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# Concentration Estimation from Flow Cytometry Exosome Data Protocol.

## Standard Curve

Create a standard curve for the target exosome by plotting the mean fluorescence (y axis) against the protein concentration (x axis). Draw a best fit curve through the points in the graph (we suggest that a suitable computer program be used for this).

It is recommended including a standard on each experiment batch to provide a standard curve for each day of use of the cytometer.

We recommend using a sample of known concentration as a positive control. The concentration of the positive control sample should be within the linear section of the standard curve in order to obtain valid and accurate results.

## Material Needed

1. Reagents: ExoStep™ family
2. Kit Components:

| Description | Components | Amount |
|---|---|---|
| Superparamagnetic Capture Beads | Anti-tetraspanin capture beads. Polystyrene micro-particles with Mean Diameter (µm) 6.5±0.2 (CV<5%), having discrete fluorescence intensity characteristics | According TDS |
| Primary detection antibody | Anti-tetraspanin biotin or anti-tetraspanin PE depending on your kit | |
| Secondary detection reagent<br>*(only if necessary)* | Streptavidin-Phycoerythrin (PE) is useful for detecting biotinylated antibodies. The excitation of PE by 488 nm laser light induces a light emission maximum of 578 nm. | |
| Assay Buffer 10X | PBS 10% BSA, pH 7,4 - 10x . Do not freeze. Dilute to 1X for use in this assay. | |

3. Tested application: Flow Cytometry
4. Exosome sample well characterized (n° vesicles / µl)

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## Assay

Tubes to be prepared and conditions:

| Tube | | Capture bead | Beads Volume | Exosomes PC3 (µl) | Detector Antibody | Incubation time | Incubation Temp. |
|---|---|---|---|---|---|---|---|
| 1 | Control (-) | Anti-tetraspanin magnetic beads | 100 µl | 0 | Biotin anti-tetraspanin | O/N | Room Temperature (RT) |
| 2 | Standard | Anti-tetraspanin magnetic beads | 100 µl | 0.125 | | O/N | RT |
| 3 | | | | 0.25 | | | |
| 4 | | | | 0.5 | | | |
| 5 | | | | 1 | | | |
| 6 | | | | 2 | | | |
| 7 | | | | 4 | | | |
| 8 | | | | | | | |
| 9 | | | | 8 | | | |

## Protocol

1. Prepare exosome sample to be used such as standard at two different concentrations: **1 µg /µl (± vol 25 µl) and 0,1 µg /µl (± vol 10 µl). Keep on ice.**
2. Prepare eleven 12x75 mm Polystyrene Round Bottom tube (cytometer tube) and label them with: 0,003125; 0,0625; 0,125; 0,25; 0,5; 1; 2; 4; 6 and 8.
3. Resuspend the capture beads by vortex for approximately 20 seconds.
4. Add 50 µL of the capture bead to each tube.
5. Add the exosomes as shown in the following table:

| µg | Vol [1 µg/µl] | Vol [0,1 µg/µl] |
|---|---|---|
| 0 | 0 | 0 |
| 0,03125 | | 0,3125 |
| 0,0625 | | 0,625 |
| 0,125 | | 1,25 |
| 0,25 | | 2,5 |
| 0,5 | | 5 |
| 1 | 1 | |
| 2 | 2 | |
| 4 | 4 | |
| 6 | 6 | |
| 8 | 8 | |

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6. Mix the reactives gently by pipetting up and down several times with a pipette and vortexing for few seconds.
7. Incubate in the dark overnight at room temperature (RT). NO STIRRING.
8. After overnight incubation, Add the suggested volume indicated (5µL) of the Primary detection antibody to the bead-bound exosomes tube.
9. Incubate in the dark 60 minutes at 2-8°C. NO STIRRING.
10. Wash the sample (bead-bound exosomes) by adding 1 ml of Assay Buffer 1X.
11. Collect the Magnetic beads by placing tubes on a magnetic rack and incubate 5 minutes.
12. Remove supernatant from tubes by Hand-decanting (Fig.1) or by aspiration. Take care not to disturb the microspheres, and make sure not to leave more than 100 ul of supernatant in the tube.

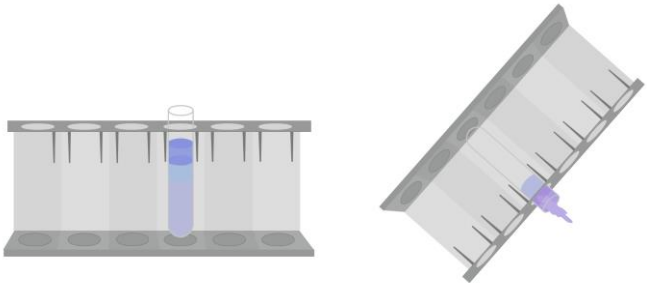

Figure 1: Hand-decanting supernatant using a Magnetic Rack

13. Remove the tubes from the magnetic rack and resuspend the microspheres in the remaining 100 µL of Assay Buffer 1X. Mix gently by pipetting.
14. Add 5 µL of the Secondary detection reagent to each tube. Mix the reactives gently by pipetting up and down several times with a pipette.
15. Incubate in the dark 30 minutes at 2-8°C, without stirring.
16. Wash the sample (bead-bound exosomes) by adding 1 ml of Assay Buffer 1X.
17. Collect the Magnetic beads by placing tubes on a magnetic rack and incubate 5 minutes. Remove supernatant from tubes by Hand-decanting (Fig. 1) or by aspiration. Take care not to disturb the microspheres, and make sure not to leave more than 100 ul of supernatant in the tube.
18. Resuspend the sample in 350 µL Assay Buffer 1X and Acquire on a flow cytometer or store in the dark max up to 2 hours at 2-8°C, until the analysis is carried out.

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- **Assay Acquisition**

An adequate gating strategy FSC / SSC and FL4 / FL6, helps to bead population identification and discrimination of doublets on flow cytometer.

1. Gate on the single population(s) on a Forward Scatter vs. Side Scatter plot in linear scale. (Figure 2, A)
2. Gate on the single population(s) on a FL4 vs. FL6 channel (bead auto fluorescence) in logarithmic scale (Figure 2, B).
3. Using the FL2 channels, determine whether or not any bead populations tested "positive" for the exosome. Note: A positive bead will produce a fluorescent peak in the FL2 channel.

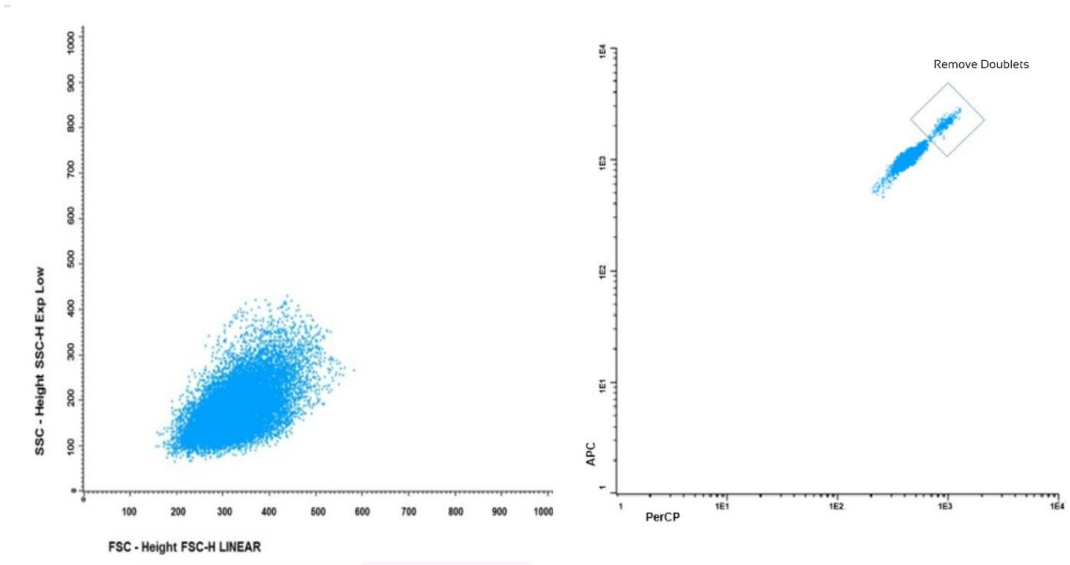


Figure 2 Dot-plot gating strategy for acquisition and analysis. FSC vs SSC and PerCP vs APC.

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- **Concentration of target exosome in the sample**

Several measurements of multiple concentrations exosomes were analyzed across the reportable range of the kit.

Results were observed to be linear within the range of 0.03 - 8 µg of exosomes in conventional flow cytometers, allowing concentration or quantity estimation of exosomes by flow cytometry with ExoStepTM kits.

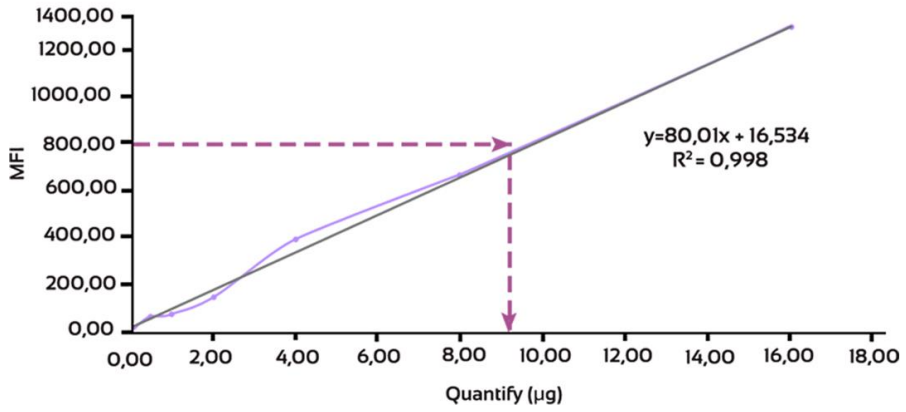


Figure 3: Linearity of assay was determined with the ExoStep™ kit on exosomes isolated from PC3 Cell Culture Media by conventional flow cytometry