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Title: Human Urine R-Plex Protocol on MSD

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Document History			
Date	**Comment**	**Reviewer**	**Signature**
11/05/2020	New SOP	Venkata Sabbisetti	
11/05/2020		Manasi Jogalekar	
11/05/2020		Joseph V. Bonventre	

1. Purpose

This Standard Operating Procedure (SOP) describes the process for the quantitative assessment of the ectodomain of Uromodulin (UMOD) in human urine specimens on the Meso Scale Discovery (MSD) platform.

2. Introduction

Uromodulin is excreted in urine in abundant quantities as a result of the proteolytic cleavage in the kidney. This 105kDa protein consists of 616 amino acids. Uromodulin serves as a critical indicator of kidney function as the urine levels of this protein play a key role in the diagnosis and prognosis of kidney diseases. This SOP describes an assay for studies designed to evaluate the utility of uromodulin as a biomarker to predict the progression of kidney disease.

3. Sample Collection, Handling & Storage

Frozen urine samples received from investigators by the central laboratory will be immediately stored after ID QC evaluation at -80°C. 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. Sample dilution ratio of 1:100 will be used. Diluent 37 (MSD® Cat# R50AF-6) will be used for sample dilution before measurement.

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 microplate (Fisher Scientific, catalog # 125655010)
- BioTek EL406 Washer/Dispenser
- Refrigerated Centrifuge (Eppendorf 5430R Rotor F-35-6-30)
- Vortex
- VIAFLO II Electronic pipettes
    - VIAFLO Electronic Pipets: single (Part # 4013), 8 (Part # 4624) and 12 (Part # 4632 and 4633) channels.
- 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)
- Reagent Reservoirs
    - 100 ml reagent reservoir (Integra, catalog # 4322)
    - 25 ml reagent reservoir (Integra, catalog # 4312)
- Orbital Plate shaker (IKA MTS 2/4D51, Max speed 11000 rpm)
- 37°C incubator (Thermolyne, Type 41900)
- Tween 20 (Promega, catalog # H5151)
- 10X Phosphate Buffered Saline Solution (PBS) (Fisher Scientific, catalog #BP399-1)
- MSD Quickplex SQ 120 Reader (AI0AA-0)

- Diluent 37 (MSD, catalog # R50AF-6)
- Diluent 100 (MSD, catalog # R50AA-2)
- R-Plex Human Uromodulin Antibody Set (5 Plate Size) (MSD, catalog # F215G-3)
  - Biotin Human Uromodulin Antibody (catalog # C215G-3)
  - SULFO-TAG™ Human Uromodulin Antibody (catalog # D215G-3)
  - Human Uromodulin Calibrator (catalog # C015G-2)
- MSD GOLD Read Buffer (catalog # R92TG-4) or MSD Read Buffer T (4X) with Surfactant (catalog # R92TC-3)
- MSD GOLD 96-well Small Spot SA SECTOR Plate Pack (5 Plate) (MSD, catalog # L45SA-2)
- Nunc adhesive sealing tape (Thermo Scientific, catalog # 236370)
- MilliQ water (Type 1, reagent grade water) (MilliQ Academic, Cat # ZMQ600017)
- Control Urine Samples: three control urine samples that contain high, medium, low levels of Uromodulin. Samples obtained from Sushrut Waiker's kidney clinic patients, aliquoted (500 ul) and stored at – 80 °C.

4.3 Buffer preparation

- Washing buffer (0.05% Tween 20 1X PBS): 200 ml of 10X PBS + 1800 ml distilled H_2O + 1ml Tween 20
- Read Buffer (2X): If MSD GOLD Read Buffer is used, then no dilution is needed. If Read Buffer T is used, then mix 10 mL of Read Buffer T (4x) with 10 mL of DI water before use.

4.4 Preparation of the R-plex plate

- Combine 200 µL of the biotinylated capture antibody to 3.3 ml Diluent 100. Mix by vortex.
- Add 25 µL L of the above solution to each well of the provided MSD GOLD plate. Seal the plate with adhesive cover and shake at 700-900 rpm for 1h.
- Wash plate 3 times with 300 µL/well 1X PBS using Biotek plate washer protocol.
- Remove excess fluid. Plate is ready to use.

4.5 Sample preparation

- Sample tubes are arranged in 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 should be cold to touch after the samples are thawed).
- Vortex the tubes for 15 sec.
- Centrifuge tubes at 4°C in carbon fiber fixed angle rotor at 3000 rpm for 5 min.
- Samples will be diluted 1 in 100 by adding 198 µL of Diluent 37 to a dilution plate first, then 2 µL of sample, and mixing 10 times.

4.6 Calibrator preparation

- Thaw calibrator vials on ice for 30 min.
- Take out ice and place on vial holder for 5 min.
- Add to the assay diluent to make the calibrator solutions below (7 calibrator solutions plus a zero calibrator):
  - Prepare the highest calibrator by adding 15 µL of calibrator blend to 285 uL of Diluent 37.
  - Mix well by vortex.

- Prepare the next calibrator by transferring 60 µL of the highest calibrator to 180 µL of Diluent 37. Mix well by vortex. Repeat the 4-fold series dilutions 4 additional times to generate 7 calibrators.
- Use Diluent 37 as the zero calibrator.

Standard	UMOD (pg/ml)	Dilution (x)	Volume (µL)	Source	Diluent 37 (µL)
STD1	1000000	20	15	Calibrator (20x)	285
STD2	250000	4	60	STD1	180
STD3	62500	4	60	STD2	180
STD4	15630	4	60	STD3	180
STD5	3910	4	60	STD4	180
STD6	980	4	60	STD5	180
STD7	240	4	60	STD6	180
STD8	0	-	-	Diluent 37	180

4.7 Detection antibody

- Add 60 µL of 100X SULFO-TAG Anti-hu UMOD Antibody to 5940 uL of Diluent 37.

4.8 Assay procedure

- Bring all reagents to room temperature before starting.
- Prepare the calibrator and samples as mentioned above.
- Coat the plate with capture antibody as mentioned above. Cover with the adhesive strip, place on microplate shaker and shake at 700-900 rpm. Incubate for 1 hour at room temperature.
- Wash plate 3 times with 300 µL/well 1X PBS using Biotek plate washer protocol. Remove excess fluid by blotting on paper towels.
- Using a multi-channel pipette, transfer 25 µL of Diluent 37 to each well. Tap the plate gently on all sides.
- Using a multi-channel pipette, transfer 25 µL of standards, samples, controls to the Primary plate (follow plate map). Cover with adhesive strip, keep on microplate shaker and shake at 700-900 rpm. Incubate for 1 hour at room temperature. Note: 30 mins before the end of incubation, prepare the detection antibody solution.
- Wash the plate 3 times with 150 µL /well wash buffer. Blot the plate on paper towels
- Add 50 µL of detection antibody to each well. Cover with adhesive strip, keep on microplate shaker and shake at 700-900 rpm. Incubate for 1 hour at room temperature.
- Wash plate 3 times with 300 µL/well 1X PBS using Biotek plate washer protocol. Remove excess fluid by blotting on paper towels.
- Add 150 µL of 2x Read Buffer T to each well. No incubation required. Read the plate on the MSD instrument immediately. Make sure there are no bubbles in wells.

4.8.1 BioTek EL406 plate washer protocol:

Adjust Heights to 4 cm of bottom of U-PLEX plate
Program for 3x Wash
Plate Type: 96

Wash Component: Method
No. Cycles: 3
Wash Format
Shake/Soak? Y
Soak Duration (sec): 0
Shake before soak? Y
Shake Duration (sec) 2
Shake Intensity: 3 (17 cycles/s)
Prime After Soak? N
WashComponent: Dispense
Dispense Vol. (µl/well):300
Disp. Flow Rate: 4
Disp. Height: 120 (15.24 mm)
Horiz. Disp. Position: -37 (-1.692 mm)
Horiz. Y Disp. Position: 0 (0 mm)
Bottom Wash First?: Y
Bottom Disp. Vol (µl/well): 300
Bottom Disp. Flow Rate: 4
Bottom Disp. Height: 060 (7.620 mm)
Bottom Horiz. Position: 0 (0 mm)
Bottom Horiz. Y Position: 0 (0 mm)
Prime Before Start? N
Wash Component: Aspirate
Aspirate Height * ---
Horiz. Asp. Position: 50 (2.286 mm)
Horiz. Y Asp. Position: 0 (0 mm)
Asp. Rate: 2 (3.4 mm/s)
Asp. Delay (msec): 1500
Crosswise Asp? Y
Crosswise On: Final
Crosswise Height * ---
Crosswise Horiz. Position:0 (0 mm)
Crosswise Horiz. Y Position: 0 (0 mm)
Final Asp.? Y
Final Asp. Delay (msec): 1500

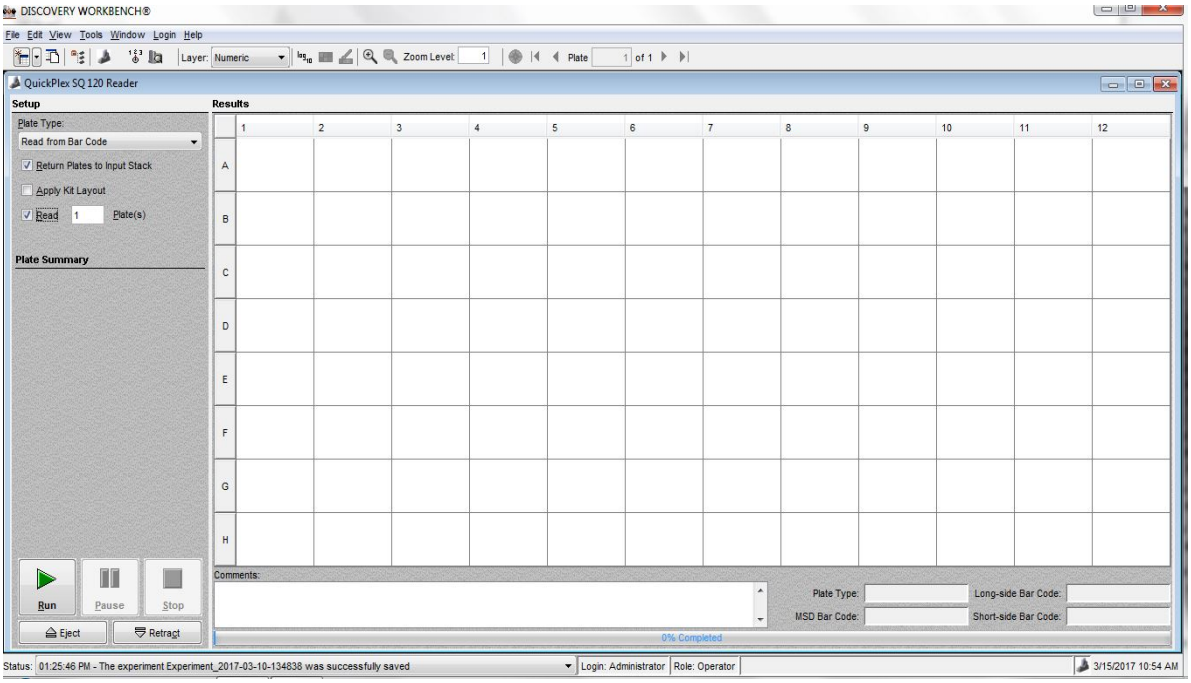
4.9 Equipment (MSD QuickPlex SQ120)

- The instrument should be turned on 30 min before starting the experiment and calibrated using an Electronic plate (shown below), which has specific wells that will activate upon reading. This plate should be read everytime before reading the experimental plate.
- The signal for calibration plate wells should be >1000 and that for experimental samples should be <1 million. Signal higher than that value indicates saturation of the reader.
- The instrument can be turned off when not in use.

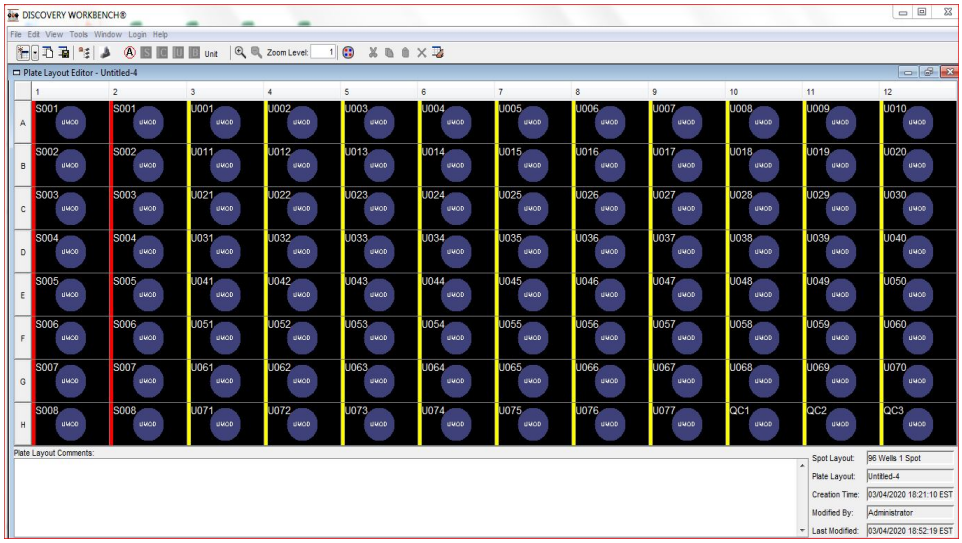
4.10 Plate layout

Initiation of QuickPlex SQ 120 MSD Discovery Workbench Software: MSD Discovery Workbench program is started by clicking the Bio-Plex icon on the desktop. The instrument is already on, and can be checked by clicking the Quickplex SQ 120 icon in the top menu bar.

Then once the QuickPlex SQ 120 Reader screen pops up:



Go to the **Tools** in the menu bar, and select **Status**. Here is where you can check on the instrument status, and the temperature should be -24°C to -27°C. 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:

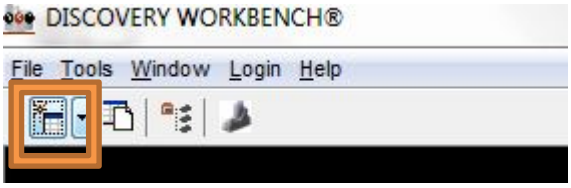


S001-S007: Standards
S008: Blank
U001-U077: Unknown Samples
QC1-QC3: Quality Controls

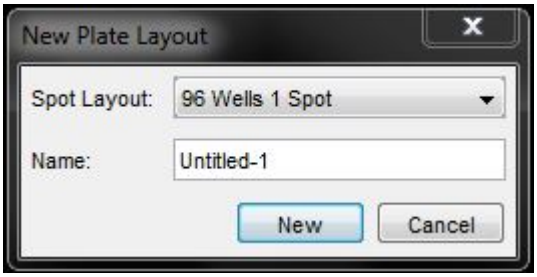
Note: It is very important that you be careful to highlight the correct cells when setting up the template to assign the order and duplicates.

To acquire the above plate format, the following procedure should be followed:

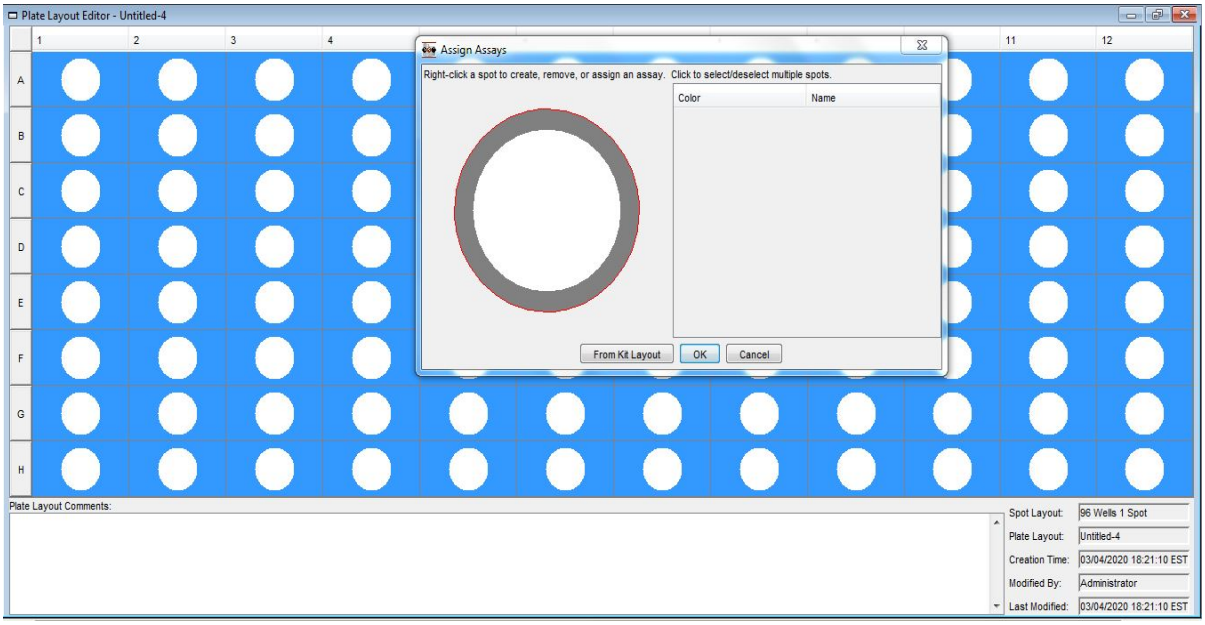
- Click on File
- Select **New Plate Layout in Library**



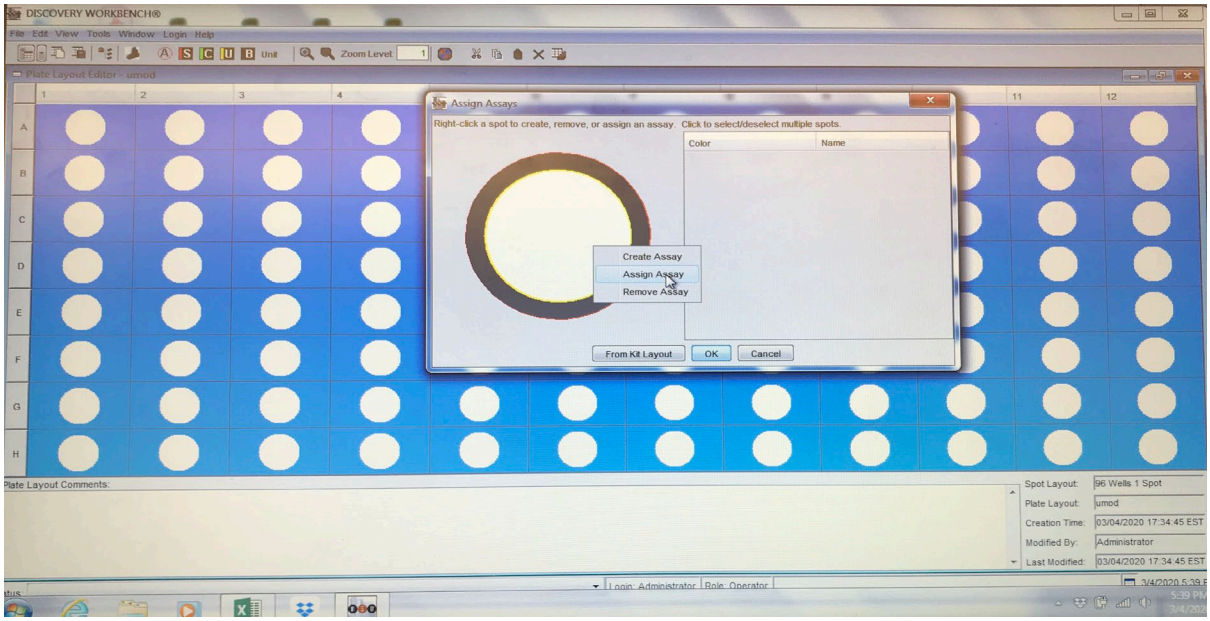
- The following dialog box will appear. Select '96 Wells 1 Spot' as your spot layout and name the file.



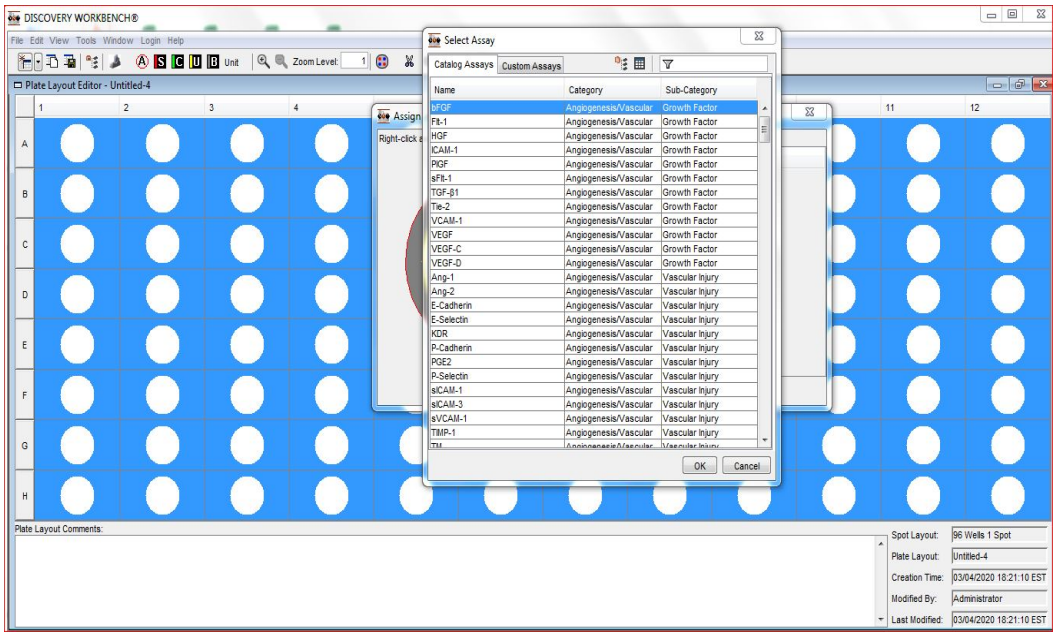
- A 1-Spot layout will appear. You must select the entire plate, right click and then click ‘Assign Assays’. The following screen will appear.



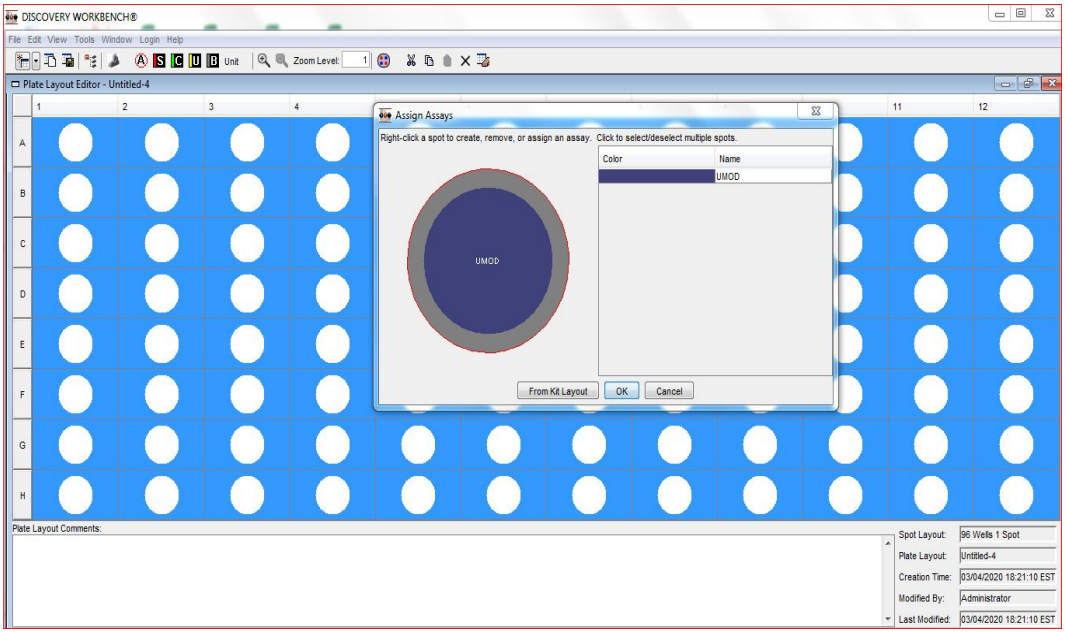
- Right click on the white circle and either select ‘ Assign Assay’ or Create Assay (if the assay is not available), as shown below.



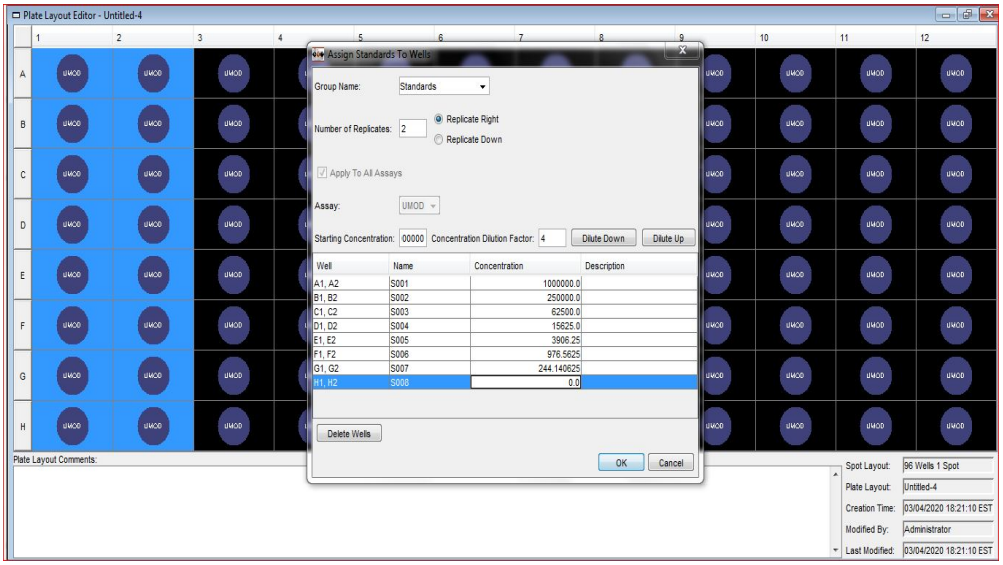
- Once you click 'Assign Assay', the following screen will appear.



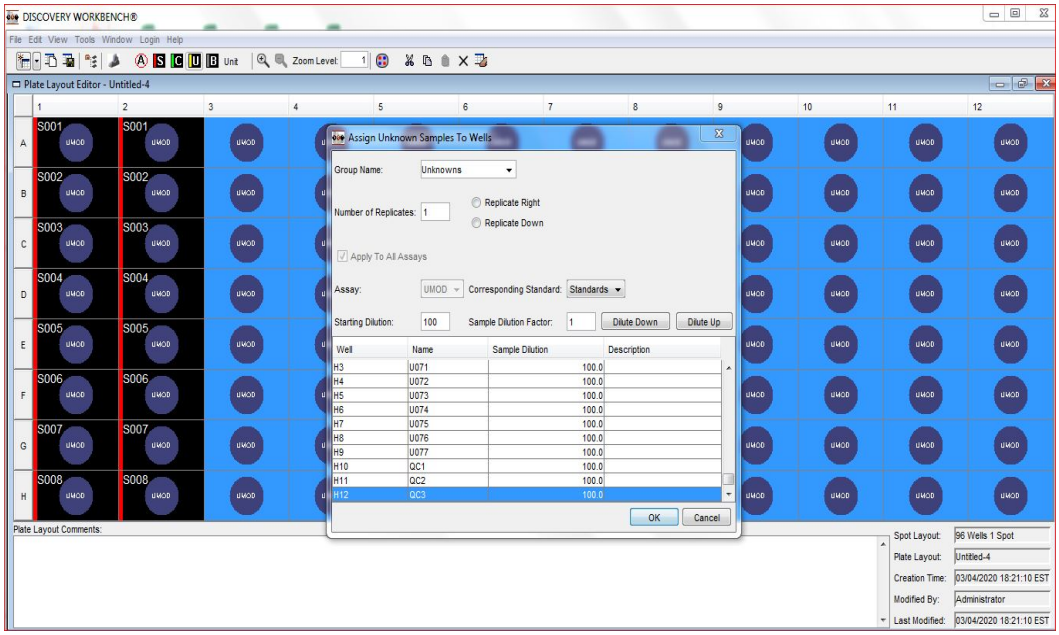
- In the top right corner, type 'UMOD'. Select the UMOD assay that pops up and click 'OK'. The assay will be assigned to the well (as shown below). Click 'Ok' and the assay will be assigned to all of the wells. In this assay, we will only be measuring Uromodulin (UMOD) levels in urine. You can follow the same procedure to assign other assays to wells.



- Now highlight the cells from A1 to H2 (this will highlight the columns 1 & 2) and the click the **red S** icon in the menu bar. This will assign the standards. You must add **2** in the **Number of Replicates** and then check **Replicate Right** making sure this is completed for all assays.
- Then you must enter 1,000,000 in 'starting concentration' field (this number will change depending upon the type of biomarker) and 4 in 'dilution' field. Select 'Dilute Down' and the concentration for all standards will be auto-filled. Then, assign 0.0 for S008 since this is a blank. **Check that this assignment is correct and select OK (see below).**

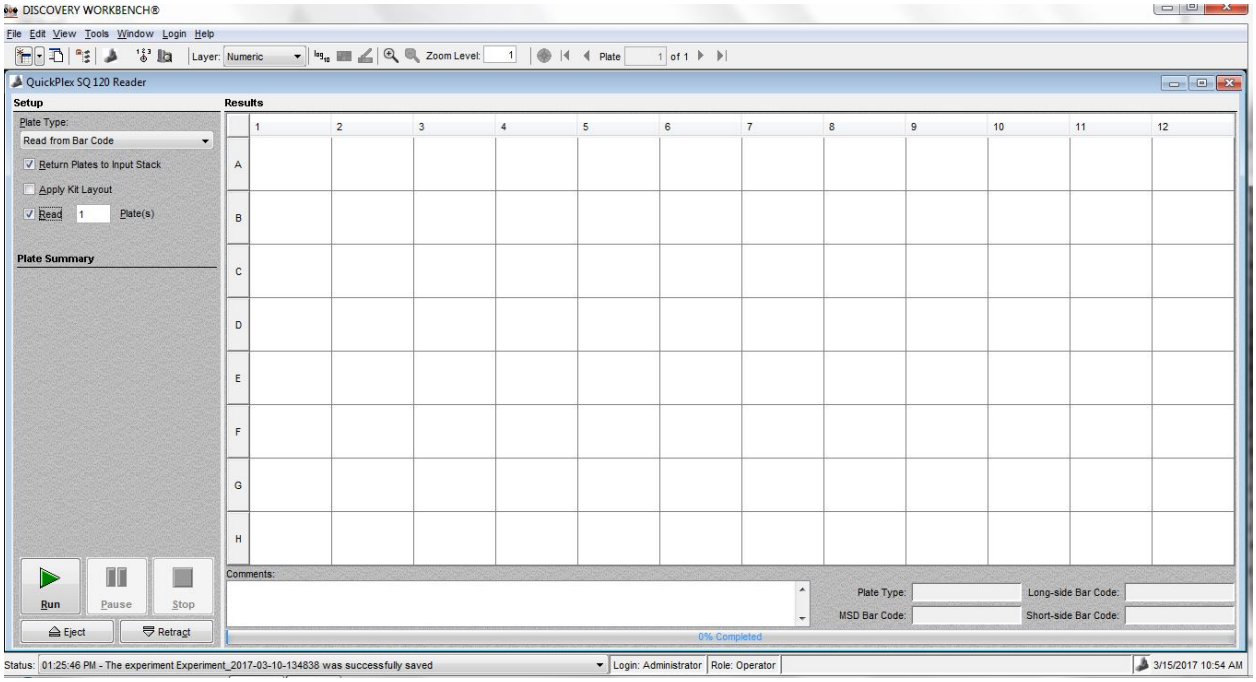


- Now, we will assign ‘Unknowns’. In this case, we will treat quality control (QC) samples as ‘Unknown’ since their concentration is not known. To assign “**Unknowns**” on the plate, follow these steps (See figure below):
  - Highlight wells A3 to H12.
  - Select the **yellow U** icon in the menu bar.
  - Set **Number of Replicates** to **1** since we run duplicates in different plates.
  - Add the **Starting Dilution** as **100** and the **Sample Dilution Factor** as **1**. This will give all samples a dilution of 100.

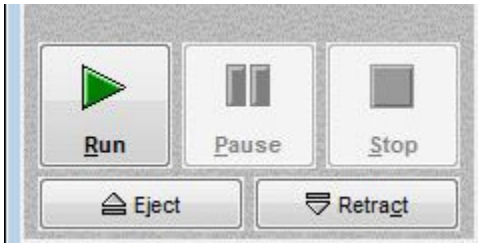


- The information of the standards, description of the samples (sample IDs), QCs will be incorporated into the template. Once the protocol is ready, save the Plate Layout with a specific name (month/day/Year- Project number - sample number) (eg: 03052020-PN156-1-40).
- Once the plate layout is saved, open the Quickplex SQ120 Reader using this icon.

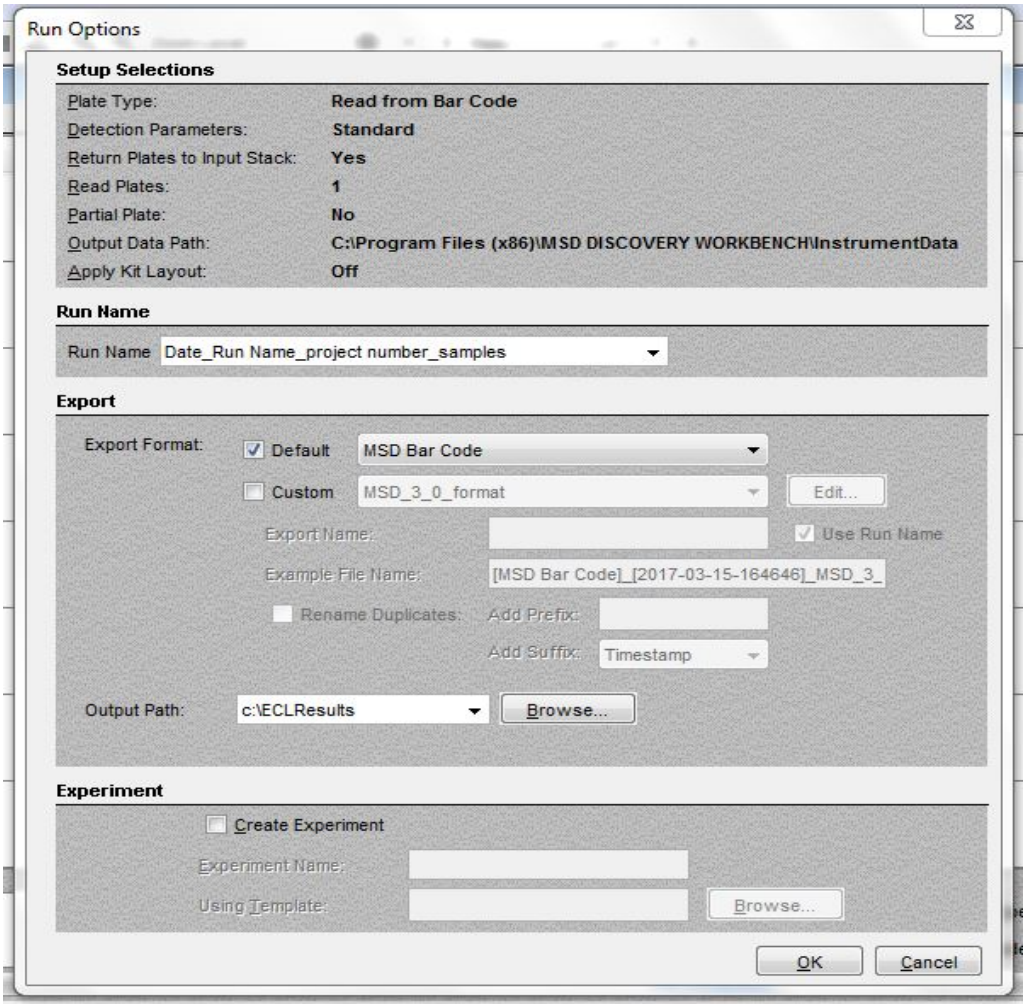
- The QuickPlex SQ120 Reader plate will pop up:



- Select the correct 'Plate Type' from the drop-down menu (depending upon whether you are using small spot, 7-assay or 10-assay plate). Check the **Return Plate to Input Stack,** and **Read 1 plate.**
- Then keep the plate on the plate holder on the right hand side with well A1 in the upper right position of the plate holder, the barcode of the plate should be on the right, and select **Run**.



- The following screen will appear:



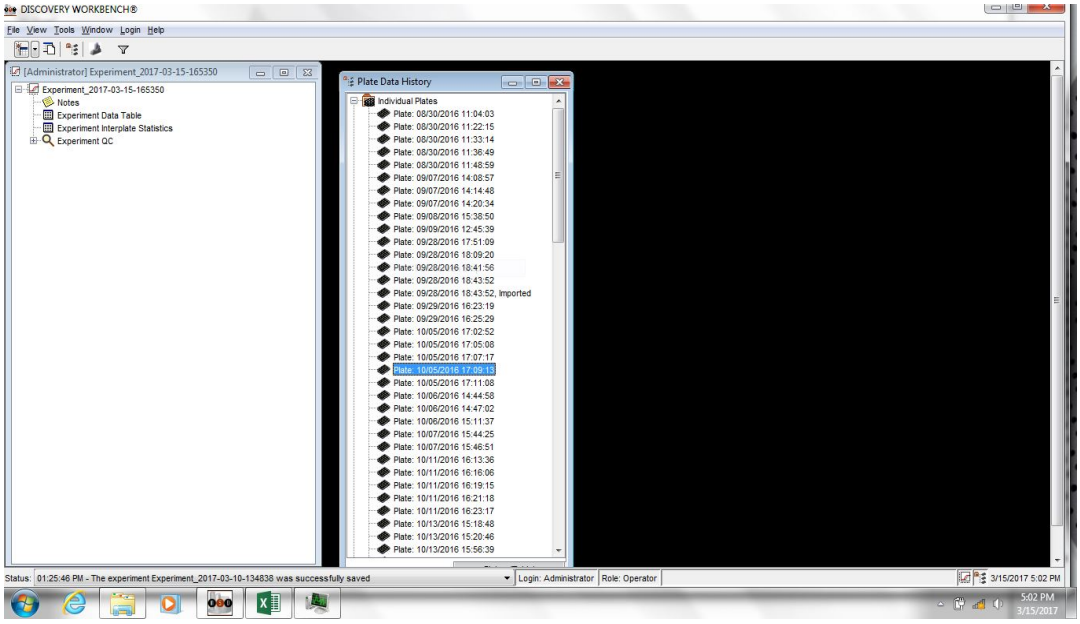
- Enter the (month/day/Year- Project number - sample number) (eg: 03052020-PN156-1-40).
- Select OK and the plate will be taken inside the MSD and read.
- The output screen is saved in the plate data history, so you can close the output window.

5. Data Generation & Processing

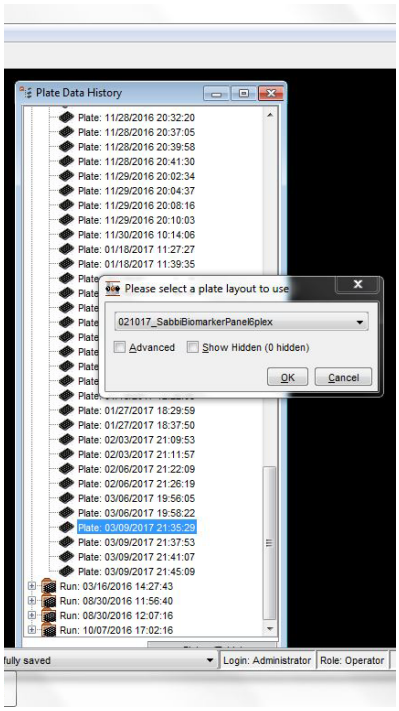
5.1 Data output

- Data is analyzed using Mesoscale Discovery Workbench integrated instrument operating software.
- From the menu bar, select the **plate data history** icon.

- The plates ordered by date and time run will appear (see below).

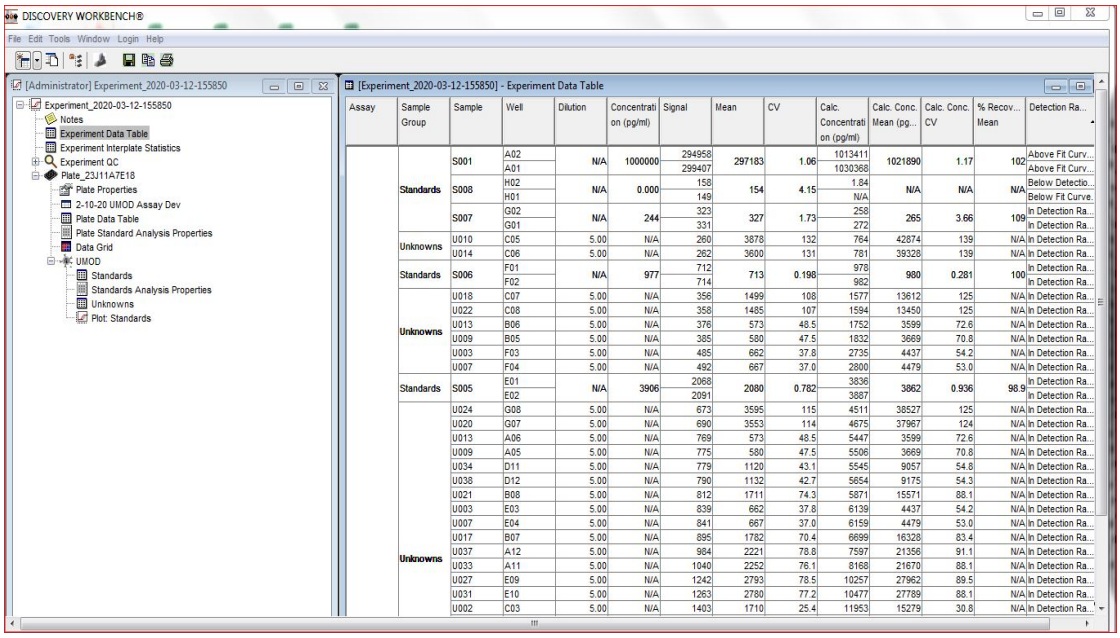


- Right click on the plate, which you wish to analyze. Select ‘Analyze plates’ option from the drop down menu.
- The following dialog box will appear. Select the plate layout that you created previously and click OK.



- The experiment with plate information will appear on the screen based on the layout you selected.

- You can click on the **Plate Data Table and Data Grid** to maneuver through the data generated, and open the individual biomarker links to observe standard curves and specific biomarker information.



Assay	Sample Group	Sample	Well	Dilution	Concentration (pg/ml)	Signal	Mean	CV	Calc. Concentration (pg/ml)	Calc. Conc. Mean (pg...	Calc. Conc. CV	% Recov... Mean	Detection Ra...
	Standards	S001	A02	N/A	1000000	294958	297183	1.06	1013411	1021890	1.17	102	Above Fit Curv...
			A01			299407			1030368				Above Fit Curv...
		S008	H02	N/A	0.000	158	154	4.15	1.84	N/A	N/A	N/A	Below Detectio...
			H01			149			N/A				Below Fit Curve.
		S007	G02	N/A	244	323	327	1.73	258	265	3.66	109	In Detection Ra...
			G01			331			272				In Detection Ra...
	Unknowns	U010	C05	5.00	N/A	260	3878	132	764	42874	139	N/A	In Detection Ra...
		U014	C06	5.00	N/A	262	3600	131	781	39328	139	N/A	In Detection Ra...
	Standards	S006	F01	N/A	977	712	713	0.198	978	980	0.281	100	In Detection Ra...
			F02			714			982				In Detection Ra...
	Unknowns	U018	C07	5.00	N/A	356	1499	108	1577	13612	125	N/A	In Detection Ra...
		U022	C08	5.00	N/A	358	1485	107	1594	13450	125	N/A	In Detection Ra...
		U013	B06	5.00	N/A	376	573	48.5	1752	3599	72.6	N/A	In Detection Ra...
		U009	B05	5.00	N/A	385	580	47.5	1832	3669	70.8	N/A	In Detection Ra...
		U003	F03	5.00	N/A	485	662	37.8	2735	4437	54.2	N/A	In Detection Ra...
		U007	F04	5.00	N/A	492	667	37.0	2800	4479	53.0	N/A	In Detection Ra...
	Standards	S005	E01	N/A	3906	2068	2080	0.782	3836	3862	0.936	98.9	In Detection Ra...
			E02			2091			3887				In Detection Ra...
	Unknowns	U024	G08	5.00	N/A	673	3595	115	4511	38527	125	N/A	In Detection Ra...
		U020	G07	5.00	N/A	690	3553	114	4675	37967	124	N/A	In Detection Ra...
		U013	A06	5.00	N/A	769	573	48.5	5447	3599	72.6	N/A	In Detection Ra...
		U009	A05	5.00	N/A	775	580	47.5	5506	3669	70.8	N/A	In Detection Ra...
		U034	D11	5.00	N/A	779	1120	43.1	5545	9057	54.8	N/A	In Detection Ra...
		U038	D12	5.00	N/A	790	1132	42.7	5654	9175	54.3	N/A	In Detection Ra...
		U021	B08	5.00	N/A	812	1711	74.3	5871	15571	88.1	N/A	In Detection Ra...
		U003	E03	5.00	N/A	839	662	37.8	6139	4437	54.2	N/A	In Detection Ra...
		U007	E04	5.00	N/A	841	667	37.0	6159	4479	53.0	N/A	In Detection Ra...
		U017	B07	5.00	N/A	895	1782	70.4	6699	16328	83.4	N/A	In Detection Ra...
		U037	A12	5.00	N/A	984	2221	78.8	7597	21356	91.1	N/A	In Detection Ra...
		U033	A11	5.00	N/A	1040	2252	76.1	8168	21670	88.1	N/A	In Detection Ra...
		U027	E09	5.00	N/A	1242	2793	78.5	10257	27962	89.5	N/A	In Detection Ra...
		U031	E10	5.00	N/A	1263	2780	77.2	10477	27789	88.1	N/A	In Detection Ra...
		U002	C03	5.00	N/A	1403	1710	25.4	11953	15279	30.8	N/A	In Detection Ra...

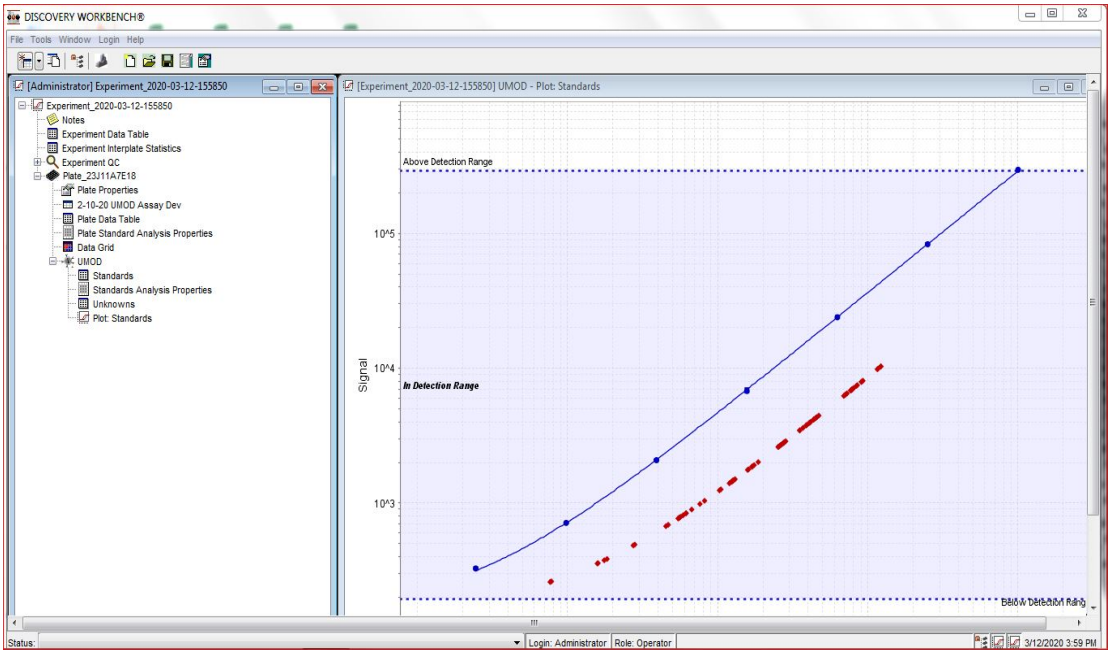
- Standard curve recovery is a practical parameter commonly used to determine the overall accuracy of an assay. Mesoscale Discovery Workbench includes a mechanism for assessing the fit of a standard curve to its individual standard points. This is the recovery percentage. For each analyte standard, an observed concentration is back calculated by plotting the electrochemiluminescence 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), which is the upper expected concentration of the standard curve, in which an assay is both precise and accurate. The Mesoscale Discovery Workbench analyzes the curve fit based on observed/expected ratio.

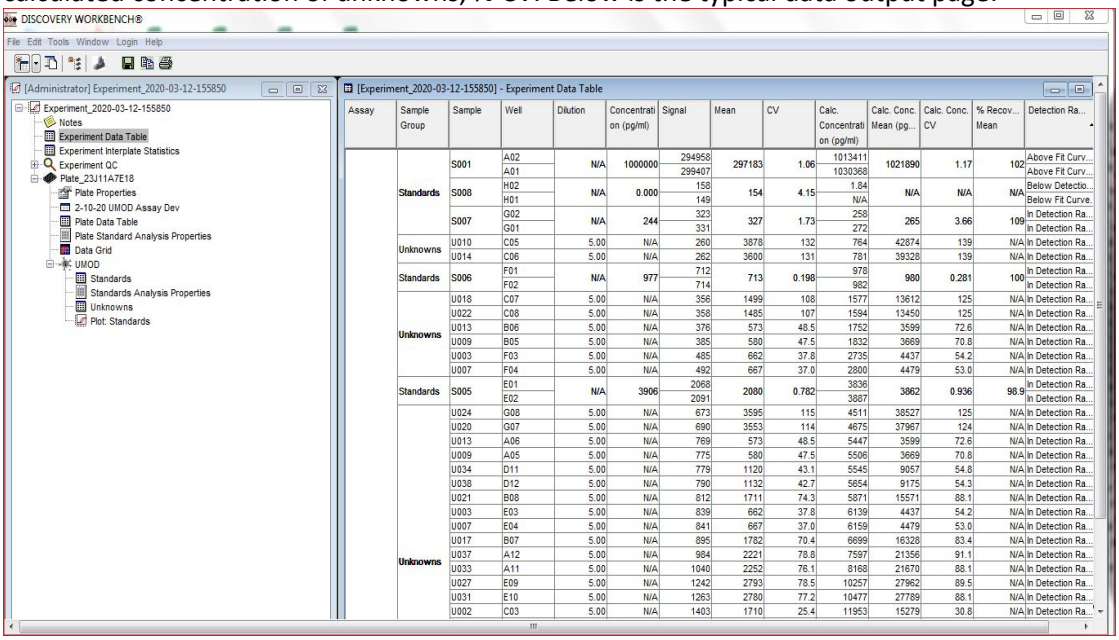
5.2 Standard Curve generation

- The Mesoscale Discovery Workbench 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:



5.3 Data Processing and Data Calculations

- The Mesoscale Discovery Workbench automatically generates the value of unknowns based on the standard curve, and the template selected. The data generated includes the signal, calculated concentration of unknowns, % CV. Below is the typical data output page:



Assay	Sample Group	Sample	Well	Dilution	Concentration (pg/ml)	Signal	Mean	CV	Calc. Concentration (pg/ml)	Calc. Conc. Mean (pg...	Calc. Conc. CV	% Recov... Mean	Detection Ra...
		S001	A02	N/A	1000000	294958	297183	1.06	1013411	1021890	1.17	102	Above Fit Curv...
			A01			299407			1030368				Above Fit Curv...
	Standards	S008	H02	N/A	0.000	158	154	4.15	1.84	N/A	N/A	N/A	Below Detectio...
			H01			149			N/A				Below Fit Curve.
		S007	G02	N/A	244	323	327	1.73	258	265	3.66	109	In Detection Ra...
			G01			331			272				In Detection Ra...
	Unknowns	U010	C05	5.00	N/A	260	3878	132	764	42874	139	N/A	In Detection Ra...
		U014	C06	5.00	N/A	262	3600	131	781	39328	139	N/A	In Detection Ra...
	Standards	S006	F01	N/A	977	712	713	0.198	978	980	0.281	100	In Detection Ra...
			F02			714			982				In Detection Ra...
	Unknowns	U018	C07	5.00	N/A	356	1499	108	1577	13612	125	N/A	In Detection Ra...
		U022	C08	5.00	N/A	358	1485	107	1594	13450	125	N/A	In Detection Ra...
		U013	B06	5.00	N/A	376	573	48.5	1752	3599	72.6	N/A	In Detection Ra...
		U009	B05	5.00	N/A	385	580	47.5	1832	3669	70.8	N/A	In Detection Ra...
		U003	F03	5.00	N/A	485	662	37.8	2735	4437	54.2	N/A	In Detection Ra...
		U007	F04	5.00	N/A	492	667	37.0	2800	4479	53.0	N/A	In Detection Ra...
	Standards	S005	E01	N/A	3906	2068	2080	0.782	3836	3862	0.936	98.9	In Detection Ra...
			E02			2091			3887				In Detection Ra...
	Unknowns	U024	G08	5.00	N/A	673	3595	115	4511	38527	125	N/A	In Detection Ra...
		U020	G07	5.00	N/A	690	3553	114	4675	37967	124	N/A	In Detection Ra...
		U013	A06	5.00	N/A	769	573	48.5	5447	3599	72.6	N/A	In Detection Ra...
		U009	A05	5.00	N/A	775	580	47.5	5506	3669	70.8	N/A	In Detection Ra...
		U034	D11	5.00	N/A	779	1120	43.1	5545	9057	54.8	N/A	In Detection Ra...
		U038	D12	5.00	N/A	790	1132	42.7	5654	9175	54.3	N/A	In Detection Ra...
		U021	B08	5.00	N/A	812	1711	74.3	5871	15571	88.1	N/A	In Detection Ra...
		U003	E03	5.00	N/A	839	662	37.8	6139	4437	54.2	N/A	In Detection Ra...
		U007	E04	5.00	N/A	841	667	37.0	6159	4479	53.0	N/A	In Detection Ra...
		U017	B07	5.00	N/A	895	1782	70.4	6699	16328	83.4	N/A	In Detection Ra...
		U037	A12	5.00	N/A	984	2221	78.8	7597	21356	91.1	N/A	In Detection Ra...
		U033	A11	5.00	N/A	1040	2252	76.1	8168	21670	88.1	N/A	In Detection Ra...
		U027	E09	5.00	N/A	1242	2793	78.5	10257	27962	89.5	N/A	In Detection Ra...
		U031	E10	5.00	N/A	1263	2780	77.2	10477	27789	88.1	N/A	In Detection Ra...
		U002	C03	5.00	N/A	1403	1710	25.4	11953	15279	30.8	N/A	In Detection Ra...

- Data is exported from Mesoscale Discovery Workbench to Microsoft excel by right clicking and selecting copy and then paste in excel.

- Evaluation of duplicates: First, the plate layout is created in the software. Then, two plates (each containing a singlicate) are analyzed using the plate layout created previously. The data are exported as the excel sheet and CVs are calculated in Excel. For assay validation, duplicates were run on the same plate. Therefore, the software was able to calculate the CV. When we run duplicates on 2 different plates, the software will show N/A in CV column. We calculate CV later in Excel after putting together the data from 2 plates. In the current example, a calculated conc CV is used for 15% cutoff.
- Data output can also be customized to include desired parameters by right-clicking data table headers and selecting 'Column Selections'. After making changes, the table layout can be saved by clicking 'file' and selecting 'Set Table Profile as Default'. That way, all future experiments will have the same data table profiles.
- 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.

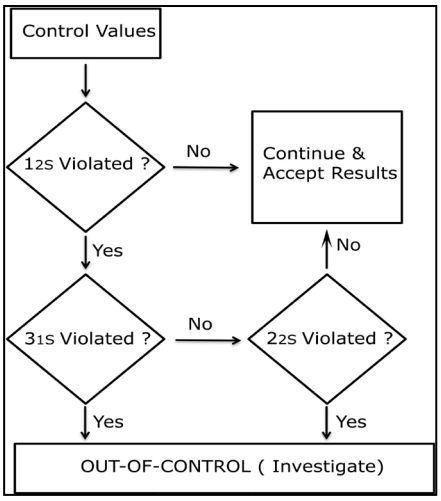
6. Quality Control

6.1. Quality control for the assay

- We will ensure that biomarker levels obtained using the assay on control specimens are within the mean ± 2 S.D. limit established from the Validation Protocol.
- If both samples are within the mean ± 2 S.D. limit, we will accept the data. If one of the two control values is outside mean ± 2 S.D., 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 the mean ± 2 S.D. limits, then we will follow the westgard three-quality control rule and not accept the data.
- Please see below for Westgard quality control rules.

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 ± SD on the same assay or two consequtive values of one control is outside of mean ± SD

Levey-Jenning's plots of controls:



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.
- Samples with a concentration higher than the upper limits of detection (ULOD) will be remeasured at a higher dilution.

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 both controls.
- If the quality control values pass the above described Westgard rules, then proceed with unknown testing.
- If the quality control fails again, then prepare fresh reagents (e.g. recombinant protein) and then rerun controls. Also, document the problem.
- If the quality control indicates that Westgard rules are passed, proceed with the testing of the unknown samples.
- If QC fails again, recalibrate the assay (use a 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, analyte values of unknown samples with their corresponding sample ID, date of measurement, mean value, %CV, and SD will be copied and pasted into a master excel data workbook 'Sheet 1'. Biomarker values, %CV, S.D., and date of measurement of quality control samples and proficiency samples will be copied and pasted in 'Sheet 2'. Sheets 1 & 2 will be updated with the addition of data from each run.
- The master data sheet that will go to the coordinating center will contain the following information
  1. Sample ID
  2. Date of the assay
  3. Platform and the Instrument used for the assay
  4. Reagent lot numbers, which include date of U Plax preparation, lot numbers of primary antibody, secondary antibody, and recombinant proteins
  5. Concentration of biomarker (pg/ml)
  6. If samples are below Lower limit of detection (LLOD), we will indicate as LLOD.
  7. % CV across the sample replicates
  8. Biomarker levels, SD and % CV of each control sample with the description of each control sample
  9. Biomarker 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
  10. Levey-Jennings plots of Control samples and proficiency samples (These plots will be updated and cumulative plots will be reported with each data submission).

7. Assay Validation

- Two samples were used for assay validation.

  S1: Urine Sample 1

  S2: Urine Sample 2

7.1 Determination of the linearity of dilution

Dilution	Sample (ul)	Diluent 37 (ul)	Total volume
1:75 (D1)	8	592	600
1:100 (D2)	315 ul of D1	105	420
1:125 (D3)	140 ul of D2	35	175

Plate Layout

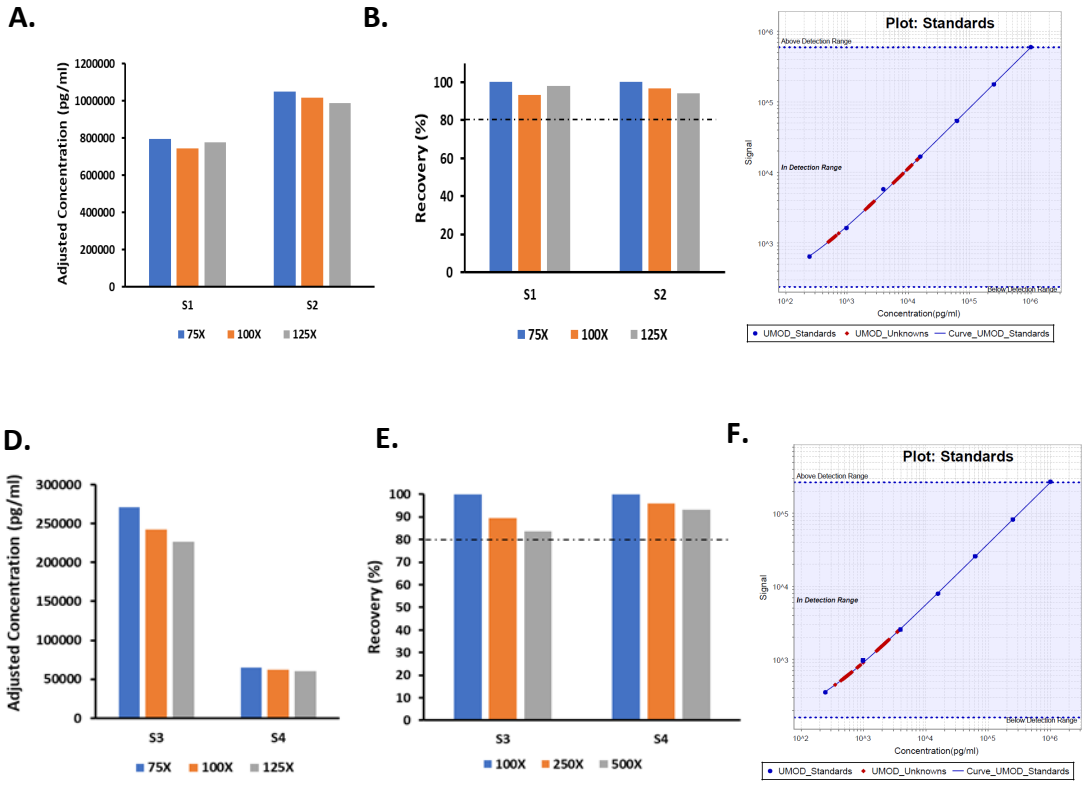
1	2	3	4	5	6	7	8	9	10	11	12
Std1	**Std1**	S1D1	S1D1	S1D1	S1D1	S1D1 (Dil.37)	S1D1 (Dil.37)	S1D1 (Dil.37)	S1D1 (Dil.37)		
Std2	**Std2**	S1D2	S1D2	S1D2	S1D2	S1D1 (G)	S1D1 (G)	S1D1 (G)	S1D1 (G)		
Std3	**Std3**	S1D3	S1D3	S1D3	S1D3	S1D1 (H)	S1D1 (H)	S1D1 (H)	S1D1 (H)		
Std4	**Std4**	S2D1	S2D1	S2D1	S2D1	S1D1 (A)	S1D1 (A)	S1D1 (A)	S1D1 (A)		
Std5	**Std5**	S2D2	S2D2	S2D2	S2D2	S1D1 (B)	S1D1 (B)	S1D1 (B)	S1D1 (B)		
Std6	**Std6**	S2D3	S2D3	S2D3	S2D3	S2D1 (Dil.37)	S2D1 (Dil.37)	S2D1 (Dil.37)	S2D1 (Dil.37)		
Std7	**Std7**	S2D1 (G)	S2D1 (G)	S2D1 (G)	S2D1 (G)	S2D1 (A)	S2D1 (A)	S2D1 (A)	S2D1 (A)		
Std8	**Std8**	S2D1 (H)	S2D1 (H)	S2D1 (H)	S2D1 (H)	S2D1 (B)	S2D1 (B)	S2D1 (B)	S2D1 (B)		

- Linearity of dilution was checked using 2 samples and 3 dilutions as shown in the above layout (Rows 1-6, columns 3-6).

- Adjusted concentration (pg/ml) and recovery (%) for each sample are shown in graphs below.

- When normal urine samples from patients without known renal disease (S1, S2) are used, the assay shows linearity across 1:75 to 1:125 dilution and the points lie close to the middle of the standard curve (Graphs A, B, C). On the other hand, when urines from patients with renal disease, having lower uromodulin levels (S3, S4), are used, the samples show decreasing concentrations at higher dilutions and the points lie along the lower end of the curve (Graphs D, E, F). In both cases, recovery (%) was between 80-100%. The goal is to use dilutions that will result in measurements close to the middle of the standard curve, and avoid the lower end as much as possible.

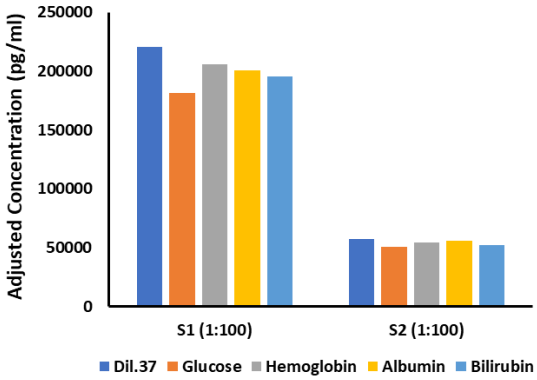
C.

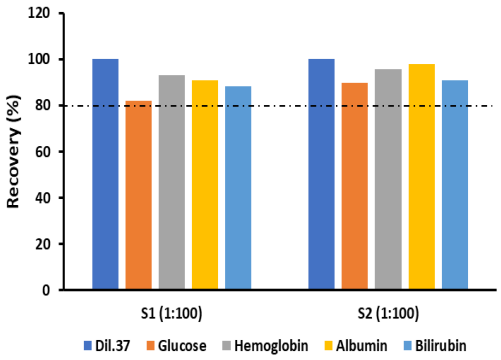


7.2 Interference study

- Sample spiking was carried out to determine if the components such as glucose, blood or proteins, usually found in disease states, interfere with uromodulin measurements. Four different interfering agents (glucose, albumin, hemoglobin and bilirubin) were used for spiking.
- First, the concentrated stocks of glucose, albumin, hemoglobin and bilirubin were prepared in Dil. 37. Appropriate volumes of these stock solutions were then mixed with urine samples (S1, S2) to obtain the following final concentrations:
  1. Glucose (G): 500 mg/dl
  2. Hemoglobin (H): 40 mg/dl
  3. Albumin (A): 200 mg/dl
  4. Bilirubin (B): 10 mg/dl

- These sample mixtures were then diluted 1:100 with Diluent 37 (Columns 7-10 in the plate layout shown above).
- Calibrators or standards (Std1-Std8) were prepared as per the instructions on page 5 of this SOP. Adjusted concentration (pg/ml) and recovery (%) for each sample are shown in graphs below.
- All samples showed recovery between 80-100% when spiked with various agents indicating no interference.





7.3 Assay parameters

Biomarker Urine	Assay range (pg/ml)	LLOD (pg/ml)	Linearity of Dilution	Spike Recovery	Intra-assay %CV	Inter-assay %CV	Interference
UMOD	244.14-1,000,000	244.14	1:75-1:125	80-100%	5.5	6.4	No