APPLICATION NOTE

FOR EXO-NET® ISOLATION OF EVS FROM CELL-CONDITIONED MEDIUM

EXO-NET® Pan-Exosome Capture, Product # 40031, 40033, 40036
For Research Use Only.

1 SUMMARY

This document provides details on EXO-NET captured EVs from cell-conditioned medium (CCM). In this Application Note, EVs captured from MCF-7 (human breast cancer) and PC-3 (human prostate cancer) CCM were used to confirm the presence of common EVs tetraspanin markers CD9 and Flotillin-1 (in accordance with the minimal information for studies of EVs, MISEV guidelines from the International Society for Extracellular Vesicles 1). Two types of 'EV-Collection' media (E8 and MEG) were utilized to demonstrate versatility of EXO-NET for isolating EVs from CCM. For each marker, EVs were isolated from 20 mL CCM using EXO-NET and analyzed by Western blot as described in more detail below. Cells were analyzed for viability and total live counts at CCM harvest (Figure 1). Total protein from EXO-NET captured EVs was quantified by Nanodrop analysis (Figure 2) prior to Western immunoblotting. Western blot analysis demonstrated that EXO-NET captured EVs were positive for CD9 as well as Flotillin-1 (Figure 3) confirming successful isolation of CCM-derived EVs. As concentration of EVs in CCM displays cell-specific variation, optimization of the EXO-NET to CCM volume ratio is recommended.

The use of EXO-NET for isolating EVs from CCM and the downstream analysis of EV-associated proteins, nucleic acids and lipids can be achieved using 20-50 mL CCM and does not require CCM to be concentrated prior to EV isolation. Nor does it require sample processing post-EV fractionation (*e.g.,* concentration using Amicon filters). Conservatively, using EXO-NET to isolate and characterized CCM-EV reduces total isolation time from 8 h per batch to 60 min.

2 RESULTS

2.1 CELL HARVEST DATA

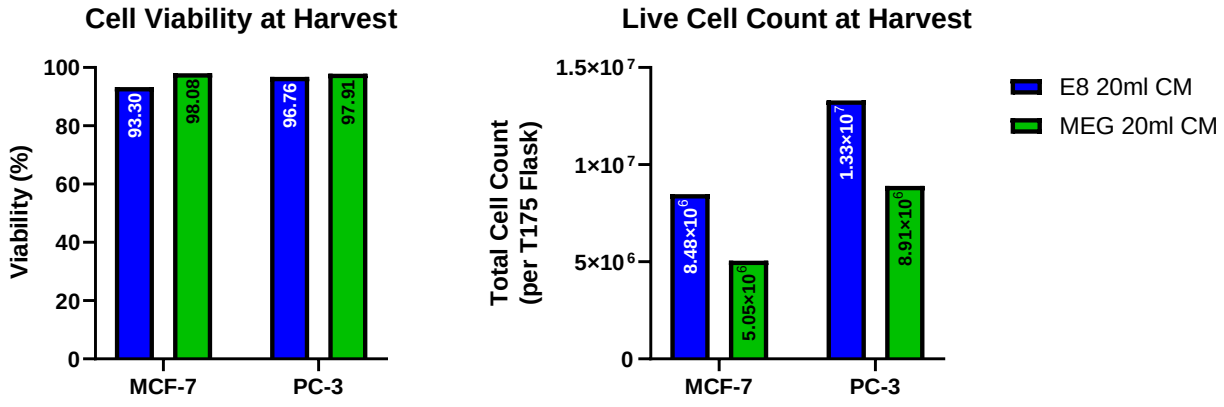

Figure 1. Cell viability and total number of live cells from samples at CCM harvest. Data are an average of duplicate counts using Trypan blue exclusion analysis.

2.2 PROTEIN YIELD

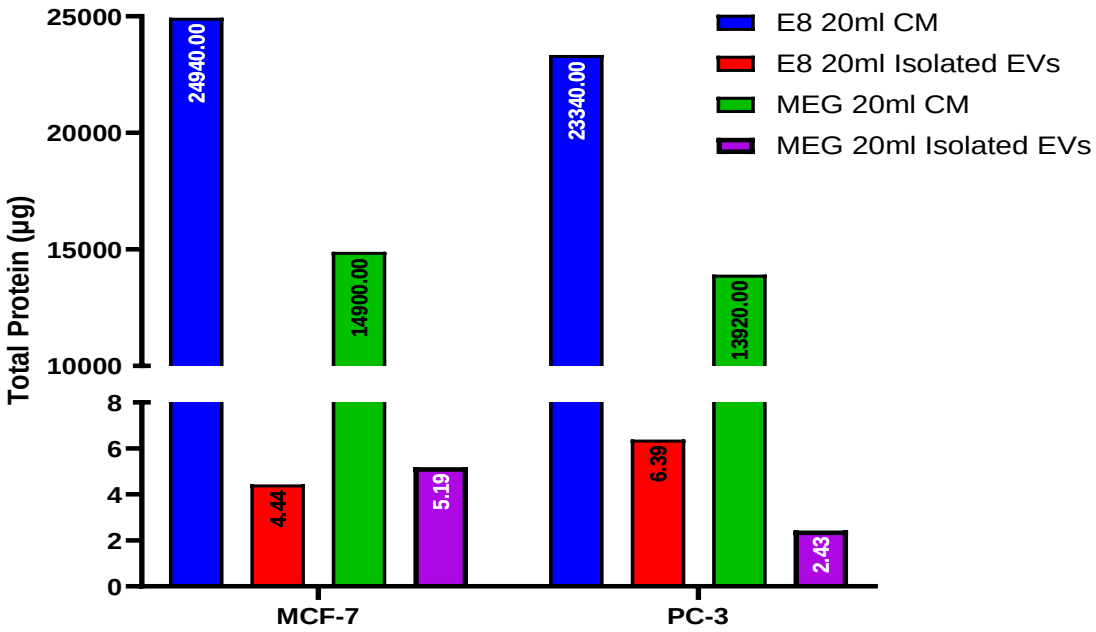

Figure 2. Total protein yield obtained from MCF-7 and PC-3 CCM was quantified using Nanodrop. Cells were grown in complete media (MED +10% FBS) to 80% confluence. Media was then replaced with two different EV-Collect media (E8 and MEG). CCM was collected after 24 h.

2.3 WESTERN BLOT

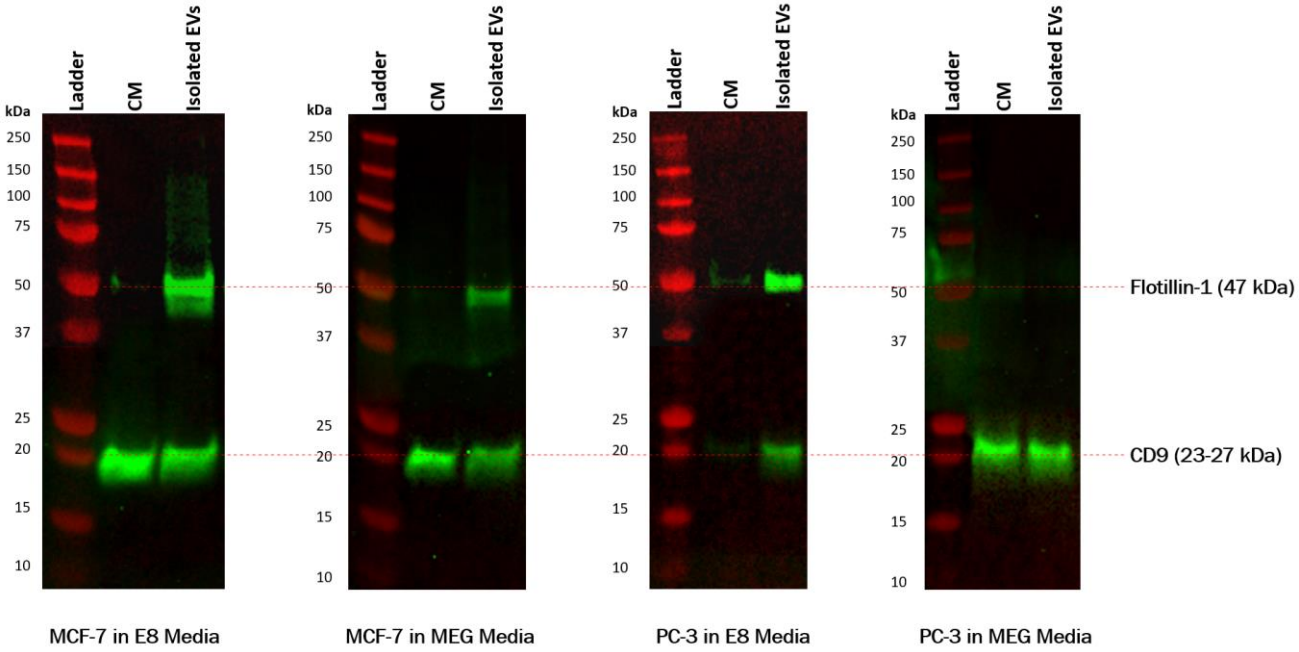

Figure 3. Western blot analysis of EXO-NET captured CCM-EVs. Samples were volume-matched, with input sample (CCM) and EXO-NET enriched EVs analyzed in parallel. EXO-NET-captured EVs from MCF-7 and PC-3 cells incubated in E8 or MEG were positive for CD9 (~22 kDa) and Flotillin-1 (~49 kDa).

3 MATERIALS AND EQUIPMENT

LIST OF CELL CULTURE REAGENTS	
Growth Medium	
Minimum Essential Medium Eagle	Sigma, Cat# M5650
GlutaMAX™ Supplement (100X)	Gibco, Cat# 35050-061
Fetal Bovine Serum	Gibco, Cat# 1099-141
EV Collect Medium	
E8	
Essential 8™ Basal Medium	A15169-01
Essential 8™ Supplement (50X)	A15171-01
MEG	
Minimum Essential Medium Eagle	Sigma, Cat# M5650
GlutaMAX™ Supplement (100X)	Gibco, Cat# 35050-061
Additional Reagents	
TE (Trypsin/EDTA Solution 1x)	Gibco, Cat# R-001-100
PBS pH 7.4 (1X)	Gibco, Cat# 100-10-023

LIST OF REAGENTS FOR EV ISOLATION	
EXO-NET®	INOVIQ, Cat# 40031, 40033, 40036
MagnaRack™ Magnetic Separation Rack, Invitrogen	Invitrogen, Cat # CS15000
MagJET™ Separation Rack, 4 x 50 mL vial	ThermoFisher, Cat # MR04
Vortex	
Pipettes/Pipette tips	
Serological Pipette/Stripette Tips	
NanoDrop™ One	ThermoFisher, Cat # 840274100
Vacuum Filtration Systems	ThermoFisher, Cat # 10040-436
1X Dulbecco's Phosphate Buffered Saline (DPBS)	ThermoFisher, Cat # 02-0119-1000
Protein LoBind® Tubes, snap cap, Protein LoBind®, 0.5 mL	Eppendorf, Cat # 0030108094
Protein LoBind® Tubes, snap cap, Protein LoBind®, 2.0 mL	Eppendorf, Cat # 0030108132
Protein LoBind® Tubes, screw cap, Protein LoBind®, 50 mL	Eppendorf, Cat # 0030122240

LIST OF PRIMARY AND SECONDARY ANTIBODIES	
CD9	Cell Signaling Technology, Cat # 13174T
Flotilin-1	Cell Signaling Technology, Cat # 18634T
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology, Cat # 7074P2

LIST OF BUFFERS AND REAGENTS FOR WESTERN BLOT	
Bolt™ MES SDS Running Buffer (1x) Mix 50 mL 20x Bolt™ MES SDS Running Buffer with 950 mL Milli-Invitrogen™ Cat # B0002 Q® water.	
Lysis Buffer 1% SDS Lysis Buffer	 Sigma Cat # L3771-100G
Master Mix 7.5 µL of Sample buffer (Bolt LDS Sample Buffer (4X)) 3 µL of Reducing agent (Bolt Sample Reducing Agent (10X))	 Invitrogen™ Cat # B0007 Invitrogen™ Cat # B0009
Wash Buffer Gently mix 500 µL of Tween 20 LR Polysorbate 20 and 500 mL ofCSA Scientific Cat # TL020-500M TBS.	
Blocking Buffer (2.5%) Dissolve 1.25 g of BSA in 50 mL of PBS/TBS plus Tween 20 (0.1%)Sigma; A9647-50G	

Note 1: Other blocking buffers such as 5% Skim Milk or other commercial blocking buffers can be used, however, optimization may be required.

4 PROTOCOL

4.1 GENERATION OF CELL CULTURE CONDITIONED MEDIUM

4.1.1 Seeding of EV Producer Cells:

- Seed cells (PC-3 at $2xE^4/cm^2$ or MCF-7 at $5xE^4/ cm^2$) in a T175 flask in 25 mL growth medium (Min Eagle's + 10% FBS + 1X GlutaMAXTM Supplement) and allow to grow over 2 days to reach ~80% confluency.

4.1.2 Media Change for EV Collection:

- Rinse the monolayer with 10 mL 1X PBS, twice.
- Carefully add 25 mL of EV collect medium (E8 – reconstituted according to the manufacturer's recommendations or MEG – Min. Eagle's + 1X GlutaMAXTM Supplement) and allow cell culture to incubate overnight.

4.1.3 Collection of CCM for EV Isolation:

- The following day (~24h post-incubation), harvest the EVs by collecting the cell culture supernatant into a 50 mL centrifuge tube.
- Count the cells by first rinsing the monolayer with 1X PBS, followed by trypsinization and subsequent resuspension in growth medium. Analyze the cell viability and number of total live cells.

4.1.4 Clarification of EV Conditioned Medium

- Centrifuge the CCM at 300 x g for 5 min. Transfer the supernatant into a new tube.
- Centrifuge again at 3,000 x g for 10 min at 4°C.
- Collect the resulting supernatant and proceed to EV isolation.

4.2 EV ISOLATION FROM CELL CONDITIONED MEDIUM USING EXO-NET

4.2.1 Preparation of sample and EXO-NET beads

- If the cell conditioned media is frozen, defrost by incubating in a water bath at 35°C until thawed.
- Aliquot the required volume of EXO-NET beads into a microfuge tube and keep at RT.
- Label a 50 mL, a 2 mL and a 0.5 mL centrifuge tubes (preferably Eppendorf Protein LoBind® Tubes) for each sample and place them in a nonmagnetic rack.
- Add 20 mL of cell conditioned media into the previously labelled 50 mL centrifuge tube.

4.2.2 EXO-NET Isolation of EVs

- Resuspend EXO-NET beads by gently pipetting 10 times immediately prior to adding to sample tubes.
- Add 30 µL of EXO-NET beads to the tube containing 20 mL of CCM.
- Cap the tubes. Mix the beads and CCM by gently flicking the tube 10 times. Avoid forming bubbles.
- Incubate the mixture for 30 min at RT on a rotisserie or shaking platform (ensure the equipment is set to a low-speed setting to not form air bubbles).
- Place the tubes in the magnetic rack for at least 10 min or until the liquid is clear.
- Remove the supernatant carefully using a serological pipette while the tube is still in the magnetic rack. Guide the pipette tip towards the clear side of the tube to avoid bead loss.
- Resuspend beads in 1000 µL filtered DPBS to wash. Gently target the bead pellet while dispensing the buffer to bring the beads in suspension. Do not invert or vortex the tube.
- Transfer the resuspended pellet into the previously labelled 2 mL microcentrifuge tube.
- Repeat the above resuspension step to obtain a total of 2 mL of resuspended beads solution.
- Place the tubes in the magnetic rack for 5 min or until the liquid is clear.
- Carefully remove the supernatant using a p1000 pipette. Guide the pipette tip towards the clear side of the tube to avoid bead loss. Discard the supernatant in the appropriate waste stream.
- Perform DPBS wash two additional times with 1 mL of DPBS each time. Remove the maximum volume of wash solution after each wash.

4.2.3 On-bead Lysis of EVs

- Add 30uL of 1% SDS to the final pellet, briefly centrifuge and keep it for 15 min at RT.
- Place the tubes in the magnetic rack, for at least 5 min or until the liquid is clear.
- Carefully transfer the lysate using a p1000 pipette to the previously labelled 0.5 mL microfuge tube and proceed with protein quantification using Nanodrop One. (Other protein quantification methods such as BCA or micro-BCA can be used, however, it may require optimization).

4.2.4 Protein Quantification

- Turn on NanoDrop One.
- Before the first measurement, clean both pedestals with a new laboratory wipe
- From the instrument Home Screen, select the Protein A280 application and set the Curve type to BSA.
- Keep Baseline correction unselected, and press done.
- Run a blanking cycle to verify pedestals are clean.
- Measurements are taken by raising the arm, loading 1-2 µL of ultrapure water onto the lower platform, then lowering the arm.

- After each new measurement, clean both pedestals with a new wipe to prevent any carryover.
- Clean pedestals again and proceed with loading and measurement of 1–2 µL of the selected sample.
- Clean the pedestals in between samples.
- After the last sample, press End Experiment and clean both platforms for one last time.
- The results can be written down or extracted from the equipment with a USB drive, or via a wired or wireless connection (determined when equipment is installed)
- Turn off the equipment.

4.3 WESTERN BLOT

4.3.1 Sample Preparation and Lysis

- Switch on the heating block to 95ºC.
- Mix 19.5 µL of lysate with 10.5 µL of the Master Mix and mix it well (Vortex, 3 pulses)
- Keep it on a heating block (95°C) for 3 min.
- After 3 min, briefly open the microcentrifuge tubes to release pressure and close it again.
- Centrifuge the microcentrifuge tubes for 20 seconds and wait until it cools down for 5 min.
- Proceed with loading and running the gel as described below.

4.3.2 Loading and Running the SDS-Page Gel

- Prepare the Bolt™ MES SDS Running Buffer (1x)
- Place a Bolt™ 4 to 12% Bis-Tris Mini protein gel in the tank (Bolt Mini Gel Tank – Invitrogen). Do not forget to remove the white tape to allow the electric current to flow.
- Lock the gel (see Figure 2).

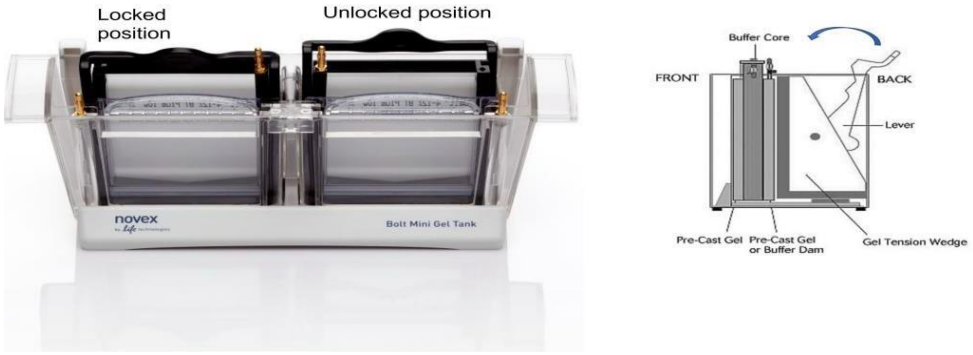

Figure 2. Invitrogen Mini Gel Tank.

- Pour enough Bolt™ MES SDS Running Buffer (1x) to reach the mark indicated on the side of the tank.
- Remove the combs carefully to avoid damaging the wells.
- Load 30 µL of the lysate.
- Run the gel at 150 V for 40 min (check other recommendations for the gel and optimize it if necessary). The time and voltage may require optimization. We recommend following the manufacturer's instructions.

4.3.3 iBlot Transfer

- Once the run is completed, proceed with the transfer of the proteins from the gel to a membrane using iBlot™ 2 Dry Blotting System.
- Unseal the Transfer Stack and separate into top and bottom halves. Set the Top Stack to one side. Make sure the membrane is not stuck to the separator. Keep the Bottom Stack in the plastic tray.
- Place the Bottom Stack (in the plastic tray) on the blotting surface. Align electrical contacts on the tray with the corresponding electrical contacts on the blotting surface of the iBlot 2 Gel Transfer Device. Ensure the tray is centered so that the electrical contacts are not obstructed.
- Wet the pre-run gel(s) and place it on the transfer membrane of the Bottom Stack.
- Place a pre-soaked (in deionized water) iBlot Filter Paper on the gel and remove air bubbles using the Blotting Roller
- Remove the white separator and place the Top Stack over the pre-soaked filter paper.
- Remove air bubbles using the Blotting Roller.
- Place the Absorbent Pad on top of the Top Stack such that the electrical contacts are aligned with the corresponding electrical contacts on the blotting surface of the iBlot 2 Gel Transfer Device.
- Close and latch the lid of the device carefully to ensure that the Transfer Stack and electrical contacts do not shift.
- Select the desired method and make sure the parameters are correct. Select Start Run, or if desired, use the Start Last Run icon. (Note: the results herein were obtained using program P3).
- Once transfer begins, the elapsed time is displayed on the screen.
- An audible alarm and a message on the digital display indicates the end of the transfer.
- Select Done to end the run.
- Turn off the iBlot 2 Gel Transfer Device and open the lid.
- Discard the Absorbent Pad and Top Stack.
- Carefully remove and discard the gel and filter paper. Remove the transfer membrane from the stack and proceed with the blocking procedure or stain the membrane (Note: transfer efficiency can be corroborated by Coomassie stain of the gel).
- Discard the Bottom Stack.
- Clean the instrument surfaces and electrodes with a damp cloth or paper tissue.
- At this point, the iBlot 2 Gel Transfer Device is ready for another run (no cooling period is required). If you are not using the device, turn off the power switch.
- Proceed to the next step with blocking and antibody incubation.

4.3.4 Blocking and Antibody Incubation

- Transfer the membrane into Blocking Buffer and incubate at RT on rocking platform for 1 h.
- After 1-h blocking, remove and incubate the membrane with the primary antibody.
- For incubation with antibodies, wet-chamber method was used.
- Use a chamber or plastic box as a humidified chamber. Place a piece of soaked absorbent paper towel.
- Cut pieces of parafilm to a size large enough to contain the membranes and place the parafilm in the chamber.
- Slightly stretch the parafilm to attach it to the surface of the chamber.
- Dilute primary antibodies in 1:500.

- Pipette on the parafilm the antibody solution and gently place the membrane face down over the solution creating a thin layer of solution between membrane and parafilm.
- Incubate overnight at 4°C (optional: RT for at least 2 h).
- After incubation, remove the membrane and proceed to wash 3 times for 5 min each with Wash Buffer.
- After washing, dilute the secondary antibody in 1:2000.
- In the same fashion, incubate with the secondary antibody at RT for 1 h.
- Next, wash the membrane 2 times with Wash Buffer for 15 min each.
- Place the membrane in a plastic/parafilm/cling wrap.
- Add visualizing substrate, Amersham ECL™ Western Blotting Detection Reagents (protect from light) over the membrane, cover the surface completely.
- Leave for 3-5 min in the dark.
- In the meantime, set the imaging system and proceed with the Imaging section next.

4.3.5 Imaging

Note: This protocol utilizes the BioRad ChemiDoc MP Imaging System.

-
- Open Image Lab software.
- A window with protocols will automatically open, click “New Multichannel”.
- The first window opens in “Gel Imaging”.
- Select Channel 1 and press “Configure”.
- Next in “Application” and select “Blots”.
- In this channel, select “Chemi”.
- The default setting will automatically appear.
- At the bottom of the page, deselect “Highlight saturated pixels” and adjust the channel color to “Green”.
- Press “OK”.
- For channel 2, follow the same steps but change application to “Colorimetric” and Channel
- color to “Red”.
- Once the settings are set, proceed to position the membrane.
- Click on “Run protocol”.
- Once the final image is generated, adjust contrast if needed and save the final image in the desired format.

5 PRODUCT ORDERING INFORMATION

Cat No.	Product Name	Isolations*	Size
40031	EXO-NET® Pan-Exosome Capture	60	1.0mL
40033	EXO-NET® Pan-Exosome Capture	30	0.5mL
40036	EXO-NET® Pan-Exosome Capture	15	0.25mL

** Isolations based on 15µl of EXO-NET® per 200µl plasma*

To learn more about EXO-NET® Pan-Exosome Capture visit inoviq.com

Contact Information

USA	orders-US@inoviq.com
ROW	info@inoviq.com

6 REFERENCES

1. Théry, Clotilde, et al. "Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines." *Journal of extracellular vesicles* 7.1 (2018): 1535750.