Mouse ExoStep™

Exosome FACS analysis and purification kit of exosomes derived from Mouse

REF			RUO
MO2ExoS-25-C9	25 test	Includes CD9 biotin as primary detection antibody	

1. INTRODUCTION

Exosomes are small extracellular vesicles that are released from cells upon fusion of an intermediate endocytic compartment, the multivesicular body (MVB)[1], with the plasma membrane. They are thought to provide a means of intercellular communication[2,3] and of transmission of macromolecules between cells allowing the spread of proteins, lipids, mRNA, miRNA and DNA and as contributing factors in the development of several diseases. Exosomes can also modulate cancer microenvironment[4] and the immune response [5,6].

2. PRODUCT DESCRIPTION

The kit is a simple immunobead assay for isolation/detection of exosome, using a bead-bound anti-CD63 capture antibody and a fluorochrome conjugated anti-CD9 detection antibody. The kit provides reproducible results and can be run in parallel to exosome immunophenotyping.

- Tested application: Flow Cytometry Species reactivity: Rodent
- Storage buffer: aqueous buffered solution containing protein stabilizer and 0.09% sodium azide (NaN3).
- Recommended usage: Immunostep's Rodent ExoStep, is intended for the immunoisolation (immunomagnetic or FACS) and Flow Cytometry analysis of pre-enriched CD63+ rodent exosomes from cell culture media.
- Presentation: liquid

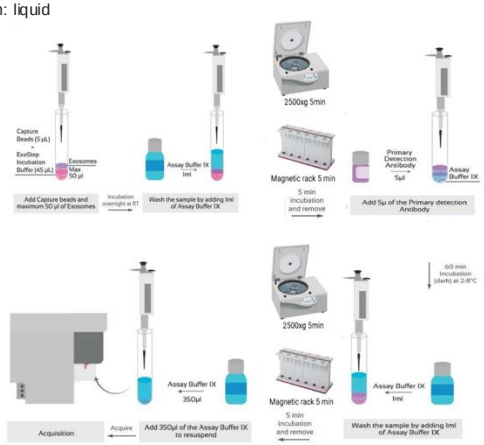


Figure 1: Immunobead assay for isolation/detection of exosome workf low.

3. REAGENTS PROVIDED

DESCRIPTION	COMPONENTS	AMOUNT	Σ
Superparamagnetic Capture Beads	CD63+ (Clone NVG-2 capture beads. Polystyrene micro-particles with Mean Diameter (µm) 6.5± 0.2 (CV<5%), having discrete fluorescence intensity characteristics	6000 beads/test (5 µl/test)	25 test
ExoStep Incubation Buffer	Incubation buffer for ExoStep kits	1.2ml (45 µl/test)	25 test
Primary detection antibody	Anti-CD9 biotin (Clone MZ3)	(5 µl/test)	25 test
Secondary detection reagent	Streptavidin-Phycoerythrin (PE) is useful for detecting biotinylated antibodies. The excitation of PE by 488nm laser light induces a light emission maximum of 575 nm	(5 µl/test)	25 test
Assay Buffer 10X	PBS 10% BSA, pH 7.4 - 10X. Do not freeze. Dilute contents of the 10X Assay Buffer to 1X (PBS 1% BSA) in PBS, for use in this assay	10 ml	

4. APPROPRIATE STORAGE AND HANDLING CONDITIONS

Store in the dark, refrigerated between 2 ºC and 8 ºC. DO NOT FREEZE. The kit is stable until the expiry date stated on the vial label if kept at 2-8ºC. Do not use af ter the date indicated.

5. REAGENTS NOT PROVIDED

- Pre-enriched exosomes by ultra-centrifugation.
- Magnetic Rack; MagneSphere(R) Mag. Sep. Stand 12-hole, 12x75mm (PROMEGA, Ref Z5343).
- 12x75 mm Polystyrene Round Bottom Tubes (cytometer tubes).
- Sterile syringe filter with a 0.45 µm pore (EMD Millipore Millex, Ref: SLHV033RS).
- Syringe of adequate volume.

6. EVIDENCE OF DETERIORATION

Reagents should not be used if any evidence of deterioration is observed. For more information, please contact our technical service: tech@immunostep.com

7. RECOMMENDATIONS AND WARNINGS

a. Avoid microbial contamination of the reagent. Assay buffer 1X can be filtered before use.
b. Microspheres and reagents should be protected from prolonged exposure to light throughout this procedure.
c. Microspheres are internally dyied with a fluorescent dye (fluorescent in PerCP, PerCP-Cy5, PerCP-Cy5.5 and APC). For exosome staining protocol ensure that the detector antibody does not occupy these fluorescent channels.
d. The samples should be treated with appropriate handling procedures.
e. Depending on the type of exosomes used, the number of exosomes may vary with respect to the concentration of the protein.
f. Do not use after the expiry date indicated on the vial.
g. Deviations from the recommended procedure could invalidate the analysis results.
h. Before acquiring the samples, it is necessary to make sure that the flow cytometer is calibrated and compensated.
i. The isolation and detection success is dependent on the quality of the sample pre-enrichment process.
j. Pay attention if the kit is used in combination with annexin assays. The buffer used to work with annexin generates non-specificity with the Exostep kit.

8. WARRANTY

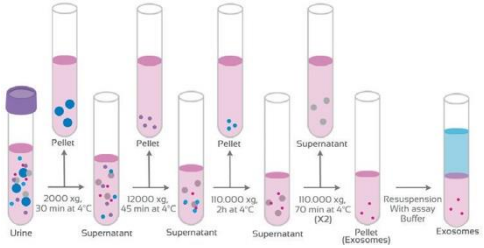
Warranted only to conform to the quantity and contents stated on the label or in the product labelling at the time of delivery to the customer. Immunostep disclaims hereby other warranties. Immunostep`s sole liability is limited to either the replacement of the products or refund of the purchase price.

9. SAMPLE PREPARATION

Exostep allows the detection of isolated exosomes from differential ultracentrifugation, precipitation or size exclusion purification as well as direct detection in the sample without previous isolation and just with simple pretreatment.

a) Purification of Exosomes by Differential Ultracentrifugation

The kit has been validated for pre-enriched human exosomes from cell culture and body fluids, such as serum/plasma, and urine, through an ultracentrifugation protocol (Fig. 2). The principle for exosome purification is the same for cell culture and body fluids, but due to the viscosity of some fluids it is necessary to dilute them with an equal volume of PBS, before centrifugations.



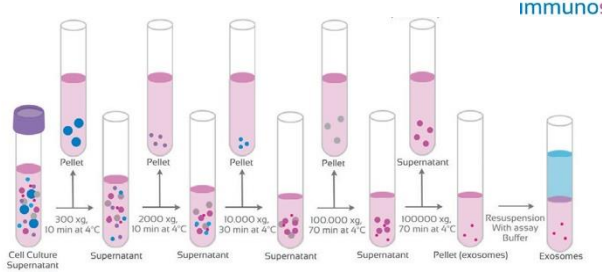


Figure 2: Workflow for the exosome pre-enrichment based on differential ultracentrifugation.

If it is planned to quantify the exosome preparation by Bradford assay to obtain the amount of total protein, do not resuspend in Assay Buffer as it contains BSA which will interfere with the measurement. For this purpose it is recommended to resuspend in PBS.

b) Sample pretreatment for direct exosome detection on cell-culture supernatant

The sample pretreatment for direct exosome detection from cell culture supernatant is not recommended for detection of exosomes from body fluids.
Specific sample pretreatment protocols are available for body fluids (plasma, urine) each optimized for its specific type of biological sample. To ensure that detected exosomes originate from your cells of interest, culture the cells with exosome depleted fetal bovine serum (FBS), because normal FBS contains extremely high levels of exosomes that will contaminate the cells derived exosomes.

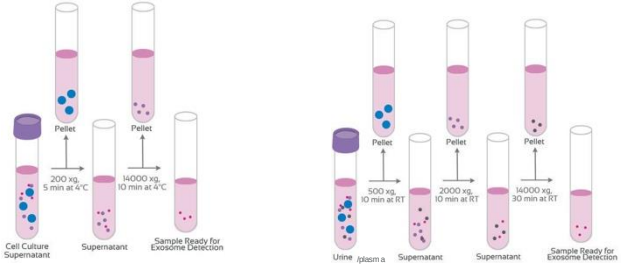


Figure 3: Cell Culture, human plasma and urine pretreatment workflow for direct exosome detection.

10. PROTOCOL

Isolate CD63+ exosomes

1. Resuspend the capture beads by vortex for approximately 20 seconds.
2. Add 5µL of the capture bead to each 12x75 mm Polystyrene Round Bottom tube (cytometer tube).
3. Add 45µL of ExoStep Incubation Buffer and a vortex for 20 seconds.
4. Add between 10-15ug of exosomes isolated by differential ultracentrifugation or until 100ul for direct exosomes. Previously prepared according to "Sample Preparation" to the appropriate tubes. Mix the reactions gently by pipetting up and down several times with a pipette and vortexing for few seconds.
5. Incubate in the dark overnight at room temperature (RT). NO STIRRING.
6. After overnight incubation wash the sample (bead-bound exosomes) by adding 1ml of Assay Buffer 1X.
7. Collect the Magnetic beads by placing tubes on a magnetic rack and incubate 5 minutes or by centrifugation at 2500xg for 5 minutes. Remove supernatant from tubes by Hand-decanting in the case of using the magnetic rack (Fig. 4) or by aspiration. Take care not to disturb the microspheres, and make sure not to leave more than 100ul of supernatant in the tube.

Stain exosomes for Flow Cytometry

8. After overnight incubation, add the suggested volume indicated of the primary detection antibody (5µL of the supplied antibody) to the bead-bound exosomes tube. Mix gently by pipetting and/or by tapping. It is advisable to prepare an additional tube with the appropriate isotype control or without exosomes, for background determination.
9. Incubate in the dark 60 minutes at 2-8ºC, w ithout stirring.
10. Wash the sample (bead-bound exosomes) by adding 1ml of Assay Buffer 1X.

11. Collect the magnetic beads by placing tubes on a magnetic rack and incubate 5 minutes or by centrifugation at 2500xg for 5 minutes. Remove supernatant from tubes by hand-decanting in the case of using the magnetic rack (Fig.4) or by aspiration. Take care not to disturb the microspheres, and make sure not to leave more than 100ul of supernatant in the tube.

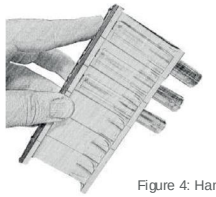


Figure 4: Hand-decanting supernatant using a magnetic rack.

12. Remove the tubes from the magnetic rack and resuspend the microspheres in the remaining 100µL of Assay Buffer 1X. Mix gently by pipetting.
13. Add 5µL of the secondary detection reagent to each tube. Mix the reactions gently by pipetting up and down several times with a micropipette.
14. Incubate in the dark 30 minutes at 2-8ºC, without stirring.
15. Wash the sample (bead-bound exosomes) by adding 1ml of Assay Buffer 1X.
16. Collect the magnetic beads by placing tubes on a magnetic rack and incubate 5 minutes or by centrifugation at 2500xg for 5 minutes. Remove supernatant from tubes by hand-decanting decanting in the case of using the magnetic rack (Fig.4) or by aspiration. Take care not to disturb the microspheres, and make sure not to leave more than 100ul of supernatant in the tube.
17. Resuspend the sample in 350µL Assay Buffer 1X and acquire on a f low cytometer or store in the dark max up to 2 hours at 2-8ºC, until the analysis is carried out.

11. ASSAY ACQUISITION

An adequate gating strategy FSC / SSC for 6 micron bead size and FL3 / FL4, helps bead population identif ication and discrimination of doublets on f low cytometer.

1. Gate on the single population(s) on a forward scatter vs. side scatter plot in linear scale. (Fig. 5A).
2. Gate on the single population(s) on a FL3 vs. FL4 channel (bead auto fluorescence) in logarithmic scale (Fig. 5B).
3. Using the FL2 channels, determine whether or not any or not any bead populations tested "positive" for the exosome. Note: A positive bead will produce a fluorescent peak in the FL2 channel.

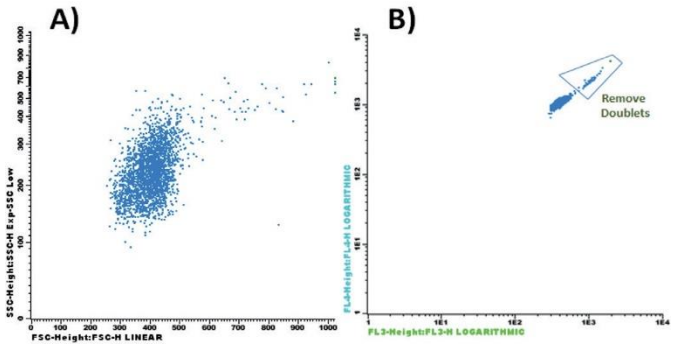


Figure 5: Dot-plot gating strategy for acquisition and analysis. FSC vs SSC (A) and FL3 vs FL4 (B).

12. FLOW CYTOMETRY ANALYSIS

ExoStep has been used for detection of exosome derived from different sources

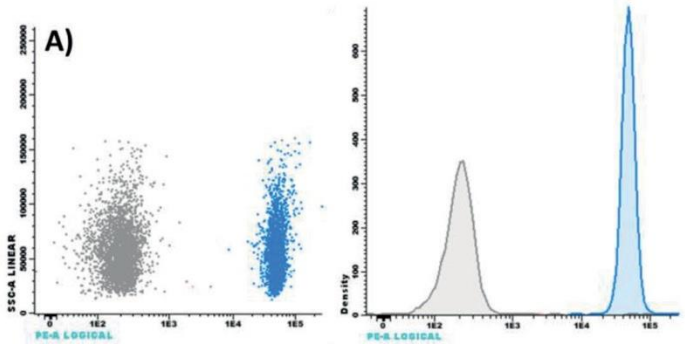
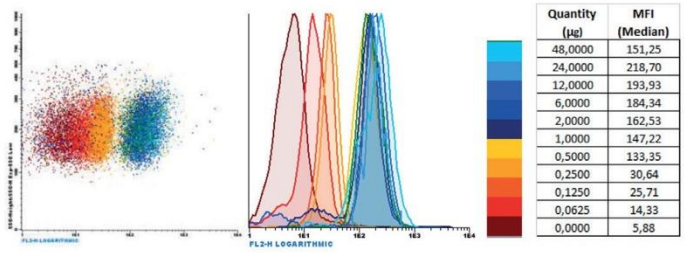


Figure 6 Flow analysis of exosomes bound to ExoStep. Cell culture exosomes, pre-enriched using total exosome isolation from PC3 cell culture media (A), were resuspended in PBS and bound to CD63-capture beads during an overnight incubation. The following day the bead-bound exosomes were indirect stained with primary detection antibody (CD9 Biotin/CD81 Biotin) and Streptavidin-PE and analyzed by flow cytometry.

13. PERFORMANCE DATA

Limit of Detection (LOD), dynamic range and linearity of exosome kit was assessed at Immunostep.

LOD is the lowest quantity of exosomes that is distinguished from the absence of analyte (a blank value), and as reference, was determined in >0,25µg which corresponds with >1.75 * 10^8 vesicles. Whilst the upper limit or saturation level was established in 24µg. For both technical specifications were used exosome form B16F10 cell culture media (7 * 10^8 particles /µl). (Fig. 7).



Quantity (µg)	MFI (Median)
48,0000	151,25
24,0000	218,70
12,0000	193,93
6,0000	184,34
2,0000	162,53
1,0000	147,22
0,5000	133,35
0,2500	30,64
0,1250	25,71
0,0625	14,33
0,0000	5,88

Figure 7: Dynamic range of the assay analyzed by flow cytometry. Relationship between background noise and specific signal at different exosome concentrations.

Several measurements of multiple concentrations of lyophilized exosomes were analyzed across the reportable range of the kit, finding the linearity of the kit in a broad range of concentrations, allowing fluorescence interpolation in the estimation of concentrations.

Please, refer to our website technical support http://www.immunostep.com/content/34-protocols for further information.

14. ADDITIONAL INFORMATION

For research use only. Not for diagnostic use.

Not for resale. Immunostep will not be responsible of violations that may occur with the use of this product. Any use of this product other than the specified in this document is strictly prohibited.

Unless otherwise indicated by Immunostep by written authorization, this product is intended for research only and is not to be used for any other purpose, including without limitation, for human or animal diagnostic, therapeutic or commercial purposes.

Please, refer to www.immunostep.com technical support for more information.

15. EXPLANATION OF SYMBOLS

	Fluorochrome
REF	Product reference
Σ	Content for <n> analysis
	Regulatory Status
	Description
RUO	Research Use Only
	Manufacturer

16. REFERENCES

1. Yáñez-Mó M, Siljander P, Andreu Z, Bedina Zavec A, Borràs F, Buzas E et al. Biological properties of extracellular vesicles and their physiological functions. Journal of Extracellular Vesicles. 2015;4(1):27066.
2. Pitt JM, André F, Amigorena S, Soria JC, Eggermont A, Kroemer G, Zitvogel L. Dendritic cell-derived exosomes for cancer therapy. J Clin Invest. 2016.
3. Tkach M, Théry C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. 2016 Cell 10;164(6):1226-32.
4. Becker A, Thakur BK, Weiss JM1, Kim HS, Peinado H, Lyden D Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. Cancer Cell 2016 Dec 12;30(6):836-848.
5. López-Cobo S, Campos-Silva C, Valés-Gómez M. Glycosyl-Phosphatidyl-Inositol (GPI)-Anchors and Metalloproteases: Their Roles in the Regulation of Exosome Composition and NKG2D-Mediated Immune Recognition. Front Cell Dev Biol. 2016 Sep 12;4:97.
6. Jonathan M. Pitt, Guido Kroemer, Laurence Zitvogel Extracellular vesicles: masters of intercellular communication and potential clinical interventions. 2016 J Clin Invest. 2016;126(4):1139-1143.
7. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. Current Protocols in Cell Biology. 2006.

17. MANUFACTURED BY

IMMUNOSTEP S.L.
Address: Avda. Universidad de Coimbra, s/n
Cancer Research Center (C.I.C)
Campus de Unamuno
37007 Salamanca (Spain)
Telf./fax: (+34) 923 294 827
E-mail: info@immunostep.com
www.immunostep.com