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► To cite this version:

Sylvie Cointe, Coralie Judicone, Stephane Robert, Mj Mooberry, Philippe Poncelet, et al.. Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop. *Journal of Thrombosis and Haemostasis*, 2017, 15 (1), pp.187-193. 10.1111/jth.13514 . hal-01459831

HAL Id: hal-01459831

<https://hal-amu.archives-ouvertes.fr/hal-01459831>

Submitted on 9 May 2018

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Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop.

Running Head : Standardization of MP counts by flow cytometry

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1 **Essentials**

- 2 • The clinical enumeration of microparticles (MPs) is hampered by a lack of
3 standardization.
- 4 • A new strategy to standardize MP counts by flow cytometry was evaluated in a
5 multicenter study.
- 6 • No difference was found between instruments using forward or side scatter as the
7 trigger parameter.
- 8 • This study demonstrated that beads can be used as a standardization tool for MPs.

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1 **Summary**

2 **Background:** Microparticles are extracellular vesicles resulting from the budding of cellular
3 membranes that have a high potential as emergent biomarkers; however, their clinical
4 relevance is hampered by methodological enumeration concerns and a lack of
5 standardization. Flow cytometry (FCM) remains the most commonly used technique with
6 the best capability to determine the cellular origin of single MPs. However, instruments
7 behave variably depending on which scatter parameter, (Forward (FSC) or Side
8 scatter (SSC)), provides the best resolution to discriminate submicron particles. To overcome
9 this problem, a new approach, based on two sets of selected beads adapted to FSC or SSC
10 optimized instruments, was recently proposed to reproducibly enumerate platelet-derived MP
11 counts among instruments with different optical systems. **Objective:** The objective was to
12 evaluate this strategy in an international workshop that included 44 laboratories accounting
13 for 52 cytometers of 14 types. **Methods/Results:** Using resolution capability and background
14 noise level as criteria to qualify the instruments, the standardization strategy proved to be
15 compatible with 85% (44/52) instruments. All instruments correctly ranked the PMP levels of
16 two platelet-free plasma samples. The inter-laboratory variability of PMP counts was 37%
17 and 28% for each sample. No difference was found between instruments using forward or side
18 scattered light as the relative sizing parameter. **Conclusions:** Despite remaining limitations,
19 this study is the first to demonstrate a real potential of bead-based strategies for
20 standardization of MP enumeration across different FCM platforms. Additional
21 standardization efforts are still mandatory to evaluate MP clinical relevance at a multicenter
22 level.

23 **Keywords**

24 Cell-derived microparticles, extracellular vesicles, flow cytometry, multicenter study,
25 standardization.

1 **Introduction**

2 Among extracellular vesicles, microparticles (MPs) are sub-micron sized vesicles
3 released by blebbing from cell membranes in response to activation or apoptosis. MPs
4 originate from blood and vascular cells, and plasma levels are elevated in a variety of
5 prothrombotic and inflammatory disorders, cardiovascular diseases, autoimmune disorders,
6 infectious diseases, and malignancies [1].

7 Although MP counts may provide useful diagnostic/prognostic information,
8 assessment of their pathophysiological relevance in multicenter studies is hampered by
9 methodological concerns and a lack of standardization. Among the various methodologies
10 available to measure MPs in biological samples, flow cytometry (FCM) remains the most
11 commonly used technique with the highest potential to determine the cellular origin of single
12 MPs [2]. Over the past few years, significant improvements have been made in the sensitivity
13 of flow cytometers to detect vesicles of smaller size, and thus have confirmed this
14 methodology as the most promising for routine enumeration of MP subsets [3-5].

15 Six years ago, a first collaborative workshop defined the inter-laboratory
16 reproducibility of platelet MP (PMP) counts using FCM [6]. The standardization strategy was
17 based on the forward light scatter (FSC) signal of size-calibrated latex beads to set a common
18 MP window of analysis [7]. However, the variety of optical designs among flow cytometer
19 (FCMr) sub-types impeded a universal standardization strategy for PMP enumeration. Since a
20 better resolution and a more homogeneous response of instruments was observed in a
21 subgroup of FCMrs using the light scatter signal measured at 90° (Side scatter, SSC) rather
22 than FSC, a new set of beads was selected to better suit the design of these SSC-oriented
23 instruments [8]. Correspondence between the two sets of beads was accurately determined so
24 that similar PMP counts were obtained on both types of FCMrs. Thus, a new standardization
25 strategy is proposed based on the use of two types of beads, each adapted to instruments of

1 different optical design. Based on this strategy, the International Society on Thrombosis and
2 Haemostasis (ISTH) Vascular Biology Standardization Subcommittee organized an additional
3 workshop to evaluate the inter-instrument reproducibility of PMP counts among different
4 platforms.

5

6 **Materials and methods**

7 **Study design**

8 The study was conducted in two stages over a two-year period. The first stage was
9 aimed at qualifying the instruments for the standardization strategy according to required
10 performance levels of scatter resolution and background noise. This step led to acceptance or
11 rejection of the tested instrument(s). In the second stage, the inter-instrument reproducibility
12 of 3 different platelet free plasma (PFP) samples, prepared by the core laboratory and
13 featuring defined levels of PMP subsets, was evaluated using common reagents and the
14 standardized protocol.

15

16 **Cytometers**

17 The study included 44 laboratories from 17 different countries, accounting for 52 registered
18 cytometers. The tested instruments included 11 FACSCanto (I/II), 6 FACSCalibur, 2
19 FACSVerse, 5 FACS Aria (I/II), 4 LSR II, 3 LSR Fortessa, 1 Influx and 2 Accuri C6 from
20 Becton-Dickinson (BD, Franklin lakes, NJ, US), 1 EPICS XL, 2 FC500 and 12
21 Gallios/Navios from Beckman-Coulter (BC, Miami, FL, US), 1 Apogee A50 micro (Apogee
22 System, Hertfordshire, UK), 1 Guava EasyCyte (Millipore, Hayward, CA, US) and 1
23 Stratedigm S1000 EXi (Stratedigm, San Jose, CA, US).

24

25

1 **Standardization beads**

2 Megamix-Plus FSC or SSC beads were provided by BioCytex (Marseille, France) to the core-
3 lab that distributed them to participants according to their instrument's characteristics.
4 Megamix-Plus SSC is a ready-to-use mix of fluorescent [polystyrene](#) beads of various
5 diameters (0.16 μm , 0.20 μm , 0.24 μm and 0.5 μm) dedicated to flow cytometers using SSC
6 as the best resolving size-related parameter. Megamix-Plus FSC is a mix of fluorescent
7 [polystyrene](#) beads of various diameters (0.1 μm , 0.3 μm , 0.5 μm and 0.9 μm) dedicated to
8 FCMrs using FSC as the best resolving size-related parameter. The intrinsic numerical ratio of
9 2:1 from the 0.3 μm to 0.5 μm beads facilitates fine-tuning of the FSC threshold [3].
10 According to the instrument characteristics, standardization beads were tested as follows: 1)
11 Megamix-Plus FSC: Gallios, Navios, FC500, Epics XL and Guava. 2) Megamix-Plus SSC:
12 FACSAria (I/II), LSR II (+/- Fortessa), FACSCanto (I/II), FACSVerse, FACSCalibur, Accuri
13 C6 and Megamix-Plus FSC and Megamix- Plus SSC: Influx, Apogee A50 and Stratadigm.

14

15 **Flow cytometry reagents**

16 The common flow cytometry reagents for PMP staining were annexinV-FITC (fluorescein)
17 (Tau Technologies, Kattendijke, Netherlands) and its associated binding buffer, CD41-PE
18 (phycoerythrin; clone PL2-49) and its concentration-matched isotype control IgG1-PE (clone
19 2DNP-2H11/2H12), both from BioCytex. Counting beads (3 μm , MP-Count beads, prototype
20 version) were from BioCytex.

21

22 **Platelet-free plasma preparation**

23 [All Platelet-free plasma \(PFP\) were prepared at the core laboratory. Briefly, blood from](#)
24 [healthy donors, who signed an informed consent, was collected](#) with a 21-gauge needle in
25 0.129 M citrated tubes after discarding the first 2 ml. PFP was prepared according to a

1 published protocol using two successive centrifugations, each of 15 minutes at 2,500g [9, 10]
2 with the following modifications: Sample A was prepared after agitation of the blood tubes at
3 room temperature on a rotating wheel for 2 hours. Sample C was prepared after a 2 hour delay
4 without agitation. Samples A and C were prepared from a pool of 10 donors, whereas sample
5 B was from a unique donor. Aliquots (200 μ l) of PFP were stored at -80°C until use (less than
6 6 months). Inter-aliquot variability of PFP samples was measured on a single instrument
7 (Gallios) by the core-lab over a 2 month period, yielding values with CVs of 14%, 8% and
8 24% (n=18) for samples A, B and C, respectively. The variability of a triplicate measurement
9 of one aliquot was also found to be acceptable, resulting in CVs of 10%, 8% and 16% (n=6).
10 Given the high variability of PMP counts on sample C, results with this sample were
11 retrospectively excluded from the study. The preparation of this sample as a mixture of
12 plasma from different blood groups may have generated MP aggregates with an impact on
13 MP count reproducibility.

14

15 **Instrument qualification**

16 Instrument qualification to enable the proposed strategy was based on 2 criteria [8]. First, a
17 sufficient resolution was required to resolve small beads whose size depends on the selected
18 scatter parameter (0.3 μ m and 0.5 μ m for FSC and 0.16 μ m and 0.2 μ m for SSC). This was
19 attested by a scatter sensitivity index > 3 ($SSI = (\text{Median bead A} - \text{Median bead B}) / (\text{SD bead}$
20 $A + \text{SD bead B})$ [11]. The second criterion was based on a background noise ratio (BNR)
21 which was defined as the ratio between the number of events per second measured in the
22 protocol settings and the maximal number of events per second acceptable by the instrument
23 without significant abort rate (FACSCanto I/II = 4000, FACSCalibur = 2000, FACSaria I/II
24 = 4000, LSR II (+/- Fortessa) = 4000, FACSVerse = 4000, Apogee A50 = 2000, Stratadigm =
25 4000, Gallios/Navios = 5000, Influx = 15,000, defined according to both instrument

1 [specifications and core lab validation](#)). BNR was evaluated on filtered distilled water and
2 should be lower than one in order to avoid impeding the instrument's electronic system.

3

4 **Protocol setting**

5 The standardization protocols were set according to the manufacturer's instructions for SSC
6 and FSC Megamix beads. For FSC-optimized instruments, the MP analysis region was
7 defined as follows: 1. the upper boundary was determined by the edge of the 0.9 µm bead
8 cloud, and 2. the lower boundary was defined by the threshold on FSC that allowed inclusion
9 of 50% of the 0.3 µm beads in the analysis. A range of 48% to 52% was considered
10 acceptable [3]. For SSC-optimized instrument, the upper boundary of the MP analysis region
11 was determined by the end of the 0.5 µm bead peak (e.g. 99th percentile). The lower
12 boundary was set according to the product insert following the formula: Low SSC-H level =
13 Median 0.16 µm beads + (0.3 x (Median 0.20 µm beads – Median 0.16 µm beads)) [8]. The
14 [MP protocol settings were optimized as follows](#): a) Scatter settings were optimized recording
15 PEAK (= HEIGHT) signals. b) Low flow rate was selected and acquisition time was
16 optimized according to the MP count beads (60 s when the total number of MP Count beads
17 in 1 minute ranged from 500 to 2,000 or 120 s if MP Count beads were < 500). c)
18 Fluorescence settings were optimized by setting FL1 and FL2 PMT voltages to reach pre-
19 defined target values (median intensities) for single fluorescence positive beads ("Fluo-
20 Setting-Beads" (FSB), designed by BioCytex for this exercise). Briefly, blank beads as well as
21 high intensity FITC-labelled and PE-labelled beads were mixed extemporaneously, and
22 staining reagents (AnnV-FITC + CD41-PE) were added at the same final concentrations as in
23 plasma samples, thus providing a comparable level of non-specific fluorescence background.
24 d) Compensation settings were set up using single fluorescence labelling of PFP samples. e)
25 Positive and negative region boundaries were defined using concentration-matched isotype

1 control and AnnV-FITC in filtered PBS without calcium so that <0.1% of events were
2 included in the positive gates. [Detailed instructions for optimization of the MP protocol](#)
3 [setting can be found in supplemental document 1.](#)

4

5 **PMP counting experiments**

6 Three PMP counting experiments were performed for each PFP sample operated in
7 independent series. Before running each series of samples, standardized scatter settings were
8 checked with Megamix-Plus and fluorescence target channels assessed with Fluo-
9 SettingBeads. 30 μ l of PFP were incubated for 20 minutes with 10 μ l of AnnV-FITC and 5 μ l
10 of CD41-PE, and then diluted in 1 mL of Binding Buffer. A negative control was performed
11 for each PFP, by incubating 30 μ l of PFP with 10 μ l of AnnV-FITC and 5 μ l of IgG1-PE, and
12 diluting the sample in PBS without calcium. In order to derive absolute PMP counts per μ L of
13 plasma, 30 μ l of counting beads (MP-Count beads) were added before running the samples.
14 PMP concentration in plasma was calculated according to the formula: events/ μ L = Double
15 positive events x Counting bead concentration/ Number of Counting Beads. Non-specific
16 events/ μ L in the control tube were subtracted from the PMP counts.

17

18 **File transfer and re-analysis**

19 All electronic raw data (listmode) files corresponding to instrument qualification, protocol
20 setting and PFP analysis were sent to the core laboratory in fcs (flow cytometry standard
21 format) 2.0 or fcs 3.0. Files were re-analyzed by the core-lab using the same software (Kaluza
22 v1.2 software, Beckman Coulter). In the event of irreversible discrepancies with the protocol
23 instructions, data were not accepted for final analysis.

24

25

1 **Statistical analysis**

2 All statistical analyses were performed using GraphPad Prism software v.5.0 (GraphPad
3 Software, San Diego, CA). Each PFP was analyzed in triplicate and the mean of this triplicate
4 (xi) was considered for further analysis. The robust mean (X^*) and robust standard deviation
5 (SD^*) of these data were calculated, taking into account only the results from cytometers with
6 values between median \pm SD [12]. The Mann-Whitney test was used to compare instrument
7 families. A result was considered significant if $p < 0.05$.

8

9

10 **Results**

11 **Instrument qualification**

12 Instruments were qualified for the standardization strategy according to their
13 resolution capability and low background noise. As illustrated in table 1, with the exception of
14 two Accuri C6 and one FACSAria, most instruments using exclusively the SSC strategy
15 showed an SSI > 3 , indicating that the resolution was sufficient to enable the proposed
16 strategy. Among these instruments, LSRII (\pm LSRII Fortessa) showed the best resolution
17 (SSI = 7.9 ± 1.3 , $n=7$). All SSC-FCMr (except a FACSCalibur) showed a background noise
18 that was acceptable in the standardization protocol settings. Overall, combining both criteria,
19 87% of the instruments that used SSC as the preferred parameter were found to be qualified.
20 Regarding instruments that used FSC, only the last generation of FCMr (Gallios/Navios,
21 Stratedigm and BD-Influx) showed a SSI > 3 (table 1). Among these instruments, BD influx
22 and Apogee A50 showed the best resolution (SSI = 11.3 , $n=2$). Regarding background noise,
23 five (of twelve) Gallios/Navios showed a BNR > 1 . This noise proved to be of optical origin,
24 and was reversible by externally cleaning the flow cell from dust deposits. **Therefore, these**
25 **instruments were incorporated in the second stage of the study.** The qualification step resulted

1 in a 75% qualification rate for FSC-optimized instruments. Finally, the standardization
2 strategy proved to be compatible with 44/52 instruments (85%).

3

4 **Inter-instrument variability**

5 In the second stage of the protocol, participating labs with qualified instruments
6 enumerated PMPs on 3 PFP samples prepared by the core laboratory. Because of the one year
7 delay between the two stages of the workshop, it was required to check SSI and BNR of the
8 instruments again before analyzing the samples. The qualification criteria were same as in
9 first stage. As a result, with the exception of two Navios with significant background noise,
10 all instruments re-qualified. The standardized protocol was set up optimizing the scatter
11 settings, flow rate, fluorescence and compensation settings, and region boundaries as detailed
12 in Methods. After analysis of the FCM raw data files by the core- lab, data from 3 instruments
13 were rejected due to irreversible discrepancies with the protocol instructions. Also, plasma
14 sample C was excluded from analysis due to its inherent heterogeneity leading to high PMP
15 count CVs at the core-lab. Each PFP was analyzed in triplicate. The mean CV for each
16 triplicate of the validated PMP counts were 15% and 12% for samples A and B, respectively.
17 Individual results showing a triplicate CV > 50% -- suggestive of a manipulator-dependent
18 bias -- were not considered valid (3 instruments for sample A and no instrument for sample
19 B). Finally, among the 32 results received by the core lab for samples A and B, 81% and 91%
20 were considered valid, respectively.

21 As shown in figure 1, all instruments with validated results for the two samples (n=26)
22 correctly discriminated the two PMP levels. The inter-instrument variability of the ratio
23 between sample A and B was 30.2% and was not significantly different between the
24 instruments using SSC or FSC as the preferred scatter parameter (35.1 +/- 4.9 vs. 39.3 +/-
25 14.3, respectively, p = 0.7). As illustrated in figure 2A for sample A, 58% of instruments

1 provided comparable PMP counts within a restricted range of values (robust mean +/- robust
2 SD). This result was better with sample B (69%, figure 2B). 15/26 instruments (58%) gave
3 results within the robust mean +/- robust SD range. However, some individual instruments
4 (LSR II Fortessa, Apogee A50) were systematically outside the robust mean +/- robust SD
5 range for both samples. In the specific case of Apogee A50, the discordance with expected
6 values was clearly due to an inappropriate choice of the set of beads (FSC instead of SSC).
7 Finally, the inter-laboratory variability of PMP counts was 37% and 28% for samples A and B,
8 respectively (figure 2C) with mean and 95% confidence interval at 8490 [7190-9790] PMP/ μ l
9 and 3075 [2745-3400] PMP/ μ l for samples A and B, respectively. Interestingly, we found no
10 significant difference in PMP counts between instruments using SSC or FSC as the preferred
11 trigger (sample A: 8900 +/- 4000 PMPs/ μ l vs. 8000 +/- 2000 PMPs/ μ l, p = 0.8; sample B:
12 3100 +/- 980 PMPs/ μ l vs. 2800 +/- 550 PMP/ μ l, p = 0.5). However, the inter-instrument
13 variability was higher for SSC instruments compared to FSC-oriented instruments (sample A:
14 46% vs. 25%; sample B: 31% vs. 19%) probably due to a greater diversity of tested models.

15

16 Discussion

17 This study is the first to demonstrate that standardization is possible for MP
18 enumeration by flow cytometry. We also demonstrated that size-calibrated polystyrene beads
19 can be used as a standardization tool for MP enumeration, provided that instrument intrinsic
20 behaviors for size-related measurements have been taken into account. Bead-based strategies
21 have been criticized because the relationship between bead and MP sizes is not obvious and
22 highly depends on the size-related scatter parameter used and on the refractive index [13-16].
23 Therefore, the beads should not be used as calibrators to derive absolute size values for MPs.
24 Other standards with refractive indices closer to those of MPs may be a better alternative.
25 However, such a standardization strategy awaits similar multi-center validation. Moreover,

1 whereas we focused in this study on the use of scatter for triggering MP analysis, several
2 other groups focus on fluorescence as a preferred threshold [4, 17-20]; however, thresholding
3 on fluorescence currently encounters several practical limitations. Although generic labels
4 have been proposed, e.g. lipophilic fluorescent labels such as PKH dyes, the labeling
5 procedure of MPs in complex body fluids such as plasma is hardly applicable, necessitating
6 protocols that use specialized lab equipment to get rid of free dye and prevent measurement of
7 artifacts. Indeed, non-specific fluorescent background due to the staining of lipoprotein
8 particles present in plasma added to the variability in fluorescence sensitivity among
9 instruments, remain two major limitations to define any clear-cut, reproducible, fluorescent
10 threshold level that could be generally applied. Most probably, both fluorescence and scatter
11 triggering strategies will have to be combined.

12 In contrast to the previous ISTH standardization study [6], the proposed bead-based
13 strategy is now applicable on most commercially available instruments. No significant
14 variability was observed between instrument families measuring PMPs with different optical
15 systems. These results open the way for multicenter studies comparing MP counts in clinical
16 samples. Although only PMP were measured in this workshop, it can be anticipated that the
17 same strategy could be extended to other clinically-relevant MP subsets. However, this
18 standardization strategy displays several limitations: 1) It still addresses only a small fraction
19 of MPs, a large part being below the detection limit of instruments; 2) Homogeneous re-
20 treatment of raw data by the core laboratory was still required. Thus specific training is still
21 needed for data treatment; 3) It was mainly focused on harmonizing the scatter-based MP
22 gates. Although the conditions of fluorescence detection were tentatively harmonized in this
23 study using specifically designed Fluo-Setting-Beads to be set in similar target channels, the
24 complete standardization of fluorescence measurements would require more sophisticated

1 approaches [21]; 4) The strategy has to be challenged on future instruments with different
2 optical design.

3 Despite having still significant limitations, this study is the first to demonstrate a real
4 potential for standardization of MP enumeration across different FCM platforms. [Additional](#)
5 [standardization efforts](#) are mandatory to allow the evaluation of the clinical relevance of MP
6 counts at a multicenter level, and should accompany the continuous improvement in the
7 sensitivity of instruments to detect progressively smaller MPs.

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1 **Addendum:** Authors' contribution

2 S. Cointe, C. Judicone, and S. Robert performed the research, collected the data and analyzed
3 and interpreted the data. M. Mooberry, and P. Poncelet designed the research and reviewed
4 the manuscript. M. Wauben and R. Nieuwland reviewed the manuscript, N. S. Key and F.
5 Dignat-George supervised the work and reviewed the manuscript and R. Lacroix designed the
6 research, supervised the work, analyzed and interpreted the data and wrote the manuscript.

7

8 **Appendix:** ^xISTH SSC Workshop

9 Amirkhosravi A.¹, Annichino-Bizzacchi J.², Arkesteijn G.J.A.³, Bene M-C.⁴, Bailly N.⁵,
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- 16 42. Onze Lieve Vrouwe Gasthuis, Hematologisch Klinisch Chemisch Laboratorium,
17 Amsterdam, The Netherlands
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19

20 **Acknowledgements**

21 This study received a financial support from the International Society on Thrombosis and
22 Haemostasis. Research reported in this publication was supported by the Office of the
23 Director, National Institutes of Health under award number S10OD012052.

24

25 **Disclosure of conflict of interests**

1 C. Judicone and P. Poncelet are full-time employees of BioCytex.
2 For the workshop, BioCytex provided Megamix beads, and non commercial reagents
3 including counting and fluo setting beads. Separate from this study; F. Dignat-George and R
4 Lacroix declare a collaboration contract with Stago (on fibrinolytic microparticles: licensed
5 patent).

6

7 **Figure legends**

8 **Figure 1: Inter-instrument variability of the PMP ratio between samples.**

9 PMP counts of sample A was fixed at 100% and counts in sample B was displayed as a
10 percentage of sample A for both groups of instruments, using side scatter (SSC) or forward
11 scatter (FSC) as preferred parameter to define the MP gate of analysis.

12

13 **Figure 2: Inter-instrument variability of PMP counts.**

14 A and B: Platelet-derived microparticle (PMP) counts determined as events/ μ l in sample A
15 (A) or sample B (B) by each qualified flow cytometer using either side scatter (SSC) or
16 forward scatter (FSC) as the preferred parameter. The grey area is defined by the robust mean
17 (X^*) +/- the robust standard deviation (SD^*). X^* and SD^* were calculated taking into
18 account only results from cytometers with values between the median +/- SD. **C:** Inter-
19 instrument variability (CV) of PMP counts. $p < 0.05$ was considered significant.

20

21 **References**

22 1 Revenfeld AL, Baek R, Nielsen MH, Stensballe A, Varming K, Jorgensen M.
23 Diagnostic and prognostic potential of extracellular vesicles in peripheral blood. *Clin Ther.*
24 2014; **36**: 830-46.

1 2 van der Pol E, Coumans FA, Grootemaat AE, Gardiner C, Sargent IL, Harrison P,
2 Sturk A, van Leeuwen TG, Nieuwland R. Particle size distribution of exosomes and
3 microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle
4 tracking analysis, and resistive pulse sensing. *J Thromb Haemost.* 2014; **12**: 1182-92.

5 3 Robert S, Lacroix R, Poncelet P, Harhoury K, Bouriche T, Judicone C, Wischhusen J,
6 Arnaud L, Dignat-George F. High-sensitivity flow cytometry provides access to standardized
7 measurement of small-size microparticles--brief report. *Arterioscler Thromb Vasc Biol.* 2012;
8 **32**: 1054-8.

9 4 Hoen EN, van der Vlist EJ, Aalberts M, Mertens HC, Bosch BJ, Bartelink W,
10 Mastrobattista E, van Gaal EV, Stoorvogel W, Arkesteijn GJ, Wauben MH. Quantitative and
11 qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles.
12 *Nanomedicine.* 2011; **8**: 712-20.

13 5 Zhu S, Ma L, Wang S, Chen C, Zhang W, Yang L, Hang W, Nolan JP, Wu L, Yan X.
14 Light-scattering detection below the level of single fluorescent molecules for high-resolution
15 characterization of functional nanoparticles. *ACS Nano.* 2014; **8**: 10998-1006.

16 6 Lacroix R, Robert S, Poncelet P, Kasthuri RS, Key NS, Dignat-George F.
17 Standardization of platelet-derived microparticle enumeration by flow cytometry with
18 calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC
19 Collaborative workshop. *J Thromb Haemost.* 2010; **8**: 2571-4.

20 7 Robert S, Poncelet P, Lacroix R, Arnaud L, Giraudo L, Hauchard A, Sampol J,
21 Dignat-George F. Standardization of platelet-derived microparticle counting using calibrated
22 beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies?
23 *J Thromb Haemost.* 2009; **7**: 190-7.

1 8 Poncelet P, Robert S, Bouriche T, Bez J, Lacroix R, Dignat-George F. Standardized
2 counting of circulating platelet microparticles using currently available flow cytometers and
3 scatter-based triggering: Forward or side scatter? *Cytometry A*. 2016; **89**: 148-58.

4 9 Lacroix R, Judicone C, Poncelet P, Robert S, Arnaud L, Sampol J, Dignat-George F.
5 Impact of pre-analytical parameters on the measurement of circulating microparticles:
6 towards standardization of protocol. *J Thromb Haemost*. 2012; **10**: 437-46.

7 10 Lacroix R, Judicone C, Mooberry M, Boucekine M, Key NS, Dignat-George F.
8 Standardization of pre-analytical variables in plasma microparticle determination: results of
9 the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J*
10 *Thromb Haemost*. 2013; **11**: 1190-3.

11 11 Bigos M. Separation index: an easy-to-use metric for evaluation of different
12 configurations on the same flow cytometer. *Curr Protoc Cytom*. 2007; **Chapter 1**: Unit1 21.

13 12 Huber P. Robust statistics. Wiley, 1981.

14 13 Robert S, Poncelet P, Lacroix R, Raoult D, Dignat-George F. More on: calibration for
15 the measurement of microparticles: value of calibrated polystyrene beads for flow cytometry-
16 based sizing of biological microparticles. *J Thromb Haemost*. 2011; **9**: 1676-8.

17 14 Mullier F, Bailly N, Chatelain C, Dogne JM, Chatelain B. More on: calibration for the
18 measurement of microparticles: needs, interests, and limitations of calibrated polystyrene
19 beads for flow cytometry-based quantification of biological microparticles. *J Thromb*
20 *Haemost*. 2011; **9**: 1679-81.

21 15 Konokhova AI, Yurkin MA, Moskalensky AE, Chernyshev AV, Tsvetovskaya GA,
22 Chikova ED, Maltsev VP. Light-scattering flow cytometry for identification and
23 characterization of blood microparticles. *J Biomed Opt*. 2012; **17**: 057006.

- 1 16 Gardiner C, Shaw M, Hole P, Smith J, Tannetta D, Redman CW, Sargent IL.
 2 Measurement of refractive index by nanoparticle tracking analysis reveals heterogeneity in
 3 extracellular vesicles. *J Extracell Vesicles*. 2014; **3**: 25361.
- 4 17 Arraud N, Gounou C, Linares R, Brisson AR. A Simple Flow Cytometry Method
 5 Improves the Detection of Phosphatidylserine-Exposing Extracellular Vesicles. *J Thromb*
 6 *Haemost*. 2014.
- 7 18 Arraud N, Gounou C, Turpin D, Brisson AR. Fluorescence triggering: A general
 8 strategy for enumerating and phenotyping extracellular vesicles by flow cytometry. *Cytometry*
 9 *A*. 2015.
- 10 19 Nolan JP. Flow Cytometry of Extracellular Vesicles: Potential, Pitfalls, and Prospects.
 11 *Curr Protoc Cytom*. 2015; **73**: 13 4 1- 4 6.
- 12 20 van der Vlist EJ, Nolte-t Hoen EN, Stoorvogel W, Arkesteijn GJ, Wauben MH.
 13 Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and
 14 qualitative analysis by high-resolution flow cytometry. *Nat Protoc*. 2012; **7**: 1311-26.
- 15 21 Stoner SA, Duggan E, Condello D, Guerrero A, Turk JR, Narayanan PK, Nolan JP.
 16 High sensitivity flow cytometry of membrane vesicles. *Cytometry A*. 2016; **89**: 196-206.

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18

19 **Table**

| | Instrument type | SSI mean [min-max] | BNR mean [min-max] | Qualified instruments |
|------------------------|-------------------------|---------------------------|---------------------------|------------------------------|
| SSC instruments | Accuri C6 | 0 | ND | 0/2 |
| | Apogee A50 ^a | 7.7 | 0.03 | 1/1 |
| | FACSAria | 5.2 [2.8-7.1] | 0.07 [0-0.22] | 4/5 |
| | FACSCalibur | 4.6 [3-6.5] | 0.58 [0-2.59] | 5/6 |
| | FACSCanto | 4.5 [3.4-7] | 0.07 [0.01-0.2] | 11/11 |
| | FACSVerse | 7.1 [7-7.3] | 0.12 [0.06-0.19] | 2/2 |
| | Influx ^a | 2.4 | 0.43 | 0/1 |
| | LSR Fortessa | 8 [7.2-9.1] | 0.02 [0.02-0.05] | 3/3 |

| | | | | |
|----------------------------|-------------------------|---------------|-------------------------------------|----------------------------|
| | LSR II | 7.9 [5.7-10] | 0.02 [0-0.03] | 4/4 |
| | Stratedigm ^a | 4.3 | 0.02 | 1/1 |
| FSC instruments | Apogee A50 ^a | 11.3 | 0 | 1/1 |
| | Epics XL | 0 | ND | 0/1 |
| | FC500 | 0 | ND | 0/2 |
| | Guava | 0 | ND | 0/2 |
| | Influx ^a | 11.3 | 0.01 | 1/1 |
| | Navios/Gallios | 5.6 [2.8-7.6] | 1.53 [0-6.2] (0.03) ^b | 7/12 (12/12) ^b |
| | Stratedigm ^a | 4 | 1 | 1/1 |
| Total | | | | 39/52 (44/52) ^b |

1 ^a Instruments tested both in SSC and FSC; ^b Results after flow cell wash; Sensitivity index (SSI) =
2 (Median bead A- Median bead B)/ (SD bead A+ SD bead B) where bead A = 0.2µm and bead B =
3 0.16µm for SSC FCMrs, and bead A = 0.5 µm and bead B = 0.3 µm for FSC FCMrs. SSI > 3 was
4 required to be compatible with the standardization strategy. Background noise ratio (BNR) is the ratio
5 between the number of events per second measured in the protocol settings and the instrument specific
6 maximal number of events per second keeping abort rate at a low level (FACSCanto I/II = 4000,
7 FACSCalibur = 2000, FACSaria I/II = 4000, LSR II (+/-Fortessa) = 4000, FACSVerse = 4000,
8 Apogee A50 = 2000, Stratedigm = 4000, Gallios/Navios = 5000, Influx = 15 000, defined according to
9 both instrument specifications and core lab validation).

10

11 **Table 1: Instrument qualification according to resolution and background noise.**

12

13