First class of lyophilized controls: production and characterization

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> INTRODUCTION

The use of exosomes in clinical research requires a sensitive, reproducible and high-performance method for characterization and quantification of samples, but also is needed controls or reference materials to assess the validity of the results, calibrating equipment, to verify or validate methods, to determine bias and to estimate uncertainty, reducing development time and cost of any assay and increasing its reproducibility. However, the use of exosomes as reference material in biomedical research requires the exosomes to be well characterized, what is not common in commercialized exosomes. The aim of this work was to standardize production conditions, including extensive characterization of the exosomes so that they can be used as reference materials.

> MATERIAL AND METHODS

To produce and characterise the lyophilized standars the following materials and equipment are used:

For **cell culture** method, hollow fiber biorreactor (#C2011; FiberCell System) supplied with appropriate culture media with serum exo-free, antibiotics and antifungals. The cell culture is maintained with [gluc > 90 mg/dL]. For exosome **purification**, the first centrifugations is performed in Eppendorf 5810R centrifuge (300xg-10min; 2000xg-10min), subsequently ultracentrifuge HIMAC CR30NX is used, the rotor employed to isolate exosomes is R30AT (10.000xg- 30min; 2x 100.000xg-10min). To increase purity in complex samples, like serum or plasman, SEC columns are utilized (#SEC7012; SEC3512; Immnostep S.L, Salamanca). Bio-Rad Protein Assay Dye Reagent Concentrate is used to **bradford assay**. For particle concentration and size, Nanoparticle tracking analysis (NTA) is used (Nanosight NS300) . Different antibodies are used in **Western Blot** as primary antibody CD9, CD63, CD81 (1:1000) (#P9PU-01MG; #P63PU-01MG; #P81PU-01MG; Immunostep S.L, Salamanca) and TSG101 (1:400)(Santa Cruz Biotecnology). As secondary antibody it is used a goat anti-mouse IgG DyLight 680 (1:10000) (#UC284771; Thermo). For **Flow Cytometry** #Exostep-25-C9 or #Exostep-25-P81: Immunostep S.L, Salamanca are used in an Aurora cytometer (Cytek). Finally, the freeze-dryer employed to **lyophilize** is Epsilon 2-4 (LCSplus) (Martin Christ GmbH) equipment.

> RESULTS

1. Upstream cell culture - Exosome source.

The cell lines used to produce exosomes are Mycoplasma-free and are routinely genotyped and characterized to assure their authenticity. For serum- or plasma-derived exosomes, donor samples are pre-tested for HIV, HBV and HCV, ensuring user safety.

2. Cell culture method.

For exosome production, the chosen method involves the use of a hollow fiber bioreactor, which offers many advantages over conventional culture contributing to the higher yield, purity and control (limited passages, environmental, CO2, and glucose monitoring) during exosome production. In addition, cells grown in hollow fiber bioreactors are easily adapted to sustained growth in protein-free medium, which also facilitates the purification of exosomes without serum contaminants. Volume of fluid, and/or cell number is recorded.

3. Exosome purification.

Differential ultracentrifugation as method of choice that provides a relatively high purity and is very suitable large sample volumes and is currently considered the "gold standard" method. Differential ultracentrifugation is combined with size exclusion chromatography column (SEC) to obtain higher purity and is suitable for isolation of exosome plasma/serum derived.

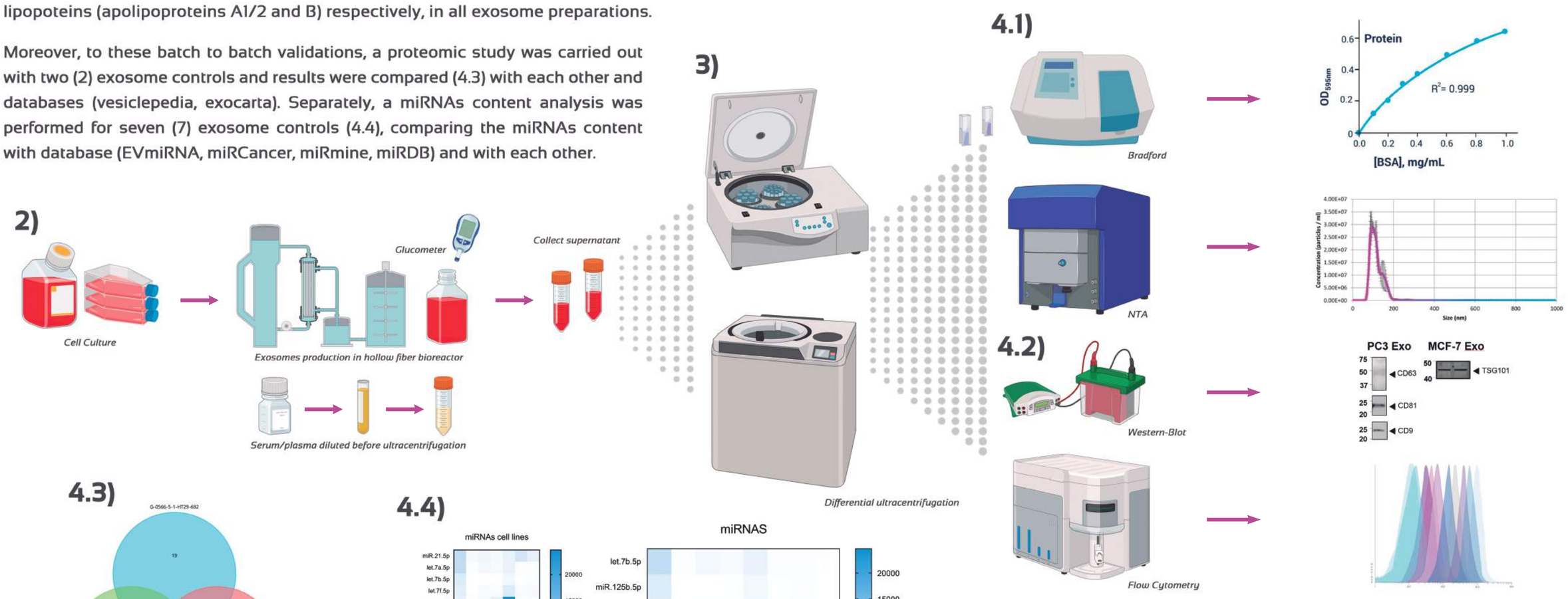
4. Downstream exosome purification - Exosomes characterization.

All exosome manufactured batches are consistently and extensive characterized according MISEV2018.

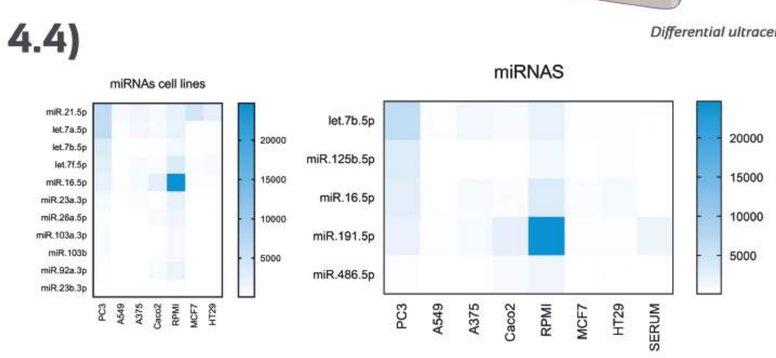
4.1. Global quantification by methods: protein amount and particle number: Total protein amount is measured by colorimetric assay [bradford] while particle number and size distribution is measured by nanoparticle tracking analysis (NTA).

4.2. Global characterization by western-blot, flow cytometry and ELISA to assess presence of transmembrane proteins such as tetraspanins (CD63, CD9, CD81), cytosolic proteins like Alix and TSG101 and lipopoteins (apolipoproteins A1/2 and B) respectively, in all exosome preparations.

Moreover, to these batch to batch validations, a proteomic study was carried out with two (2) exosome controls and results were compared (4.3) with each other and databases (vesiclepedia, exocarta). Separately, a miRNAs content analysis was performed for seven (7) exosome controls (4.4), comparing the miRNAs content with database (EVmiRNA, miRCancer, miRmine, miRDB) and with each other.



FunRich comparison of all Homo sapiens entries from the Vesiclepedia database in Exosomes versus MCF7 versus HT29.



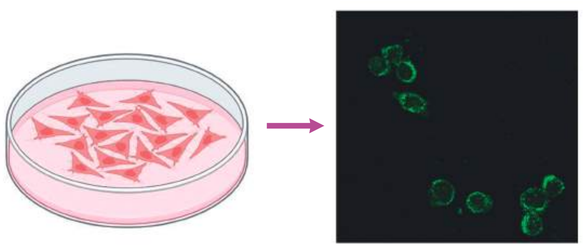
miRNAs that share all (7) cell line-derived exosomes (top) and miRNAs that share cell line-derived exosomes with serum/plasma-derived exosomes (bottom).

Product attributes	Immunostep	Competitor 2	Competitor 3	Competitor 4
NTA	✔	✔	✔	✔
Protein markers of EVs	✔	X	X	✔
Cytosolic protein	✔	X	X	✔
Total protein amount	✔	X	X	✔
Quantification of total lipids	✔	X	X	X
Functional data	✔	X	X	X
miRNAS study	✔	X	X	✔
Proteomic study	✔	X	X	X
Frozen or lyophilized	Lyophilized	Lyophilized	Lyophilized	Lyophilized

Table I: Comparison of Immunostep and competitor exosomes

5. Functional studies.

Cellular uptake and internalization of exosomes labeled with PKH67 and DAPI assays has been performe in adipose-derived Mesenchymal stem/stromal cells (MSCs) derived exosomes for its therapeutic interest.



6. Storage conditions - Lyophilization process.

Lyophilization (freeze-drying) is used for the prolonged storage of exosome controls. The buffer and excipients used have been carefully selected to maintaining their structure and functionality. For instance, 2-8% trehalose or 10% w/v sorbitol and 2-10 % sucrose are suitable for exosome freeze-drying. Lyophilized exosomes can be stored between 2°C and 8°C for up to 2 years without functional compromise and can be delivered at room temperature

Packaging: 1 vial each containing 100 µg total protein, ~1E12 particles/ml.

> CONCLUSIONS

- Exosome standards by Immunostep are manufactured over standardize protocols and are subject to quality control characterization batch to batch, guaranteeing traceability and reproducibility of the assays.
- Exosome standards provided by Immunostep have the most extensive characterization on the market, when it is compared with other competitors (Table 1).
- The use of these standards help to mantaing the quality of your research, saving time and cost.

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