Urinary extracellular vesicle analysis and detection of low abundance immunoglobulin oligomers in samples isolated from patients with significant urine proteinuria

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Method Article

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Abstract

Urinary extracellular vesicles (uEVs) have been used as a biomarker tool for detection of low abundance immunoglobulin-based targets in uEVs with heterogeneous high protein mixtures. This protocol outlines a pre-incubation method to minimize non-specific secondary antibody binding. Additionally, we include our approach for standardization of uEV amounts used for sample comparison. We propose that these and knowledge of the detection limit for target proteins are strategies applicable to other laboratories working with complex heterogeneous samples.

Introduction

Urinary extracellular vesicles (uEVs), including but not limited to exosomes and microvesicles, are lipid bilayer encased nanoparticles released by nephron and urinary tract epithelial cells[1-3]. Our group has previously shown unique features from uEVs from patients with proteinuria [4,5]. The biomarkers identified are low abundance immunoglobulin light chain oligomers (>250kDa), resistant to SDS and heat.

The issue of immunoglobulin contamination in extracellular vesicles preparations from urine, serum and/or plasma is increasingly recognized[6,7], with a further complication in the experimental methodology when the proposed biomarker is an immunoglobulin. The presence of immunoglobulins in uEV samples can cause issues of assay interference, particularly non-specific binding of secondary antibodies during Western Blot analysis.

The problem of immunoglobulin interference is two-fold. First, the size of immunoglobulin light chain (25kDa) and immunoglobulin heavy chain (50kDa) overlap in size with other uEV proteins. Non-specific binding of secondary antibodies interferes with protein blotting[8]. Second, oligomer biomarker detection relies on antibody specificity and confidence that detected species are actually immunoglobulin light chain complexes and not artifacts due to high sample concentrations required to detect low abundance targets.

Urine is extremely variable in content and concentration. Total protein content has proven problematic for uEV researchers due to the presence of albumin[9,10]. Depending on the disease state, the % albumin in the urine can have high variability. Calculating the amount of non-albumin uEV protein allowed us to obtain the most meaningful values to standardize our samples.

The details of the calculations are detailed in a manuscript submitted for publication. However, the pre-incubation method and strategy for sample standardization are outlined here and applicable for other groups studying low abundance targets from complex samples. Additionally, we have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV190061; First Author COOPER) (Van Deun J, et al. *EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research.* Nature methods. 2017;14(3):228-32).

Reagents

Name: Methanol

Manufacturer: Mayo Inventory Center

Risk: Flammable, Irritant

Name: Nanopure Water

Manufacturer: Made in Lab

Risk: None

Name: TRIZMA Base

Manufacturer: Sigma Chemical

Catalog Number: T-6066

Formula Weight (FW): 121.14

Name: Sodium Chloride (NaCl)

Manufacturer: Sigma Chemical

Catalog Number: S-9888

Formula Weight (FW): 58.44

Name: TWEEN 20

Manufacturer: Sigma Chemical

Catalog Number: P7949-500ml

Name: BSA

Manufacturer: Sigma Chemical

Catalog Number: A4503

Name: Glycine

Manufacturer: Sigma Chemical

Catalog Number: G-8790

Formula Weight (FW): 75.07

Name: Sodium dodecyl sulfate (SDS)

Manufacturer: Sigma Chemical

Catalog Number: L-4390

Formula Weight (FW): 288.4

Name: 10X Tris/Glycine/SDS Buffer

Name: Criterion Precast Gel (4-15%, 18well, 30µL/well)

Manufacturer: BioRad

Catalog Number: 161-0732

Manufacturer: BioRad

Catalog Number: 345-0028

Name: Sodium Azide

Manufacturer: Sigma

Catalog Number:S2002-25G

Formula Weight (FW): 65.1

Name: SuperSignal West Pico Luminol Enhancer Solution

Manufacturer: Thermo Scientific

Catalog Number: PI-34078

Equipment

· 3.5kDa molecular weight cut-off SpectraPor membranes (Spectrum Labs #132725, Rancho Dominguez CA)

· Nalgene Rapid Flow filtration unit with a 0.2 µm aPES membrane (568-0020 Thermo Scientific, Rockford IL)

· Complete® EDTA-free protease inhibitor (A32965 Thermo-Pierce, Rockford IL)

· 29.9 mL Beckman Optiseal Tubes 361625

· 70 Ti fixed angle rotor in a Beckman Coulter Optima L100 XP Ultracentrifuge (Beckman Coulter, Indianapolis IN)

· cell culture grade 1× PBS (Cellgro without calcium or magnesium Corning, Manassas VA)

· Parafilm and Saran wrap

· Power supply

· Blue Max 15mL Polypropylene Conical Tubes (Becton Dickinson: catalog #352097)

· Blue Max 50mL Polypropylene Conical Tubes (Becton Dickinson: catalog #352098)

· Membrane incubation boxes (4 total)

· Criterion Precast Gel (4-15%, 18well, 30µL/well; BioRad: catalog # 345-0028)

· Immobilon-P Transfer Membrane (Millipore: catalog #IPVH00010; Filter Type: PVDF; Pore Size: 0.45µm)

· Precision Plus Protein Dual Color Standards (BioRad: catalog# 161-0374)

· Criterion Blotter Filter Paper (BioRad: catalog# 170-4085)

· Classic Blue Autoradiography Film BX (13 x 18 cm: MIDSCI; catalog #6045538) and film cassette

· 2X SDS Loading Dye 4% SDS and 10% beta-mercaptoethanol(Prepared in MRA Lab; -20°C Freezer)

· Pyrex glass dish (8 X 11.5inches)

· Primary and secondary anti-bodies

o Sheep anti-human kappa free light chain (Binding Site PX016, San Diego CA)

o Sheep anti-human lambda free light chain (Binding Site PX018, San Diego CA)

o Rabbit anti-sheep HRP (Abcam ab7111 Cambridge MA)

· Criterion Electrophoresis Cell and Transfer box (BioRad)

· RP X-omat system (Eastman Kodak, Rochester NY)

· Shaker or Rotator (Room temperature and 4°C)

· Cold room or refrigerator

· Scissors/razor blade

· Heating block set to 95°C

· Timer

· 1.5mL centrifuge tubes

Procedure

Urine Sample Collection and Storage

1. Obtain clinical residual urine taken from 24 hour urine collections of plasma cell dyscrasia patients.

2. Supplement urine samples with 10% sodium azide preservative to a final concentration of 0.02% (1:50 dilution).

3. Store the urine samples at 4°C until further processing.

4. Obtain control urine sample (a first of the morning void or 24hr collection if possible) from a healthy volunteer…Preserve and store in the same manner.

Urine Sample Dialysis and Filtration

1. Dispense 4L purified H2O into an appropriately-sized beaker and add a magnetic stir bar.

2. Cut an 8 inch length of dialysis tubing (Spectra/Por #132725 3.5 kD MWCO) and pre-wet the membrane in H2O.

3. Secure a dialysis clip to one end of the tubing, then loosen the opposite end of the tubing with a gloved hand to open.

4. Dispense 100 ml urine into the open end of the dialysis tubing using a 50 ml serological pipet.

5. Secure the open end of the tubing with a dialysis clip, leaving small amount of air over the top of the urine sample.

6. Position a foam float onto the top dialysis clip.

7. Deposit the urine-filled dialysis tubing into the beaker containing 4L purified H2O.

8. Cover the beaker with aluminum foil, place the beaker on a magnetic stirrer and dialyze the sample overnight at 4°C.

9. After dialysis, remove the dialyzed urine sample from the beaker and rinse the membrane with H2O.

10. Gently remove the foam float and top clip from the dialysis tubing, ensuring not to spill the urine sample.

11. Pour the urine into a 250 ml 0.2 micron filtration unit (Thermo #568-0020) and apply a vacuum to filter the sample.

12. After filtration, add 10% sodium azide to a final concentration of 0.02%.

13. Add ½ protease inhibitor tablet (Pierce #A32965).

Vesicle Isolation

1. Pre-cool an ultracentrifuge and Ti70 rotor to 4°C.

2. Dispense urine sample into paired 29.9 ml Optiseal tubes (Beckman Coulter #361625) with a 25 ml serological pipet.

3. Balance the tubes in opposite pairs using 1X PBS.

4. Seal the tubes by inserting the vendor-supplied plug seals.

5. Load the tubes into the pre-cooled Ti70 rotor.

6. Insert tube spacers, insuring that they are fully seated over the Optiseal tubes, and secure the rotor cover.

7. Centrifuge the samples at 100,000 x g for 90 minutes at 4°C.

8. Remove the tube spacers using a threaded spacer removal tool.

9. Remove the Optiseal tubes from the rotor using an extraction tool.

10. Remove the plug seals from the tubes using a forceps.

11. Remove and discard about 1/3 of the urine sample volume from the tube using a disposable plastic bulb transfer pipet.

12. Carefully remove the top of the tube by cutting with a razor blade.

13. Pour off the remainder of the sample into a collection beaker and dab any remaining liquid onto a paper towel.

14. Place the tube on ice for the remainder of the processing steps.

15. Add 375 µl storage solution (45 ml 1X-PBS + 1 protease inhibitor tablet Pierce #A32965 stored at 4°C) to one tube of each pair.

16. Seal the top of the tube with parafilm and vortex on maximum speed for 45 seconds.

17. Transfer the liquid resuspension from the first to the second tube of each pair…Repeat the vortex step.

18. Transfer/combine the contents of both tubes into a 2 ml screw-cap microtube. NOTE: Processed vesicle stocks prepared from ~60 ml urine, with resuspension of the post-centrifugation pellets in a total of 375 ul, is considered a 1X stock. Vesicle concentration can be modified based on user preference.

19. Label the microtubes appropriately and store vesicle stocks at 4°C for immediate use. Stocks may also be frozen at -80°C for long-term preservation.

Sample protein is quantified via Bradford assay and results utilized in conjunction with clinical data to determine sample loading for Western blotting.

SOLUTION PREPARATION

Preparation of Transfer Buffer (2L)

1. Prepare at least 24 hours prior to use (or at least long enough to chill well).
2. Add 400mL of methanol (MeOH) to a 4L beaker.
3. Add 600mL of nanopure water to the beaker.
4. Weigh and add 57.7g of glycine to the beaker and mix.
5. Weigh and add 12.0g TRIZMA Base to the beaker and mix.
6. Mix solution until all solids have gone into solution (Add nanopure water to help dissolve).
7. Add solution to 2L graduated cylinder and adjust volume to 2L with nanopure water and pour the solution into 2 L bottles.
8. Add 1 mL of 20% SDS solution to achieve 1:2000 dillution. Mix well (watch foam).
9. Store in the refrigerator (4°C) until use.

Preparation of 5M NaCl (1L) for Wash Buffer

1. Weigh 292.2g of NaCl and add to a 1L beaker
2. Add 500ml nanopure water to the beaker and allow the solution to mix.
3. Slowly add nanopure water to the 900 mL mark on the beaker and mix.
4. Once everything is dissolved, empty the contents of the beaker into a 1L graduated cylinder
5. Bring the volume to 1L with nanopure water
6. Pour the solution into 1-1L bottle or 2-500mL bottles
7. Store at room temperature

Preparation of Blocking Buffer (4%w/v;100mL)

1. Add 100mL wash buffer to a 200mL beaker

2. Weigh and add 4g of albumin to the 100mL wash buffer

3. Mix the solution completely on a stir-plate to ensure all the albumin has gone into solution

4. Mixing of the blocking buffer can be done during the transfer step (or a stock solution can be prepared and stored at (4°C) with 0.01% sodium azide.

5. Add the blocking buffer to membrane(s) **(DO NOT POUR DIRECTLY ON MEMBRANE)**

Preparation 1X Tris/Glycine/SDS Buffer (1L per gel)

1. Add 100 mL of 10X Tris/Glycine/SDS Buffer to a 1000mL graduated cylinder

2. Add 900 mL nanopure water to 1000 mL graduated cylinder

3. Place Parafilm over the mouth of the graduate cylinder and invert to mix

4. Store at room temperature and save extra for future use.

Preparation of 2X SDS Sample Buffer

1. NOTE: Prepare in a fume hood. Beta-mercaptoethanol produces strong, toxic fumes

2. Add 15 ml purified water to a 50 ml graduated cylinder

3. Add 6.25 ml 1M Tris-HCl ph 6.8

4. Add 200 µl Beta-mercaptoethanol

5. Add 10 ml 20% SDS solution

6. Add 10 ml Glycerol

7. Add 30 mg Bromophenol Blue powder

8. Bring to final volume of 50 ml with the addition of purified water.

Western Blot Procedure

Sample Preparation for Running on PAGE Gel (Performed for each sample)

1. Determine uEV protein concentration and calculate required sample amount for assay detection limit. For oligomer biomarker detection, 15µg non-albumin uEV protein is needed.

2. Vortex the uEV sample prior to pipetting it. Mix the the uEV sample protein in a 1:1 ratio with 2X SDS Loading Dye in a 1.5mL centrifuge tube by pipetting the mixture up and down.

3. Heat sample(s) at 95°C for 5 minutes (Prepare gel for running prior to heating samples)

Running Samples on a PAGE Gel

1. Unwrap the Criterion Precast Gel (4-15% Gradient; 18-well; 30µL/well)

2. Remove gel from plastic dish

3. Remove the tape at the bottom of the Criterion gel

4. Place the Criterion gel into the Criterion electrophoresis cell

5. Fill the Criterion gel box, to the fill-line, with 1X Tris/Glycine/SDS Buffer (TGS)

6. Fill the top of the Criterion gel with 1X Tris/Glycine/SDS Buffer

7. Remove the well comb

8. Load 20µL of the ladder to the first well of the Criterion gel

9. Load 20µL of sample to separate wells in the Criterion gel

10. To empty wells, add 20µL 2X SDS Loading Dye

11. Run the gel at 100 volts.

12. Run the gel until the blue dye front runs reaches the bottom of the gel and turn off.

Transferring Protein to a PVDF Transfer Membrane using a Wet System

1. Fill the Criterion transfer box with cold transfer buffer

2. Cut a piece of Immobilon-P Transfer Membrane the size of the running gel (index card is good approximation)

3. Label membrane in a corner with a methanol-proof marker or pencil to maintain orientation during processing.

4. Remove Criterion gel from Criterion electrophoresis cell

5. Remove the Criterion gel from the plastic running cassette

6. Break seal with spatula and watch for flying debris.

7. Place the gel in a plastic dish containing cold transfer buffer to rinse for about 5 minutes

8. Fill the Pyrex dish with cold transfer buffer

9. Open the transfer cassette and placed the negative side flat in a Pyrex dish

10. Wet one cassette sponge with cold transfer buffer and place it on the negative side of the open cassette

11. Wet one piece of Blotter paper in cold transfer buffer and place it on top of the sponge

12. Place the gel on top of the Blotter paper.

13. Make sure all the air bubbles were out from underneath the gel

14. Place the membrane in a plastic dish containing methanol for 30 to 60 seconds to activate

15. Rinse the activated membrane in cold transfer buffer

16. Place the membrane on the gel (Make sure there are no air bubbles)

17. Gently smooth over the membrane with the back of finger to help remove any air bubbles

18. Place a piece of wet Blotter paper, soaked in cold transfer buffer, over the membrane

19. With the broken serological pipette, roll the bubbles out of the membrane/gel sandwich.

20. Place the second cassette sponge, soaked in cold transfer buffer, on top of the Blotter paper

21. Close the cassette (Positive over membrane, Negative under Gel)

22. Place the transfer cassette in the Transfer box with the membrane facing you (Positive Electrode; Red side of the cassette facing you)

23. Add an ice pack to the back of the Transfer box. Close latch over cold pack. Place Transfer box apparatus into the ice bucket.

24. Transfer at 100V for 60 minutes. Make sure the mA are set to 500. Voltage should be in the 90’s to upper 70’s by the end of the hour.

Blocking the PVDF Transfer Membrane

1. After the transfer is complete, remove transfer cassette from the Transfer box

2. Open transfer cassette with POSITIVE side up. Remove the top piece of filter/blotter paper.

3. The back of the membrane faces up. With a Fisher marker, mark the bands of the ladder. If the ladder ran crooked, use inside edge to get best approximation of location.

4. Remove the membrane and place in plastic dish (tweezers at edges). Rinse 2x with MilliQ water to remove excess marker without running water directly on membrane.

5. Pour off final rinse and add the blocking buffer to the PVDF transfer membrane (DO NOT pour directly on membrane)

6. Cover plastic dish securely with lid.

7. Place dish on the shaker in the cold room.

8. Adjust the shaker speed so that it is slow enough not to splash the blocking buffer all over but fast enough to keep the buffer moving.

9. Block overnight at 4°C

10. Proceed to the Antibody Pre-incubation step the next morning.

Western blot Analysis (Antibody Pre-Incubation)

1. Prepare 1:10,000 sheep anti-Kappa free light chain antibody(or 1:1000 sheep anti-lambda free light chain antibody) and 1:20,000 Abcam rabbit anti-sheep pre-adsorbed secondary antibody in a 50 mL conical tube, diluting with wash buffer. For 20 mL of preincubation volume, use 2 mL of sheep anti kappa free light chain antibody (or 20 mL of sheep anti lambda free light chain antibody) and 1 mL of secondary anti-sheep antibody.

2. Incubate the tube on the membrane rocker for 2 hours at room temperature to allow the primary and secondary antibodies to bind to each other. Hand mix periodically (every 20 minutes).

3. When incubation is 5 minutes from completion, prepare the membrane for blotting.

4. Remove blocking buffer. Store at 4°C for reuse. Do not re-use more than 3 times.

5. Transfer the membrane to a clean container and wash at least twice with wash buffer to make sure that all of the azide has been removed from the system. The azide from the blocking buffer will interfere with the enzymatic activity of HRP conjugated secondary antibody.

6. Add the antibody mixture to the transfer membrane (DO NOT pour directly on membrane)

7. Incubate for 2 hours at room temperature on the shaker

8. Following the incubation, wash twice for 5 minutes with a generous amount of wash buffer, then 3X for 20 minutes each at room temperature. Use a large volume of wash buffer and longer washes to reduce background.

Western blot Analysis (Development)

1. Place a sheet of plastic wrap on the bench-top
2. Prepare the ECL Developing reagents at a 1:1 ratio according to package directions
3. Add 6.0mL of the solution in the white bottle to the 15mL conical tube
4. Add 6.0mL of the solution in the black bottle to the 15mL conical tube
5. Mix the 15mL conical tube by inverting
6. Transfer PVDF membrane to a clean plastic dish
7. Pour the ECL solution into the dish. (DO NOT pour directly on membrane)
8. Agitate the ECL solution over the membrane for 5 minutes. Solution is stable for 24 hours so it can be re-used if developing multiple blots in 1 day.
9. Remove the blot from the ECL solution
10. Remove any excess ECL by gently dabbing on a paper tissue (Corner of membrane only)
11. Place the blot on the plastic wrap protein side down
12. Wrap the blot in the plastic wrap and transfer it to a film cassette
13. Tape the outer edges of the blot so that it stays in place during transport
14. Proceed to Western blot Film Exposure and Development step.

Western blot Analysis (Film Exposure and Development)

1. Gather the film cassette, a permanent black marker, a timer and box of film
2. Go to the dark room.
3. With lights off, and safety light on, open the cassette

4. Place one piece of film on top of the blot with the film edge matched to the bottom left corner of the cassette. Be careful that the film does not move around on top of the blot too much during this step

5. Close cassette lid. Start timer for desired exposure time.

6. After the desired exposure time has finished, open cassette, gently remove film and immediately feed into the Exomat.

7. Once the Exomat develops the film, label the film with exposure time, date, primary used, secondary used and developer used. Also, label all steps of the ladder as seen on the blot.

8. After the first exposure, it may be helpful to mark the ladder locations on the plastic wrap surrounding the blot with a small black dot.

9. Repeat steps 3-7 for differing exposure times.

10. Take exposures at 1, 5, 30, and 60 minutes before exposing overnight. Use the longest exposure that contains a negative control sample for interpretation of bands above 250kDa. Label films immediately with exposure time, antibody concentrations, and incubation conditions.

11. When you get all the exposures you need and you are finished with the membrane, store transfer membrane wrapped in a refrigerator.

Troubleshooting

Optimizing the ratios of primary to secondary antibody and determining the detection range of the protein of interest are critical for reliable results.

Running a negative control is key!

For longer run times, gel electrophoresis may be run with gel box in an ice bucket. Chilling will prevent the gel edges from curling and interfering with good transfer.

Time Taken

Days

Anticipated Results

Oligomers larger than 250kDa should be present for the involved light chain in patients with active disease.

Dissociated IgG will likely be present, but no species larger than 150kDa in controls or patients in remission.

Non-specific binding to sample immunoglobulin should be minimized.

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