Simoa Extracellular Vesicle assays

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1 Works for me
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ABSTRACT

Extracellular Vesicles (EV) are reservoirs of biomarkers such as mRNA and post-translationally modified proteins, which may be of use in diagnosing disease. However, major challenges still remain in the isolation and characterization of EVs in complex biofluids such as plasma and cerebrospinal fluid. We developed a method for quantifying EVs in complex biofluids by measuring three commonly expressed EV transmembrane proteins (CD9, CD63 and CD81). Due to the low abundance of EVs in biofluids, we used single molecule array (Simoa) technology in order to quantify these transmembrane proteins. By using all three, we reduce the chance of missing a rare population of EVs and instead get a composite measure of total EVs. This protocol describes how to prepare the reagents necessary for Simoa analysis of EVs as well as how to set up and run the assay on the Quanterix HD-1 or HD-X instrument.

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KEYWORDS

Single Molecular Arrays, Simoa, Tetraspanins, Extracellular Vesicles, Exosomes

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MATERIALS TEXT

Antibodies: Abcam AB195422, R&D Systems MAB5048, Abcam AB79559, Abcam AB58989, BD Biosciences 556019, Biolegend 349502

Recombinant Protein Standards: CD9 (Abcam ab152262), CD63 (Origene TP301735), CD81 (Origene TP317508)

Quanterix Neuroplex 3A sample diluent (Quanterix 102002)

Quanterix Sample Diluent E (Quanterix 101579)

50kD Centrifugal Filters (Millipore Sigma UFC505096)

Quanterix Homebrew Assay Development Kit (Quanterix 101354)

Quanterix Enzyme Substrate Kit (Quanterix 101361)

Quanterix Disc Kit (Quanterix 103347)

EDC (Thermo Fisher A35391)

Z2 (Beckman Coulter 6605700)

Z-Pac (Beckman Coulter 8320312)

Biotin (EZ-Link NHS-PEG4-Biotin, No Weigh Format Thermo Fisher A39259)

Benchtop Centrifuge

Dynamag (Thermo Fisher 12321D)

Multiplex "Helper" Beads: 647 (Quanterix 101985), 700 (Quanterix 101986), 750 (Quanterix 101987)

Quanterix HD-X analyzer

ABSTRACT

Extracellular Vesicles (EV) are reservoirs of biomarkers such as mRNA and post-translationally modified proteins, which may be of use in diagnosing disease. However, major challenges still remain in the isolation and characterization of EVs in complex biofluids such as plasma and cerebrospinal fluid. We developed a method for quantifying EVs in complex biofluids by measuring three commonly expressed EV transmembrane proteins (CD9, CD63 and CD81). Due to the low abundance of EVs in biofluids, we used single molecule array (Simoa) technology in order to quantify these transmembrane proteins. By using all three, we reduce the chance of missing a rare population of EVs and instead get a composite measure of total EVs. This protocol describes how to prepare the reagents necessary for Simoa analysis of EVs as well as how to set up and run the assay on the Quanterix HD-1 or HD-X instrument.

Bead Coupling Protocol 5h

1 This beads coupling protocol is based on the coupling protocols developed by Quanterix. The manual for these can be found at Quanterix.com. 5h

Each bead coupling procedure below has been optimized for the given antibody and assay. Where differences arrise between the procedures they are noted.

1.1 Thaw 100ug of antibody: CD9 (Abcam AB195422), CD63 (R&D Systems MAB5048), CD81 (Abcam AB79559) 30m

Raise the volume of the antibody to 500uL (0.2mg/mL) using 1x PBS and allow to rotate on a mixer (such as Hula Mixer) for ~10 minutes with gentle rotation.

Place the 500uL solution in an Amicon Ultra-0.5mL (50kD) Centrifugal Filter (Millipore Sigma

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UFC505096).

Centrifuge at 14,000xg for 5 minutes.

Discard the flow through and add 450uL of Bead Conjugation Buffer (Quanterix 101357).

Centrifuge at 14,000xg for 5 minutes.

Discard the flow through and add 450uL of Bead Conjugation Buffer (Quanterix 101357).

Centrifuge at 14,000xg for 5 minutes.

Discard the flow through and flip the tube into a clean tube (provided with the UFC505096 spin filter).

Centrifuge at 1,000xg for 2 minutes.

Flip the tube back and add 25uL of Bead Conjugation Buffer (Quanterix 101357) to each side of the filter (50uL total) and the flip back to collect the fluid.

Centrifuge at 1,000xg for 2 minutes.

Measure the concentration of the antibody in solution using a NanoDrop. Use the protein A280 Setting, select IgG. Blank the NanoDrop with the Bead Conjugation Buffer and then measure the concentration of the antibody.

Measure the volume in the tube and transfer it to a new 1.5mL tube.

Record the volume and concentration and multiply them for the ug recovered.

Raise the volume of each antibody to 300uL in Bead Conjugation Buffer. Place the antibody either on ice or in the 4C refrigerator.

1.2 Wash and activate the Beads (use Singleplex 488 non-dye-encoded beads Quanterix 103207) : 45m

Place appropriate number of beads in a tube and place on a dynamag (Thermo Fisher 12321D):
CD9: 4e8 beads
CD63: 2.8e8 beads
CD81: 4e8 beads

Remove EDC (Thermo Fisher A35391) from the -20 and let sit at room temperature for 10 minutes.

Repeat 2x: Remove the supernatant and remove the tube from the Dynamag magnet, add 200uL Wash Buffer (Quanterix 101355), vortex for 3 seconds at high speed and place back on the magnet.

Repeat 2x: Remove the supernatant and remove the tube from the magnet, add 200uL Bead Conjugation Buffer (Quanterix 101357), vortex for 3 seconds at high speed and place back on the magnet.

Remove the supernatant and remove the tube from the magnet, add 300uL Bead Conjugation Buffer (Quanterix 101357)

Reconstitute the EDC in 100uL of Bead Conjugation Buffer and vortex for 10 second. Add the appropriate amount of EDC to each tube of beads:

CD9: 2uL EDC solution
CD63: 5uL EDC solution

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CD81: 5uL EDC solution

Put the beads in a Hula Mixer (Thermo Fisher 15920D) with rotation and intermittent shaking for 30 minutes at the appropriate temperature:

CD9: 4C
CD63: Room Temperature
CD81: 4C

1.3 After 30 minutes remove the beads from the hula mixer and spin them down before placing them on the DynaMag magnet. For the following steps do each bead one at a time to minimize the time any bead tube spends on the magnet. 2h

Remove the supernatant for each tube, remove the tube from the magnet, add 200uL Bead conjugation buffer and vortex for 3 seconds.

Place the tube on the magnet, remove the tube from the magnet, add the 300uL of purified antibody in Bead Conjugation Buffer and vortex for 3 seconds.

Put the beads in a Hula Mixer with rotation and intermittent shaking for 2 hours at the appropriate temperature:

CD9: 4C
CD63: Room Temperature
CD81: 4C

1.4 After 2 hours remove the beads from the Hula Mixer and spin them down before placing them on the DynaMag. 45m
For the following steps do each bead one at a time to minimize the time any bead tube spends on the magnet.

Remove the supernatant for each tube, place it in a tube labeled Supernatant and add 200uL Bead Wash Buffer (Quanterix 101355) and vortex for 3 seconds. Place back on the magnet.

Remove the supernatant for each tube, place it in a tube labeled Wash 1 and add 200uL Bead Wash Buffer (Quanterix 101355) and vortex for 3 seconds. Place back on the magnet.

Repeat 2x: Discard the supernatant for each tube and add 200uL Bead Wash Buffer and vortex for 3 seconds. Place back on the magnet.

Discard the supernatant for each tube, add 200uL Bead Blocking Buffer (Quanterix 101356)and vortex for 3 seconds. Put the beads in a Hula mixer with rotation and interemittent shaking for 30 minutes at room temperature.

1.5 After 30 minutes remove the beads from the hula mixer and spin them down before placing them on the Dynamag. 5m

Remove the supernatant, remove the tube from the magnet and add 200uL of Bead Wash Buffer. Vortex for 3 seconds. Place back on the magnet.

Remove the supernatant, remove the tube from the magnet and add 200uL of Bead Diluent. Vortex for 3 seconds. Remove the 200uL solution including all the beads from the tube and place in a new clean tube for long term storage.

1.6 Characterize the bead coupling efficiency: 5m

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Measure the supernatant and Wash 1 from the reserved tubes. Do this using a nanodrop. Use the protein A280 Setting, select IgG. Blank the nanodrop with the Bead Conjugation Buffer for the supernatant and then measure the concentration of the antibody. Then Blank the nanodrop with the Bead Wash Buffer for the Wash 1 and then measure the concentration of the antibody.

Calculate the efficiency of antibody capture:

ug in original solution before coupling as measured by the nanodrop - ug in supernatant as measured by the nanodrop
-ug in wash1 as measured by the nanodrop= coupled antibody

Percent coupling efficiency= ug coupled antibody/ug original antibody

Optimally coupling efficiency is above 50%, but these assays will work with coupling efficiency as low as 10%. Sensitivity may be reduced with lower coupling efficiency.

1.7 Characterize the bead aggregation and quantity: 5m

Using a Coulter Counter Z2 (Beckman Coulter 6605700), count the bead concentration and the % monomeric beads. This can be done by blanking the Z2 with a clean solution of Z-Pac (Beckman Coulter 8320312) and then measuring the beads at a 2000x dilution (place 5uL bead solution in 10mL of Z-Pac). The bead concentration should be noted as well as the monomeric bead concentration. Ideally at least 80% of the beads should be monomeric.

Biotinalation Protocol 1m

2 1m

This step prepares the detection antibodies for the Simoa assay:

Thaw 100ug of antibody: CD9 (Abcam AB58989), CD63 (BD Biosciences 556019), CD81 (Biolegend 349502), also bring 1 vial of Biotin (EZ-Link NHS-PEG4-Biotin, No Weigh Format Thermo Fisher A39259) to room temperature from the -20C to thaw.

2.1 Raise the volume of each antibody to 500uL (0.2mg/mL) using Biotinylation Reaction Buffer (Quanterix 101358)and allow to rotate on a Hula mixer for ~10 minutes. 30m

Place the 500uL solution in an Amicon Ultra-0.5mL (50Kd) Centrifugal filter (Millipre Sigma UFC505096).

Centrifuge at 14,000xg for 5 minutes.

Remove the supernatant and add 450uL of Biotinylation Reaction Buffer (Quanterix 101358).

Centrifuge at 14,000xg for 5 minutes.

Remove the supernatant and add 450uL of Biotinylation Reaction Buffer (Quanterix 101358).

Centrifuge at 14,000xg for 5 minutes.

Flip the tube into a clean tube (provided with the UFC505096 spin filter).

Centrifuge at 1,000xg for 2 minutes.

Flip the tube back and add 25uL of Biotinylation Reaction Buffer (Quanterix 101358) to each side of the filter (50uL total) and the flip back to collect the fluid.

Centrifuge at 1,000xg for 2 minutes.

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Measure the concentration of the antibody in solution using a NanoDrop. Use the protein A280 Setting, select IgG. Blank the NanoDrop with the Biotinylation Reaction Buffer (Quanterix 101358) and then measure the concentration of the antibody.

Measure the volume in the tube and transfer it to a new 1.5mL tube. Add Biotinylation Reaction Buffer (Quanterix 101358) to bring the concentration down to 1mg/mL and record the volume (which should be equal to the ug of antibody calculated).

2.2 Calculate the amount of Biotin needed for 40x excess Biotin: 35m

To get 40x Biotinylation: Resuspend the vial of Biotin in 100uL of Ultra Pure H_20 and vortex for 10 seconds. Remove 30.6uL of this solution and dilute it in 69.4uL of Ultra Pure H_20. Of this solution, you should add to each antibody:

0.02564 * uL of antibody @1mg/mL = uL of Biotin solution to add in (this will generally be between ~2-3uL if starting with 100ug of antibody).

Put the appropriate amount of biotin in the antibody solution, vortex for 5 seconds and spin down briefly. Let sit at room temperature without shaking for 30 minutes.

2.3 After 30 minutes, remove the excess biotin that is not bound to the antibody. 30m

Bring each antibody up to 500uL in (Quanterix 101358). Place the 500uL solution in an Amicon Ultra-0.5mL (50Kd) Centrifugal filter (Millipore Sigma UFC505096).

Centrifuge at 14,000xg for 5 minutes.

Remove the supernatant and add 450uL of Biotinylation Reaction Buffer (Quanterix 101358).

Centrifuge at 14,000xg for 5 minutes.

Remove the supernatant and add 450uL of Biotinylation Reaction Buffer (Quanterix 101358).

Centrifuge at 14,000xg for 5 minutes.

Flip the tube into a clean tube (provided with the UFC505096 spin filter).

Centrifuge at 1,000xg for 2 minutes.

Flip the tube back and add 25uL of Biotinylation Reaction Buffer (Quanterix 101358) to each side of the filter (50uL total) and the flip back to collect the fluid.

Centrifuge at 1,000xg for 2 minutes.

Measure the concentration of the antibody in solution using a NanoDrop. Use the protein A280 Setting, select IgG. Blank the NanoDrop with the Biotinylation Reaction Buffer (Quanterix 101358) and then measure the concentration of the antibody.

Running The Calibration curve and Samples. 2h 45m

3 Calculate the number of measurements to be made for each assay. This will be: 2h

#Jobs= (# of points in your calibration curve + # of samples to be measured)* number of replicates.

In general a minimum of 2 replicates should be used for both the curve and the samples.

3.1 Calculate the volume of beads to be used per assay: 5m

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#jobs*35uL+700uL = total volume of bead solution to be inserted in the insturnment

All of the EV assays use healper beads at a ratio of 1:1:1:1. This means that a quarter of the beads used will be the assay beads attached to the capture antibody and then the other 3/4 will be dye encoded beads without capture antibody. These are dye encoded at the following wavelengths 647 (Quanterix 101985), 700 (Quanterix 101986), 750 (Quanterix 101987). To calculate (X) how much of each bead to spike into the bead diluent:

5000beads/UL* total volume in uL= X uL* bead concentration in beads/uL.

Since 4 bead types are in use this will yield a solution with 20,000 beads/uL.

Using an HD1/HDX brown reagent bottle (Quanterix 102411) put in the total volume of bead diluent needed and then put in the uL of each of the 4 bead types.

3.2 Calculate the volume of detector to be used per assay: 5m

#jobs*30uL+700uL = total volume of detector solution to be inserted in the insturnment

The concentration of each respective antibody for each EV assay should be as follows:

CD9= 3.2ug/mL
CD63= 2.175ug/mL
CD81= 2.175ug/mL

Amount of Biotinylated detection antibody (X) to spike into sample/detector diluent:

For CD9:

3.2ug/mL* total volume of detector solution= X uL * concentration of biotinylated antibody

For CD63:

2.175ug/mL* total volume of detector solution= X uL * concentration of biotinylated antibody

For CD81:

2.175ug/mL* total volume of detector solution= X uL * concentration of biotinylated antibody

Spike in the appropriate uL of biotinylated antibody into the total volume of solution in Quanterix detector/sample diluent (Quanterix 101359)

3.3 Calculate the volume of SBG solution to be used per assay: 5m

#jobs*110uL+700uL = total volume of SBG solution to be inserted in the insturnment

The SBG concentration (Quanterix 103397) of each respective EV assay should be as follows:

CD9= 120pM
CD63= 120pM
CD81= 150pM

Generally, the SBG stock concentration is at 50nM (but if not this equation should be adjusted):

For CD9:

120pM* total volume of SBG solution= X uL SBG stock* 50,000pM

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For CD63:

120pM* total volume of SBG solution= X uL SBG stock* 50,000pM

For CD81:

150pM* total volume of SBG solution= X uL SBG stock* 50,000pM

Spike in the appropriate uL of SBG stock into the total volume of solution in Quanterix SBG diluent (100376)

3.4 Calculate the amount of RGP for all jobs for all assays in a given run. The insturnment uses 25uL of RGP per job. A given bottle of RGP (Quanterix 103159) contains enough RGP for 96 jobs. 5m

3.5 Create calibration curves for each assay in the correct diluent. Each diluent has been optimized to work for spike and recovery and dilution linearity in both cerebrospinal fluid and plasma. While the calibration curve can be adjusted to any needed concentration range, the following calibration curves work well for biological samples: 20m

The curves below are written for the following stock concentrations of recombinant protein:

CD9 (Abcam ab152262)= 40ug/mL
CD63 (Origene TP301735)= 50ug/mL
CD81 (Origene TP317508)= 120ug/mL

The diluent used for each assay are:

CD9= 30% Quanterix Neuroplex 3A sample diluent (Quanterix 102002) and 70% Sample/Detector Diluent (Quanterix 101359)
CD63= Quanterix Sample Diluent E (Quanterix 101579)
CD81= Quanterix Sample Diluent E (Quanterix 101579)

CD9 in 30% Neuroplex 3A Diluent/70% Sample/Detector Diluent
9) 1.215µL*40µg/mL = 600µL*81ng/mL
8) 200µL*81ng/mL = 600µL*27ng/mL
7) 200µL*27ng/mL = 600µL*9ng/mL
6) 200µL*9ng/mL = 600µL*3ng/mL
5) 200µL*3ng/mL = 600µL*1ng/mL
4) 200µL*1ng/mL = 600µL*333.3pg/mL
3) 200µL*333.3pg/mL = 600µL*111.1pg/mL
2) 200µL*111.1pg/mL = 600µL*37pg/mL
1) 200µL*37pg/mL = 600µL*12.34pg/mL
0) 400µL Dil

CD63 in Diluent E
10) 2µL*50µg/mL = 100µL*1µg/mL
9) 5.4µL*1µg/mL = 600µL*9ng/mL
8) 200µL*9ng/mL = 600µL*3ng/mL
7) 200µL*3ng/mL = 600µL*1ng/mL
6) 200µL*1ng/mxL = 600µL*333.3pg/mL
5) 200µL*333.3pg/mL = 600µL*111.1pg/mL
4) 200µL*111.1pg/mL = 600µL*37pg/mL
3) 200µL*37pg/mL = 600µL*12.3pg/mL
2) 200µL*12.3pg/mL = 600µL*4.11pg/mL
1) 200µL*12.3pg/mL = 600µL*1.37pg/mL
0) 400µL Dil

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CD81 in diluent E
10) 2µL*120µg/mL = 240µL*1µg/mL
9) 5.4µL*1µg/mL = 600µL*9ng/mL
8) 200µL*9ng/mL = 600µL*3ng/mL
7) 200µL*3ng/mL = 600µL*1ng/mL
6) 200µL*1ng/mxL = 600µL*333.3pg/mL
5) 200µL*333.3pg/mL = 600µL*111.1pg/mL
4) 200µL*111.1pg/mL = 600µL*37pg/mL
3) 200µL*37pg/mL = 600µL*12.3pg/mL
2) 200µL*12.3pg/mL = 600µL*4.11pg/mL
1) 200µL*12.3pg/mL = 600µL*1.37pg/mL
0) 400µL Dil

Plate each of these putting 360uL in each well of the 96 well plate.

Dilute each sample in the diluent for the assay for which it is being used. Each sample should be diluted at minimum 4x in the respective sample diluent. This dilution can be done by hand or on the HD-1 or HD-X insturnment.

3.6 Setting up the instrument:

5m

Create a new HD-X assay, all EV simoa assays are 2-step with 25uL RGP. Set the liquid volume to 160uL, all other parameters should be left as is. Assign each reagent a number (e.g. for CD9 Use "bead 1", "detector 1", "sbg1" and for CD63 Use "bead 2", "detector 2", "sbg2"

In the 'Plexes' tab create 4 plexes with 10 Calibrators each. Enter the concentration of each calibrator for each plex. The 4 plexes should be

1) 488 L0 with all other plexes off
2) 488 L0 with 647 L1 and all other plexes off
3) 488 L0 with 700 L1 and all other plexes off
4) 488 L0 with 750 L1 and all other plexes off

Load the reagents and plate into the insturnment assigning each well to the appropriate assay and hit start run!

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