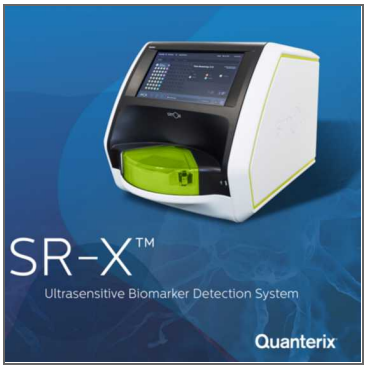

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Protocol status: Working
We use this protocol and it's working

Created: Dec 27, 2023

Last Modified: Dec 27, 2023

SiMOA pT73-Rab10 Homebrew Assay

Forked from Simoa Quanterix pT73-Rab10 Homebrew Assay Development

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Duke University

andrew.west

ABSTRACT

Assay for the detection of pT73-Rab10 in biofluids and lysates

MATERIALS

Reagent and Material:

Antibody for Capture Beads:

- Recombinant Anti-RAB10 (phospho T73) antibody [MJF-R21] - BSA and Azide free **Abcam Catalog #ab231707**

Antibody for Detector:

- Recombinant Anti-RAB10 antibody [MJF-R23] **Abcam Catalog #ab237703**

Standards:

- Laboratory prepared HEK-293T protein lysates was used as standard in this Homebrew Assay.

Reagent:

- Pierce™ Bovine Gamma Globulin Standard Ampules, 2 mg/mL **Thermo Fisher Catalog #23212**
- Pierce™ BCA Protein Assay Kit **Thermo Fisher Catalog #23227**
- EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) **Thermo Fisher Catalog #77149**

PROTOCOL integer ID:
92762

Keywords: ASAPCRN

Funders Acknowledgement:
NIH/NINDS
Grant ID: NS064934
Michael J. Fox Foundation for Parkinson's Disease Research
Grant ID: MJFF-022434

- EZ-Link™ NHS-PEG4-Biotin, No-Weigh™ Format **Thermo Fisher Catalog #A39259**
- Carboxylated Paramagnetic Beads **Quanterix Catalog #100458**
- Bead Conjugation Buffer **Quanterix Catalog #101357**
- Bead Wash Buffer **Quanterix Catalog #101355**
- Bead Blocking Buffer **Quanterix Catalog #101356**
- Bead Diluent **Quanterix Catalog #100458**
- Homebrew Detector/Sample Diluent **Quanterix Catalog #101359**
- SBG Concentrate 50nM **Quanterix Catalog #103397**
- SBG Diluent **Quanterix Catalog #101376**
- Resorufin ß-D- galactopyranoside (RGP) **Quanterix Catalog #101736**
- Biotinylation Reaction Buffer **Quanterix Catalog #101358**
- Simoa Wash Buffer A (SR-X) **Quanterix Catalog #103078**
- Simoa Wash Buffer B (SR-X) **Quanterix Catalog #103079**

Material:

- 50kDa MWCO filter **Merck Millipore (EMD Millipore) Catalog #UFC505096**
- Disposable tips for SR-X **Quanterix Catalog #102919**
- Simoa Disks **Quanterix Catalog #100001**
- Magnetic Rack **V&P Scientific Catalog #VP 772F11A**
- 15-mL Conical tubes
- 1.7-mL Conical tubes

Equipment:

Equipment

SR-X NAME

Quanterix BRAND

SR-X Biomaker Detection System SKU

file 

Equipment

Simoa Microplate Washer NAME

Quanterix BRAND

Simoa Microplate Washer SKU

file

Equipment

Simoa Microplate Shaker NAME

Quanterix BRAND

Simoa Microplate Shaker SKU

file

Equipment

Rotator motor drive and base NAME

Glas-Col BRAND

099A RD4512 SKU

- Mini benchtop centrifuge

Equipment

Eppendorf® microcentrifuge 5424 NAME

Centrifuge TYPE

Eppendorf BRAND

Z722960 SKU

PROTOCOL MATERIALS

Recombinant Anti-RAB10 (phospho T73) antibody [MJF-R21] - BSA and Azide fre
Abcam Catalog #ab231707

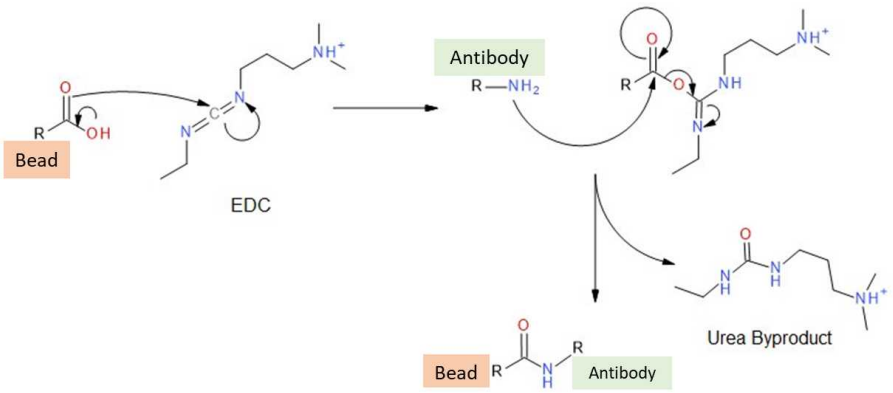
Step 1

Recombinant Anti-RAB10 antibody [MJF-R23] **Abcam Catalog #ab237703**

Step 4

Prepare Capture Beads Concentrate (5-6 hours)

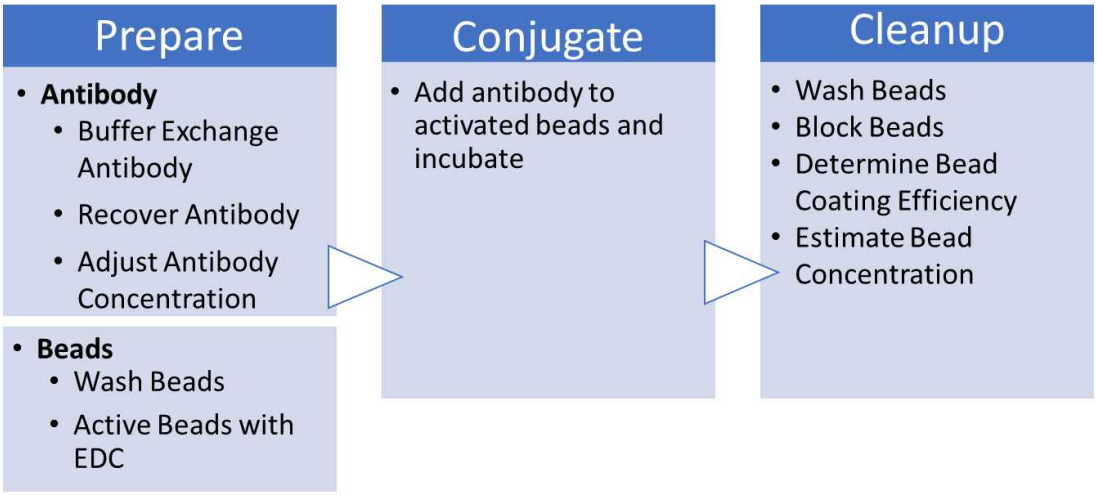
1 Prepare capture beads using a two-step EDC coupling protocol. Reaction occurs between the antibody primary amino groups (-NH_2) and the carboxyl groups (-COOH) on the beads. 80ug of antibody is required for 0.2 mg/ml buffer exchanged antibody, producing 4.2 x 10^8 beads, sufficient for 500 tests at 5 x 10^5 beads/test.



The general mechanistic steps for EDC-mediated coupling of carboxylic acids and amines under acidic conditions.

Capture antibody for Homebrew pT73-Rab10 Assay is

Recombinant Anti-RAB10 (phospho T73) antibody [MJF-R21] - BSA and Azide free
Abcam Catalog #ab231707



Workflow for preparing capture beads concentrate

1.1 Prepare the Antibody: Buffer Exchange the Antibody into Bead-Coating Buffer

11m

Note

Always keep Bead Conjugation Buffer and Bead Wash Buffer On ice during the antibody conjugation process.

1.1.1 Block Amicon Filters

- Insert one Amicon filter device into the supplied Amicon microcentrifuge tube, ensuring that the filter membranes align with the tube cap strap.
- Dilute IgG stock (2 mg/mL) with 1X PBS to 1 mg/mL.
- Add 500 uL of 1 mg/mL of IgG to the filter.
- Centrifuge at 17000 x g for 00:01:00 at room temperature.
- Remove flow thru and liquid from filter.

1.1.2 Determine stock antibody concentration with BCA assay (with IgG standards) and A280.

1.1.3 Calculate Antibody mass for a 150-uL reaction with 30% excess of antibody (e.g., 0.04 mg required for a coating concentration of 0.2 mg/mL). Calculate volume of stock antibody based on desired antibody coating concentration (0.2 mg/mL).

Note

e.g. If stock antibody concentration is 1 mg/mL, volume of stock antibody required = 0.04 mg/1 mg/mL*1000= 39 uL

1.1.4 Remove the antibody storage buffer

- In a fresh 1.7 mL tube, add the calculated amount of Antibody.
- Add sufficient Bead Conjugation Buffer to the Antibody tube to a total volume of 500 µL.
- Vortex and spin down.
- Transfer to the pre-blocked Amicon filter and cap the filter.
- Place the filter device in the centrifuge, aligning the cap strap toward the center of the rotor. Counterbalance with a similar device. Centrifuge at 14000 x g for 00:05:00 at Room temperature.
- Keep the flow through in a new separate 1.7 mL tube, then reinsert the filter.

Note

The antibody is now concentrated on the inner walls of the filter. Proceed immediately to the next step.

1.1.5 Wash with Bead Conjugation Buffer x2 to buffer exchange:

- Add 450 µL of BCB to the filter, centrifuge at 14000 x g for 00:05:00 at Room temperature, and keep the flow through(s) in new separate 1.7 mL tube (labeled "Wash 1")

Note

Do not discard. "Wash 1" will be used later to determine the antibody coating efficiency.

- Repeat this buffer exchange procedure once, discard the flow through.

Note

If volume in the filter is greater than 50 µL, repeat the procedure once more, for a total of 3 buffer exchanges.

1.2 Recover the Buffer Exchanged Antibody

4m

1.2.1 Invert the filter and place it inside a clean Amicon microcentrifuge tube (the cap will not close). Centrifuge at 1000 x g for 00:02:00 at Room temperature to transfer the washed antibody from the filter to a new 1.7 mL tube (labeled "Buffer Exchanged Antibody").

1.2.2 Wash filter membranes:

- Add 50 µL of BCB to the filter. Pipette the retained volume multiple times to wash both of the membranes on the filter.
- Carefully invert the filter into the Amicon microcentrifuge tube.
- Centrifuge at 1000 x g for 00:02:00 at Room temperature
- Transfer the rinsate from the filter to the buffer exchanged antibody tube.
- Remove the filter from the tube and discard the filter.

1.2.3 Estimate the volume of buffer exchanged antibody, using a P-100 pipette(The antibody should be in a 70~100 µL concentrated volume).

1.3 Dilute the buffer Exchanged Antibody with Bead Conjugation Buffer

1.3.1 Calculate the volume of buffer exchanged antibody required

- Measure and record the buffer exchanged antibody concentration using a BCA assay.
- Calculate the volume of buffer exchanged antibody solution required:

$$Ab.Coating\ Conc.(mg/mL) \times Batch\ Size\ (\mu L)\ /\ Measured\ Ab.Concentration\ (mg/mL) = Volume\ of\ Buffer\ Exchanged\ Ab.(\mu L)$$

Note

$$\frac{Ab.Coating\ Conc.(mg/mL)\ \times Batch\ Size\ (\mu L)}{Measured\ Ab.Concentration\ (mg/mL)} = Volume\ of\ Buffer\ Exchanged\ Ab.(\mu L)$$

- Calculate the required volume of Bead Conjugation Buffer required for the total conjugation batch volume (i.e., 150 µL):

$$Batch\ Size\ (\mu L) - Volume\ of\ Buffer\ Exchanged\ Ab.(\mu L) = Required\ Volume\ of\ Bead\ Conjugation\ Buffer\ (\mu L)$$

Note

$$Batch\ Size\ (\mu L) - Volume\ of\ Buffer\ Exchanged\ Ab.\ (\mu L) = Required\ Volume\ of\ Bead\ Conjugation\ Buffer\ (\mu L)$$

- Measure the calculated volume of ice-cold Bead Conjugation Buffer into a 1.7-mL tube.
- Add the calculated volume of antibody.
- Vortex the tube to mix.
- Measure and record the diluted antibody concentration using a spectrophotometer at OD 280, blanked with Bead Conjugation Buffer to confirm the final reaction concentration.
- Place the tube of diluted antibody on ice until use.

2 Prepare the Beads

2.1 2.1.1 Calculate the volume of beads required for the total conjugation batch volume

10m

- Consult the Certificate of Analysis online (via Link: *http://www.quanterix.com/sds-and-coas/*) using the lot number to determine the stock concentration of the supplied bead concentrate.
- Calculate the volume of well-mixed beads required to supply 1.4 x 10^9 beads per mL of conjugation reaction.

$$Batch\ Volume\ (\mu L) \text{ x } 1.4\times10\text{^}9\ Beads/mL / Stock\ Bead\ Concentration\ (Beads/mL) = Volume\ of\ Stock\ Beads\ (\mu L)$$

Note

$$\frac{Batch\ Volume\ (\mu L) \cdot 1.4 \times 10^9 \frac{Beads}{mL}}{Stock\ Bead\ Concentration\ \left(\frac{Beads}{mL}\right)} = Volume\ of\ Stock\ Beads\ (\mu L)$$

e.g. If the Stock Bead concentration = 1.22x10^9 Bead/mL, Batch Volume=150uL, The Volume of Stock Beads required=150(uL) x 1.4x10^9(Bead/mL)/ 1.22x10^9(Bead/mL)= 172.13uL

- Vortex the vial of Paramagnetic Carboxylated Beads for 30 seconds, then place on a rotator at

100 rpm at Room temperature for at least 00:10:00.

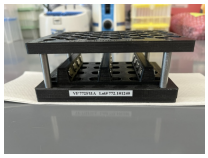
- Add the calculated volume of well mixed beads to a 1.7 mL microtube.

2.2 Wash Beads

> **Note**
>
> **488 Dyed-beads are light sensitive. Avoid light while long term incubation and storage!**

2.2.1 Wash Beads with Bead Wash Buffer x3:

- Place the tube in the magnetic separator for at least 1 minute.
- Aspirate the supernatant.
- Add Batch Volume (i.e. 150 µL) Bead Wash Buffer to the beads.
- Cap the tube and vortex for 5 seconds to disperse the beads. Pulse spin.
- Place the tube in the magnetic separator for at least 1 minute to collect the beads to the side of the tube.
- Aspirate the supernatant with a pipette.
- Remove the tube from the magnet and add Batch Volume (i.e.,150 µL) Bead Wash Buffer to the beads.
- Repeat washing procedure for a total of 3 washes.



Magnetic Separator/Rack

2.2.2 Wash the beads with cold Bead Conjugation Buffer x2:

- Place the tube in the magnetic separator for at least 1 minute to collect the beads to the side of the tube.
- Aspirate the supernatant.
- Add Batch Volume (i.e. 150 µL) cold Bead Conjugation Buffer to the beads.
- Cap the tube and vortex for 5 seconds to disperse the beads. Pulse spin.
- Repeat this washing procedure once, for a total of 2 washes.

2.2.3 Re-suspend the beads:

- Place the tube in a magnetic separator for at least 1 minute.
- Aspirate all of the supernatant. Make sure no residual volume is left.
- Calculate the volume of EDC required to activate the beads for 0.3 mg/mL EDC concentration.

$$Batch\ Size\ (mL) \times 0.3 mg/mL\ EDC\ in\ Activation\ Reaction / 10 mg/mL\ Concentrated\ EDC = Required\ EDC\ Volume\ (mL)$$

Note

$$\frac{Batch\ Size\ (mL) \times 0.3 \frac{mg}{mL} EDC\ in\ Activation\ Reaction}{10 \frac{mg}{mL}\ Concentrated\ EDC} = Required\ EDC\ Volume\ (mL)$$

e.g. Batch Size= 150uL (0.15mL), Required EDC Volume (mL)= 0.15 mL x 0.3 mg/mL EDC/ 10 mg/mL= 0.0045 mL (or 4.5 uL)

- Resuspend the beads in the Batch Volume (150 uL) of cold Bead Conjugation Buffer, minus the volume of EDC required as calculated above.
  $Batch\ Size\ (mL) - Required\ EDC\ Volume\ (mL) = Bead\ Resuspension\ Volume\ (mL)$

Note

$$Batch\ Size\ (mL) - Required\ EDC\ Volume\ (mL) = Bead\ Resuspension\ Volume\ (mL)$$

e.g. Batch Size= 150uL, $Required$ Bead Conjugation Buffer Volume (mL)=150uL- 4.5uL EDC=145.5uL

Add calculated volume of EDC

- Remove the tube from the magnet and vortex it to mix the beads.
- Pulse spin and place the tube on ice.

3 Conjugate

3.1 Activate Beads

30m

3.1.1 Prepare the 10 mg/mL EDC solution:

Note

Timing is critical for this step. Allow for no idle time after the vial of EDC has been opened. Use ice-cold buffers for all steps.

Note

It is important that you do not lose any EDC when you open the vial. This can easily happen due to static charges on your gloves or in the work area.

- Remove the 10-mg vial of EDC from storage. Tap the vial on the bench top gently to force the powder towards the bottom of the vial.
- Carefully open the vial of EDC and slowly add 1 mL of ice-cold Bead Conjugation Buffer.
- Cap the vial and vortex it until the EDC is completely dissolved.

3.1.2 Activate the beads:

- Vortex the beads well (~30 seconds), and immediately add the required volume of the 10 mg/mL EDC stock (calculated above) to the tube of beads.

Note

Vortex beads well before and after adding EDC!!!

- Cap the tube and vortex for 10 seconds.
- Place the tube in a rotator for 00:30:00 00:00:00 at 2-8 °C . Visually ensure that the mixing is adequate for a homogenous bead solution during activation.

Note

488 Dyed-beads are light sensitive!
Parafilm tube and cover the tube with aluminum foil to avoid light wile incubation.

3.2 Wash Beads

Note

Timing is critical for this step. Allow for no idle time after the beads have been activated. Use ice-cold buffers for all steps.

3.2.1 Wash the activated beads with Beads Conjugation Buffer:

- Briefly centrifuge the tube for 1–2 seconds to collect all the liquid into the tube.
- Place the tube in the magnetic separator for at least 1 minute to collect the beads to the side of the tube.
- Aspirate and discard the supernatant.
- Remove the tube from the magnet and add the Batch Volume (i.e., 150 µL) of ice-cold Bead Conjugation Buffer to the tube.
- Vortex for 5 seconds to disperse the beads. Briefly centrifuge the tube for 1–2 seconds to collect all the liquid into the tube.
- Place the tube in a magnetic separator with the bead pellet adjacent to the magnet for approximately 1 minute, to collect the beads to the side of the tube.
- Aspirate and discard the supernatant completely.

3.3 Conjugate the antibody to the activated beads: 2h

- Remove the tube of activated beads from the magnet.
- Add the Batch Volume (i.e., 150 µL) ice-cold buffer exchanged capture antibody to the beads.
- Cap the tube and mix by vortexing for 10 seconds.
- Place the tube in the rotator and mix for 02:00:00 at 2-8 °C . Visually ensure that the mixing is adequate for a homogenous bead solution during activation.

3.4 Clean Up: Wash & Block the Beads 45m

3.4.1 Collect the reaction supernatant:

- Briefly centrifuge the reaction tube of activated beads and antibody.
- Place the tube in a magnetic separator for at least 1 minute to collect the beads to the side of the tube.
- Aspirate the supernatant and dispense it into a new 1.7-mL tube labeled "Supernatant."

> **Note**
>
> Do not discard. This supernatant will be used later to determine the antibody coating efficiency.

3.4.2 Wash the beads 2 times with Bead Wash Buffer:

- Remove the tube from the magnet.
- Add the Batch Volume (i.e., 150 µL) of Bead Wash Buffer to the tube.
- Vortex for 5 seconds to disperse the beads. Briefly centrifuge the tube.
- Place the tube in a magnetic separator for at least 1 minute to collect the beads to the side of the tube.
- Aspirate the wash liquid and dispense it into a new 1.7-mL tube labeled "Wash 1."

Note

Do not discard. This wash liquid will be used later to determine the antibody coating efficiency.

- Repeat this washing procedure once, for a total of 2 washes. Discarding the second wash.

3.4.3 Block the beads:
- Remove the tube from the magnetic separator.
- Add the Batch Volume (i.e., 150 µL) Bead Blocking Buffer to the washed beads.
- Vortex for 5 seconds to disperse the beads. Briefly centrifuge the tube.
- Place the tube on a rotator and incubate for 00:45:00 at Room temperature . Visually ensure that the mixing is adequate for a homogenous bead solution during activation.

Note

488 Dyed-beads are light sensitive! Avoid light while blocking!

3.4.4 Wash the beads with Bead Wash Buffer:
- Briefly centrifuge the tube.
- Place the tube in a magnetic separator for at least 1 minute to collect the beads to the side of the tube.
- Aspirate the supernatant.
- Remove tube from the magnet and add Batch Volume (i.e., 150 µL) of Bead Wash Buffer to the beads.
- Vortex for 5 seconds to disperse the beads.
- Briefly centrifuge the tube.
- Place the tube in a magnetic separator for at least 1 minute to collect the beads to the side of the tube.
- Aspirate and discard the supernatant.

3.4.5 Perform the final wash and re-suspend the beads in Bead Diluent:
- Remove the tube from the magnet and add Batch Volume (i.e., 150 µL) Bead Diluent to the beads.
- Vortex for 5 seconds to disperse the beads.
- Briefly centrifuge the tube.
- Place the tube in a magnetic separator for at least 1 minute to collect the beads to the side of the tube.
- Aspirate the supernatant.
- Remove the tube from the magnet and add Batch Volume (i.e., 150 µL) Bead Diluent.
- Mix and then briefly centrifuge the tube to remove beads from the cap.

- Store the beads at 2-8 °C until you are ready to use them.

3.5 Clean Up: Determine Antibody Coating Efficiency

3.5.1 Measure the antibody concentration in both the supernatant and the "Wash 1" samples using a spectrophotometer at OD 280 and record the concentration values in the table below.

- For the supernatant sample, blank using Bead Conjugation Buffer.
- For the wash sample, blank using Bead Wash Buffer.

3.5.2 Calculate the amount of antibody coated onto the beads.

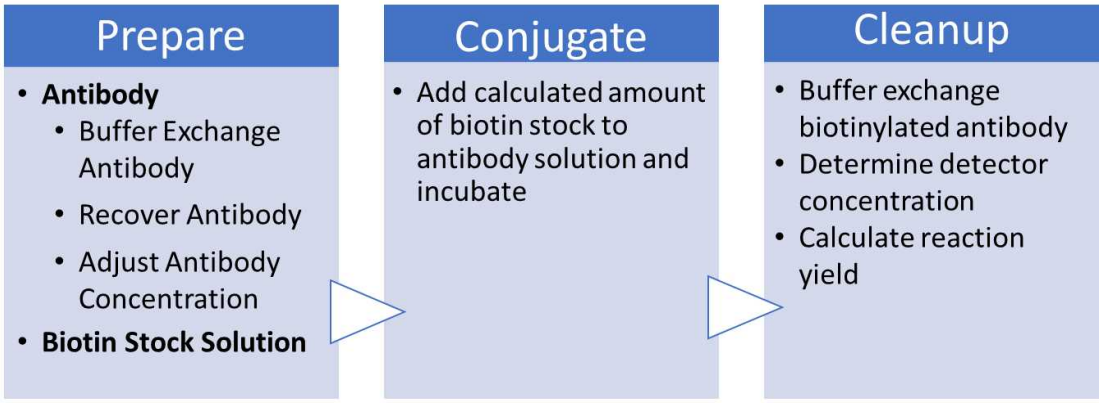
Calculation	Value
Initial Antibody in Reaction	$Reaction\ Ab\ Concentration\ \left(\frac{mg}{mL}\right) \times Batch\ Size\ (mL)$
Residual Antibody: Supernatant	$Supernatant\ Ab\ Concentration\ \left(\frac{mg}{mL}\right) \times Batch\ Size\ (mL)$
Residual Antibody: Wash 1	$Wash\ 1\ Ab\ Concentration\ \left(\frac{mg}{mL}\right) \times Batch\ Size\ (mL)$
Total Antibody Coated	$Initial - Residual\ (supernatant) - Residual\ (wash\ 1)$
% Antibody Coated	$\frac{Total\ Antibody\ Coated}{Initial\ Antibody\ in\ Reaction}$

Prepare Detector Antibodies (4 hours)

4 This protocol describes how to prepare biotinylated detector antibodies (IgG). The NHS-PEG4-biotin used in this protocol is an amine-reactive ester that couples to primary amine groups (-NH_2) of proteins. The hydrophilic polyethylene glycol (PEG) spacer improves water solubility. Other reactive groups (carboxyl, –SH) and spacers can be tried, but some optimization will be needed. This guide specifies preparing and testing an initial lot of detector with 1 mg/mL of antibody and a 40:1 ratio of biotin to antibody. Each biotinylation reaction requires 130 µg of antibody and will produce approximately 50 to 80 µg of detector.

Detector antibody for Homebrew pT73-Rab10 Assay is

Recombinant Anti-RAB10 antibody [MJF-R23] **Abcam Catalog #ab237703**



Workflow for Preparing Detector Antibody

4.1 Prepare the Antibody

14m

4.1.1 Follow instructions in Section 1 to block Amicon filters.

4.1.2 Remove the detector antibody from storage and allow it to warm to room temperature.

- Determine stock Ab concentration with BCA assay (with IgG standards) and A280.
- Calculate the mass of antibody required for a 100-µL reaction with 30% excess of antibody (e.g., 0.13 mg).
- Calculate the volume of antibody solution required to obtain the desired antibody mass. Use the stock antibody label concentration or measure the concentration by a spectrophotometer at OD 280, blanked with the antibody storage buffer.

$$Required\ Ab\ Mass\ (mg) Ab\ Stock\ Concentration\ (mg/mL) = Required\ Ab\ Stock\ Volume\ (mL)$$

Note

$$\frac{Required\ Ab\ Mass\ (mg)}{Ab\ Stock\ Concentration\ (mg/mL)} = Required\ Ab\ Stock\ Volume\ (mL)$$

4.1.3 Remove the antibody storage buffer:

- Insert one Amicon filter device into a supplied Amicon microcentrifuge tube, ensuring that the filter membranes align with the tube cap strap.
- Mix the antibody vial by vortexing. Pulse spin.
- Add the calculated antibody volume to the filter.
- Add sufficient Biotinylation Reaction Buffer to the filter for a total volume of 500 µL and cap the filter.
- Place the filter device in the centrifuge, aligning the cap strap toward the center of the rotor.
- Centrifuge the tube at 14000 x g for 00:05:00.
- Remove the filter device from the centrifuge and separate the filter from the tube.

- Discard the flow through and reinsert the filter into the tube.

Note

The antibody is now concentrated on the filter. Proceed immediately to the next step.

4.1.4 Buffer exchange the antibody using 2 washes with Biotinylation Reaction Buffer:

- Add 450 µL of Biotinylation Reaction Buffer to the filter and cap it.
- Place the filter device in the centrifuge, aligning the cap strap toward the center of the rotor.
- Centrifuge the tube at 14000 x g for 00:05:00.
- Remove the filter device from the centrifuge and separate the filter from the tube.
- Discard the flow through.
- Repeat this buffer exchange procedure once, for a total of 2 buffer exchanges. If volume in the filter is greater than 50 µL, repeat the procedure once more, for a total of 3 buffer exchanges.

4.1.5 Recover the antibody:

- Invert a clean Amicon microcentrifuge tube onto the top of the filter and invert the assembled device to collect the purified antibody.
- Centrifuge the device (with filter inverted) at 1000 x g for 00:02:00 to transfer the washed antibody from the filter to the tube.
- Add 50 µL of Biotinylation Reaction Buffer to the filter.
- Pipette up and down multiple times washing both membranes in the filter assembly.
- Carefully invert the filter into the same Amicon microcentrifuge tube from step b.
- Centrifuge the tube inverted at 1000 x g for 00:02:00 to transfer the rinsate from the filter to the tube.
- Remove the filter from the tube and discard the filter.
- Cap the tube and retain it.

4.1.6 Adjust the antibody concentration to 1 mg/mL:

- Measure and record the buffer exchanged antibody concentration using a spectrophotometer at OD 280, blanked with biotinylation reaction buffer.
- Estimate the volume of buffer exchanged antibody, using a P-100 pipette.
- Calculate and record the amount of Biotinylation Reaction Buffer to add to adjust the antibody (Ab) concentration to 1 mg/mL.
  $[Ab\ Conc.(mgmL) \times Ab\ vol.(\mu L)/1\ mg/mL] - Ab\ vol.(\mu L) = Reaction\ Buffer\ vol.(\mu L)$

Note

$$\frac{Ab\ Conc.\left(\frac{mg}{mL}\right) \times Ab\ vol.\ (\mu L)}{1\ mg/mL} - Ab\ vol.\ (\mu L) = Reaction\ Buffer\ vol.\ (\mu L)$$

- Add the calculated volume of Biotinylation Reaction Buffer to the concentrated antibody. Cap the tube and vortex for 5 seconds to mix.

4.1.7 Confirm the antibody concentration and volume and record the findings:
- Measure and record the antibody concentration using a spectrophotometer at OD 280, blanked with Biotinylation Reaction Buffer. The target concentration is 0.95 – 1.05 mg/mL.
- Estimate the volume of antibody using a P-100 pipette.

4.2 Conjugate

30m

4.2.1 Remove 1 vial of NHS-PEG4-Biotin from –20°C storage and allow it to warm to room temperature.

Note

- NHS-PEG4-Biotin is moisture-sensitive. Do not open the vial until it has reached room temperature.
- NHS-PEG4-Biotin must be fully dissolved to reach the correct concentration.

4.2.2 Reconstitute the NHS-PEG4-Biotin to a 8.9 mM stock concentration:
- Immediately before use, unscrew the yellow cap.
- Add 383 µL of deionized water.
- Mix by pipetting up and down. Alternatively, the vial can be vortexed for a few seconds to ensure a homogeneous solution.

4.2.3 Measure into a new 1.7-mL tube the required volumes of 1 mg/mL antibody, reconstituted NHS-PEG4-Biotin, and Biotinylation Reaction Buffer for the detector concentration that you want to prepare, according to the following table. NOTE: use values correlating to a 40x ratio to start.

Molar Biotinylation Ratio	20x	40x	60x
Detector antibody at 1 mg/mL (µL)	100	100	100
Reconstituted 8.9 mM NHS-PEG4-Biotin (µL)	1.5	3	4.5
Biotinylation Reaction Buffer (µL)	3	1.5	0
Final volume (µL)	104.5	104.5	104.5

4.2.4 Mix the reaction by vortexing.
4.2.5 Centrifuge the tube for 2 seconds to collect all the liquid into the bottom of the tube.
4.2.6 Incubate the tube for 00:30:00 minutes at r Room temperature

Note

- Shaking is not required in this step.
- The antibody is now biotinylated. Purify the biotinylated antibody now.

4.3 Purify the Biotinylated Antibody

14m

4.3.1 Concentrate the antibody:

- Follow instructions in Section 1 to block Amicon filters.

Note

Ensuring that the filter membranes align with the tube cap strap.

- Add all of the conjugated antibody to the blocked Amicon filter.
- Add sufficient Biotinylation Reaction Buffer to the filter for a total volume of 500 µL (approximately 400 µL) and cap the filter.
- Place the filter device in the centrifuge, aligning the cap strap toward the center of the rotor. Centrifuge at 14000 x g for 00:05:00.
- Remove the filter device from the centrifuge and separate the filter from the tube.
- Discard the flow through and reinsert the filter into the tube.

4.3.2 Purify the antibody using 4 washes with Biotinylation Reaction Buffer:

- Add 450 µL of Biotinylation Reaction Buffer to the filter and cap it.
- Place the filter device in the centrifuge, aligning the cap strap toward the center of the rotor. Centrifuge at 14000 x g for 00:05:00.
- Remove the filter device from the centrifuge and separate the filter from the tube.
- Discard the flow through.
- Repeat this washing procedure 3 times, for a total of 4 washes.

Note

The antibody is now concentrated on the filter, and free biotin has been removed.

4.3.3 Recover the antibody and Storage:

- Invert a clean Amicon microcentrifuge tube onto the top of the filter and invert the assembled device to collect the purified antibody.
- Centrifuge the device (with filter inverted) at 1000 x g for 00:02:00 to transfer the washed antibody from the filter to the tube.
- Add 50 µL of Biotinylation Reaction Buffer to the filter.

- Pipette up and down multiple times washing both membranes in the filter assembly.
- Carefully invert the filter into the same Amicon microcentrifuge tube from step b.
- Centrifuge the tube inverted at 1000 x g for 00:02:00 to transfer the rinsate from the filter to the tube.
- Remove the filter from the tube and discard the filter.
- The final detector concentrate should be in a 90-µL volume. Store the concentrate at 4 °C until use.

4.3.4 Characterization

- Measure and record the concentration of biotinylated antibody using a spectrophotometer at OD 280, blanked with Biotinylation Reaction Buffer.
- Calculate the antibody Biotinylation reaction yield:

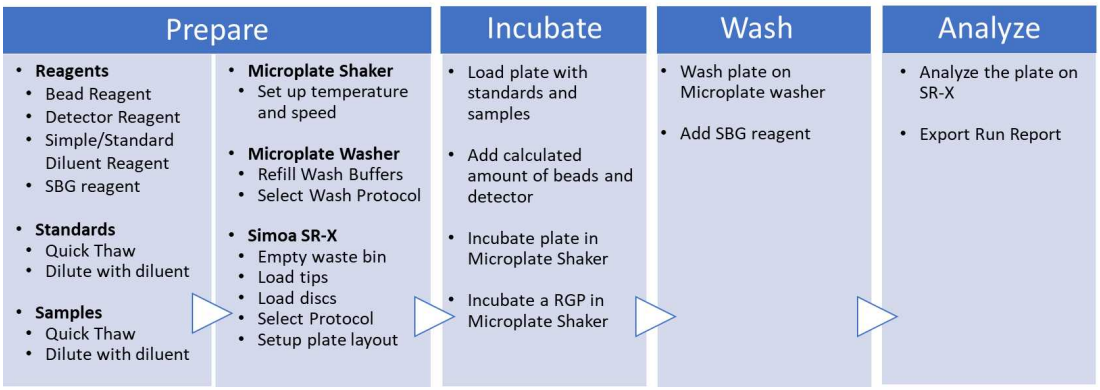
$$[Final\ Detector\ Volume\ (\mu L)\ \text{x}\ Detector\ Concentration\ (mg/mL)]/\ [Reaction\ Volume\ (\mu L)\text{x}Reaction\ Concentration\ (mg/mL)]\times 100\%$$

Note

$$\frac{Final\ Detector\ Volume\ (\mu L) * Detector\ Concentration\ \left(\frac{mg}{mL}\right)}{Reaction\ Volume\ (\mu L) * Reaction\ Concentration\ \left(\frac{mg}{mL}\right)} \times 100\%$$

Perform Simoa Assay (5 hours)

5



Workflow for performing Simoa Assay on SR-X

5.1 Preparing Reagent Solutions

5.1.1 Determine the number of individual samples that will be run, including calibrators and samples that will be diluted manually on the SR-X Analyzer.

5.1.2 Prepare Bead Reagent

- Based on the bead concentration determined previously (1.4 x 10^9 beads /mL), calculate the volume of bead concentrate required to yield a solution of sufficient volume (calculated below) at a working concentration of 2.0 x 10^7 beads/mL (25 uL bead reagent per test/reaction, which gives 500k beads per reaction).

$$((Reagent\ Volume\ Required\ per\ test + 10\ \mu L) \times Number\ of\ tests) x\ 25\ uL\ Bead$$

$$Vol.Concentrated\ Bead\ Stock\ Req.(mL) = [Working\ Vol\ Bead\ Reagent\ Req.\ (mL) \times Working\ Bead\ Concentration(beads/mL)] / Bead\ Stock\ Concentration\ (beads/\ mL)$$

Note

$$Vol.Concentrated\ Bead\ Stock\ Req.(mL) = \frac{Working\ Vol\ Bead\ Reagent\ Req.\ (mL) \times Working\ Bead\ Concentration\left(\frac{beads}{mL}\right)}{Bead\ Stock\ Concentration\ \left(\frac{beads}{mL}\right)}$$

- Vortex the bead stock for at least 30 seconds to completely resuspend beads.
- Add calculated volume of bead stock to 1.7-mL tube.
- Add 300 uL of Bead Diluent to the beads. Vortex to mix.
- Vortex this tube briefly (followed by a spin if beads get lodged into the lid), then place on the magnetic rack.
- Wait for ~60 seconds (when all beads have pelleted to the side of the tube) then carefully remove supernatant.
- Take tube off magnet and add back ~300uL bead diluent, repeat these steps x2.
- In final wash leave the beads in the 300uL diluent.
- Add the appropriate/calculated volume of Bead Diluent (Working Vol. Bead Reagent Req.)to the bead stock and vortex to mix. Keep this On ice until needed.

5.1.3 Prepare Detector Reagent

- Based on the detector stock concentration determined previously, calculate the volume of detector stock required to yield a solution of sufficient volume (calculated above) at the desired working concentration. A final detector concentration of 0.1-0.3 µg/mL may be appropriate for a preliminary test of untested reagents.

$$Vol.Detector\ Stock\ Req.(mL) = Working\ Vol\ Detector\ Reagent\ Req.(mL) \times Working\ \square etector\ Conc.\ (\square g/mL) / Detector\ Stock\ Conc.(\square g/mL)$$

Note

$$Vol.\,Detector\ Stock\ Req.\,(mL) = \frac{Working\ Vol\ Detector\ Reagent\ Req.\,(mL)\ \times\ Working\ Detector\ Conc.\ \left(\frac{\mu g}{mL}\right)}{Detector\ Stock\ Conc.\left(\frac{\mu g}{mL}\right)}$$

- Spike the volume of detector stock calculated above into the appropriate volume of Detector Diluent in a 1.7 uL tube.
- Keep this On ice until needed.

5.1.4 Prepare SBG Reagent

Note

Prepare SBG Reagent 30 minutes earlier/warm up to room temperature before needed.

- Prepare a 150 pM solution of SBG with the fill volume calculated below using SBG Concentration (stock concentration 50mM) by spiking the required volume concentrate into the appropriate volume of SBG Diluent in a 15 mL tube.
  $Volume\ SBG\ Stock\ Required\ (mL)$= [$Vol\ SBG\ Solution\ Req.(mL)$× 150 pM/ $SBG\ Stock$ ▯ ▯$onc.\ (nM)$]× [1 nM1000 pM]

Note

$$Volume\ SBG\ Stock\ Required\ (mL) = \frac{Vol\ SBG\ Solution\ Req.\,(mL)\ \times\ 150\ pM}{SBG\ Stock\ Conc.\ (nM)} \times \frac{1\ nM}{1000\ pM}$$

- Label the tube and store at Room temperature until needed.

5.1.5 Prepare Homebrew Sample Diluent Reagent

- Base on the number of samples and dilution factors needed for samples, calculate the volume of sample diluent required.
- Spike in appropriate amount of sodium dodecyl sulfate, sodium deoxycholate and freshly prepared dithiothreitol to Homebrew Sample Diluent Buffer to the desired concentration described in the table below.

	Ingredients Spike in Homebrew Sample Diluent	Final Concentration in Homebrew Sample Diluent Buffer

	Ingredients Spike in Homebrew Sample Diluent	Final Concentration in Homebrew Sample Diluent Buffer
	Sodium Dodecyl Sulfate	0.1%
	Sodium Deoxycholate	0.05%
	Freshly Prepared Dithiothreitol	0.4mM

5.2 Prepare standards and samples

5.2.1 Prepare standards

Laboratory prepared HEK-293T protein lysates was used as standard in this Homebrew Assay. Use Homebrew Sample Diluent Buffer to produce a dilution series (ranging from 1000 pg/mL to 0.1 pg/mL). Use Homebrew Sample diluent as the zero standard (0 pg/mL).

Note

HEK-293T lysates were generated through transient transfection of plasmids expressing R1441G-LRRK2 with human Rab10. Lysates were harvested 24-hours after transfection. Phos-tag immunoblot analysis was used to determine the proportion of Rab10 protein phosphorylated at position Thr73 by LRRK2 protein in HEK-293T lysate.

- Transfer 100 µL of each standard to the desired wells of the 96-well assay plate.

5.2.2 Prepare samples

Note

Total buffer volume is around 100 uL in the cuvette during each incubation.

Note

- Serum and plasma samples require 10-fold dilution. A suggested 10-fold dilution is 10 uL of sample + 90 uL of Homebrew Sample Diluent Buffer.
- Urine Exosome samples require 3 to 5-fold dilution. A suggested 5-fold dilution is 20 uL of sample + 80 uL of Homebrew Sample Diluent Buffer.

- Quick thaw samples in water bath at 37 °C.
- Immediately transfer sample tubes On ice.

- For each sample to be tested, transfer the appropriate volume of sample diluent to the desired wells of the 96-well assay plate. Add the appropriate volume of well mixed sample (e.g. 5ul samples + 95 uL Sample Diluent Buffer) and mix briefly by pipetting up and down.

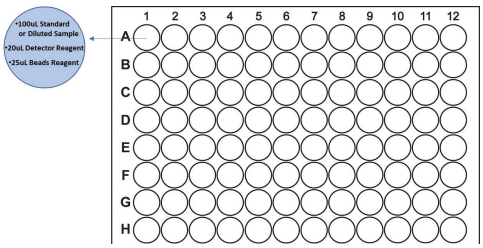
5.3 Load Bead and Detector Reagents to the plate

Add the appropriate volume of beads and detectors to desired wells of the assay plate.
For 2-step Dispense Protocol, 25 uL of Beads and 20 uL of Detector reagent were loaded to each well.

Note

For optimal performance, it is important to transfer samples and reagents to the assay plate with minimal delays between columns or rows. Ensure that all reagents are ready for transfer as incubation times near completion.

Reagent	Recommended 2-Step Dispense Volumes (µL)	Recommended 3-Step Dispense Volumes (µL)
Beads	25	25
Detector	20	100



96-Well Microplate

5.4 Starting the Assay

1h

5.4.1 Incubate plate and RGP Reagent on the Microplate Shaker.

Note

Recommended incubation times for initial experiments are:
3-step protocol: 30 min beads/sample, 10 min detector, 10 min SBG
2-step protocol: 30 min beads/sample/detector, 10 min SBG

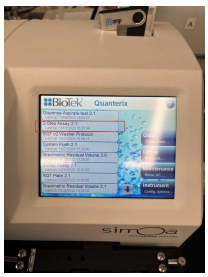
- Set up temperature and shaking speed on Microplate shaker. (optimized 2-step protocol is used in this assay in West lab).
- Incubate the samples, calibrators, and bead solution with detector solution at RT on the microplate shaker at 800 rpm for 01:00:00. Cover plate with lid or aluminum foil during incubation.
- Prior to use, RGP must be solubilized fully by heating with constant vigorous shaking. Incubate RGP bottle in the plate shaker at 800 rpm. Minimum required shaking time at specific temperature is shown below:

	Temp (C)	Time (min)
	< 25	120
	25	60
	30	30
	35	20

RGP shaking temperature and time required.

5.4.2 Wash the plate on the Microplate Washer.

- Check and refill Wash Buffer in A and B and Milli-Q water in C.
- Empty the waste bottle.
- After incubation completed, transfer the plate to the Microplate washer, remove lid.
- Begin the semi-automated 2-step assay on the Simoa Washer.



- After the first wash, incubate samples with 100 uL SBG for 10 minutes at 37 °C at 800 rpm on the Microplate shaker.

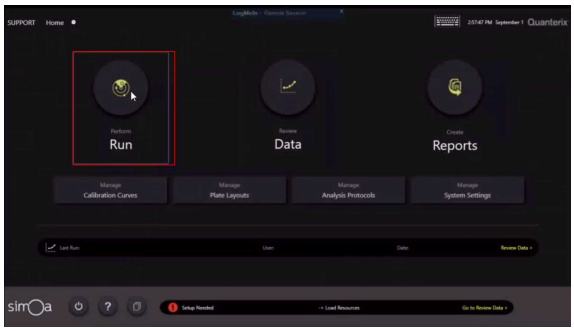
Note

For best results transfer detector and SBG with a multi-channel repeat pipettor.

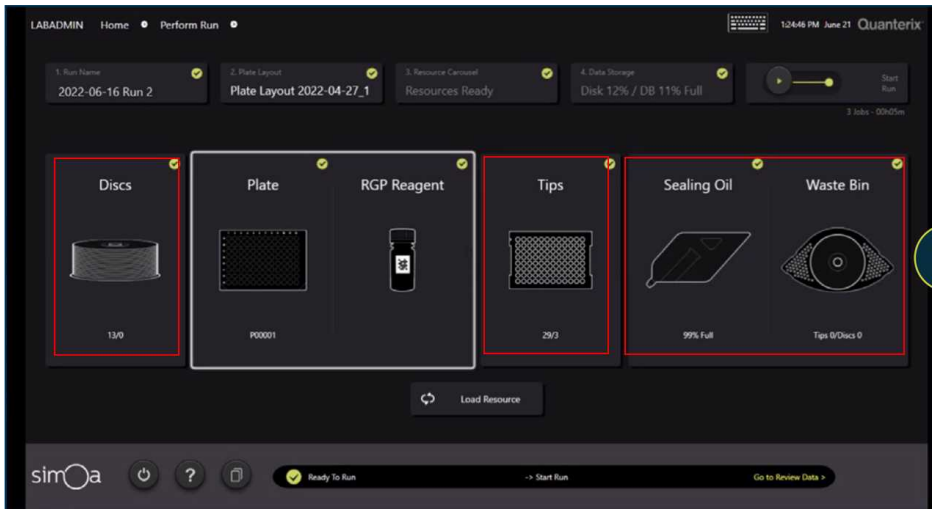
- When the 2-step assay wash protocol has been completed, check the positions of the bead pellets in the wells. If the pellets are too high, manually add buffer B to the well, shake, place back on magnet, and aspirate buffer.

5.4.3 Analyze the plate on Quanterix SR-X (2 hours)

- Turn on the SR-X.
- Select "Run" on the home page.



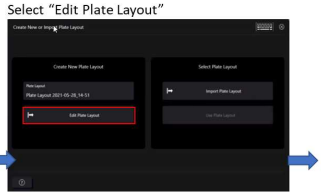
- Load the tips, micro-discs and RGP onto the instrument, ensure the waste is emptied and sealing Oil is enough (2-3% of sealing oil will be used per run).



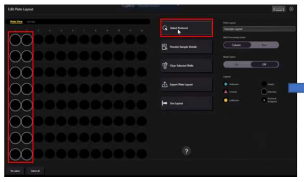
- Choose a proper Homebrew analysis protocol and define plate layout.

Select “Plate Layout”

Select “Edit Plate Layout”

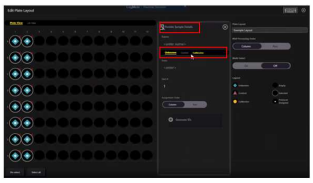


Select wells need to be assigned, then click “Select Protocol”.

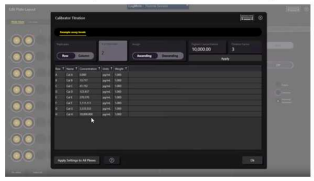


Select “Dyed Beads Homebrew” from the drop box

Provide Sample Details



For Calibrators, titration factors need to be provided

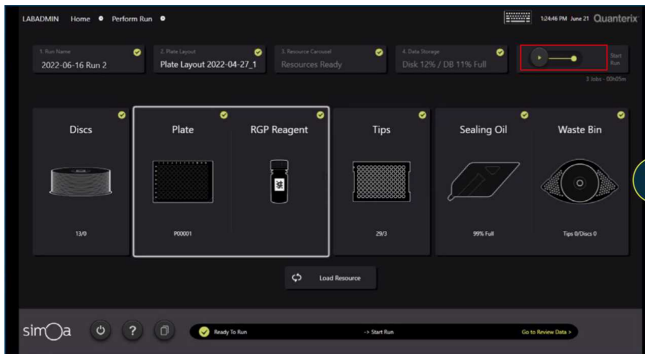


- When the semi-automated protocol has been completed and the assay plate is dry, transfer the plate to the Quanterix SR-X.

Note

Run the plate on the SR-X within one hour of drying.

- Start the run.

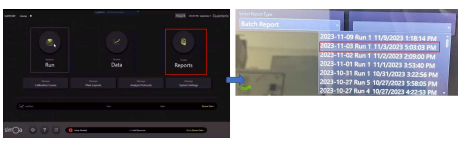


Optimize Assay Conditions

6 Review Results

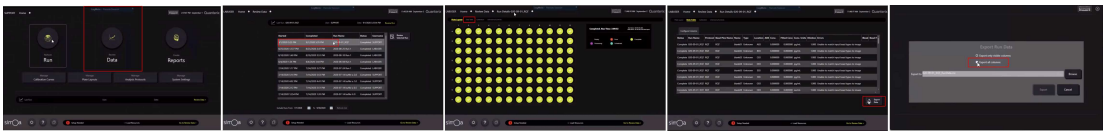
6.1 Export run report and data to an external flash drive.

- Select "**Report**" on home page of SR-X.
- Select the run need to be generated a report from the drop box, then click on "Generate report"
- View the report and.
- Export report (PDF or Excel) and to an external hard drive.



- Select "**Data**" on home page of SR-X.

- Select the run need to be generated a run data.
- Select "data table" on the top left corner.
- Select "Export data".
- Select "Export all columns"
- Export run data (csv) and to an external hard drive.



6.2 Evaluate LOD, LLOQ, and Signal-to-Background Ratio

- Open "Run report" excel file.
- Check AEBs for standards.
- Limit of Detection (LOD) is the lowest amount of analyte in a sample that can be detected with acceptable statistical certainty but not quantitated as an exact value. Quanterix calculates LOD at 2.5 x STD above Cal A Average AEB, and typical Quanterix kits use 4PL curve fitting with 1/y2 weighting.
- LLOQ is the lower limit of quantitation, calculated as the lowest concentration at which the CV's are less than 20%.
- The signal-to-background ratio between Calibrator A (background) and Calibrator B should be between 2.5–3.

6.3 Ideal assay characteristics

- Open "Run data" csv file.
- Check "fon" column.
- There should be a low background, less than 0.02 AEB (fon <~ 0.02). The ideal level is 0.005–0.01 AEB (fon ~ 0.005–0.01)
- High signal-to-background ratio (robust dose-response slope) with an upper range of target signal on the order of ~16 AEB. Levels above 20 AEB should be avoided. Above this, the optics will begin to exhibit saturation at the current setting of exposure time. Signal saturation
- will manifest as a reduction in the expected dose-response of the calibrator level(s) exhibiting saturation.
- Approximately 4 logs of signal range are typical for a Simoa assay (0.005–16 AEB). A low slope indicates a larger dynamic range (+) but greater dose read-back imprecision at a given signal (-). A higher slope indicates a smaller assay range due to the camera ceiling (-) but minimizes dose imprecision at a given signal (+).

6.4 For low-abundance analytes, it is more important to maximize sensitivity. The objective is to maximize signal-to-background ratios with the highest possible calibration curve slope and the lowest possible background.