Journal of Visualized Experiments

Flow Cytometric Analysis of Extracellular Vesicles from Cell-Conditioned Media

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59128R2
Full Title:	Flow Cytometric Analysis of Extracellular Vesicles from Cell-Conditioned Media
Keywords:	Exosomes Microvesicles Flow Cytometry Exosomes Phenotyping Beads Conditioned medium Extracellular Vesicles
Corresponding Author:	Lucio Barile Cardiocentro Ticino Taverne, Ticino SWITZERLAND
Corresponding Author's Institution:	Cardiocentro Ticino
Corresponding Author E-Mail:	lucio.barile@cardiocentro.org
Order of Authors:	Carolina Balbi
	Sara Bolis
	Giuseppe Vassalli
	Lucio Barile
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Switzerland

Cover Letter

Dr. Lucio Barile
Cardiologia Molecolare
T +41 (0)91 805 33 84
F +41 (0)91 805 38 13

Cardiocentro Ticino
via Tesserete 48
CH-6900 Lugano

Lucio Barile, PhD
Lab coordinator, head of project
Cellular and Molecular Cardiology Laboratory
Fondazione Cardiocentro Ticino
and Swiss Institute for Regenerative Medicine
Via Tesserete 48
6900 Lugano (Switzerland)

lucio.barile@cardiocentro.org

Phillip Steindel, Ph.D.

Review Editor

JoVE, Biology

Re: **Protocol for Flow Cytometric Analysis of Extracellular Vesicles from Cell-Conditioned Media (JoVE59128-R2)**

Dear Dr. Steindel

Thank you for reviewing our manuscript.

We have addressed Editor's comments, all changes made in the text are tracked by red color. Essential steps of the protocol for the video are highlighted in yellow.

On behalf of all of the authors, I thank you for considering our revised manuscript for publication in JoVE.

Best regards

Lucio Barile, PhD

Lucio Barile

Firmato digitalmente da Lucio Barile
ND: cn=Lucio Barile, o=CCT, ou, email=lucio.barile@cardiocentro.org, c=CH
Data: 2018.11.14 11:45:34 +01'00'

Lugano, Switzerland;

November 14, 2018

TITLE:

Flow Cytometric Analysis of Extracellular Vesicles from Cell-Conditioned Media

AUTHORS & AFFILIATIONS:

Carolina Balbi*[1], Sara Bolis*[1], Giuseppe Vassalli,[1,2] and Lucio Barile[1]

[1]Cellular and Molecular Cardiology Laboratory, Cardiocentro Ticino Foundation and Swiss Institute for Regenerative Medicine (SIRM), Lugano, Switzerland

[2]Molecular Cardiology Institute, Dept. of Cardiology, University of Zürich, Zürich, Switzerland

*These authors contributed equally.

Email Addresses of Co-authors:

Carolina Balbi (carolina.balbi@cardiocentro.org)

Sara Bolis (sara.bolis@cardiocentro.org)

Corresponding Authors:

Lucio Barile

lucio.barile@cardiocentro.org

Giuseppe Vassalli

giuseppe.vassalli@cardiocentro.org

KEYWORDS:

exosomes, microvesicles, flow cytometry, exosomes phenotyping beads, conditioned medium, extracellular vesicles

SUMMARY:

The protocol describes a reproducible method designed for use with cell culture supernatants to detect surface epitopes on small extracellular vesicles (EV). It utilizes specific EV immunoprecipitation using beads coupled with antibodies that recognize surface antigen CD9, CD63, and CD81. The method is optimized for downstream flow cytometry analysis.

ABSTRACT:

Flow cytometry (FC) is the method of choice for semi-quantitative measurement of cell-surface antigen markers. Recently, this technique has been used for phenotypic analyses of extracellular vesicles (EV) including exosomes (Exo) in the peripheral blood and other body fluids. The small size of EV mandates the use of dedicated instruments having a detection threshold around 50-100 nm. Alternatively, EV can be bound to latex microbeads that can be detected by FC. Microbeads, conjugated with antibodies that recognize EV-associated markers/Cluster of Differentiation CD63, CD9, and CD81 can be used for EV capture. Exo isolated from CM can be analyzed with or without pre-enrichment by ultracentrifugation. This approach is suitable for EV analyses using conventional FC instruments. Our results demonstrate a linear correlation between Mean Fluorescence Intensity (MFI) values and EV concentration. Disrupting EV through

sonication dramatically decreased MFI, indicating that the method does not detect membrane debris. We report an accurate and reliable method for the analysis of EV surface antigens, which can be easily implemented in any laboratory.

INTRODUCTION:

Cells secrete extracellular vesicles (EV) of different sizes including microvesicles (MV) and exosomes (Exo). The latter can be distinguished from MV by both size and the subcellular compartment of origin. MV (200-1,000 nm in size) are released from parent cells by shedding from the plasma membrane. Conversely, Exo (30- 150 nm) originate from endosomal membranes and are released into the extracellular space when the multivesicular bodies (MVB) fuse with the cell membrane[1,2].

EV are increasingly used as diagnostic biomarkers as well as, potentially, therapeutic tools in many fields including oncology, neurology, cardiology, and musculoskeletal diseases[3-5]. A vast majority of ongoing studies using EV as therapeutic agents exploit the isolation of vesicles from cell-conditioned medium (CM) of in vitro cultured cells. Mesenchymal stem cells (MSCs) exert beneficial effects in several contexts, and MSC-derived EV have shown benefits in models of myocardial ischemia/reperfusion injury[6] and brain injury[7]. MSC-derived EV also exhibit immune modulatory activities that can be exploited to treat immune rejection, as demonstrated in a model of therapy-refractory graft-versus-host disease[8]. Amniotic fluid stem cells (hAFS) actively enrich CM with MVs and Exo, heterogeneously distributed in size (50–1,000 nm), which mediate several biological effects, such as proliferation of differentiated cells, angiogenesis, inhibition of fibrosis, and cardioprotection[4]. We have recently shown that EV, and particularly Exo, secreted by human cardiac-derived progenitor cells (Exo-CPC) reduce myocardial infarct size in rats[5,9].

Exo share a common set of proteins on their surface, including tetraspanins (CD63, CD81, CD9) and major histocompatibility complex class I (MHC-I). In addition to this common set of proteins, Exo also contain proteins specific for the EV subset of the producer cell type. Exo markers are gaining paramount importance because they play a crucial role in inter-cellular communication, thereby regulating many biological processes[5,10]. Because of their small size, finding an easy way to analyze EV using classical flow cytometry (FC) remains a challenging task.

Here, we present a simplified protocol for EV analysis using FC, which can be applied to pre-enriched samples obtained through ultracentrifugation or directly to CM (**Figure 1**). The method uses beads coated with a specific antibody that binds canonical Exo-associated surface epitopes (CD63, CD9, CD81) without additional washes. FC analyses can be performed using a conventional cytometer with no need for adjustments prior to measurements. Methods for the characterization of antigens on individual small particles using flow cytometers have been described by other groups with respect to various applications[11–13]. Here, we used functionalized magnetic beads for the capture of small particles and Exo, followed by phenotyping of captured particles by FC. Although this method can be used to characterize the antigenic composition of small vesicles released by any cell type in vitro, here we provided specific cell culture conditions that apply for the culture of human cardiac progenitor cells (CPC) and the most appropriate environment for the production of EV by these cells.

PROTOCOL:

1. Collection and processing of conditioned media

1.1. Coat 55 cm^2 Petri dishes with 0.02% porcine skin gelatin in PBS.

1.2. Plate CPC (8,000/cm^2) in pre-coated dishes with 7 mL of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS (Fetal Bovine Serum) and 1% Penicillin/Streptomycin (P/S).

NOTE: The term "CPC" refers to human explant derived cells that have been described elsewhere[14]. CM can be collected from different cell types cultured in specific culture conditions. Wear gloves and work under a biological hood.

1.3. Once cells reach about 80% confluence, remove the culture medium, wash cells twice with Dulbecco's PBS (PBS) Ca- and Mg-free and replace it with 10 mL of serum-free Exo-production medium (Dulbecco's Modified Eagle's Medium [DMEM] High Glucose, 4.5 g/mL).

NOTE: Proceed with a progressive decrease of serum concentration in culture medium, to gradually adapt cells to serum-free condition (SF). For cells that are sensitive to serum-free medium prepare a medium containing FBS but depleted of EV. Prepare medium supplemented with all nutrients, plus 10%–20% (v/v) FBS. Centrifuge the medium overnight at 100,000 × *g*, 4 °C. Be aware that this procedure does not guarantee 100% removal of FBS-derived EV.

1.4. After 7 days, collect the CM in a polypropylene centrifuge tubes.

NOTE: The time for medium conditioning depends on the cell type. For cells that are sensitive to serum-free medium, increase the initial volume of medium and decrease the time for CM collection to 24-48 h.

1.5. Clear CM from cell debris by centrifuge at 3,000 x *g* for 20 min at 4–10 °C. Collect the supernatant in a 100 kDa centrifuge filter tube.

1.6. Concentrate cleared CM by spinning the tube at 2,000 x *g* for 20 min at 4–10 °C.

NOTE: This step will reduce the initial volume of CM allowing the use of small-volume tubes for high speed centrifuge steps and will contribute to eliminate small protein aggregates.

1.7. Collect the concentrate in a microcentrifuge tube. Centrifuge at 10,000 x *g* for 15 min at 4–10 °C.

1.8. Collect the supernatant and proceed to section 2 (EV enrichment). If an ultracentrifuge is not available proceed directly to section 3 (Nanoparticle Tracking Analysis (NTA) quantification).

NOTE: Pre-enrichment of EV fraction improves final read-out in fluorescence intensity (see representative results and **Figure 4**). Cleared CM (from point 1.10) can be stored at 4 °C for no longer then 1-2 days or alternatively at −80°C for several months.[5] CM should be pre-cleared to remove any cellular debris before storage at -80°C in order to ensure that exosome-sized debris is not formed in the freeze-thaw process.

2. Extracellular vesicles enrichment by ultracentrifuge (optional)

2.1. Place the supernatant from step 1.8 in a polycarbonate thick wall ultracentrifuge tube. Fill the tube with 1x phosphate buffer saline (PBS) until it reaches the maximal capacity (3.2 mL).

2.2. Load samples in a titanium fixed-angle rotor (8 x 3.2 mL, k-Factor 13). Load the rotor in a tabletop ultracentrifuge. Ultracentrifuge at 100,000 x *g* for 3 h at 4–10 °C.

2.3. Discard the supernatant and resuspend the pellet in 100 µL of 1x PBS.

3. Nanoparticle tracking analysis (NTA) quantification

3.1. Dilute 1 µL of sample (from either step 2.3 or 1.8) in 999 µL of 1x PBS. Load the sample in a 1 mL syringe, avoiding bubble formation. Load the syringe in the inlet port of the examination chamber.

3.2. Turn on the laser. Open the camera with the **Capture** button. Adjust the focus.

3.3. Record at least 3 different frames of 60 s each.

3.4. Analyze the 3 different acquisitions using the **Batch Process** option in the software.

NOTE: If the NTA technology is not available, perform a protein quantification by the Bradford assay (1 x 10^8 particles correspond to 1-2 µg of total protein after ultracentrifugation or to 50-60 µg total protein if quantification is performed directly for CM as it includes proteins contaminants different from EV; see **Table 1**)[5].

4. Sample preparation

4.1. Prepare a pool of the 3 types of capture beads (CD9 beads, CD63 beads, and CD81 beads at a 1:1:1 ratio; see **Table of Materials**) and vortex.

NOTE: The pool has been tested to be stable for 1 month. A single type of capturing bead may be used, this will allow the detection of EV sub-populations not discriminable with pool of beads. By using single antigen capturing beads, it will be possible to contemporary demonstrate the presence of tetraspanin proteins, such as CD9, CD81, or CD63, and the antigen of interest detected by fluorescent antibody. Alternative aldehyde-sulfate latex beads are also commercially

177 available. These beads present hydrophobic surface for the adsorption of MV and EV.
178
179 4.2. In a round bottom tube, add the amount of volume corresponding at 1 x 10^8 particles plus 1
180 µL of the bead pool, corresponding to 1.2 x 10^5 beads in total (for each test).
181
182 4.3. Prepare a tube containing 1 µL of beads without EV that will serve as negative control, hereby
183 referred as "Beads".
184
185 4.4. Adjust the volume to 100 µL with 1x PBS. Place the tube in a thermo-mixer, and shake at 400
186 rpm overnight at 4-10 °C.
187
188 4.5. The next day, move the samples to a round bottom 96-well plate (or FC tubes).
189
190 4.6. Add the antibody (10 µg/mL of CD9_FITC, 10 µg/mL of CD63_PE, and 5 µg/mL of CD81_PE).
191
192 4.7. Incubate 1 h at 4–10 °C.
193
194 4.8. Add 100 µL of 1x PBS to each well.
195
196 4.9. Proceed to acquisition.
197
198 **5. Acquisition**
199
200 5.1. Open the acquisition software and start up the instrument (**Cytometer > System Startup**
201 **Program**). Open a new experiment.
202
203 5.2. Create an experimental template, then select **Plate** and then **Add Plate**. Select the position
204 of the samples on the plate and then press **Set As Sample Well**.
205
206 5.3. Enter the sample name (i.e. CPC#1_CD9FITC) in the **Naming Rules**. Open the **Channel** Tab
207 and switch on the FITC and PE Channels.
208
209 5.4. Load the plate in the instrument.
210
211 5.5. Press **Dot Plot** and create a new dot plot (P1). Set Forward Scatter Area (FSC-A) versus Side
212 Scatter-Area (SSC-A).
213
214 5.6. Select **Initialize** to start the laser and the fluidics. Press **Acquire** to start the acquisition.
215
216 5.7. Adjust the scale to show the population in the middle of the P1. In P1 draw the "BEADS" gate
217 around the whole population (excluding debris) (**Figure 2A**). Press **Stop**.
218
219 5.8. Press **Dot Plot** and create a new dot plot (P2). Set Forward Scatter-Height (FSC-H) versus FSC-
220 A. Gate the new dot plot on "BEADS." In P2 draw a new gate around the bigger population and

name it “SINGLETS” (**Figure 2A**).

NOTE: This strategy is designed in order to avoid as much as possible the inclusion of beads aggregates in both acquisition and analysis steps.

5.9. Press **Dot Plot** and create two different dot plots; one FITC versus SSC-A and one PE versus SSC-A. Gate the new dot plot on “SINGLETS.”

5.10. Select the number of **Events to record** (e.g., 20,000). Select **Record** and start the experiment.

6. Data analysis

6.1. Load the acquisition files into the analysis software.

NOTE: The following steps have to be applied to every sample including beads only and every unknown sample.

6.2. Open a new protocol and create dot plots following the same strategy described in step 5.

6.3. Set all scatter axes to linear scale and all the fluorescence axes to log scale.

6.4. Show the Fluorescence Geometric Mean (X-Gmean-MFI) in FITC and PE channels.

6.5. Calculate the ratio X-G_{mean} of the samples / X-G_{mean} of the beads stained with antibody without exosome.

6.6. Compare the fold change MFI of different EV preparations.

7. Extracellular vesicle number titration

NOTE: Sections 7 to 10 can be performed to set up the number of particles, antibodies concentration and specificity, but can be skipped if the cell type, from which EV are derived, and antibodies remain the same.

7.1. Dilute particles, obtained from step 2.3, in 1x PBS to obtain a series of suspensions ranging from 5×10^5 to 2.5×10^8 particles.

7.2. For each suspension add in a round bottom tube 1 µL of capture bead pool.

7.3. Follow the protocol from step 4.3.

8. Antibody titration

NOTE: Antibody titration is usually performed by adding a single antibody for each tube.

8.1. Keep the number of EV (total particle or protein content) constant in all samples.

NOTE: The appropriate number is empirically determined as described above in section 7.

8.2. Follow the protocol from steps 4.1 to 4.5.

8.3. Test a range of antibody concentrations above and below the amount recommended by the supplier. For example, for an antibody with a suggested concentration of 10 µg/mL per test, test 1, 2, 5, 10, 20 and 50 µg/mL. Include a sample with "beads only", adding the highest concentration of antibody.

8.4. Incubate for 1 h at 4–10 °C.

8.5. Add 100 µL of 1x PBS for each sample.

8.6. Acquire the samples.

9. Incubation time

9.1. Verify the incubation time performing acquisitions at different time points (e.g., 1 h, 2 h, and 5 h).

9.2. Follow the protocol starting from section 4.

9.3. Incubate samples at 4–10 °C for 1 h.

9.4. Acquire samples.

9.5. Incubate samples at 4–10 °C for 3 h.

9.6. Acquire samples.

10. Protocol validation

10.1. EV binding

10.1.1. In a round bottom tube, add the amount of volume corresponding to 1 x 10^8 particles or the corresponding amount of total protein.

10.1.2. Adjust the volume to 100 µL with 1x PBS.

10.1.3. Disrupt the EV membrane by sonication (alternatively a heat shock step can be applied)

according to manufacturer's instructions. Set frequency and intensity. The sonication period can be empirically established setting a time-course experiment (i.e., 0 s, 30 s, 1 min, 5 min, etc.).

10.1.4. Add 1 µL of the bead pool.

10.1.5. Follow the protocol starting from step 4.4.

10.2. Antibody specificity

10.2.1. Prepare one tube for each fluorochrome-conjugated IgG and add the complex of beads-EV as described in step 4.6.

NOTE: The fluorochrome-conjugated Isotype must match with the IgG of the used antibody.

REPRESENTATIVE RESULTS:

Total number of particles for single staining

Since a single bead can bind more than one particle, we tested different conditions to set the smallest amount of total EV (single antibody per tube) to reach the early exponential phase of MFI curve. A fixed concentration of antibody was used while the total number of particles ranged from 5×10^5 to 2.5×10^8. As shown in **Figure 3A**, the number of particles that allows us to ensure that the antibody performs within an acceptable MFI, avoiding the use of an excess of EV, is 1×10^8 particles/staining.

Antibody titration

We selected the proper concentration of antibody resulting in the highest signal preventing the nonspecific antibody binding. This test has been optimized for 1×10^8 particles, as determined in the previous setting. Anti-CD9_FITC, anti-CD63_PE, and anti-CD81_PE were tested with concentrations ranging from 1 to 50 µg/mL (**Figure 3B**). Anti-CD9_FITC and anti-CD63_PE antibodies gave a good resolution of signal (7.5 and 130-fold change of MFI vs. beads alone, respectively) when used at concentration of 10 µg/mL while the selected concentration for the anti-CD81_PE antibody was 5 µg/mL (465.3 Fold Change MFI).

Method validation

In order to confirm that our method is suitable to analyze only "cup-shaped" extracellular vesicles and not membrane debris, we applied different sonication steps, at 10% of amplitude, to the solution containing particles. 100 µL of 1x PBS solution containing 1×10^8 particles underwent different sonication steps from 30 seconds to 5 minutes and the resulting preparation containing both broken and well-shaped EV were analyzed as described (experimental protocol from point 3.3). As a result, we found that 30 seconds of sonication decrease MFI that was completely dumped after 1 minute. At this timepoint, no fluorescence is detectable for any of the EV markers (**Figure 3D**).

Flow cytometry characterization of HEK293- and CPC-derived exosomes

These results were generated following the protocol presented above with the ultracentrifuge isolation method. The isolated exosomes are quantified by NTA technology and loaded overnight with 1 µL of mixed beads (1x anti-CD9:1x anti-CD63:1x anti-CD81). The complex beads + Exo were stained with the proper amount of antibody anti-CD9_FITC, anti-CD63_PE, and anti-CD81_PE (**Figure 3E,F** and **Table 2**).

Furthermore, by using EV-CPC we compared FC analysis with or without pre-enrichment by ultracentrifugation. **Figure 4** shows that both methods are suitable to profile Exo surface markers. Pre-enrichment of extracellular vesicles fraction greatly improves fluorescence intensity especially for CD63_PE and CD81_PE staining (**Figure 4** and **Table 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol and NTA plots. (**A**) Schematic representation of the experimental protocol. (**B**) Representative NTA plots for Conditioned Media, Pre-Ultracentrifuge and Post-Ultracentrifuge step.

Figure 2: Acquisition and data analysis. (**A**) Flow cytometry analysis begins with creating a first gate to the whole beads population "beads" (excluding debris) and then a second gate to distinguish "singlets" events. Singlets are gated on a plot set up with FSC-H as x-axis and FSC-A as y-axis. (**B-D**) Representative dot plots showing right-shift of fluorescence intensity for the positive populations of beads-exosomes complexes (green, CD9+; red CD63+; brown CD81+). Isotype control (violet) overlap negative gray colored beads.

Figure 3: Titration curves for number of particles (arrows show the selected amount of particles, 1 x 10^8). (**A**) Antibody concentrations (arrows show the selected concentration for each used antibody). (**B**) Both number of particles and antibody concentration are plot vs. mean fluorescence intensity (MFI). (**C**) Curve showing 3 different acquisition of the preparation with 1 h – 2 h or 5 h of antibody incubation. (**D**) Curve showing the decrease of fluorescence following sonication at different time-points. (**E-F**) Fold change (mean ± SD) of MFI for CD9, CD63 and CD81 vs negative control (beads + antibodies, no EV) are shown for HEK293 EV (n = 3 independent replicates) and for CPC EV (n = 3 primary cell lines from 3 different patients)

Figure 4: MFI analysis and comparison between two procedures: direct EV-binding with beads (Capture Beads Isolation) and pre-enrichment with ultracentrifuge (Ultracentrifuge Isolation). Data are shown as fold change (mean ± SD) of MFI for (**A**) CD9, (**B**) CD63, and (**C**) CD81 vs. negative control (beads + antibodies, no EV). N = 3 primary cell lines from 3 different patients.

Table 1: Comparison between NTA concentration and protein concentration for 3 different patient derived CPC before and after ultracentrifuge.

Table 2: Value of fold change (mean ± SD) of MFI for HEK293 EV (n = 3 independent replicates) and CPC EV (n = 3 primary cell lines from 3 different patients).

Table 3: Value of fold change of MFI (mean ± SD) for CPC EV (n = 3 primary cell lines from 3 different patients) isolated by capture beads or ultracentrifuge.

Table 4: Single product specification.

DISCUSSION:

Conventional FC technique remains the most straightforward analytic method to characterize markers expressed onto the surface of EV. In this regard, selecting the most appropriate protocol is crucial to obtain useful information on individual particle fractions of interest by avoiding limitations due to instrument sensitivity. We described a method using magnetic particles coupled with antibodies that recognize Exo and small EV surface antigens which are suitable for downstream FC application. We validated the method using two different cell types: primary human CPC that are emerging as a major cell source for Exo-based therapeutic approaches for heart disease; and HEK293 cells, an immortalized cell line widely used in cell biology research because of reliable cell growth and plasticity.

The method can be applied to ultracentrifuge-enriched EV and, for faster analysis, also directly on in vitro cell–derived CM with no pre-enrichment by ultracentrifugation. The starting material is critical when comparing samples. Adding capturing beads directly to the CM will speed up the procedure but at the same time decrease fluorescence intensity, as shown in **Figure 4A**. It is also critical to use an appropriate amount of PBS to mix beads and EV during the "capturing" step 4.4. When using a constant incubation time, an increased volume will decrease fluorescence intensity due to inefficient EV coupling.

A limitation of the protocol is that a single capture bead can bind multiple EV/Exo particles on its surface. This will limit the possibility of identifying subsets of EV expressing peculiar combination of antigens using a multiple staining. The bead-based method therefore yields semi-quantitative data. Using beads carrying a single capturing Ab (CD9, CD63 or CD81) will allow at maximum the characterization of particles that express two epitopes: the one present on the bead and recognized by the capturing antibody and the one detected by the antibody that is subsequently added.

The current gold standard for Exo analyses using FC is a protocol developed by van der Vlist et al. in 2012[15]. It allows for a high-resolution analysis of EV using an optimized configuration of the commercially available high-end FC (e.g., BD Influx). This protocol is extremely detailed and useful but still needs a complex hardware setting with specific FC calibration before use. Three years later, Pospichalova et al.[16] proposed a simplified protocol for FC analysis of Exo using a dedicated cytometer specifically developed for analysis of small particles (e.g., Apogee A50 Micro)[17]. With respect to this protocol and others that have used special threshold setting[11], here we propose a basic protocol to perform small EV phenotyping using magnetic binding beads that is suitable for conventional FC instruments and does not require any special setting. Different protocols have described bead-based methods to characterize small EV found in bodily fluids by FC[12]. Here, we show the immunocapture of discrete sub-populations of vesicles positive for CD9, CD63, and CD81 that are commonly used as Exo markers[18]. Aldehyde-sulfate latex[19,20] and polystyrene[12]

beads remain valid alternatives for binding of EV present in CM and blood plasma fluid; however, aldehyde groups exposed on the surface of the polymer particle enable coupling of unspecific proteins and other materials to the latex particles, thus increasing the risk of contamination by lipoprotein or apoptotic bodies during isolation and detection process[21,22].

Beads used in the protocol bind only entire, well-shaped EV. We proposed to disrupt EV structure by sonication to quench the signal (section 4, "protocol validation"). Indeed, one minute of sonication results in diminished fluorescence intensity, thus showing that a positive signal cannot be affected by membrane debris adsorbed on beads.

ACKNOWLEDGMENTS and FUNDING:

L.B. was supported by research grants of Helmut Horten Stiftung and Velux Stiftung, Zurich (Switzerland). G.V. was supported by research grants of Swiss National Science Foundation, the Cecilia-Augusta Foundation, Lugano, and the SHK Stiftung für Herz- und Kreislaufkrankheiten (Switzerland)

DISCLOSURES:

Nothing to declare.

REFERENCES:

1 Barile, L., Vassalli, G. Exosomes: Therapy delivery tools and biomarkers of diseases. *Pharmacology & Therapeutics.* doi:10.1016/j.pharmthera.2017.02.020 (2017).

2 Thery, C. Exosomes: secreted vesicles and intercellular communications. *Molecular Biology Reports.* **3**, 15, doi:10.3410/B3-15 15 (2011).

3 Yadav, D. K. et al. Liquid biopsy in pancreatic cancer: the beginning of a new era. *Oncotarget* **9**, 26900-26933, doi:10.18632/oncotarget.24809 (2018).

4 Balbi, C. et al. First Characterization of Human Amniotic Fluid Stem Cell Extracellular Vesicles as a Powerful Paracrine Tool Endowed with Regenerative Potential. *Stem Cells Translational Medicine.* **6**, 1340-1355, doi:10.1002/sctm.16-0297 (2017).

5 Andriolo, G. et al. Exosomes From Human Cardiac Progenitor Cells for Therapeutic Applications: Development of a GMP-Grade Manufacturing Method. *Frontiers in Physiology.* **9**, doi:10.3389/fphys.2018.01169 (2018).

6 Lai, R. C. et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Research.* **4**, 214-222, doi:S1873-5061(09)00141-X [pii] 10.1016/j.scr.2009.12.003.

7 Doeppner, T. R. et al. Extracellular Vesicles Improve Post-Stroke Neuroregeneration and Prevent Postischemic Immunosuppression. *Stem Cells Translational Medicine.* **4**, 1131-1143, doi:10.5966/sctm.2015-0078 (2015).

8 Kordelas, L. et al. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia.* **28**, 970-973, doi:10.1038/Leu.2014.41 (2014).

9 Barile, L. et al. Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction. *Cardiovascular Research.* **103**, 530-541, doi:10.1093/cvr/cvu167 (2014).

10 Longatti, A. et al. High affinity single-chain variable fragments are specific and versatile

targeting motifs for extracellular vesicles. *Nanoscale.* **10**, 14230-14244, doi:10.1039/c8nr03970d (2018).

11 Menck, K., Bleckmann, A., Schulz, M., Ries, L., Binder, C. Isolation and Characterization of Microvesicles from Peripheral Blood. *Journal of Visualized Experiments*. doi:10.3791/55057 (2017).

12 Inglis, H., Norris, P., Danesh, A. Techniques for the analysis of extracellular vesicles using flow cytometry. *Journal of Visualized Experiments*. doi:10.3791/52484 (2015).

13 Arakelyan, A. et al. Flow Virometry to Analyze Antigenic Spectra of Virions and Extracellular Vesicles. *Journal of Visualized Experiments*. doi:10.3791/55020 (2017).

14 Chimenti, I. et al. Isolation and expansion of adult cardiac stem/progenitor cells in the form of cardiospheres from human cardiac biopsies and murine hearts. *Methods Molecular Biology.* **879**, 327-338, doi:10.1007/978-1-61779-815-3_19.

15 van der Vlist, E. J., Nolte-'t Hoen, E. N., Stoorvogel, W., Arkesteijn, G. J., Wauben, M. H. Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry. *Nature Protocols.* **7**, 1311-1326, doi:10.1038/nprot.2012.065 (2012).

16 Pospichalova, V. et al. Simplified protocol for flow cytometry analysis of fluorescently labeled exosomes and microvesicles using dedicated flow cytometer. *Journal of Extracellular Vesicles.* **4**, 25530, doi:10.3402/jev.v4.25530 (2015).

17 van der Pol, E. et al. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *Journal of Thrombosis and Haemostasis.* **12**, 1182-1192, doi:10.1111/jth.12602 (2014).

18 Witwer, K. W. et al. Updating the MISEV minimal requirements for extracellular vesicle studies: building bridges to reproducibility. *Journal of Extracellular Vesicles.* **6**, 1396823, doi:10.1080/20013078.2017.1396823 (2017).

19 Thery, C., Amigorena, S., Raposo, G., Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current Protocols in Cellular Biology.* **Chapter 3**, Unit 3.22, doi:10.1002/0471143030.cb0322s30 (2006).

20 Suarez, H. et al. A bead-assisted flow cytometry method for the semi-quantitative analysis of Extracellular Vesicles. *Scientific Reports.* **7**, 11271, doi:10.1038/s41598-017-11249-2 (2017).

21 Sodar, B. W. et al. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. *Scientific Reports.* **6**, 24316, doi:10.1038/srep24316 (2016).

22 Sluijter, J. P. G. et al. Extracellular vesicles in diagnostics and therapy of the ischaemic heart: Position Paper from the Working Group on Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovascular Research.* **114**, 19-34, doi:10.1093/cvr/cvx211 (2018).

Figure 1

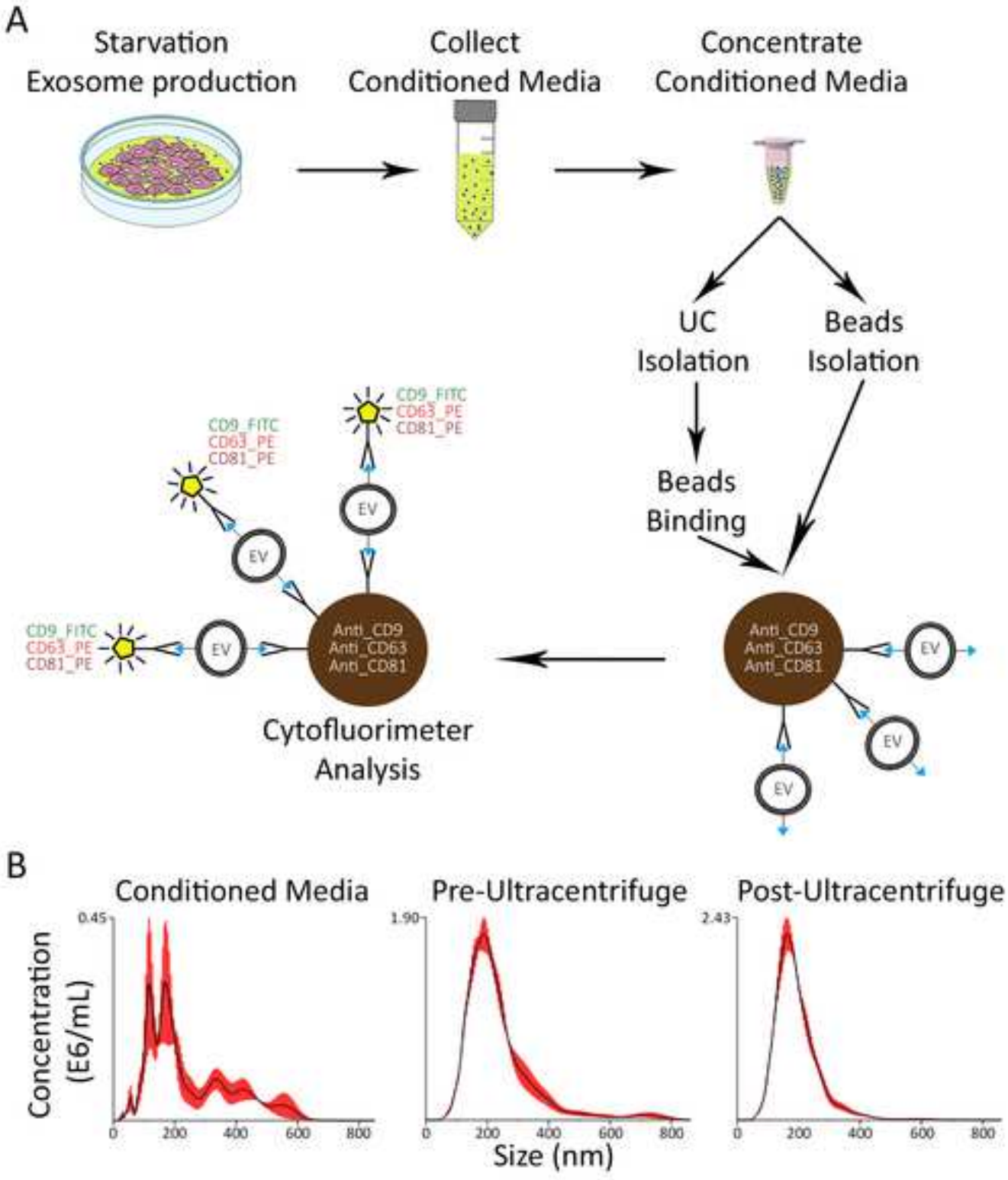

Figure 2

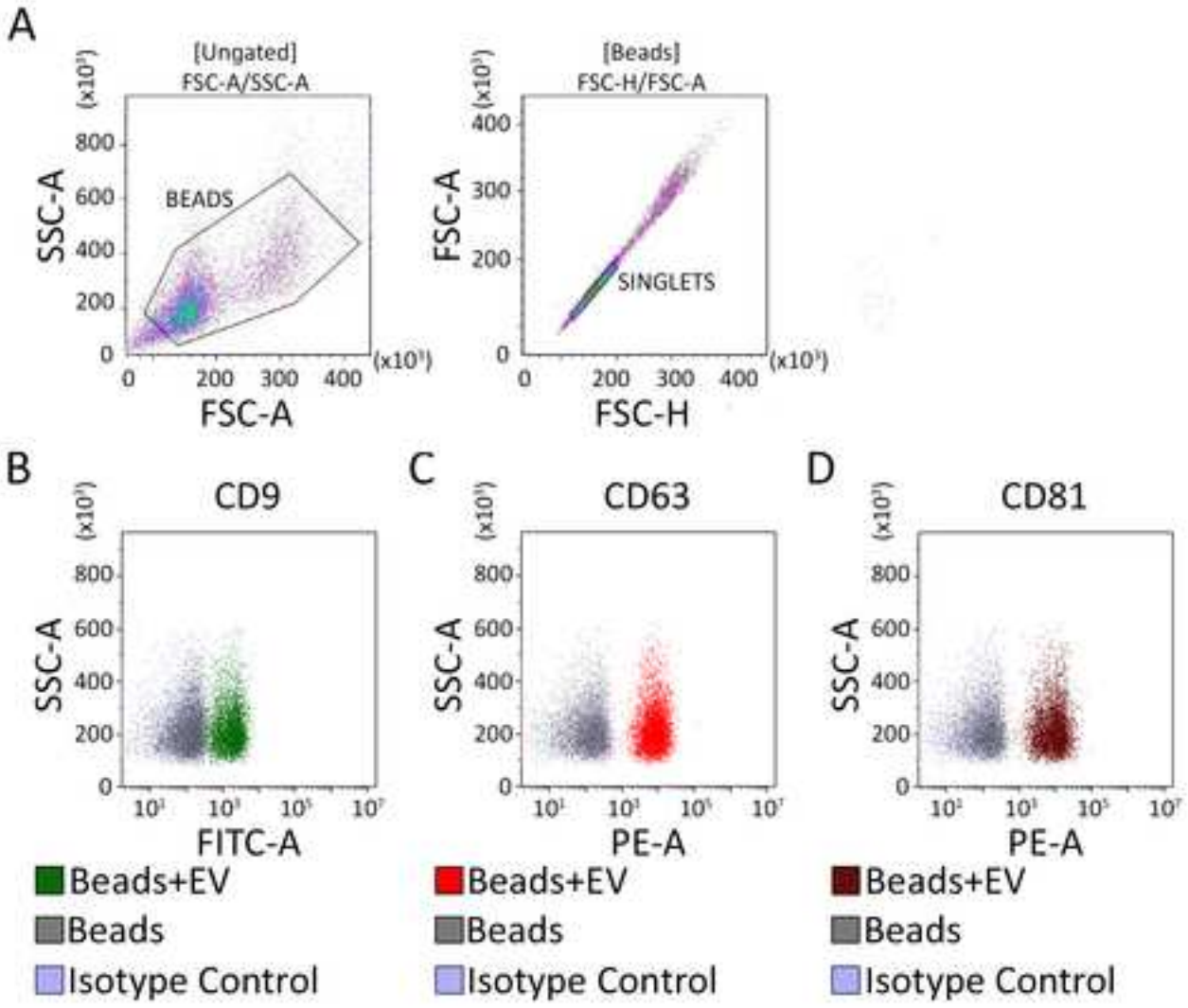

Figure 3

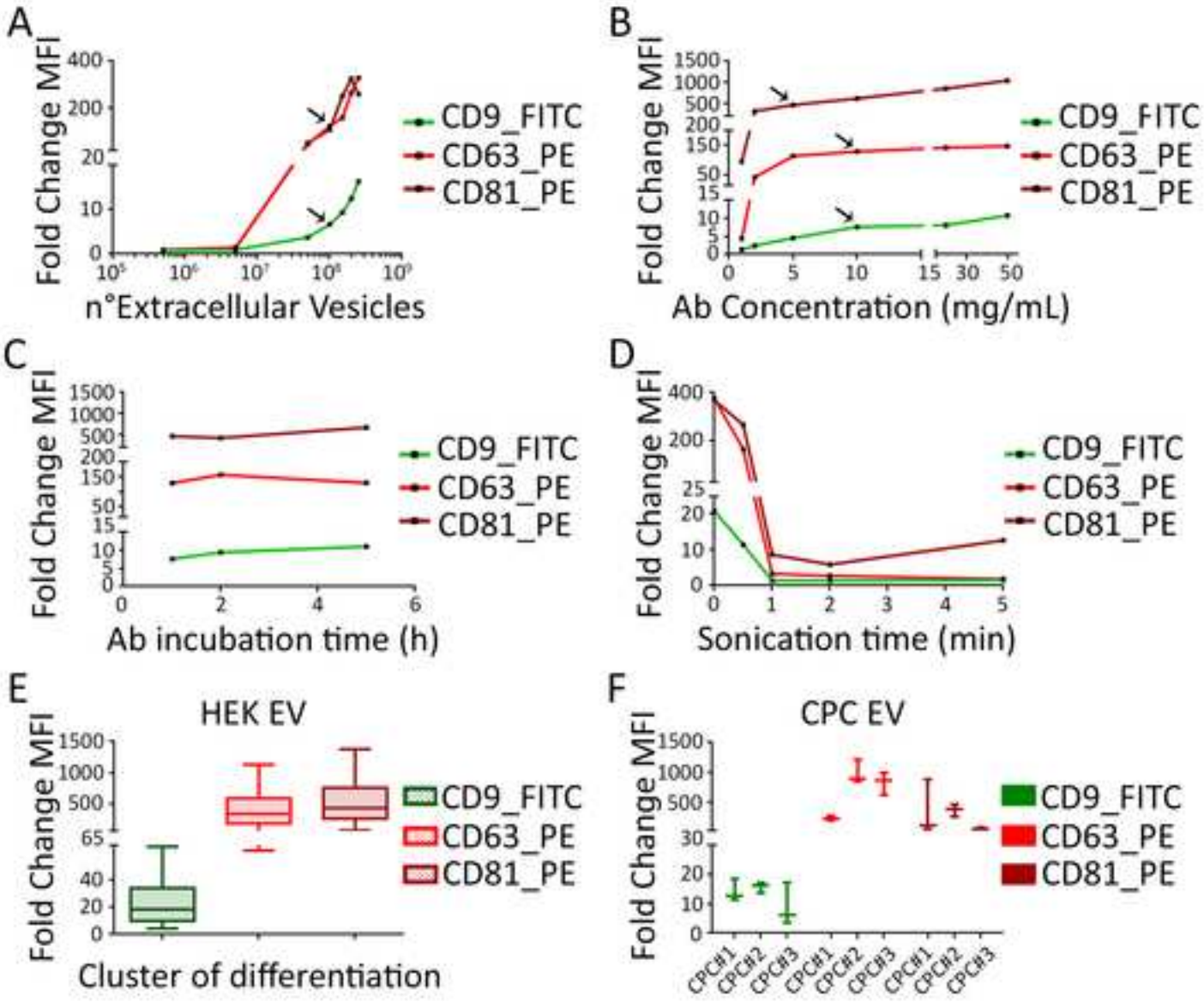

Figure 4

Click here to access/download;Figure;FIG4_FINALE.tif

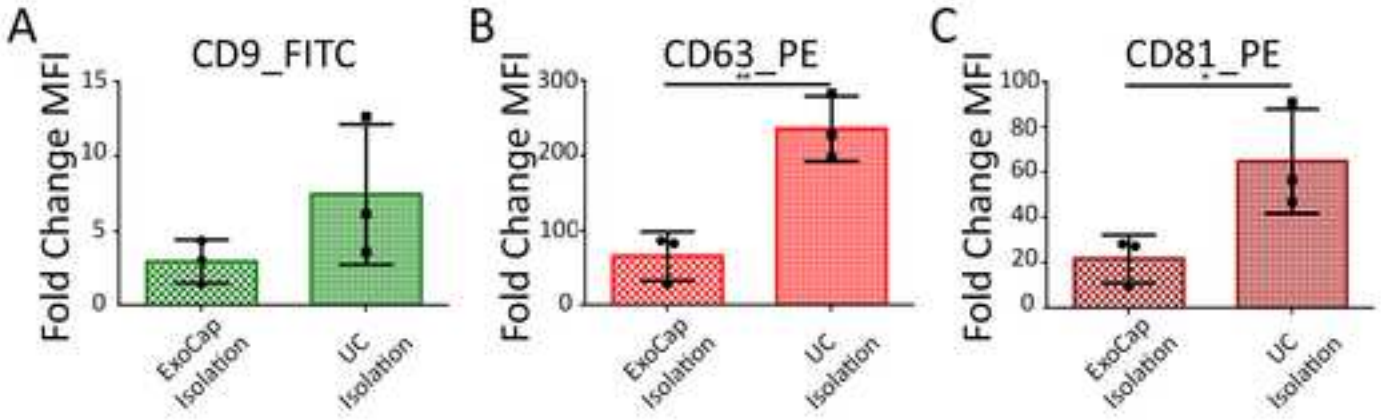

Table 1

CPC	CONC NTA (part/µL)	CONC (µg/µL)
CPC#1 Pre-Ultracentrifuge	5.02E+06	2.01
CPC#1 Post-Ultracentrifuge	6.10E+07	1.04
CPC#2 Pre-Ultracentrifuge	5.74E+06	2.30
CPC#2 Post-Ultracentrifuge	7.43E+07	0.79
CPC#3 Pre-Ultracentrifuge	2.02E+06	1.90
CPC#3 Post-Ultracentrifuge	2.91E+07	0.42

Table 2

FOLD CHANGE OF MFI ± SD	CD9	CD63	CD81
HEK293	24.44 ± 19.17	430.7 ± 344.9	535.2 ± 410.3
CPC#1	14.15 ± 3.72	236.05 ± 43.40	353.30 ± 452.43
CPC#2	15.76 ± 1.87	983.06 ± 195.63	374.45 ± 108.05
CPC#3	8.94 ± 7.19	830.50 ± 184.73	60.05 ± 23.18

Table 3

FOLD CHANGE OF MFI ± SD	CD9	CD63	CD81
Capture Beads Isolation	2.96 ± 1.45	65.65 ± 18.87	21.85 ± 6.12
Ultracentrifuge Isolation	7.47 ± 2.71	236.00 ± 25.06	65.05 ± 13.38

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

NAME OF MATERIAL / EQUIPMENT	COMPANY
IMDM	Gibco
Amicon Ultra-15, PLHK Ultracel-PL Membran, 100 kDa	Millipore
CytoFlex, Flow Cytometer Platform	Beckman Coulter
DMEM, high glucose, HEPES, no phenol red	Gibco
Dulbecco's PBS (PBS) Ca- and Mg-free	Lonza
ExoCap CD63 Capture Kit	JSR Life Sciences
ExoCap CD81 Capture Kit	JSR Life Sciences
ExoCap CD9 Capture Kit	JSR Life Sciences
Exosome-Depleted FBS	Thermofisher
Exosome-depleted FBS Media Supplement	SBI
FBS-Fetal Bovine Serum	Gibco
FITC anti-human CD9 Antibody	Biolegend
Flow Cytometer analysis software	Beckman Coulter
NanoSight LM10	Malvern
NanoSight Software	Malvern
Optima Max-XP	Beckman Coulter
PE anti-human CD63 Antibody	Biolegend
PE anti-human CD81 (TAPA-1) Antibody	Biolegend
Penicillin-Streptomycin	Gibco
Thermomixer C	Eppendorf
TLA-110	Beckman Coulter

CATALOG NUMBER	NOTES
12440061	
UFC910024	
CytoFlex	
21063045	
BE17-512F	
Ex-C63-SP	
Ex-C81-SP	
Ex-C9-SP	
A2720801	
EXO-FBS-250A-1	
10270106	
312104 RRID: AB_2075894	
Kaluza	
NanoSight LM10	
NTA 2.3	
393315	
353004 RRID:AB_10897809	
349505 RRID:AB_10642024	
15140122	
5382 000 015	
TLA-110 rotors	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Protocol for flow cytometry analysis of exosomes from cell-conditioned media

Author(s): Carolina Balbi, Sara Bolis, Giuseppe Vassalli and Lucio Barile.

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Lucio Barile
Department:	Cellular and Molecular Cardiology Laboratory
Institution:	Cardiocentro Ticino c/o Swiss Institute for Regenerative Medicine
Article Title:	Protocol for flow cytometry analysis of exosomes from cell-conditioned media
Signature:	Lucio Barile Firmato digitalmente da Lucio Barile ND: cn=Lucio Barile, o=CCT, ou, email=lucio.barile@cardiocentro.org, c=CH Data: 2018.09.19 16:00:45 +02'00'
Date:	September 19, 2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Reply to Reviewers and Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. What is the 'SETUP METHOD' section for? It is not referenced anywhere else. Please either continue the protocol with these steps or provide as a separate supplemental section (in a different file).

The "setup method" is thought to be performed to find the best working condition. It is not necessary to repeat these steps if EV type and antibodies do not change. However we think that are important before to start analysis, so we changed the numeration and included this part in the main protocol.

2. It is unclear which samples NTA quantification are done for. Right now, you direct users to possibly skip to sample prep (the current step 4) if an ultracentrifuge is not available.

It is expected to skip the ultracentrifuge step but not the total number quantification by NTA (or alternatively by protein quantification). This was not clear enough before, now we edited the text accordingly.

3. Figure 2A: You show gating for both beads and singlets here, but only mention gating for singlets in the legend; please clarify.

Legend has been now revised accordingly.

4. Figures 3E,F and 4: Please define the error bars in the legends. Also, what is N in Figure 4?

Legend has been now revised accordingly.

5. Tables 2-3: Please use US standards for decimals here (periods, not commas). Also, what are the errors?

Tables has been now revised accordingly.