Megamix-Plus FSC

Beads for cytometer settings in microparticle analysis

Kit for 50 Tests **Ref. 7802**

For Research Use Only.
Not for Use in Diagnostic Procedures.

1 INTRODUCTION

Biological microparticles ("MP") are cellular vesicles of heterogeneous size ranging from 0.1 to 1 µm. Due to such small size, MP analysis requires working conditions close to the size-related sensitivity limit of the cytometers. Standardization of MP count requires to master this limit for an optimal compromise between MP analysis and background exclusion. This limit has significantly progressed with the advent of the latest generations of instruments from 0.5 down to 0.3 µm-eq.* (ref.1). Also, experience with Megamix have shown that FCM instruments may not all behave the same in terms of scatter response to sub-micron particles, some being optimized to use Forward Scatter (FS or FSC), e.g. Beckman-Coulter (BC) Gallios/Navios and others making best use of Side Scatter (SS or SSC) parameter, e.g. Becton-Dickinson FACS and LSRs (ref.2).
Megamix-Plus FSC reagent is most appropriate for use with FSC-optimized cytometers.

* µm bead-equivalents, special arbitrary unit used to remind that FCM can not provide absolute measurement of MP size but only reference points to standardize analysis (ref.1).

2 PRINCIPLE

Megamix-Plus FSC is a mix of fluorescent beads of varied diameters selected to cover the theoretical MP size range (0.1 to 1 µm). Beads acquisition according to the following procedure allows setting the cytometer to study MP within a constant size region (0.3 - 1 µm-eq.) and getting reproducible MP counts. The intrinsic numerical ratio of 2:1 from the 0.3 µm to 0.5 µm beads helps fine tuning FSC threshold for those instruments which only provide coarse discontinuous adjustment (e.g. BC Gallios). Finally, Megamix-Plus FSC also helps to transfer both the protocol and scatter settings from one cytometer to another and to check the stability over-time.

<u>Limitation:</u> flow cytometers using different optical designs for scatter collection may not feature enough FSC resolution to be used for standardized MP counts. This will lead to a bad resolution between 0.3 and 0.5 µm beads (no clear-cut separation in Fig. 3). Some of them may work better using SSC as the prime size-related threshold parameter. For these instruments, standardized MP analysis may rather be done using Megamix-Plus SSC beads (ref. 7803) that are optimized for the use of SSC. This alternative approach should provide similar MP counts despite using an other parameter and different reference beads (ref.2).

3 REAGENT PROVIDED

1 vial of 25 mL of beads.
Megamix-Plus FSC is a mix of beads with the following diameters: 0.1 µm, 0.3 µm, 0.5 µm and 0.9 µm.

4 MATERIAL REQUIRED BUT NOT PROVIDED

- Cytometer, FSC-optimized model (e.g. BC Gallios/Navios).
- Stirring machine type Vortex.
- Cytometry tubes (similar to those recommended for MP analysis, i.e. polypropylene tubes, with low dust and protein adsorption).
- Adjustable pipette with disposable tips (500 µL).

5 REAGENT PREPARATION AND STORAGE

Unopened vial remains stable until expiration date printed on the label when stored at 2-8°C. Do not freeze.
The reagent is ready for use.

Resuspend this reagent by vortexing for 10 seconds before use.
Stability after opening: until expiration date printed on the label at 2-8°C when free of contamination.

6 PROCEDURE

The reagent must be at room temperature for the procedure.
We recommend to run Megamix-Plus FSC beads before each series of MP analysis.

6.1 Bead preparation

Label a cytometry tube.
Pipette into it **500 µL** of Megamix-Plus FSC reagent after shaking vigorously the reagent vial for at least **10 secondes by vortexing.**

6.2 Generation of cytometric protocol and instrument settings

Refer to the Operator's Manual of the cytometer for instructions on how to perform cytometric readings.
Vortex the tube just prior to analysis.

For the acquisition protocol of Megamix-Plus FSC beads, proceed to the following settings:
- Scale: Log
- Time of analysis: 1 to 2 minutes
- Speed of analysis: the lowest available ("LOW", around 10-15µL/mn).

Then create :
- one cytogram FL1 Log x SS Log (density plot) and four rectangular regions A, B, C and D (Fig.1)
- one histogram FS Log x count gated by the Boolean gate "A or B or C" (Fig.2)
- one histogram FS Log x count gated by the Boolean gate " B or C" and two cursors E and F (Figs 3 and 4)
- one cytogram SS Log x FS Log, one autogate G and one rectangular region "MP" (Fig.5).

The following analysis has been performed on Gallios cytometer. The suggested settings below apply for this type of instrument and must be optimized for each individual cytometer:
- parameters: select signal integral ("XX INT") for all parameters
- specific FS option: W²
- gain SS at 7.5 and PMTv near 600v
- gain FS at 20 and PMTv near 440v (may widely vary for fine discriminator setting, see § 6.2.4)
- gain FL1 at 1 and PMTv near 520v

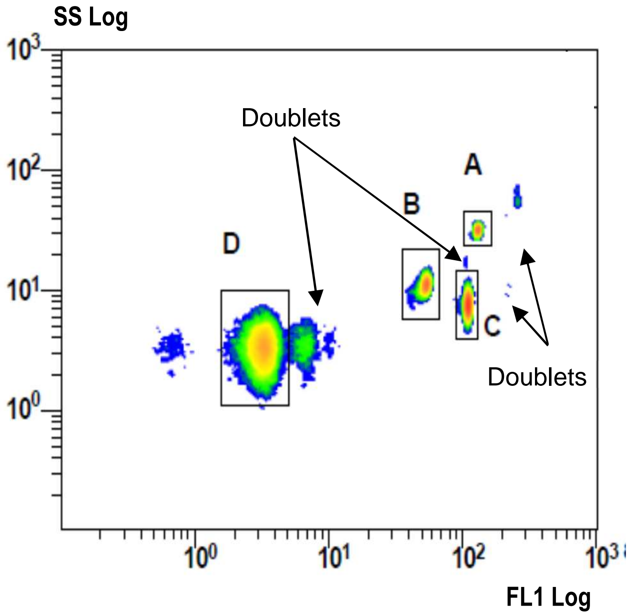
6.2.1 Settings of FL1 PMT and SS PMT (Fig.1)

- Proceed to acquisition of Megamix-Plus FSC beads (Mgx+).
- Add a **discriminator on FL1, threshold = 1** to only analyze FL1+ events and to eliminate non fluorescent events (background of the instrument).

On the FL1 Log x SS Log cytogram:

- Set regions "A", "B", "C" and "D" around each of the singlet bead populations as shown. Take care to gate each region around the dense cloud of beads corresponding to the singlets and not include doublets.
- Adjust the FL1 PMT voltage in order to set the 0.9 µm bead cloud at the beginning of the 4th FL1 decade (MFI ~ 100 to 200 a.u.).
- Adjust the SS PMT voltage in order to set the 0.9 µm bead cloud in the middle of the 3rd SS decade (MFI ~ 30 to 40 a.u.).

Fig.1: Settings of FL1 PMT and SS PMT



Region A: 0.9 µm beads Region B: 0.5 µm beads
Region C: 0.3 µm beads Region D: 0.1 µm beads

Check that the number of events in region C (0.3 µm singlets) is twice the number of events in region B (0.5 µm singlets, see also § 6.2.3), ratio 0.3/0.5 µm acceptable within range of 1.7 to 2.7.
Outside this range, refer to §8: Troubleshooting.

Option: Settings of other fluorescence PMT

Only 3 detectors (FS, SS and FL1/FITC) are specifically needed to run Megamix-Plus FSC beads. Additional detectors (FL2, FL3 … FL9…) may be needed to analyze stained MP and counting beads. These fluorescence detectors will need to be optimized with biological samples (see Fig.6 and ref.1) as well as counting beads.

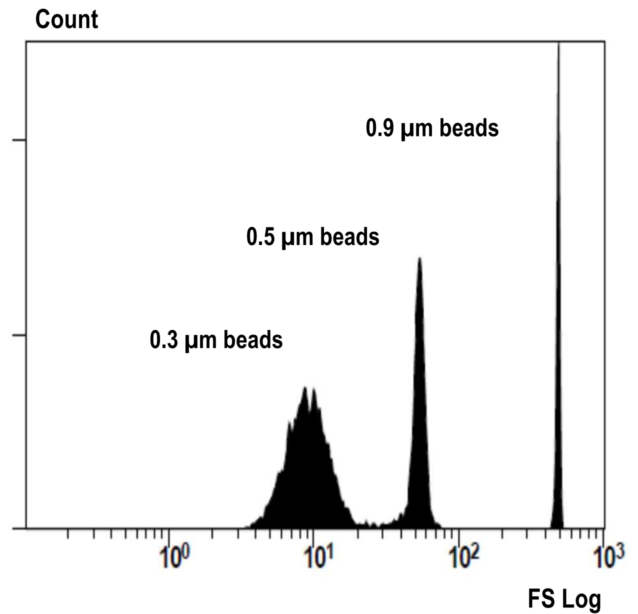
Note: Due to the necessary use of very high scatter settings for MP analysis, the diameter of counting beads should not exceed 7 µm (e.g. MP-Count Beads, BioCytex ref. 7804; see Fig. 7).

6.2.2 Evaluation of FS resolution (Fig.2)

- On the FS Log x Count histogram gated on 0.3, 0.5 and 0.9 µm bead singlets (gate "A or B or C"), check that 3 individual peaks can be discriminated, as illustrated in Fig.2. Overlapping of 0.3 µm with 0.5 µm peak would suggest a too low resolution impeding standardized MP analysis.

Option: FS parameter resolution can be described by a numerical parameter (see § 7).

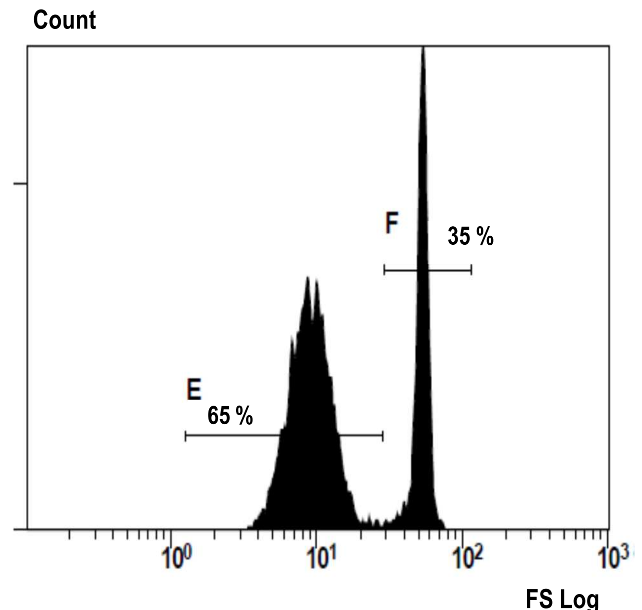
Fig.2: Beads distribution in FS parameter



- On the FS Log x Count histogram gated on 0.3 µm and 0.5 µm bead singlets (gate " B or C"), check that the 0.3 µm/0.5 µm bead ratio is close to 2. This corresponds to a percentage of nearly 68% (63% to 73% acceptable range) in region E and thus nearly 32% in the complementary region F (where E+F = 100%).

At that level, it is recommended to store the analysis as an electronic file (e.g. "Mgx+ FSC sFL1 xxx.LMD"). This helps monitoring instrument's behaviour over-time, see option above) or requesting technical support if needed.

Fig.3: Ratio of 0.3/0.5 µm beads with discriminator on FL1

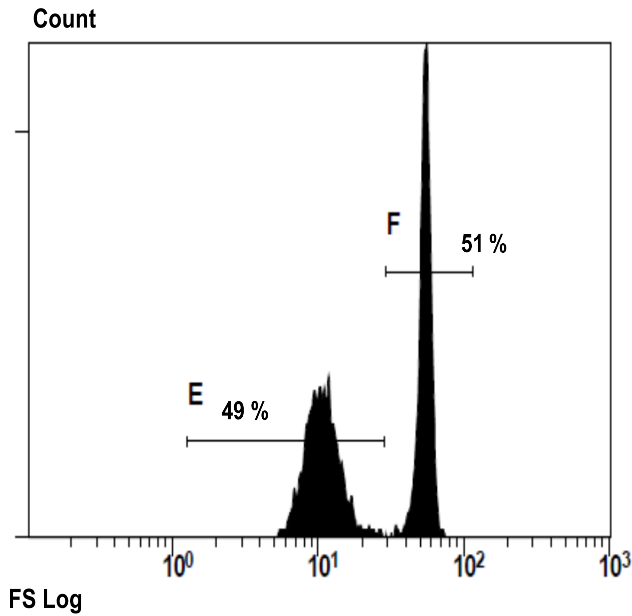


6.2.3 Settings of FS PMT (Fig.4)

- Run Megamix-Plus FSC beads once again.
- **Switch discriminator on FS, threshold ~ 8** (6 to 10, to be optimized).
- On the FS Log x Count histogram gated on 0.3 µm and 0.5 µm bead singlets (gate "B or C"), adjust FS gain and PMTv in order to obtain **50% of 0.3 µm bead** (region E) and thus 50% of 0.5 µm bead (region F). To decrease the 0.3 µm percentage, decrease FS PMTv and conversely.

A range of 48% to 52% is acceptable.

Fig.4: Ratio of 0.3/0.5 µm beads with discriminator on FS



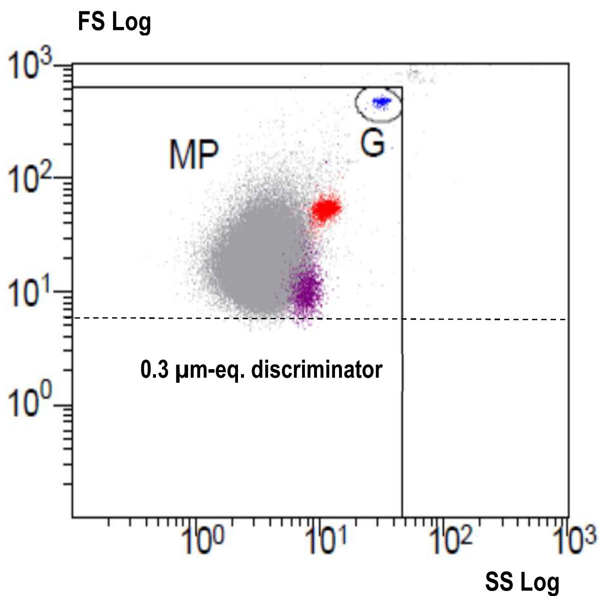
6.2.4 <u>MP analysis region determination</u> (Fig.5)

The MP analysis region is defined as follows:
- the lower side is defined by the threshold on FS (here shown as a dashed line) allowing acquisition of events of at least 0.3 µm-eq. (settings already performed at § 6.2.3).
- the upper side is the end of the 0.9 µm bead cloud.

To achieve this, on the SS Log x FS Log cytogram (Fig.5), set the autogate "G" (maximum sensitivity i.e. "0.11") around the 0.9 µm bead cloud and set the "MP" gate tangent to the upper and right sides of the autogate "G".

For other instruments/softwares, autogate may be replaced by a contour plot with "log density".

Fig.5: MP gate
Blue: 0.9 µm beads Red: 0.5 µm beads
Violet: 0.3 µm beads Grey: background



6.3 Routine analysis of Megamix-Plus FSC

Acquisition protocol being created (cf paragraph 6.2) proceed to acquisition of Megamix-Plus FSC bead tube.

- <u>Using FL1 threshold, check that the beads are located within the pre-existing regions</u> (A, B, C and D). If not, adjust FL1 PMT and/or SS PMT to achieve it. Option: store electronic file

- <u>Using FS threshold, check</u> on FS Log x Count histogram that the indicated <u>0.3 µm bead percentage is close to 50%</u> (region E: 48-52%). If not, adjust FS PMT to achieve it.

- Check that the <u>"MP" gate is still correctly set</u>. If not, adjust its position.

After checking these 3 conditions, cytometer settings are optimal and allow a standardized acquisition of MP above 0.3 µm-eq.

6.4 Example of platelet MP staining: PMP (Figs 6)

Here is an example of PMP analysis made on a platelet-free plasma sample following AnnexinV-FITC/CD41-PE staining and appropriate fluorescence compensation settings. Figs 6 illustrate the presence of 2 subsets of PMP, a so-called "large PMP" subset which shows the highest levels of both Annexin V and CD41 staining as well as of dual scatter parameters and a so-called "small PMP" subset with the lowest levels of all size-related and fluorescence parameters. FS level of the gap between both subsets is generally close to 0.5 µm bead-eq, as illustrated by the "AC" region (low FS level = median of 0.5 µm beads) in Fig.6d.

Fig.6a: Density plot of TMP (total MP) gated on "MP gate"
PMP subsets in the upper right quadrant

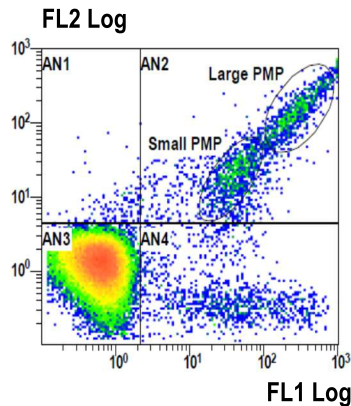


Fig.6b: Density plot of PMP subsets size distribution
PMP from upper right quadrant of Fig.6a

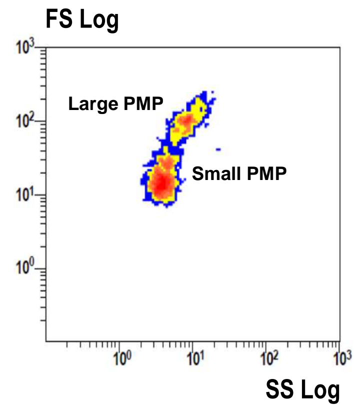


Fig.6c: Color dot-plot of TMP (total MP) gated on "MP gate".
Blue: "small PMP" Red: "large PMP"
Grey: background & non PMP events

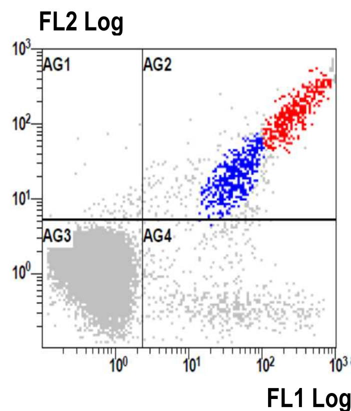


Fig.6d: Color dot-plot of PMP subsets size distribution.
Blue: "small PMP" Red: "large PMP"

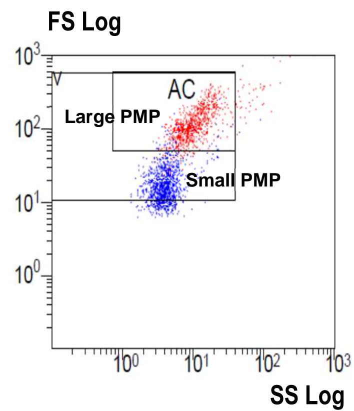
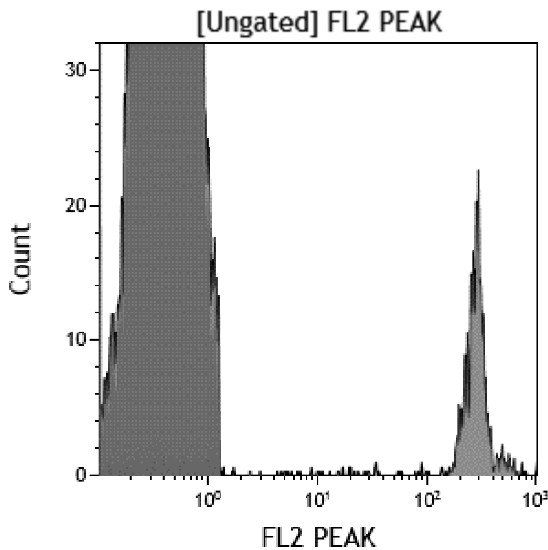


Fig.7: Counting beads MP-Count Beads
(FL2 ungated)



7 Monitoring of FS resolution

FS parameter resolution can be described by a numerical parameter called Separation Index or S.I. (adapted from ref.3). This provides opportunity for instrument monitoring (e.g. Levy-Jennings plots) or inter-instrument comparisons in terms of FS resolution.

$S.I._{0.5\text{-}0.3} = [Md_{0.5\ \mu m} - Md_{0.3\ \mu m}] / [SD_{0.5\ \mu m} + SD_{0.3\ \mu m}]$ where:
$Md_{0.5\ \mu m}$ = Median FS of the 0.5 µm (resp. 0.3 µm) beads
$SD_{0.5\ \mu m}$ = Standard Deviation FS of the 0.5 µm (resp. 0.3 µm) beads.

$S.I._{0.5\text{-}0.3}$ is currently in the range of 3 to 8 a.u.* using BC Gallios/Navios.
A value below 3 is sign of an unacceptable FS resolution. This may suggest requesting a microparticle-dedicated technical service from Beckman-Coulter, with optics cleaning and realignment.
* This range of values should be determined for any other instrument.

$S.I._{0.9\text{-}0.5} = [Md_{0.9\ \mu m} - Md_{0.5\ \mu m}] / [SD_{0.9\ \mu m} + SD_{0.5\ \mu m}]$
This index has been studied for standard cytometers from previous generation (e.g. BC FC500) using Megamix beads (ref. 7801).

$S.I._{0.9\text{-}0.5}$ is currently in the range of 25 to 35 a.u. using a BC Gallios/Navios in optimal FS resolution status.

8 Troubleshooting

If the ratio is lower than 1.7:

- check that bead subsets have been properly assigned, based on the typical pattern illustrated in Fig.1, with 0.3 µm beads being located between 0.5 µm and 0.9 µm beads in the FL1 scale (Megamix-Plus FSC typical pattern).

If the ratio is higher than 2.7:

- check that bead subsets have been properly assigned, based on the Megamix-Plus FSC typical pattern illustrated in Fig.1. One typical error would be to assign 0.5 µm and 0.9 µm singlets to 0.9 µm singlet and doublet clouds, respectively.
- check that FL1 settings (threshold and PMTv) do not induce a cut among the 0.5 µm bead cloud. In such a case (very high FL1 discriminator or very low FL1 PMTv) the 0.1 µm bead subsets would be invisible and the 0.5 µm/0.9 µm ratio << 2 (loss of 0.5 µm beads).
- check that the instrument in use does not miss the biggest and brightest 0.9 µm subset in the mix. In such a case, 0.9 µm would progressively appear when increasing FS (or FL1) threshold level. Although there is no such risk using BC Gallios, this kind of thresholding artefact has been encountered with some new instruments.

If you are using polystyrene tubes (e.g. Falcon 4 mL FACS tubes), check that the bead suspension has not spent more than 30 min waiting in this tube (risk for differential disappearance of beads sticking to the tube wall).

REFERENCES

1) Robert S et al, Artherioscler Thromb Vasc Biol, 2012; 32: 1054-58.
2) Poncelet P et al, Transf Apher Sci 2015,53(2):110-26.
3) Poncelet P et al, Cytometry A. 2016;89(2):148-58.
4) Cointe S et al, J Thromb Haemost 2016, 14: 1-7.

BIOCYTEX
140 ch. DE L'ARMEE D'AFRIQUE
13010 MARSEILLE
FRANCE
TEL : +33 (0) 4 96 12 20 40
FAX : +33 (0) 4 91 47 24 71

Version April 2017