

## Concentration Estimation from Flow Cytometry Exosome Data Protocol

### 1. 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.

### 2. MATERIAL NEEDED

- Reagents: ExoStep™ (Ref: : ExoS-25)
- Kit Components:

Description	Components	Amount	N° test
Superparamagnetic Capture Beads	CD63+ (Clone TEA3/18) capture beads. Polystyrene micro-particles with Mean Diameter ( $\mu\text{m}$ ) $6.5 \pm 0.2$ (CV<5%), having discrete fluorescence intensity characteristics	6000 beads/test (100 $\mu\text{l}$ /test)	25
Primary detection antibody	Anti-CD9 biotin (Clone VJI/20)	2 $\mu\text{g}$ /test (5 $\mu\text{l}$ /test)	25
Secondary detection reagent	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.	5 $\mu\text{l}$ /test	25
Assay Buffer 10X	PBS 10% BSA, pH 7,4 - 10x . Do not freeze. Dilute to 1X for use in this assay.	40 ml	

- Tested application: Flow Cytometry
- Exosome sample well characterized ( $n^\circ$  vesicles /  $\mu\text{l}$ )

### 3. ASSAY

Tubes to be prepared and conditions:

TUBE	Capture bead	BEADS VOLUME	EXOSOMES PC3 ( $\mu$ l)	Detector Antibody ( $\mu$ l)	Incubation time	Incubation T <sup>o</sup>
Control (-)	CD63 magnetic beads	100 $\mu$ l	0	CD9-BIOT (3)	O/N	Room Temperature (RT)
Standard	CD63 magnetic beads	100 $\mu$ l	0.125	CD9-BIOT (3)	O/N	RT
			0.25	CD9-BIOT (3)		
			0.5	CD9-BIOT (3)		
			1	CD9-BIOT (3)		
			2	CD9-BIOT (3)		
			4	CD9-BIOT (3)		
			8	CD9-BIOT (3)		

### 4. PROTOCOL

- Prepare exosome sample to be used such as standard at two different concentrations:
  - 1  $\mu$ g /  $\mu$ l ( $\pm$  vol 25  $\mu$ l) and 0,1  $\mu$ g /  $\mu$ l ( $\pm$  vol 10  $\mu$ l). Keep on ice.
- 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.
- Resuspend the capture beads by vortex for approximately 20 seconds.
- Add 50  $\mu$ L of the capture bead to each tube.
- Add the exosomes as shown in the following table:

$\mu$ g	Vol [1 $\mu$ g/ $\mu$ l]	Vol [0,1 $\mu$ g/ $\mu$ l]
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	

- Mix the reagents gently by pipetting up and down several times with a pipette and vortexing for few seconds.
- Incubate in the dark overnight at room temperature (RT). NO STIRRING.
- After overnight incubation, Add the suggested volume indicated (5 $\mu$ L) 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 IX.
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  $\mu$ l of supernatant in the tube.

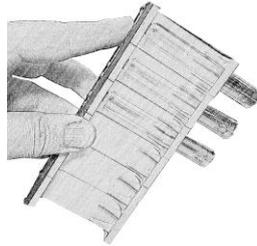


Figure 1: Hand-decanting supernatant using a Magnetic Rack

13. Remove the tubes from the magnetic rack and resuspend the microspheres in the remaining 100  $\mu$ L of Assay Buffer IX. Mix gently by pipetting.
14. Add 5  $\mu$ L of the Secondary detection reagent to each tube. Mix the reactivities 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 IX.
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  $\mu$ l of supernatant in the tube.
18. Resuspend the sample in 350  $\mu$ L Assay Buffer IX 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.

## 5. 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).

- 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.

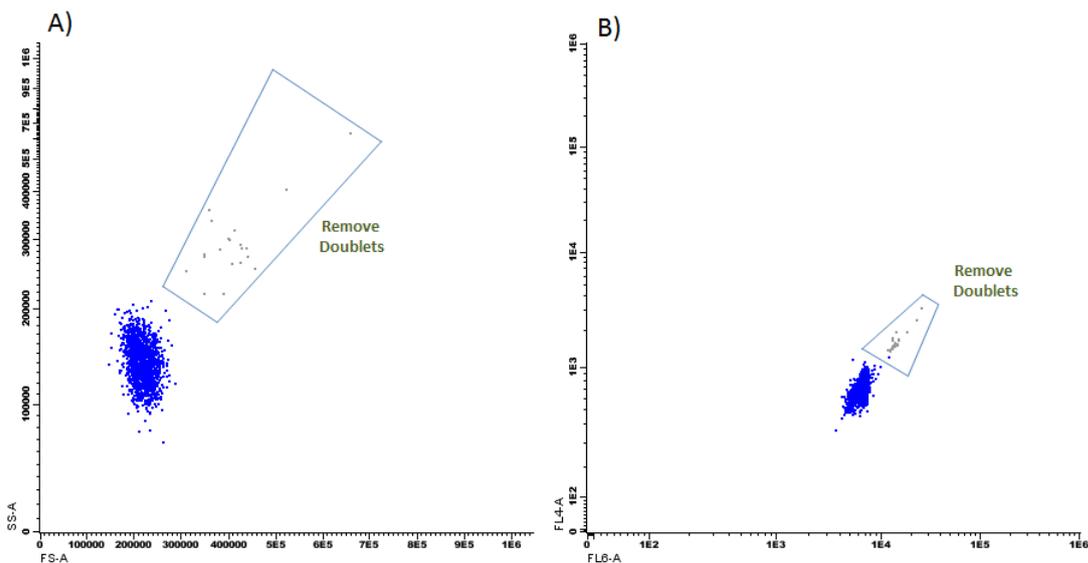


Figure 2. Dot-plot gating strategy for acquisition and analysis. FSC vs SSC (A) and FL4 vs FL6 (B)

## 6. CONCENTRATION OF TARGET EXOSOME IN THE SAMPLE

To determine the concentration of target exosome concentration in each sample, first find the mean fluorescence value of the sample. From the Y axis of the standard curve graph, extend a horizontal line from this fluorescence value to the standard curve.

For example, if the fluorescence reading is 5000, extend the line from this fluorescence point on the Y axis, and at the point of intersection, extend a vertical line to the X axis and read the corresponding concentration (Fig 3).

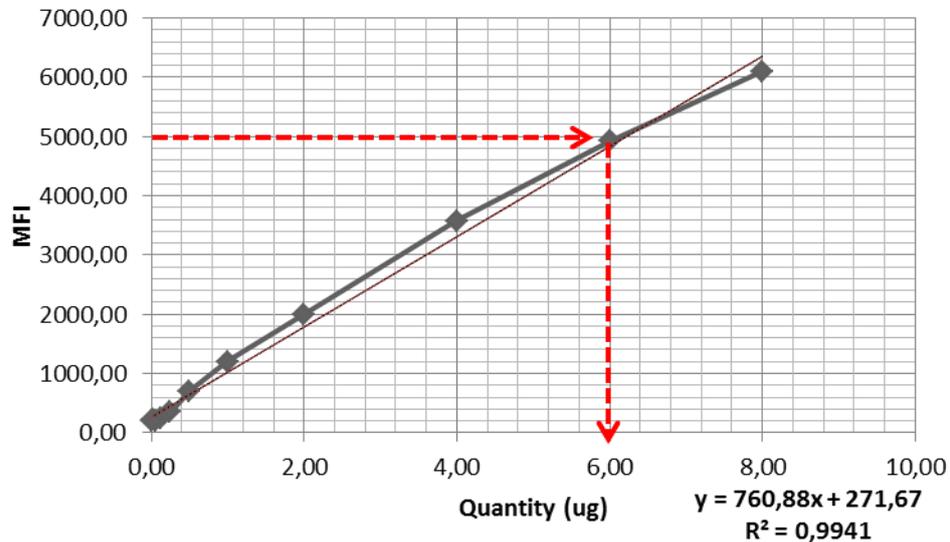


Figure 3. Linearity of assay was determined with the ExoStep kit on exosomes isolated from PC3 Cell Culture Media by conventional flow cytometry

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 ExoStep™ kits.