Exosome detection and characterization based on flow cytometry.

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BACKGROUND

Exosomes are small (~50-150 nm) extracellular vesicles (EVs) released from all cells and found in body fluids and cell culture supernatant. Exosomes are generated by fusion of a specialized endosome, the multivesicular body (MVB) (1), with the plasma membrane.

It is thought that exosomes provide an intercellular communication way of macromolecules between cells, allowing the spread of proteins, lipids, mRNA, miRNA and DNA as contributing factors to important functions in different biological processes, including apoptosis, antigen presentation, angiogenesis, inflammation, and coagulation; playing an important role in the development of several diseases, and specifically, modulating cancer microenvironment and the immune response (2,3).

In addition, exosomes have recently emerged as a new source of potential biomarkers for various diseases, since they can be easily obtained from body fluids such as urine, blood, saliva or breastmilk and their composition may be directly dependent on the physiological and/or pathological state of the patient. Even the number of secreted exosomes can change with the onset of different pathologies, so the detection of quantity variations could be of great relevance for diagnosis, especially in patients with cancer.

Isolation and characterization of exosomes from body fluids can provide very valuable information for early detection, disease monitoring and development of effective treatments against cancer, and autoimmune diseases, among others. Moreover, a deeper knowledge of the exosome biology can also accelerate the use of these extracellular vesicles in fields such as regenerative medicine, vaccines and monoclonal antibodies, where they could play an important role as delivery systems, helping to increase the effectiveness of the treatments.

Research in exosomes as a potential source of biomarkers of human diseases has grown rapidly in recent years and consequently there is a large number of techniques for the isolation and characterization of this type of EVs. However, many of the current techniques are poorly standardized. Furthermore, the use of exosomes in diagnostic tests or clinical research, requires a sensitive, reproducible and high performance method for detection, characterization and quantification of exosome sample.

LIMITATIONS OF CURRENT METHODS AND UNMET NEEDS

Nowadays, most researchers (87%) process between 1 to 50 samples per month while only a 4% process more than 100 samples per month. On the other hand, 71% of these same researchers process between 5 - 100 ml of starting sample volume, while the other 29% work with sample volumes <5 ml. In fact, 100% of researchers who work with biological fluids process sample volumes lower than 1 ml, except for those who work with urine. Within the biological fluids analyzed, plasma is the most frequently used, followed by serum, urine and cerebrospinal fluid, although there are also researchers working with saliva or milk among others (4).

The initial sample volume has a considerable effect on the isolation and detection techniques used. In this sense, ultracentrifugation remains by far the most widely used primary isolation method for all applications, while the Western Blot (WB), the Nanoparticle Tracking Analysis (NTA) (5) and electron microscopy, in this order, are the techniques for the analysis and characterization of exosomes. None of these detection techniques meets the premise of being reproducible and offering high performance potential, for diagnosis or clinical research application.

In contrast, flow cytometry is a technique well adapted to the reproducible analysis of clinical samples, allowing the analysis of different physical and chemical characteristics of cells and particles in suspension, which are passed through a beam of light (laser). However, conventional flow cytometers do not allow the detection of particles <300 nm based on forward scattered light (FSC), and therefore do not allow the direct detection of exosomes (6).

In this scenario, most researchers usually use more than one method for the characterization of exosomes in their experiments. Specifically, the quantification and size

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analysis of the vesicles is done by NTA and dynamic light scattering (DLS), although these techniques are limited to the definition of objectively define the size range and concentration of the exosomes without contributing much to phenotype information. Likewise, to determine the protein composition, most researchers perform WB technique, despite being a laborious and non-quantitative method that sometimes yields poor results, due to the quality of the antibodies used and/or to low exosome abundance in the sample.

In summary, the diagnostic use of the detection and characterization of circulating nanovesicles derived from pathological cells has been technically limited by the lack of methods to measure and characterize exosomes.

APPLICATION FIELD

In order to overcome some of the above limitations, here, we describe a specific, sensitive and easily scalable method and kit for exosome detection and characterization from supernatants of cell cultures and biological fluids by CMFflow cytometry.

This method is based on the use of magnetic beads coated with antibodies against tetraspanins, as CD63, a common marker used for exosome characterization, which allows exosome detection in conventional cytometers. This same approach is also used by some kits already marketed by competitors, but the sensitivity of these assays is very limited.

Sensitivity and reproducibility of the kit is the result of the detailed study of certain critical factors, such as the volume and concentration of beads and samples, incubation times and conditions, as well as the concentration of the reagents, which was carried out during its development.

Kit description

Specifically, the kit is composed of an exosome capture reagent consisting of 6 micron size magnetic beads coated with anti-human CD63 antibody, as a solid support detectable by the cytometer, which can be recognized by the FSC (Forward Scatter) and SSC (Side Scatter) cytometry parameters (Fig. 1 A). On the other hand, the kit also contains a biotin anti-human CD9 antibody, which together with streptavidin conjugated in phycoerythrin (PE) conform the detection reagent, allowing the detection of exosomes located on the surface of the beads by the FL2 (PE) (585/40) detector fluorescent channel of the cytometer.

Additionally, the capture beads are also detectable by the fluorescent detectors FL3 (PerCP) (670 LP) and FL4 (APC) (675/25) of a conventional cytometer, helping to identify bead population and to remove doublets during analysis (Fig. 1B).

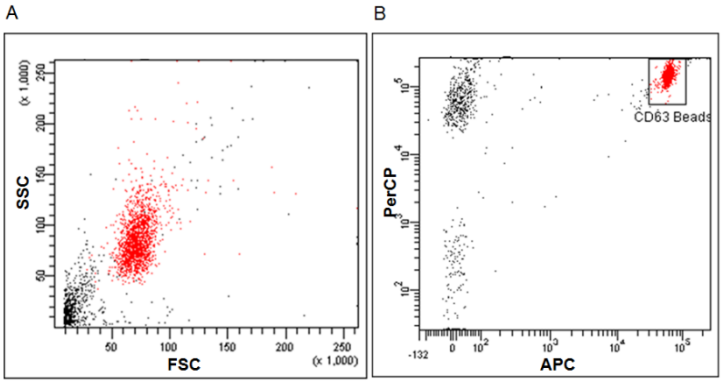
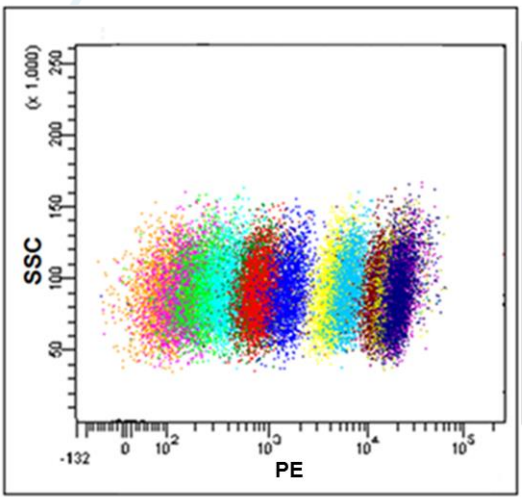
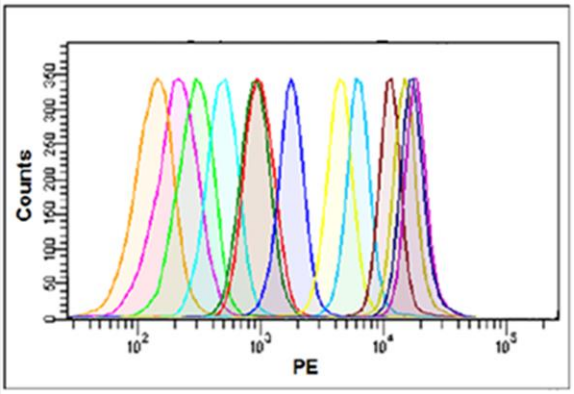


Figure 1.- Flow cytometric analysis of capture beads. (A) Scatter Dot-plot of beads, FSC vs SSC. (B) Gating strategy on FL3 vs FL4 for flow cytometry acquisition and analysis, in order to remove doublets. Low-speed Flow Cytometry acquisition is recommended.

Finally, the kit is supplied with a couple of buffers, one of them for washing steps and another one for biological fluids pretreatment before direct exosomes assays. (Fig. 2).

Figure 2. ExosStep Kit components. The reagents supplied: CD63+ (Clone TEA3/18) capture beads. Polystyrene micro-particles with Mean Diameter (µm) 5.5±0.2 (CV<5%), having discrete fluorescence intensity characteristics; Primary detection antibody, Anti-CD9 biotin (Clone VJ1/20); Secondary detection reagent, Streptavidin-Phycoerythrin (PE) for detecting biotinylated antibodies. Excitation of PE by 488 nm laser light induces a light emission maximum of 575 nm; Assay Buffer 10X, PBS 10% BSA, pH 7,4 for washing steps and Pretreatment Buffer, for direct detection on plasma/serum (HBS-BSA 2%) or urine samples (1 M DTT) .





Sensitivity

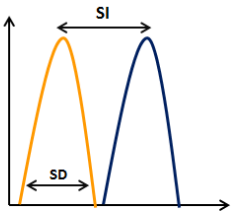
µg	MFI	SD*
0,0000	8,74	4,06
0,0625	14,20	8,49
0,1250	20,91	14,19
0,2500	36,85	52,61
0,5000	74,32	32,18
1,0000	79,86	82,30
2,0000	158,20	137,14
4,0000	380,00	248,41
8,0000	661,17	257,49
16,0000	1286,40	342,05
32,0000	1746,58	519,01
64,0000	2206,73	537,59
128,0000	2035,14	593,98

* Standard Desviation.

Figure 3. Flow cytometric analysis. (A) Scatter Dot-plot and histogram (B) of exosomes captured with anti-human CD63 antibody (Clone TEA3/18) coated beads and detected with biotin anti-human CD9 antibody (Clone VJ1/20) stained with Streptavidin-PE. Each population (dot plot) or peak (histogram) corresponds to a different exosome quantity ranging from 0 µg (orange peak), to 128 µg (violet peak), serially diluted in PBS and incubated overnight with capture beads. Next day exosome captured were stained using the CD9 biotin + Streptavidin-PE, and analyzed by Flow cytometry. (C) We show Mean Fluorescence Intensity (MFI) and Standard Deviation (SD) data related with every exosome quantity point. Exosomes of the human prostate cancer cell lines PC3 were isolated, from cell culture supernatant recovered and centrifuged at 400 g for 5 minutes and at 2,000 g for 10 minutes to remove cells and cell debris. Supernatants were centrifuged again at 17,000 g for 20 minutes and then at 100,000 g for 2 h. The 100,000 g pellet was then washed with PBS and centrifuged again for 2 h at 100,000 g. Exosome were suspended and concentrated in PBS, at 12 * 10^8 vesicles / µl analyzed by NTA.

Method Sensitivity

In order to evaluate the efficiency of the kit, we perform a series of assays. Firstly, the method dynamic range was performed, for which we analyzed different exosome concentrations from same type of sample. Initially, exosomes from the culture supernatant of the human prostate cancer cell lines PC3, isolated by ultracentrifugation and filtration were used. The assay was performed incubating increasing dilutions of exosome sample with capture beads and detection probe, keeping the reagents at constant concentrations. The objective of the analysis was to establish the limit of detection (LOD), defined as the smallest amount of exosomes that can be reliably measured. The result was 0.0625 µg, which in this case corresponded with $7.5 \cdot 10^7$ vesicles. The limit of quantification (LOQ) was also determined, which is defined as the lowest concentration at which exosomes can be reliably detected with a Stain Index (SI) > 1, where SI is a measure of flow cytometry assay sensitivity, defined as the ability to detect differences between stained and unstained bead populations , helping to normalize the results independently of the relative intensity for a fluorochrome on a given instrument (7). LOQ was estimated in 0.125 µg.



Stain Index = (Median of Positive - Median of Negative) / (SD of Negative * 2)

Finally, the fluorescence intensity (MFI) reached its highest value when 64 µg of exosomes were used, and higher amounts saturate the test. This was confirmed by the fact that 128 µg produced a slight decrease of MFI , which is due to an excess of antigen, when both the capture and detection antibodies are saturated by the high concentration of analyte, also called Hook effect.

Quantitative analysis of exosome samples.

In addition to the sensitive detection, because of the good results, we proceeded to evaluate if there was a linear correlation between MFI and exosome concentration, which would allow the use of the kit, in combination with a standard, as a quantification method of exosomes. This would be extremely useful, because most of the current experimental approaches for the exosome quantification are time-consuming, require

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specialized instrumentation and are quite inaccurate. In this sense, the analysis revealed a good linear behavior, with very high r2 values, for a wide range of samples of purified exosomes, allowing an accurate and reproducible estimation of the exosomes concentration by flow cytometry, thanks to the kit (Fig 4).

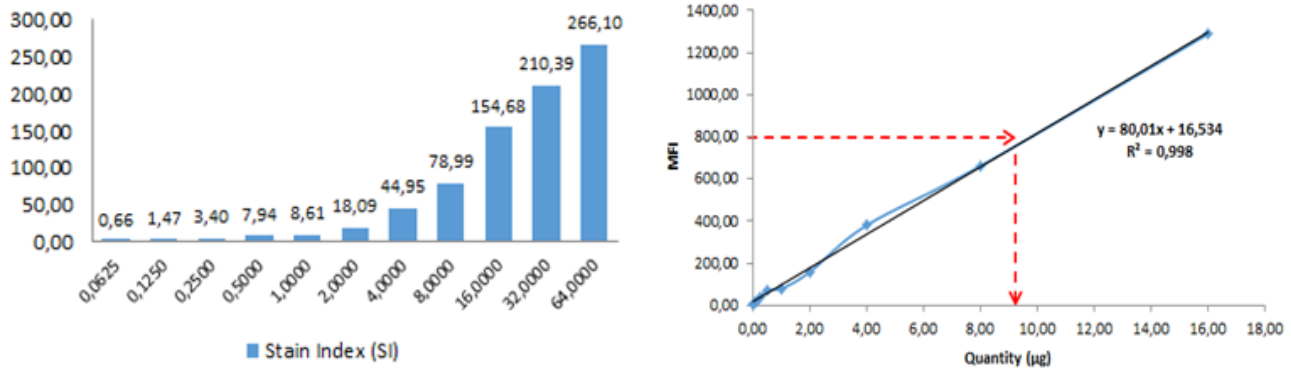

Figure 4. Sensitivity and linearity analysis. (A) Flow cytometry analysis of sensitivity (Stain Index) of different quantities (0,0625 to 64 µg) of exosomes against the negative control (0 µg) . (B) Correlation between exosome quantity and CD9 MFI. Exosome quantity was plotted against MFI, resulting in a linearity correlation between 0 -16 µg. R^2=0,99. Exosomes isolated from cell culture supernatant of the human prostate cancer cell lines PC3 were used.

Comparative sensitivity of the kit versus WB

Then, keeping in mind the previous results, we wanted to compare the sensitivity of the method against WB, as the most common method of detection. In this way, we analyzed the same exosome amounts from the culture supernatant of the PC3 cell line, from the same batch used for assay. The results show that the LOD is around 2 µg for WB (Fig. 5).

The results show that the flow cytometry detection capacity of our kit, it is much greater than that of the WB, in particular 16 times higher, allowing the use of smaller quantity of sample for analysis, that is especially relevant in clinical settings.

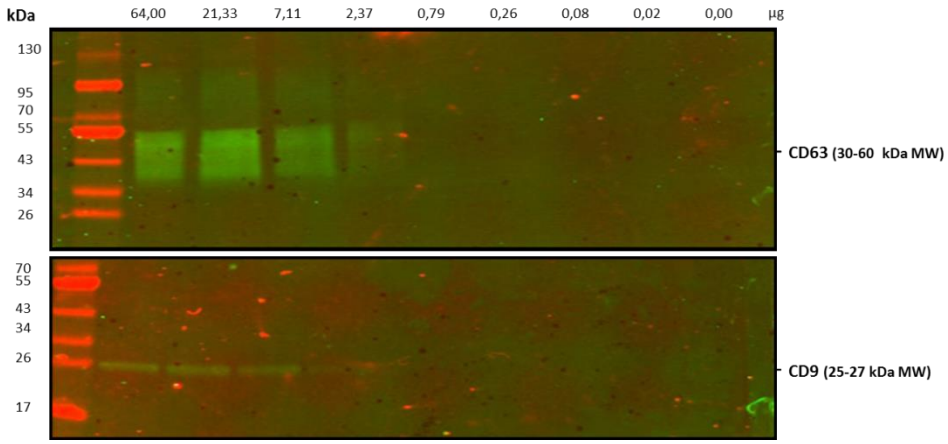

Figure 5. Western blot (WB). Sensitivity analysis by native conditions WB on serial diluted (0-64 ug) exosomes isolated from cell culture supernatant of the human prostate cancer cell line PC3. Primary antibodies: Anti-human CD9 (Clone VJ1/20) (bottom) and anti- human CD63 (Clone TEA3/18) (top), at 1:1000 dilution from a concentration of 0,2 mg/ml. Conjugated secondary antibody: Dylight 800 anti-mouse IgG.

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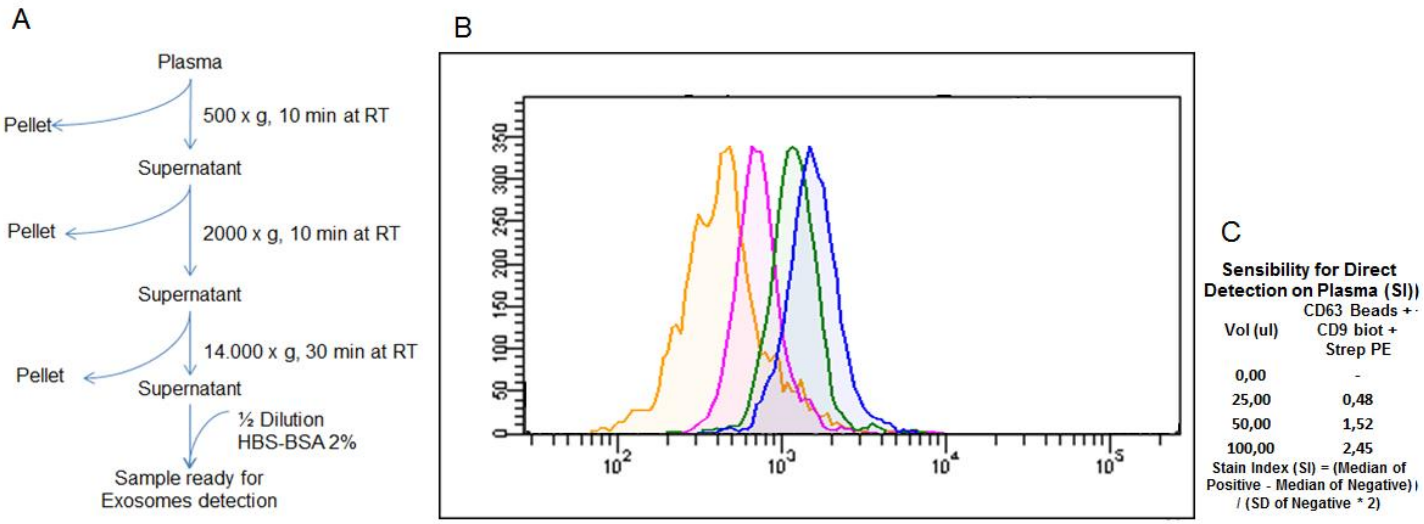

Figure 6: Direct detection of exosomes by flow cytometry. (A) Plasma pretreatment workflow previous tor direct exosome detection. (B) Histogram of exosomes detected on normal plasma, each peak corresponds to different plasma volume (0 µl, 25 µl, 50 µl and 100 µl). (C) Flow cytometry data analysis of sensitivity (Stain Index) for each plasma volume tested.

Direct exosome detection in biological fluids

As mentioned previously, exosomes are becoming important biomarkers for many pathologies, and because of the high sensitivity achieved by the kit , we wanted to find out if it would be possible direct exosome detection in body fluids, without the need to purify or concentrate the fluid, which would provide a fast and easily scalable protocol. For this purpose, several samples of pretreated plasma were analyzed using flow cytometry (Fig. 6). During the study, increasing plasma volumes were used, while the amount of reagents was kept constant, according to the kit specifications. The data show that the kit enables the direct detection of exosomes in plasma from 25 ul of sample, without the need to precipitate or purify the exosomes, simplifying the detection of exosomes in body fluids and contributing to the potential use of these type of EVs in the diagnosis of diseases.

Analysis of exosome markers in isolated exosome samples: multiplexed phenotyping.

Considering exosome direct detection results in body fluids, we want to anticipate researcher needs for exosome subpopulations characterization through specific markers. In order to optimize the protocol, we performed an immunophenotyping assay simultaneously with the exosome detection. For this analysis, exosomes purified by ultracentrifugation and filtration, from the culture supernatant of the PC3 cell line, were also used (8). On the other hand, in relation to the reagents used, as in previous experiments, capture beads coated with anti-human CD63 antibody were used. While for exosome detection the probe was moved to FL1 channel, using biotin anti-human CD9 antibody, combined with Streptavidin FITC. This protocol optimization, made possible to include in the FL2 channel (the most sensitive), the antibody against the marker of interest, which in this particular case was PE anti-human CD81 antibody (Clone MEM38). The suitability of the kit for the specific detection of exosomes was confirmed. Further it was possible to characterize their phenotype, by labeling with other markers of interest. The results show the simultaneous expression of CD81 and CD9 on the population of exosomes captured by the CD63-coated beads, with high sensitivity detection in a wide range of quantities (Fig. 7).

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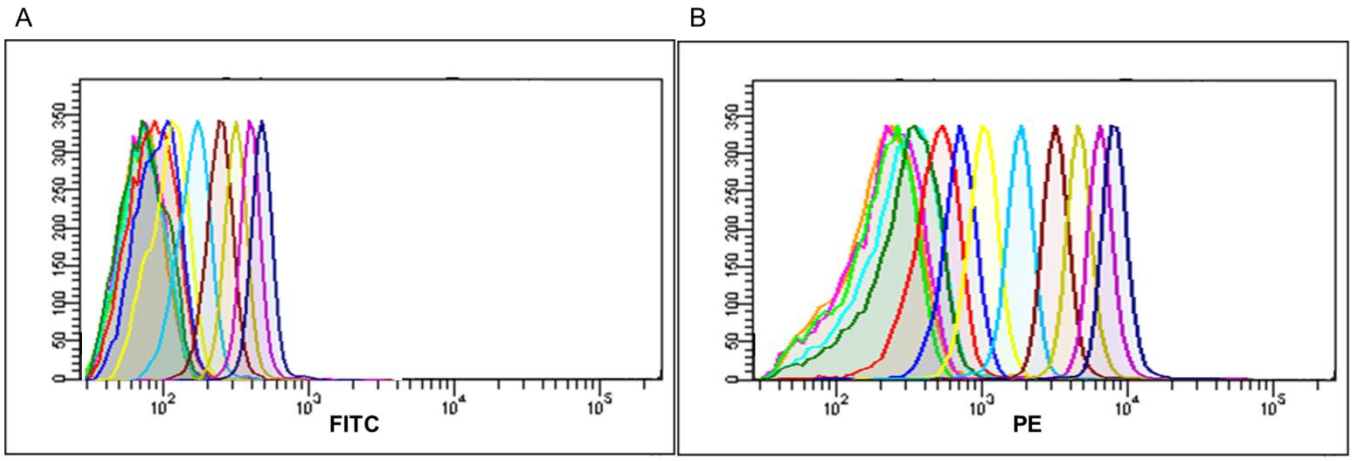

C

Immunophenotyping (SI*)		Sensitivity
µg	CD9-FITC	CD81- biot + Strep PE
0,0000	-	-
0,0625	-0,24	0,02
0,1250	-0,24	-0,04
0,2500	-0,30	0,30
0,5000	-0,24	0,45
1,0000	0,12	1,30
2,0000	0,24	2,46
4,0000	0,73	4,39
8,0000	2,06	9,65
16,0000	4,30	20,46
32,0000	6,54	33,62
64,0000	9,56	51,72
128,0000	13,77	69,17

*Stain Index (SI) = (Median of Positive - Median of Negative) / (SD of Negative * 2)

Figure 7. Simultaneous CD81 and CD9 immunophenotyping of the population of exosomes captured on the surface of the CD63-beads. Fluorescence histogram corresponding to exosomes captured on CD63 beads surface, and simultaneous stained with (A) CD9-FITC (Clone VJ1/20) and (B) CD81-PE (Clone MEM38) respectively. Each peak histogram corresponds to a different exosome quantity ranging from 0 µg (orange peak), to 128 µg (dark blue peak), serially diluted in PBS. (C) Stain Index (SI) values obtained by flow cytometry analysis on simultaneous exosome labeling for each quantity and marker.

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Sensitivity compared of the kit versus others available in the market.

Finally, we proceeded to compare the results of our kit against competitors' kits. For this, up to three different kits, from different manufacturers (ThermoFisher, System Biosciences and Hansa Biomed) were evaluated in parallel and on the same sample. All of them based on the same principle of the use of beads as a solid support detectable by the cytometer, but with different approaches for the capture and subsequent detection. Thus, regarding Thermo Fisher, the "Exosome- Human CD63 Isolation / Detection kit" was used, consisting of 6 micron size magnetic anti-human CD63 coated beads, for the exosome captured. In this specific case as a detection reagent our CD9 biotin + Streptavidin-PE was used. On the other hand, in the case of System Biosciences, the "Exo-FLOW Exosome Purification Kit" was tested. It is a kit designed in principle for the isolation of exosomes and its subsequent verification to the flow cytometer. This kit contains 9.1 micron size magnetic beads coated with streptavidin, which together with a biotinylated anti-human CD9 antibody, form the capture reagent. For detection, the kit uses a reagent called Exo-FITC, whose principle is based on the detection of glycosylated proteins on the surface of the exosome, so it is worth to highlight it is not a specific marker for exosomes. Finally, concerning to Hansa Biomed, we tested the kit called "Exo-FACS, Exosome marker identification via FACS analysis", consisting on 4 micron size Aldeheyde-Sulfate latex beads, for the non-specific exosome capture, which in combination with a Purified anti-human CD63 primary antibody, and an Alexa 488 anti-mouse IgG secondary antibody, forms the detection reagents. In this case, it is also worth to emphasize that latex beads can capture other things besides exosomes, since the union has a hydrophobic character, providing a strong physical adsorption, thus it is a non-specific binding. Manufacturer's protocols and exosomes previously isolated by ultracentrifugation from culture supernatant of the PC3 line were used for kits comparison. In relation with the protocols, some differences were observed, such as the fact that, only Immunostep kit does not require stirring incubation or that in the case of Hansa Biomed kit, the washing steps can only be carried out by centrifugation, because the beads are not magnetic. In summary, simplest and best adapted protocol corresponds to our kit.

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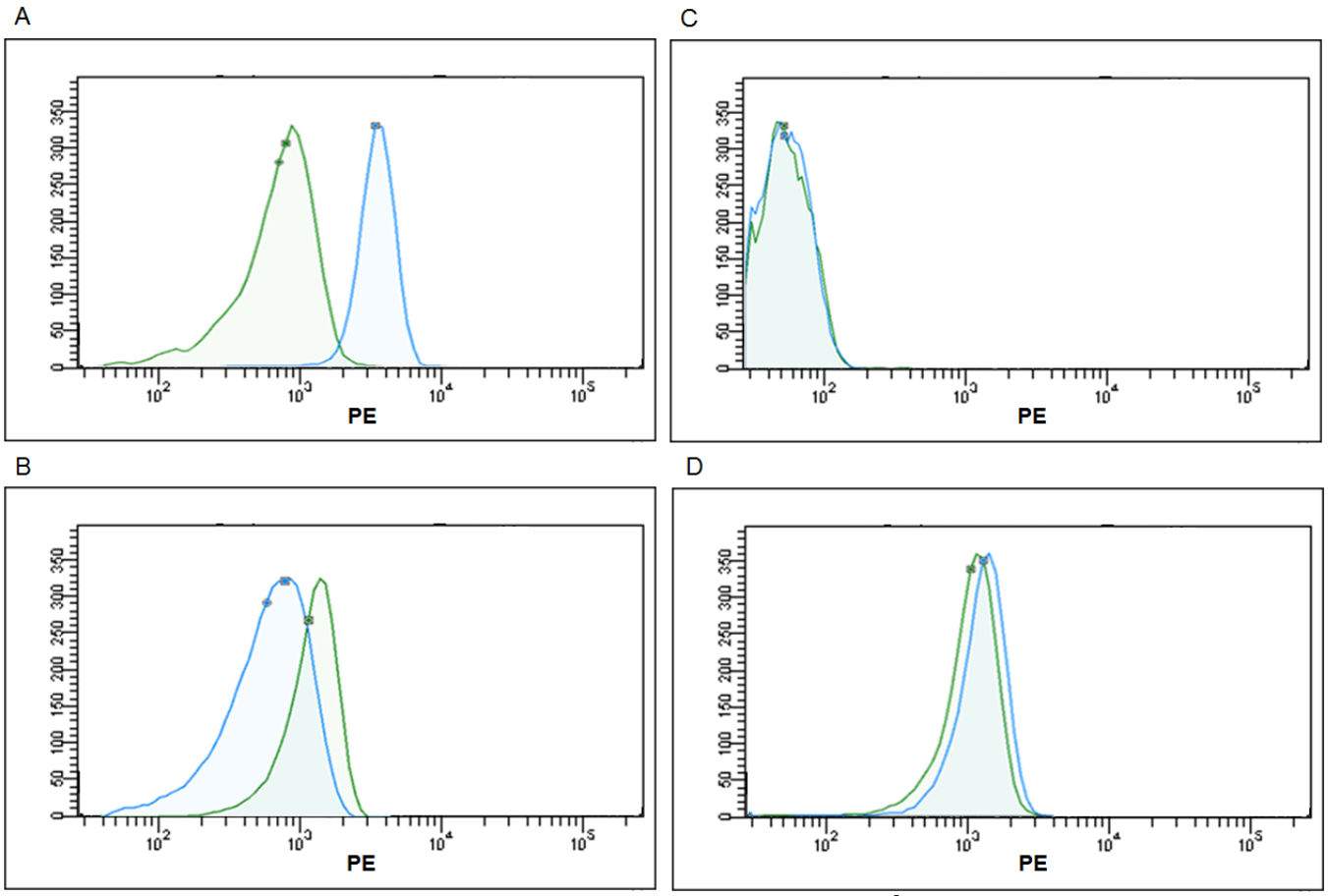

Figure 8. Representative histograms for detection of same exosome quantity (2µg - 2,40x10^9) with most popular flow cytometry kits. Each kit was utilized according their technical instructions; result in different signal-to noise ratios. (A) high brightness from Immunostep, (B) medium brightness from Thermo Fisher, (C) low brightness from SBI and (D) no signal from HMB.

According to the data resulting from the comparison test, we found that the Immunostep kit show higher MFI and sensitivity (Fig 5). Specifically, it is up to eight times more sensitive than the second most sensitive (Thermo Fisher)(Fig. 6). In the same way, neither the Hansa Biomed kit nor the System Bisociences kit are able to distinguish among the different exosome quantities tested, because potentially LOD would be much lower. Additionally, although the data are not shown here, of the four kits tested, Immunostep also has a lower background, making the analysis easy and intuitive.

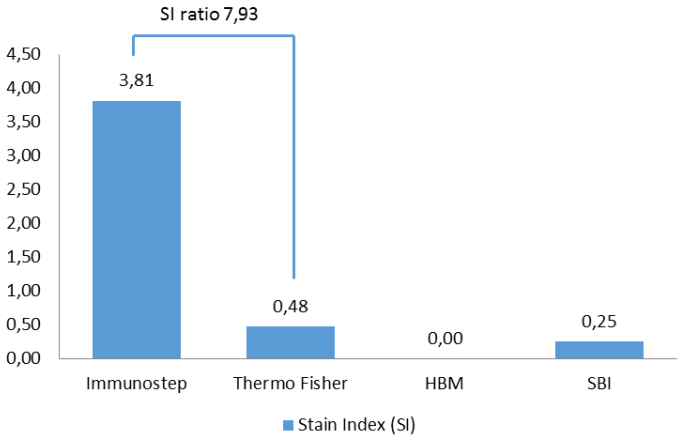

Figure 9. Sensitivity comparison. Data correspond to Stain Index (SI) values obtained by flow cytometry analysis from the 4 different kits on same sample.

ADVANTAGES OF IMMUNOSTEP KIT

Here, we have described a reliable method associated with a kit for the sensitive detection of exosomes by

FCM, which in contrast to other methods, also enable quantification, simultaneous characterization and direct detection of exosomes, all using a small sample quantity, overcoming many of the limitations of current methods. In addition, comparing the kit with other methods available in the market, we found that the kit described here presents a better sensitivity and accuracy than some of the other most popular kits intended for similar applications.

Therefore, the proposed kit is a superior alternative for the sensitive detection of exosomes compared to the most commonly used methods, besides being easy to implement and analyze for any laboratory that has access to a conventional flow cytometer.

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