ExoStep™ Cell Culture

Exosome FACS analysis for exosomes derived from human cell lines

REF	Σ	
ExoS-25-C9	25 test	Includes CD9 PE 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.

The use of IgG1 as a control recommended. It is compatible with this kit so it can be used simultaneously. Isotypes controls are monoclonal antibodies produced against a synthetic hapten, which is normally not present in humans or animals using inf flow cytometry. They are often used as negative controls.

- **Tested application:** Flow Cytometry[7,8].
- **Species reactivity**: Human
- **Storage buffer**: aqueous buffered solution containing protein stabilizer and 0.09% sodium azide (NaN3).
- **Recommended usage**: Immunostep's ExoStep, is intended for the immunoisolation (immunomagnetic or FACS) and Flow Cytometry analysis of pre-enriched CD63+ human exosomes from biofluids (plasma, urine) or cell culture media.
- **Presentation**: Liquid

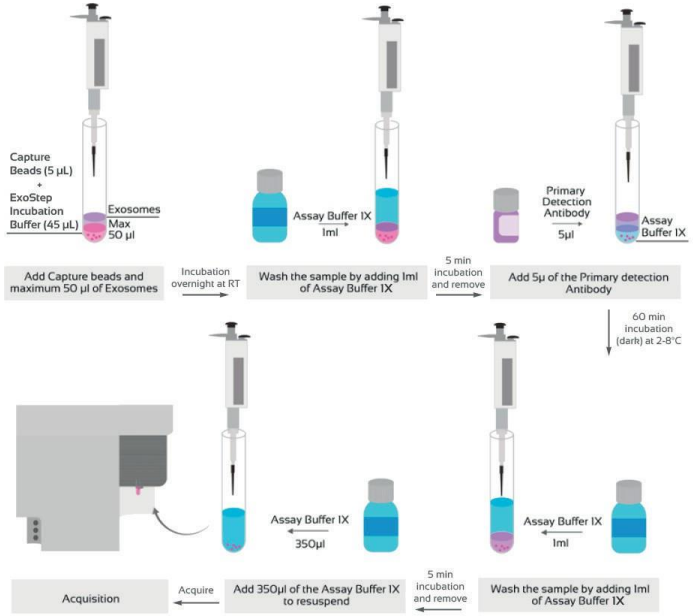

Figure 1: Immunobead assay for isolation/detection of exosome workflow.

3. REAGENTS PROVIDED

DESCRIPTION	COMPONENTS	AMOUNT	Σ
Superparamagnetic Capture Beads	CD63+ (Clone TEA3/18 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	1,2 ml (45 µl/test)	25 test
Primary detection antibody	Anti-CD9 PE (Clone VJ1/20)	(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. 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.

5. REAGENTS NO PROVIDED

- Isotype control IgG1b. IgG1b capture beads can be purchased. It appears in the catalog with IGG1CB-25 reference.
- 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.

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 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 (a) as well as direct detection in the sample without the need for ultracentrifugation, 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 culture 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.

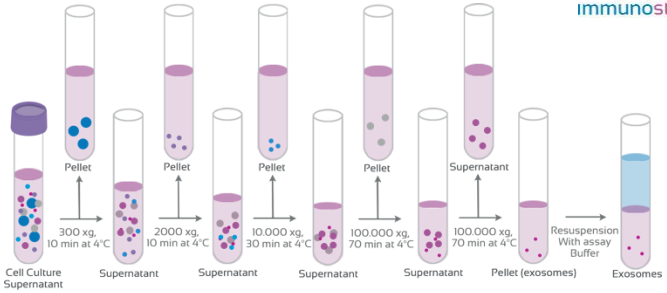

Figure 2: Workflow for the exosome pre-enrichment based on differential ultracentrifugation.

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.

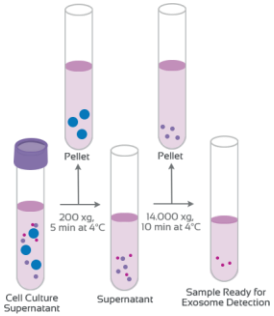

Figure 3: Cell Culture Supernatant pretreatment workflow for direct exosome detection.

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.

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 50µL of sample previously prepared according to "Sample Preparation" to the appropriate tubes. Mix the reactions gently by pippeting up and down several times with a pipette and vortexing for few seconds.
5. 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.
6. Incubate in the dark overnight at room temperature (RT). NO STIRRING.
7. After overnight incubation wash the sample (bead-bound exosomes) by adding 1ml of Assay Buffer 1X.
8. 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**

7. Add 5ul of the primary detection 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.
8. Incubate in the dark 60 minutes at 2-8ºC, without stirring.
9. Wash the sample (bead-bound exosomes) by adding 1ml of Assay Buffer 1X.
10. 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
11. Resuspend the sample in 350µL Assay Buffer 1X 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.

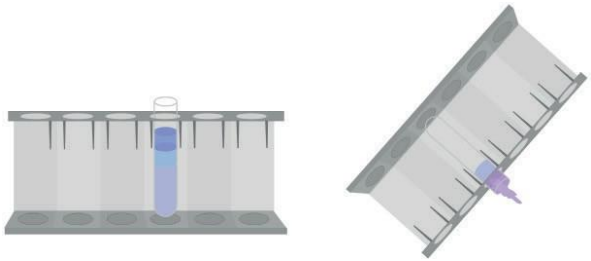

Figure 4: Hand-decanting supernatant using a Magnetic Rack.

11. ASSAY ADQUISITION

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 discrimination of doublets on flow 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 PerCP vs. APC channel (bead auto fluorescence) in logarithmic scale (Fig. 5B)

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.

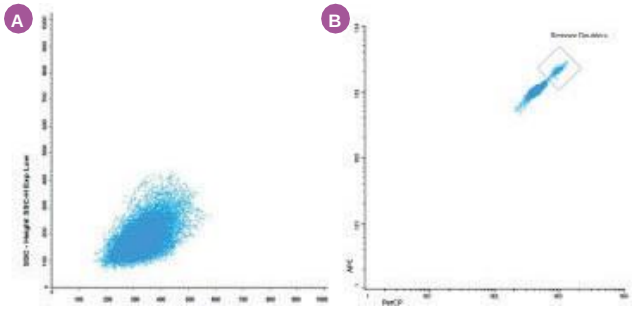

Figure 5: Dot-plot gating strategy for acquisition and analysis. FSC vs SSC and A PerCP vs APC B

12. FLOW CYTOMETRY ANALYSIS

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

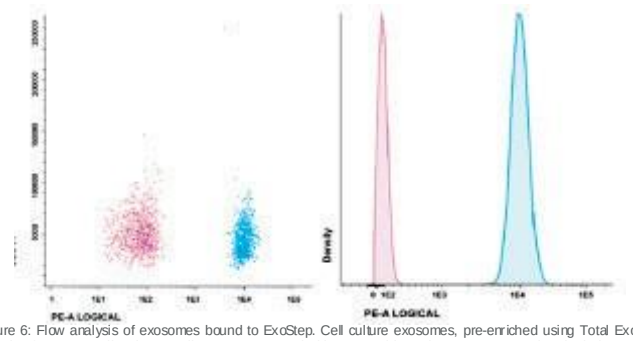

Figure 6: Flow analysis of exosomes bound to ExoStep. Cell culture exosomes, pre-enriched using Total Exosome Isolation from PC3 Cell Culture Media, were resuspended in PBS and bound to CD63-capture beads during an overnight incubation. The following day the bead-bound exosomes were direct stained with primary detection antibody (CD9 -PE) and analyzed by Flow Cytometry.

13. REPRODUCIBILITY

- Sample: PC3-derived exosomes.
- Batch: 3 different batches.
- Cytometer: Acquired every day in 2 different cytometers.
- Replicate: 4 replicates assayed for 3 days not necessarily consecutive.
- Protocol: Bead-Based Flow Cytometric Assays.

Intra assay: It was determined calculating the deviation and the CV for each of the samples by batch. Was analyzed the mean of all typical deviations and CVs of 3 days for each lot. Finally, was obtained the mean of the standard deviation and the CV of the three lots.

Inter assay: It was determined the mean of the 4 repetitions for each day and compare them between each batch taking the standard deviation and the CV. Was calculated the mean deviation thus obtained and the CV of the three days.

	CV (%)
Intra Assay	10%
Inter Assay	11%

14. 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 >0,125 µg which corresponds with >1.25 * 10^9 vesicles. Whilst the upper limit or saturation level was established in 10µg. For both technical specifications were used exosome form PC3 cell culture media (1*106 vesicles/µl). (Fig. 7).

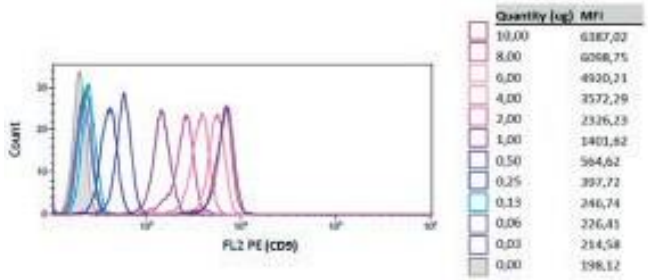

Figure 7: Dynamic range of the assay analyzed by flow cytometry. Relationship between background noise and specific signal at different exosome concentrations.

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 www.immunostep.com.

* **PE (Ex-Max 496 nm/Em-Max 578 nm); Excitation laser line 488 nm**
* **FITC (Ex-Max 494 nm/Em-Max 519 nm); Excitation laser line 488 nm**
* **CF-Blue (Ex-Max 401 nm/Em-Max 452 nm); Excitation laser line 405 nm**

15. REFERENCES

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