



**EV-FACS Flow Cytometry Kit
PRODUCT INFORMATION & MANUAL**

NBP3-18360 (Plasma)

NBP3-18361 (Urine)

NBP3-18362 (Serum)

NBP3-18363 (Cell Culture Media)

For research use only.

Not for diagnostic or therapeutic procedures.

TABLE OF CONTENTS

Product description	4
Product content	4
Storage information	5
EV-FACS Ready to Use Kit procedure	6
Data analysis	10

PRODUCT DESCRIPTION

Product overview

EV-FACS allows exosome isolation from biofluids or cell culture media and FACS analysis of exosome markers. The kit consists of EV Precipitation reagent, for exosome isolation, 4 µm beads used for the overall capture of pre-isolated exosomes, lyophilized exosomes from cell culture supernatants or human biological fluids, as positive control. The characterization of exosomal proteins (membrane expressed or internal) is subsequently performed using appropriate detection antibodies against exosome associated antigens.

About Exosomes

Exosomes are small endosome derived lipid nanoparticles (50-120 nm) actively secreted by exocytosis by most living cells. Exosome release occurs either constitutively or upon induction, under both normal and pathological conditions, in a dynamic, regulated and functionally relevant manner. Both amount and molecular composition of released exosomes depend on the state of a parent cell.

Exosomes have pleiotropic physiological and pathological functions and an emerging role in diverse pathological conditions such as cancer, infectious and neurodegenerative diseases.

PRODUCT CONTENT

Reagents provide in each kit:

Description	Component	Amount
Sample buffer 5X	Buffer for antibody dilution and incubation	1 bottles (10 ml)
EV Precipitation	Exosome isolation reagent (plasma, serum, saliva)	1 bottle (3 ml)
	Exosome isolation reagent (cell and urine)	1 bottle (20 ml)
FACS-Beads	4 µm Aldehyde-Sulfate latex beads	1 vial (120 µl)
Lyophilized Exosome Standards	Exosome Standards, from human biofluids of certified healthy donors (plasma, serum, urine, saliva) or from different cell culture media.	1 vial (100 µg)
Primary Antibody	Primary antibody against CD9 or CD63 for exosome marker detection to use as positive control.	1 vial (20 µl)
Secondary Antibody	Secondary antibody Alexa 488	1 vial (3 µl)

Other material required

- Single-use and/or pipettes with disposable tips 2-100 μ l
- Polypropylene tubes
- Pipettes 1 ml and 5 ml for reagent preparation
- Deionized water
- PBS
- BSA or FBS or FCS
- Disposable pipetting reservoirs

STORAGE INFORMATION

All reagents provided within the EV-FACS Ready To Use Kits can be stored at +4°C for up to 12 months, if unopened.

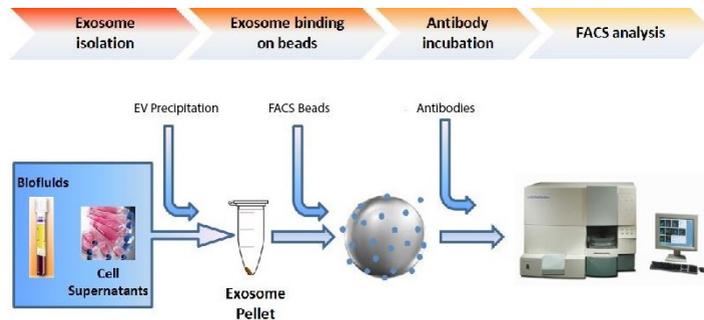
DO NOT FREEZE!

Open and reconstituted components	
Exosome standards	The remaining reconstituted standard stock solution should be aliquoted into polypropylene vials (preferably low binding) and stored at -20°C for up to one month or at -80°C for up to six months. Strictly avoid repeated freeze-and-thaw cycles.
FACS-Beads	Store opened and diluted reagents at +4°C up to 12 months
Primary detection antibody	
Sample buffer 5X	
Secondary Antibody	
EV Precipitation Solution	

EV-FACS READY TO USE KIT PROCEDURE

Overview

EV-FACS allows exosome isolation from biofluids or cell culture media and FACS analysis of exosome markers. The kit consists of EV Precipitation reagent for exosome isolation, 4 µm beads used for the overall capture of pre-isolated exosomes, lyophilized exosomes from cell culture supernatants or human biological fluids as positive control. The characterization of exosomal proteins (membrane-expressed or internal) is subsequently performed using appropriate detection antibodies against exosome associated antigens.



STEP A: Sample preparation

1- Human plasma and serum

Prepare samples by 3 centrifugation steps to eliminate red blood cells and cellular debris. After each step, transfer the supernatant to a new tube and discard the pellet.

- 10' at 300 g (save supernatant; discard pellet).
- 20' at 1200 g (save supernatant; discard pellet).
- 30' at 10 000 g (save supernatant; discard pellet).

2- Cell culture medium

Prepare cell supernatants by 3 centrifugation steps:

- 10' at 300 g (save supernatant; discard pellet).
- 20' at 1600 g (save supernatant; discard pellet).
- 30' at 10000 g (save supernatant; discard pellet).

3- Human urine

- Centrifuge at 350 g for 10' at room temperature and save the supernatant.

4- Human saliva

Add PBS 1X in saliva samples in ratio 1/1 (5 ml of saliva + 5 ml of PBS 1X). Mix together and proceed to the following centrifugation steps:

- 15' at 2600 g
- 20' at 15550 g
- filter through 0.22 µm filter

Step B: Exosome isolation from biological sample:

1- From human plasma and serum

Fluid	Minimum volume required	Volume suggested
Plasma	100 µl	100 µl -250 µl
Serum	200 µl	250 µl - 500 µl
Saliva	200 µl	250 µl - 500 µl

- Add EV Precipitation solution to your sample in ratio 1/4 (i.e. 100 ul of plasma + 25 ul of EV Precipitation)
- Mix well by pipetting and inverting tube
- Incubate on ice for 1 hour
- Centrifuge 20 minutes at 10000 g (centrifuge can be performed at 4°C or at RT)
- Discard the supernatant
- Centrifuge for 2 minutes at 1500 g to eliminate entirely the supernatant
- Resuspend the pellet in 100 ul* of PBS 1x

* Volume of resuspension can be defined by the user.

2- From cell culture medium

Fluid	Minimum volume required	Volume suggested
Cell medium	1 ml	1 ml - 5 ml

- Add EV Precipitation solution to your sample in ratio 1/1 ((i.e 1 ml of cell medium + 1 ml of EV Precipitation solution)
- Mix well by pipetting and inverting tube
- Incubate on ice for 1 hour
- Centrifuge 20 minutes at 10000 g (centrifuge can be performed at 4°C or at RT)
- Discard the supernatant
- Centrifuge for 2 minutes at 1500 g to eliminate entirely the supernatant
- Resuspend the pellet in 100 ul* of PBS 1x
- Resuspended exosomes can be used for analysis or stored at -20°C.

* Volume of resuspension can be defined by the user on the base of downstream analysis.

Final exosome yield can be dependent on the cell line used. Different cell lines produce different quantity of exosomes. If exosome yield is poor, increase the volume of medium, mantaining the ratio with EV Precipitation 1/1 (2 ml of cell medium + 2 ml of EV Precipitation solution).

3- From human urine

Fluid	Minimum volume required	Volume suggested
Urine	5 ml	8 ml -20 ml

- Add EV Precipitation solution to your sample in ratio 1/4 ((i.e 5 ml of urine + 1.2 ml of EV Precipitation solution)
- Mix well by pipetting and inverting tube
- Incubate on ice for 1 hour
- Centrifuge 20 minutes at 10000 g (centrifuge can be performed at 4°C or at RT)
- Discard the supernatant
- Centrifuge for 2 minutes at 1500 g to eliminate entirely the supernatant
- Resuspend the pellet in 100 μ l* of PBS 1x
- Resuspended exosomes can be used for analysis or stored at -20°C.

* Volume of resuspension can be defined by the user

Step C: Lyophilized Exosome Standard reconstitution:

Reconstitute lyophilized exosome standard by adding 100 μ l of deionized water to get a final concentration of 1 μ g/ μ l.

Resuspend exosomes pipetting the solution up and down 10-15 times, avoiding bubbles.

Vortex the reconstituted standard for 60 seconds. Briefly centrifuge the tubes containing the standard to ensure that the solution is collected at the bottom of the tube. Pipette the solution up and down 10 times, avoiding the introduction of bubbles. After this step, the standard is ready to use.

Use 5 μ l of reconstituted Exosome Standard for each reaction.

Step D: Exosome binding onto latex FACS-Beads:

- Dilute 5 times Sample buffer 5x with PBS 1x (Sample buffer 1x).
 - It is recommended to prepare the complex Exosome-Beads in one single tube, then to divide in single reactions before the antibody incubation.
 - Latex FACS-Beads are ready to use. Resuspend well FACS-Beads prior to use by vortexing or pipetting several times.
 - For each reaction mix together 5 μ l of Exosome Standards and 5 μ l of FACS-beads in an eppendorf tube (preferably low binding). Mix well by pipetting 5-6 times.
 - *Example: if you want to run 10 reactions, mix into the same eppendorf low binding tube 50 μ l of Exosome Standards and 50 μ l of FACS-Beads.*
 - For exosome isolated by EV Precipitation, mix 5 μ l of FACS-Beads with the volume of resuspended exosomes suggested (volumes are indicative only; the user should define the appropriate volumes on the base of exosome yield).
 - Plasma and serum: 20 μ l - 50 μ l /reaction
 - Urine: 50 μ l - 100 μ l /reaction
 - Cell medium: 50 μ l - 100 μ l /reaction
 - Incubate for 15 minutes at room temperature (RT).
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- Add 0.7 ml of PBS 1x and incubate in rotator or shaker for 2 hours at RT or overnight (ON) at 4°C.
 - Centrifuge the complex Exosomes-Beads (Exo-Beads) for 5 minutes at 4500 g at 4°C and discard the supernatant.
 - Add 1 ml of Sample Buffer 1x, resuspend Exo-Beads for 5-6 times and incubate for 15 minutes at RT.
 - Centrifuge 5 minutes at 4500 g at 4°C, discard the supernatant.
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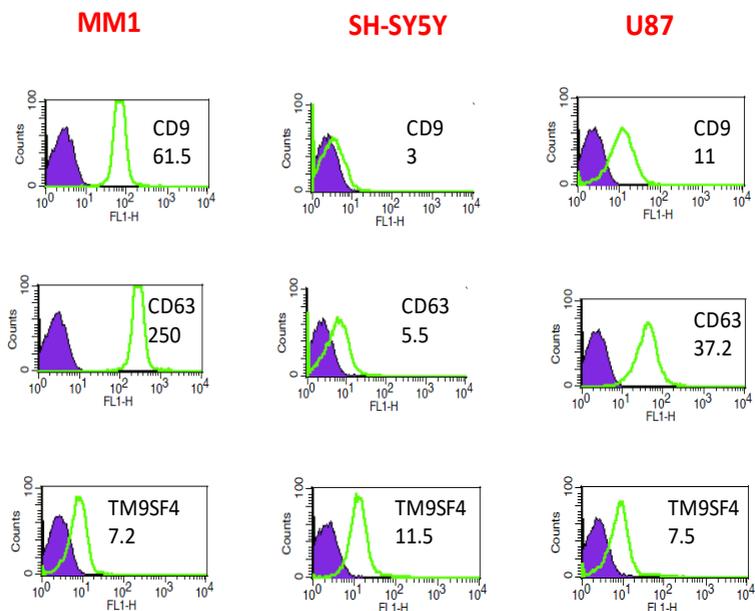
Step E: Antibody incubation:

- Prepare the Washing buffer (not provided in the kit) diluting 2% of FBS (or FCS) in PBS 1x (consider that you need 8 ml of washing buffer for each reaction). Alternatively, if you don't have FBS or FCS, prepare the Washing buffer diluting 0,5% of BSA in PBS 1X. Keep on ice.
 - Resuspend the Exo-Beads in 100 µl of Sample buffer 1x for each reaction.
Example: if you are running 10 reactions resuspend Exo-Beads in 1 ml of Sample buffer.
 - Prepare the FACS tubes (not provided in the kit), one tube for each reaction.
 - Divide the Exo-Beads resuspended in sample buffer in each FACS tube, pipetting 100 µl of suspension in each tube.
 - Spin the antibody vials 30 seconds at 10000 g in order to collect all the material on the bottom of the tube.
 - Add primary antibody in ratio 1:200 (0.5 µl per each FACS tube).*
**If other primary antibodies are used the correct dilution must be defined by the user.*
 - Incubate 2 hours at 4°C in the dark. For negative control can be used PE or FITC-anti-Mouse IgG1 isotype or FITC or PE anti-Rabbit IgG1. (isotype controls not provided in the kit) Otherwise incubate control samples with secondary Abs only (either leave in ice or directly add sec Abs).
 - Add 4 ml of prepared Washing buffer to each FACS tube.
 - Centrifuge 5 minutes at 4000 g and discard the supernatant.
 - Resuspend beads pellet into the FACS tubes in 100 µl of Sample buffer 1x.
 - Add secondary antibody in ratio 1:1000. (mix 38 µl of Sample buffer 1x with 2 µl of secondary antibody; add 2 µl of the received solution per each FACS tube to obtain the right dilution of antibody).
 - Incubate for 1 hour in the dark at 4°C.
 - Add 4 ml of Washing buffer in each FACS tube, centrifuge for 5 min at 4000 x g , 4°C. Discard supernatant by pouring it out. Vortex what has remained inside the FACS tubes. If not use immediately, put in the dark at 4°C.
 - Add 500 µl of Washing buffer per FACS tube.
 - Analyse the samples.
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DATA ANALYSIS

- Read 10000 events from the gated first population.
- Alexa 488 is read in FL1 channel (green)

EV-FACS was used for a protein marker profile in exosomes derived from different sources. Exosome binding on FACS-beads was performed by incubation at 4°C over night. Exosome-bead complex is ready to be labeled with fluorophore-conjugated antibodies for specific exosome markers. In figure 7 is shown a profile of expression of three different exosome markers in exosomes purified* from Melanoma (MM1), Neuroblastoma (SH) and Glioblastoma (U87) cell supernatants.



FACS profiling of exosomal markers CD9, CD63 and TM9SF4 in purified exosomes from MM1, SH-SY5Y and U87 cell lines.