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🌐 Ultrafiltration and purification of conditioned media (Pall Jumbossep and Izon qEV-10)

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Protocol status: Working

We use this protocol and it's working

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Disclaimer

This protocol summarizes key steps for a specific type of method, which is one of a collection of methods and assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

Abstract

Protocol for ultrafiltration of cell culture conditioned media using Pall Jumbosep products and purification of EVs from conditioned media concentrate using Izon qEV-10 size exclusions columns.

Guidelines

- All handling of conditioned media for isolation and purification of EVs should be done in sterile conditions
- Each additional 60 mL of conditioned media that is filtered through the Jumbosep device insert increases the time required to concentrate subsequently added media to the device

Materials

MATERIALS

⊗ 0.5M NaOH

⊗ DPBS, no calcium, no magnesium Thermo Fisher Catalog #14190136

⊗ Ethanol

For concentrating conditioned media:

- An unopened sterile 500 mL bottle of Gibco DPBS
- 70 mL of HPLC or other pure water
- 30 mL of Ethanol
- Pall Jumbosep filtrate receiver, sample reservoir, and 100k membrane insert
- 3 Eppendorf 5 mL low protein binding tubes
- Centrifuge inserts to hold Jumbosep devices

Jumbosep™ Centrifugal Devices

Starter Kits with Omega PES Membrane

Product No.	Description	Packaging
FD000K65	Generic starter kit, (no membrane or inserts)	4/pkg
FD003K65	3K starter kit, gray	4/pkg
FD010K65	10K starter kit, blue	4/pkg
FD030K65	30K starter kit, red	4/pkg
FD100K65	100K starter kit, clear	4/pkg
FD300K65	300K starter kit, orange	4/pkg

Jumbosep Device Omega PES Membrane Inserts

Product No.	Description	Packaging
OD003C65	3K membrane insert, gray	12/pkg
OD010C65	10K membrane insert, blue	12/pkg
OD030C65	30K membrane insert, red	12/pkg
OD100C65	100K membrane insert, clear	12/pkg
OD300C65	300K membrane insert, orange	12/pkg

Accessory Products

Product No.	Description	Packaging
FD001X65	Filtrate receiver and cap	12/pkg
FD002X65	Sample reservoir and cap	12/pkg
FD003X65	Insert release	24/pkg

Table of available Pall Jumbosep products (Jan 2020)



For concentrate purification with qEV column:

- Izon qEV-10 Column (70 nm pore size)
- New 500 mL of bottle Gibco sterile DPBS
- 14 × 5 mL LoBind Eppendorf tubes
- ~ 20 mL of filtered 20% ethanol
- ~ 5 mL of filtered 0.5 M NaOH

Troubleshooting

Preparing the Pall Jumbosep device

- 1 Make up 70% ethanol with HPLC or other pure water
- 2 Filter at least 20 mL of the 70% ethanol with a 0.02 μm syringe-driven filter, or other small pore size filter
- 3 Obtain a Jumbosep sample reservoir, a Jumbosep filtrate receiver, and a Jumbosep insert
- 4 Snap the reservoir onto the receiver
- 5 Without contacting the inside of the reservoir or the insert, place the insert into the reservoir
- 6 Use the blunt end of an aspirating tip to firmly push the insert into place
- 7 Fill the reservoir with at least 10 mL of the prepared filtered 70% ethanol
- 8 Place the reservoir cap onto the reservoir
- 9 Swirl the device around to maximize ethanol contact and let sit for a 5 minutes
- 10 Spin the device (with an appropriate swinging bucket rotor and adaptor insert, along with another balancing device, as needed) at 2500 RCF for 5 minutes to concentrate ethanol down to the device's dead volume of 5 mL
- 11 To separate the assembled device, remove the reservoir cap, clasp hands behind the device, place both thumbs on the lip of the sample reservoir, and press upward and backward.
- 12 Use a tissue to blot any fluid which may be present on rim of reservoir and receiver



- 13 Flush the reservoir with ~10 mL of sterile PBS and aspirate all volume from the receiver
- 14 Assemble the device, add 60 mL of sterile PBS to the reservoir and spin at 3000 RCF for 5 minutes
- 15 Aspirate all volume from the receiver, and the device is ready to have conditioned media loaded onto it

Cell culture media clearing

- 16 Cell culture media is ready to be harvested from cells when they are 50% to 80% confluent, typically 48 hours after cells have been seeded into phenol-red-free, bovine EV-depleted complete media
- 17 Using a 50 mL serological pipet tip, transfer the complete volume of media in each flask of cells to a sterile 50 mL conical tube
- 18 Spin the tubes at 500 RCF for 10 minutes
- 19 Transfer the supernatants from the 50 mL conical tubes to a new set 50 mL conical tubes, leaving behind a small but visible pellet of cells and cell debris
- 20 Spin the tubes at 2500 RCF for 10 minutes
- 21 Transfer the supernatants from the 50 mL conical tubes (leaving behind 1 to 2 mL of fluid at the bottom of the tubes) to a sterile media storage bottle for temporary storage during the subsequent ultrafiltration process

Concentrating cleared conditioned media

- 22 Wipe down with 70% ethanol two 750 mL centrifuge bucket inserts and the insides of the 750 mL centrifuge buckets
- 23 Load conditioned media into the reservoir until the volume reaches the 60 mL mark



- 24 Spin the device at 3000 RCF for ~20 minutes checking to ensure only the ~5 mL dead volume of fluid remains in the sample reservoir after the spin
- 25 Disassemble the device, aspirate the filtrate, and reassemble the device before addition of conditioned media
- 26 Add conditioned media to the sample reservoir until it reaches the 60 mL mark
- 27 If you are trying to balance two devices but have less than 60 mL of media for each device, PBS can be added to one of the devices to ensure the volumes are equal
- 28 Spin at 3000 RCF for 30 minutes at room temperature or until only ~5 mL of fluid remains in the sample reservoir
- 29 Continue the process of adding additional cleared conditioned media until all media has been transferred to the sample reservoir, increasing the centrifuge time as necessary (typically 5 to 10 minutes more per additional spin)
- 30 If the last volume of media remaining to be filtered is not 60 mL, PBS can be added to the reservoir to fill it to the 60 mL marker
- 31 After the last spin with added cleared media, fill the sample reservoir with 60 mL of PBS, ensuring resuspension of any material adhered to the bottom of the reservoir and insert, and complete the final 3000 RCF spin at RT, so that there is a ~5 mL dead volume

Note

This is a wash step to remove any soluble protein <100 kDa that may be remaining in the 5 mL dead volume.

- 32 Being careful not to spill concentrate or create excessive bubbles, use a P1000 micropipette to thoroughly resuspend the concentrate which is sitting on top of the insert in the sample reservoir
- 33 Transfer the ~5 mL from the top of the sample reservoir to Eppendorf Low-protein binding 5.0 mL tubes, placing a maximum volume of 4 mL in each tube to prevent overflow
- 34 Label and store the conditioned media concentrate at 4 °C overnight, do not store for more than a short period of time without purifying the concentrate



Size exclusion chromatography of conditioned media concentrate

- 35 Remove a new qEV-10 column from its 4°C storage location at the start of work day to allow to warm to room temperature
- 36 Use a ring stand and metal clamp to position the column vertically
- 37 Rinse 5 mL of PBS from a new, unopened, room temperature bottle of PBS through the loading chamber into a waste container
- 38 Unscrew the top cap from the column
- 39 Fill the column with PBS until it fills the column
- 40 Press the loading chamber into the top of the column, ensuring a tight seal between the two units
- 41 Fill the loading chamber to the top with PBS
- 42 Unscrew the bottom cap from the column, PBS should flow through the column and into a waste container
- 43 Once around half the PBS volume in the loading chamber has flushed through the column, fill it back up to the top with PBS
- 44 While the column is being flushed, label 14, 5mL low protein-binding Eppendorf tubes with the a fraction number (1 through 14) and date. A marker can be used to highlight the 5.0 mL volume graduation on the tube
- 45 As soon as all of the loaded PBS has moved through the loading chamber, position the "Fraction 1" 5.0 mL tube under the colum. Gently vortex and invert the concentrated media and add it to the loading chamber 1 mL at a time, ensuring to pipette the media directly onto the reservoir filter and not the walls of the chamber. The time elapsed from the last drop of PBS eluting to loading the cell culture media concentrate onto the column should be as short as possible.



- 46 As soon as all of the loaded concentrated media has passed through the loading chamber, the loading reservoir can be filled to the top with PBS
- 47 Continue collecting fractions in 5.0 mL increments until all fractions have been collected, refilling the loading chamber to the top with PBS when the PBS level falls by about half
- 48 While PBS continues to flush through column, into a waste container, collected fraction tube lids can be closed and set aside
- 49 Once the entire volume of PBS in the loading chamber has run through, add 5 mL of filtered 0.5 M NaOH to the chamber and allow it to pass through the column. The NaOH can be added by pipetting along the walls of the reservoir to wash away any contaminants
- 50 Fill the buffer reservoir to the top with PBS and allow it all to pass through the column except for the last ~1 mL to prevent drying the column
- 51 Refill the buffer reservoir to the top with PBS
- 52 After the majority of the buffer has eluted use a pH test strip to ensure the eluting buffer is neutral
- 53 Fill the column up with 5 mL of filtered 20% EtOH and allow it to flush through column
- 54 Place the bottom cap back on the column
- 55 Pipette filtered 20% EtOH into the top of the column until it is filled to the top with EtOH.
- 56 Place the top cap back on the column
- 57 Allow the loading chamber to dry in the hood overnight
- 58 Return all column components to the box they came from and label the box with date used, cell line used on, and volume of concentrated media loaded into the column.

