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Comparison of six commercial serum exosome isolation methods suitable for clinical laboratories. Effect in cytokine analysis

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Abstract

Background: Exosomes are nanovesicles released by cells that can be detected in blood. Exosomes contain several molecules, such as cytokines that have potential utility as disease biomarkers. The aim of the present work is to compare six different commercial kits suitable for the clinical laboratory in relation to the efficiency and purity of exosome isolation, and their effect in subsequent cytokines analysis.

Methods: Serum exosomes were obtained from 10 volunteers using six commercial kits: exoEasy, ExoQuick, Exo-spin, ME kit, ExoQuick Plus and Exo-Flow. Exosome concentrations and size distributions were quantified by nanoparticle tracking analysis. Exosome markers CD63, CD9 and TSG101 were determined by Western blot. ApoB and albumin were measured using nephelometry. S100A9, CXCL5 and CXCL12 were measured using a Luminex assay.

Results: The concentration of particles obtained between different kits varied by a factor of 100. There was no correlation in particle concentrations extracted between different kits, except between ExoQuick and Exo-Flow.

The highest exosome purity was achieved with ExoQuick Plus and exoEasy, while the lowest were achieved with ME and ExoQuick. Albumin was present in all exosome extracts analyzed and ApoB in all except those extracted with Exo-Flow and ME. Cytokine detection varied depending on the purification kit used and there was no correlation in cytokine concentrations between samples obtained with different kits.

Conclusions: Both the sample and the type of commercial kit used affect the efficiency and purity of exosome isolation. In addition, the exosome purification method deeply affects the capability to detect and quantify cytokines.

Keywords: CXCL5; CXCL12; cytokines; exosomes; purification; S100A9.

Introduction

Exosomes are small microvesicles of 50–200 nm with endosomal origin, produced by most cells, and are actively released by fusion of the microvesicular bodies with the plasma membrane [1]. These exosomes carry different molecules, such as nucleic acids, including DNA, mRNAs, and miRNAs and proteins [2]. Some of these proteins have been used for exosome identification or purification, especially the tetraspanin proteins CD63, CD9 and CD81 or the component of the ESCRT-I complex TSG101 [2].

Exosomes have been associated with cell-to-cell communication as a system for molecules interchange between exosome-producing cells and target cells [3]. These exosomes also carry inflammatory mediators, such as S100A9, that can be involved in different pathologies [4, 5]. Particularly in cancer, exosomes can take part in the modification of the tumor microenvironment, favoring tumor progression and metastasis [3, 6]. Additionally, some exosome cargo molecules can be biomarkers in multiple diseases, including cancer [7–9], multiple sclerosis [10] and graft rejection [11, 12].

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As potential biomarkers in diseases, researchers have analyzed exosomes isolated from many different biological fluids, including blood [13], urine [14] and others [4]. Traditionally, exosomes have been purified using ultracentrifugation-based methods [15]. However, ultracentrifugation is very laborious and employs equipment usually not available in a clinical laboratory setting. In addition, ultracentrifugation purifies exosomes with low efficiency and purity [16]. Other alternative manual methods have been proposed such as size exclusion chromatography [15], with lower turnaround times and more purified exosome fraction. However, this method has great technician-dependent variability and the purified exosome fraction is small and diluted [17].

Exosomes isolation from serum or plasma is especially difficult because of the small volume usually available, its high viscosity, the high concentration of proteins and the presence of other particles, mainly lipoproteins that have a diameter in the range of exosomes [18]. In recent years different commercial isolation kits have been developed to rapidly and easily obtain exosomes from small serum volumes [17, 19–21]. In addition, these isolation kits should be capable of reducing the exosomes' co-isolation with other particles and protein aggregates in order to consistently identify exosome biomarkers [16].

Some papers have compared some of these exosome isolation kits in relation to their effect in the subsequent mRNA [16, 22] and proteomic [23] analysis. However, no study has been performed in relation to their effect in proteins that are in very low concentration, such as cytokines [24]. For this reason, the aim of the present work was to compare six different commercial kits as regards to the efficiency of exosome isolation, the purity of isolated exosomes and their effect in cytokine analysis. We studied the effect of exosome isolation in the analysis of low molecular weight cytokines S100A9, CXCL5 and CXCL12, which were detected in these exosomes [5, 25, 26], as described in the exosomal studies database Exocarta (www.exocarta.org), and whose concentration are in

the range of $\mu\text{g/L}$, about 10^6 lower than the total protein content in serum.

Materials and methods

Blood

Samples were collected from 10 healthy donors. Blood was drawn into 10 mL-serum tubes and centrifuged at 3500 g at room temperature. A second high-speed centrifugation at 16,060 g was performed and samples were subsequently aliquoted and stored at -80°C until further analysis. Due to the limitation of blood volume, some experiments could not be performed with samples from all the volunteers. The protocol was approved by the local Ethics Committee and all participants signed an informed consent.

Exosome isolation

Serum samples were filtered through a 0.45 μm syringe filter (Teknokroma, Spain) before applying into the commercial exosomes isolation kits. For each sample, six different kits were used: exoEasy (Qiagen, Venlo, The Netherlands), a membrane-affinity-based method [19], ExoQuick (System Biosciences, Palo Alto, CA, USA), a polymer-based precipitation method [20], Exo-spin (Cell Guidance System, Cambridge, UK), a column-based chromatographic method [17], ME kit (New England Peptide, Gardner, MA, USA), a peptide binding method [21], and two kits, ExoQuick Plus and Exo-Flow, in which polymer precipitation (ExoQuick) is followed by an extra-step with immunoaffinity capture beads. In the case of Exo-Flow a positive selection was performed with beads coated with anti-CD63 antibody, whereas in the case of ExoQuick Plus there was a negative selection employing beads to reduce protein carry-over. All isolation procedures were performed according to manufacturer's instructions and the characteristics of each one are detailed in Table 1.

Nanoparticle tracking analysis

Particles size and concentration were measured with a NanoSight LM20 (NanoSight, Malvern, UK). Concentrations were reported in particles/L (P/L), and adjusted to 1 L of serum.

Table 1: Characteristics of each isolation procedure.

Method	Principle	Serum volume, μL	Time, min	Complexity
ExoQuick	Precipitation (polymer)	250	65	Easy
+ ExoQuick Plus	+ Immunoaffinity (beads)		97	Easy
+ Exo-Flow	Negative selection + Immunoaffinity (beads) Positive selection		199 + Overnight	More complex
Exospin	Size exclusion chromatography	100–500	152	Easy
exoEasy	Membrane-affinity	200–4000	18	Easy
ME Kit	Precipitation (peptides)	1000	75–165	Easy

Protein concentration

Protein concentration was quantified in the exosome purified solution by NanoDrop® ND-1000 (Thermo Scientific). Concentrations were adjusted to 1 L of serum sample. In some samples, protein concentration was also analyzed with Bradford assay kit (BioRad Laboratories, Hercules, CA, USA).

Gel electrophoresis and Western blot

Isolated exosomes (20 µg) were boiled in SDS-containing loading buffer (Life Technologies, Paisley, UK) with 20 mM dithiothreitol, and subjected to electrophoresis on Mini-PROTEAN TGX precast 10% gels (BioRad). In some experiments, proteins in gels were stained with Coomassie Blue following the supplier's instructions (Bio-Rad).

For Western blot, proteins were transferred to polyvinylidene difluoride membranes, then blocked in TBS containing 0.5% (w/v) Tween-20 and 3% (w/v) skimmed milk, then incubated overnight with the corresponding antibodies: mouse monoclonal for CD63 (clone MX-49.129.51:200 dilution, Santa Cruz Technology, Dallas, TX, USA), CD9 (clone C-4 1:200 dilution, Santa Cruz Technology) and TSG101 (clone C-2 1:200 dilution, Santa Cruz Technology), and rabbit polyclonal against Argonaute 2 (Ago2) (1:500 dilution, Abcam, Cambridge, UK) and Calnexin (1:1250 dilution, Abcam). After washing, immunoblot analysis was performed with horseradish peroxidase conjugated antibodies (1:5000; Amersham Biosciences, Amersham, UK) and developed with the ECL kit (Amersham Biosciences).

ApoB quantification

Levels of apoB were determined by nephelometry using a commercial kit (Beckman Coulter, Nyon, Switzerland) in an Image 800 analyzer (Beckman Coulter).

Albumin quantification

Albumin was also measured by nephelometry using a commercial kit (Beckman Coulter) in an Image 800 analyzer (Beckman Coulter).

Cytokine quantification

The cytokines S100A9, CXCL5 and CXCL12 were measured in purified exosomes using a Human Magnetic Luminex Assay (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Fluorescence intensities were measured in a Luminex 200 system (Luminex xMAP Technology, USA) and cytokine concentrations were obtained from a comparison with the corresponding calibration curves. The detection limits were: 6.39 ng/L for S100A9, 8.2 ng/L for CXCL5 and 17 ng/L for CXCL12.

Statistical analysis

Data were expressed as median and interquartile range (Q1–Q3). Non-parametric statistical analysis was performed using Graph-Pad Prism version 6.07 (La Jolla, CA, USA). For comparisons, the Kruskal-Wallis test was used followed by Dunn's multiple comparison test, while for correlations Spearman's test was used. A two-tailed p-value of <0.05 was considered to be statistically significant.

Results

Characterization of isolated exosomes

The median size of the isolated particles ranged between 130 nm (Q1–Q3: 122–186 nm) for those obtained with ExoQuick and 154 nm (Q1–Q3: 140–188 nm) for those obtained with exoEasy. There were no differences in the size of the particles obtained using different kits (Figure 1A). Western blot analysis used antibodies against the characteristic exosome protein markers TSG101, CD63 and CD9, and showed the bands at the corresponding molecular weights (Figure 1B). Based on these results, we could conclude that the serum extracts obtained with the six kits were enriched in exosomes.

The concentration of particles obtained between different kits varied by a factor of 100. The median concentration of particles obtained with ExoQuick (median: 44×10^{13} P/L; Q1–Q3: $29\text{--}91 \times 10^{13}$ P/L), ExoQuick Plus (median: 59×10^{13} P/L; Q1–Q3: $28\text{--}148 \times 10^{13}$ P/L) and Exo-spin (median: 64×10^{13} P/L; Q1–Q3: $23\text{--}102 \times 10^{13}$ P/L) were similar (Figure 2A). exoEasy yielded less concentration of particles (median: 18×10^{13} P/L; Q1–Q3: $8\text{--}64 \times 10^{13}$ P/L), although the difference with previous kits did not reach significance. Also, exosome purification with Exo-Flow was significantly less efficient than with Exo-spin, ExoQuick and ExoQuick Plus (median: 10×10^{13} P/L; Q1–Q3: $5\text{--}14 \times 10^{13}$ P/L; $p < 0.05$). The lowest concentration of particles was observed when exosomes were purified with ME kit (median: 0.13×10^{13} P/L; Q1–Q3: $0.06\text{--}0.18 \times 10^{13}$ P/L; $p < 0.01$ related to Exo-spin, ExoQuick, ExoQuick Plus and exoEasy). Very interestingly, there was no correlation in particles concentration extracted between different kits, except between ExoQuick and Exo-Flow ($p < 0.05$). An example of the variability in the particles concentration depending on the purification kit used and the sample is shown in Figure 2B.

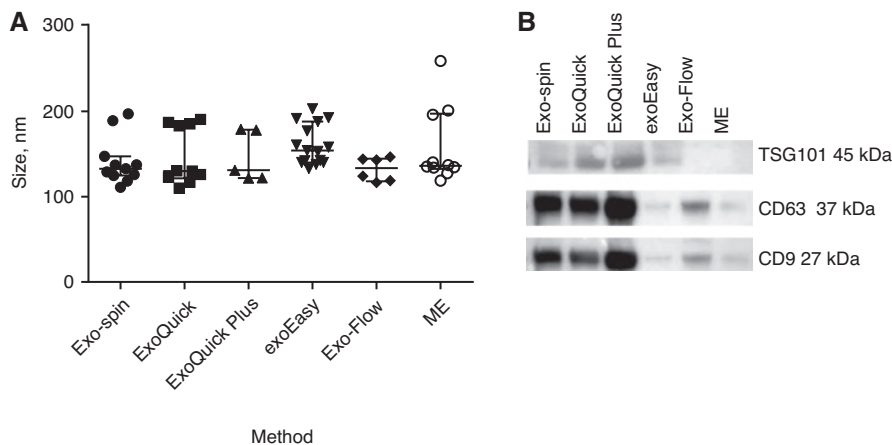


Figure 1: Identification of exosomes.

Size distribution (A) and representative experiment (B) of Western blot incubated with anti-TSG101, anti-CD63 and anti-CD9, of the exosomes isolated from human serum using the different commercial kits.

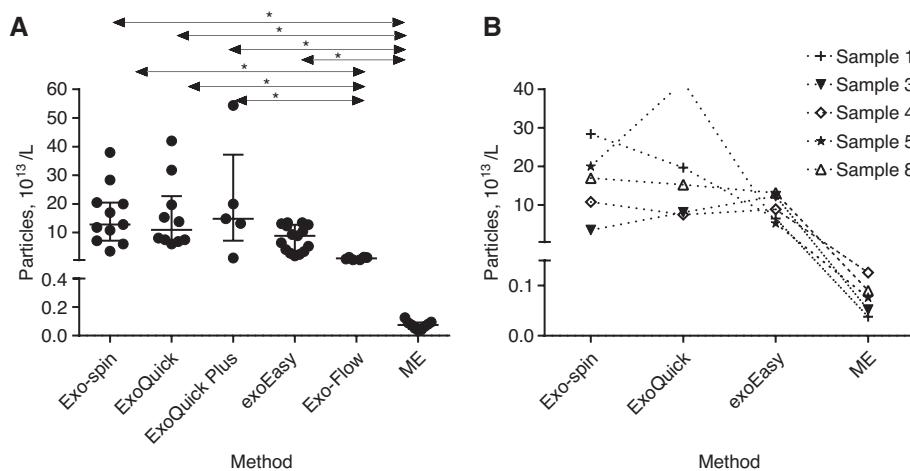


Figure 2: Effect of isolation kits on particle yield.

Comparison of different methods for exosome purification in the concentration of particles obtained (A) and in individual samples (B). * $p < 0.05$.

Purity of the exosomes

There was a relationship between the concentration of proteins and the particles in the extracted solution ($r = 0.579$; $p < 0.01$). The proteins concentration was significantly lower with the ME, Exo-Flow and exoEasy kits. We used the ratio between particle concentration and protein to determine the exosomes purity [27]. The highest purity in exosome solution (ratios higher 3×10^{16} P/g protein) was observed with ExoQuick Plus and exoEasy, while the preparations with lower ratios (less than 1×10^{16} P/g protein) were achieved with ME and ExoQuick kits (Figure 3). Considering ExoQuick, the second step of

purification achieved with ExoQuick Plus, but not with Exo-Flow, increased exosomes purity about 3 times.

We could detect the presence of apoB in exosome fraction obtained using ExoQuick (5/5 samples), Exo-spin (4/5 samples) ExoQuick Plus (1/5 samples) and exoEasy (3/5 samples). ExoQuick was the method in which lipoprotein contamination was higher (median = 0.50 g/L; Q1-Q3 = 0.28–0.82 g/L). No apoB was detected in the extracts from Exo-Flow (0/5 samples) and ME (0/5 samples). Similarly, albumin was present in the exosome extracts from all commercial kits, especially with ExoQuick (median = 3.32 g/L; Q1-Q3 = 1.69–10.47 g/L). In the case of ExoQuick, further exosome purification with beads

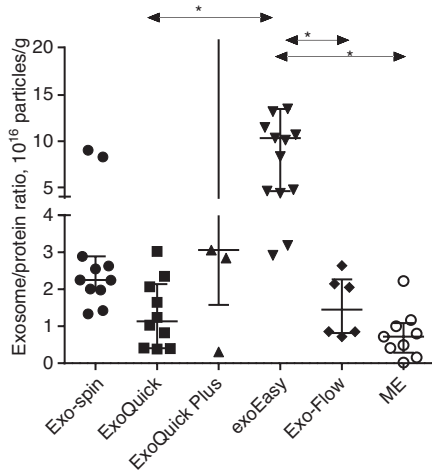


Figure 3: Analysis of particle to protein ratio of the exosomes isolated from human serum using the different commercial kits. * $p < 0.05$.

(ExoQuick Plus and Exo-Flow) resulted in a decreased apoB and albumin contamination.

Cytokine detection and quantification

We also analyzed three cytokines (S100A9, CXCL5 and CXCL12) in exosomes purified with ExoQuick, exoEasy, Exo-Flow and ME from four controls (Figure 4). The three cytokines could be detected in exosomes obtained with exoEasy, but not with the other kits. S100A9 was detected in all samples, except in exosomes purified with Exo-Flow (Figure 4A). CXCL5 could be only detected with ExoQuick and exoEasy (Figure 4B). CXCL12 was detected in the exosomes purified with exoEasy, Exo-Flow and ME, but not with ExoQuick (Figure 4C). In addition, cytokine concentrations were very different depending on the type of purification kit used. The highest concentrations of both S100A9 and CXCL5 were observed in the exosome fraction

obtained with ExoQuick. There was no correlation in exosome cytokine concentrations in the samples obtained with different kits.

Discussion

When being used in clinical laboratories, methods for serum exosome isolation should be rapid, standardized, produce a high yield of pure exosome from small serum volumes and be capable of being used with a large number of samples. To the best of our knowledge, this is the largest comparison study of exosome purification kits [15, 17, 19, 22, 23]. The methods studied here are not complex, do not need special equipment, and several samples can be processed simultaneously and in a rational short time. All the methods were suitable for exosome isolation, as demonstrated by particle size (130–154 nm) and the Western blot detection of the exosome markers CD63, CD9 and TSG101 [28]. We have observed certain dispersion in particle counts, already described by other authors [19, 22, 27]. This dispersion may be probably due to: (i) almost all cells can produce exosomes and the interindividual variability could be very high; (ii) blood is a rather complex medium, and (iii) methods do not exclusively isolate exosomes and some impurities could account for the dispersion observed in particle concentration measured with NanoSight [27].

Regarding impurities, although we have not detected the presence of contaminant debris from endoplasmic reticulum or an RISC complex marker [28] (Supplementary material, Figure 1), we have detected lipoprotein and albumin contamination. LDL and VLDL lipoproteins have a size in the range of exosomes [18]. Lipoprotein contamination is a problem with most of the kits studied here, except for ME and Exo-Flow. Previously, it was suggested that lipoproteins were not retained in exosomes purified with the exoEasy kit [29], but we have detected apoB in half of the samples, so this contaminant cannot

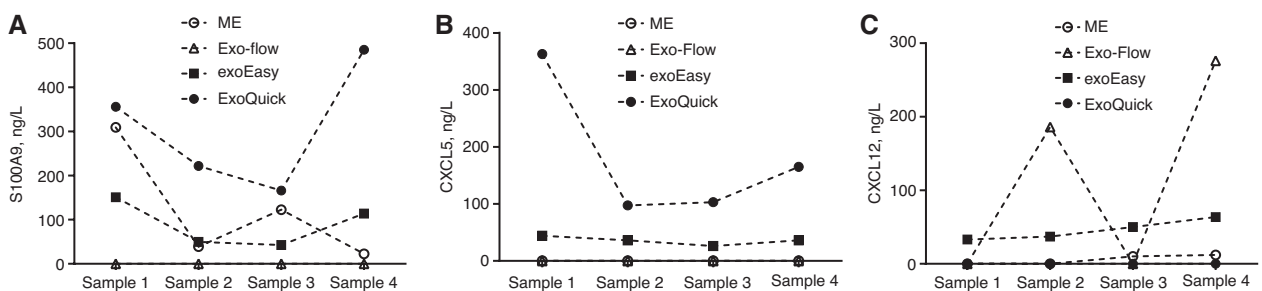


Figure 4: Effect of isolation kits on subsequent cytokine analysis. Concentrations of S100A9 (A), CXCL5 (B) and CXCL12 (C) in exosomes from four different serum samples obtained with ME, Exo-Flow, exoEasy and ExoQuick.

be excluded [19]. Another serum contaminant detected in all exosome samples is albumin, which is the main protein present in serum. Albumin is not included in the exosome proteome [23], and therefore, its presence indicates protein contamination. This protein can be either non-specifically bound to exosomes or dragged during the process of purification.

Although ExoQuick performance is better than ultracentrifugation [20], it was the one with most contaminants in the exosome fraction from all kits analyzed. Probably, the polymer-based precipitation facilitates co-precipitation of other particles, such as lipoproteins. However, further purification steps with specific exosome antibodies noticeably increased the purity. On the contrary, the membrane-affinity column method exoEasy seems to be the one with the highest exosome purification, considering the particle/protein ratio [27]. It is interesting to note that the exosome extraction yield was especially low with the use of ME, a method that uses a peptide against the heat shock proteins present in exosomes [21].

Gel electrophoresis of the exosomes purified already showed a different pattern of proteins depending on the kit used for exosome isolation (Supplementary material, Figure 2). This could be due to the fact that the isolation method could determine the type and quantity of exosomes, and also, the carry-over of non-exosomal proteins as we mentioned before. For example, some methods employ antibodies against exosome proteins, such as CD63, so they could exclude those particles that do not show this marker or it is in low concentration [30]. Also, the intensity of the bands of CD63, CD9 and TSG101 varied with the purification method used [22]. Consequently, biomarker analysis in exosomes can be method dependent. As serum is a very complex and viscous fluid with protein concentration around 60–80 g/L, it is very challenging to isolate exosomes for measuring proteins that are in the range of 10^{-6} g/L [24]. For this reason, it is important, not only to have a method that can purify exosomes efficiently, but also one that does not interfere with subsequent biomarker measurements [9]. Here, when measuring cytokines in exosomes obtained with different kits we can observe that, depending on the kit used, some cytokines could be detected, while others were missed. For example, CXCL12 was readily detected with exoEasy as it was previously shown by other authors [26]. However, this cytokine was not detected using exosomes purified with a polymer (ExoQuick), although the use of a second step of purification with anti-CD63 (Exo-Flow) allowed its detection in some samples. The difference can be due to the presence of some substances from the ExoQuick kit that

later interfere in the immunoassay. Also, sample characteristics in relation to protein, lipoproteins content, etc., could influence the efficiency of exosome purification, resulting in the lack of correlation of particle and cytokine concentration observed between samples obtained with different kits [20]. Very probably, these observations with three cytokines, S100A9, CXCL5 and CXCL12 could be extrapolated to other cytokines.

In summary, after analyzing six different kits for serum exosome purification, we have observed that all of them can extract exosomes, but with very different degrees of efficiency and contamination with lipoprotein and albumin. Three methods obtained similar particle counts: Exo-spin, exoEasy and ExoQuick, and any of those methods can be adequate alternatives. However, if the highest purity possible is mandatory, then exoEasy or ExoQuick Plus seem to be the more adequate methods. Nevertheless, it is essential to check that the exosome isolation method does not interfere with subsequent analyses to be performed in isolated exosomes. For example, the type of extraction method deeply affects the concentration of cytokines measured, which could influence later data interpretation. Therefore, comparison of studies analyzing cytokines in exosomes can be difficult if exosomes were purified with different methods.

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