Tech Tip: Exosome Isolation and Staining Protocols

Written by Alesha Cox-Harris (Research Associate at Biotium)

In this tech tip, Biotium scientists provide detailed protocols for isolating exosomes from cell supernatants using PEG precipitation or size exclusion chromatography (SEC). The tech tip also includes protocols for staining purified or bead-bound exosomes using dyes and antibodies for analysis by flow cytometry.

Contents

Cell Plating and Conditioned Medium Collection	1
30% PEG Isolation Method (Enriched Exosomes)	2
qEV SEC Isolation Method (qEV Exosomes)	2
Staining Enriched or Purified Exosomes with Dyes	3
Staining Enriched or Purified Exosomes with Antibodies	3
Analyzing Enriched or Purified Exosomes By Flow Cytometry	3
Isolating and Staining Bead-Bound Exosomes	4

Cell Plating and Conditioned Medium Collection

The exact culturing conditions will vary depending on the cell type, but these conditions are provided as a guideline.

Materials needed:

- Complete growth medium
- Serum-free growth medium
- Sterile 0.2 um cellulose acetate (CA) syringe filter

1. Culture adherent cells in a flask of at minimum T75 size, with 20 mL complete cell culture media. Allow the cells to become as confluent as they can tolerate. For suspension cells, culture the cells in a minimum volume of 20 mL complete cell culture medium, until they reach a high density (e.g., for Jurkat cells, around 2 x 10^6 cells/mL).
2. Two days before exosome isolation, replace the medium in the flask with serum-free medium. For suspension cells, do this by pelleting the cells and resuspending in serum-free medium.
3. Incubate the cells in serum-free medium for 2 days (1 or 3 days should also work), and then collect at least 20 mL of cell supernatant. For suspension cells, pellet the cells and collect the supernatant. Use sterile technique if you plan to store the supernatant.
4. Centrifuge the cell supernatant at 2,000 x g for 10 minutes at room temperature to remove cells and large debris.
5. Syringe filter the supernatant with 0.2 um CA filter. This will be your conditioned medium.
6. Proceed to exosome isolation or store the conditioned medium at 4°C for up to a few days.

Print this Tech Tip for quick access in the lab!

Exosome Isolation Methods

30% PEG Isolation Method (Enriched Exosomes)

Materials needed:

- Polyethylene Glycol (PEG) 6,000. Prepare a 30% solution in dH2O or sterile-filtered PBS.
- Sterile-filtered PBS + 0.1% BSA

Day 1:

1. Retrieve fresh or stored conditioned medium.
2. Add 0.5 volumes of 30% PEG 6000 to the conditioned medium (i.e., 10 mL of 30% PEG to 20 mL of conditioned medium).
3. Mix the solution well by inverting the tube until the solution is homogeneous.
4. Incubate the mixture at 4°C overnight without rocking.

Day 2:

5. Centrifuge the mixture at 10,000 x g for 1 hour at 4°C.
6. Carefully pour off the supernatant, then remove as much as possible of the remainder by placing tube upside down on an absorbent towel.
7. Resuspend the pellet in filtered 0.1% BSA in 1X PBS (1 mL per 20 mL of starting sample).
8. Aliquot the enriched exosomes at 500 uL per tube and store at -80°C until ready to be used. The exosomes can also be used immediately without freezing.

qEV SEC Isolation Method (qEV Exosomes)

Materials needed:

- Vivaspin® 20, 10 kD columns (Vivaproducts Cat. No. VS2002)
- qEVoriginal 70 nm columns (IZON Cat. No. SP1)
- Sterile-filtered PBS

Day 1:

1. Bring the qEV column to room temperature.
2. Retrieve conditioned medium.
3. Concentrate the conditioned medium from 20 mL to 500 uL using a Vivaspin® tube (spin at 3700 x g for 10 minutes, repeat as necessary until the volume reaches 500 uL).
4. Set up the qEV column in a rack such that buffer and sample can flow-through the column by gravity and be collected (Fig. 1).
5. Apply 10 mL of PBS to the column. Let it flow out into a waste tube. Discard this flow-through.
6. Apply 0.5 mL of concentrated conditioned medium to the loading frit of the column.
7. Once the sample has all been loaded on the column, fill the loading frit with buffer (about 4 mL of PBS) and start collecting.
8. The first 3 mL collected in the waste tube is the void volume (waste).
9. After the 3 mL waste volume has passed through, replace the waste tube with a clean collection tube. The next 2 mL collected is your exosome sample.
10. Once the 2 mL exosome sample has been collected, put the waste tube back underneath the column and run 1.5 column volumes (15 mL) of PBS to flush the column. Store the column at 4°C to be used again if desired.
11. Aliquot the exosome sample at 500 uL per tube and store at -80°C.

Staining and Analyzing Purified Exosomes

Staining Enriched or Purified Exosomes with Dyes

Materials needed:

- Sterile-filtered PBS
- ExoBrite™ EV Membrane Stains Cat. No. 30111-30114 (or other fluorescent dyes)

1. Thaw exosomes, if they had been stored at -80°C.
2. Prepare staining buffer: Dilute your dye at 1X final concentration in PBS, making enough for 900 uL per sample.
3. Aliquot 100 uL of exosomes into flow tubes, and store any remaining exosomes at -80°C. Always include a tube with dye only (no exosomes) as a background control.
4. Add 900 uL of staining buffer to the appropriate tubes and vortex.
5. Incubate for 30 minutes at room temperature in the dark.

Staining Enriched or Purified Exosomes with Antibodies

Materials needed:

- Sterile-filtered PBS
- Primary antibodies (i.e., ExoBrite™ CD9 Flow Antibody, ExoBrite™ CD63 Flow Antibody, or ExoBrite™ CD81 Flow Antibody)
- Isotype control antibodies (i.e., ExoBrite™ IgG1 Isotype Control Flow Antibody)

1. Thaw exosomes, if they had been stored at -80°C.
2. Aliquot 100 uL of exosomes into flow tubes, and store any remaining exosomes at -80°C.
3. Add the desired amount of labeled antibody to each tube with exosomes and mix well by vortexing. It is recommended to also include a tube of exosomes stained with an isotype control antibody labeled with the same dye.

   Note: For ExoBrite™ Flow Antibodies, use 5 uL. For other primary antibodies, we suggest 0.1 ug as a starting point, but you may need to titrate the antibody. The ExoBrite™ Isotype Control Flow Antibody can be used with the ExoBrite™ Flow tetraspanin antibodies.
4. For each antibody and concentration tested, include a tube with PBS and antibody alone (no exosomes) as a background control.
5. Incubate for 30 minutes at room temperature in the dark.
6. Add 900 uL PBS before running the samples on flow.

Analyzing Enriched or Purified Exosomes By Flow Cytometry

1. If desired, review the references listed in the references section on page 5 for tips on analyzing exosome samples by flow cytometry.
2. Set the configuration of the flow cytometer so that SSC is detected off the violet laser (V-SSC).
3. Run fluorescent sizing beads and use the smallest distinguishable beads to set the V-SSC threshold.
4. Run samples with a slow flow rate for 2 minutes, analyzing in the appropriate channels for the dyes tested.
5. To analyze the exosome staining, create a plot with the fluorescent dye channel on the x-axis and V-SSC on the y-axis. Stained exosomes will usually appear as a diagonal population, with the larger particles (i.e., higher V-SSC signal), staining more brightly with the dye (Fig. 2). Draw a gate around this population and copy the same gate to the "dye alone" or "antibody alone" plots. The percent of particles present inside the stained exosome gate can give you a relative idea of the concentration of exosomes in your sample. The percent of particles inside the gate in the "dye alone" sample should be low, ideally < 1%, indicating that the dye is not forming aggregates of similar size to exosomes, which can complicate analysis.

Isolating and Staining Bead-Bound Exosomes

Materials needed:

- Magnetic Immunoaffinity Isolation/Detection beads (i.e., beads with tetraspanin antibodies)
- A magnet stand for use with microcentrifuge tubes
- Sterile-filtered PBS

Day 1:

1. Thaw enriched exosomes or use freshly enriched exosomes. You will need 100 uL of exosomes for each test antibody and isotype control.
2. Resuspend beads by vortexing for 30 seconds.
3. Transfer 40 uL of beads per microfuge tube, one tube for each test antibody and isotype control you intend to use.
4. Remove the tubes from the magnet and wash the magnetic beads by adding 300 uL of PBS and mix well by pipetting.
5. Place tubes on the magnet for 1 minute and discard the supernatant with a pipette.*
6. Remove the tubes and add 100 uL of the enriched exosomes to each tube.
7. Incubate the tubes overnight at 4°C on rocker.

Day 2:

1. Place the tube on the magnet for 1 minute and discard the supernatant with a pipette.*
2. Remove the tubes from the magnet and wash the bead-bound exosomes by adding 300 uL of PBS, mix gently by pipetting.
3. Place the tube on the magnet for 1 minute and discard the supernatant.*
4. Resuspend the bead-bound exosomes in 100 uL of PBS.
5. Add 1 ug of primary antibody conjugate. Include isotype controls.

   Note: Lipophilic dyes cannot be used with bead-bound exosomes, because the dye will stick directly to the beads. ExoBrite™ EV Membrane Stains can be used to stain bead-bound exosomes.
6. Incubate tubes for 1 hour at 4°C on a rocker, in the dark.
7. Place the tubes on the magnet for 1 minute and discard the supernatant using a pipette.*
8. Remove the tubes from the magnet and wash the bead-bound exosomes by adding 300 uL of PBS, mix gently by pipetting.
9. Place the tube on the magnet for 1 minute and discard the supernatant with pipette.*
10. Resuspend the beads in 500 uL of PBS and transfer to flow tubes.
11. Run samples on the flow cytometer at slow speed, collecting at least 2000 events per sample.

**If you're having trouble seeing the beads try centrifuging the tubes at 2,000 x g for 3-5 seconds and place back on the magnet.*

Figures

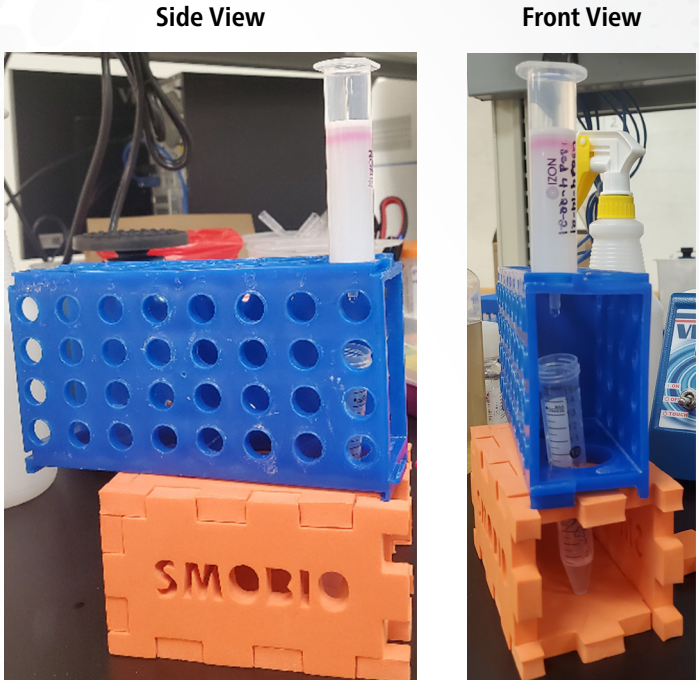

Figure 1. qEV column set-up.

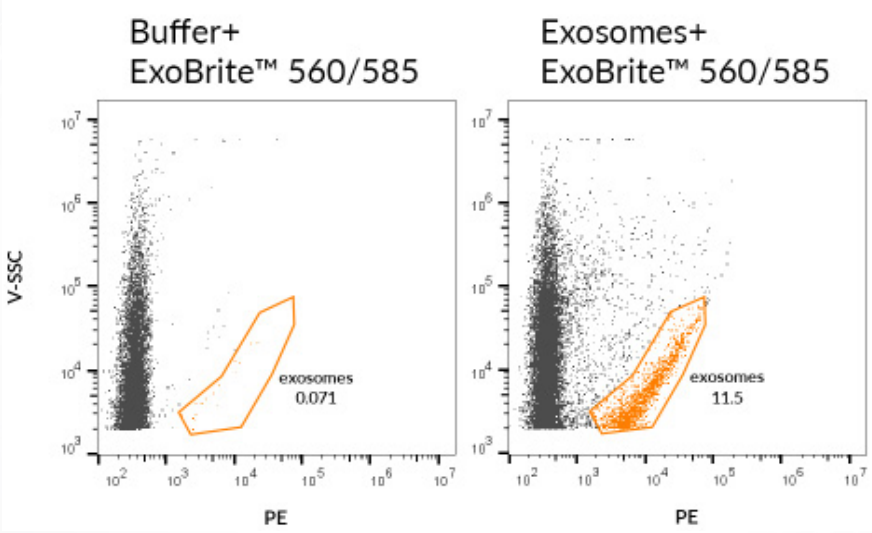

Figure 2. ExoBrite™ 560/585 EV Membrane staining of exosomes, compared to ExoBrite™ 560/585 run in buffer alone.

References

1. Application Note: Set-Up of the CytoFLEX for Extracellular Vesicle Measurement (2015). https://www.selectscience.net/application-articles/set-up-of-the-cytoflex-for-extracellular-vesicle-measurement/?artid=38228
2. Tech Tip: Fluorescent Detection of Exosomes by Flow Cytometry (2022). https://biotium.com/tech-tips/tech-tip-fluorescent-detection-of-exosomes-by-flow-cytometry/