APPLICATION NOTE

FOR WESTERN BLOT
EXO-NET® Pan-Exosome Capture, Product # 40031, 40033, 40036
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1 WESTERN BLOT APPLICATION FOR EXO-NET® CAPTURED HUMAN EXTRACELLULAR VESICLES

This document provides details on Western blotting procedure for detection of known extracellular vesicles (EVs) protein markers from EXO-NET® captured EVs. In the example, normal human plasma has been used to confirm the presence of the common EVs tetraspanins markers (CD9, CD63, CD81), TSG101 and Flotillin-1 according to the minimal information for studies of EVs (MISEV) guidelines from the International Society for Extracellular Vesicles[1]. For each marker, EVs were isolated from 0.2 mL pooled normal human plasma using EXO-NET and analysed by Western blot as described in more detail below. Western blot analysis demonstrated that EXO-NET captured EVs were positive for all the above- mentioned common EV markers (Figure 1).

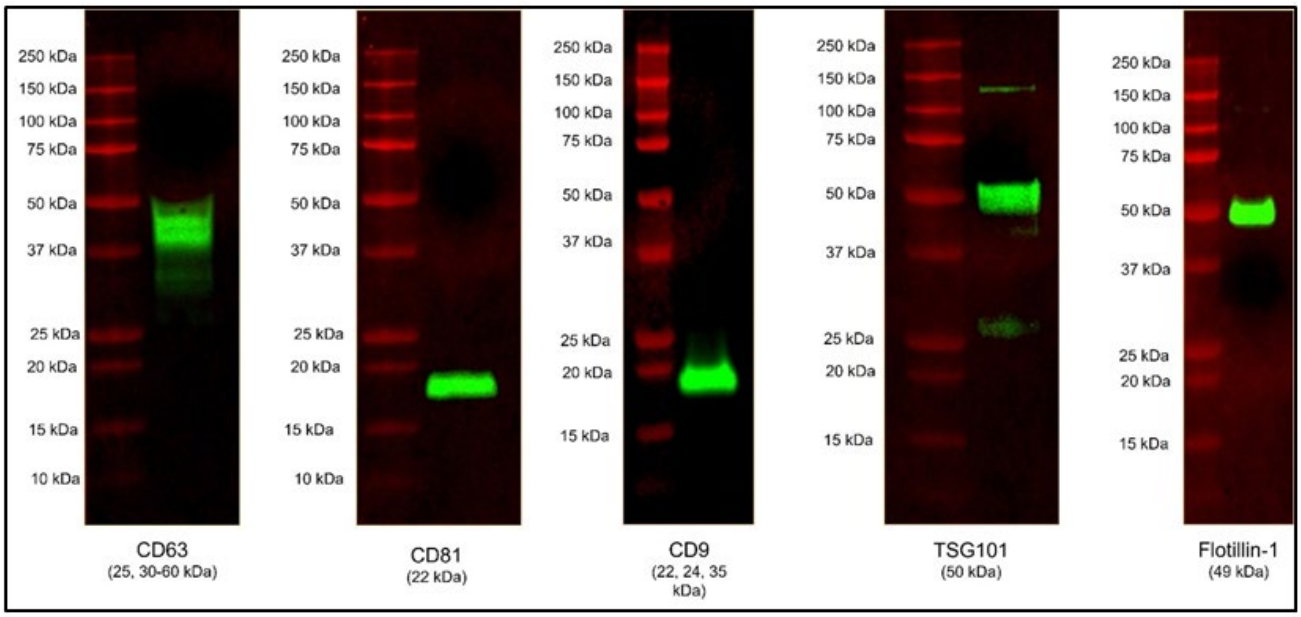

Figure 1. Western blot analysis of EXO-NET captured EVs. EXO-NET captured EVs from a pooled normal human plasma were positive for CD63, CD81, CD9, TSG101 and Flotillin-1.

2 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

3 MATERIALS AND EQUIPMENT

LIST OF REAGENTS FOR EV ISOLATION	
EXO-NET®	INOVIQ, Cat# 40031, 40033, 40036
MagnaRack™ Magnetic Separation Rack, Invitrogen	Invitrogen, Cat # CS15000
Vortex	
Pipettes/Pipette tips	
NanoDrop™ One Microvolume UV-Vis Spectrophotometer	ThermoFisher
Vacuum Filtration Systems	VWR, Cat # 10040-436, or similar
1X Dulbecco's Phosphate Buffered Saline (DPBS), filtered through VWR® Vacuum Filtration Systems (0.2µ)	ThermoFisher, Cat # 02-0119-1000 or similar

LIST OF PRIMARY AND SECONDARY ANTIBODIES	
CD9 (Cell Signaling - 13174T)	Cell Signaling Technology, Cat # 13174T
Flotilin-1 (Cell Signaling - 18634T)	Cell Signaling Technology, Cat # 18634T
TSG101 (Cell Signaling - 72312)	Cell Signaling Technology, Cat # 72312
CD81 (Cell Signaling - 52892)	Cell Signaling Technology, Cat # 52892
CD63 (Cell Signaling - 52090)	Cell Signaling Technology, Cat # 52090
Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling; 7074P2)	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-Q® water.	Invitrogen™ Cat # B0002
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 of TBS.	CSA Scientific Cat # TL020-500M
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 EV ISOLATION USING EXO-NET

- Defrost human plasma by incubating at RT for ~ 15 min.
- Warm the EXO-NET beads to RT.
- Label 1.5 mL microfuge tubes for each sample and place them in a nonmagnetic rack.
- Add 200 µL of plasma into 1.5 mL microfuge tubes.
- Centrifuge plasma for 5 min at 10000 x g, RT.
- Transfer the supernatant to a new tube.
- Resuspend EXO-NET beads by gently pipetting 10 times to disperse EXO-NET beads.
- Once homogenous, add 15 µL of EXO-NET beads to each tubes containing 200 µL of plasma.
- Cap the tubes. Mix the beads and plasma cocktail by gently flicking the tube 10 times. Avoid forming bubbles.
- Incubate the mixture for 15 min at RT.
- Place the tubes in the magnetic rack, for at least 5 min or until the liquid is clear.
- Remove the supernatant carefully 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.
- 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 tubes.
- Place the tubes in the magnetic rack for 5 min or until the liquid is clear.
- Remove the supernatant carefully 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. Remove the maximum volume of wash solution after each wash.
- 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.
- Transfer the lysate carefully using a p1000 pipette to a new 1.5 mL microfuge tube and proceed with protein quantification using Nanodrop One. (Other protein quantification methods such as BCA or microBCA can be used, however it may require optimization).
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4.2 WESTERN BLOT

4.2.1 Sample Preparation and Lysis

- Switch on the heating block to 95°C.
- Mix 19.5 µL of lysate (10µg-20µg of protein) 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.

4.2.2 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).

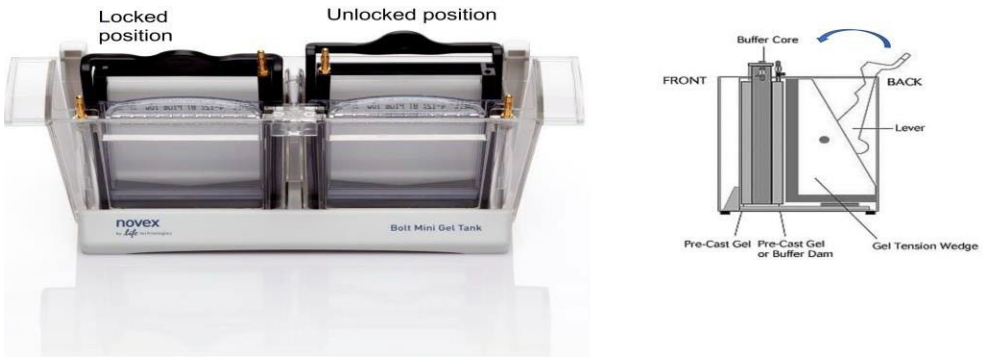

Figure 2. Invitrogen Mini Gel Tank.

- 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.

4.2.3 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.

4.2.4 Blocking and Antibody Incubation

- Transfer the membrane into Blocking Buffer and incubate at RT on rocking platform for 1 hour.
- After 1-hour 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^{o}C (optional: RT for at least 2 hours).
- 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 hour.
- 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.

4.2.5 Imaging

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

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- 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 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.

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

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