Capture Beads

Immunobeads for Exosome capture and flow detection

REF	Σ	
9CB-25 63CB-25 81CB-25 274CB-25 326CB-25 MO63CB-25 (mouse) IGG1CB-25 (isotype control) IGG2ACB-25 (isotype control)	25 test	RUO

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 product consists of a simple bead population, coated with a capture antibody for isolation/ detection of exosomes.

- **Tested application:** Flow Cytometry[7,8].
- **Species reactivity**: Human / Mouse (MO63CB-25)
- **Storage buffer**: aqueous buffered solution containing protein stabilizer and 0.09% sodium azide (NaN3).
- **Recommended usage**: Capture Beads are intended for the immunoisolation (immunomagnetic or FACS) and Flow Cytometry analysis of pre-enriched human exosomes from biofluids (plasma, urine) or cell culture media.
- **Presentation**: Liquid

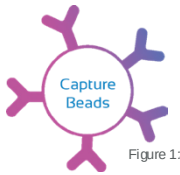

Figure 1.Superparamagnetic Capture Bead Graphical Representation.

3. REAGENTS PROVIDED

CAPTURE BEADS CLONE	DESCRIPTION	AMOUNT
9CB-25 (VJ/20) 63CB-25 (TEA3.18) 81CB-25 (M38) 274CB-25 (29E.2A3) 326CB-25 (VU-1D9) MO63CB-25 (NVG-2) IGG1CB-25 (B11/6) IGG2ACB-25 (B12/8)	Superparamagnetic Capture Beads Polystyrene micro-particles with Mean Diameter (µm) 6.5±0.2 (CV<5%), having discrete fluorescence intensity characteristics	600 0 beads/test (5µL/test)

4. ISOTYPES CONTROLS

Immunostep's isotypes controls IgG1 (clone B11/6) and IgG2a (clone B12_8) are monoclonal antibodies produced against a synthetic hapten, which is normally not present in humans or animals using in flow cytometry. These are often used as negative controls.
IgG1 is coated in the population beads number while IgG2a is in population number 2. Both populations do not differ in size but in average fluorescence intensity.

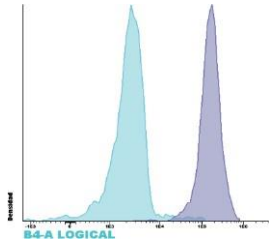

Figure 2 : IgG1+(CD63 or CD81) capture beads

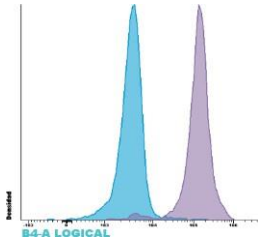

Figure 3 : IgG2a+CD9 capture beads

5. APPROPIATE 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 after the date indicated.

6. REAGENTS NOT PROVIDED

- Pre-enriched exosomes by ultra-centrifugation.
- Magnetic Rack; MagneSphere(R) Mag. Sep. Stand 12-hole, 12x75mm (PROMEGA, Ref Z5343).
- 12x75mm Polystyrene Round Bottom Tubes (cytometer tubes).
- Sterile syringe filter with a 0.45 µm pore (EMD Millipore Millex, Ref: SLHV033RS).
- Syringe of adequate volume.
- Assay Buffer: PBS-BSA 1%pH 7.4. Assay buffer 10X can be purchased as it appears in the catalog with the following reference: IMS0515.
- ExoStep Incubation Buffer (reference EISTEP).

7. 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

8. RECOMMENDATIONS AND WARNINGS

a. Avoid microbial contamination of the reagent. Assay buffer 1Xcan 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 Capture Beads.

9. 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.

10. SAMPLE PREPARATION

Exostep allows the detection of isolated exosomes from differential ultracentrif ugation (a) as well as direct detection in the sample without the need for ultracentrif ugation, just with simple pretreatment (b).

a) Purification of Exosomes by Differential Ultracentrifugation

The kit has been validated for pre-enriched human exosomes from cell culture and bodily fluids, such as serum/plasma, and urine, through an ultracentrifugation protocol (Fig. 2)[9].

The principle for exosome purification is the same for cell cult ure and bodily fluids, but due to the viscosity of some fluids it is necessary to dilute them with an equal volume of PBS, before centrifugations.

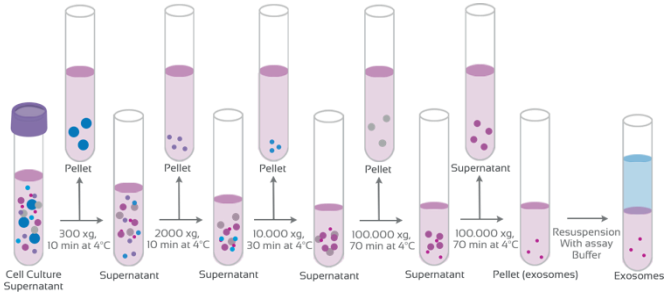

Figure 4 : Workflow for the exosome pre-enrichment based on differential ultracentrifugation.

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

The sample pretreatment required for direct exosome detection varies depending on the type of biological sample. Specific pretreatment protocols are available for each type of sample (e.g., plasma, urine), optimized according to their unique characteristics. Below, two sample pretreatment procedures are described: one for cell culture supernatant and another for plasma. 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 cell derived exosomes.

I) Cell culture

Prepare the samples for the following centrifugation steps:

- 5 min at 200xg and 4ºC. Collect supernatant and discard pellet.
- 10 min. at 14000xg and 4ºC. Collect supernatant and discard pellet.

II) Plasma, serum and urine

About 100–1000µL of plasma/urine typically provides enough exosomes for most standard types of analysis.

- 10 min. at 500xg collect supernatant and discard pellet.
- 10 min. at 2000xg collect supernatant and discard pellet.
- 30 min. at 14000xg collect supernatant and discard pellet.

11. PROTOCOL

Isolate exosomes

1. Resuspend the capture beads by vortex for approximately 20 seconds.
2. Add 5µL of the capture bead to each 12x75mm Polystyrene Round Bottom tube (cytometer tube). If an isotypic control needs to be added, please, do it at this point.
3. Add 45µL of ExoStep Incubation Buffer and a vortex for 20 seconds. The addition of this buffer is not mandatory, but it is recommended, as it will help to avoid unspecific binding.
4. Add between 10±5 µg of exosomes isolated by differential ultracentrifugation or up to 100 µL of direct exosomes. If protein quantification is not performed, the theoretical maximum binding capacity per bead has been estimated at 6,420 EVs per bead. This corresponds to approximately 3.85×10^{7} exosomes per 5 µL bead reaction. However, experimental results indicate that saturation occurs at 3.6×10^{9} EVs per test, suggesting that not all available exosomes bind during a single incubation step. Prepare the samples as described in the "Sample Preparation" section and transfer them to the appropriate tubes. Gently mix the reactions by pipetting up and down several times, followed by brief vortexing for a few seconds.
5. Incubate in the dark overnight at room temperature (RT). NO STIRRING. After incubation if only exosome isolation is needed, whithout FACS analysis, go directly to step 12.

Stain exosomes for flow cytometry

6. After overnight incubation, add the suggested volume indicated of the primary detection antibody (not supplied. Visit our website: www.immunostep.com) 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.
7. Incubate in the dark 60 minutes at 2-8ºC, without stirring.
8. Wash the sample (bead-bound exosomes) by adding 1ml of assay buffer 1X.
9. 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. 5A) or by aspiration. Take care not to disturb the microspheres, and make sure not to leave more than 100ul of supernatant in the tube.

   Go to step 13 for direct stain protocol (using direct detection antibody labeled with fluorochrome) for indirect stain protocol go to next step (10).

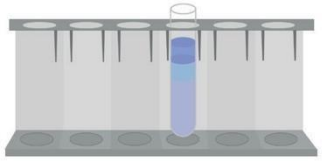

Figure 5A: Hand-decanting supernatant using a Magnetic Rack.

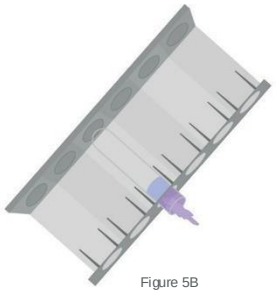

Figure 5B

10. Add 5µL of the secondary detection reagen (not supplied) t to each tube. Mix the reactions gently by pipetting up and down several times with a micropipette.
11. Incubate in the dark 30 minutes at 2-8ºC, without stirring.
12. Wash the sample (bead-bound exosomes) by adding 1ml of PBS-BSA 1%pH 7.4.
13. 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. 5A) or by aspiration. Take care not to disturb the microspheres, and make sure not to leave more than 100 ul of supernatant in the tube. For exosome magnetic isolation without FACS analysis, protocol ends her, on the contrary, continue with next step.
14. Resuspend the sample in 350µLPBS-BSA 1%pH 7.4. and Acquire on a flow cytometer or store in the dark max up to 2 hours at 2-8ºC, until the analysis is carried out.

12. ASSAY ACQUISITION

An adequate gating strategy FSC / SSC for 6 micron bead size and PerCP/APC, PerCP-Cy5/ APC or PerCP-Cy5.5/APC helps bead population identification and discriminat ion of doublets on flow cytometer.

1. Gate on the single population(s) on a Forward Scatter vs. Side Scatter plot in linear scale. (Fig. 6A).
2. Gate on the single population(s) on a PerCP vs. APC channel (bead auto fluorescence) in logarithmic scale (Fig. 6B)
3. Using the PE channels, determine whether or not any bead populations tested "positive" for the exosome. Note: A positive bead will produce a fluorescent peak in the PE channel.

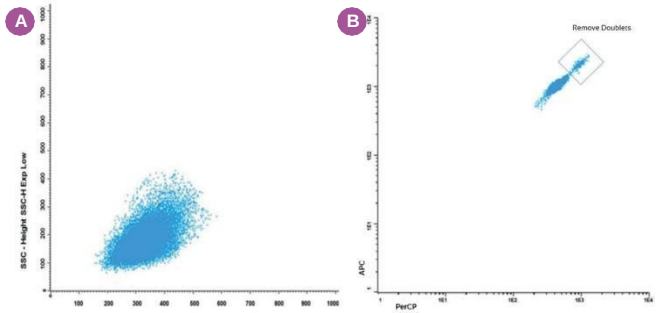

Figure 6: Dot-plot gating strategy for acquisition and analysis. FSC vs SSC and A PerCP vs APC B

13. 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.

14. EXPLANATION OF SYMBOLS

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

15. 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 Vesides. 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. JClin Invest. 2016.
3. Tkach M, Théry C. Communication by Extracellular Vesides: 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. Campos S, Suárez H, Jara-Acevedo R, Linares-Espinós E, Martínez-Piñeiro L, Yáñez-Mó M, Valés-Gómez M. High sensitivity detection of extracelular vesicles immune-captured from urine by conventional flow citometry. Sci Rep. 2019; Feb 14;9(1):2042.
8. Jara-Acevedo R, Campos-Silva C, Valés-Gómez M, Yáñez-Mó M, Suárez H, Fuentes M. Exosome beads array for multiplexed phenotyping in cancer. J Proteomics. 2019; Apr 30;198:87-97.
9. 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.

16. MANUFACTURED BY

IMMUNOSTEP S.L.
Address: Avda. Universidad de Coimbra, s/n
Cancer Research Center (C.I.C)
Campus de Unamuno
3700 7 Salamanca (Spain)
Telf./fax: (+34) 923 294 827
E-mail: info@immunostep.com
www.immunostep.com