Exo-Flow™ 2.0 EV Analysis for Tissue Culture Media

Cat # EXOFLOW2-200A-TC, EXOFLOW2-205A-TC, EXOFLOW2-210A-TC, EXOFLOW2-250A-TC, EXOFLOW2-BASICA-TC

User Manual

Storage: Store Kits at +4°C upon receipt

Version 1
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Contents

Product Description

Take your EV insights to the next level by focusing on specific subpopulations

> *"One of the biggest challenges for the EV field at this stage is addressing heterogeneity of secreted EVs, and heterogeneity within EV populations."*[1]

Gain powerful insights into extracellular vesicle (EV) biology and analyze EV subpopulations for therapeutics development, biomarker discovery, and more with SBI's Exo-Flow™ 2.0 EV Analysis Kits (Table 1). With Exo-Flow 2.0 technology, we've re-engineered our original Exo-Flow beads (Figure 1) to deliver virtually undetectable background binding. The result is highly specific antibody-based capture of EVs so you can easily analyze specific EV subpopulations by flow cytometry, western blot, or other downstream technology. Take your EV insights to the next level with Exo-Flow 2.0.

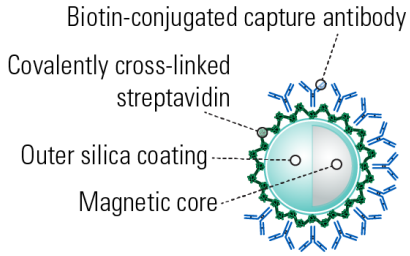

Figure 1. Exo-Flow 2.0 beads have an outer silica coating which delivers undetectable background binding.

- Powerful—analyze EV subpopulations for deeper insights into EV biology and biomarker discovery
- Flexible—the basic kit extends the power of Exo-Flow 2.0 beads by giving you the ability to use the biotinylated antibody of your choice.
- Efficient—our Exo-Flow 2.0 beads can capture even small subpopulations of EVs to maximize your discoveries
- Low-background—Exo-Flow 2.0 beads deliver undetectable background binding for targeted analysis of specific EV subpopulations
- Easy-to-use—the magnetic bead-based workflow translates into quick and easy EV capture

> *"The new Exo-Flow 2.0 beads are much easier to see during the processing steps (sample capturing, washing, staining, etc.) which prevents accidental loss of the sample. I am also able to acquire more events with the flow cytometer with the new Exo-Flow 2.0 beads."*
>
> *—Ananthu Pucha, DeKalb Veterans Affairs Hospital at Emory University*

Our Exo-Flow 2.0 technology is available as both individual tetraspanin (CD63, CD9, and CD81) and combination kits, as well as a basic kit that can be used with the biotinylated antibody of your choice. Note that unlike the original Exo-Flow technology, Exo-Flow 2.0 kits are specific for isolating EVs from either serum/plasma or tissue culture medium, so be sure to choose the right kit for your needs.

TABLE 1. Available Exo-Flow 2.0 Kits

Biotinylated Antibody	Exo-Flow 2.0 Kit for EV Isolation from Tissue Culture Media Cat.#	Exo-Flow 2.0 Kit for EV Isolation from Tissue Culture Medium Cat.#
CD63	EXOFLOW2-100A-SP	EXOFLOW2-200A-TC
CD9	EXOFLOW2-105A-SP	EXOFLOW2-205A-TC
CD81	EXOFLOW2-110A-SP	EXOFLOW2-210A-TC
Tetraspanin Combo (CD63, CD9, and CD81)	EXOFLOW2-150A-SP	EXOFLOW2-250A-TC
Basic Kit without antibody	EXOFLOW2-BASICA-SP	EXOFLOW2-BASICA-TC

The Exo-Flow 2.0 workflow uses a simple, four-step workflow:

1. Couple your biotinylated antibody to the magnetic streptavidin Exo-Flow 2.0 beads
2. Use the antibody-coupled magnetic beads to capture already-isolated EVs (compatible with ExoQuick®, SmartSEC™-HT, ultracentrifugation, or any other EV isolation method)
3. Wash away unbound EVs
4. Prep for analysis
    - **For flow cytometry**—stain with the included Red or Green Dye
    - **For western blotting**—resuspend beads in M-PER/RIPA lysis buffer (not included), add sample loading buffer load onto gel
    - **For RNA analysis**—resuspend beads in RNA lysis buffer of your choice and process

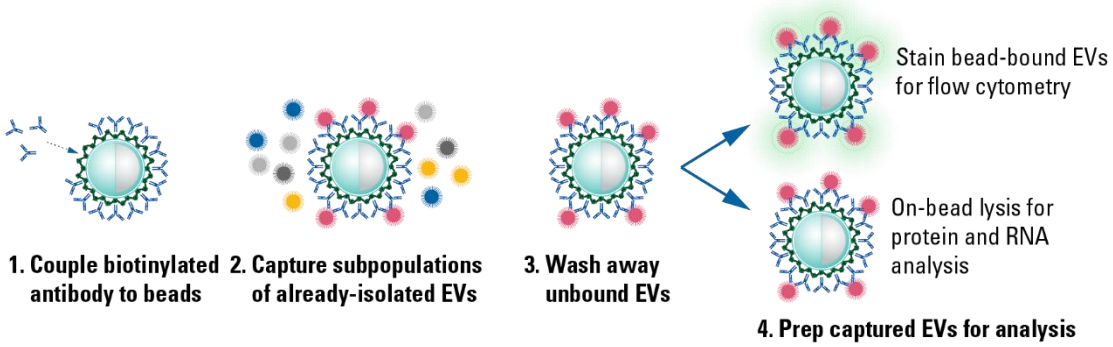

Figure 2. The simple Exo-Flow 2.0 workflow.

REFERENCE

1. Willms E, et al. Extracellular Vesicle Heterogeneity: Subpopulations, Isolation Techniques, and Diverse Functions in Cancer Progression. Front Immunol. 2018; 9: 738. PMCID: PMC5936763.

List of Components

Table 2. Components for the individual Exo-Flow 2.0 EV Analysis Kits for Tissue Culture Media: CD63 (Cat.# EXOFLOW2-200A-TC), CD9(Cat.# EXOFLOW2-205A-TC), and CD81 (Cat.# EXOFLOW2-210A-TC)*

Component	Qty/Volume	Storage Temperature
ExoFlow 2.0 Magnetic Capture Beads	150 µL	4°C
Monoclonal Capture Antibody	10 µL	
Wash Buffer Component A (20X)	5 mL	
Wash Buffer Component B (10X)	10mL	
Blocking Buffer	10 mL	
TC Green dye	100 µL	
TC Red dye	100 µL	
EV Binding Buffer	10 mL	

*Each kit contains enough reagents to process 10 reactions of 150 – 300 µg protein equivalent of EVs (the actual amount of input protein equivalent depends on your EV isolation method, see the protocol)

Table 2. Components for the Exo-Flow 2.0 Tetraspanin Combo (CD63, CD9, CD81) EV Analysis Kit for Tissue Culture Media (Cat.# EXOFLOW2-150A-SP)*

Component	Qty/Volume	Storage Temperature
ExoFlow 2.0 Magnetic Capture Beads	450 µL	4°C
Monoclonal CD63 Capture Antibody	10 µL	
Monoclonal CD9 Capture Antibody	10 µL	
Monoclonal CD81 Capture Antibody	10 µL	
Wash Buffer Component A (20X)	15 mL	
Wash Buffer Component B (10X)	30 mL	
Blocking Buffer	30 mL	
TC Green dye	300 µL	
TC Red dye	300 µL	
EV Binding Buffer	30 mL	

*Each kit contains enough reagents to process 10 reactions of 150 – 300 µg protein equivalent of EVs (the actual amount of input protein equivalent depends on your EV isolation method, see the protocol) for each of the three tetraspanins.

Table 3. Components for the Exo-Flow 2.0 Basic EV Analysis Kit (Streptavidin beads + Reagents) for Tissue Culture Media (Cat.# EXOFLOW2-BASICA-TC)*

Component	Qty/Volume	Storage Temperature
ExoFlow 2.0 Magnetic Capture Beads	450 µL	4°C
Wash Buffer Component A (20X)	15 mL	
Wash Buffer Component B (10X)	30 mL	
Blocking Buffer	30 mL	
TC Green dye	300 µL	
TC Red dye	300 µL	
EV Binding Buffer	30 mL	

*Each kit contains enough reagents to process 30 reactions of 150 – 300 µg protein equivalent of EVs (the actual amount of input protein equivalent depends on your EV isolation method, see the protocol).

Additional Required and Optional Equipment Not Included in Kit

- Scientific Vortex Mixer
- Magnet stand for molecular applications
- 1.5 ml non-stick Eppendorf tube(s)

Before you start:

Prepare the working Wash Buffer: Dilute 20X Wash Buffer Component A to 1X with DI Water, and use it to dilute Wash Buffer component B to 1X to obtain the final working Wash Buffer. For example, prepare 100 mL 1X Wash Buffer Component A by diluting 5 mL 20X Wash Buffer Component A in 95 mL DI Water. To 90 mL of the 1X Wash Buffer Component A, add 10 mL of Wash Buffer Component B to obtain the working Wash Buffer.

Protocol

Exo-Flow FACS Magnetic bead preparation

1. Vortex bead slurry briefly and transfer 15 µL of bead slurry solution into a 1.5 mL tube (1 tube per sample). Add 200 µL of Wash Buffer
2. Place bead-containing tubes on magnetic stand for 2 minutes
3. Carefully remove the supernatant after 2 minutes. Make sure to not disturb the magnetic bead pellets.
4. Remove tubes from magnetic stand and add 250 µL of Wash buffer. Invert a few times.
5. Place tubes back on magnetic stand and repeat steps 2-4 for a total of 2 washes.
6. Remove all liquid so only beads are left on the side of the tube

Binding of Capture Antibody to Beads

7. Remove tubes from magnetic stand. Mix 1 µl of the biotinylated capture antibody with 250 µL of Blocking Buffer and add the entire content to tube containing the ready-to-use beads.
8. Place tubes on a rotating rack in 4°C for 1 hour
9. Place tubes on magnetic stand for 2 minutes
10. Carefully remove the supernatant after 2 minutes. Make sure to not disturb the magnetic bead pellets.
11. Remove tubes from magnetic stand and add 250 µL Wash buffer. Invert a few times; DO NOT VORTEX. Flick tubes to dislodge the pellet.
12. Repeat steps 9-11 for a total of 2 washes.
13. Resuspend capture antibody-beads with 250 µL of Blocking Buffer and incubate at room temperature with rotation for 30 minutes
14. Repeat steps 9-11 for an additional 2 washes. Remove the Wash buffer and proceed to step 15.

EV Capture

15. Add 150-300 µg of isolated exosomes to each bead preparation in a final volume of 250 µL of Blocking Buffer

    Note: The input (in terms of protein equivalent) will vary depending on the EV isolation method. We recommend performing titrations to determine the optimal EV input for your assay.

16. Incubate on a rotating rack at 4°C overnight for capture

Note: TC-derived EV capture can be performed at RT for 1 hour; however, for optimal specificity and EV binding we recommend O/N.

17. Place samples on magnetic stand for 2 minutes
18. Carefully remove the supernatant after 2 minutes. Make sure to not disturb the magnetic bead pellets
19. Remove samples from magnetic stand and add 250 µL Wash buffer. DO NOT VORTEX. Flick tubes to mix do a quick spin down
20. Place the tubes on a rotating rack at room temperature for 3 minutes for the wash step
21. Remove tubes from the rack and place the samples on magnetic stand and repeat steps 17-20 for a total of 3 washes. The captured EVs are now ready for downstream applications.

APPLICATIONS

I. Flow cytometry

EV Staining for Flow Cytometry for Tissue Culture Media-derived EVs

1. Prepare 1X EV Binding buffer from the 5X buffer (provided) in 1X PBS
2. Resuspend the EV-bound beads in 200 µL of 1X Binding buffer
3. Add 10 µL of TC Green/Red dye for a final volume of 210 µL
4. Incubate the sample at RT for 15 minutes
5. Add 500 µL of 1X EV Binding buffer to the sample (final volume 710 µL). Sample is ready for flow cytometry.

II. Western Blotting

1. After EV capture (step 21), place samples on magnetic stand for 2 minutes
2. Carefully remove the supernatant after 2 minutes. Make sure to not disturb the magnetic bead pellets
3. Resuspend the beads in 30 µl of M-PER/RIPA lysis buffer (not provided)
4. Add 10 µl of sample loading buffer (5X) (not provided) per sample and pipette up and down to mix
5. Incubate the sample at 95^{o}C for 4 minutes. Cool on ice for 5 minutes. Sample is now ready to be loaded onto the into the wells of the SDS-PAGE gel or it can be stored at -20^{o}C for future use

III. RNA analysis

1. After step 20, place samples on magnetic stand for 2 minutes

2. Carefully remove the supernatant after 2 minutes. Make sure to not disturb the magnetic bead pellets

3. Resuspend the beads in RNA lysis buffer of choice. Once lysis is complete as per the manufacturers protocol, carefully remove the lysate from the magnetic beads by placing the samples on the magnetic rack and then proceed with RNA extraction according to your method of preference.

Example Data and Applications

Exo-Flow 2.0 delivers undetectable background binding when analyzed by western blot

We isolated EVs from HEK293 cells and serum using ExoQuick®-TC and ExoQuick Exosome Isolation Reagents, captured specific EV subpopulations using Exo-Flow 2.0 beads coated with either anti-CD63, anti-CD9, anti-CD81, or a no-antibody control, and analyzed the captured EVs by western blot probing for the exosome-specific marker TSG101 (Figure 3). The absence of detectable signal in the no-antibody control lane demonstrates the low background binding of Exo-Flow 2.0 technology. The varying signal intensity seen in Figure 3 also highlights the heterogeneity of EV subpopulations.

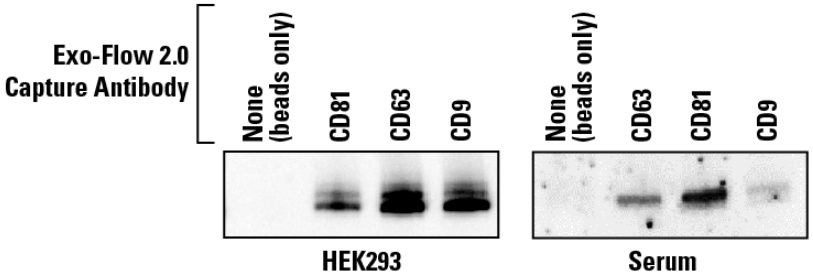

Figure 3. Exo-Flow 2.0 delivers undetectable background binding when analyzed by western blot.

Exo-Flow 2.0 delivers undetectable background binding when analyzed using flow cytometry

We isolated EVs from HEK293 cells using ExoQuick-TC and captured the subpopulation of EVs expressing CD9 (Figure 4A, dark red trace) and the subpopulation of EVs bearing CD63 (Figure 4A orange trace). We also isolated EVs from serum using the SmartSEC HT EV Isolation system and captured the subpopulation of EVs bearing CD81 (Figure 4B). After staining bead-bound EVs and washing away excess stain, we analyzed the EV subpopulations by flow cytometry. In both plots, distinct subpopulations of EVs can be seen—in Figure 4A, the peak seen on the red trace indicates a subpopulation of EVs bearing CD9 and the separate peak seen on the orange trace indicates a

subpopulation of EVs bearing CD63. In Figure 4B, the peak seen on the green trace indicates a subpopulation of EVs bearing CD81.

In both plots, the overlap of signal in the beads-only and no-antibody traces demonstrates the low background binding of Exo-Flow 2.0 technology.

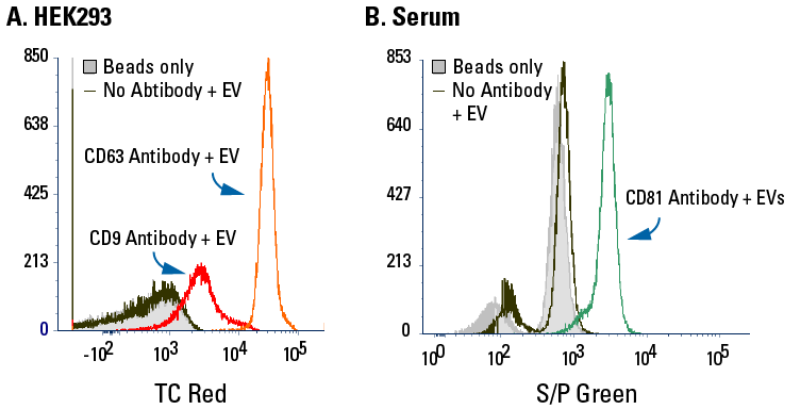

Figure 4. Exo-Flow 2.0 delivers undetectable background binding when analyzed using flow cytometry.

Understand surface marker differences between EV subpopulations with Exo-Flow 2.0

We isolated EVs from serum using SmartSEC HT, captured subpopulations of EVs using Exo-Flow 2.0 beads coupled with either biotinylated CD9 (Figure 5A) or CD63 (Figure 5B) antibodies, and analyzed these subpopulations for the presence of CD14, a myeloid lineage marker, using flow cytometry (Figure 5). The data show that EVs captured using anti-CD9 Exo-Flow 2.0 beads are more likely to also bear CD14 than EVs captured using anti-CD63- Exo-Flow 2.0 beads, demonstrating the applicability of Exo-Flow 2.0 technology for EV subpopulation analysis and enabling better biomarker screening.

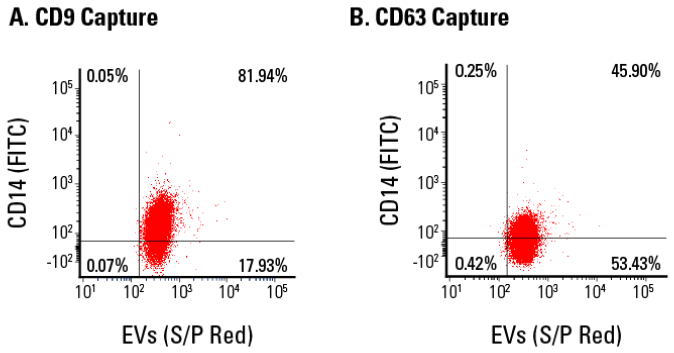

Figure 5. Exo-Flow 2.0 supports flow cytometric analysis of EV subpopulations.

Use Exo-Flow 2.0 to understand differences in cargo, such as miRNA levels, between EV subpopulations

We isolated EVs from HEK293-conditioned medium using ExoQuick-TC, captured subpopulations of EVs using Exo-Flow 2.0 beads coupled with either biotinylated CD63 or CD81 antibodies, and analyzed these subpopulations for the presence of miR-122 (Figure 5). The data show a statistically significant difference in the amount of miR-122 present in these EV subpopulations, with ~3.6-fold more miR-122 found in CD81 EVs than CD63 EVs, demonstrating the applicability of Exo-Flow 2.0 technology for EV subpopulation analysis.

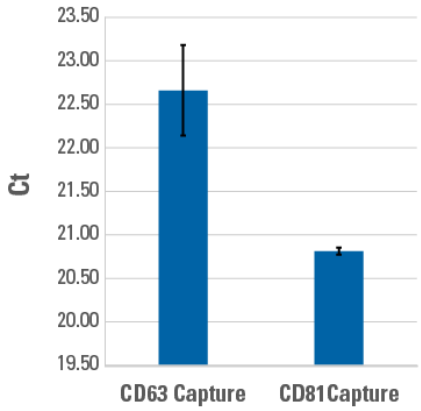

Figure 6. Use Exo-Flow 2.0 to understand differences in cargo, such as miRNA levels, between EV subpopulations.

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