phosphate buffer solution and aliquoted into small volumes and stored at -80 °C
- Sample Diluent (HBS/BSA/Tween: 5.95g HEPES, 1.46g NaCl, 2.5g BSA, 0.25ml Tween-20, 250ml H<sub>2</sub>O adjust final pH to 7.4; filter prior to use, and store at 4°C)
- Anti-KIM-1 coupled microbeads (antibody is from RnD systems catalog # AF1750, Bio-Plex polystyrene beads are from Bio-Rad catalog # 171-506015)
- Anti-KIM1 detection Antibody (RnD Systems, catalog # BAF1750)
  - Reconstitute the antibody (Lyophilized powder, 50 µg) with 500 µL of sterile 0.1% BSA in PBS solution.
  - Antibodies should be stored at 4 °C
- Streptavidin-Phycoerythrin Conjugate Solution (Conc: 1 mg/ml, Invitrogen, catalog # S-866), protected from light and stored at 4 °C.
- Wash Buffer (0.05% Tween-20 in 1X PBS), stored at 4 °C.

#### 4.3. Buffer preparation

- The following buffers should be filtered and stored at 4°C.

KIM-1 microbead based assay buffers	
Buffer	Components
Wash buffer (Filtered) 1X Tween-PBS solution	1X Phosphate Buffered Solution <ul style="list-style-type: none"> <li>• 100 ml of 10X PBS solution (Fisher Scientific, Catalog # BP</li> </ul>

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	399-1) will be mixed with 900 ml of MilliQ water (Millipore) 1X Tween-PBS solution <ul style="list-style-type: none"> <li>• 500 µl of Tween-20 (Fisher Scientific, catalog # BP 337-500) will be added to 1 L of 1X PBS and mixed for 5 min.</li> <li>• Filter Sterilized using 0.2 µm filtration bottles (Corning)</li> <li>• Buffer will be stored at 4 °C</li> </ul>	
Sample Diluent (Filtered)	Total volume 250 ml	
	HEPES (Fisher Scientific, Catalog # BP310-500)	5.95g
	NaCl (Fisher Scientific, Catalog # BP337-500)	1.46g
	BSA (Roche, Catalog# 9048-46-8)	2.5g
	Tween (Fisher Scientific, Catalog # BP 337-500)	0.25 ml
	Millipore water	Up to 250 ml
	pH is adjusted to 7.4 using 1 N HCl, filter sterilized using 0.2 µm filtration bottles, and stored at 4 °C.	

#### 4.4. Preparation of anti-KIM-1 conjugated microbeads

**Reagents:** Bio-Plex Amine Coupling Kit (Bio-Rad, catalog # 171-406001)

EDC (Thermo Scientific, catalog # 77149)

Sulpho NHS (Thermo Scientific, Catalog # 24510)

Anti-KIM-1 antibody (RnD systems, AF 1750)

- 0.5 mL of sterile PBS will be added to the antibody (100 µg lyophilized powder) to make a stock concentration of 0.2 mg/ml. Aliquots of antibody will be stored at -80 °C.

C00H Bio-Plex beads # 25 (Bio-Rad, Catalog # 171-506025)

**Principle:** Carboxyl groups on the surface of the polystyrene beads are first activated with the carbodimide derivative EDC (Thermo Scientific) to form an intermediate that is stabilized with S-NHS (Thermo Scientific). The intermediate then reacts with primary amine of antibodies to form an amide bond. The protein-coupled beads are stable for 1 year when stored at 4 °C and protected from light.

- All the buffers used in this protocol come with the coupling kit.
- Place EDC and S-NHS in desiccator at room temperature (RT) for one hour (until ready to use).
- Vortex Bio-Plex beads ( $1.25 \times 10^7$  beads in 1 ml) for 40 seconds followed by bath sonication (25-30 pulses).
- Aliquot 500 µL ( $6.25 \times 10^6$  beads) to a reaction tube.
- Centrifuge @ 14,000 rpm (Eppendorf 5430R, Rotor FA-45-30-11) for 4 minutes at 4 °C.
- Discard supernatant carefully so as not to disturb pellet.
- Add 200 µL bead wash buffer to pellet and vortex for 20 seconds followed by bath sonication (25-30 pulses).

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- Centrifuge @ 14,000 rpm (Eppendorf 5430R, Rotor FA-45-30-11) for 4 minutes at 4 °C and discard the supernatant.
- Re-suspend the beads in 50 µL of bead activation buffer and vortex for 20 seconds followed by bath sonication (25-30 pulses).
- Add 50 µL EDC (10 µg of EDC dissolved in 200 µL of bead activation buffer) and 50 µL Sulpho NHS (10 µg of EDC dissolved in 200 µL of bead activation buffer) to beads and vortex for 40 seconds.
- Cover tube with aluminum foil and rotate at room temperature for 40 minutes.
- After incubation, add 150 µL PBS buffer to the tube and centrifuge at 14,000 rpm for 4 minutes at 4 °C.
- Discard supernatant, re-suspend beads in 150 µL PBS buffer, vortex for 40 seconds, and centrifuge at 14,000 rpm (Eppendorf 5430R, Rotor FA-45-30-11) for 4 min at 4 °C.
- Re-suspend the beads in 475 µL PBS, vortex for 20 seconds and add 25 µL of antibody.
- Cover the tube with aluminum foil and rotate overnight at 4 °C.
- After overnight incubation, centrifuge the tube at 14,000 rpm (Eppendorf 5430R, Rotor FA-45-30-11) for 4 minutes at 4°C, discard supernatant and re-suspend 500 µL PBS (pH 7.4) and repeat the washings with PBS 2 more times.
- Re-suspend the beads in 250 µL blocking buffer, vortex for 20 seconds, cover the tube with aluminum foil, and incubate at room temperature for 45 minutes at 4 °C, discard the supernatant and re-suspend in 500 µL storage buffer
- Repeat the washing step with storage buffer and re-suspend the beads in 150 µL storage buffer in amber colored centrifuge tube. Count the number of beads using a Cellometer (Nexcelom), and store the coupled beads at 4 °C.

**Coupling Validation:** Typically we get 70-80% of bead recovery after the bead coupling reaction. Based on bead recovery, we calculate the volume of the bead solution that contains 5000 beads. We add 5000 beads per well. To validate the coupling reaction, each batch of freshly made beads are analyzed by determining the KIM-1 levels in control urine samples using these beads. If the KIM-1 values are within the  $\pm 2SD$ , then we keep the beads for the analysis of the samples. If any of the control KIM-1 values are outside of  $\pm 2SD$ , we will repeat the analysis procedure again. If both the KIM-1 values are within  $\pm 2SD$  range after re-run we will keep the beads. If the beads fail again, then we will re-couple the beads and repeat the procedure.

#### 4.5. Experimental Procedure

- **Sample thawing:** Sample tubes will be arranged in open tube racks with an empty space between each tube for better air circulation. Place these racks in 30°C incubator until all samples are thawed (The tubes will be still cold after the samples are thawed). Vortex the tubes for 5 sec, centrifuge at 3000 RPM using RTH-250 rotor on Sorvall bench top centrifuge for 5 min at 4°C, keep on ice, and proceed for the analysis.
- Quality Control Urines that contain high and low urine KIM-1 values are thawed.
- Prepare human recombinant KIM-1 (rhKIM-1) standard from stock aliquot (100 µg/mL).
  - Add 2 µL of rhKIM-1 stock to 98 µL of sample diluent buffer to make a final concentration of 2 µg/mL
  - Add 20 µL of 2 µg/mL rhKIM-1 solution to 980 µL of sample diluent buffer to get a solution of 40 ng/mL rhKIM-1 (Standard 1).
  - Use serial dilution approach to generate 40ng, 20ng, 10ng, 5ng, 2.5ng, 1.25ng, 625 pg, 312.5 pg, 156.3pg, 78.1pg, 39.1pg, 19.53pg, 9.76pg,

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4.8pg/ml using Standard 1 and sample diluent buffer in eppendorf tubes. Store on ice until ready to use.

- Turn on vacuum manifold.
  - Check that vacuum is  $\leq 3$  psi
- Remove plate cover, and add 100  $\mu$ l sample diluent to each well.
- Place plate on vacuum manifold without cover for two seconds, then blot bottom of plate against clean paper towels.
- Vortex pre-coupled beads for 40 seconds. Add appropriate volume of beads (5000 beads per well) to the sample diluent in covered tube. Invert 15 times.
- Add 50  $\mu$ l bead solution to each well with multichannel pipette
- Place plate on vacuum manifold without cover for two seconds, and then blot.
- Add 100  $\mu$ l wash buffer to each well.
- Place plate on vacuum manifold without cover for two seconds.
- Repeat steps 8 and 9 two times, for a total of 3 washes, and then blot.
- Add 30  $\mu$ l of sample to each well.
- Seal plate and wrap in aluminum foil.
- Shake at 1100rpm for 30 seconds on plate shaker.
- **Let sit for 1 hour at 300 rpm on plate shaker.**
- Dilute BAF1750 secondary antibody (20  $\mu$ l in 10 ml sample diluent) and mix it gently.
- Unwrap plate and remove seal.
- Place plate on vacuum manifold for two seconds, and then blot.
- Add 100  $\mu$ l wash buffer to each well.
- Place plate on vacuum manifold for two seconds.
- Repeat the wash steps for 2 more times for 100  $\mu$ l wash buffer.
- Add 95  $\mu$ l BAF1750 secondary antibody solution to each well.
- Seal plate and wrap in aluminum foil.
- Shake at 1100 rpm for 30 seconds on plate shaker.
- **Let sit for 45 min at 300 rpm on plate shaker.**
- Add 12  $\mu$ l of streptavidin, R-phycoerythrin (PE) conjugate solution (stock: 1 mg/ml, Invitrogen) to 6 ml of sample diluent buffer to get a final concentration 2  $\mu$ g/ml just before the incubation period is over. Protect from light by covering the tube with aluminum foil and store on ice until ready to use.
- Unwrap plate and remove seal.
- Place plate on vacuum manifold for two seconds, and then blot.
- Add 100  $\mu$ l wash buffer to each well.
- Place plate on vacuum manifold for two seconds.
- Repeat the wash steps two more times
- Add 50  $\mu$ l Streptavidin-PE to each well.
- Seal plate and wrap in aluminum foil.
- Shake at 1100rpm for 30 seconds on plate shaker.
- **Let sit for 15 minutes at 300 rpm on shaker.**
- Unwrap plate and remove seal.
- Place plate on vacuum manifold for two seconds.
- Repeat two times, for a total of three washes, and then blot.
- Add 125  $\mu$ l of sample diluent to each well.
- Seal plate and wrap in aluminum foil.
- Shake at 1100rpm for 30 seconds on plate shaker.
- **Let sit at 300 rpm for 1 minute on plate shaker.**

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- Unwrap plate and remove seal.
- Blot gently, and then load plate into Luminex Bio-Plex 200 machine.

#### 4.6 Equipment

- Bio-Plex 200
- Machine is calibrated every day using Bio-Plex calibration kit (Bio-Rad, Catalog #171-203060)
- Machine is validated every month using Bio-Plex validation kit (Bio-Rad, Catalog # 171-203001). The kit validates following parameters:

*Optical Alignment:* Confirms assay sensitivity and well-to-well precision and verifies that array reader optics are properly aligned

*Reporter performance:* Measures linearity, instrument threshold, dynamic range, slope and accuracy

*Classification accuracy:* Measures efficiency of determining bead regions and ensures proper bead classification

*Fluidics Integrity:* Monitors fluids performance and prevents cross-contamination.

If the machine fails validation or calibration procedure, we will repeat the procedure again. If the problem persists, we will contact the technical support.

- Bio-Plex 200 Luminex machine undergoes preventive maintenance every 6 months

#### 4.7 Plate layout in the Bioplex-200 instrument

*Initiation of Bio-Plex Software.* Bio-Plex program will be started by clicking the Bio-Plex icon on the desktop. The instrument will be initiated by clicking the start up program, followed by 30 min laser warm up, and then calibrated using the Bio-Rad calibration kit. Once the instrument is ready, the plate to be analyzed is loaded into the instrument and the assay protocol template created in the format given below:

**Plate Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	St01	Standards St01	St09	St09	UN01	UN01	Unknowns UN01	UN09	UN09	UN09	Proficiency Pr01	Pr03
B	St02	St02	St10	St10	UN02	UN02	UN02	UN10	UN10	UN10	Pr01	Pr03
C	St03	St03	St11	St11	UN03	UN03	UN03	UN11	UN11	UN11	Pr01	Pr03
D	St04	St04	St12	St12	UN04	UN04	UN04	UN12	UN12	UN12	Pr02	
E	St05	St05	St13	St13	UN05	UN05	UN05	UN13	UN13	UN13	Pr02	
F	St06	St06	Blank BL	BL	UN06	UN06	UN06	UN14	UN14	UN14	Pr02	
G	St07	St07	Controls Co01	Co01	UN07	UN07	UN07	UN15	UN15	UN15		
H	St08	St08	Co02	Co02	UN08	UN08	UN08	UN16	UN16	UN16		

St01-St13: Standards

Un01-Un16: Unknown Samples

Co01-Co02: Urine Controls

Pr01-Pr03: Proficiency Samples

BL: Blank (Sample diluent buffer)

**Note:** It is very important that you be careful to highlight the correct cells when setting up the template to assign the order and duplicates.

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To acquire the above plate format the following procedures should be followed

- Click on new plate
- Select KIM-1 analyte from the dropdown list
- Select plate format
  - To assign standards on the plate follow these steps (See figure below)



- Click on the “123 icon with arrow pointing toward right side”
- Select “2” from the drop down menu
- Select “**circled S icon**”
- Now highlight the cells from A1 to H2 (this will highlight the columns 1 & 2) and the software automatically assigns St01-St08 as shown in the plate template above. **Check that this assignment is correct.**
- Now highlight the cells from A3 to E4 and the software automatically assigns St09-St13 as shown in the plate

- To assign Blanks on the plate follow these steps (See figure below)



- Click on the “123 icon with arrow pointing toward right side”
- Select “2” from the drop down menu
- Select “**B icon**”
- Now highlight the cells from F3 to F4 and the software automatically assigns “BL” as shown in the plate template above. **Check that this assignment is correct.**

- To assign “**Controls**” follow these steps (See figure below)



- Click on the “123 icon with arrow pointing toward right side”
- Select “2” from the drop down menu
- Select “**C**” icon
- Now highlight the cells from G3 to H4 (G3, G4, H3 H4) and the software automatically assigns Co01-Co02 as shown in the plate template above. **Check that this assignment is correct.**

- To assign “**Unknowns**” on the plate follow these steps (See figure below)



- Click on the “123 icon with arrow pointing toward downwards”
- Select “3” from the drop down menu
- Select “**X**” icon
- Now highlight the cells from A5 to H7 and the software automatically assigns UN01-UN08 as shown in the plate template above. **Check that this assignment is correct.**

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- Now highlight the cells from A8 to H10 and the software automatically assigns UN09-UN16 as shown in the plate template above. **Check that this assignment is correct.**
- To assign “**Proficiency Samples**” on the plate follow these steps (See figure below)



- Click on the “123 icon with arrow pointing toward downwards”
  - Select “3” from the drop down menu
  - Select “X “ icon
  - Now highlight the cells from A11 to F11 and the software automatically assigns Pr01-Pr02 as shown in the plate template above. **Check that this assignment is correct.**
  - Now highlight the cells from A12 to C12 and the software automatically assigns Pr03 as shown in the plate template above. **Check that this assignment is correct.**
- The information of the standards, description of the samples (sample IDs), controls (C1-C2) will be incorporated into the template. Once the protocol is ready, the analysis will be initiated by clicking the RUN button.
  - In addition to barcode scanning, each sample tube will be numerically labeled for subsequent cross verification in the order we aliquot the samples on the plate.

## 5. Data Generation & Processing

### 5.1 Data Output

- Data will be analyzed using Bio-Plex Manager Software integrated instrument operating software.
- Standard curve recovery is a practical parameter commonly used to determine the overall accuracy of an assay. Bio-Plex Manager includes a mechanism for assessing the fit of a standard curve to its individual standard points. This is the recovery percentage. For each analyte standard, an observed concentration is back calculated by plotting the fluorescence intensity of the standard on the standard curve. This is divided by the expected concentration and multiplied by 100 to give a recovery percentage. The measurement can be performed automatically by the software. The output is derived from the assigned (expected) concentration of each standard and the actual (observed) concentration using the formula:

**Observed concentration/Expected concentration] x 100.**

- Working assay range is defined as the range between the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ) in which an assay is both precise and accurate. The Bio Plex manager analyzes the curve fit based on observed/expected ratio.

### 5.2 Standard Curve generation

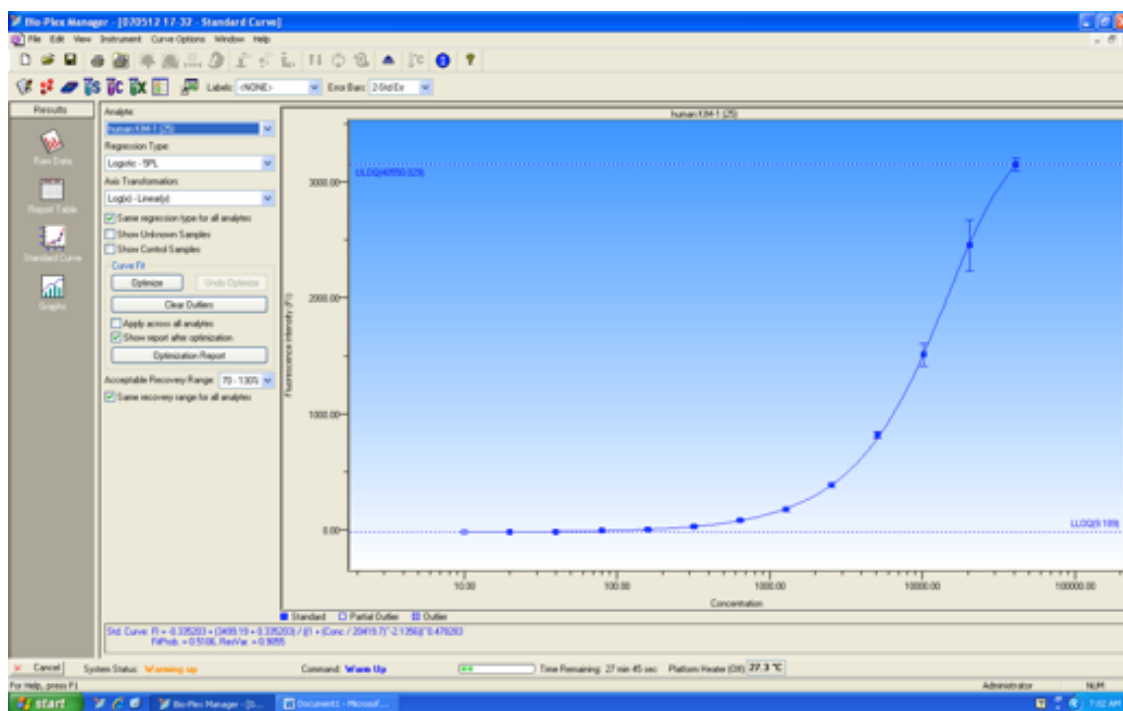
- The Bio-Plex manager uses the serially diluted standards to generate the standard curve. The software has options to choose the type of regression analysis. We use 5 parametric logistic regression analyses to predict the concentrations of unknown samples. (Below is the typical standard curve)

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### 5.3 Data Processing and Data Calculations

- The Bio-Plex manager automatically generates the value of unknowns based on the standard curve. The data generated (see below) includes the fluorescence Intensity, predicted concentration of unknowns, standard error, % CV, and errors in the run of all duplicate or triplicate samples (below is the typical data output page).

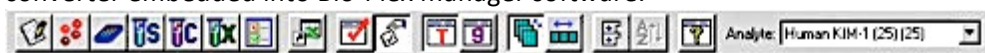
Bio-Plex Manager - [Results4 - Report Table]													
File Edit View Instrument Table Options Window Help													
Analyte: human KIM-1 (25)													
Results	Type	Well	Outlier	Description	FI	FI - Bkgd	Std Dev	%CV	Conc in Range	Obs Conc	Exp Conc	(Obs/Exp) * 100	Dilution
1	B	F3,F4			1.5	1.5	0.71	47.14					1.00
2	S1	A1,A2			1559.5	1558.0	89.10	5.71	45074.07	45074.07	40000.00	113	1.00
3	S2	B1,B2			928.8	927.3	261.98	28.21	16486.87	16486.87	20000.00	82	1.00
4	S3	C1,C2			744.5	743.0	37.48	5.03	11626.96	11626.96	10000.00	116	1.00
5	S4	D1,D2			404.3	402.8	3.18	0.79	5086.23	5086.23	5000.00	102	1.00
6	S5	E1,E2			217.3	215.8	8.84	4.07	2492.96	2492.96	2500.00	100	1.00
7	S6	F1,F2			110.3	108.8	1.06	0.96	1244.26	1244.26	1250.00	100	1.00
8	S7	G1,G2			52.0	50.5	2.83	5.44	610.49	610.49	625.00	98	1.00
9	S8	H1,H2			24.8	23.3	3.89	15.71	309.88	309.88	312.50	99	1.00
10	S9	A3,A4			11.5	10.0	0.71	6.15	151.43	151.43	156.25	97	1.00
11	S10	B3,B4			7.3	5.8	0.35	4.88	94.40	94.40	78.13	121	1.00
12	S11	C3,C4			3.5	2.0	0.71	20.20	35.34	35.34	39.06	90	1.00
13	S12	D3,D4			2.5	1.0	0.71	28.28	14.67	14.67	19.53	75	1.00
14	S13	E3,E4			2.5	1.0	0.71	28.28	14.67	14.67	9.77	150	1.00
15	C1	G3,G4		Quality control 1	1.0	-0.5	0.00	0.00	OR <	OR <	0.00	***	1.00
16	C2	H3,H4		Quality control 2	77.5	76.0	109.60	141.42	886.22	886.22	0.00	***	1.00
17	X1	A5,A6,A7		XXXXXX	237.5	236.0	9.99	4.21	2746.49	2746.49			1.00
18	X2	B5,B6,B7		XXXXXX	12.3	10.8	0.58	4.68	162.04	162.04			1.00
19	X3	C5,C6,C7		XXXXXX	16.3	14.8	2.02	12.37	211.39	211.39			1.00
20	X4	D5,D6,D7		XXXXXX	107.0	105.5	3.61	3.37	1208.37	1208.37			1.00
21	X5	E5,E6,E7		XXXXXX	19.0	17.5	1.73	9.12	243.20	243.20			1.00
22	X6	F5,F6,F7		XXXXXX	12.8	11.3	2.36	18.41	168.34	168.34			1.00
23	X7	G5,G6,G7		XXXXXX	66.0	64.5	10.69	16.20	761.84	761.84			1.00
24	X8	H5,H6,H7		XXXXXX	11.0	9.5	1.00	9.09	144.99	144.99			1.00
25	X9	A8,A9,A10		XXXXXX	57.2	55.7	6.05	10.58	666.40	666.40			1.00
26	X10	B8,B9,B10		XXXXXX	28.0	26.5	0.50	1.79	346.75	346.75			1.00
27	X11	C8,C9,C10		XXXXXX	93.2	91.7	12.69	13.62	1056.56	1056.56			1.00
28	X12	D8,D9,D10		XXXXXX	127.5	126.0	12.12	9.51	1436.45	1436.45			1.00
29	X13	E8,E9,E10		XXXXXX	37.0	35.5	4.50	12.16	446.91	446.91			1.00
30	X14	F8,F9,F10		XXXXXX	52.3	50.8	2.36	4.52	614.10	614.10			1.00
31	X15	G8,G9,G10		XXXXXX	29.7	28.2	2.89	9.73	365.49	365.49			1.00
32	X16	H8,H9,H10		XXXXXX	34.2	32.7	2.02	5.91	415.62	415.62			1.00
33	X17	A11,B11,C11		proficiency sample 1	9.5	8.0	1.50	15.79	125.30	125.30			1.00
34	X18	D11,E11,F11		proficiency sample 2	40.7	39.2	4.91	12.07	487.17	487.17			1.00
35	X19	A12,B12,C12		proficiency sample 3	97.5	96.0	30.83	31.62	1103.96	1103.96			1.00

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- Data is exported from Bio-Plex manager to Microsoft excel using the automatic converter embedded into Bio-Plex manager software.



- The program will export information from the run including sample Type, information of the wells, sample description, fluorescence intensity (FI), FI-Background, standard deviation, %CV, concentration in Range, observed concentration, and dilution. The QC samples and proficiency samples are evaluated for quality control.

File Name: Untitled													
Analyte	Type	Well	Outlier	Description	FI	FI - Bkgd	Std Dev	%CV	Conc in Range	Obs Conc	Exp Conc	(Obs/Exp) * 100	Dilution
human KIM-1 (25)	B	F3,F4	0		1.5	1.5	0.71	47.14					1
human KIM-1 (25)	S1	A1,A2	0		1559.5	1558	89.1	5.71	45074.07	45074.07	40000	113	1
human KIM-1 (25)	S2	B1,B2	0		928.8	927.3	261.98	28.21	16486.87	16486.87	20000	82	1
human KIM-1 (25)	S3	C1,C2	0		744.5	743	37.48	5.03	11626.96	11626.96	10000	116	1
human KIM-1 (25)	S4	D1,D2	0		404.3	402.8	3.18	0.79	5086.23	5086.23	5000	102	1
human KIM-1 (25)	S5	E1,E2	0		217.3	215.8	8.84	4.07	2492.96	2492.96	2500	100	1
human KIM-1 (25)	S6	F1,F2	0		110.3	108.8	1.06	0.96	1244.26	1244.26	1250	100	1
human KIM-1 (25)	S7	G1,G2	0		52	50.5	2.83	5.44	610.49	610.49	625	98	1
human KIM-1 (25)	S8	H1,H2	0		24.8	23.3	3.89	15.71	309.88	309.88	312.5	99	1
human KIM-1 (25)	S9	A3,A4	0		11.5	10	0.71	6.15	151.43	151.43	156.25	97	1
human KIM-1 (25)	S10	B3,B4	0		7.3	5.8	0.35	4.88	94.4	94.4	78.13	121	1
human KIM-1 (25)	S11	C3,C4	0		3.5	2	0.71	20.2	35.34	35.34	39.06	90	1
human KIM-1 (25)	S12	D3,D4	0		2.5	1	0.71	28.28	14.67	14.67	19.53	75	1
human KIM-1 (25)	S13	E3,E4	0		2.5	1	0.71	28.28	14.67	14.67	9.77	150	1
human KIM-1 (25)	C1	G3,G4	0	Quality control 1	1	-0.5	0	0	0	0	0	***	1
human KIM-1 (25)	C2	H3,H4	0	Quality Control 2	77.5	76	109.6	141.42	886.22	886.22	0	***	1
human KIM-1 (25)	X1	A5,A6,A7	0	XXXXXX	237.5	236	9.99	4.21	2746.49	2746.49			1
human KIM-1 (25)	X2	B5,B6,B7	0	XXXXXX	12.3	10.8	0.58	4.68	162.04	162.04			1
human KIM-1 (25)	X3	C5,C6,C7	0	XXXXXX	16.3	14.8	2.02	12.37	211.39	211.39			1
human KIM-1 (25)	X4	D5,D6,D7	0	XXXXXX	107	105.5	3.61	3.37	1208.37	1208.37			1
human KIM-1 (25)	X5	E5,E6,E7	0	XXXXXX	19	17.5	1.73	9.12	243.2	243.2			1
human KIM-1 (25)	X6	F5,F6,F7	0	XXXXXX	12.8	11.3	2.36	18.41	168.34	168.34			1
human KIM-1 (25)	X7	G5,G6,G7	0	XXXXXX	66	64.5	10.69	16.2	761.84	761.84			1
human KIM-1 (25)	X8	H5,H6,H7	0	XXXXXX	11	9.5	1	9.09	144.99	144.99			1
human KIM-1 (25)	X9	A8,A9,A10	0	XXXXXX	57.2	55.7	6.05	10.58	666.4	666.4			1
human KIM-1 (25)	X10	B8,B9,B10	0	XXXXXX	28	26.5	0.5	1.79	346.75	346.75			1
human KIM-1 (25)	X11	C8,C9,C10	0	XXXXXX	93.2	91.7	12.69	13.62	1056.56	1056.56			1
human KIM-1 (25)	X12	D8,D9,D10	0	XXXXXX	127.5	126	12.12	9.51	1436.45	1436.45			1
human KIM-1 (25)	X13	E8,E9,E10	0	XXXXXX	37	35.5	4.5	12.16	446.91	446.91			1
human KIM-1 (25)	X14	F8,F9,F10	0	XXXXXX	52.3	50.8	2.36	4.52	614.1	614.1			1
human KIM-1 (25)	X15	G8,G9,G10	0	XXXXXX	29.7	28.2	2.89	9.73	365.49	365.49			1
human KIM-1 (25)	X16	H8,H9,H10	0	XXXXXX	34.2	32.7	2.02	5.91	415.62	415.62			1
human KIM-1 (25)	X17	A11,B11,C11	0	proficiency sample 1	9.5	8	1.5	15.79	125.3	125.3			1
human KIM-1 (25)	X18	D11,E11,F11	0	proficiency sample 2	40.7	39.2	4.91	12.07	487.17	487.17			1
human KIM-1 (25)	X19	A12,B12,C12	0	proficiency sample 3	97.5	96	30.83	31.62	1103.96	1103.96			1

- In addition to barcode scanning, each sample tube will be numerically labeled for cross verification in the order we aliquot the samples on the plate. Once the data are transferred and compiled, we will again cross check the numerical number on the tube with the barcode scan.
- Another member in the facility will perform a secondary check to further validate the sample order.

## 6 Quality Control

### 6.1. Quality control of the Assay.

- We will ensure that KIM-1 levels obtained using the assay on control specimens are within mean  $\pm$  2SD limit established from the Validation Protocol.
- If both samples are within mean  $\pm$  2SD limit, we will accept the data. If one of the two control values is outside mean  $\pm$  2SD, we will employ the Westgard 2 rules to determine if the analysis run results can be accepted.

Reference: human urine KIM-1/SOP/001

Version: 1.1

Author: Venkata S. Sabbisetti

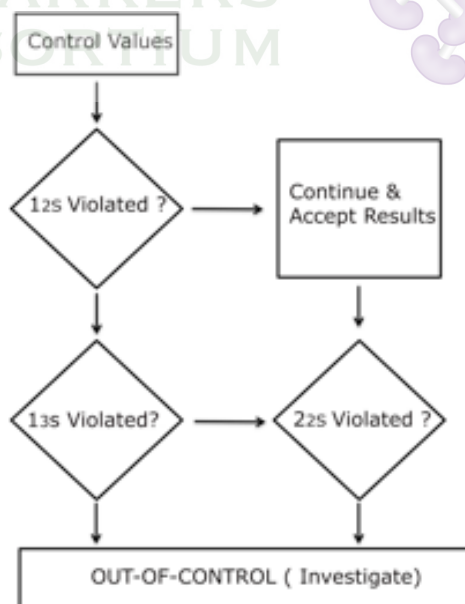
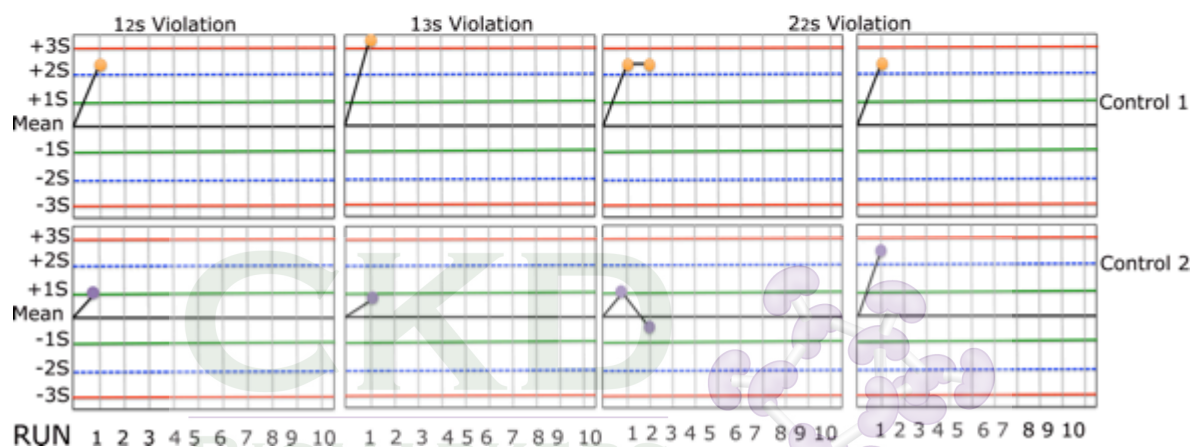
Page 12 of 15



- If neither of the assayed control results is within mean  $\pm$  2SD limits, then we will follow the westgard three-quality control rule and not accept the data.

RULE	QUALITY CONTROL RULE EXPLANATION
12S	One value of any of the two controls used in the assay is outside of the mean+2 SD or mean-2 SD of that control
13S	One value of any of the two controls used in the assay is outside of the mean+3 SD or mean-3 SD of that control
22S	Both controls used in the assay are outside of their mean $\pm$ SD on the same assay or two consecutive values of one control is outside of mean $\pm$ SD

Levey-Jenning's plots of controls:



Reference: human urine KIM-1/SOP/001

Version: 1.1

Author: Venkata S. Sabbiseti



## 6.2 Quality Control for each sample

- Sample measurements will be repeated if the %CV of the sample is > 15%
- Sample measurements will be repeated if the instrument produces any run errors for a sample.

## 6.3 Quality Control troubleshooting procedure

If the controls fail the Westgard quality control procedure, then we will employ the following procedure to resolve the issue:

- Contact the Lab Director
- Rerun Control
- If the quality control values pass the above described westgard rules proceed with unknown testing
- If the quality control failed again, make up fresh reagents (couple new beads) and rerun controls and document the problem
- If the quality control indicates that the westgard rules are passed, proceed with testing
- If QC fails again, recalibrate the assay (use new standard lot)
- If after calibration the QC determinations have passed westgard criteria, proceed with testing and document correction
- If Westgard criteria were not met after preparation of fresh reagents, do not proceed with the analysis and contact the Laboratory Director.

## 6.4 Data storage and reporting

- After the analysis has passed the quality control test KIM-1 values of unknown samples with their corresponding %CV and SD will be copied and pasted into a master excel data workbook in the sequential order in sheet 1. KIM-1 values, %CV, SD of quality control samples and proficiency samples will be copied and pasted in sheet 2. Both sheet 1 & 2 will be updated with addition of data from each run.
- The master data sheet that will go to coordinating center will contain the following information
  1. Sample ID (study e.g. Biocon ID, ROSE ID, etc)
  2. Date of the assay
  3. Platform and the Instrument used for the assay
  4. Reagent lot numbers, which include date of microbead preparation, lot numbers of primary antibody, secondary antibody, substrate (PE-SAPE), and recombinant proteins
  5. Concentration of KIM-1 (pg/ml)
  6. % CV across the sample replicates
  7. KIM-1 levels, SD and % CV of each control sample with the description of each control sample
  8. KIM-1 levels, SD and %CV of each proficiency samples with the description of each proficiency sample (the cells will be left blank if the proficiency samples are not run on that plate)

Reference: human urine KIM-1/SOP/001

Version: 1.1

Author: Venkata S. Sabbiseti

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9. Levey-Jennings plots of Control samples and proficiency samples (These plots will be updated and cumulative plots will be reported with each data submission.

#### 6.5 Assay parameters

Assay Range	9.7 pg/ml-40 ng/ml
LLOD	9.7 pg/ml
LLOQ	4.4 pg/ml
Recovery	90-100%
Linearity of Dilution	1:2, 1:10, 1:20 dilution
Intraassay %CV	< 10%
Interassay %CV	< 12%
Reference range in healthy volunteers*	9.7-650 pg/mg uCr

\* Based on assumptions regarding “normality”

#### 7. References

1. Vaidya VS, Waikar SS, Ferguson MA, Collings FB, Sunderland K, Gioules C, Bradwin G, Matsouaka R, Betensky RA, Curhan GC, Bonventre JV. Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. Clin Transl Sci. 2008 Dec;1(3):200-8.
2. Koyner JL, Vaidya VS, Bennett MR, Ma Q, Worcester E, Akhter SA, Raman J, Jeevanandam V, O'Connor MF, Devarajan P, Bonventre JV, Murray PT. Urinary biomarkers in the clinical prognosis and early detection of acute kidney injury. Clin J Am Soc Nephrol. 2010 Dec;5(12):2154-65
3. Vaidya VS, Niewczas MA, Ficociello LH, Johnson AC, Collings FB, Warram JH, Krolewski AS, Bonventre JV. Regression of microalbuminuria in type 1 diabetes is associated with lower levels of urinary tubular injury biomarkers, kidney injury molecule-1, and N-acetyl- $\beta$ -D-glucosaminidase. Kidney Int. 2011 Feb;79(4):464-70



#### DESCRIPTION

**Source** Mouse myeloma cell line, NS0-derived  
Ser21-Thr288, with a C-terminal 6-His tag  
Accession # Q96D42

**N-terminal Sequence Analysis** Ser21

**Predicted Molecular Mass** 29.6 kDa

#### SPECIFICATIONS

**SDS-PAGE** 85-105 kDa, reducing conditions

**Activity** Measured by its ability to inhibit anti-CD3-induced proliferation of stimulated human T cells. Human T lymphocytes cultured for 72 hours with PHA were incubated for an additional 3 days in 96 well plates coated with 500 ng/mL anti-CD3 and rhTIM-1.  
The ED<sub>50</sub> for this effect is typically 0.2-0.8 µg/mL.  
**Optimal dilutions should be determined by each laboratory for each application.**

**Endotoxin Level** <1.0 EU per 1 µg of the protein by the LAL method.

**Purity** >90%, by SDS-PAGE under reducing conditions and visualized by silver stain.

**Formulation** Lyophilized from a 0.2 µm filtered solution in PBS. See Certificate of Analysis for details.

#### PREPARATION AND STORAGE

**Reconstitution** Reconstitute at 100 µg/mL in sterile PBS.

**Shipping** The product is shipped at ambient temperature. Upon receipt, store it immediately at the temperature recommended below.

**Stability & Storage** **Use a manual defrost freezer and avoid repeated freeze-thaw cycles.**

- 12 months from date of receipt, -20 to -70 °C as supplied.
- 1 month, 2 to 8 °C under sterile conditions after reconstitution.
- 3 months, -20 to -70 °C under sterile conditions after reconstitution.

\* This product is covered by one or more of the following US Patents 7,300,652; 7,041,290; 6,664,385 and other US and foreign patents pending or issued.

#### BACKGROUND

TIM-1 (T cell-Immunoglobulin-mucin; also KIM-1 and HAVcr-1) is a 100 kDa, type I transmembrane glycoprotein member of the TIM family of immunoglobulin superfamily molecules (1 - 3). This gene family is involved in the regulation of Th1 and Th2-cell-mediated immunity. Human TIM-1 is synthesized as a 359 amino acid (aa) precursor that contains a 20 aa signal sequence, a 270 aa extracellular domain (ECD), a 21 aa transmembrane segment and a 48 aa cytoplasmic domain (4 - 6). The ECD contains one V-type Ig-like domain and a mucin region characterized by multiple PTTTTL motifs. The mucin region undergoes extensive O-linked glycosylation. The TIM-1 gene is highly polymorphic and undergoes alternate splicing (1). For instance, the presence of a six aa sequence (MTTTPV) at position #137 of the mature molecule is associated with protection from atopy in people with a history of hepatitis A (7, 8). There are two cytoplasmic alternate splice forms of TIM-1. One is a long (359 aa) kidney form termed TIM-1b, and one is a short (334 aa) liver form termed TIM-1a. Both are identical through the first 323 aa of their precursors. TIM-1b contains a tyrosine phosphorylation motif that is not present in 1a (6). TIM-1 is also known to circulate as a soluble form. Constitutive cleavage by an undefined MMP (possibly ADAM33) releases an 85 - 90 kDa soluble molecule (6). The ECD of human TIM-1 is 50% and 43% aa identical to mouse and canine TIM-1 ECD, respectively. The only two reported ligands for TIM-1 are TIM-4 and the hepatitis A virus (4, 9). However, others are believed to exist, and based on the ligand for TIM-3, one may well be an S-type lectin (10). TIM-1 ligation induces T cell proliferation and promotes cytokine production (1, 10).

#### References:

1. Meyers, J.H. *et al.* (2005) Trends Mol. Med. **11**:1471.
2. Kuchroo, V.K. *et al.* (2003) Nat. Rev. Immunol. **3**:454.
3. Mariat, C. *et al.* (2005) Phil. Trans. R. Soc. B **360**:1681.
4. Feigelsstock, D. *et al.* (1998) J. Virol. **72**:6621.
5. Ichimura, T. *et al.* (1998) J. Biol. Chem. **273**:4135.
6. Bailly, V. *et al.* (2002) J. Biol. Chem. **277**:39739.
7. Umetsu, D.T. *et al.* (2005) J. Pediatr. Gastroenterol. Nutr. **40**:S43.
8. Gao, P-S. *et al.* (2005) J. Allergy Clin. Immunol. **115**:982.
9. Zhu, C. *et al.* (2005) Nat. Immunol. **6**:1245.
10. Meyers, J.H. *et al.* (2005) Nat. Immunol. **6**:455.

## DESCRIPTION

<b>Species Reactivity</b>	Human
<b>Specificity</b>	Detects human TIM-1/KIM-1/HAVCR in ELISAs and Western blots. In sandwich immunoassays, less than 0.1% cross-reactivity with recombinant mouse (rm) TIM-1, recombinant rat TIM-1, and recombinant human (rh) TIM-4 is observed.
<b>Source</b>	Polyclonal Goat IgG
<b>Purification</b>	Antigen Affinity-purified
<b>Immunogen</b>	Mouse myeloma cell line NS0-derived recombinant human TIM-1/KIM-1/HAVCR (R&D Systems, Catalog # 1750-TM) Ser21-Thr288 Accession # Q96D42
<b>Formulation</b>	Lyophilized from a 0.2 µm filtered solution in PBS with Trehalose. See Certificate of Analysis for details.

## APPLICATIONS

**Please Note:** Optimal dilutions should be determined by each laboratory for each application. *General Protocols* are available in the Technical Information section on our website.

	<b>Recommended Concentration</b>	<b>Sample</b>
<b>Western Blot</b>	0.1 µg/mL	Recombinant Human TIM-1/KIM-1/HAVCR (Catalog # 1750-TM)
<b>Human TIM-1/KIM-1/HAVCR Sandwich Immunoassay</b>		<b>Reagent</b>
<b>ELISA Capture</b>	0.2-0.8 µg/mL	Human TIM-1/KIM-1/HAVCR Antibody (Catalog # AF1750)
<b>ELISA Detection</b>	0.1-0.4 µg/mL	Human TIM-1/KIM-1/HAVCR Biotinylated Antibody (Catalog # BAF1750)
<b>Standard</b>		Recombinant Human TIM-1/KIM-1/HAVCR (Catalog # 1750-TM)

## PREPARATION AND STORAGE

<b>Reconstitution</b>	Reconstitute at 0.2 mg/mL in sterile PBS.
<b>Shipping</b>	The product is shipped at ambient temperature. Upon receipt, store it immediately at the temperature recommended below.
<b>Stability &amp; Storage</b>	<p><b>Use a manual defrost freezer and avoid repeated freeze-thaw cycles.</b></p> <ul style="list-style-type: none"> <li>12 months from date of receipt, -20 to -70 °C as supplied.</li> <li>1 month from date of receipt, 2 to 8 °C, reconstituted.</li> <li>6 months from date of receipt, -20 to -70 °C, reconstituted.</li> </ul>

\* This product is covered by one or more of the following US Patents 7,300,652; 7,041,290; 6,664,385 and other US and foreign patents pending or issued.

## BACKGROUND

TIM-1 (T cell-immunoglobulin-mucin; also known as KIM-1 and HAVCR) is a 100 kDa, type I transmembrane glycoprotein member of the TIM family of immunoglobulin superfamily molecules (1-3). This gene family is involved in the regulation of Th1 and Th2-cell-mediated immunity. Human TIM-1 is synthesized as a 359 amino acid (aa) precursor that contains a 20 aa signal sequence, a 270 aa extracellular domain (ECD), a 21 aa transmembrane segment and a 48 aa cytoplasmic domain (4-6). The ECD contains one V-type Ig-like domain and a mucin region characterized by multiple PTTTTL motifs. The mucin region undergoes extensive O-linked glycosylation. The TIM-1 gene is highly polymorphic and undergoes alternate splicing (1). For instance, the presence of a six aa sequence (MTTTPV) at position #137 of the mature molecule is associated with protection from atopy in people with a history of hepatitis A (7, 8). There are two cytoplasmic alternate splice forms of TIM-1. One is a long (359 aa) kidney form termed TIM-1b, and one is a short (334 aa) liver form termed TIM-1a. Both are identical through the first 323 aa of their precursors. TIM-1b contains a tyrosine phosphorylation motif that is not present in 1a (6). TIM-1 is also known to circulate as a soluble form. Constitutive cleavage by an undefined MMP (possibly ADAM33) releases an 85-90 kDa soluble molecule (6). The ECD of human TIM-1 is 50% and 43% aa identical to mouse and canine TIM-1 ECD, respectively. The only two reported ligands for TIM-1 are TIM-4 and the hepatitis A virus (4, 9). However, others are believed to exist, and based on the ligand for TIM-3, one may well be an S-type lectin (10). TIM-1 ligation induces T cell proliferation and promotes cytokine production (1, 10).

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<b>Purification</b>	Antigen Affinity-purified
<b>Immunogen</b>	Mouse myeloma cell line NS0-derived recombinant human TIM-1/KIM-1/HAVCR (R&D Systems, Catalog # 1750-TM) Ser21-Thr288 Accession # Q96D42
<b>Formulation</b>	Lyophilized from a 0.2 µm filtered solution in PBS with BSA as a carrier protein. See Certificate of Analysis for details.

## APPLICATIONS

**Please Note:** Optimal dilutions should be determined by each laboratory for each application. [General Protocols](#) are available in the Technical Information section on our website.

	<b>Recommended Concentration</b>	<b>Sample</b>
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<b>Human TIM-1/KIM-1/HAVCR Sandwich Immunoassay</b>		<b>Reagent</b>
<b>ELISA Capture</b>	0.2-0.8 µg/mL	Human TIM-1/KIM-1/HAVCR Antibody (Catalog # AF1750)
<b>ELISA Detection</b>	0.1-0.4 µg/mL	Human TIM-1/KIM-1/HAVCR Biotinylated Antibody (Catalog # BAF1750)
<b>Standard</b>		Recombinant Human TIM-1/KIM-1/HAVCR (Catalog # 1750-TM)

## PREPARATION AND STORAGE

<b>Reconstitution</b>	Reconstitute at 0.2 mg/mL in sterile Tris-buffered saline, pH 7.3 (20 mM Trizma base, 150 mM NaCl) containing 0.1% bovine serum albumin.
<b>Shipping</b>	The product is shipped at ambient temperature. Upon receipt, store it immediately at the temperature recommended below.
<b>Stability &amp; Storage</b>	<p><b>Use a manual defrost freezer and avoid repeated freeze-thaw cycles.</b></p> <ul style="list-style-type: none"> <li>12 months from date of receipt, -20 to -70 °C as supplied.</li> <li>1 month from date of receipt, 2 to 8 °C, reconstituted.</li> <li>6 months from date of receipt, -20 to -70 °C, reconstituted.</li> </ul>

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6. Bailly, V. *et al.* (2002) J. Biol. Chem. **277**:39739.
7. Umetsu, D.T. *et al.* (2005) J. Pediatr. Gastroenterol. Nutr. **40**:S43.
8. Gao, P-S. *et al.* (2005) J. Allergy Clin. Immunol. **115**:982.
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10. Meyers, J.H. *et al.* (2005) Nat. Immunol. **6**:455.

**Translational Core Laboratory (TCL)**  
**Institute for Translational Medicine and Therapeutics (ITMAT)**  
Andrew Mutch Building / PUH  
Philadelphia, PA 19104

## Laboratory Protocol

### Quantitation of Human L-FABP via Sandwich ELISA

**Date:** 15 May 2013

**Version Number:** 3.9

**Brief Summary of Procedure:** This document describes an automated method to quantify human Liver Fatty Acid Binding Protein (hL-FABP) in human urine using a sandwich ELISA in a 96 well microplate. The assay requires about 4.5 hours to perform.

**Expected Outcome of Procedure's Use:** The user will understand how to set up and execute the automated ELISA procedure to quantify hL-FABP in human urine using CMIC's ELISA kit. Detailed instructions are included to allow for the procedure to be performed with sufficient training and adequate experience in executing automated ELISAs using the Beckman FXp liquid handling robot.

### Method Review and Approval

Date of Review and Approval	Signatures	
	T.E. Mifflin, Ph.D.	S.R. Master, M.D., Ph.D.



## SOP Training and Awareness Signoff List

My signature below indicates that I have read and understand the contents of this document. If I locate or find errors and items to be corrected, I will notify my supervisor or the laboratory director as soon as possible.

### Distribution and Signoff List

Name of Laboratory Personnel	Training Date	SOP Version	Signature

## 2. Principle:

The procedure described here is an ELISA (Enzyme-Linked-ImmunoSorbent Assay) or 2-step sandwich method to quantify human liver fatty acid binding protein (hL-FABP) in urine. hL-FABP Standards, controls, and urine samples (all in duplicate) are first incubated with Pretreatment Solution which enhances the antigen's recognition and reactivity to anti-hL-FABP antibody. These treated samples are then transferred into an hL-FABP Antibody-Coated Microplate containing Assay Buffer and incubated while covered. During this incubation, hL-FABP in the treated solution binds to the immobilized antibody. After washing, the Ab-POD Conjugate is added as the secondary antibody and incubated, thereby forming sandwich of the hL-FABP antigen between the immobilized antibody and conjugate antibody. After incubation, the plate is washed and Substrate is added for enzyme reaction. The enzymatic reaction color develops proportional to the quantity of hL-FABP antigen present in the sample. The absorbance is measured using a microplate reader and a calibration curve is prepared based on the absorbance values of the hL-FABP calibrator concentrations. Finally, the concentration of hL-FABP concentration in controls and unknown samples is determined by interpolation of the calibration curve using a log-logit curve.

**PLEASE NOTE: Take necessary precautions while handling the following hazardous substances.**

- Assay Buffer, Standard Diluent (0ng/mL) and hL-FABP Standard (400ng/mL) contain sodium azide as a preservative (0.1% w/v, 0.05% w/v and 0.05% w/v, respectively).
- Substrate contains o-phenylenediamine dihydrochloride (13mg/tablet).
- Stop Solution contains sulphuric acid (4.9% w/v).

## 3. Specimen Handling and Collection:

### 3.1 Specimen Handling and Collection:

**3.1.1** Blood-borne pathogen safety training (offered online by UPENN's EHRS) must be completed before any work in the laboratory commences. **Use Universal Precautions and treat blood and blood products as potentially infectious materials. It is not known if specimens contain HBV, HIV, and other blood borne pathogens.**

**3.1.2** Wear proper protective equipment, lab coat and gloves.

**3.1.3** Dispose all tips and materials that come in contact with biological agents into proper biological waste containers.

### 3.2 Specimen Collection:

#### 3.2.1 Urine:

Test samples should be measured soon after collection. For storage of test samples, store them frozen (- 80°C) and do not subject samples to repeat freeze/thaw cycles. Thaw the test samples by leaving them at 2-8°C in open air racks in a refrigerator. After thawing, ensure the lids are still on the

tubes and vortex for 10-15 secs. Then invert each tube and vortex again for another 20 secs. Centrifuge samples if opaque to remove particulates prior to analysis. Unknown samples stored frozen at - 80°C are stable for one year.

#### 4. Reagents, Recipes and Equipment:

##### 4.1 Reagents:

- 4.1.1 hL-FABP ELISA Assay kit. The ELISA kit for quantifying hL-FABP is obtained from CMIC Corporation at the address below:

CMIC HOLDINGS Co., Ltd.  
7-10-4 Nishigotanda, Shinagawa-ku  
Tokyo 141-0031 JAPAN  
E-mail: [sayaka-yamada@cmic.co.jp](mailto:sayaka-yamada@cmic.co.jp)  
CMIC website: <http://www.fabp.jp/e/>

Kit contents are listed below. Store at 4-8°C or as indicated on each container.

##### Reagents

	Description	Quantity	Form
1	L-FABP Antibody Coated Microplate	96 Well x 1	96-well strip plate coated with anti human L-FABP mouse monoclonal antibody (CloneL)
2	Pretreatment Solution	6 mL x 1	0.1M CHES-LIOH (pH9.5) 0.2M LiCl 0.01M EDTA(EDTA Free acid) 2% SDS 0.2mg/mL blue dye
3	Assay Buffer	12 mL x 1	0.1M HEPES-NaOH pH7.5 0.15M NaCl 1% BSA 0.1% NaN <sub>3</sub>
4	The 2nd Ab-POD Conjugate	12 mL x 1	Anti human L-FABP mouse monoclonal antibody (Clone2) conjugated to horseradish peroxidase 0.05M Phosphate buffer pH7.2 0.15M NaCl 1% BSA 0.5% Casein-Na 0.1% ProClin300
5	Substrate	2 tablets	o-phenylenediamine dihydrochloride (13mg/ Tab)
6	Substrate Diluent	12 mL x 2	0.05M Citrate buffer pH4.1 0.015% H <sub>2</sub> O <sub>2</sub>
7	Wash Agent (x40 concentrate)	50 mL x 1	0.4M Phosphate buffer pH7.5 2% Tween20 0.5% ProClin300
8	Stop Solution	12 mL x 1	1N H <sub>2</sub> SO <sub>4</sub>
9	Standard Diluent (0ng/mL)	2.5 mL x 1	Dulbecco's Phosphate buffered saline (Ca free, Mg free) pH7.4 1% BSA 0.05% NaN <sub>3</sub>
10	L-FABP Standard (400ng/mL)	0.5 mL x 1	Recombinant human L-FABP 400ng/mL... Dulbecco's Phosphate buffered saline (Ca free, Mg free) pH7.4 1% BSA 0.05% NaN <sub>3</sub>

- 4.1.2 hL-FABP purified protein. The highly purified form (~95+ %) of hL-FABP is obtained from CMIC Corporation at the address below:

CMIC HOLDINGS Co., Ltd.  
7-10-4 Nishigotanda, Shinagawa-ku  
Tokyo 141-0031 JAPAN

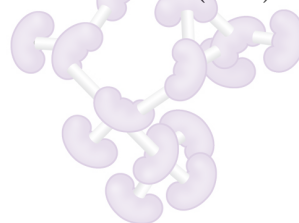
E-mail: [sayaka-yamada@cmic.co.jp](mailto:sayaka-yamada@cmic.co.jp)  
CMIC website: <http://www.fabp.jp/e/>

Upon receipt, store @ -80°C until ready for use.

- 4.1.2.1 Reconstitute the purified hL-FABP according to manufacturer's instructions.
- 4.1.2.2 Mix thoroughly and dispense into small aliquot volumes (e.g., 200- 250 uL) into labeled cryovials.  
(NOTE: *These are use-once and discard aliquots.*)
- 4.1.2.3 Store at -80°C until needed.

- 4.1.3 Human urine. Obtain as pooled material from multiple donors and mixed gender and ethnicities. Material is to be checked for and found negative for these viruses using the methods indicated in parentheses: HIV-1 (PCR), HIV I/II Ab, HBsAG and HCV (PCR)

Source: **Biochemed**  
172 Linden Drive  
Suite 101  
Winchester, VA USA  
Tel: 888-628-7948



Thaw the 1 liter of urine at 4-8°C by storage in refrigerator for 24hrs. Mix well by inversion several times and divide into 250 mL aliquots. Store aliquots @ -80°C until needed.

- 4.1.4 Human urine controls with hL-FABP (for daily QC).
- 4.1.4.1 Thaw a volume of human urine (e.g., ~150-200 mL) in refrigerator overnight.
- 4.1.4.2 Assay several aliquots on several different hL-FABP ELISA plates. Average the found conc of BASELINE hF-LABP in human urine.
- 4.1.4.3 Add an amount of additional hL-FABP to achieve the desired concentration of hL-FABP in the final product.
- 4.1.4.4 Mix well and dispense 0.5 mL aliquots into 0.6 mL labeled cyrovials. Store @ -80°C in 0.5 mL aliquots until needed.
- 4.1.4.5 Create at least two hL-FABP controls with known concentrations of hL-FABP.

## 4.2 Recipes:

### 4.2.1 Substrate solution :

One Substrate tablet is reconstituted in 12mL of Substrate Diluent (1 bottle) in the dark and used as a substrate solution. The substrate solution is prepared 15 min prior to use and used within 30 min after the preparation. Do not store or reuse.

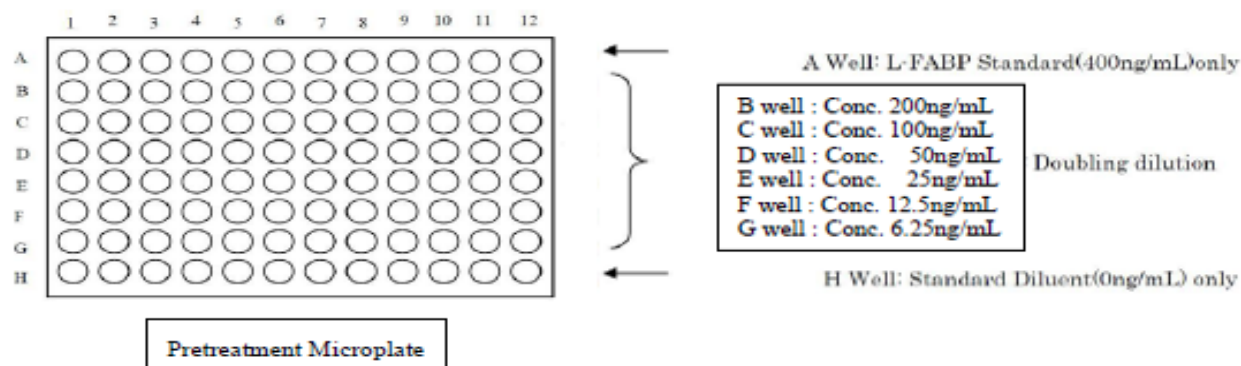
### 4.2.2 Wash Solution:

Dilute whole volume of Wash Agent (x40 concentrate) with Type I water from Millipore “MilliQ” water system to make 2,000 mL of wash solution

### 4.2.3 Preparation of hL-FABP Standard Solution

*(Note: Preparation of calibration curve is done on Biomek FXP. These instructions are provided for background information only)*

- Use the first column (“A1 - H1” wells) of Pretreatment Microplate for the preparation.
- Add 50µL of Standard Diluent (0ng/mL) to the “B1, C1, D1, E1, F1, G1 and H1” wells of the Pretreatment Microplate.
- Add 50µL of hL-FABP Standard (400ng/mL) to the “A1” well.
- Also, add 50µL of hL-FABP Standard (400ng/mL) to the “B1” well and mix gently (ten times pipetting).
- Then take 50µL of this solution and add to the “C1” well. Mix it as well.
- Take 50µL from the “C1” well and add to the “D1” well in the same manner as the above.
- Complete the steps of double dilution as taking and discarding 50µL from the “G1” well after mixing.
- Set the “H1” well as the “blank”.
- The final layout should be as follows:



### 4.3 Equipment:

Biomek FXp Robotic Liquid-Handling Workstation  
 Microplate reader (Paradigm Detection Platform) capable of measuring absorbance at 450 nm, with the correction wavelength of 540 nm / 570 nm.  
 Horizontal orbital microplate shaker (Liconic HRBT) (0.12" orbit) capable of speed of  $500 \pm 50$  rpm.  
 Automated plate washer (Biotek ELx405, Biotek Instruments)  
 Mecour incubation blocks on Biomek FXp deck.  
 Variable volume pipettes (calibrated within 6 mos.)  
 Multichannel pipette (calibrated within 6 mos.)  
 Fisher Dry block incubator, set @ 30°C  
 Sterile Corning Reagent reservoirs (Fisher Cat. #: 11-842-78)  
 Pipette tips, 200uL  
 Appropriate size graduated cylinders  
 Vortex  
 Nitrile or Latex Gloves  
 Cluster tubes (Micronics Tubes) (Fisher Cat. #: 11-842-85)  
 Cluster tube caps (Micronics Tubes) (Fisher Cat. #: 11-842-78)  
 12x75 polypropylene tubes and caps (Fisher Cat. #: 14-959-16)  
 Kimwipes (Fisher Cat. #: 06-666A)  
 NIST-traceable timer

## 5. Procedural Steps:

***Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls and standards be assayed in duplicate.***

### 5.1 Stamp Plate Creation:

- 5.1.1 Place 38 Unknown (CRIC) Urine aliquots and 1 set of Controls (1 Low and 1 High) in the Dry Block incubator for 5min at 30°C
- 5.1.2 Remove, invert, vortex for 3 seconds.
- 5.1.3 Place in centrifuge (*Thermo Legend X1R*) for 5 minutes at 2500rpm and 18°C.
- 5.1.4 Using SPS, scan the barcode located on the Mecour plate to bring up a 8X12 template (Rows A-H, Columns 1-12)
- 5.1.5 Starting with the first sample, scan the sample's 2-D barcode and place in duplicate positions A1 & A2 of the Mecour plate. The corresponding position in SPS will populate with the barcoded information (PID, Visit# sample type, shipment type, UUID, etc.).
- 5.1.6 Repeat step 5.1.5 for the remaining 37 samples. Columns 1&2 will populate first from position A1&A2 to H1&H2 and continue to the right so that F9&F10 will be the final sample position)
- 5.1.7 Scan Low and High LFABP Controls into positions G9-G10 and H9-H10 respectively.
- 5.1.8 Leave wells A11&A12-H11-H12 blank as they will be populated with the calibrator IDs when scanned by the barcode reader.
- 5.1.9 Repeat for each additional group of 38 Unknown urine samples, creating a separate Stamp plate for each group of 38 Unknowns.
- 5.1.10 Be sure to position the Unknowns in the same relative position so to allow for placement of controls and calibrators.



- 5.1.11 Place Stamp Plates in locations 1-7 in Hotel 1 of the Cytomat.
- 5.1.12 Uncap urine specimens in column 1 placing the lids next to each cryovial and, using a multichannel pipette, transfer 50µL of urine into the column 1 of the pre-treatment microplate.
- 5.1.13 Repeat process for columns # 2-10.
- 5.1.14 Place barcoded microplate lid on pre-treatment microplate and place in positions 1-10 of plate hotel #1 in the Cytomat.
- 5.1.15 Recap urine aliquots and scan back into 9X9 freezer storage boxes. Update freeze/thaw count by 1 and subtract 100µL from the aliquot volume. Scan freezer box back into freezer.

### Reagent Preparation

- 5.1.16 After preparing the wash buffer as detailed in step 4.2.2, Transfer volume into 5 gallon reservoir and connect to valve A of ELX-405.
- 5.1.17 Empty contents of the hL-FABP conjugate into a 300mL diamond bottom reservoir and place on position P4.
- 5.1.18 Empty contents of hL-FABP Assay Buffer into a 300mL diamond bottom reservoir and place in position P1.
- 5.1.19 Empty contents of hL-FABP Pretreatment Solution into a 300mL diamond bottom reservoir and place in position P2.
- 5.1.20 Empty contents of hL-FABP Substrate solution (prepared using steps 4.2.1.) into a 300mL diamond reservoir and place in position P7
- 5.1.21 Place one full rack of p1000 1mL tips on position P13.
- 5.1.22 Place calibrator (as prepared in steps 4.2.3) on position P10 with position A1 oriented to the upper left.
- 5.1.23 Place barcoded lidded stamp plates in hotel 1 slots 1-10.
- 5.1.24 Place lidded assay plates in hotel 1 slots 12-21.
- 5.1.25 Place p200 multichannel tips in the following Cytomat positions:
  - hotel 2 Slots 1-7
  - hotel 3 Slots 1-3
  - hotel 4 Slots 1-3
  - hotel 5 Slots 1-7
  - hotel 6 Slots 1-3
  - hotel 7 Slots 1-7
  - hotel 8 Slots 1-3
- 5.1.26 Place 100mL of Stop solution into a 300mL diamond bottom reservoir and place in hotel 9 slot 1.

### 5.2 Bio-Mek Preparation:

- 5.2.1 Turn on Bio-Mek devices included in integrated platform.
- 5.2.2 Open BIOMEK software. Home the device by selecting INSTRUMENT>HOME ALL AXES.
- 5.2.3 Purge the plate washer by selecting INSTRUMENT>DEVICE EDITOR>ELx405>ACTION COMMANDS>BC\_DAY\_PURGE.
- 5.2.4 Open SAMI WORKSTATION EDITOR. Select L\_FABP method.
- 5.2.5 Select SCHEDULE and choose the number of stamp plates to be assayed (1-10/run).
- 5.2.6 Once a schedule has been successfully created, click RUN. This will open the RUNTIME SAMI software.

- 5.2.7 Confirm that all of the labware is in the correct positions on the Biomek deck and filled with the proper volume of reagents.
- 5.2.8 Click START and watch initial actions of Biomek to ensure that it is performing correctly.

### Tabular Summary of ELISA Method's Operation Protocol

Activity	Test Sample / Control	Calibrator	Blank
Pretreatment	Test Sample / Control 50 µL	Calibrator 50 µL	Blank 50 µL
	Pretreatment Solution, 50 µL		
Mix solutions	Mix solutions thoroughly by plate mixer for ~ 5 min		
Add Assay Buffer	100 µL	100 µL	100 µL
Mix solutions	Mix solutions thoroughly by plate mixer for ~ 5 min		
Pipet Mixed Samples to Microplate	20 µL	20 µL	20 µL
Mix solutions	Mix solutions thoroughly by plate mixer for ~ 5 min		
Incubate	Incubate for 55 mins at 20 – 28 °C		
Wash microplate	Wash microplate wells with 350 µL Wash Buffer (3) times		
Add 2 <sup>nd</sup> Ab-HRP Conjugate	100 µL	100 µL	100 µL
Mix solutions	Mix solutions thoroughly by plate mixer for ~ 5 min		
Incubate	Incubate for 55 mins at 20 – 28 °C		
Wash microplate	Wash microplate wells with 350 µL Wash Buffer (3) times		
Add HRP Substrate	100 µL	100 µL	100 µL
Incubate with light protection	Incubate for 25 mins at 20 – 28 °C		
Add HRP Substrate	100 µL	100 µL	100 µL
Add STOP Solution	100 µL	100 µL	100 µL
Mix solutions	Mix solutions thoroughly by plate mixer for ~ 2-3 min		
Read abs @ 490nm & ref @ 620nm within 30 min of adding STOP soln	Measure Abs	Measure Abs	Measure Abs

### 5.3 Data Processing and Data Calculations

- 5.3.1 Activate Data processing software
- 5.3.2 Calculate mean values of each pair of duplicate abs data sets
- 5.3.3 Calculate the Standard Deviation and CV of every pair of Abs values measured from each microplate.
- 5.3.4 Record these values for later evaluation if needed.
- 5.3.5 Subtract the mean of the BLANK abs values from all of the remaining mean Abs values in each plate.
- 5.3.6 Using the processing software, plot the log-logit transform of the calibration curve abs vs. corresponding concentration
- 5.3.7 Interpolate each control and unknown sample's mean Abs value to determine conc.

## 6. Results and Recording of Data:

### 6.1 Results - Plate reader method:

- 6.1.1 In SPS software, select the desired set of results by clicking; ASSAY TYPE=hL-FABP, SAMPLE TYPE=24H URINE, SHIPMENT TYPE=TRANSCOLD, and specify the date the assay was run.
- 6.1.2 Create a CSV export of specimen IDs, well positions and corrected raw O.D. values
- 6.1.3 Open MASTERPLEX analysis software and select desired .csv file. Choose hL-FABP format under TEMPLATE. This will populate the plate data with expected standard concentrations, and specimen assignment (i.e. unknown, control or standard)
- 6.1.4 Click BEST FIT and select 4-PL for curve fit equation. Observe the standard curve.
- 6.1.5 Save file as "Assay Name\_Run Date.xml" (e.g. LFABP\_12.13.11.xml)

### 6.2 Results - Combining Run Data:

- 6.2.1 Open \*.xml file and copy all of the plate information.
- 6.2.2 Open file hL-FABP COMBINED RESULTS.xlsx in the desktop folder hL-FABP. Paste the daily results at the bottom of the combined results. Label results with a run date and plate #.

## 7. Quality Control

### 7.1 Standard Curve:

- 7.1.1 Examine the curve fit to the calibrators to determine if it accurately represents the calibrators. Enter the result into the Daily ELISA Result Checklist.
- 7.1.2 Standard curve generated for each plate should be a near linear curve. Do not extend or extrapolate this curve below 6.25ng/mL or above 400 ng/mL of hL-FABP.

### 7.2 Frequency of Assaying L-FABP controls.

The recommended control requirements for the CMIC L-FABP ELISA is a single sample of EACH control level to be tested at least once on every microplate. It is also recommended that a single sample of each Biocon Proficiency material be tested at least once every 8 hours (or once per run every day).

### 7.3 List of L-FABP Controls

- 7.3.1 In-house preparation of Low L-FABP control
- 7.3.2 In-house preparation of High L-FABP control

The values of these controls must be within the acceptable ranges as determined during the Method Validation procedure. If a control is out of its specified limit, the associated unknown test results may be invalid. The following steps must be followed during the troubleshooting process when an invalid control result is obtained.

### 7.4 Steps in Processing QC Results

- 7.4.1 In a stepwise manner, evaluate each control material to determine whether the L-FABP values obtained using this ELISA method are within  $\pm 2SD$  limit established from the Validation Protocol.
- 7.4.2. If both are within the stated  $\pm 2SD$  limits, accept the run and transfer the unknown results to the reporting worksheet
- 7.4.3. If one control's value is within the  $\pm 2SD$  limits and the other is outside, then follow the Westgard 2 Control evaluation algorithm depicted on the next page before deciding how to determine if the analysis run results can be accepted.
- 7.4.4. If neither of the assayed control L-FABP control results are within limits, then following the stepwise evaluation tree listed below the Westgard diagram on the next page.

Rule	Quality-Control Rule Explanation
1 <sub>2s</sub>	The 1 <sub>2s</sub> rule describes a situation where one control value for any of the two levels of control is outside the mean $\pm$ 2 SD.
1 <sub>3s</sub>	The 1 <sub>3s</sub> rule describes a situation where one control value for any of the two levels of control is outside the mean $\pm$ 3 SD.
2 <sub>2s</sub>	The 2 <sub>2s</sub> rule describes a situation where both control fluids or two consecutive control values of one fluid are outside the mean by either +2 SD or -2 SD.

Table 1. Quality-Control Rules

The three quality-control rules just described are used together when the mean value for the quality-control fluids has been established. The diagram below should help you better understand the three rules.

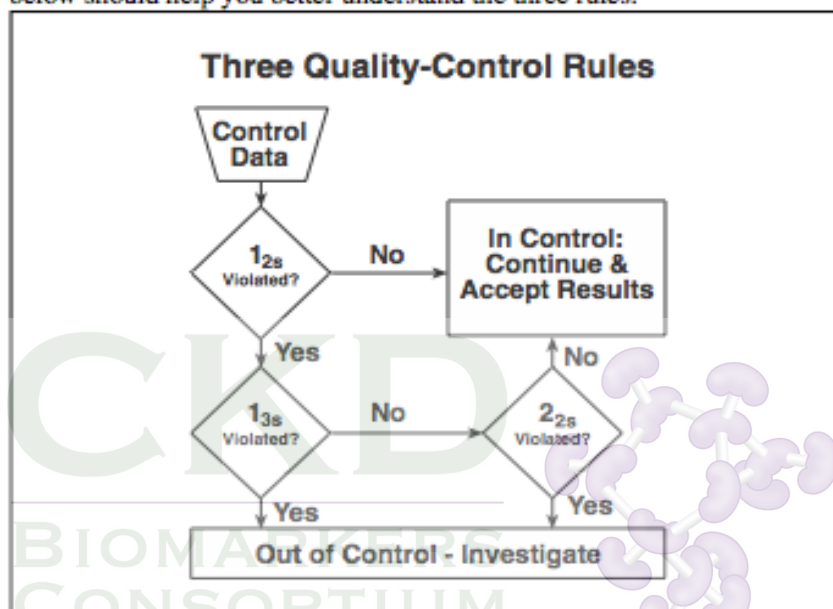


Figure 11. Three Quality-Control Rules

## 7.5 Quality Control Troubleshooting Procedure:

- 7.5.1. Rerun controls
- 7.5.2. If QC is within specified range document correction and proceed with unknown testing.
- 7.5.3 If QC is **Not** within specified range, document it, make up fresh QC fluid and rerun controls.
- 7.5.4. If controls are within specified range, proceed with testing.
- 7.5.5 If QC is **Not** within specified range, recalibrate the assay.
- 7.5.6. If after calibration the QC is within specified range, proceed with testing and document correction.
- 7.5.7. If QC in **Not** within specified range after calibration, place a fresh reagent on analyzer and recalibrate.
- 7.5.8. If QC is within range after calibrating with fresh reagent, proceed with patient testing and document correction.
- 7.5.9 If QC is **Not** within specified range after using fresh reagent and control fluid, do not proceed with unknown testing and contact the Laboratory Director.

## 7.6 Procedure for Reporting L-FABP Results

- 7.6.1 After the analysis has been accepted, the final hL-FABP concentrations of the unknown samples can be transferred to the Sample Processing System (SPS).
- 7.6.2 This is done by opening SPS, selecting RESULT TRANSFER, and selecting destination of result file.
- 7.6.3 Next, select the source file (e.g., today's "Daily ELISA Result Checklist") that contains the accepted results.
- 7.6.4 Activate SPS to transfer (upload) the results. Note that ALL of the results including the unknown concentrations as well as the controls and their concentrations will be uploaded into SPS.
- 7.6.5 ALL unknown LFABP concentrations will be transferred to SPS, regardless of their value. This includes values that are interpolated below the lowest LFABP calibrator concentration.
- 7.6.6 Save the Daily ELISA Result Checklist in the Month fold it was analyzed.
- 7.6.7 Shut off the PC.
- 7.6.8 **Minimum and Maximum Values:** Samples run in this assay should have values within the standard curve range.

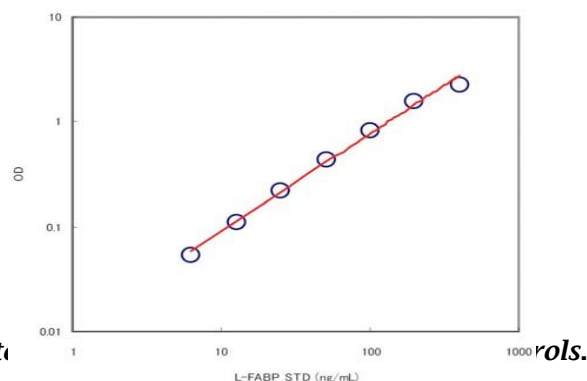
Minimum Value: 6.25 ng/mL  
Maximum Value: 400 ng/mL

## 8. Typical Results and Performance of hL-FABP ELISA

### 8.1 Typical Calibration Results & Graph (results below provided from CMIC)

#### Calibrator Curve Values and Graph of Typical hL-FABP Calibration Results

L-FABP concentration (ng/mL)	Absorbance (492nm)
400	2.614
200	1.492
100	0.742
50	0.384
25	0.207
12.5	0.127
6.25	0.080
0 (blank)	0.034



**NOTE:** This curve is not to be used to calculate

### 8.2 Limit of Detection (results below provided from CMIC)



Measured serial concentrations of Blank (0.0), 1.0, 2.0, 3.0, 4.0, and 5.0 ng/mL L-FABP for eight replicates. Calculated mean and SD for each conc of L-FABP. Limit of Detection set as lowest L-FABP conc - 3xSD not less than mean of 0.0 L-FABP + 3xSD.

$$\text{LOD} = 2.4 \text{ ng/mL L-FABP}$$

### 8.3 Recovery Studies

(results from normal human urines below provided from CMIC)

#### 8.3.1 100 ng/mL of L-FABP added to 3 urine normal subject samples (8 replicates of each urine assayed)

AVERAGE Recovery: 109.2 %  $\pm$  10.2 %  
(Recovery %  $\pm$  2SD)

#### 8.3.2. 25 ng/mL of L-FABP added to 3 urine normal subject samples (8 replicates of each urine assayed)

AVERAGE Recovery: 97.6 %  $\pm$  8.9 %  
(Recovery %  $\pm$  2SD)

(results below from human CKD urines below provided from Univ Penn)

#### 8.3.3 63 ng/mL of L-FABP added to Biocon Proficiency urine Sample 1 (6 replicates of each urine assayed)

AVERAGE Recovery: 105.3 %  $\pm$  12.3 %  
(Recovery %  $\pm$  2SD)

#### 8.3.4 46 ng/mL of L-FABP added to Biocon Proficiency urine Sample 2 (6 replicates of each urine assayed)

AVERAGE Recovery: 79.4 %  $\pm$  3.6 %  
(Recovery %  $\pm$  2SD)

#### 8.3.5 25 ng/mL of L-FABP added to Biocon Proficiency urine Sample 5 (6 replicates of each urine assayed)

AVERAGE Recovery: 115 %  $\pm$  5.2 %  
(Recovery %  $\pm$  2SD)

Average Recovery (5 determinations) = 101.4  $\pm$  16.2 %

### 8.4 Reference Range

(results below provided from CMIC)

Adult (urine) range obtained after log transformation of L-FABP concentrations from 412 urine samples

**Mean:** 1.6 µg L-FABP/g creatinine

**Range:** 0.3 – 8.4 µg L-FABP/g creatinine

## 8.5 Imprecision of Method (results below provided from CMIC)

### 8.5.1 Within-Day Imprecision

Sample	#1	#2	#3
Number (replicates same day)	8	8	8
Mean L-FABP, ng/mL	9.85	52.4	240.9
Standard Deviation, ng/mL	0.67	4.15	8.53
Coefficient of Variation, CV, %	6.8	7.9	3.5

### 8.5.2 Between-Day Imprecision

Sample	#1	#2	#3
Number days assayed	8	8	8
Mean L-FABP, ng/mL	11.02	46.7	227.1
Standard Deviation, ng/mL	1.31	5.27	21.87
Coefficient of Variation, CV, %	11.9	11.3	9.6

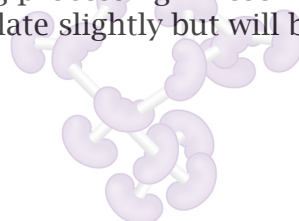
## 9. References

- 9.1 L-FABP Product Insert (Release date: Feb, 2012). CMIC Holdings, Ltd, Tokyo, JAPAN.
- 9.2 Kamijo-Ikemorif A, Sugaya T, Kimura K. Clin Chim Acta 2006;374:1-7.
- 9.3 Pelsers MM. Scand J Clin Lab Invest 2008;68:73-7.
- 9.4 Zaffanello M, Antonucci R, Cussolin L, Cataldi L, Fanos V. J Mater-Fetal Neonat Med 2009;22:62-6.
- 9.5 Yokoyama T, Kamijo-Ikemorif A, Sugaya T, Hoshino S, Kimura K. Am J Path 2009;174:2096-2106.
- 9.6 McMahon BA, Murray PT. Kid Intl 2010;77:657-9.
- 9.7 Ferguson MA, Vaida VS, Waikar SS et al. Kid Intl 2010;77:708-14.
- 9.8 Matsui K, Kamijo-Ikemorif A, Sugaya T, Yasuda T, Kimura K. Am J Path 2011;178:1021-32.
- 9.9 Matsui K, Kamijo-Ikemorif A, Sugaya T, Yasuda T, Kimura K. Circ J 2012;76:213-20.

## 10. Procedural Notes and Comments

- 10.1 Test samples should be diluted with Standard Diluent if required. If the measurement result exceeds 400ng/mL, dilute and measure again.
- 10.2 In case of dividing and using hL-FABP Antibody Coated Microplate for more than one time, put an unused portion of hL-FABP Antibody Coated Microplate in a bag with a desiccant and zip the bag. Then seal the bag tightly with tape and store it at 2 - 8°C until next use.
- 10.3 Use test samples in neutral pH range. The presence of organic solvent may affect the measurement.
- 10.4 During incubations in particular, ensure that plate covers are in place to reduce evaporation.
- 10.5 Use only Wash Agent contained in this kit for washing hL-FABP Antibody Coated Microplate. Insufficient washing may lead to inaccurate concentration measurements.
- 10.6 Substrate will be uniformly dissolved in Substrate Diluent without stirring in approximately 10 min. However, do not form an airtight seal with the container because the substrate solution will develop pressure.
- 10.7 Confirm there is no dirt on the bottom of the plate prior to the measurement of absorbance values. The measurement should be conducted as quickly as possible, although it is confirmed that the values will not change for 2 hours after the addition of Stop Solution.
- 10.8 Do not use a common calibration curve for each day's run. Instead, use the calibration curve developed on each microplate to calculate the results for that microplate because every microplate will encounter slightly different conditions during processing. These minor changes will affect the outcome of each microplate slightly but will be consistent within each microplate.

BIOMARKERS  
CONSORTIUM



*Reviewed by: Theodore E. Mifflin, Ph.D., DABCC*

*Date reviewed: 20 May 2013*



Current Written By:	Original Date Adopted:	Supersedes Document #:
April Horne, BS		New <input checked="" type="checkbox"/> Previous Doc / Version #

Signature: Original in 150 Hamilton Hall	
New: <input checked="" type="checkbox"/>	Date Approved: Division Director: Haifeng M. Wu, M.D.
Revised: <input type="checkbox"/>	

Staff – sign to indicate that you have read and understand procedure as written.		
Name	Date	Signature

## 1. PRINCIPLE

- 1.1. Monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, MCAF and TDCF, is a heparin-binding, 10-14 kDa member of the beta or CC family of chemokines. Human MCP-1 is synthesized as a 99 amino acid (aa) precursor that contains a 23 aa signal sequence coupled to a 76 aa mature region (1-3). The mature region contains a receptor binding and dimerization N-terminus plus a glycosaminoglycan (GAG)-binding C-terminus (4-5). MCP-1 circulates as a monomer and is also suggested to form dimers and/or multimers (6-8). The monomer is considered the predominant form and demonstrates full chemotactic activity (6-7). A wide variety of cells secrete MCP-1, including endothelial cells (EC), monocytes, fibroblasts, vascular smooth muscle cells and endothelial cells (1-3, 9), mast cells, and astrocytes. MCP-1 is best known for its chemotactic activity on monocytes. Several different lines of evidence utilizing *in vivo* animal models suggest that MCP-1 is an important player in inflammatory processes. Blocking MCP-1/IE activity can suppress models of endotoxemia, delayed-type sensitivity reactions, and inflammatory arthritis, while over-expression enhances the recruitment of monocytes and lymphocytes (10-11). The knockout studies also show that MCP-1/IE-deficient mice exhibit suppressed inflammation-related macrophage, monocyte, NK cell, NKT cell, and/or  $\gamma\delta$  T cell infiltration in several different contexts including models of pulmonary infection, stroke, blood vessel injury, renal tubule injury, autoimmune



disease, uveitis, and wound healing (12-13). In clinical studies, elevated MCP-1 levels in humans have been associated with sepsis, Crohn's disease, lupus nephritis, amyotrophic lateral sclerosis, multiple sclerosis, rheumatoid arthritis, acute pancreatitis, and atherosclerosis (14-19).

- 1.2. Principle of the assay: The Human MCP-1 Immunoassay from R&D systems employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for MCP-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any MCP-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for MCP-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of MCP-1 bound in the initial step. The color development is stopped and the intensity of the color is measured.
- 1.3. This immunoassay has been shown to accurately quantitate recombinant human MCP-1. Based on the data from vender, the results obtained using natural human MCP-1 showed linear curves that were parallel to the standard curves obtained using the kit standards. In our own evaluation, both recovery study and immunodepletion study showed that the assay kit can indeed accurately detect the analyte of MCP-1 in urine samples. Detailed analytical validation analysis indicated that the technical characteristics of the assay are acceptable for measurement of human MCP-1 in clinical urine specimens (please refer to the document "analytical validation" for details).

## 2. SPECIMEN COLLECTION:

### 2.1. Specimen type: Urine

2.1.1. **Samples obtained at OSU:** Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge at 2000g for 10min, at 4°C in order to remove particulate matter. Assay immediately or aliquot and store at -80 °C. Avoid repeated freeze-thaw cycles.

2.1.2. **Samples from other sites:** Samples will arrive frozen and should be stored at -80 until thawed once immediately before testing. The information on the number of freeze-thaw cycles prior to the shipment should be recorded.

2.1.3. Minimum sample volume: 250uL

2.1.4. 250 µL of samples will be mixed with equal volume of 1X Calibrator Diluent RD5L which will yield 500 µL of final volume. All the samples will be assayed by duplicate.

## 3. EQUIPMENT: VersaMax Tunable Microplate Reader (Molecular Devices)

## 4. REAGENTS/SUPPLIES:

4.1. **For all kit components, follow expiration date guidelines in kit. DO NOT mix reagents from different kits.**

4.2. **Bring all reagents to ambient temperature before use.**

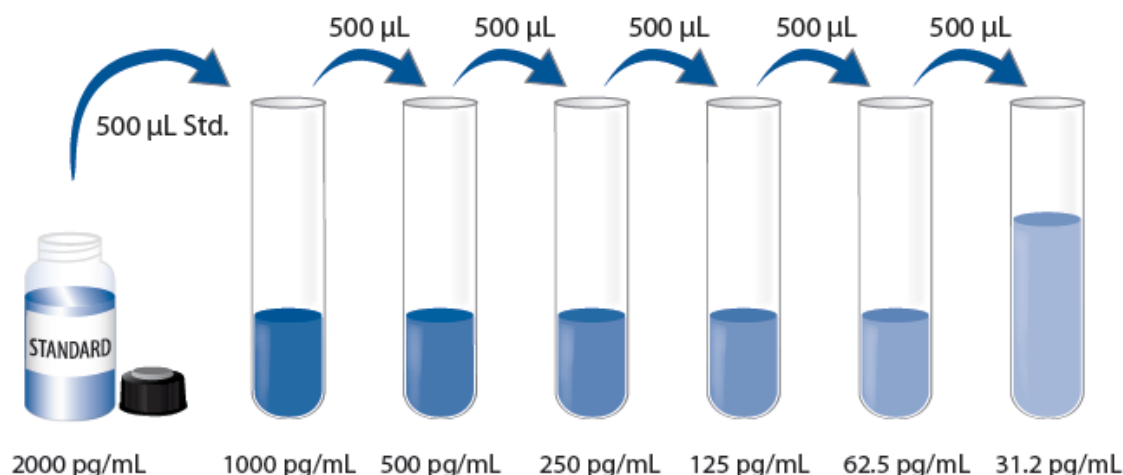
4.3. **Human MCP-1 Immunoassay Quantikine ELISA kit. (R&D Systems #DCP00)**

4.3.1. MCP-1 Microplate

- a. Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8°C.

4.3.2. MCP-1 Standard

- a. Original aliquot and store for up to 1 month at  $\leq -20^{\circ}\text{C}$  in a manual defrost freezer. Avoid repeated freeze-thaw cycles.
- b. Reconstitute the MCP-1 Standard with 5.0 mL of Calibrator Diluent RD5L (1X). This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. **Use polypropylene tubes.**
  - 4.3.2.b.1. Pipette 500 µL of the appropriate Calibrator Diluent into each tube.
  - 4.3.2.b.2. Use the stock solution to produce a dilution series (fig 1 below).
  - 4.3.2.b.3. Mix each tube thoroughly before the next transfer.
  - 4.3.2.b.4. The undiluted standard serves as the high standard (2000 pg/mL). The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL). Diluted standards are made freshly for each run.



**FIGURE 1. Dilutions of MCP-1 Standard**

**4.3.3. MCP-1 Conjugate**

- a. May be stored for up to 1 month at 2-8 °C.

**4.3.4. Calibrator Diluent RD5L concentrate**

- a. May be stored for up to 1 month at 2-8 °C.  
b. Dilute 20 mL of Calibrator Diluent RD5L Concentrate into deionized or distilled water to yield 100 mL of Calibrator Diluent RD5L (1X).

**4.3.5. Wash buffer concentrate**

- a. May be stored for up to 1 month at 2-8 °C.  
b. If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

**4.3.6. Substrate solution:**

- a. Color Reagent A  
4.3.6.a.1. May be stored for up to 1 month at 2-8 °C.  
b. Color Reagent B  
4.3.6.b.1. May be stored for up to 1 month at 2-8 °C.  
c. Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

**4.3.7. Stop solution**

- a. May be stored for up to 1 month at 2-8 °C.

**4.3.8. Plate Sealers (adhesive films)**

**4.4. Items not included in kit:**

- 4.4.1. orbital shaker  
4.4.2. aluminum foil  
4.4.3. Pipettes and pipette tips  
4.4.4. multi-channel pipette  
4.4.5. reagent troughs for multi-channel pipette  
4.4.6. 100 mL and 500mL graduated cylinders  
4.4.7. Deionized, distilled water  
4.4.8. Polypropylene test tubes for dilution of standards and samples  
4.4.9. Control samples (see section 7.)

**5. SPECIAL SAFETY PRECAUTIONS:**

- 5.1. This assay uses human source materials and should be performed in a BSLII laboratory facility.  
5.2. Personal protective equipment:  
5.2.1. gloves





- 5.2.2. gown
- 5.2.3. optional face shield is available
- 5.3. Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.
- 5.4. The stop solution provided with this kit is an acid. Use care when working with acids and wash hands thoroughly after handling.

## 6. CALIBRATION/PROGRAMMING/MAINTENANCE:

### 6.1. Calibration

- 6.1.1. This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human MCP-1 produced at R&D Systems. The NIBSC unclassified MCP-1 preparation 92/794 (recombinant DNA Human Type) was evaluated in this kit.
- 6.1.2. A standard curve is plotted with each run of the assay. Controls with known values are analyzed, along with the standards and patients, to verify an acceptability of the assay performance.
- 6.1.3. the VERSAmax plate reader automatically performs an internal calibration before each reading.

### 6.2. Maintenance procedure

#### 6.2.1. Daily Maintenance

- a. check for spills or excessive dust and wipe outside surfaces of reader as needed.

#### 6.2.2. Weekly Maintenance

- a. Clean the outside surfaces of the VERSAmax plate reader using a paper towel that has been dampened with water. Do not use abrasive cleaners. If required, clean the surfaces using a mild dish soap solution diluted with water, or glass cleaner, and then wipe with a clean damp cloth to remove any residue. Do not spray cleaner directly onto the instrument.
- b. If needed, clean the microplate drawer with a paper towel that has been dampened with water.

#### 6.2.3. Quarterly Maintenance – Cleaning the fan filter:

- a. Turn power to the instrument OFF and then remove the power cord and cables from the back of the instrument.
- b. Make sure no plate is in the instrument. Turn the instrument over so that it rests flat on the bench.
- c. Pop the black fan cover off and remove the filter
- d. Clean the filter by blowing clean, canned air through it or by rinsing it – first with water and then with alcohol – and allowing it to dry completely.
- e. Place the clean, dry filter over the fan and replace the black cover.
- f. Turn the instrument back over. Reconnect the power cord and cables to the instrument.

#### 6.2.4. As Needed

- a. Fuse replacement – If the instrument does not seem to be getting power after switching it on, first check to see whether the power cord is securely plugged in to a functioning power outlet and to the receptacle at the rear of the VERSAmax. If power failed while the VERSAmax was already on, check that the power cord is not loose or disconnected, and that power to the outlet is functioning properly. If these checks fail to remedy the loss of power, replace the fuses. see pages 4.5 through 4.7 of VERSAmax plate reader instruction manual for further instructions and ordering information.
- b. Spills – should fluids spill in the drawer area (while the drawer is out), they will be directed to a tray at the bottom of the instrument, from which they will exit to the bench or counter beneath the instrument. Wipe up any spills immediately. Clean only the exterior of the unit and the microplate drawer if necessary. Never clean the inside of the instrument. Do not allow excess water or other fluids to drip inside the instrument.

## 7. QUALITY CONTROL:

- 7.1. Control materials are selected donor materials that have been tested multiple times to establish an acceptable range.
  - 7.1.1. Current acceptable range for normal control (NOR1): 207-288 pg/mL
  - 7.1.2. Current acceptable range for abnormal control (LUP1): 1186-1921 pg/mL
  - 7.1.3. Control samples are immediately processed upon collection: Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge at 2000g for 10min, at 4°C in



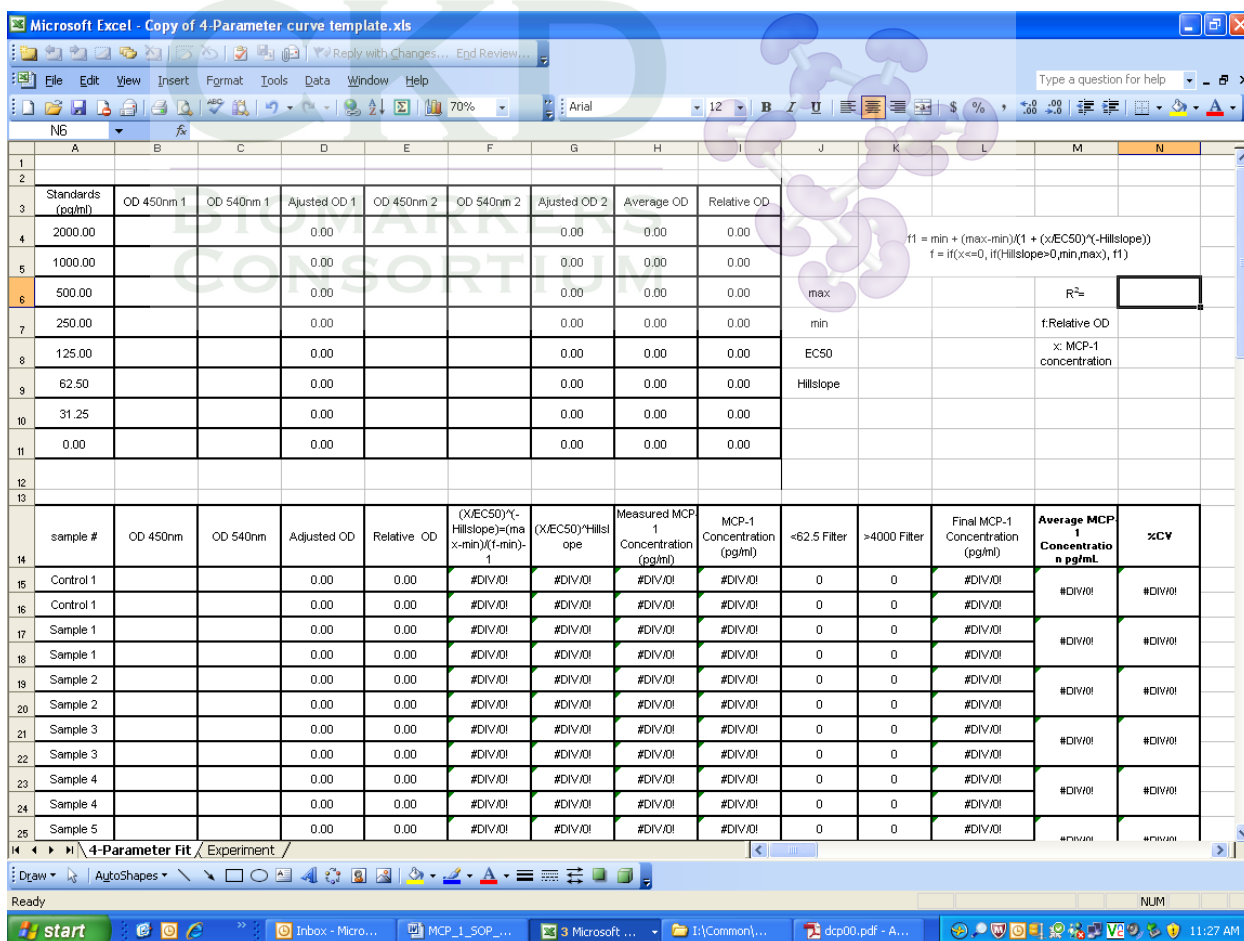
order to remove particulate matter. Small aliquots are stored in 0.5mL microcentrifuge tubes and store at -80 °C. thawed only once for assays.

- 7.2. Two levels of control, a normal and an abnormal control, are included in duplicate with each run.
- 7.3. For a run to be considered valid, both levels of control must fall into the pre-established posted range.
  - 7.3.1. If both replicates of each level are within the established  $\pm 2SD$  range, AND their CV's are  $\leq 15\%$ , then the QC is acceptable.
  - 7.3.2. If one replicate of a control value is outside the established  $\pm 2SD$  range, the Westgard 2 rules will be applied to determine the validity of the run.
    - a. ONE replicate of any two controls MAY be outside the established  $\pm 2SD$  range, CV of the replicates must still be  $\leq 15\%$ .
    - b. If one or both replicate values are outside of the  $\pm 3SD$  range, the run must be rejected and repeated.
    - c. If both replicate values are out of the established  $\pm 2SD$  range, the run must be rejected and repeated.
- 7.4. In case of a QC failure, complete the "Documentation of QC failure" form and submit to laboratory director.
- 7.5. Control values will be recorded in a log and reviewed periodically to monitor for statistical drift and shifts.
- 7.6. New lots of controls will be tested in parallel with existing lots and an acceptable range determined before placing the new lot into service and discontinuing the current lot.

## 8. TEST PROCEDURE:

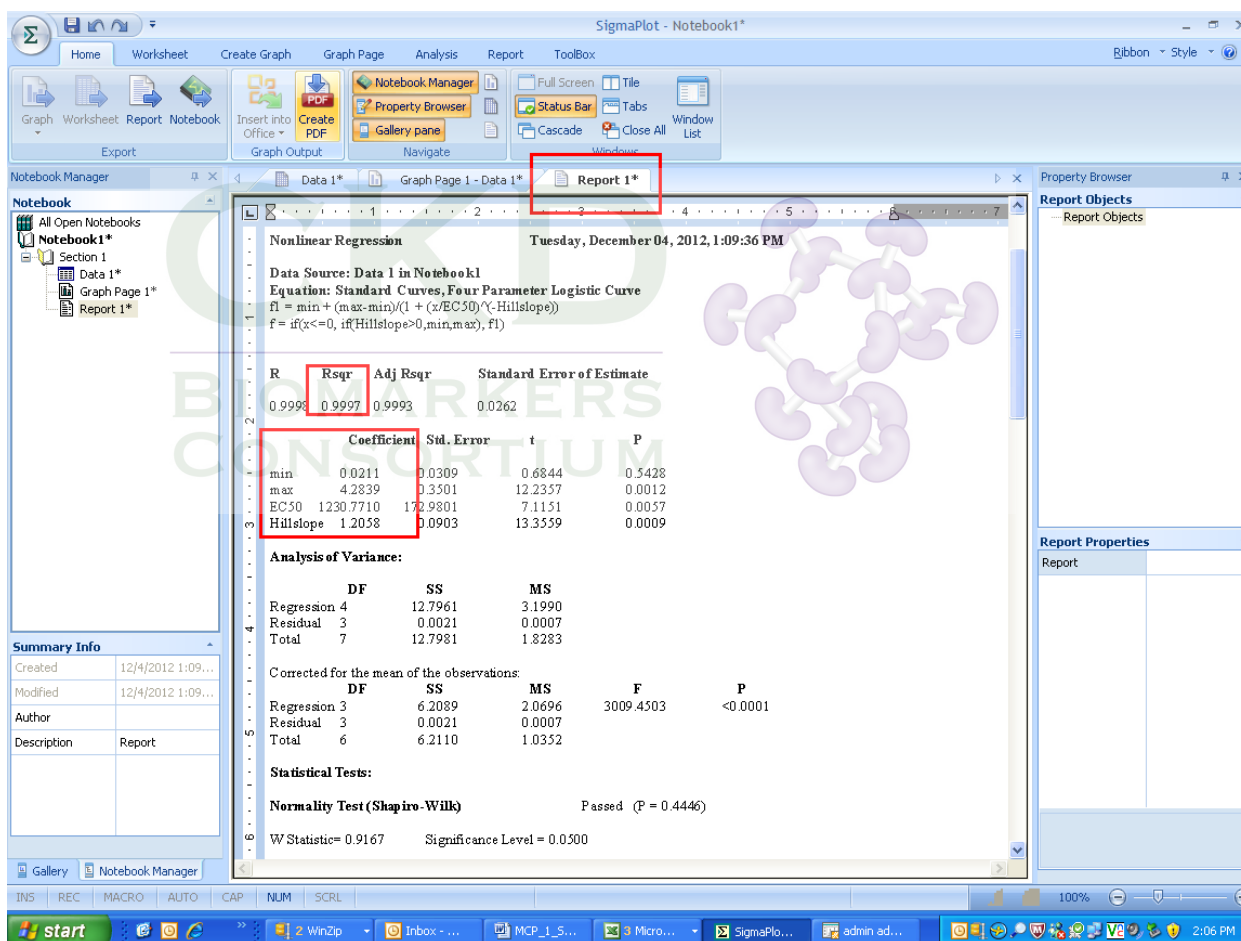
- 8.1. **Bring all reagents and samples to room temperature before use. All standards, samples, and controls will be assayed in duplicate and plotted on a plate template.**
  - 8.1.1. Sheet 2 of the Excel workbook "Blank Template MCP-1 Calculations" provides a space to map the location of each standard, control, and sample on the actual plate.
  - 8.1.2. Patient samples will be labeled on the template with SAMPLE ID.
- 8.2. Prepare all reagents, working standards, and samples as directed in the previous sections.
- 8.3. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
- 8.4. Add 200  $\mu$ L of Standard, sample\* (see 9.4), or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
- 8.5. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400  $\mu$ L) using the pipette, and invert the plate over the sink to expel the contents of the wells. Blot dry onto paper towels to avoid cross-contamination of wells. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
- 8.6. Add 200  $\mu$ L of MCP-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
- 8.7. Repeat the aspiration/wash as in step 8.5.
- 8.8. Add 200  $\mu$ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light by completely covering the plate with aluminum foil.
- 8.9. Add 50  $\mu$ L of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, mix the Stop Solution in the well by pipetting up and down until the color changes to yellow.
- 8.10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. Set wavelength correction to 540 nm.
- 8.11. The following instructions in steps 8.11.1-8.11.7 are for the VersaMax plate reader. For other plate readers, please see section 8.12.
  - 8.11.1. Turn on the VersaMax plate reader and allow it to warm up.
  - 8.11.2. Once the SOFTmax software is running, click the small picture of the plate reader in the upper left hand corner to open the "Preferences" window. Select "COM2" from the dropdown menu for Serial Port.
  - 8.11.3. When the computer connects to the plate reader, the display on the reader will say "Remote Control".

- 8.11.4. From the “Assay” tab, select “Endpt ELISA” and then “HRP and TMB”. A plate diagram will show up.
- 8.11.5. Click the “Setup” button to check the parameters. They should be as follows, (fig 4) then click OK.
  - a. Number of wavelengths: 2
  - b. Lm1: 450
  - c. Lm2: 540
  - d. Automix & Blanking
    - 8.11.5.d.1. Before: 10 Secs
    - 8.11.5.d.2. Pre-Read Plate: Off
  - e. AutoCalibrate: On
  - f. Strips: select applicable strips to be read
- 8.11.6. Click the “Read” button at the top left corner of the screen.
- 8.11.7. When the plate is read, values will appear for each well.
- 8.12. For plate readers other than the VersaMax, please utilize the following guidelines:
  - 8.12.1. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
- 8.13. Export the results into .txt format.
- 8.14. Open Excel and open the spreadsheet “4 Parameter curve template” (see image below)





- 8.15. Open the .txt file in Excel. Copy and paste the data into the correct columns of the template spreadsheet. Control 1 should be in the first two rows of the template before the patient samples, and Control 2 should be in the last 2 rows, after the patient samples. This serves as a check to ensure that the formulas used to calculate results are not accidentally altered. Use “save as” to save your days data in a new spreadsheet and not overwrite the blank template. Relative OD will be calculated by the template.
- 8.16. Open SigmaPlot v12.2 and select “create new notebook”
- 8.17. Copy the standards values from column A of the Excel spreadsheet to column 1 of the new SigmaPlot notebook.
- 8.18. Copy the relative OD of the standards from column I of the Excel spreadsheet to column 2 of the new SigmaPlot notebook.
- 8.19. Select the “create graph” tab. Create a scatter plot with no trendline using column 1 as the X axis and column 2 as the Y axis.
- 8.20. right click on any spot in the graph and select “curve fit”. Under the “standard curves” equation category, select “4 Parameter logistic curve”.
- 8.21. You will now have a “Report” tab available. (see image below) Click on it and copy the values for Rsqr, min, max, EC50. and hillslope to the Excel template.



- 8.22. The template will perform the calculations using the 4 parameter curve and generate a result in the “Final MCP-1 concentration” column.

## 9. CALCULATIONS:

- 9.1. Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. This is done on the excel spreadsheet automatically.



- 9.2. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the MCP-1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure is performed by the excel template.
- 9.3. Because urine samples have been diluted prior to the assay, the measured concentrations must be multiplied by the dilution factor. (This is done automatically by the template, dilution factors are entered in by the user.)
- 9.4. \*samples require a 2-fold dilution as directed in the Sample Preparation section. If results do not fall within the range of the standard curve, further dilutions must be run. Dilute samples as needed and repeat assay procedure.

#### 10. REPORTING RESULTS/DATA TRANSFER:

- 10.1. Reference intervals: The reference range, obtained from 40 apparently healthy local donors, is <62.5~937 pg/mL (mean+2\*SD). The study was performed under standard operation procedure.
- 10.2. Analytical Measurement Range: 31.2-2000 pg/ml
- 10.3. Clinically Reportable Range: 62.5-64000 pg/ml
- 10.4. MCP-1 final values will be reported as an average of two replicates with the corresponding CV. Values will be reported in pg/ml and rounded to one decimal point.
- 10.5. Results from valid runs will be copied from the assay template Excel sheet into a master Excel reporting workbook in sequential order. Patient data will be included on sheet 1, and control/proficiency samples will be included on sheet 2. This master workbook will be transferred securely to the coordinating site and contain the following information:
  - 10.5.1. Sample ID
  - 10.5.2. Date of the assay
  - 10.5.3. Platform and instrument used for the assay, including serial number
  - 10.5.4. reagent lot number (kit)
  - 10.5.5. Concentration of MCP-1 (pg/mL) for patient samples, controls, and proficiency samples as needed
  - 10.5.6. %CV between replicates for patient samples, controls, and proficiency samples as needed

#### 11. OTHER TECHNICAL CHARACTERISTICS:

- 11.1. Sample requirement studies indicated that common conditions for sample storage and processing usually do not cause >10% variation in detection of the analyte. However, investigators should avoid extreme conditions such as storage of urine at room temperature for > 4 hours, or at refrigerated conditions for more than 24 hours, or >3 cycles of freeze-thaw.
- 11.2. Analytical sensitivity of the assay
  - 11.2.1. LoB = 11.2 pg/ml
  - 11.2.2. LoD = 19.9 pg/ml
  - 11.2.3. LOQ = 28.8 pg/mL
- 11.3. Test precision
  - 11.3.1. Intra-assay: CV of 6.0%
  - 11.3.2. Inter-assay: CV of 7.9%
- 11.4. Test accuracy
  - 11.4.1. The kit manufacturer reported an average recovery of 92% (range 85-100%) after spiking samples at 3 levels.
  - 11.4.2. Please refer to the document "Analytical validation of MCP-1 assay" for full information about the technical performances of the assay. Briefly, we spiked urine samples with reference standard of MCP-1 at various concentrations and then performed a recovery study. Observed MCP-1 values correspond linearly to the expected MCP-1 values in a dose-responsive manner. Additionally, we performed an immuno-depletion study in which MCP-1 analyte in a known positive sample was depleted by specific MCP-1 antibody and then we then compared the MCP-1 readouts with and without immunodepletion. Addition of the antibody against MCP-1 to the urine sample selectively reduces the readout of MCP-1 signal by up to 92.7%. These studies demonstrate that the assay measures MCP-1 in human urine samples accurately.
- 11.5. Linearity of Dilution





- 11.5.1. The kit manufacturer assessed linearity by spiking samples with high concentrations of MCP-1 and diluting with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay. Their average % of expected was 92%.
- 11.5.2. Our laboratory performed our own linearity study in order to validate the manufacturer's claims. An abnormal sample was diluted 1:2, 1:4, 1:8 and 1:16 and tested, producing an average % recovery of 101%.

## 12. REFERENCES:

### 12.1. Package Insert: R&D Systems Quantikine ELISA: Human CCL2/MCP-1 Immunoassay: Catalog Number DCP00

### 12.2. References

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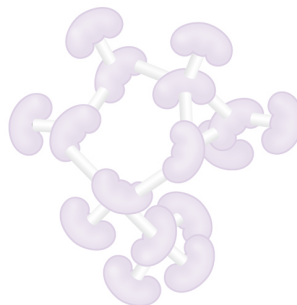




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### 13. RELATED DOCUMENTS

- 13.1. Excel Spreadsheet: "Blank Template MCP-1 Calculations"
- 13.2. **Package Insert: R&D Systems Quantikine ELISA: Human CCL2/MCP-1 Immunoassay: Catalog Number DCP00**



**BWH: Center for Kidney Diseases  
HIM 550, Harvard Institutes of Medicine  
77 Louis Pasteur Avenue, Boston, MA 02115  
Phone # 617-525-5988**

**Title: human urinary N-Acetyl- $\beta$ -D-glucosaminidase (NAG) assay**

**Reference: human urine NAG/sop/003**

**Version Number: 1.1**

**Date: December 19, 2012**

Author	Venkata Sabbiseti	Reviewed by	Kazumi Ito	Approved by	Joseph V. Bonventre
Title	Instructor in Medicine	Title	Research Fellow	Title	Chief, Renal division
Signature		Signature		Signature	
Date		Date		Date	

Document History			
Date	Comment	Reviewer	Signature
04/20/2010	New SOP	Venkata Sabbiseti	
12/19/2012	Updated SOP	Venkata Sabbiseti	

Reference: human urine NAG/SOP/003

Version: 1.1

Author: Venkata S. Sabbiseti

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## 1. Purpose

This Standard Operating Procedure (SOP) describes the process for the quantitative assessment of N-acetyl- $\beta$ -D-glucosaminidase (NAG) in human urine specimens. The concentration of NAG in urine will be determined by enzymatic assay using the commercially available Kit from Roche. In brief, 5  $\mu$ l of urine specimen will be incubated with substrate reagent that contains 3-cresolsulfonphthaleinyl-*N*-acetyl- $\beta$ -D-glucosaminide. NAG present in the urine catalyzes the hydrolysis of sodium salt of 3-cresolsulfonphthaleinyl-*N*-acetyl- $\beta$ -D-glucosaminide and produces 3-cresol purple (3-cresolsulfonphthalein, sodium salt), which can be measured at 580 nm using a spectrophotometer. The absorbance is directly proportional to the amount of NAG present in the urine. NAG levels will be interpreted using a 7-point standard curve using linear regression analysis.

Reference: human urine NAG/SOP/003

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## 2. Introduction

NAG is a lysosomal enzyme that is present abundantly in the brush border of renal tubular epithelia. NAG is the most active glycosidase found in proximal tubular cell lysosomes, and an increase in urinary excretion is a reasonably sensitive marker of tubular injury because its size precludes filtration by the glomerulus. The urinary levels of NAG have been shown to be increased in various forms of renal injury.

## 3. Sample Collection, Handling & Storage

Frozen urines (Spot, timed or 24 hr collection) obtained from investigators will be immediately stored at – 80 °C. NAG is stable for up to at least 5 freeze thaw cycles. Urine specimens that were collected at BWH will be placed on ice immediately, and centrifuged at 3000 RPM using RTH-250 rotor on Sorvall Bench top Centrifuge for five minutes to pellet the particulate matter. Supernatant will be aliquoted into 1.8 ml screw cap centrifuge tubes (generally a maximum volume of 1 ml /tube) and stored in an -80 °C freezer ideally within 1 hour of urine collection but overnight preservation on ice does not appear to alter the level of urinary NAG. All the personnel handling the specimens will have undergone Environmental Health & Safety training at Brigham & Women's Hospital. Protective gear including lab coats and gloves must be worn while working in the laboratory. The addition of preservatives to urine specimens is investigators choice. In our studies, we didn't see any significant difference in NAG levels in samples that were stored at -80 °C with and without preservatives.

## 4. Procedure

### 4.1 Location

Harvard Institutes of Medicine, Room 550

Renal Division/ Department of Medicine/ Brigham and Women's Hospital

### 4.2 Required equipment / reagents

- 96 well clear bottom micro plate (Fisher Sci, catalog # 125655010)
- Spectrophotometer (Molecular Devices SpectraMax Plus 384)
- Refrigerated Centrifuge (Sorvall Benchtop swing bucket centrifuge, Rotor RTH-250)
- Vortexer
- Electronic Multichannel pipettes
  - E4 XLS multichannel pipette (dispenses 2 µl-20 µl)(Rainin, catalog # E8-20XLS)
  - EDO 3-Pls multichannel pipette (dispenses 100 µl-1200 µl) (Rainin, catalog # E8-1200)
- Single Channel Manual Pipettes
  - Rainin Classic 0.5 µl-10 µl (catalog # PR-10)
  - Rainin Classic 10 µl-100 µl (catalog # PR-100)
  - Rainin Classic 100 µl-1000 µl (catalog # PR-1000)
- 50 ml reagent reservoir (Fisher Scientific)

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- Orbital Plate shaker (IKA & Denville Scientific, Max speed 11000 rpm)
- 30°C incubator (Thermo Scientific)
- N-Acetyl-β-D-glucosaminidase Kit (Roche Applied Science, Catalog # 10 875 406 001).
- N-Acetyl-β-D-glucosaminidase Standard (Roche Applied Science, Catalog # 10982962001)
- Two control urine samples that contain high and low levels of NAG (Obtained from kidney disease patients, aliquoted and stored at – 80 °C)
- Clear polyester adhesive sealing tape (Thermo Scientific, catalog # 236366 )
- MilliQ water (Type 1, reagent grade water)

### 4.3 Buffer preparation

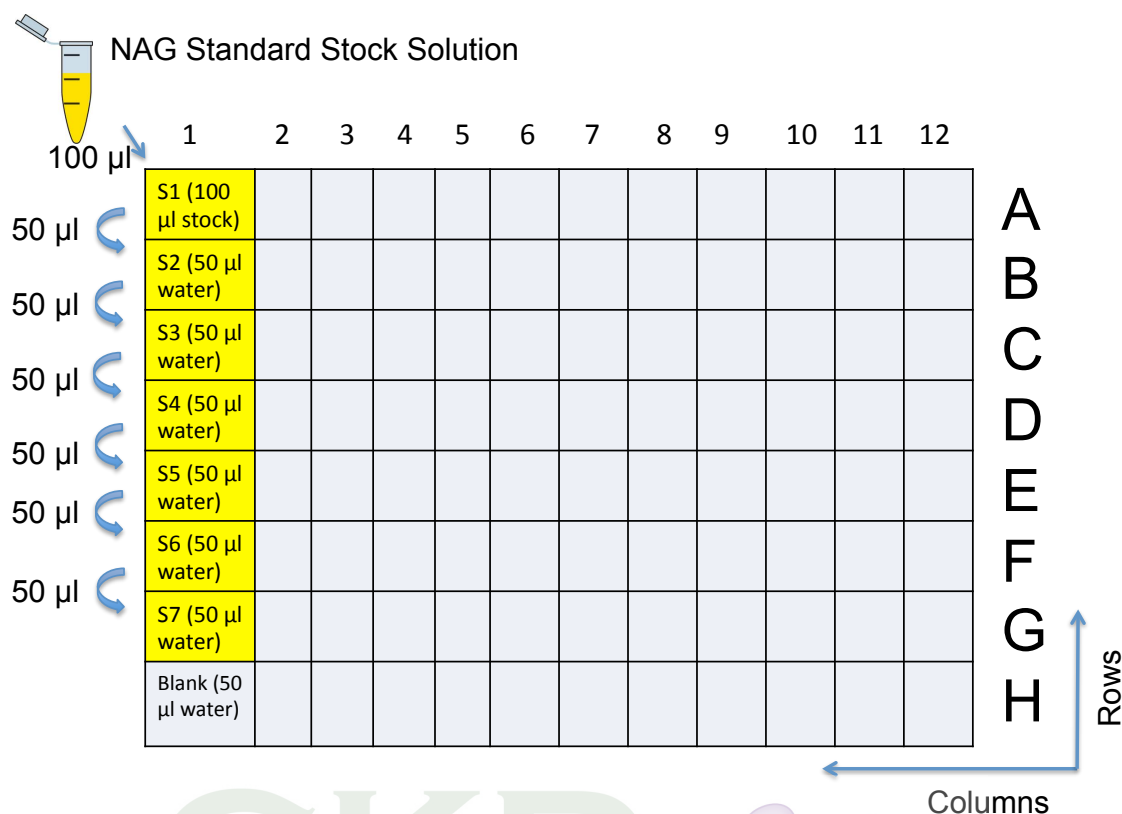
NAG assay buffers	
Buffer	Components
Buffer 1 (buffer solution)	Dissolve material in bottle 1 with 55 ml MilliQ water
Buffer 2 (Substrate Solution)	Dissolve the contents of bottle 2 with 55 ml of buffer 1. Stored at 4°C, protected from light. Stable for a month.
Buffer 3 (Stop reagent)	Dissolve the contents of bottle 3 with 110 ml MilliQ water. Stored at 4 °C, protected from light. Stable for a month.

### 4.4 Experimental Procedure

**4.4.1 Sample thawing:** Sample tubes will be arranged in open tube racks with an empty space between each tube for better air circulation. Place these racks in 30°C incubator until all samples are thawed (The tubes will be still cold after the samples are thawed). Vortex the tubes for 5 sec, centrifuge at 4°C 3000 RPM using RTH-250 rotor on Sorvall Bench top Centrifuge, and keep on ice and proceed for the analysis.

**4.4.2 NAG Standard preparation** (For complete information please see the product insert enclosed at the end)

- Dissolve the contents of one bottle with 3 ml of MilliQ water, close bottle and wait for 5 min. Dissolve contents completely by gentle swirling.
- The standard solution is stable for at least 1 month stored at 2-4 °C, protected from light.
- The stock concentration of NAG varies with each lot. **Please make sure to check the concentration of NAG on the bottle.** Record the lot number of the standards for reference. (Usually it's in the range of 24-26 mU/mL).
- Prepare fresh standard solutions using a serial dilution approach to generate 7 serially diluted standards using Milli Q water using the following approach. Dispense 100 µl of stock standard solution (usually in 24-26 mU/ml range) to a 96 well micro plate (Standards plate) into well A1. Add 50 µl of water in wells from B1-H1. Take 50 µl of standard solution from A1 and mix with 50 µl of water in well B1 by pipetting up and down for four times. Take 50 µl from well B1 and add it to C1 by mixing up and down four times with a pipette. Repeat the procedure till you reach G1. Leave H1 (water) as blank.



#### 4.4.3 Procedure

- Add 100 µl of substrate solution (buffer 2) to each well on a new 96 well assay microplate and seal it with a sealing tape.
- Incubate at 37°C for 5 min to activate the substrate
- Remove the sealing tape and dispense 5 µl of urine samples to wells (please see the plate format below) sequentially using the same tip in triplicate using repeat dispensing option using a electronic multichannel pipette (E8 XLS, Rainin) and discard the tips. (Note: The settings on the pipette will be Volume: 15.00 µl, Aliquot volume 5.00 µl & Aliquots: 3).
- Dispense 5 µl of Standards & Blank to wells (please see the plate format below) sequentially using the same tip in triplicate using repeat dispensing option using a electronic multichannel pipette (E8 XLS, Rainin) and discard the tips. (Note: The settings on the pipette will be Volume: 15.00 µl, Aliquot volume 5.00 µl & Aliquots: 3).
- Dispense 2 control urine samples in triplicate using repeat dispensing option using a single channel electronic pipette and discard the tip. (Note: The settings on the pipette will be Volume: 15.00 µl, Aliquot volume 5.00 µl & Aliquots: 3).
- Include 3 proficiency samples on the plate in triplicate (proficiency samples will be run every week).
- Place the sealing tape over the plate and press the tape firmly to make sure it adhered properly. Mix on plate shaker for 1 min at 1000 rpm to mix reagents and incubate the plate at 37°C for 45 min (Note: Check the seal to make sure that it is adhered completely. Improper adhesion leads to evaporation of the reagents and wrong results).
- Add 200 µl of stop reagent (buffer 3) to each well using a multi-channel pipette.

Reference: human urine NAG/SOP/003

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- Seal the plate again, mix on plate shaker for 1 min at 1000 rpm and allow to stand for 10 min.
- Measure the absorbance of each well on the plate at 580 nm using a Molecular Devices Spectrophotometer plate reader. Place the 96 well plate in the Spectrophotometer and press “Read” icon in SoftMax Pro software.

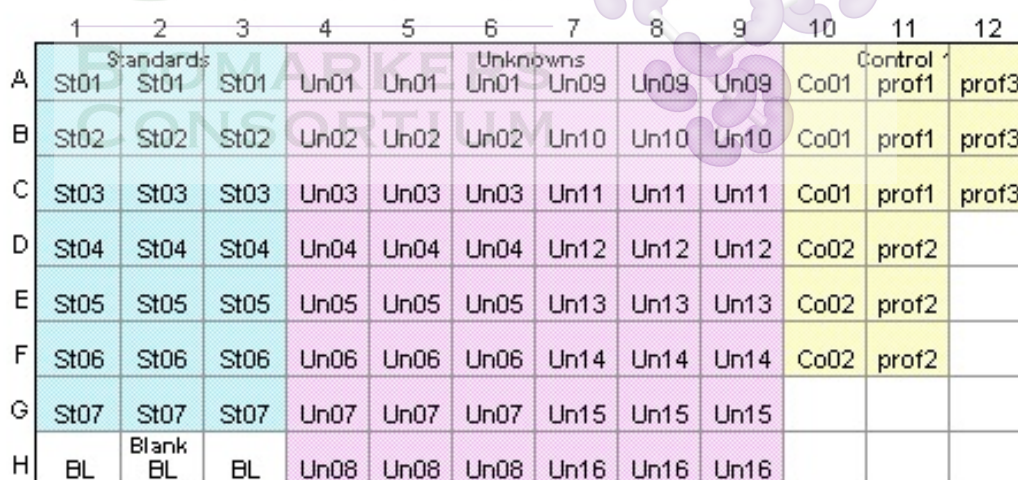
## 5. Equipment

- Spectrophotometer (Molecular Devices, Molecular Devices SpectraMax Plus 384)
- Software: Softmax Pro 5.4 (Integrated with Spectrophotometer Software)

### 5.1 Initiation of SoftMax Pro software

- The SoftMax pro software is started by clicking the SoftMax pro icon on the screen of the computer.
- Once the software is open, click on the protocols icons and select NAG folder.
- Click on the stored NAG plate template reflecting sample format as shown below.

### 5.2 Plate Lay out



	1	2	3	4	5	6	7	8	9	10	11	12
A	Standards St01	Standards St01	Standards St01	Un01	Un01	Un01	Un09	Un09	Un09	Co01	Control prof1	prof3
B	St02	St02	St02	Un02	Un02	Un02	Un10	Un10	Un10	Co01	prof1	prof3
C	St03	St03	St03	Un03	Un03	Un03	Un11	Un11	Un11	Co01	prof1	prof3
D	St04	St04	St04	Un04	Un04	Un04	Un12	Un12	Un12	Co02	prof2	
E	St05	St05	St05	Un05	Un05	Un05	Un13	Un13	Un13	Co02	prof2	
F	St06	St06	St06	Un06	Un06	Un06	Un14	Un14	Un14	Co02	prof2	
G	St07	St07	St07	Un07	Un07	Un07	Un15	Un15	Un15			
H	BL	Blank BL	BL	Un08	Un08	Un08	Un16	Un16	Un16			

St01-St-07: Standards

Un01-Un16: Unknown Samples

Co01-Co02: Urine Controls

Prof1-Prof 3: Proficiency Samples

Reference: human urine NAG/SOP/003

Version: 1.1

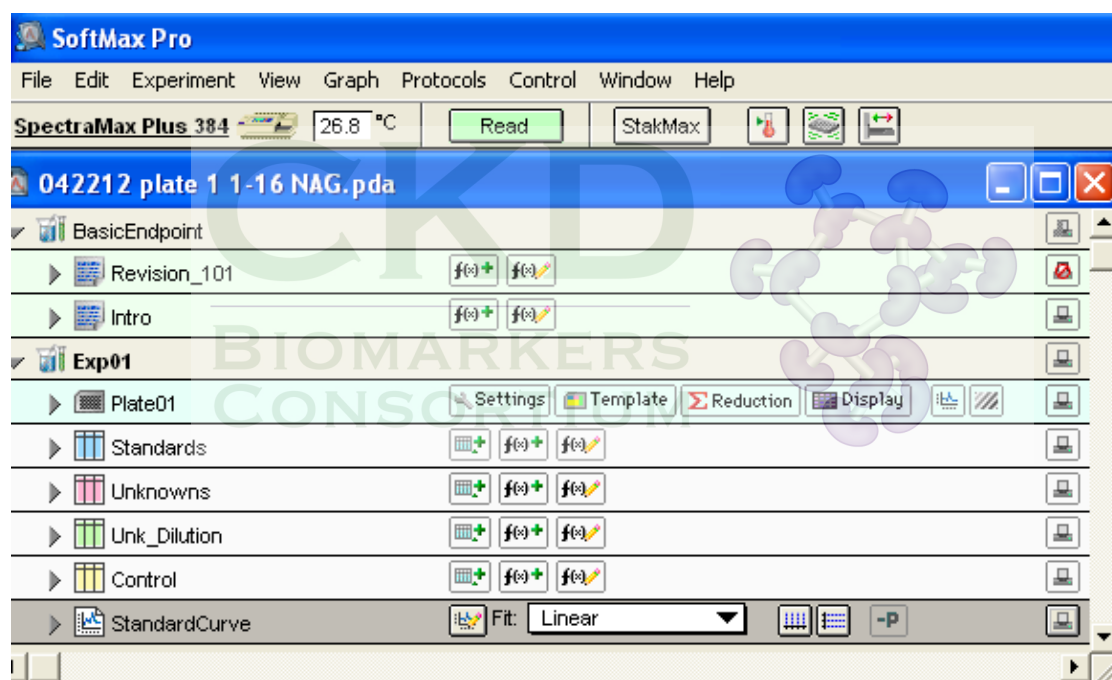
Author: Venkata S. Sabbiseti

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### 5.3 Typical Standard range:

Standards	NAG mU/mL
St01	26.4
St02	13.2
St03	6.60
St04	3.30
St05	1.65
St06	0.825
St07	0.415

## 6. Data Generation & Processing



### 6.1 Data Output

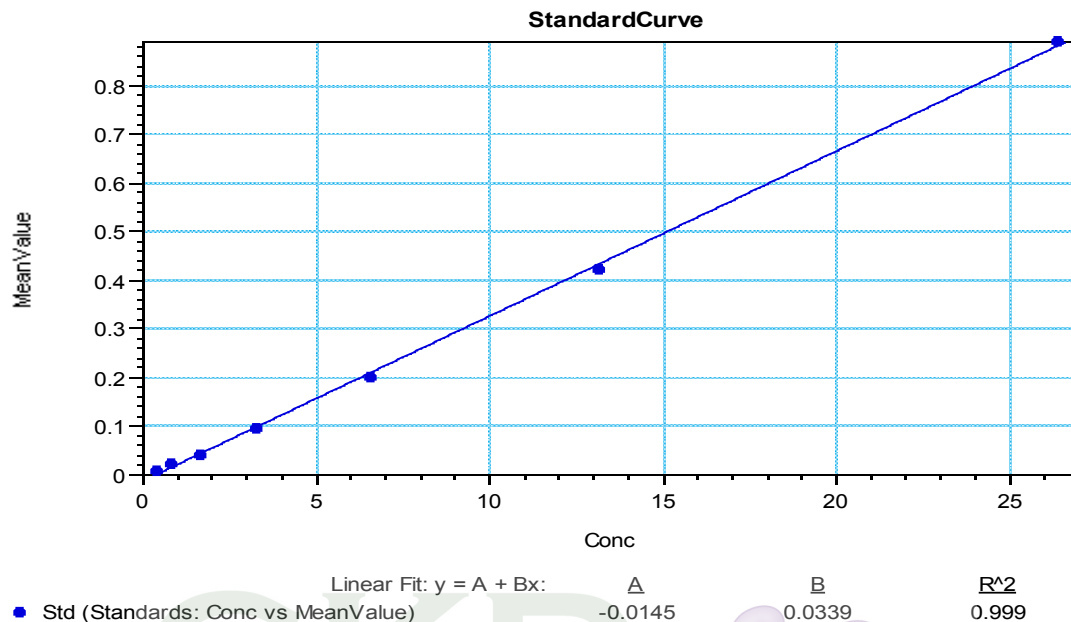
- As indicated above, the 96 well plate is placed in a Spectrophotometer and absorbance read by pressing “Read” icon in SoftMax Pro software.
- Data will be automatically generated by SoftMax Pro 5.4 software that is integrated into the instrument operating software, once the machine is done with the reading (shown below).
- The SoftMax Pro software uses serially diluted standards to generate a standard curve. The software has user-selected options to choose the type of regression analysis. We use linear regression analysis to quantitate the concentrations of unknown samples.

Reference: human urine NAG/SOP/003

Version: 1.1

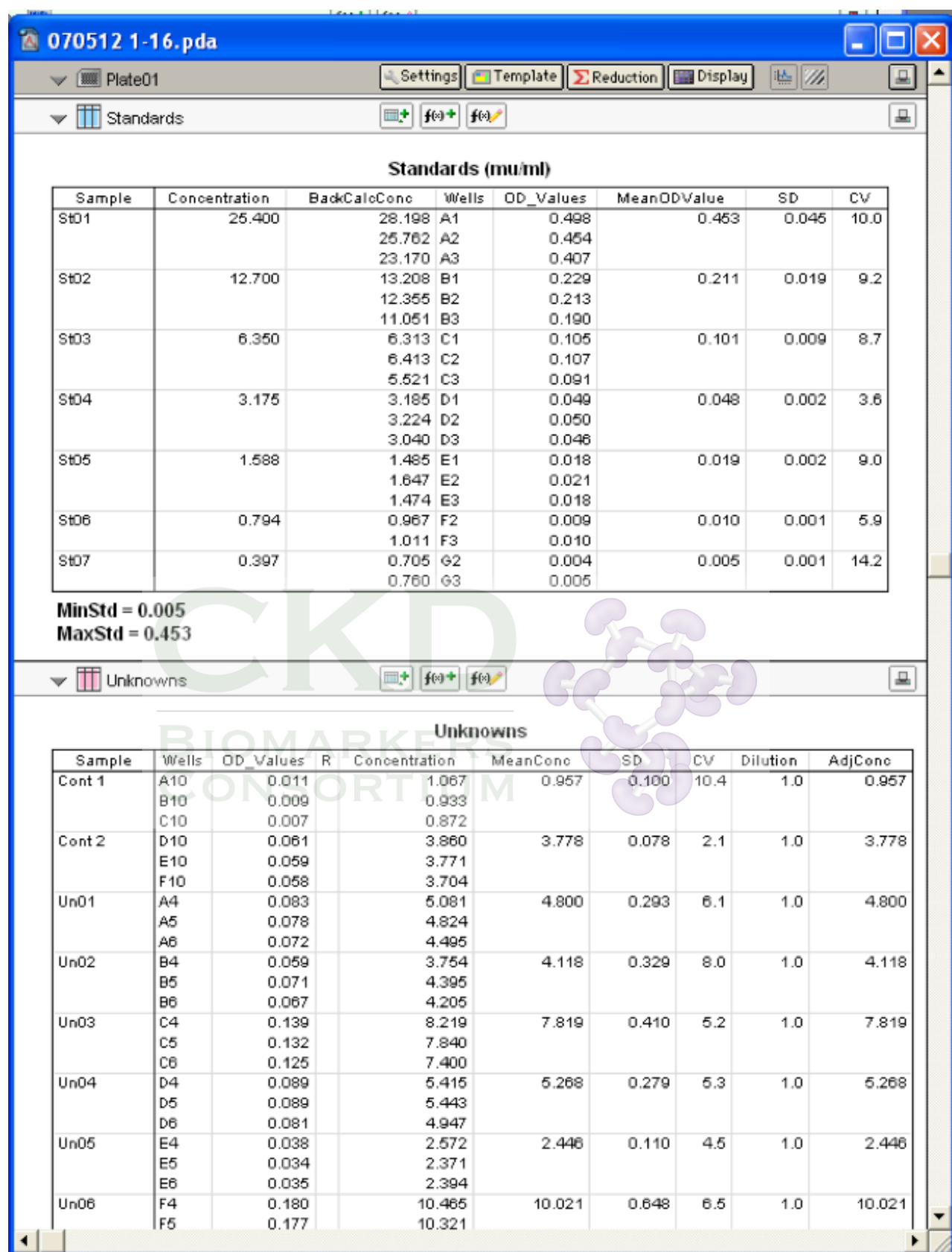
Author: Venkata S. Sabbiseti

- For each analyte standard, a concentration is back-calculated by plotting the absorbance of the standard on the standard curve. (Below is the typical standard curve)



## 6.2 Data Processing and Data Calculations

- The SoftMax automatically generates the values of unknowns based on the standard curve. The data generated includes the absorbance, predicted concentration of unknowns, standard error, % CV, and errors in the run of all samples (below is the typical data output page).
- Data will be exported from Softmax pro to Microsoft Excel using the export file option. The program will export information from the run including Sample ID, information of the wells, absorbance of each replicate, concentration of each replicate, mean concentration of the sample, standard deviation, and % CV. Two control urine specimens are evaluated on every plate. Three proficiency samples will be run every week.
- In addition to sample ID (Biocon ID), each tube will be numerically labeled for cross verification in the order we aliquot the samples on the plate. Once the data are transferred and compiled, we will again cross check the numerical number on the tube with the barcode scan.
- Another member in the facility will perform a secondary check to further validate the sample order.



Reference: human urine NAG/SOP/003

Version: 1.1

Author: Venkata S. Sabbiseti

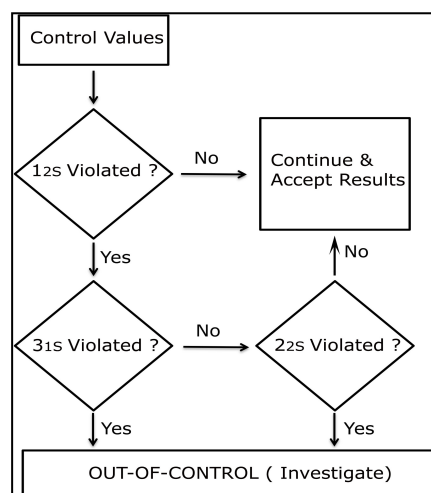
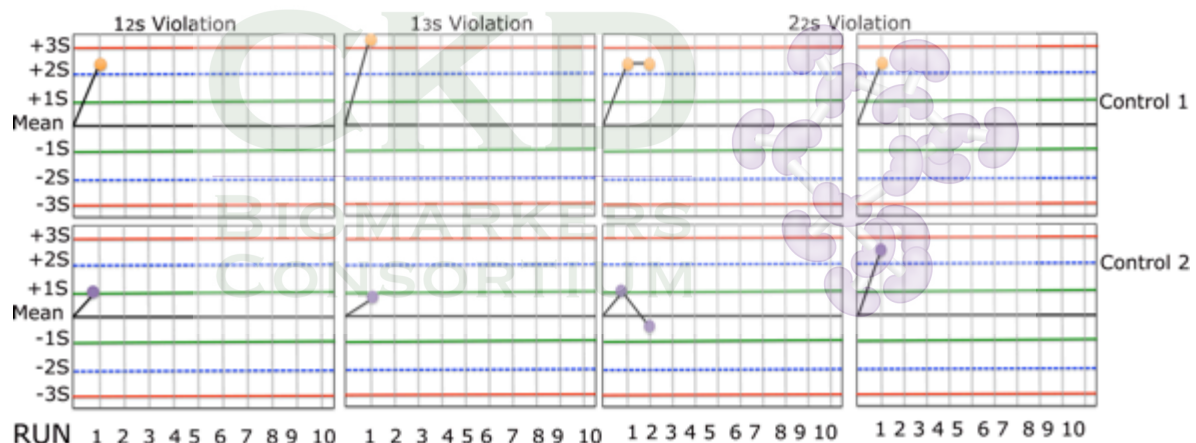
## 7 Quality Control

### 7.1. Quality control of the Assay.

- We will validate that NAG levels obtained using the assay on control specimens are
- within mean  $\pm 2$  SD limit established from the Validation Protocol.
- If both samples are within mean  $\pm 2$  SD limit, we will accept the data. If one of the two
- control values is outside mean  $\pm 2$  SD, we will employ the Westgard 2 rules to determine
- if the analysis run results can be accepted.
- If neither of the assayed control results is within mean  $\pm 2$  SD limits, then we will follow
- the following Westgard three-quality control rule.

RULE	QUALITY CONTROL RULE EXPLANATION
1 <sub>2</sub> S	One value of any of the two controls used in the assay is outside of the mean+2 SD or mean-2 SD of that control
1 <sub>3</sub> S	One value of any of the two controls used in the assay is outside of the mean+3 SD or mean-3 SD of that control
2 <sub>2</sub> S	Both controls used in the assay are outside of their mean $\pm$ SD on the same assay or two consecutive values of one control is outside of mean $\pm$ SD

Levey-Jenning's plots of controls:



Reference: human urine NAG/SOP/003

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## 7.2 Quality Control for each sample

- Sample measurements will be repeated if the %CV of the sample is > 15%
- Sample measurements will be repeated if the absorbance of the sample is below 0.020 to make sure that the low absorbance values are not due to pipetting error.

## 7.3 Quality Control troubleshooting procedure

If the controls fail the above quality control procedures, then we will employ the following procedure to resolve the issue:

- Rerun Control
- If the quality control values pass the above described westgard rules and proceed with unknown testing
- If the quality control failed again, make up fresh reagents and rerun controls and document the problem
- If the quality control indicates that the westgard rules are passed, proceed with testing
- If QC fails again, recalibrate the assay (use new standard lot)
- If after calibration the QC determinations have passed westgard criteria, proceed with testing and document correction
- If Westgard criteria were not met after preparation of fresh reagents, do not proceed with the analysis and contact the Laboratory Director.

## 7.4 Data storage and reporting

- After the analysis has passed the quality control test, NAG values of unknown samples and their corresponding %CV and SD will be copied and pasted into a master excel data sheet in the sequential order on sheet 1 in excel workbook. NAG values, %CV, SD of quality control samples and proficiency samples will be pasted on sheet 2. Both sheet 1 & 2 will be updated with addition of data from each run.
- The master data sheet that will go to coordinating center will contain the following information
  1. Sample ID (Bio Con ID)
  2. Date of the assay
  3. Platform and Instrument# used for the assay
  4. Reagent lot numbers, which includes date of NAG standard preparation, lot numbers of standard solutions, Lot numbers and date of NAG reagent preparation ( Buffer 2&3)
  5. NAG levels in the specimen (mU/ml)
  6. % CV across the sample replicates
  7. NAG levels, SD and % CV of each control sample with the description of each control sample
  8. NAG levels, SD and %CV of each proficiency samples with the description of each proficiency sample (the cells will be left blank if the proficiency samples are not run on that plate)
  9. Levey-Jennings plots of Control samples and proficiency samples (These plots will be updated and cumulative plots will be reported with each data submission.

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## 7.5 Assay parameters

Assay Range	0.2-26.4 mU/mL
LLOD	0.1 mU/mL
Recovery	90-100%
Linearity of Dilution	1:2, 1:10, 1:20 dilution
Intraassay %CV	< 9.3%
Interassay %CV	< 9.7%
Reference range in HV	0-5.3 mU/mg uCr

## 7. References

1. Vaidya VS, Waikar SS, Ferguson MA, Collings FB, Sunderland K, Gioules C, Bradwin G, Matsouaka R, Betensky RA, Curhan GC, Bonventre JV. Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. Clin Transl Sci. 2008 Dec;1(3):200-8.
2. Koyner JL, Vaidya VS, Bennett MR, Ma Q, Worcester E, Akhter SA, Raman J, Jeevanandam V, O'Connor MF, Devarajan P, Bonventre JV, Murray PT. Urinary biomarkers in the clinical prognosis and early detection of acute kidney injury. Clin J Am Soc Nephrol. 2010 Dec;5(12):2154-65
3. Vaidya VS, Niewczas MA, Ficociello LH, Johnson AC, Collings FB, Warram JH, Krolewski AS, Bonventre JV. Regression of microalbuminuria in type 1 diabetes is associated with lower levels of urinary tubular injury biomarkers, kidney injury molecule-1, and N-acetyl- $\beta$ -D-glucosaminidase. Kidney Int. 2011 Feb;79(4):464-70

# N-Acetyl-β-D-Glucosaminidase (NAG)

Colorimetric assay for the determination of N-Acetyl-β-D-Glucosaminidase in urine

**Cat. No. 10 875 406 001**

Test-Combination for approx. 50 tests

 **Version 17**

**Content version: October 2011**

Store at +15 to +25°C

## 1. What this Product Does

### Kit Contents

Vial	Content
1	<b>Buffer Substance</b> consisting of citric acid and potassium citrate.
2	<b>Lyophilized Substrate</b> consisting of sodium 3-cresolsulfon-phthaleinyl-N-acetyl-β-D-glucosaminide and borax.
3	<b>Stop Reagent</b> consisting of sodium carbonate.

### Storage and Stability

Stable when stored at +15 to +25°C until the expiration date printed on the labels.

### Application

The test is intended for research studies. It is used to explore the assumed relationship between physiological disturbances and the appearance of NAG in urine.

## 2. How to Use this Product

### 2.1 Before You Begin

#### Preparation of Solutions

- **Buffer solution:** Dissolve the contents of bottle 1 with 55 ml double dist. water.
- **Substrate solution (solution II):** Dissolve the contents of bottle 2 with 55 ml solution I.
- **Stop reagent (solution III):** Dissolve the contents of bottle 3 with 110 ml double dist. water.

#### Stability of Solutions

Solution II is stable for 1 month when stored at +2 to +8°C, protected from light.

Solution III is stable for 1 month stored at +2 to +8°C.

#### Stability of the sample

The activity determination of the N-acetyl-β-D-glucosaminidase (NAG) should be carried out directly after collecting the sample. Turbid urines should be centrifuged and the supernatant decanted. NAG is stable for one week at +2 to +8°C and for one month when stored at –15 to –25°C (1).

#### Control Reaction

The inclusion of a reagent blank is necessary for the NAG assay, as otherwise false positive values may be found. Such values are on average between 4 and 6 U/l too high.

The reagent blank rises from 4.5 to 6 U/l within 5 h and should therefore be reassayed for each series of measurements.

### 2.2 Procedure

Wavelength: 580 nm

Light path: 1 cm

Measuring volume: 3.05 ml

Incubation temperature: 37°C

Measurement against reagent blank. One reagent blank is sufficient for each series of measurements

Pipette into test tubes	sample	reagent blank
substrate solution (I)	1.00 ml	1.00 ml
<b>incubate for 5 min at 37°C, add</b>		
urine sample	0.05 ml	– <sup>1)</sup>
<b>mix and incubate for exactly 15 min at 37°C (stop watch). Add</b>		
stop reagent (III)	2.00 ml	2.00 ml
<b>mix, allow to stand for 10 min at 37°C and measure at +15 to +25°C against the reagent blank within 50 min = A<sub>sample</sub> for 15 min.</b>		

<sup>1)</sup> 0.05 ml water can be neglected.

If the NAG activity in the sample is too low, then the incubation time can be increased to 30 or 60 min. The longer incubation time must then be taken into account in the calculation.

#### Calculation

$$\frac{1000 \times V}{40.67 \times 1 \times v \times t} \times A \text{ (sample)}$$

V: total volume measured (in ml)

v: volume of urine sample (in ml)

t: incubation time (in min)

A: absorbance measured at 580 nm

#### Example

3-Cresolsulfonphthalein:

$$E_{580 \text{ nm}} = 40.67 [l \times \text{mmol}^{-1} \times \text{cm}^{-1}]$$

Volume activity

$$\frac{1000 \times 3.05}{40.67 \times 1 \times 0.05 \times 15} \times A \text{ (sample)} = 100 \times A \text{ (sample)} [\text{U/l}]$$

### 3. Additional Information on this Product

#### Principle

3-Cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide, sodium salt, is hydrolyzed by N-acetyl-β-D-glucosaminidase (NAG), releasing 3-cresolsulfonphthalein, sodium salt (3-cresol purple), which is measured photometrically at 580 nm.

3-Cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide



3-cresolsulfonphthalein + N-acetyl-glucosamine

#### Reference

Yakata, M. *et al.* (1983) *Jap. J. Clin. Path. Suppl.* **56**, 90.

### 4. Supplementary Information

#### 4.1 Changes to Previous Version

- Regulatory Disclaimer updated

#### 4.2 Ordering Information

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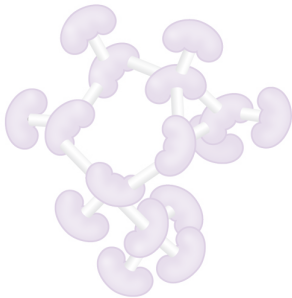
Product	Pack size	Cat. Nos.
N-Acetyl-β-D-Glucosaminidase (NAG)	for approx. 50 tests	10 875 406 001
N-Acetyl-β-D-Glucosaminidase Control (NAG Control)	3 bottles	11 164 368 001
N-Acetyl-β-D-Glucosaminidase Standard (NAG Standard)	Lyophilizate for 5 × 3 ml	10 982 962 001

#### Trademarks

All product names and trademarks are the property of their respective owners.

#### Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.



### Contact and Support

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Roche Diagnostics GmbH  
Roche Applied Science  
68298 Mannheim  
Germany

# N-Acetyl-β-D-Glucosaminidase standard (NAG-Standard)

Standard for the determination of NAG activity in urine research samples with the aid of the Test-Combination N-Acetyl-β-D-Glucosaminidase


**Cat. No. 10 982 962 001**

Lyophilizate for 5 × 3 ml

 **Version 16**

Content version: October 2011

Store at +15 to +25°C

 Store protected from light.

## 1. What this Product Does

### Contents

5 bottles, each with lyophilizate for 3 ml standard solution

### Storage and Stability


Stable at +15 to +25 °C, protected from light, until the expiration date printed on the label.

After dissolving, store at +2 to +8°C, protected from light.

### Product characteristics

#### Volume activity

The activity of the NAG standard is ca. 20 mU N-acetyl-β-D-Glucosaminidase from beef kidney per ml. For exact value see data on the label

-  The NAG standard is only to be used together with the test-combination N-Acetyl-β-D-Glucosaminidase (NAG). An automation of the test and of the evaluation is possible.

## 2. How to Use this Product

### 2.1 Before You Begin

#### Preparation of the standard solution

Dissolve contents of one bottle with 3 ml double-distilled water, close bottle and wait for 5 min. Dissolve contents completely by gentle swirling. Avoid foaming.

#### Stability of the standard solution



The solution is stable for at least 1 month stored at +2 to +8°C, protected from light

## 3. Supplementary Information

### 3.1 Text Conventions

#### Symbols

In this package insert the following symbols are used to highlight important information

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
	Important Note: Information critical to the success of the procedure or use of the product.

## 3.2 Changes to Previous Version

Editorial changes

## 3.3 Ordering Information

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Product	Pack size	Cat. Nos.
N-Acetyl-β-D-Glucosaminidase (NAG)	for approx. 50 tests	10 875 406 001
N-Acetyl-β-D-Glucosaminidase Control (NAG Control)	3 bottles	11 164 368 001
N-Acetyl-β-D-Glucosaminidase Standard (NAG Standard)	Lyophilizate for 5 × 3 ml	10 982 962 001

### Trademarks

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**[www.roche-applied-science.com/support](http://www.roche-applied-science.com/support)**

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**University of Minnesota  
Advanced Research Diagnostic Laboratory**

<b>Entity:</b>	<b>University of Minnesota</b>
<b>Department:</b>	<b>Molecular Epidemiology and Biomarker Research Laboratory</b>
<b>Manual:</b>	<b>ARDL Procedure Manual</b>
<b>Procedure:</b>	<b>VITAMIN D2, D3, AND EPI-D3, 25-HYDROXY</b>

**Principle**

Vitamin D, a hormone, is a fat-soluble compound involved in mineral homeostasis and plays an important role in calcium metabolism, parathyroid hormone (PTH) release, and osteoblast formation. As vitamin D (calciferol) enters the circulation it is metabolized to several forms, the major of these being 25-hydroxycalciferol (25-hydroxyvitamin D). The first step in the metabolism of vitamin D, 25-hydroxylation, occurs primarily in the liver. Only a small amount of 25-hydroxyvitamin D is metabolized in the kidney to 1,25-dihydroxyvitamin D and other metabolites. Since 25-hydroxyvitamin D is the predominant circulating form of vitamin D in the normal population and has the longest half-life, it is considered to be the most reliable index of a patient's vitamin D status.

Vitamin D occurs in two forms: Vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). Cholecalciferol is obtained from foods of animal origin and from ultraviolet light-stimulated conversion of 7-dehydrocholesterol in the skin. Small amounts of ergocalciferol are obtained from foods of plant origin. Both forms are used to fortify various foods and in over-the-counter supplements. Vitamin D3 and vitamin D2 are hydroxylated in the liver to 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2. These are the major circulating forms of vitamin D and precursors to the active form of vitamin D (1,25-dihydroxyvitamin D). 25-hydroxyvitamin D is accepted as the functional indicator of vitamin D status.

The measurement of 25-hydroxyvitamin D is useful in the management of patients with various disorders of calcium metabolism associated with rickets, neonatal hypocalcemia, pregnancy, nutritional and renal osteodystrophy, hyperparathyroidism and post-menopausal osteoporosis.

The internal standard, deuterated 25-hydroxyvitamin D3, deuterated 25-hydroxyvitamin D2 are added to serum or plasma samples. The analytes are extracted and precipitated from the specimen using n-heptane. Specimen are stored in -80 freezer for 2 hours. The organic layer is removed using a gasket apparatus and dried in a speed vacuum. The reconstituted specimens are injected into the LC/MS/MS. 25-hydroxyvitamin D2 and D3 and the internal standard, deuterated 25-hydroxyvitamin D3, are eluted off a Phenomenex column with a mobile phase of 71% 2.0 mmol/L ammonium acetate, in methanol 45°C then introduced via an APCI probe to monitor the compounds of interest. System control and data acquisition are performed by the Analyst software. MultiQuant software is employed for calibration and data processing.

**Purpose**

This procedure provides instruction for performing 25-hydroxyvitamin D2, D3 and epi-D3 by Liquid Chromatography/Tandem Mass Spectrometry

## Specimen

Optimum Volume: 0.5 mL serum  
Minimum Volume: 0.2 mL serum

Serum specimens are preferred. EDTA plasma specimens are acceptable. Serum separator/gel tubes are acceptable. See appendix A for laboratory performed comparisons between serum and EDTA plasma.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store frozen at less than or equal to 20° C.

EDTA Plasma – Centrifuge sample for 15 minutes at 1000 x g. Remove plasma and assay immediately or aliquot and store frozen at less than or equal to 20° C.

## Equipment

This procedure employs a Shimadzu Prominence Liquid Chromatogram or equipped with an autosampler and column oven. Samples are injected into a Beta 2.6u PFP 100A Column 100 x 3.0 m into the ionization source of a ABSceix 5500 tandem mass spectrometer. This instrument is operated using atmospheric-pressure chemical ionization (APCI probe).

Vitamin D2, Vitamin D3, Vitamin D3 epimer, deuterated Vitamin D3, and deuterated Vitamin D2 are monitored in the MRM mode using the transitions M/Z 395.3>269.3, 383.4>211.3, 389.3>211.3, 389.3>211.3, 398.3>272.3 respectively. All aspects of the system operation and data acquisition are controlled using the Analyst software with automated data processing by MultiQuant software. Nitrogen is generated using a Parker Tri Gas Station.

See LC/MS/MS Operation and Maintenance Instructions for instrument set-up, maintenance and tuning.

## Supplies

1. Autosampler 96 deep well plates:  
Obtained from Fisher; part number 12-565-606.
2. 96 Deep Well Plate Covers:  
Obtained from Fisher Scientific; part number 12-565-559
3. Eppendorf Repeater Pipette:  
Discontinued
4. Eppendorf Repeater Pipette tips:  
Combitips Plus 12.5 mL. Obtained from fisher; part number 21-381-331.  
Combitips Plus 2.5 mL, Obtained from fisher; part number 21-381-329.
5. Fisherbrand Finnpiptette 200 µL Pipette: Obtained from Fisher; part number 14-487-73.
6. Dot scientific 200 uL Pipette Tips:  
Obtained from Fisher; part number ERY-0200-GS.
7. HPLC column:  
The analytical column is a Phenomenex Beta 2.6u PFP 100A  
New Column 100 x 3.0 m part number 00D-4477-Y0

**Reagents** Do not wash bottles with detergent (this could increase background ions). Rinse bottles with methanol or Milli-Q water

1. Water:  
LC/MS Grade Obtained from Sigma-Aldrich Co., St. Louis, MO, part number 39253-4X4L-R.
2. Methanol  
LC/MS grade Obtained from Sigma-Aldrich Co., St. Louis, MO, part number 34966-4X4L

**CAUTION: Flammable liquid, Target Organ Effect, Toxic by inhalation., Toxic by ingestion, Toxic by skin absorption, Irritant.**

3. 60% Methanol:



Combine 350 mL methanol and 150 mL Milli-Q water. Stable for at least 6 months stored at room temperature.

4. Heptane CHROMASOLV® Plus, for HPLC, 99%:  
HPLC grade obtained from Sigma-Aldrich Co., St. Louis, MO, part number 650536-4L  
**CAUTION: Flammable liquid, Irritant**
5. Acetonitrile:  
LC/MS Grade Obtained from Sigma-Aldrich Co., St. Louis, MO, part number 34967-4X4L  
**CAUTION: Flammable liquid, Carcinogen, Target Organ Effect, Harmful by ingestion., Harmful by skin absorption., Irritant**
6. Ammonium acetate 99.999% trace metals basis 10:  
Certified A.C.S. grade. Dry in a 30°C oven for at least one hour and store dessicated. Part number 372331-100G  
**CAUTION: Irritant**
7. 25-hydroxyvitamin D2:  
Obtained from Sigma-Aldrich Co., St. Louis, MO, 1 mg, part number 17937-1MG. Store at -20°C.  
**CAUTION: Toxic by inhalation., Highly toxic by ingestion, Highly toxic by skin absorption**
8. 25-hydroxyvitamin D3 monohydrate:  
Obtained from Sigma-Aldrich Co., St. Louis, MO, 1 mg, part number 17938. Store at -20°C.  
**CAUTION: Target Organ Effect, Toxic by inhalation., Highly toxic by ingestion, Highly toxic by skin absorption, Teratogen**
9. 25-hydroxyvitamin D3-26,26,26,27,27,27-d6: (deuterated vitamin D3):  
Obtained from Medical Isotopes, Inc. #D2831  
**CAUTION: May be harmful if inhaled. May cause respiratory tract irritation. May be harmful if absorbed through skin. May cause skin irritation. May cause eye irritation. May be fatal if swallowed.**
10. Primary stock D2 internal standard: (100 µg/mL 25-Hydroxyvitamin D2-[2H3])  
isosciences.com #4176  
Receive 1 mg quantity, dilute to 10 mL in a volumetric flask with HPLC methanol (100 µg/mL). Store exactly 1.0 mL in amber vials under nitrogen at -70°C for 10 years.
11. Primary stock D3 internal standard: (100 µg/mL 25-OH vitamin D3,26,26,26,27,27,27-d6)  
Receive 1 mg quantity, dilute to 10 mL in a volumetric flask with HPLC methanol (100 µg/mL). Store exactly 1.0 mL in amber vials under nitrogen at -70°C for 10 years.
12. Intermediate internal standard: (1 µg/mL 25-OH vitamin D3-26,26,26,27,27,27-d6)  
Dilute 1.0 mL of each of the 100 µg/mL primary internal standards D2 and D3 in a single 100 mL volumetrically with HPLC methanol. Store in an amber bottle under nitrogen at -70°C for 10 years.
13. Working internal standard: (50 ng/mL 25-OH vitamin D3,26,26,26,27,27,27-d6)  
Dilute 1 mL stock internal standard to 100 mL with HPLC methanol volumetrically. (Also can do 10 mL to 200 mL). Store in an amber bottle at -20°C. Stable for at least 1 year.
14. Vitamin D2 stock standard: (approximately 20-25 µg/mL)  
Volumetrically transfer the contents of a 1 mg vial of 25-Hydroxyvitamin D2 into a 50 mL volumetric flask with absolute ethanol and dilute to volume. Determine the actual concentration using a spectrophotometer, measuring the absorbance of this solution and calculate the concentration using the molar absorptivity (see Note 3). Stable for at least 2 years at -20°C.
15. Vitamin D3 stock standard: (approximately 20-25 µg/mL)  
Volumetrically transfer the contents of a 1 mg vial of 25-Hydroxyvitamin D3 into a 50 mL volumetric flask with absolute ethanol and dilute to volume. Determine the actual concentration using a spectrophotometer, measuring the absorbance of this solution and calculating the concentration using the molar absorptivity (see note 3). Stable for at least 2 years at -20°C.
16. 3-Epi-25-Hydroxyvitamin D3: (approximately 20-25 µg/mL)  
Volumetrically transfer the contents of a 1 mg vial of 25-Hydroxyvitamin D3 into a 50 mL volumetric flask with absolute ethanol and dilute to volume. Determine the actual concentration using a spectrophotometer, measuring the absorbance of this solution and calculating the concentration using

the molar absorptivity (see note 3). Stable for at least 2 years at -20°C. From isosciences part number 7004.

17. **6.8 mM Ammonium Acetate:**  
Weigh 1.059 g of dried and dessicated crystalline ammonium acetate and dissolve in 2000 mL Milli-Q water in a volumetric flask. Stable for at least 1 month at room temperature.
  18. **Mobile Phase A:** (6.8 mmol/L Ammonium Acetate in Water,)
  19. **Mobile Phase B:** (100% Methanol)
  20. **Wash Solvent:** 100% Methanol
  21. **Seal Wash and Purge Solvent:** 50% mobile phase A and 50% mobile phase B.
  22. **Pooled Serum:**  
Human Serum Stripped, delipidized from Biocell; part number 1131-00
- CAUTION: Handle as if capable of transmitting any infectious agent**
23. **Serum Controls:**  
Obtained from IRIS Technologies International GmbH. There are three levels of control: Part number MS7013. They are prepared by diluting the vial with 1 mL Milli-Q water. Stable for 25 days after reconstitution at 2-8°C. Aliquot into small screw cap vials and freeze at -70°C if not to be used before 25 days. Stable at -70°C for at least one year.

### **Quality Control**

Two levels of IRIS Technology International GmbH quality control are assayed with each batch of 18 specimens. Bracket each set of 18 specimens with 2 controls, one high and one low. Review Levy Jennings plot and quality control acceptability criteria before reporting results. Control values are compared to established control ranges. Violation of Westgard Rules 1-3s or 2-2s will result in corrective action.

If QC values are outside of specified ranges, perform and document the following corrective actions steps until QC is acceptable:

1. Check reagent and quality control for appropriate lot numbers, expiration dates, preparation and storage conditions.
2. Repeat the analysis using fresh QC material.
3. Perform a calibration.
4. Perform maintenance procedures.
5. If quality control continues to fall outside of the established acceptability limits discontinue testing and notify the supervisor.

### **Procedure**

#### **1. Preparing internal standards**

- a. **Primary stock internal standard:** (100 µg/mL 25-OH vitamin D3,26,26,26,27,27,27-d6 and 25-Hydroxyvitamin D2-[2H3])
  - i. Separately receive 1 mg quantity, dilute to 10 mL in a volumetric flask with HPLC methanol (100 µg/mL).
  - ii. Store exactly 1.0 mL in amber vials under nitrogen at -70°C for 10 years.
- b. **Intermediate internal standard:** (1 µg/mL 25-OH vitamin D3-26,26,26,27,27,27-d6)
  - i. Dilute 1.0 mL of each of the 100 µg/mL primary internal standards D2 and D3 in a single 100 mL volumetrically with HPLC methanol.
  - ii. Store in an amber bottle under nitrogen at -70°C for 10 years.
- c. **Working internal standard:** (50 ng/mL 25-OH vitamin D3,26,26,26,27,27,27-d6)
  - i. Dilute 2 mL intermediate internal standard to 100 mL with 50:50 HPLC methanol: water, volumetrically. Store in an 50 mL plastic tube.
  - ii. -20°C. Stable for at least 1 year.

#### **2. Extraction on standards, controls and samples**

- a. Thaw patient samples (if frozen), controls, and working standards lines and invert 6 times to mix.
  - i. **Reagent Blank:**

1. Pipette 200  $\mu$ L of water (or saline) into well #1
2. Place additional blank after last control to balance plates.
- ii. 25-OH D3/D2 standards 0/0, 5/0.5, 10/1, 30/3, 50/10, 100/25, and 200/50
  1. Pipette 200  $\mu$ L into wells #2-8 of a deep well plate.
- iii. 25-OH D3 epimer standards 0, 0.5, 1, 2.5, 10, 30, and 100
  1. Pipette 200  $\mu$ L into wells #9-15 of the 96 deep well plate.
- iv. Controls, and Specimens:
  1. Pipette 200  $\mu$ L of the controls, and patient specimens into remaining wells of the 96 deep well plates.
  2. Bracket each set of 18 specimen with 2 controls, one high, one low. 5 sets can be run in one day.
  3. Distribute the samples evenly between 2 plates for centrifugation.
- b. Add 100  $\mu$ L of acetonitrile to each well using a Eppendorf repipetor. Cover with 96 well rubber matt, invert 20 times gently and centrifuge for 10 seconds in speed vacuum.
- c. With an Eppendorf repipetor, add 250  $\mu$ L working internal standard to each well. Exception: To the reagent blank add 250  $\mu$ L of Reconstitution Solution (60% Methanol:40% Water). Cover, invert 20 times gently and centrifuge for 10 seconds in speed vacuum.
- d. Add 0.75 mL n-heptane with a Eppendorf repipetor. Cover, invert 30 times gently.
- e. Centrifuge at 4450 rpm for 20 minutes in Allegra Centrifuge.
- f. Place 96 well plate into -80 C freezer for 2 hours.
- g. Remove rubber cover and fit rubber gasket onto plate. Fit new 96 well plate onto other side of gasket.
- h. Invert gasket and plates together and gently shake till heptanes is transferred to new plate.
- i. Dry speed vacuum.
  - i. Turn on condenser unit 30 minutes before use.
  - ii. Turn on vacuum pump before starting run.
  - iii. Place uncovered plate into centrifuge.
  - iv. Follow instruction on speed vacuum screen to start.
  - v. Wait approximately 1 hour on lowest heat setting to dry.
- j. Once the extracts are dried down, add 150  $\mu$ L of 60% methanol:40% water to each tube using a Eppendorf repipetor. Vortex for 15 seconds and centrifuge briefly.
- k. Load plate into autosample and inject 40  $\mu$ L of sample into the LC/MS/MS. Refer to ABSciex LC/MS/MS Operation and Maintenance Instructions.

### 3. Gasket Preparation

- a. Cut and place 4, 1/8<sup>th</sup> inch thick rubber spacers along sides of the mold.
- b. Mix 75 mL Dow Corning 3110 RTV Silicone Rubber with 7.5 mL S TIN NW catalyst.
- c. Pour mixture into mold up to tape mark.
- d. Place 96 well plate into rubber until it makes contact with rubber pieces.
- e. Wait 24 hours for curing
- f. Remove gasket and flip over
- g. Repeats steps 1-4 on top of first 1/2 of gasket.
- h. Bore 96 holes with #2 brass sharp borer (~ 3/16<sup>th</sup> inch)

### 4. LC and Mass Spec Setup

- a. If mass spec was last used for an assay besides 25-OH Vitamin D, follow these steps to setup the mass spec.
- b. Install ESI probe (make sure corona needle is facing away from mass spec before inserting probe)
- c. Set probe depth to "2"
- d. Tune the instrument following the tuning manual (Located both on the mass spec computer and in the mass spec maintenance folder.)
- e. Install APCI probe (make sure corona needle is facing mass spec)
- f. Set depth to "8"
- g. Prepare and load correct mobile phases
  - i. 100% Water 6.8 mM Ammonium Acetate
  - ii. 100% LC/MS Methanol

- h. Install Phenomenex Beta 2.6u PFP 100A Cut peek tubing and use new fittings.
- i. Extract and run 5 high and 5 low controls to verify assay is working.

## 5. Sample procedure

- a. This procedure uses a Phenomenex Beta column. Before injecting specimens on the column, the column should be conditioned by pumping for a minimum of 15 minutes with the mobile phase.
- b. Create a sample worksheet name after current study using excel: Make a new tab for each day (i.e. 101507).
  - i. There should be 1 wash at the beginning of the batch to condition the column and the LC/MS/MS.
  - ii. The standard line should be assayed with the lowest standard first and the rest in ascending order. The blank reagent vial **MUST** be assayed right after the highest standard (200 µg/L) to check for carryover (see Note 1). The working standard pool, in duplicate, should be assayed next and one of the pool vials re-injected again near the end of the batch.
  - iii. Bracket every 18 specimen with 2 controls, one high and one low.
- c. When the worksheet is complete, resave it ("File", "Save")
- d. Open Analyst and click on Hardware Configuration
- e. Activate "Shimadzu MS" Profile
- f. Click on Build Acquisition Batch, then add set, then add samples
- g. Select Subfolder Vitamin D, enter Data File Prefix (date and study name), and enter number of samples.
- h. Copy and paste sample table into Analyst Batch
- i. Enter rack type "Deep Well MPT 96 Standard" and paste vial numbers from sample worksheet.
- j. Select Final 25-OH Vitamin D 2,3 from method tab.
- k. Go to submit tab and select all sample and click "Submit" button
- l. Purge LC first, then click equilibrate button.
- m. After equilibration, click start button
- n. The Phenomenex Beta column should to be rinsed and stored in 100% methanol after the last batch of the day.

## 6. Data/Results Processing

Calculations from raw data collected are performed by MultiQuant software. MultiQuant uses peak retention time, MRM transitions, and integration for vitamin D2, vitamin D3, vitamin D3 epimer, deuterated vitamin D2 and deuterated vitamin D3- to locate response. Refer to appendix C for chromatograms. Results are calculated relative to the internal standard response and using a calibration line.

Because the working standard line is made in pooled serum which contains endogenous vitamin D, special result processing is necessary to calculate the blank serum pool value before calculating the standard line and patient values.

- A. Open MultiQuant
  - a. Located in left hand bar under Companion Software.
  - b. Double Click MultiQuant Icon
- B. Create New Quantitation Method
  - a. Check to see that correct Analyst Project Folder is selected. This is the 4th item located on the toolbar.
  - b. Under File, select New Quantitation New Method
  - c. Locate and select a control from the sample list to be quantitated.
  - d. Click the Integration and Regression Tab
  - e. Select each Analyte and check the auto integration for correct peak selection.
    - i. For 25 OH Vitamin D3 and 25 OH Vitamin D3 epimer, and 25-OH Vitamin D2 ONLY: uncheck integrate largest peak and RT half time to 15 seconds.
  - f. Save Method as same name as batch.
- C. Create New Results Table

- a. Under File, select Create New Results Table
- b. Locate and select sample list to be processed and click right arrow to select the entire list.
- c. Under "Choose Existing Method" click "Open"
- d. Find Quantitation Method Created in Step 2
- e. Click Finish
- D. Data Integration Review
  - a. A new window should pop up containing the data.
  - b. This window has a tool bar which contains several tools we will be using including a blue box "Displays the peak review", a trash can "deletes this pane", and a white box "hides this pane"
  - c. Click the "displays the peak review" toolbar icon
  - d. Notice a new window appears below with a similar toolbar.
  - e. Click the "hide this pane" from the toolbar of the top window.
  - f. In the window of the left hand side, choose an analyte.
  - g. Using the red arrows, scroll through the chromatographs for the analyte chosen.
  - h. Makes sure the correct peak is selected based off of retention time.
  - i. Repeat steps F-H for each of the analytes in the left hand window.
  - j. Click "delete this pane" from the toolbar
- E. Creating Raw Data Spreadsheet
  - a. Select an analyte from the lefthand window.
  - b. Copy and paste the information into excel in the format shown below.
  - c. First, copy and paste the sample list into Excel.
  - d. Next copy and paste the peak area.
  - e. Finally, copy and paste the retention time.
  - f. Repeat steps C and D for each of the analytes.
- F. Ratio Calculation
  - a. Calculate the Quantifier Ratios by dividing the Target Area by the Internal Standard Area
  - b. Calculate the Qualifier Ratios by dividing the Qualifier Area by the Target (Quantifier) Area.
- G. Standard Line Calculation
  - a. Subtract each standard line ratio by the 0 ng standard line ratio
  - b. Next to the sample list name for each standard, enter the concentration into a new excel box.
  - c. Insert a Scatter with only Markers Graph
  - d. Right click the graph and click "select data"
  - e. Remove all Legend Entries automatically entered by excel
  - f. Add new Legend Entry
  - g. For the x values, select the concentrations entered in step b.
  - h. For the y values, select the adjusted ratios calculated in step a.
  - i. Click OK, then OK again.
  - j. Right click line on graph and click Add Trendline.
  - k. Click the 3 check boxes at bottom: set intercept 0, display equation on chart, display R-squared value on chart.
  - l. Click the  $y=mx+b$  formula now shown on graph.
  - m. Change the category to Number and the decimal places to 5.
- H. Sample Concentration Calculation
  - a. Use the  $y=mx+b$  formula to calculate the concentration of target in each sample where y is equal to the Quantifier Ratio
- I. Create Final Data Spreadsheet
  - a. Copy and paste special the Sample List, Sample Concentration and Qualifier Ratios into a new tab.
  - b. Organize with Concentrations together and Qualifer Ratios together.

## Results

Vitamin D2 or Vitamin D3 results  $<5.0 \mu\text{g/L}$  are reported as such. Results  $>200 \mu\text{g/L}$  are diluted 1:2 or 1:3 with saline and reassayed. Multiply the result by the dilution factor. If the final answer is greater than  $600 \mu\text{g/L}$  report as  $>600 \mu\text{g/L}$ . The total Vitamin D is the sum of the vitamin D2 and the vitamin D3.



### **Interferences**

The LCMS method uses parent ions, daughter ions and retention times to identify Vitamin D3, D2 and the epimer. This excludes most compounds from interfering with the assay. We are aware of certain isomers of Vitamin D that can interfere. These isomers are present in low amounts and have always been included in Vitamin D measurements. We continue to monitor the assay for possible compounds that interfere. (see reference 2)

### **Ion Suppression**

We use a heavy isotope of the target for the internal standard. Any ion suppression seen in the target will be equal for the isotope compensating for the ion suppression.

### **Reference Range**

25-80 ng/mL (Total Vitamin D3 and D2 combined) (see reference 3 and appendix B)

### **Assay Performance**

Analytical Range (sum of vitamin D2 and D3)	5 – 200 ng/mL
Limit of Detection (blank + 2SD)	D2 = 0.14 ng/mL D3 = 0.82 ng/mL Epi = 0.94 ng/mL
D2 Recovery	96.7% - 111.3%
D3 Recovery	95.6% – 109.9%
Intra-assay %CV (10 within day replicates at the stated concentration levels)	D2 = 4.9 % (at conc of 9 ng/mL) D2 = 4.0% (at conc of 14 ng/mL) D3 = 5.1% (at conc of 31 ng/mL) D3 = 4.2% (at conc of 59 ng/mL) Epi = 6.1% (at conc of 7 ng/mL) Epi = 8.6% (at conc of 16 ng/mL)
Inter-assay %CV (10 between day replicates at stated concentration levels)	D2 = 6.1 % (at conc of 8 ng/mL) D2 = 4.6% (at conc of 13 ng/mL) D3 = 6.1% (at conc of 31 ng/mL) D3 = 6.7% (at conc of 56 ng/mL) Epi = 4.9% (at conc of 7 ng/mL) Epi = 6.6% (at conc of 15 ng/mL)
Within day duplicate range (2.77 x ave within day %CV)	D2 = 12% D3 = 13% Epi = 20%
Reference range* (See appendix)	25 – 80 ng/mL serum

### **Notes**

1. Carryover is checked daily. The reagent blank is injected immediately following the standard 200 µg/L. It may have a peak present, but must read lower than the 0 standard from the line. Consult the supervisor if there is carryover detected.



2. Determination of the concentration of vitamin D2 and vitamin D3 standards.

- A. Prepare stock standards: Dissolve contents of a 1 mg vial of 25-hydroxyvitamin D2 in absolute ethanol in a 50 mL volumetric flask and dilute to volume (approximate concentration will be 20 µg/mL). Also dissolve the contents of a 1 mg vial of 25-hydroxyvitamin D3 in another 50 mL volumetric flask and dilute to volume (approximate concentration will be 20 µg/mL). Determine the actual concentrations of the two stock standards by measuring the absorbance of the solutions at 265 nm and calculate the concentrations using the molar absorptivity( $\epsilon$ ).
- B. Spectrometer setup for Thermospectronic Genesys 5 Spectrophotometer:
- 1) Turn on power with the toggle switch on the lower back corner on the left side of the instrument.  
The instrument will perform a series of self checks and warm up the lamp. This takes approximately 60 seconds. (Both the Visible and the UV lamps are lit.)
  - 2) The main menu screen will display. Choose option 1 for abs/%T/conc.
  - 3) The last wavelength that was used is automatically recalled. The wavelength and the absorbance are displayed in the center of the screen. To change the wavelength enter the wavelength desired, then press **<GO to WL>** key.
  - 4) The cuvette position is displayed on the right side of the screen and will show B or a number 2 through 8.
  - 5) The B stands for blank. When you press the **<AUTO ZERO>** key the spectrophotometer will move to position B (one) and set the zero.
  - 6) Place your sample in position 2 and read the absorbance. Print the answer.
- C. Use of the spectrophotometer in vitamin D2 and vitamin D3.
- 1) This is a UV wavelength (265 nm) so quartz cuvettes must be used. Before using the cuvettes must be checked so they are matched. Fill the cuvettes with absolute ethanol. Use one of the cuvettes in sample 1 and press **<AUTO ZERO>**. Read the absorbances of the other cuvettes against this blank. The cuvettes need to read within 0.005 absorbance units of each other. If they do not, rewash the cuvettes or use the cuvettes that do read within 0.005 absorbance units of each other.
  - 2) Once a set of cuvettes is matched, read standards against an absolute ethanol blank.
  - 3) A minimum of three consecutive stable readings should be achieved before accepting an average absorbance. If absorbance readings seem to drift, re-blank and read standards again, allowing a few seconds between each read.
  - 4) Print absorbances.
  - 5) Turn off power of the instrument by the toggle switch on the back of the lower right of the instrument.
  - 6) Clean cuvettes, rinse with methanol and let air dry.
- D. Wavelengths (nm), Molar Absorptivity( $\epsilon$ ), and molecular Weight (Mol. Wt.):

<u>Substance</u>	<u>(nm)</u>	<u>(<math>\epsilon</math>)</u>	<u>Mol. Wt.</u>
25-hydroxy vitamin D2	265	18900	412.66
25-hydroxy vitamin D3	265	18300	400.66

$\epsilon$  = Absorbance of a molar solution in ethanol

- E. Calculation of standard concentration:  $\mu\text{g/mL} = \text{Abs} \times \text{Mol.Wt.} \times 1000/\epsilon$   
The concentration of the stock standard is dependent on the amount received in the vial from the supplier and how well it is volumetrically transferred to the volumetric flask. If the concentration of the stock vitamin D2 or the stock vitamin D3 standard is approximately 20 µg/mL, make a intermediate standard by diluting the stock standard volumetrically 1:2 or 1:3 to make a final concentration of the intermediate approximately 10-12 µg/mL. Check the absorbances of the intermediate stock standard as above and calculate the exact concentration. Write this concentration on the container of the standard. Each time this standard is used the concentration **must** be checked and calculated, as vitamin D2 and vitamin D3 are affected by light.

- F. To calculate the amount of stock or intermediate standard to volumetrically dilute to the amount of working standard you want to make, use the VitDStdCalc.xlsx spreadsheet that contains the following formula:

$$(C_w \times V_w)/C_s = V_s$$

$C_w$  = concentration of the working standard

$V_s$  = total volume of working standard being made

$C_s$  = concentration of the stock or intermediate standard determined spectrophotometrically

$V_s$  = volume of stock or intermediate standard to dilute with blank pooled serum to make working standards

Example: Absorbance of the Vitamin D2 stock or intermediate is 1.146  
Absorbance of the Vitamin D3 stock or intermediate is 0.439  
Concentration of Vitamin D2 is  $1.146 \times 412.66 \times 1000/18900 = 25.02 \mu\text{g/L}$   
Concentration of Vitamin D3 is  $0.439 \times 400.66 \times 1000/18300 = 9.61 \mu\text{g/L}$

Working D3 standard concentrations in blank pool serum:

Std 5.0  $\mu\text{g/L}$

Std 10.0  $\mu\text{g/L}$

Std 30.0  $\mu\text{g/L}$

Std 50.0  $\mu\text{g/L}$

Std 100.0  $\mu\text{g/L}$

Std 200.0  $\mu\text{g/L}$

Working D2 standard concentrations in blank pool serum:

Std 0.5  $\mu\text{g/L}$

Std 1.0  $\mu\text{g/L}$

Std 3.0  $\mu\text{g/L}$

Std 10.0  $\mu\text{g/L}$

Std 25.0  $\mu\text{g/L}$

Std 50.0  $\mu\text{g/L}$

Working Epimer standard concentrations in blank pool serum:

Std 0.5  $\mu\text{g/L}$

Std 1.0  $\mu\text{g/L}$

Std 2.5  $\mu\text{g/L}$

Std 10.0  $\mu\text{g/L}$

Std 30.0  $\mu\text{g/L}$

Std 100.0  $\mu\text{g/L}$

Example: To make 50 mL of working vitamin D standard with a concentration of 50  $\mu\text{g/L}$  Vitamin D2 and D3 with a measured concentration of 25.02/9.61  $\mu\text{g/L}$  respectively:

$(0.05 \text{ ng/mL} \times 50 \text{ mL})/25.02 = 0.9992 \text{ mL} = 99.92 \mu\text{L}$  Vitamin D2 standard

$(0.05 \text{ ng/mL} \times 50 \text{ mL})/9.61 = 0.26015 \text{ mL} = 260.15 \mu\text{L}$  Vitamin D3 standard

Add amounts of the standards calculated to a 50 mL volumetric flask and slowly add the pool. Let the bubbles at the top settle and dilute to volume with the pool.

Aliquot 500  $\mu\text{L}$  of working standard into small plastic vials and store at  $-70^\circ\text{C}$ . Stable for three years.

3. Qualifier Ions are monitored as a performance marker. Suspect a recovery/sensitivity problem if qualifier ratios are +/- 20% from controls and high std line points.



4. LC/MS/MS Parameters:

These parameters can be changed to better optimize the assay.

A. ABSciex 5500

Solvent A 6.8 mmol/L ammonium acetate, in  
Milli-Q water

Solvent B Methanol

Wash solvent 100% methanol

Flow 0.550

Stop Time 10

Degasser On

Column Temperature (°C) 35.0

Equilibration Time 0.00

Inject Type Sequential

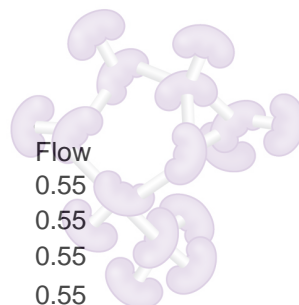
Fill Mode Partial Loop

Sample temperature (°C) 4

Wash Frequency Inject

Gradient Timetable:

Time	A%	B%	C%	D%	Flow
0.00	29	71	0.0	0.0	0.55
8	29	71	0.0	0.0	0.55
8.1	5	95	0.0	0.0	0.55
9.1	5	95	0.0	0.0	0.55
9.2	29	71	0.0	0.0	0.55
10.00	29	71	0.0	0.0	0.55



Diverter Valve

Valve	Time
A	5.5
B	9

B. ABSciex 5500 ms/ms instrument Parameters:

Polarity APCI  
Calibration Static 2  
Entrance Potential 10  
Curtain Gas 35  
CAD Gas 7  
Ionization 5500  
Temperature 450  
Gas 1 45  
Gas 2 0

# MRM Experiment Settings

Type  
Ion mode

MRM  
APCI

## Channel Transition:

Ch	Prnt(Da)	Dau (Da)	CE	CXP
1	383.4	211.3	36	14
2	389.3	211.3	36	12.00
3	395.3	269.3	27	17.00
4	383.4	211.3	30	14
5	398.3	272.3	47	16
6	395.3	55.0	77	10
7	383.4	91.0	71	10
8	383.4	91.0	71	10

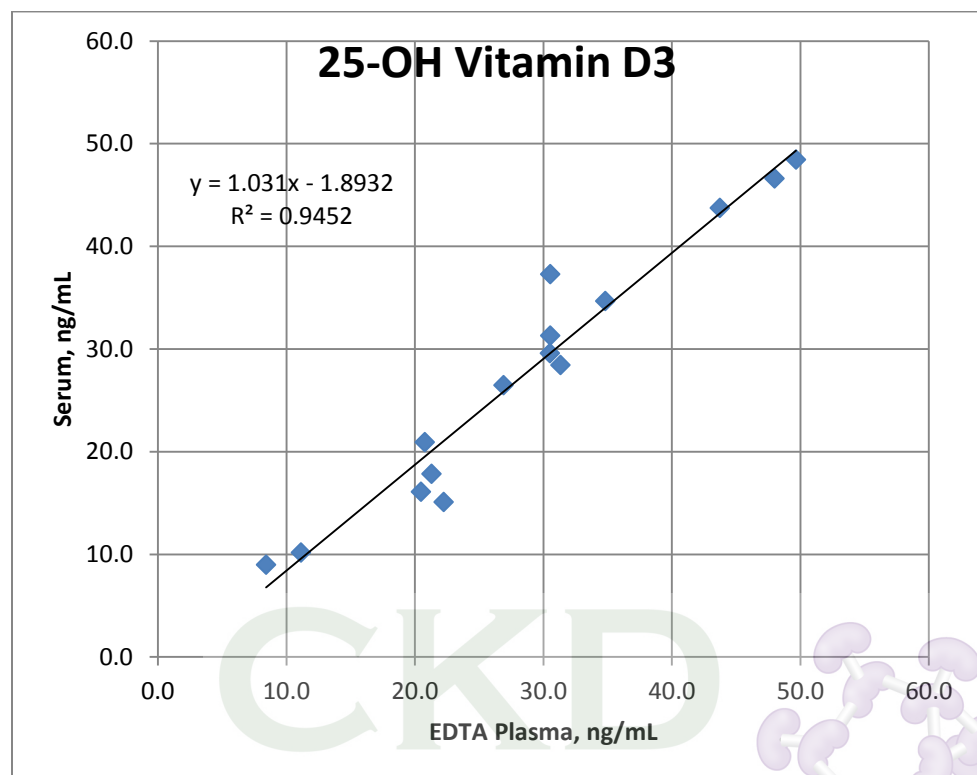
## References

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3. Mayo Medical Laboratories  
<http://www.mayomedicallaboratories.com/articles/hottopics/transcripts/2009/2009-1b-vitamind/1b-14.html>
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<b>External Ref:</b>	See Above
<b>Internal Ref:</b>	
<b>Appendix:</b>	A. Comparison of serum vs. EDTA plasma B. Reference range validation
<b>Date Written:</b>	R. Hecker 08/11
<b>Approved by:</b>	Anthony Killeen, M.D., PhD.
<b>Approved by:</b>	
<b>Date Effective:</b>	12/7/2011
<b>Date Revised:</b>	12/4/2012
<b>Date Reviewed:</b>	
<b>Date Retired:</b>	

## Appendix A

### Comparison of Serum vs EDTA Plasma 4/2013



25-OH Vitamin D3 only has been validated for serum and EDTA plasma. We do not have access to samples with adequate amount of 25-OH vitamin D2 to validate EDTA plasma. Similarly, the amount of 25-OH vitamin D3 epimer in the serum/plasma pairs was too low to provide meaningful data.

## Appendix B

### Reference Range Validation

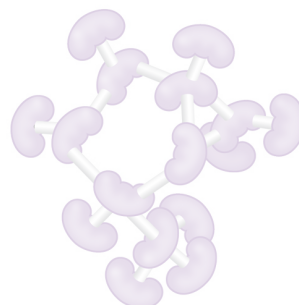
Reported Reference range:

25-80 ng/mL (Total Vitamin D3 and D2 combined)

Mayo Clinic Reference Ranges found at [www.mayomedicallaboratories.com](http://www.mayomedicallaboratories.com)

Laboratory validation of reference range based on “healthy” participants. The average Vit D level of the 20 participants was 28.2 ng/mL. This may be due to seasonal lowering of Vit D levels in the winter.

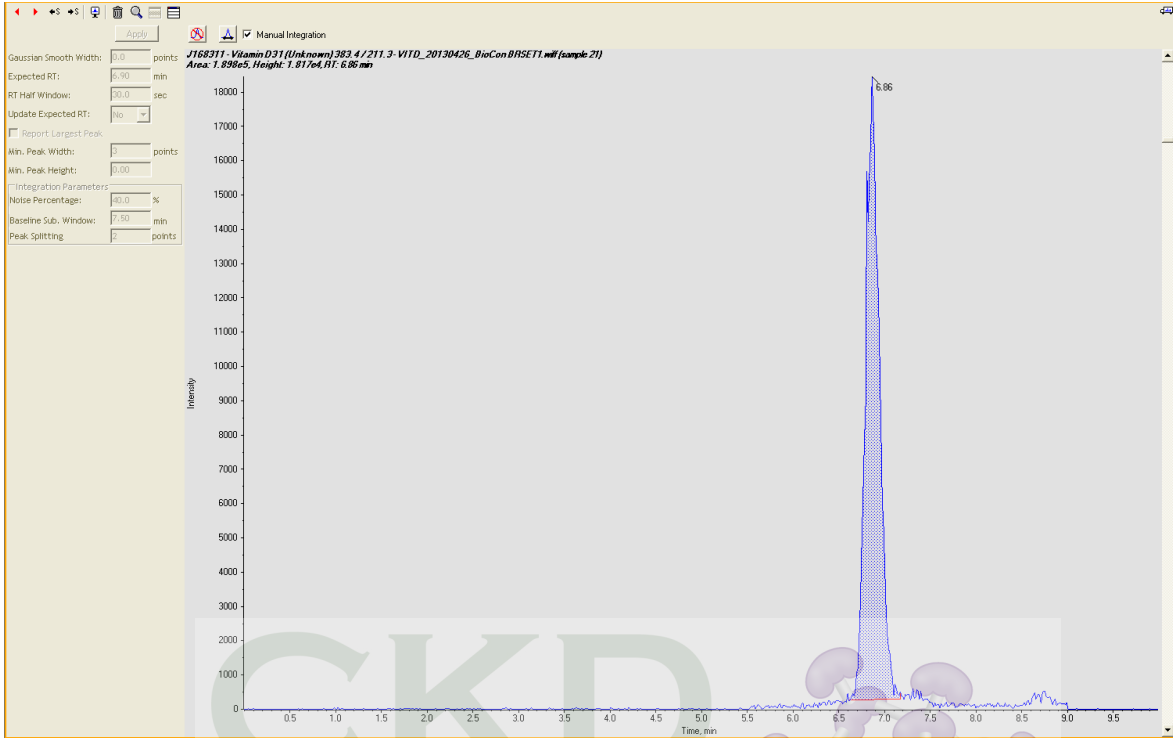
Date analyzed 1/13/12	
Total Vitamin D (ng/mL)	
1	36.4
2	30.1
3	37.6
4	19.7
5	37.0
6	30.6
7	25.7
8	18.7
9	30.8
10	35.2
11	27.3
12	28.9
13	28.3
14	26.7
15	16.4
16	14.5
17	26.8
18	25.2
19	28.1
20	40.8
Ave	28.2
SD	7.1
N	20



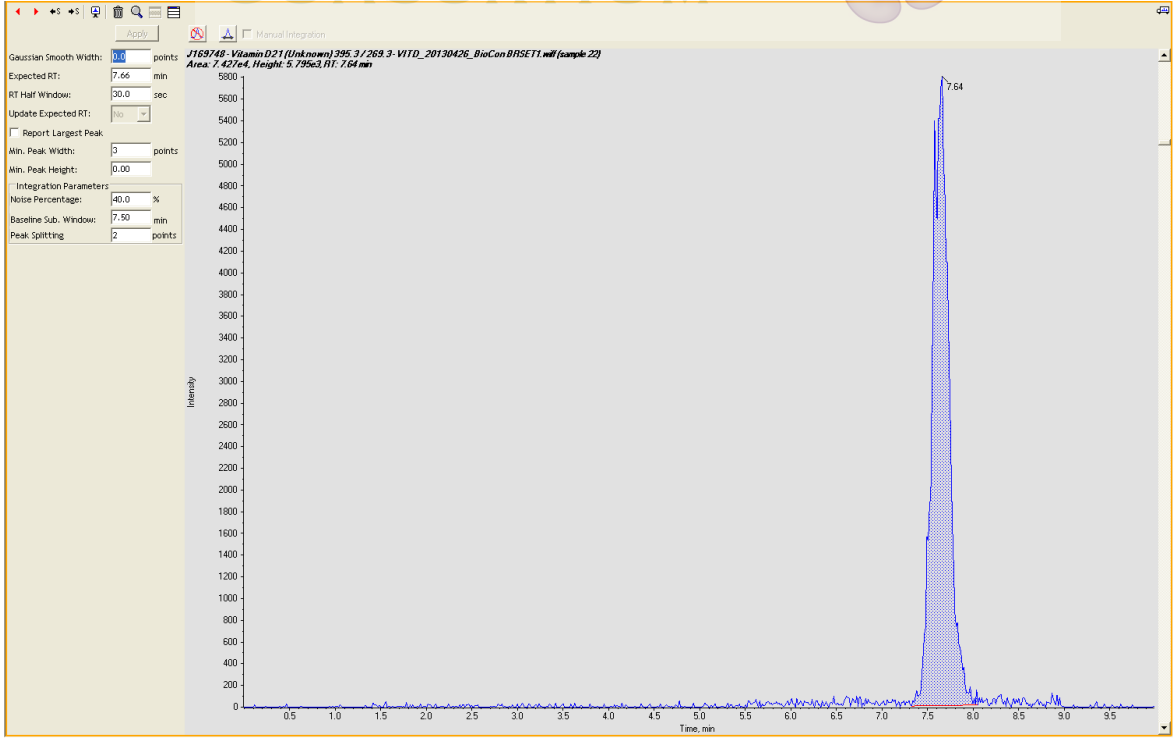


Appendix C

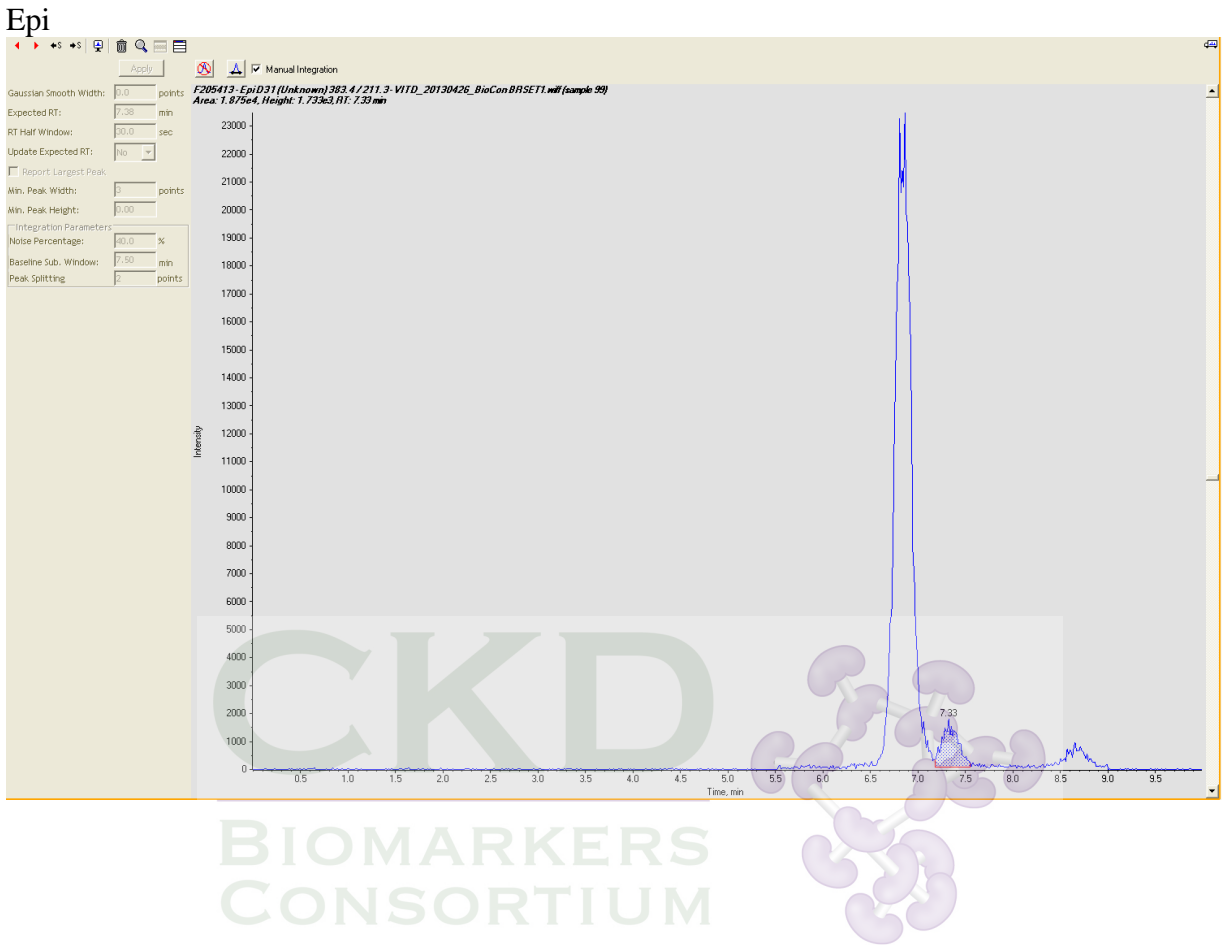
Vitamin D3



Vitamin D2



Appendix C



**University of Minnesota  
Advanced Research Diagnostic Laboratory**

<b>Entity:</b>	<b>University of Minnesota</b>
<b>Department:</b>	<b>Advanced Research and Diagnostic Laboratory</b>
<b>Manual:</b>	<b>ARDL Procedure Manual</b>
<b>Procedure:</b>	<b>Human Vitamin D Binding Protein Immunoassay</b>

**Principle**

This assay employs the quantitative sandwich enzyme immunoassay technique. Refer to the appended manufacturer package insert from Quantikine® ELISA, Human Vitamin D BP Immunoassay, R&D Systems, Inc, Minneapolis, MN.

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VitD BP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VitD BP present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for VitD BP is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VitD BP bound in the initial step. The color development is stopped and the intensity of the color is measured. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

**Specimen Type and Handling**

Serum and EDTA plasma are acceptable. See appendix A for laboratory performed comparisons between serum and EDTA plasma. Store samples at less than or equal to -20° C. Avoid repeated freeze-thaw cycles.

Minimum volume: 75µL in a 2mL microcentrifuge tube (10µL for test and additional 65µL for dead space)

**Specimen Collection**

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 10 minutes at 3000 x g. Remove serum and assay immediately or aliquot and store at -80° C.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 10 minutes at 3000 x g within 30 minutes of collection. Remove plasma and assay immediately or aliquot and store at -80° C.

**Interferences**

No significant cross-reactivity or interference indicated by manufacturer.

## Procedure

Refer to appended manufacturer's procedure for complete instructions. Note:

- Prepare daily worksheet. Refer to ARDL General ELISA procedure and see appended example (Appendix C).
- Use MilliQ water for reconstitution of the standard and preparation of the wash buffer.
- Prepare a 1:2100 sample and pooled serum quality control dilutions manually according to the following scheme:
  - Label 1.5mL Eppendorf snap-cap tubes or 2mL microcentrifuge tubes with well number (H2, A3, etc) and place in 12 x 8 rack.
  - Prepare a 1:70 dilution by pipetting 690uL calibrator diluent RD6-11 into labeled tube, followed by 10uL sample. Cap, then mix samples by inverting tubes at least 6 times.
  - Prepare a 1:30 dilution from the 1:70 dilution to yield a 1:2100 dilution using the Bioexpress 500uL v-bottom 96-well plate.
    - Pour calibrator diluent into reagent reservoir and pipette 290uL diluent into each well of dilution plate that will contain a sample.
    - Invert (1:70) sample dilution tube at least 2 times before pipetting 10uL of sample to the appropriate plate well indicated on tube label. This will result in a 1:2100 dilution.
    - After all samples are pipetted, mix samples in the 96-well plate by using a multi-channel pipette set at 200uL to pipette up and down 5 times; avoid foaming while mixing.
  - Prepare standards in 2mL microcentrifuge tubes as indicated in the kit insert. Transfer ~300uL of the prepared standards to the appropriate wells of the dilution plate.
  - Pour assay diluent into reagent reservoir and use multichannel pipet to add 100uL to each well of ELISA plate.
  - Use multichannel pipet to add 50uL of standards, controls and samples from the dilution plate to the ELISA plate. Pipet up and down 3 times to mix with assay diluent; avoid foaming while mixing.

### Manufacturer Kit Instructions:

1. Add 100 uL of Assay Diluent RD1-19 to each well.
2. Add 50 uL of Standard, control, or sample per well.
3. Cover with the adhesive strip provided.
4. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker set at 450 rpm.
5. Remove adhesive strip.
6. Using a multi-channel pipette aspirate the reaction solution.
7. Add 400 uL of Wash Buffer and then aspirate and discard.
8. Invert plate and blot on clean paper towel after all plate wells have been washed.
9. Repeat steps 7 and 8 three more times for a total of four washes. Note: work quickly with a multi-channel pipette to avoid drying out of the wells and make sure the pipette tips do not scratch the well bottoms.
10. Add 200 uL of VitD BP Conjugate to each well.
11. Cover with a new adhesive strip.
12. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker set at 450 rpm.
13. Remove adhesive strip.

14. Aspirate the reaction solution.
15. Add 400 uL of Wash Buffer and then aspirate and discard.
16. Invert plate and blot on clean paper towel after all plate wells have been washed.
17. Repeat steps 15 and 16 three more times for a total of four washes. Note: work quickly with a multi-channel pipette to avoid drying out of the wells and make sure the pipette tips do not scratch the well bottoms.
18. Add 200 uL of Substrate Solution to each well.
19. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
20. Add 50 uL of Stop Solution to each well.
21. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
22. Determine the optical density of each well by placing the plate on the SpectraMax 190 plate reader set at 450 nm within 30 minutes. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

### Dilutions

Serum and EDTA plasma samples are diluted 1:2100 in calibrator diluent RD6-11. Samples are re-diluted as needed to assay on standard curve.

### Calibration

Standard material included with the kit is used to prepare a standard curve for each assay. The immunoassay is calibrated against a highly purified natural vitamin D binding protein from human source material by the kit manufacturer.

### Quality Control

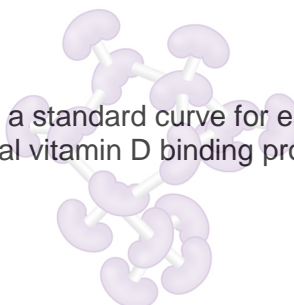
Two levels of pooled human serum controls are assayed in duplicate on each ELISA plate. Control values from each plate are compared to established control ranges. Review Levey-Jennings plots and acceptability criteria for each quality control result daily before reporting results. Violation of Westgard Rules 1-3s or 2-2s will result in corrective action.

If quality control fails the 1-3s or 2-2s Westgard rule take the following corrective action steps:

- With a new assay kit that includes controls and additional samples, reanalyze six samples from the failed plate that represent a range of results. If the controls from the new batch pass the Westgard rules and the repeat sample results are within the acceptable duplicate range, accept the batch, document the corrective action and proceed with testing.
- If the quality control fails after using a new kit, document corrective action, discontinue testing and notify the laboratory supervisor.

### New Lot Verification

A single lot is purchased to complete each study. If it is not possible to complete all testing on single lot a correlation of 5-20 samples between the two lots is performed, and a factor to harmonize results is used if kits are >10% different.



## Maintenance

Refer to *ARDL SpectraMax 190 plate reader procedure* and *ARDL General ELISA procedure* for maintenance procedures.

## Reagents and Supplies

- Human Vitamin D BP Immunoassay kit. Store unopened kit at 2 - 8°C. Opened and reconstituted reagents may be stored up to one month at 2 - 8°C. Do not use past the kit expiration date.
  - VitD BP Microplate (Part 893305) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against VitD BP.
  - VitD BP Conjugate (Part 893306) - 21 mL of a mouse monoclonal antibody against VitD BP conjugated to horseradish peroxidase with preservatives.
  - VitD BP Standard (Part 893307) - 1000 ng of natural human VitD BP in a buffer with preservatives; lyophilized.
  - Assay Diluent RD1-19 (Part 895467) - 11 mL of a buffered protein base with preservatives.
  - Calibrator Diluent RD6-11 (Part 895489) - 4 vials (21 mL/vial) of a buffered protein base with preservatives.
  - Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.
  - Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.
  - Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).
  - Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.
  - Plate Covers - 4 adhesive strips.
- Pooled Normal Serum control, 2 levels (Solomon Park Research Laboratories, 12815 NE 124th St. Suite 1, Kirkland, WA 98034). Store at -70°C for up to 4 years, at refrigerated temperature for 1 day and at room temperature for up to 4 hours. Allow the control to reach room temperature (18-25°C) and mix thoroughly by inversion before use.
- MilliQ water
- 2mL microcentrifuge tubes and caps
- 1.5mL Eppendorf snap-cap tubes
- Rainin p20, p250 and p1000 pipets and pipet tips
- Bioexpress 500uL 96-well plate
- Orbital microplate shaker (Labnet International, Woodbridge NJ)
- Rainin 12-channel pipet and pipet tips
- Reagent reservoirs

## Results

1. Quality control results must be within acceptable ranges.
2. Review plate reader printout for result flags and take appropriate action. The plate reader will flag samples with an "R" in the "R" column that have an OD that is less than the lowest standard or greater than the highest standard. Note in the results sheet that the sample requires re-dilution.
3. Report all values calculated by the plate reader software. For values <15.6 ug/mL, indicate that result is "below the sensitivity of the assay stated by the manufacturer."
4. Results are downloaded from the plate reader onto a spreadsheet, see *ARDL General ELISA procedure*.



- Results must be reviewed for accuracy by a second technologist before reporting, see *ARDL General ELISA procedure*.

### Analytical Range

15.6 - 250 ug/mL. Samples are initially diluted 1:2100 for assay. If a sample result is less than the lowest standard, it is re-assayed at a 1:700 dilution. If a sample result is greater than the highest standard, it is re-assayed at a 1:4200 dilution. ARDL has determined the maximum dilution is 1:8400.

### Critical Values

None

### Assay performance

Analytical Range	15.6 – 250 ug/mL
Limit of Detection (blank + 2SD) (Determined by the manufacturer measuring the zero standard 20 times and adding two SD above the mean. See appendix D – kit package insert)	0.65 ug/mL
Recovery (As determined by the manufacturer. See appendix D-kit package insert)	93% - 112%
Recovery, normal samples (Performed on 5 normal serum samples and spiked with one part standard at concentration 250 pg/mL to four parts normal sample )	94% - 104%
Recovery, uremic samples (Performed on 6 uremic serum samples and spiked with one part standard at concentration 250 pg/mL to four parts uremic sample )	94% - 96%
Intra-assay %CV (10 within day replicates at a concentration of 116 ng/mL)	5.72%
Inter-assay %CV (10 between day replicates at a concentration of 203 ng/mL)	5.66%
Within day duplicate range, serum	11%
Within day duplicate range, EDTA plasma	13.5%
Reference range* (See Appendix B)	55.9 – 476 serum 42.8 – 415 EDTA plasma

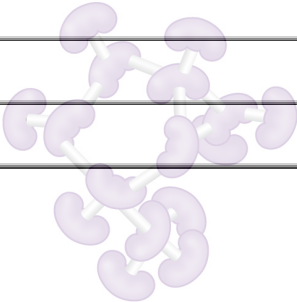
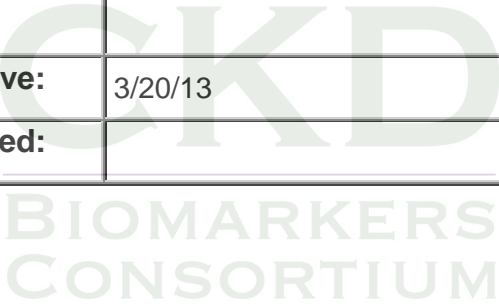
### Computer Entry

- Results are downloaded from the plate reader onto a spreadsheet, see *ARDL General ELISA procedure*.
- Report all values calculated by the plate reader software. For values <15.6 ng/ml, indicate that result is “below the reportable range of the assay as stated by the manufacturer.”

### References

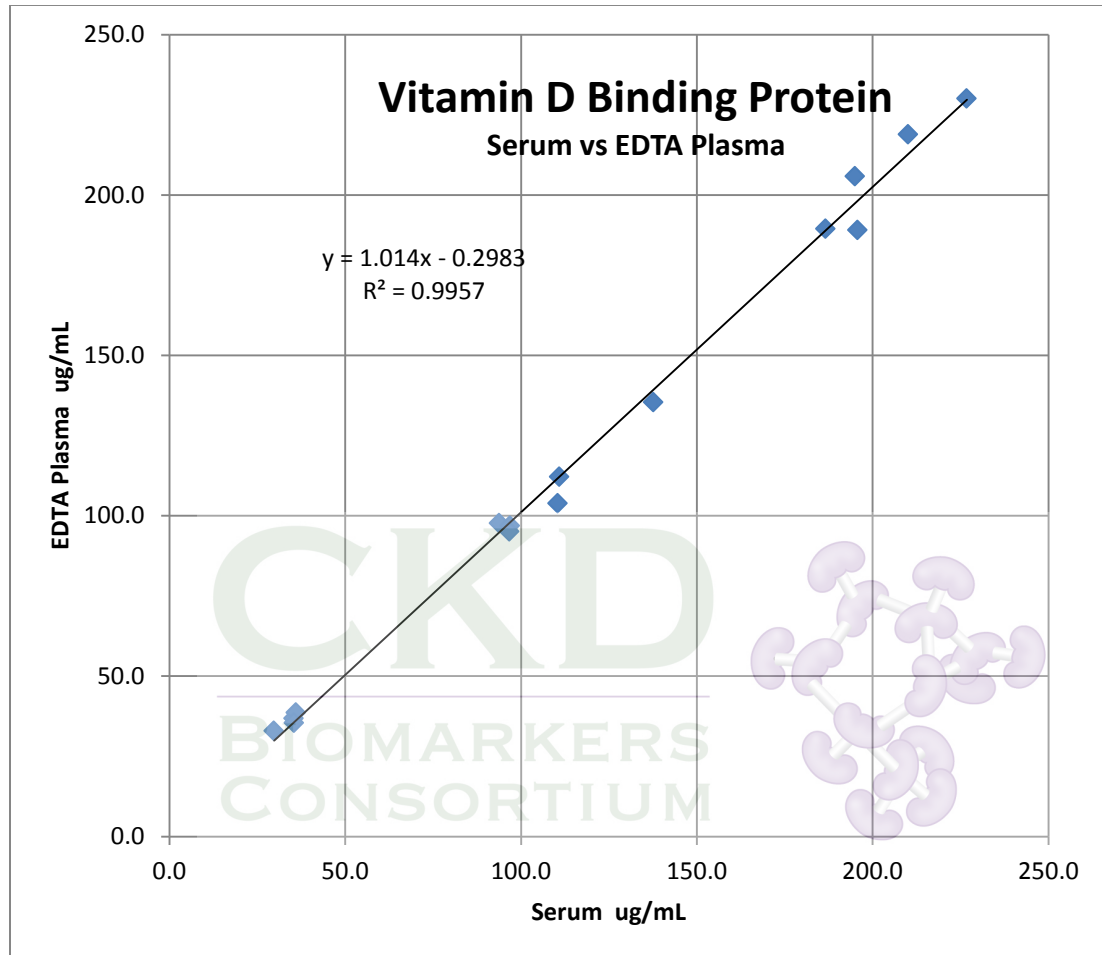
- Quantikine ELISA, Human Vitamin D BP Immunoassay, Catalog Number DVDBPO R&D Systems, Inc, Minneapolis, MN.\
- Armbruster DA, Pry T. Limit of the Blank, Limit of Detection and Limit of Quantitation. Clin Biochem Rev Vol 29 Suppl (i) August 2008.

<b>External Ref:</b>	
<b>Internal Ref:</b>	ARDL SpectraMax 190 plate reader procedure ARDL General ELISA procedure
<b>Appendixes</b>	A. Comparison of Serum and EDTA plasma B. Reference Range Validation C. Daily Worksheet D. Package insert Quantikine® Human Vitamin D Binding Protein Human Vitamin D BP Immunoassay
<b>Written By and Signature:</b>	Chris Zaun, Naomi Hanson, Valerie Arends
<b>Date Written:</b>	3/2013
<b>Approved by:</b>	Anthony Killeen, MD, PhD
<b>Date Effective:</b>	3/20/13
<b>Date Retired:</b>	



## Appendix A

### Comparison of Serum vs EDTA Plasma 4/2013



## Appendix B

### Reference Range Validation

\*\*Package insert reference range from apparently healthy volunteers

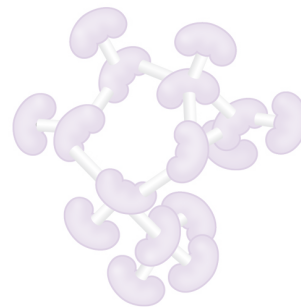
Sample Type	Number	ug/mL	ug/mL	SD ug/mL
Serum	35	248	55.9 - 473	102
EDTA Plasma	35	236	42.8 - 415	94.6

Local "healthy" (no known diseases) volunteers

Sample Type	Number	Mean	Range	SD ug/mL
Serum	15	251.6	62.4 - 476.3	145.4
EDTA Plasma	15	254.5	69.1 - 483.1	147.8

sample ID	serum	EDTA plasma
	ug/mL	ug/mL
1	411.1	397.0
2	232.9	235.5
3	197.0	205.1
4	476.3	483.1
5	203.4	203.5
6	231.9	218.1
7	74.2	77.3
8	289.1	284.3
9	409.6	432.2
10	62.4	69.1
11	75.5	81.0
12	441.2	459.6
13	74.5	74.4
14	392.0	397.8
15	203.1	199.6

Ave	251.6	254.5
Min	62.4	69.1
Max	476.3	483.1
SD	145.4	147.8



## Appendix C

### Example documentation of Daily Worksheet

#### Human Vitamin D binding protein R&D Quantikine Immunoassay Worksheet

assay set up manually

Reagent	Lot	Date
VDP kit	303948	7/22/2013
Microplate	299110	7/22/2013
Conjugate	299892	8/12/2013
Standard	299111	7/23/2013
Assay Diluent R01-12	295312	9/23/2013
Calibrator dil R06-11	298434	10/24/2013
Color reagent A	298398	1/10/2014
Color reagent B	298399	1/14/2014
Wash buffer	302111	5/2/2014
Stop solution	301920	4/22/2014

plate shaker #: \_\_\_\_\_

Sample dilution: **1:2100**

1:70 dilution 10uL sample + 690uL diluent

1:2100 dilution 10uL (1:70) sample + 290uL diluent  
need 100uL sample per well

	1 (1h)	2 (2h)	200uL conjugate	200uL substrate
Incubation	1 (1h)	2 (2h)		3 (30min)
Temp	RT (shaker 450 rpm)	RT (shaker 450 rpm)		RT (dark)
In/Out	4 washes	4 washes		50uL stop

Spectrophotometer	
$\lambda_1$	450
$\lambda_2$	540
4-parameter standard curve	

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	0	4 serum	8 serum	12 serum	pool 10X	pool 10X	4 serum	8 serum	12 serum	1 serum	CN
B	Std 5 (250)	0	4 serum	8 plasma	12 plasma	pool 10X	pool 10X	4 plasma	8 plasma	12 plasma	1 plasma	CN
C	Std 4 (125)	1 serum	5 serum	9 serum	13 serum	pool 10X	16 serum	5 serum	9 serum	13 serum	2 serum	CQ
D	Std 3 (62.5)	1 plasma	5 plasma	9 plasma	13 plasma	pool 10X	16 plasma	5 plasma	9 plasma	13 plasma	2 plasma	CQ
E	Std 2 (31.3)	2 serum	6 serum	10 serum	14 serum	pool 10X	CN	6 serum	10 serum	14 serum	16 serum	CQ
F	Std 1 (15.6)	2 plasma	6 plasma	10 plasma	14 plasma	pool 10X	CN	6 plasma	10 plasma	14 plasma	16 plasma	CQ
G	Pool CQ	3 serum	7 serum	11 serum	15 serum	pool 10X	3 serum	7 serum	11 serum	15 serum	CN	0
H	Pool CQ	3 plasma	7 plasma	11 plasma	15 plasma	pool 10X	3 plasma	7 plasma	11 plasma	15 plasma	CN	0

*Appendix D Package insert for Quantikine® Human Vitamin D Binding Protein Immunoassay  
manufactured by R&D Systems, Minneapolis, MN 55413*

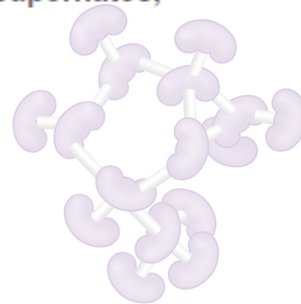
# Quantikine®

## Human Vitamin D Binding Protein Immunoassay

Catalog Number DVDBP0

**For the quantitative determination of human Vitamin D Binding Protein (VitD BP) concentrations in cell culture supernates, serum, plasma, saliva, urine, and human milk.**

CKD  
BIOMARKERS  
CONSORTIUM



***This package insert must be read in its entirety before using this product.***

**FOR RESEARCH USE ONLY.  
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**



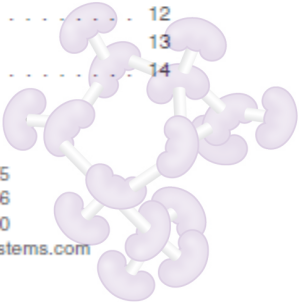
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## INTRODUCTION

Vitamin D binding protein (VitD BP), also known as DBP and Gc-globulin, is a 58 kDa glycoprotein that circulates at a high concentration in the serum and serves as a carrier protein for vitamin D. The transport of vitamin D by VitD BP is important for the function of a wide variety of tissues, and alterations in VitD BP activity contribute to the development of many diseases (1). VitD BP binds both the 25(OH) and the hormonally active 1,25(OH)<sub>2</sub> forms of vitamin D. There are three dominant alleles of VitD BP (Gc1f, Gc1s, and Gc2) and a large number of minor polymorphisms (1 - 3). VitD BP is structurally related to the major serum proteins albumin and  $\alpha$ -fetoprotein. These proteins share an internal disulfide bond pattern which divides the molecules into three domains (4, 5). Mature human VitD BP shares 77% amino acid (aa) sequence identity with mouse and rat VitD BP. VitD BP is primarily expressed in hepatocytes and to a lesser extent in the kidney (6). It delivers vitamin D into cells by Megalin-mediated endocytosis (7, 8). VitD BP is differentially O-glycosylated depending on the isoform (9 - 12). A selectively deglycosylated form of VitD BP known as macrophage activating factor (MAF) is generated by the sequential removal of carbohydrates by B cell  $\beta$ -galactosidase followed by T cell sialidase (10). In addition to promoting macrophage activation and differentiation, MAF blocks the angiogenic effects of FGF basic, VEGF, and Angiopoietin-2 on vascular endothelial cells in a CD36-dependent process (14 - 16). MAF administration in mouse xenograft models leads to reduced neovascularization and tumor regression (13). Complete deglycosylation of VitD BP destroys its anti-angiogenic effect (13).

VitD BP enhances the chemotaxis of monocytes and neutrophils to the activated complement component C5a or C5a des Arg (a C-terminally processed form of C5a) (17, 18). It does not enhance movement toward the monocyte chemoattractant f-Met-Leu-Phe or function as an independent chemotactic factor (17). VitD BP binding to C5a des Arg allows a greater number of C5a molecules to bind to the neutrophil (19). Neutrophil activation results in a dramatic increase of binding sites for VitD BP and neutrophil chemotaxis (20). VitD BP interacts with the chondroitin sulfate portion of CD44 on neutrophils and monocytes. CD44 as well as Annexin A2 are required for VitD BP to enhance chemotaxis (21). Thrombospondin-1, which is released by platelets during clotting and acts through CD36, is required to develop the full chemotactic cofactor function of VitD BP (18). The chemotactic cofactor property of VitD BP is eliminated by binding to 1,25(OH)<sub>2</sub> vitamin D, but it is not altered by binding to 25(OH) vitamin D or actin (22). VitD BP binds monomeric G-actin released from necrotic cells and clears it from the circulation (23, 24).

Circulating levels of VitD BP are decreased in liver failure, liver disease, and cystic fibrosis due to more rapid clearance (25 - 27). Patients with various cancers have an elevated serum level of alpha-N-acetylgalactosaminidase, an enzyme which removes the N-linked carbohydrates on VitD BP (28). This action does not alter the level of VitD BP protein but prevents the formation of the anti-angiogenic MAF (28).

The Quantikine Human Vitamin D Binding Protein immunoassay is a 3.5 hour solid phase ELISA designed to measure VitD BP in cell culture supernates, serum, plasma, saliva, urine, and human milk. It contains natural human VitD BP Standard. The Quantikine Human Vitamin D Binding Protein kit can be used to determine mass values for naturally occurring human VitD BP.

## PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VitD BP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VitD BP present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for VitD BP is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VitD BP bound in the initial step. The color development is stopped and the intensity of the color is measured.

## LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by ligands, proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine immunoassay, the possibility of interference cannot be excluded.

## MATERIALS PROVIDED

**VitD BP Microplate** (Part 893305) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against VitD BP.

**VitD BP Conjugate** (Part 893306) - 21 mL of a mouse monoclonal antibody against VitD BP conjugated to horseradish peroxidase with preservatives.

**VitD BP Standard** (Part 893307) - 1000 ng of natural human VitD BP in a buffer with preservatives; lyophilized.

**Assay Diluent RD1-19** (Part 895467) - 11 mL of a buffered protein base with preservatives.

**Calibrator Diluent RD6-11** (Part 895489) - 4 vials (21 mL/vial) of a buffered protein base with preservatives.

**Wash Buffer Concentrate** (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

**Color Reagent A** (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

**Color Reagent B** (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

**Stop Solution** (Part 895032) - 6 mL of 2 N sulfuric acid.

**Plate Covers** - 4 adhesive strips.

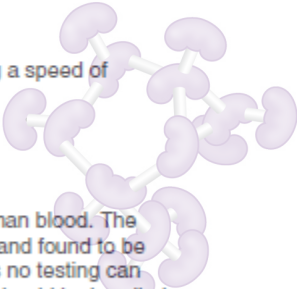
**STORAGE**

<b>Unopened Kit</b>	Store at 2 - 8° C. Do not use past kit expiration date.	
<b>Opened/ Reconstituted Reagents</b>	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1-19	
	Calibrator Diluent RD6-11	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*

\*Provided this is within the expiration date of the kit.

**OTHER SUPPLIES REQUIRED**

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Dilution tubes.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution.



**PRECAUTIONS**

The VitD BP Standard contains Vitamin D Binding Protein derived from human blood. The source material was tested at the donor level using FDA licensed methods and found to be non-reactive for anti-HIV-1/2, anti-HCV, and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, the Standard should be handled as if capable of transmitting infection.

Calibrator Diluent RD6-11 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

High concentrations of VitD BP are found in saliva. Take necessary precautions to protect kit reagents.

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

## SAMPLE COLLECTION AND STORAGE

**Cell Culture Supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Plasma** - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Note:** Citrate plasma has not been validated for use in this assay.

**Saliva** - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer and assay immediately, or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Note:** Saliva values are decreased when a Salivette<sup>®</sup> or other collection device is used. When stored at  $2 - 8^{\circ}\text{C}$ , saliva sample values increase over time.

**Urine** - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter and assay immediately or aliquot and store at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Human Milk** - Centrifuge for 15 minutes at 10,000 x g at  $2 - 8^{\circ}\text{C}$ . Collect the aqueous fraction and repeat this process twice for a total of 3 times. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

## SAMPLE PREPARATION

Serum and plasma samples require a 2000-fold dilution. A suggested 2000-fold dilution can be achieved by adding 20  $\mu\text{L}$  of sample to 980  $\mu\text{L}$  of Calibrator Diluent RD6-11. Complete the 2000-fold dilution by adding 25  $\mu\text{L}$  of the diluted sample to 975  $\mu\text{L}$  Calibrator Diluent RD6-11.

Saliva samples require a 2-fold dilution. A suggested 2-fold dilution is 100  $\mu\text{L}$  of sample + 100  $\mu\text{L}$  of Calibrator Diluent RD6-11.

Human milk samples require at least a 20-fold dilution. A suggested 20-fold dilution is 20  $\mu\text{L}$  of sample + 380  $\mu\text{L}$  of Calibrator Diluent RD6-11.

Salivette<sup>™</sup> is a registered trademark of Sarstedt, Inc.

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

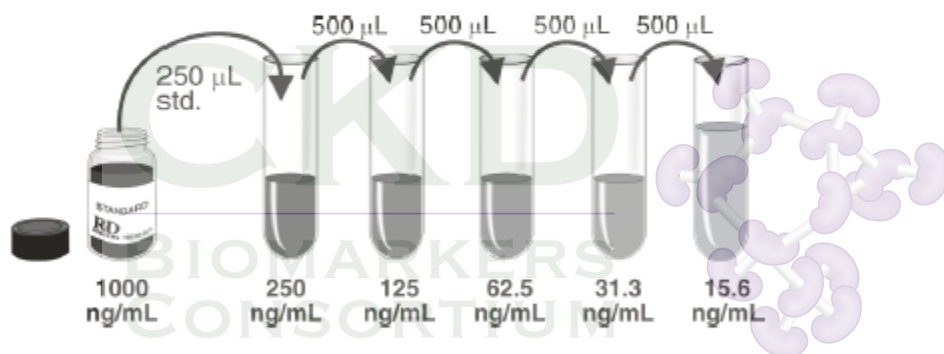
**Note:** *High concentrations of VitD BP are found in saliva. Take necessary precautions to protect kit reagents.*

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200  $\mu$ L of the resultant mixture is required per well.

**VitD BP Standard** - Reconstitute the VitD BP Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 1000 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 750  $\mu$ L of Calibrator Diluent RD6-11 into the 250 ng/mL tube. Pipette 500  $\mu$ L of Calibrator Diluent RD6-11 into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 250 ng/mL standard serves as the high standard. Calibrator Diluent RD6-11 serves as the zero standard (0 ng/mL).





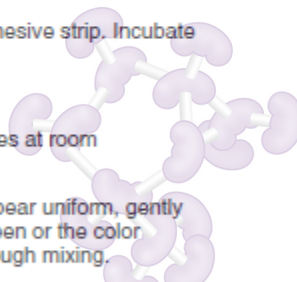
## ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

**Note:** High concentrations of VitD BP are found in saliva. Take necessary precautions to protect kit reagents.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100  $\mu$ L of Assay Diluent RD1-19 to each well.
4. Add 50  $\mu$ L of Standard, control, or sample\* per well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at  $500 \pm 50$  rpm. A plate layout is provided as a record of standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400  $\mu$ L) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.
6. Add 200  $\mu$ L of VitD BP Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200  $\mu$ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. **Protect from light.**
9. Add 50  $\mu$ L of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

\*Samples may require dilution. See Sample Preparation.



## ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.



2. Add 100  $\mu$ L Assay Diluent RD1-19 to each well.



3. Add 50  $\mu$ L Standard, control, or sample\* to each well.  
Incubate for 1 hour at RT on the shaker.



4. Aspirate and wash 4 times.



5. Add 200  $\mu$ L Conjugate to each well. Incubate for 2 hours at RT on the shaker.



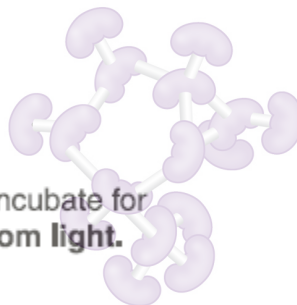
6. Aspirate and wash 4 times.



7. Add 200  $\mu$ L Substrate Solution to each well. Incubate for 30 minutes at RT on the benchtop. Protect from light.



8. Add 50  $\mu$ L Stop Solution to each well.  
Read at 450 nm within 30 minutes.  
 $\lambda$  correction 540 or 570 nm



\*Samples may require dilution. See Sample Preparation.

CALCULATION OF RESULTS

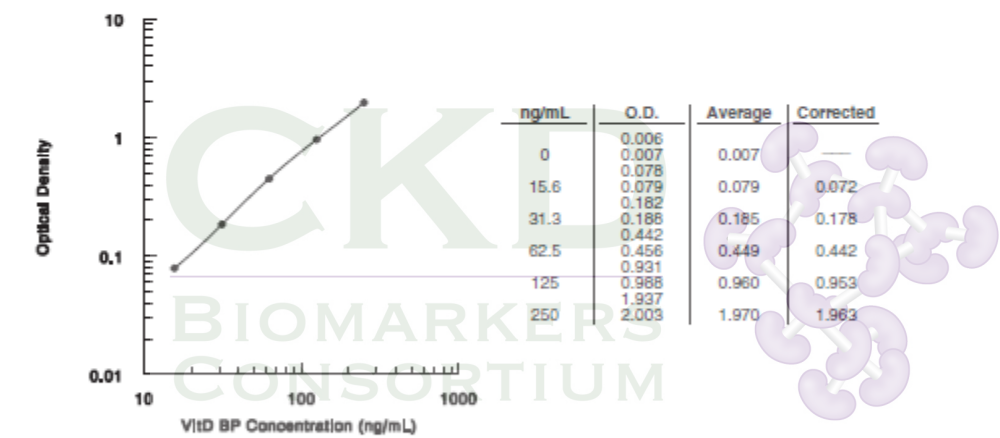
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the VitD BP concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



### TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

### PRECISION

#### Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

#### Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	33.0	128	180	52.9	105	164
Standard deviation	1.88	7.42	11.2	2.70	6.30	12.1
CV (%)	5.7	5.8	6.2	5.1	6.0	7.4



### RECOVERY

The recovery of VitD BP spiked to three different levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	104	93 - 112%

### LINEARITY

To assess the linearity of the assay, samples containing or spiked with high concentrations of VitD BP were serially diluted with Calibrator Diluent RD6-11 to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	Heparin plasma* (n=4)	EDTA plasma* (n=4)
1:2	Average % of Expected	100	99	100	100
	Range (%)	98 - 103	97 - 101	98 - 102	97 - 103
1:4	Average % of Expected	98	98	97	99
	Range (%)	97 - 99	94 - 100	94 - 102	96 - 104
1:8	Average % of Expected	97	96	98	96
	Range (%)	94 - 100	89 - 102	94 - 103	93 - 100

		Saliva* (n=4)	Urine (n=4)	Human milk* (n=4)
1:2	Average % of Expected	96	104	99
	Range (%)	90 - 103	91 - 114	98 - 101
1:4	Average % of Expected	99	104	98
	Range (%)	96 - 103	86 - 116	96 - 99
1:8	Average % of Expected	96	105	98
	Range (%)	—	85 - 115	97 - 101

\*Samples were diluted prior to assay as described in the Sample Preparation section.

### SENSITIVITY

Forty six assays were evaluated and the minimum detectable dose (MDD) of VitD BP ranged from 0.15 - 3.74 ng/mL. The mean MDD was 0.65 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

### CALIBRATION

This immunoassay is calibrated against a highly purified natural Vitamin D Binding Protein from human source material.

## SAMPLE VALUES

**Serum/Plasma/Saliva/Urine/Human Milk** - Samples from apparently healthy volunteers were evaluated for the presence of VitD BP in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Serum* (n=35)	248	55.9 - 473	102
Heparin plasma* (n=35)	237	41.9 - 471	99.0
EDTA plasma* (n=35)	236	42.8 - 415	94.6

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Saliva* (n=5)	306	153 - 724	236
Human milk* (n=5)	5386	3283 - 10,000	2851

\*Samples were diluted prior to assay as described in the Sample Preparation section.

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Urine (n=19)	101	79%	ND - 250

ND = Non-detectable

**Cell Culture Supernates** - Human peripheral blood leukocytes (PBL) were cultured in DMEM supplemented with 5% fetal calf serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed on days 1 and 6 and assayed for levels of human VitD BP. All samples measured below the lowest standard, 15.6 ng/mL.

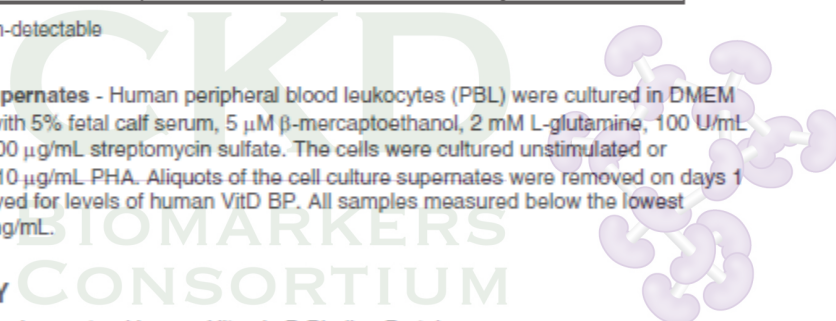
## SPECIFICITY

This assay recognizes natural human Vitamin D Binding Protein.

Recombinant human AFP was prepared at 50 ng/mL in Calibrator Diluent and in diluted human serum and assayed. No significant cross-reactivity or interference was observed.

Recombinant human Albumin was prepared at 20 mg/mL in Calibrator Diluent and in diluted human serum and assayed. No significant cross-reactivity or interference was observed.

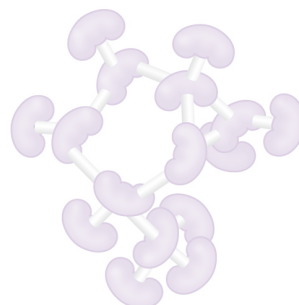
Human Vitamin D3 was prepared at 10 µg/mL in Calibrator Diluent and in diluted human serum and assayed. No significant cross-reactivity or interference was observed.





## REFERENCES

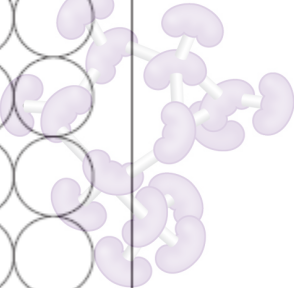
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**PLATE LAYOUT**

Use this plate layout to record standards and samples assayed.

1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	
9																	
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11																	
12																	
	A	B	C	D	E	F	G	H									



## CKD BioCon Specimen Labeling and Processing

04/05/2012 version 3.0

The goal of the BioCon sample labeling and processing SOP is to ensure that samples prepared at laboratories of origin and assayed at performance laboratories are prepared accurately, can be tracked centrally, and that the assay results can be incorporated efficiently and accurately into the central database for subsequent analysis. To achieve this goal, each sample created for BioCon will need a unique BioCon ID number (barcode) that is created by the Coordinating Center. An overarching principle of this SOP is to establish procedures that protect against errors in sample identities while remaining sufficiently flexible to permit operation within established workflow processes of the various laboratories. The general approach to sample labeling, processing and identity management is as follows:

### **Procedures for laboratories of origin**

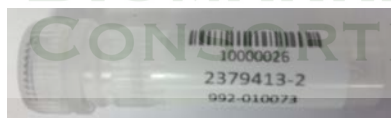
In summary, the laboratories of origin will be processing parent samples from a variety of studies. These parent samples will be aliquoted into tubes that will be labeled with unique BioCon identifiers and barcodes that are generated by the Coordinating Center. Additionally, the laboratories of origin will place the BioCon aliquots into boxes, which will also be labeled with unique BioCon identifiers and barcodes that are generated by the Coordinating Center, and ship them to performance labs for assaying and/or storage. To ensure that parent samples are aliquoted into tubes with the appropriate BioCon labels, a QC check will be implemented to validate that aliquots are properly matched to parent samples. A QC check will also be performed to ensure that aliquots are properly boxed and sent to the appropriate performance labs. To facilitate this process, the following procedures should be followed:

1. Protocol and project specific spreadsheets containing information related to parent samples to be aliquoted will be sent to the Coordinating Center. At a minimum, the spreadsheets should contain the following information:
  - a. Unique sample identifier (such as PID, sample barcode, etc.)
  - b. Additional identifying information as available (such as collection date, visit number, etc.)

In addition to these parent sample identifiers, the number of aliquots into which each parent sample will be divided, and the performance lab destination of each aliquot should be indicated. These data will be loaded into the central BioCon database at the Coordinating Center.

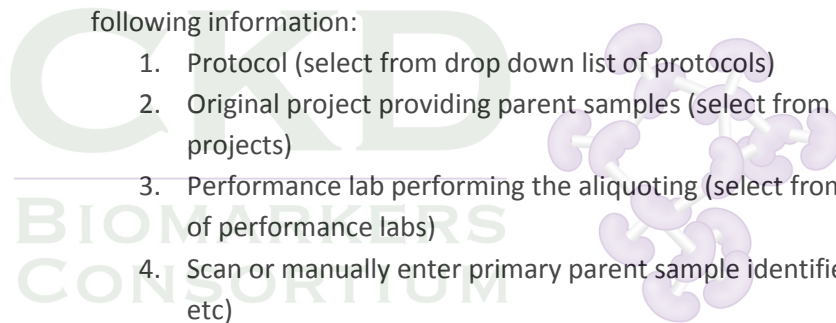
2. Once this information is loaded into the central BioCon database, a set of aliquot records will be created with the following information:
  - a. Unique BioCon Aliquot ID
  - b. Identifier that links to parent sample
  - c. Origin Lab identifier
  - d. Origin project Identifier
  - e. BioCon Protocol Identifier

3. Once the aliquot records have been created in the database, a set of records will be created for the boxes that will be used to transfer the aliquots to the performance labs. The number of box records created will be based on the number of aliquots that will be transported to each performance lab. Box records will contain the following information:
  - a. Unique BioCon Box ID
  - b. Origin Lab Identifier
  - c. Origin Project Identifier
  - d. BioCon Protocol Identifier
  - e. Performance Lab Identifier
  
4. Once the aliquots and box records have been defined in the database a, set of standard delimited text files will be created and sent to either the BioCon Central Lab or directly to the lab of origin, depending on which entity is physically creating the labels. The lab performing the aliquoting will need to inform the Coordinating Center the order in which the labels should be printed (if printed at the Fisher Central Lab) or the order according to which labels should be represented in the label file. These files will be used to inform the label making software wherever the labels are being printed, and will permit creation of a set of aliquot and box labels to be used during the aliquoting process. These labels will contain the following information:
  - a. Aliquots
    - i. Unique BioCon Aliquot ID and barcode
    - ii. Identifier 1 linking back to parent sample (for example, PID, original barcode, ...)
    - iii. Identifier 2 linking back to parent sample (for example, Collection date, Vnum, ...)
  - b. Boxes
    - i. Unique Box ID and barcode
    - ii. Box Number and Destination (Performance Lab)



control measures must be followed to ensure that parent samples are being aliquoted into tubes containing aliquot labels that were created specifically for the parent sample. The origin lab can either follow an internal QC process using its own technology and infrastructure, or the lab can follow a QC process defined by the Coordinating Center that utilizes a real time checking mechanism while connected to BioCon's central database.

- a. If the lab follows its own QC process, that process must be defined ahead of time and approved by the QC Committee. In addition, electronic data must be given to the Coordinating Center that denotes which parent samples were matched to each aliquot. These electronic data will then be compared to the data in the central database to ensure that aliquots were matched the appropriate parent samples. Additionally, electronic data must be provided that indicate which aliquots were placed into each of the BioCon boxes to be sent the performance labs. These data will also be given to the Coordinating Center for QC to ensure that that each performance lab is getting their designated aliquots.
- b. If a lab of origin is going to follow the QC process defined by the Coordinating Center, the following steps should be executed:
  - i. Connect to the BioCon central Data Management System (DMS) and enter the following information:
    1. Protocol (select from drop down list of protocols)
    2. Original project providing parent samples (select from drop down list of projects)
    3. Performance lab performing the aliquoting (select from drop down list of performance labs)
    4. Scan or manually enter primary parent sample identifier (PID, barcode, etc)
  - ii. Scan aliquot ID into aliquot field on data entry screen.
  - iii. Press "check aliquot" button.
  - iv. If the scanned aliquot ID matches an aliquot ID predefined for the parent, the aliquot record in the central database will be updated with information related to the user that performed the scan and when the scan occurred. If the aliquot ID does not match an aliquot ID predefined for the parent, an error message will be displayed.
  - v. The scanned aliquots will then be placed into boxes with BioCon labels.
  - vi. The lab technician will subsequently perform the following steps to associate the aliquot samples with the box into which they were placed:
    1. Open BioCon Box module in the DMS
    2. Select the following information from the pull down lists on the screen: Protocol ID, Project ID, Performance Lab
    3. Scan the box barcode into the "Box ID" field on the data entry screen and press the "Get Box" button.
    4. Pull aliquot from box, scan it into the Aliquot ID field and press "Check Aliquot" button.

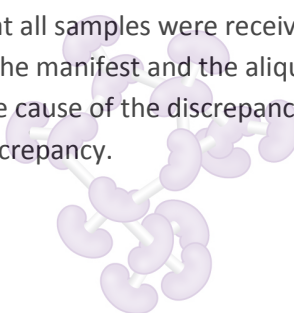


5. Place aliquot back in box, pull next aliquot, and scan it into the Aliquot ID field and press “Check Aliquot” button. Continue this process until all aliquots in a box are scanned and thus associated with the box.
  6. For the next box, repeat step 3 above, then scan each aliquot in that box as indicated in steps 4 and 5. Continue until all boxes and aliquots are scanned.
- c. Once the parent samples are aliquoted and boxed, and the appropriate QC has been performed, as defined above, the lab of origin will create a shipping manifest and send the samples to the performance labs. As part of this process, the manifest will be sent to both the performance lab and the Coordinating Center, and a copy of the manifest will be included with the shipment itself.

#### **Procedure for performance laboratories**

The performance laboratories will receive the aliquot shipments and log them into the performance lab’s local specimen tracking system that is routinely used at the laboratory as standard practice. As part of this process, the laboratory will compare the physical aliquots received to the electronic manifest and ensure that all of the expected samples were received. Once this task is complete, the performance lab will provide the Coordinating Center with confirmation that all samples were received and accounted for as expected. If there is a discrepancy between the manifest and the aliquots received, the performance lab will contact the lab of origin to determine the cause of the discrepancy. The performance lab will notify the Coordinating Center of any discrepancy.

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CONSORTIUM





## Prospective Laboratory QC Evaluation

This document represents an expansion of the recommendations found in the Biocon QC Committee Policies. It provides a more comprehensive and detailed description of proposed data flows within the consortium and the types of data and analyses that are needed to maximize data quality and integrity.

### Performance Laboratories:

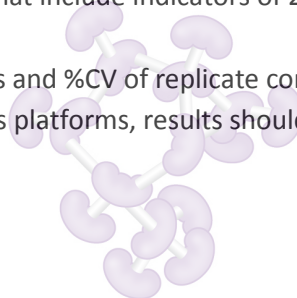
1. Each performance laboratory will provide to the QC Committee written descriptions of the data transfer processes within their laboratory and the format of the data to be transmitted to the Coordinating Center. This report should include a brief description of each step in data handling from generation of the results at the instrument to the transmission of the final results to the Coordinating Center. Particular attention should be paid to the steps in the process that are done manually, where data are transferred from one type of database to another, and that represent changes from usual laboratory practice (implemented explicitly for Biocon), as these are the steps where errors are most likely to occur. The performance laboratories are encouraged to the extent possible to use their standard data streams from their instruments as the source for data to be transmitted to the Coordinating Center rather than creating custom data files as this will reduce the chance of error in data transmission. These reports will be reviewed by the QC Committee and posted to the Biocon website.
2. Each performance laboratory will transmit Biocon data from their laboratory to the Coordinating Center at appropriate intervals. For large studies involving many samples, monthly (or more frequent) data transmissions will be completed. In the case of smaller studies, reports will be sent upon completion of all samples from a particular laboratory of origin or protocol. Prior approval by the QC Committee will be needed when the latter approach is used. The data transmitted for each sample should include:
  - a. Assay values linked to the appropriate Biocon ID
  - b. Assay date
  - c. Platform used for the assay—even if a performance laboratory has several identical instruments performing the same assay, the specific instrument should be identified.
3. Each performance laboratory shall collect and maintain the lot numbers of all reagents and calibrator materials that were used for each measurement and the dates when lots were changed, and shall provide this information to the Coordinating Center if questions arise regarding a particular analyte.
4. Each laboratory shall collect and review their internal quality control data according to their standard operating procedures and shall provide these reports and data to the Coordinating Center if questions arise regarding a particular analyte.
5. Each laboratory shall enroll in external proficiency testing programs (e.g., CAP Surveys, American Association of Bioanalysts, CDC proficiency testing programs, etc.) where such programs are available, and shall report their results upon request of the Coordinating Center if questions

arise regarding a particular analyte. Results of proficiency testing using pooled Biocon samples shall be reported to the Coordinating Center

**Coordinating Center:**

1. Once the Coordinating Center receives the data from any performance laboratory, the data will be merged with existing data previously provided by that laboratory as well as data previously received from all relevant laboratories of origin. Discrepancies related to sample identity will be reported to the appropriate laboratories (performance or origin) for resolution.
2. External quality control reports will be generated within one week of data receipt for review by the performance laboratory, the protocol PIs, the PI from the laboratory of origin, and the QC Committee Chair. These reports will include:
  - a. Plots of relative frequency distributions of the assays for the primary sample results
  - b. Scatter plots of the raw assay results for the blind replicate samples with appropriate correlation measures (e.g., intra-class correlations, Deming regression, or Passing-Bablok regression—to be determined in discussion with laboratory directors).
  - c. Bland-Altman plots of the replicate samples that include indicators of 2 SD and 3 SD from the mean.
  - d. Raw outputs of replicate data including values and %CV of replicate comparisons.
  - e. For data measured across assay runs or across platforms, results should also be presented by date and platform.

CKD  
BIOMARKERS  
CONSORTIUM



**The following are considered by the CKD Biomarkers Consortium to be unacceptable methods for thawing frozen samples and should not be used:**

1. Allowing samples to sit out on a bench top to thaw at room temperature
2. Use of uncontrolled heat sources

**The following are considered by the CKD Biomarkers Consortium to be acceptable methods for thawing frozen samples:**

1. Placing samples in a circulating water bath at a specified temperature
2. Placing samples in an incubator at a specified temperature until samples are thawed, but still cold
3. Placing samples in a heating block at a specified temperature
4. Use of heat sources such as a fan from an electronic device when the tested air temperature is between 25 and 35°C.

**The following precautionary measures should be observed when thawing samples:**

1. If samples are thawed in a water bath:
  - a. Ensure that the labels will not come off of the tubes in the water bath
  - b. Ensure the use of inks on labels and tubes that will not deteriorate when wet
  - c. Do not submerge the tubes completely in the water
  - d. Procedures should be followed to ensure that the caps will not get wet
  - e. Ensure that the water is moving and is not warmer than room temperature
  - f. Wipe the outside of the tube thoroughly after removing from the water bath to ensure that all moisture has been removed prior to removing the cap
2. If samples are thawed in a 30°C incubator:
  - a. Check thawing progress of samples at specified intervals to avoid over-warming
  - b. Remove samples as soon as they are thawed to ensure that samples remain cold
3. For all thawing methods, refer to the analyte-specific SOP for recommended thawing temperatures and for recommendations regarding mixing, vortexing, or otherwise agitating samples.