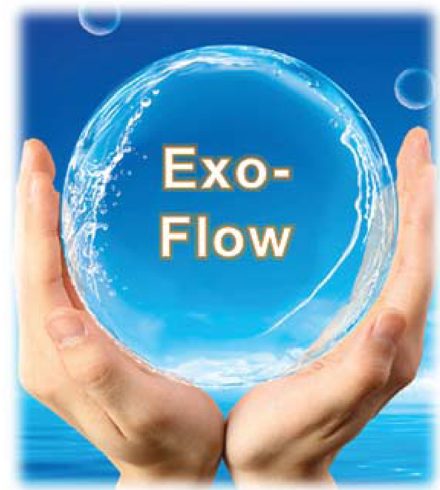
SBI
System Biosciences



Exo-FLOW™ Exosome Purification Kits

Cat #s EXOFLOWxxx

User Manual

Store Kits at +4ºC upon receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

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I. Introduction

A. Exosome Overview

Exosomes are 60 - 180 nm membrane vesicles secreted by most cell types in vivo and in vitro. These microvesicles are produced by the inward budding of multivesicular bodies (MVBs) and are released from the cell into the microenvironment following the fusion of MVBs with the plasma membrane. Exosomes are extracellular, nanoshuttle organelles that facilitate communication between cells and organs. Exosomes are found in blood, urine, amniotic fluid, breast milk, malignant ascites fluids and contain distinct subsets of RNAs and proteins depending upon the cell type from which they are secreted, making them useful for biomarker discovery. SBI has engineered tools and next-generation sequencing services to accelerate the study of exosomes, exosome protein and RNA biomarkers. The Exo-Flow kits have two major applications: 1) Purify exosome with specific surface markers for FACS analytics and purification and 2) high-throughput immunopurification (IP) of exosomes from media, serum and other biofluids in 96 well formats.

The Exo-Flow kits for FACS

These kits are for the selective capture and flow sorting to purify distinct subpopulations of exosomes, based on a particular surface marker. You will first enrich for all exosomes using ExoQuick (serum, plasma, ascites samples) or ExoQuick-TC (cell media, urine, spinal fluid). The isolated exosomes are then resuspended and bound to the magnetic beads for specific capture and subsequent FACS analysis and sorting. The Exo-Flow kits are modular, thus you can select from various pre-validated capture antibody kits, or utilize your own biotinylated capture antibody

corresponding to the exosome surface marker specific for the exosomes of interest in your model system. SBI has thoroughly tested a variety of capture antibodies that work quite well to flow-sort exosomes from either serum or cell culture samples.

Flow "Exometry"

SBI has developed a simple, one-step method for precipitation of all exosomes from biofluids using polymer formulations. The ExoQuick™ reagent can be used to isolate exosomes from serum, plasma and tumor ascites fluids. Exosomes from more dilute, larger volume fluids like cell culture media, using, cerebral spinal fluid can be efficiently isolated using SBI' ExoQuick-TC™. While ExoQuick isolation of exosomes is rapid and easy, this method will isolate every exosome from your biofluid sample. The Exo-Flow™ kits are designed to enable the selective capture and flow sorting to purify distinct subpopulations of exosomes, based on a particular surface marker. You will first enrich for all exosomes using ExoQuick (serum, plasma, ascites samples) or ExoQuick-TC (cell media, urine, spinal fluid). The isolated exosomes are then resuspended and bound to the magnetic beads for specific capture and subsequent flow exometry.

The Exo-Flow kits for High-throughput IP

The Exo-Flow96 and 32 IP kits enable the high-throughput, plate-based immunopurification of exosomes directly from serum or from concentrated exosomes from media (with ExoQuick-TC or ultracentrifugation). The magnetic beads are provided pre-coupled with either CD9, CD63 or CD81 for selective capture of distinct subsets of exosomes from either 96 or 32 samples simultaneously.

Why is this important?

Exosomes found in biofluids can originate from any tissue or cell type and end up in the serum fraction. If you are interested in just the immune-related exosomes, you would need to capture them using a specific exosome surface marker like HLA-G, same goes for tumor-derived exosome populations. The most efficient method for exosome subpopulation purification is using the Exo-Flow kits.

B. Magnetic Beads and Exosome Surface Markers

SBI has developed a magnetic streptavidin 9.1 µm Exo-Flow bead system with ultra-high exosome binding capacity. The 9.1 µm diameter of the beads *enables more exosome capture* per volume added when compared to 4 µm Dynabeads. This is significant in that some exosome subpopulations that are desired may only be present in very low numbers. The increased surface area enables the more efficient capture of these rare exosomes. ***Bigger is better.***

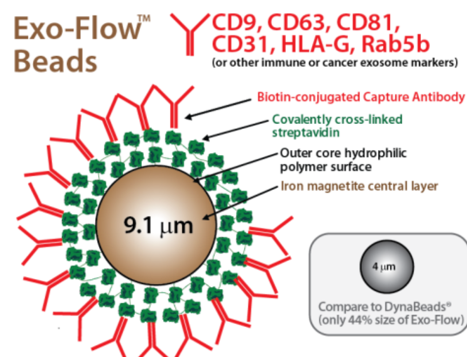


Fig. 1: Exo-Flow streptavidin magnetic beads

Depending upon the specific exosomes you wish to purify, a particular biotinylated antibody may be used to couple to the Exo-Flow streptavidin beads. The Exo-Flow kits are modular, thus you can select from various pre-validated capture antibody kits, or utilize your own biotinylated capture antibody corresponding to the exosome surface marker specific for the exosomes of interest in your model system. SBI has thoroughly tested a variety of capture antibodies that work quite well to flow-sort exosomes from either serum or cell culture samples. These are highlighted by arrows in the diagram below.

There are a few categories of known exosome surface markers based upon some functional attributes:

Marker type	Functions	Examples
Tetraspanins	Exosome formation and secretion	CD9, CD63, CD81
Targeting/Adhesion	Exosome maturation and target cell binding	Integrins, ICAM-1, CD31,CD44
Antigen Presentation	Immune modulation, anergy and priming	MHC I/II, HLA-G
Membrane transport and fusion	Exosome biogenesis, secretion and downstream cell fusion	SNAP, Rab5b

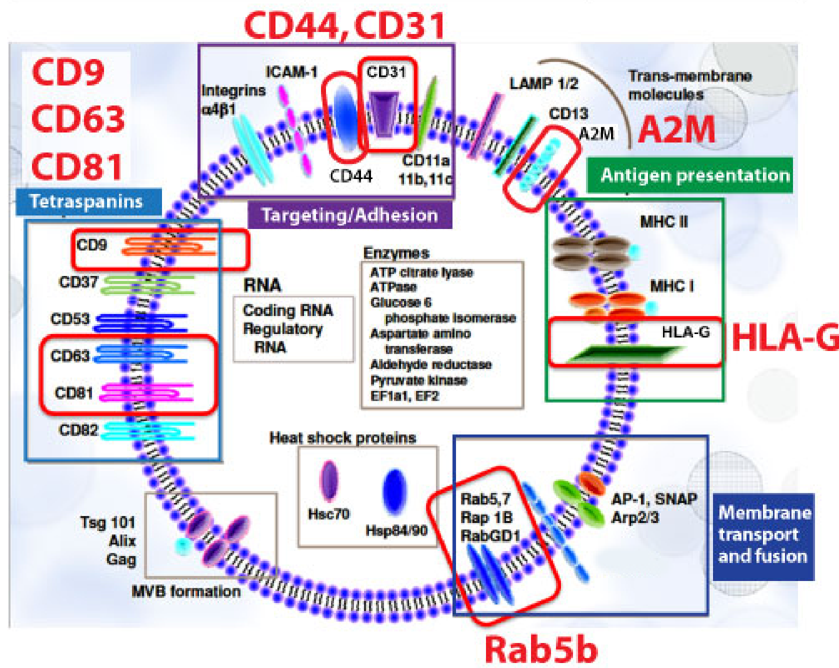
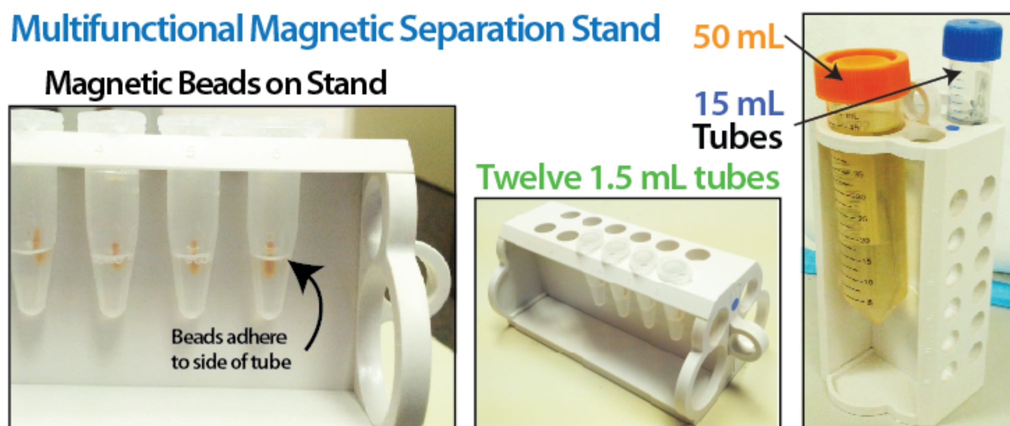


Fig. 2: Exosome surface marker examples

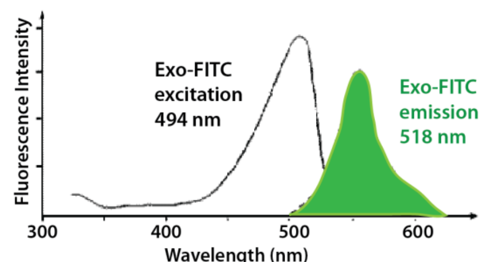
The Exo-Flow beads will appear as an orange or "pumpkin" like color which allows for them to be easily tracked during the magnetic separations and washes. The beads adhere strongly after placed on the magnetic stand such that clean separations and washes can be achieved.

The Exo-Flow kit system also offers a multifunctional magnetic stand as an accessory option (catalog# EXOFLOW700A-1). These have been validated to work very well with the Exo-Flow beads and can accommodate 12 standard 1.5 mL eppendorf tubes or the stand can be flipped and then used for two standard 15 mL or 50 mL conical tube exosome separations.



C. The Exo-FITC™ Universal and Reversible Stain

Once the desired exosomes have been captured and stabilized on the Exo-Flow beads, you will need to "stain" these exosomes with a fluorescent moiety to perform the FACS. One method is to use a second antibody conjugated to PE, APC, ALEXAs or FITC to accomplish this goal. These typically offer low staining ability and can be costly for multiple exosome sample purifications. SBI has invented a novel method to universally stain captured exosomes on the beads. The technology takes advantage of the findings that most exosome surface proteins have modifications (ex. glycosylations, carbohydrate additions, etc.). The Exo-FITC stain supplied in all kits features FITC fluorescent molecules conjugated to a protein known to universally bind these types of protein modifications.



This allows for a more general and inexpensive exosome FACS approach for captured exosome purification. The other key feature is that the ***Exo-FITC stain is also reversible***. Once the captured exosomes on the Exo-Flow beads are flow-sorted on FITC-positive gating, the Exo-FITC stain can then be washed away and exosomes eluted off of the beads with the Exosome Elution Buffer wash buffer (supplied in all kits).
Add the Exosome Elution Buffer to the exosome-bead mixture to simultaneously remove the Exo-FITC stain and liberate the intact exosomes from the beads. Then simply place the magnetic beads back on the magnetic stand, the eluted and FACS-purified exosomes will be in the supernatant. The exosome are intact and ready for downstream analysis and functional studies.

IMPORTANT NOTE:
If you want to double-stain your captured exosomes with a conjugated secondary antibody and the Exo-FITC stain, you MUST FIRST stain the exosomes on the beads with your conjugated secondary antibody, wash, then stain with the Exo-FITC stain.

D. Exo-Flow FACS Protocols

To ensure the best success of your flow exometry experiments, please follow the protocols exactly as stated below. DO NOT vortex the beads when exosomes are bound, this will shear them and break them apart leading to no flow-sorting positive results. Most steps can be performed at ambient (room) temperature unless otherwise indicated. We highly recommend using concentrated exosomes using SBI's ExoQuick or ExoQuick-TC. Exosomes isolated through centrifugation for concentration may be used as well.

Materials (10 reaction kits)

- **Streptavidin Magnetic Beads** 9.1 µm, 400 L at 10 mg/mL, 1.6x10^7 beads/mL, 1.6x10^6 beads/mg. We will use 0.4 mg per reaction, 6.4x10^6 beads per tube. Beads will have an orange "pumpkin" coloring making them

easy to track during the washings.

- **Biotinylated capture antibody**, 100 ng/µL in PBS, 100 µL provided (examples, CD9, Rab5b, HLA-G, depending upon specific kit).
- **Magnetic stand** (optional, cat# EXOFLOW700A-1).
- **Bead Wash buffer**, 60 mL.
- **Exosome Stain Buffer**, 3 mL.
- **Exo-FITC Universal exosome stain**, 100 µl provided.
- **Exosome Elution Buffer**, 3 mL provided.

ExoQuick and ExoQuick-TC are not provided in the Exo-Flow kits and can be purchased separately. The following ExoQuick products are recommended for exosome concentration prior to Exo-Flow purification.

Description	Size	Catalog #
ExoQuick Serum exosome precipitation solution (5 ml)	75 reactions	**EXOQ5A-1**
ExoQuick Plasma prep and Exosome precipitation kit (5 ml ExoQuick plus Thrombin)	75 reactions	**EXOQ5TM-1**
Thrombin Plasma prep for Exosome precipitation	100 reactions	**TMEXO-1**
ExoQuick Serum exosome precipitation solution (20 ml)	300 reactions	**EXOQ20A-1**
ExoQuick-TC for Tissue Culture Media and Urine (10 ml)	10 reactions	**EXOTC10A-1**
ExoQuick-TC for Tissue Culture Media and Urine (50 ml)	50 reactions	**EXOTC50A-1**

Exosome isolation protocol

Combine your biofluid sample containing exosomes with ExoQuick or ExoQuick-TC using the guidelines shown in the Table below. Mix the ExoQuick precipitation reagent with the biofluid sample by inversion and place at 4°C for 30 minutes to overnight, then recover the exosomes in a pellet with a low speed spin. Please refer to the ExoQuick or ExoQuick-TC User manuals for more details. Recommended amounts of exosomes provided in Table.

Biofluid	Sample volume	ExoQuick-TC volume	Resuspend exosome pellet	Volume to use in Exo-Flow
Urine	10 ml	2 ml	500 µL PBS	100 µL/rxn
Spinal fluid	10 ml	2 ml	500 µL PBS	100 µL/rxn
Culture media	10 ml	2 ml	500 µL PBS	100 µL/rxn

Biofluid	Sample volume	ExoQuick	Resuspend exosome pellet	Volume to use in Exo-Flow
Serum	250 µL	63 µL	500 µL PBS	100 µL/rxn
Plasma	250 µL	63 µL	500 µL PBS	100 µL/rxn
Ascites fluid	500 µL	120 µL	250 µL PBS	100 µL/rxn

Amount of exosomes to use

The number of exosomes in a given biofluid will vary depending upon the sample itself. There are abundant levels of exosome in serum, less in cell culture medium and urine. Use the guidelines in the Tables above as a starting point. You can typically add about 100-200 µg protein of isolated, intact exosomes per Exo-Flow reaction.

Exo-Flow FACS Magnetic bead preparation

1. Vortex bead slurry briefly and then pipette 40 µL of bead slurry solution into a 1.5 mL tube per sample.
2. Place samples 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 samples from magnetic stand and add 500 µL of **Bead Wash buffer**. Invert a few times.
5. Place samples on magnetic stand and repeat steps 2-4 for a total of 2 washes.
6. Remove all liquid so only beads are on the side of the tube.

Binding of capture antibody to beads

7. Remove tubes from magnetic stand and add 10 µL of biotinylated capture antibody (ie. CD9, CD63) per sample, using the pipette tip to move the beads to the bottom of the tube, mix by pipetting up and down three times..
8. Place tubes on ice for 2 hours. Flick the tube every 30 minutes to gently mix.
9. Add 200 µL **Bead Wash buffer** and flick to mix.
10. Place samples on magnetic stand for 2 minutes.
11. Carefully remove the supernatant after 2 minutes. Make sure to not disturb the magnetic bead pellets.
12. Remove samples from magnetic stand and add 500 µL **Bead Wash buffer**. Invert a few times; **DO NOT VORTEX**. Flick tubes to mix.
13. Place samples on magnetic stand and repeat steps 10-12 for a total of 3 washes.
14. Suspend capture antibody-beads with 400 µL **Bead Wash buffer** per sample.

Exosome capture

15. Add 100 µL of concentrated, isolated exosomes to each bead sample for a total volume of 500 µL.
16. Incubate on a rotating rack at 4°C overnight for capture.

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 500 µL **Bead Wash buffer**. Invert a 2-3 times; **DO NOT VORTEX**. Flick tubes to mix.

20. Place samples on magnetic stand and repeat steps 3-5 for a total of 2 washes.

Exosome staining

21. Add 240 µL of **Exosome Stain Buffer** and 10 µL of **Exo-FITC exosome stain** for a final volume of 250 µL per sample.

22. Place tubes on ice for 2 hours. Flick the tube every 30 minutes to gently mix.

23. Place samples on magnetic stand for 2 minutes.

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

25. Remove samples from magnetic stand and add 500 µL **Bead Wash buffer**. Invert a few times; **DO NOT VORTEX**. Flick tubes to mix.

26. Place samples on magnetic stand and repeat steps 23-25 for a total of 3 washes.

27. Resuspend samples in 300 µL **Bead Wash buffer** for flow cytometry.

28. **DO NOT VORTEX** prior to loading into FACS instrument.

Exosome Stain removal and elution (if desired)

29. Flow-sort stained exosome/bead complexes as desired.

30. Place the stained exosome/bead complexes on the magnetic stand for 2 minutes.

31. Remove buffer and add 300 µl **Exosome Elution Buffer**, Invert a few times; **DO NOT VORTEX**. Flick tubes to mix.

32. Incubate on a rotating rack or shaker at 25°C for 2 hours.

33. Place samples on magnetic stand for 2 minutes.

34. Carefully remove the supernatant containing your eluted exosomes and transfer to a fresh tube. Make sure to not disturb the magnetic bead pellets. Discard beads after use.

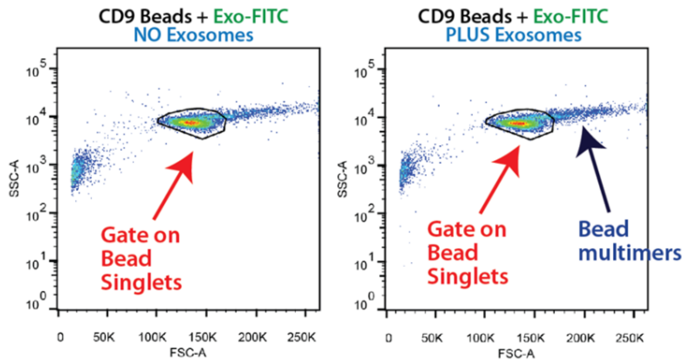
E. Sample Exo-Flow FACS data

The data below were generated on a BD LSR II instrument and are meant as examples of how to set the gate settings and perform data analysis using FlowJo software.
Information on FlowJo software can be viewed online.
http://www.flowjo.com/

Exo-Flow software gate settings

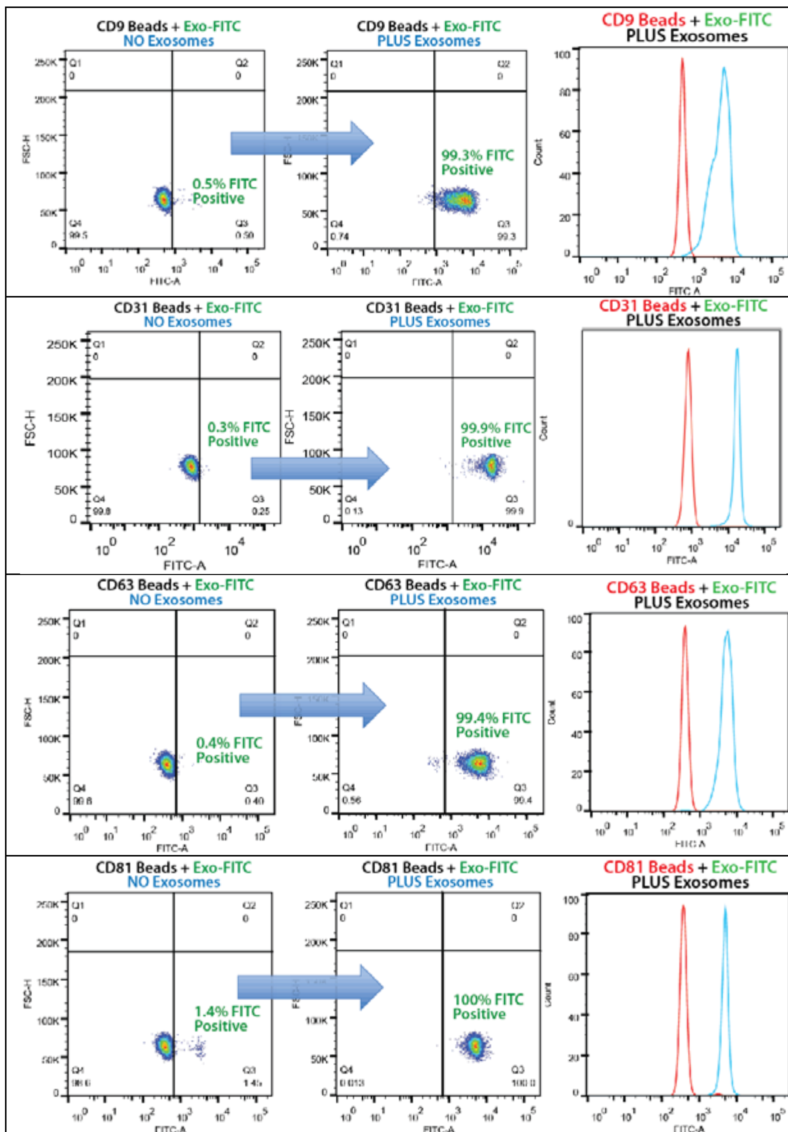
The forward and side scatter data for the 9.1 um Exo-Flow beads are shown below for samples containing no captured exosomes stained with Exo-FITC (left panel) and then data for serum CD9-captured exosomes stained with

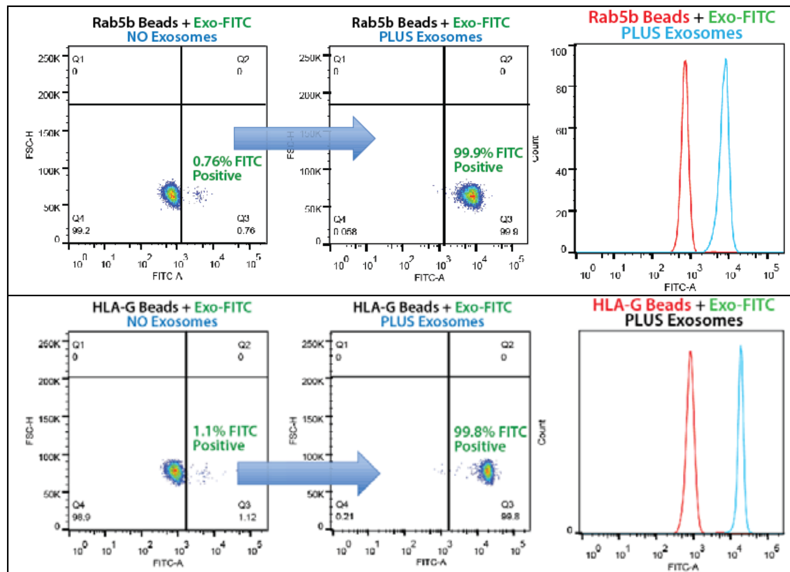
Exo-FITC. Set the gate primarily on the majority bead singlets (outlined in a black oval, red arrow pointing to the gate setting) prior to full flow analysis.



Exo-Flow exosome flow-exometry example data

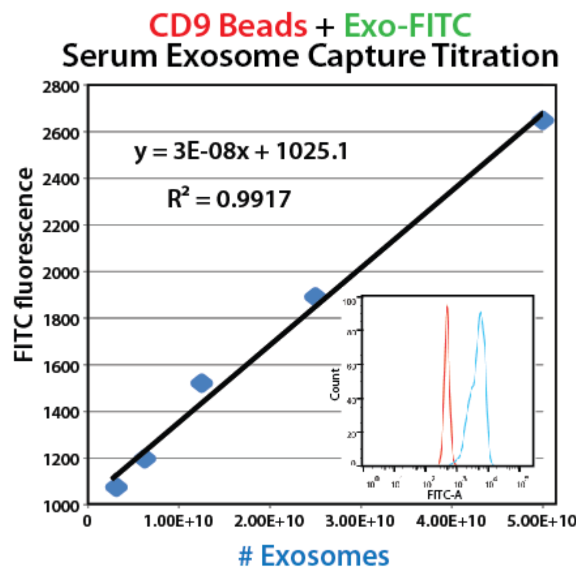
Bead flow separation data for the various capture antibodies coupled with Exo-FITC staining are shown below. The data are graphed showing forward scatter versus FITC intensity. The first panel depicts beads with no exosomes then with exosomes. The degree of flow separation is shown on the right side for each capture set.





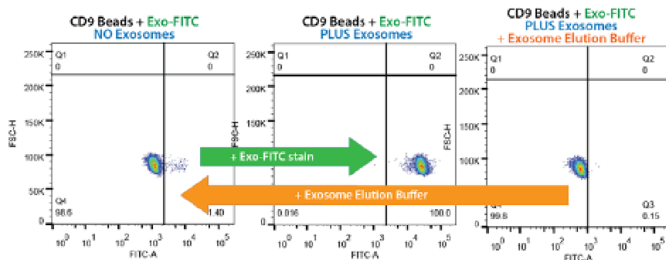
<u>Exo-Flow FACS exosome binding capacity data</u>

Human serum exosomes were isolated from 250 µl serum using ExoQuick. The exosome pellet was resuspended in 500 µl 1x PBS. The amount of exosome particles were added in two-fold dilutions starting at 50 µl and then captured using the biotinylated CD9 antibody and Exo-Flow beads. The FITC flow cytometric intensities are then plotted versus the number of exosome particles input into the flow reaction. The Exo-Flow system has the capacity to capture, flow and detect BILLIONS of exosomes.

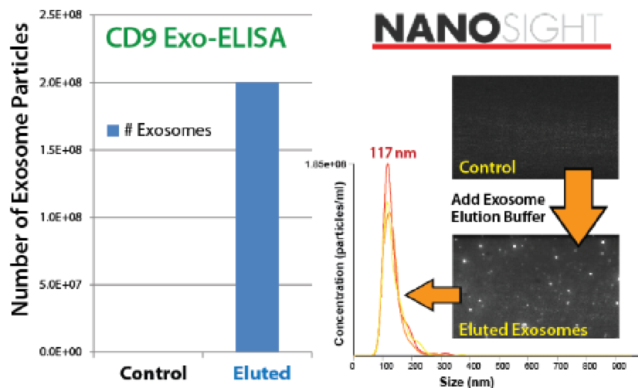


F. Exo-FITC stain removal and exosome elution

Once you have flow-sorted the captured exosomes of interest, the Exo-FITC stain can be easily removed and intact exosomes eluted safely. This can enable you to then perform downstream analysis and functional studies. The data below show the efficient Exo-FITC stain removal and exosome elution. The flow-sort results from CD9 captured exosomes, stained with Exo-FITC, and then eluted with the Exosome Elution Buffer. The entire bead population returns to pre-stain FITC intensities and NanoSight studies verify the recovery of intact exosomes.



CD9 Exo-ELISA and NanoSight data of purified exosomes eluted from the Exo-Flow beads.



G. List of Components for Flow FACS kits

The Exo-Flow kits contain enough reagents to perform up to 10 FACS flow-exometry experiments.

Item	Amount
Streptavidin Magnetic Beads, 9.1 µm	200 µL at 10 mg/mL
Biotinylated capture antibody, 100 ng/µL in PBS (multiple varieties)	100 µL
Bead Wash buffer	60 mL
Exosome Stain Buffer	3 mL
Exo-FITC Universal exosome stain	100 µL
Exosome Elution Buffer	3 mL
Magnetic stand (optional, cat# EXOFLOW700A-1)	1 Stand

H. Additional Materials Required

1) ExoQuick and/or ExoQuick-TC to concentrate exosome prior to antibody-bead capture.
2) Magnetic stand, or purchase SBI's Multifunctional stand (cat# EXOFLOW700A-1)
3) FACS instrument for flow cytometry
4) Sterile, 1.5 mL sample tubes

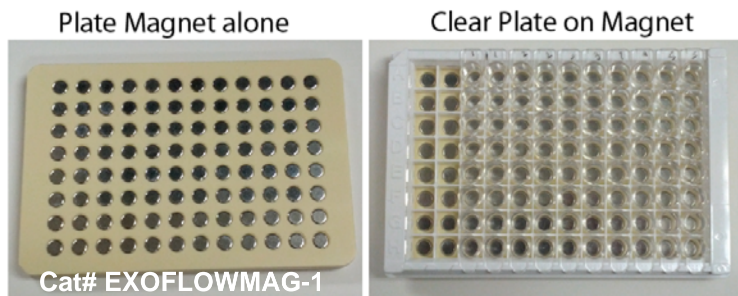
I. Exo-Flow96 and 32 Exosome IP Kits

The Exo-Flow96 and 32 IP kits enable the high-throughput, plate-based immunopurification of exosomes directly from serum or from concentrated exosomes from media (concentrated with ExoQuick-TC or ultracentrifugation). The magnetic beads are provided pre-coupled with either CD9, CD63 or CD81 antibodies for selective capture of exosomes from either 96 or 32 samples simultaneously. The Exo-Flow96 kits contain all of the reagents for exosome IP purifications from 96 samples and the Exo-Flow32 kit has all of the reagents for 32 samples. Exosomes in serum or plasma samples can be immunopurified directly by adding to the IP magnetic beads. We recommend concentrating exosomes first from media, urine and CSF before immunopurifcation. Choose from exosome IP antibodies for CD9, CD63 or CD81 surface markers. The exosome are immunopurified and eluted intact - allowing for downstream functional studies as well as for exoRNA and protein biomarker analyses.

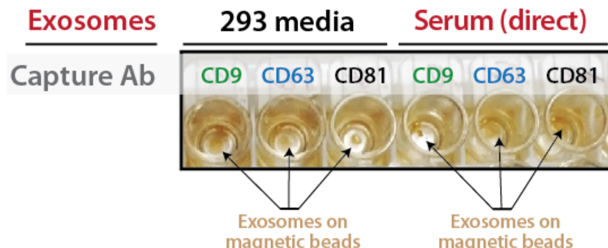
Materials provided:

- 96 well clear plate (8 well strips) and covers
- Capture Antibody pre-coupled with magnetic beads
- Wash buffer (20X stock)
- Exosome Elution Buffer
- Clearing reagent (to dissolve elution reagent away from exosomes)
- Exo-FlowMag96 magnetic 96- well rack (sold separately)

Protocol for ExoFlow96 or 32:

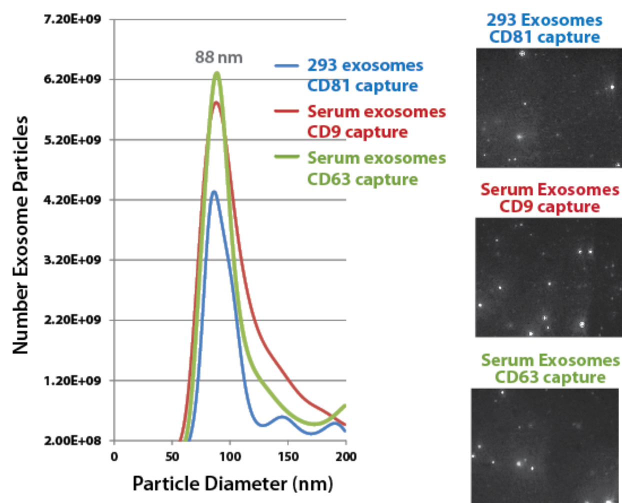


1. Add 20 µl of antibody coupled with magnetic beads to each well of the plate provided per capture reaction.
2. Add 50 µL of concentrated, isolated exosomes from media samples (isolated by ExoQuick-TC or ultracentrifugation) or directly load serum samples to each bead well for a total volume of 70 µl into the 96 well plate provided.
3. Incubate on a shaker at room temperature overnight for exosome capture.
4. Place samples on the Exo-FlowMag96 magnetic 96 well rack for 2 minutes. You should hear a subtle “click” when the plate is properly aligned with the magnetic plate.
5. Keep the clear plate on the magnetic rack and then decant the supernatant. Make sure to not disturb the magnetic bead pellets at the bottom of the wells.



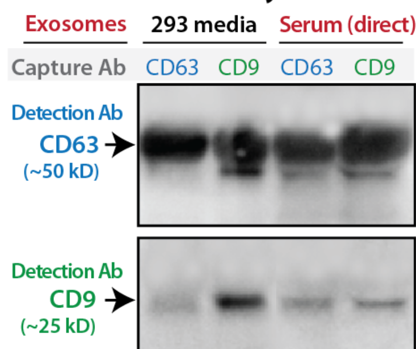
6. Remove the 96 well plate from the magnetic rack and gently add 50 µl of 1X Wash buffer (made fresh from the 20X stock provided).
7. Mix by pipetting up and down 3 times and incubate on a shaker at room temperature for 5 minutes.
8. Repeat steps 4-6 for a total of 2 washes.
9. Remove any residual Wash buffer after decanting and then add 100 µl **Exosome Elution Buffer** to each well of the 96 well plate.
10. Incubate on a shaker at room temperature for 1 hour.
11. Place samples plate on the magnetic rack for 2 minutes.
12. Carefully remove the supernatant containing eluted, intact exosomes and transfer to a fresh tube on ice. Make sure not to disturb the magnetic bead pellets. Discard the beads afterwards. The immunopurified exosomes are stable at +4°C for several days or can be frozen at -80°C for long term storage. Example data of exosome recoveries for intact exosomes and the number of exosome particles can be seen in the Figure below.

Exosome concentrations recovered using the Exo-Flow IP kits were measured by NanoSight analysis (below). The data below demonstrate the exosome recoveries, with sizes centered around 88 nm in diameter. The HEK 293 media exosomes were purified from 20 ml media and concentrated using ExoQuick-TC and resuspended in 1 mL PBS. Then 50 µl of the concentrated media exosomes were added per well for the immunopurifications. The serum exosomes were isolated from 50 µl of human serum added directly to the bead capture mixture.



Optional: Western blotting Protocol

a. To the eluted exosome supernatant containing, add 1 ul of the **clearing reagent**, this dissolves the elution buffer away from the exosomes.
b. Invert it a few times to mix it well.
c. Incubate at 37 °C for 30 minutes(the sample is now ready for Westerns).
d. Measure the protein concentration of the samples. Load 1-5 ug protein sample per well.
e. Add 10 ul of 5x loading dye to the sample in a total of 50 ul and boil for 5 minutes at 95°C.
f. Perform standard SDS-PAGE electrophoresis and Western transfer onto nitrocellulose membranes.
g. Block with 5% dry milk in Tris Buffered Saline +0.05 % Tween (TBS-T) for 1 hour or Blocking buffer for 1 hour.
h. Incubate blot overnight at 4°C with exosome-specific primary antibody (eg. Anti-CD63) at 1: 1,000 dilution (5% dry milk in TBS-T).
i. Drain the antibody solution.
j. Wash with TBS-T for 3X 5 minutes.
k. Drain the wash solution.
l. Prepare exosome validated secondary antibody at 1: 20,000 dilution (5% dry milk in TBS-T).
m. Incubate one hour at room temperature with exosome validated secondary antibody at 1:20,000 dilution (5% dry milk in TBS-T).
n. Wash with TBS-T for 3X 5 minutes.
o. Drain the wash solution.
p. Prepare detection solution.



q. Add 5 ml developing solution to the membrane.
r. Incubate at room temperature for 2 minutes.
s. Take the membrane out of the dish, drain excess detecting solution using kimwipe.
t. Place the membrane on a plastic surface for imaging.
u. Detect signals with chemi-luminescence and expose the membrane for 5-45 seconds to image signals.

J. Related Products

SBI offers a number of exosome research products. Review them here: http://www.systembio.com/exosomes

- ExoQuick exosome isolation reagents
- Exo-FBS exosome-depleted media supplement
- Detect and quantitate exosomes with Antibodies and ELISAs
- Purify exosome RNA and profile by qPCR with SeraMir
- Discover novel exoRNA biomarkers with Next-Gen sequencing services

K. Shipping and Storage Conditions for Kit

The Exo-Flow kits are shipped on blue ice and should be stored at +4°C. Avoid freeze-thawing the reagents. Shelf life of the product is 1 year after receipt if stored in +4°C.

II. Frequently Asked Questions

Q. How many exosomes should I start with before flow-sorting?

The biofluid type and cell culture media source will determine just how many exosomes are present. The best way is to first isolate and concentrate the exosomes of interest. Resuspend the isolated exosomes in a minimal volume (10% of starting volume) with 1X PBS. Take a protein concentration measurement and use approximately 50 μg to 200 μg exosome protein input per Exo-Flow capture experiment.

Q. If I want to detect the captured exosomes using a fluorescently-conjugated secondary antibody, how much should I use?

Every antibody is different and will have varying degrees of affinity. This is best determined experimentally for each antibody tried. We recommend starting with at least 50 μg secondary antibody. The Exo-FITC stain can serve as a positive control for the presence of exosomes during your FACS analysis.

Q. What are the species cross-reactivities of each of the Exo-Flow antibodies?

All of the Exo-Flow capture antibodies have been optimized for human exosome capture. Due to the high protein sequence conservation of many of these exosome surface markers, cross-species reactivity is likely, but SBI has not tested all mammalian species yet. The Rab5b biotinylated antibody has the most cross-species capture reactivity (Human, Mouse, Rat, Chicken, Pig, Cow, Horse, Rabbit, Sheep) and can be used to develop your exosome flow-sorting experiments.

III. References

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