EMPIR Grant Agreement number: 18HLT01
Project short name: METVES II

Deliverable reference number and title: D1

Report on the requirements of clinically relevant synthetic reference materials to standardise concentration measurements of EVs based on stakeholder needs

Organisation name of lead partner for the deliverable: **MTA TTK**

Due date of the deliverable: **September 2019**

Actual submission date of the deliverable: 16th **October 2019**

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1. Introduction

One goal of METVES II is to develop reference materials to calibrate size, scatter, concentration and fluorescence measurements of single extracellular vesicles (EVs). Therefore, we aim to develop reference particles of known size, refractive index, concentration, fluorescence spectrum, and fluorescence intensity. To meet the needs of potential users, an online survey was prepared to ascertain the most desired properties of reference materials to standardise EV measurements. These properties include size, concentration range, optical properties, choice of fluorochromes, and stability for flow cytometry measurements.

The survey was sent to the members of the Stakeholder Committee of METVES II and to the members of the EV Flow Cytometry Working Group which is established by flow cytometry experts from the International Society for Extracellular Vesicles (ISEV), International Society for the Advancement of Cytometry (ISAC), and International Society on Thrombosis and Haemostasis (ISTH). A list of the members of the working group can be found under http://www.evflowcytometry.org/contacts/. The survey consisted of two parts. The first part included 11 scientific questions covering the desired properties of the EV reference materials, which serves as basis for the current report. The second part of the survey with 10

scientific questions about the desired properties of stable biological test samples covers A3.1.1 of WP3. The questionnaires of activities A1.1.1 and A3.1.1 were performed within one survey because the targeted respondents were the same group of experts, and handling the responses together enabled to reveal correlations between the current measurement capacities and the desired properties of the EV reference materials.

The online survey was announced on 14th July 2019 and remained open until 6th September 2019. Two e-mail reminders were sent to the e-mail lists of the Stakeholder Committee of METVES II and the EV Flow Cytometry Working Group. A screenshot of the survey is shown in Figure 1.

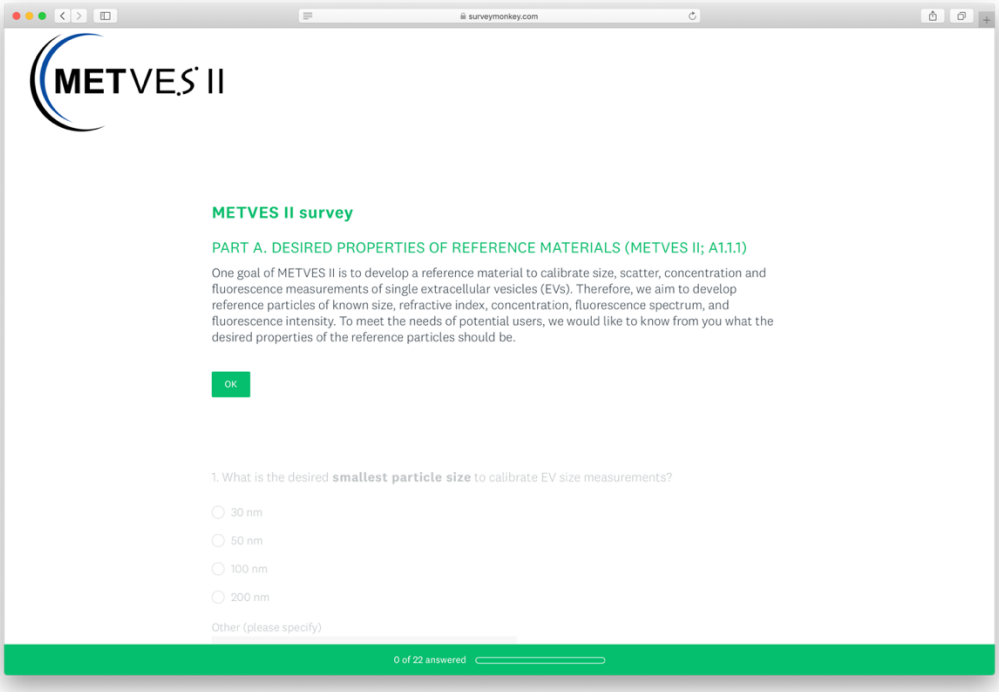


Figure 1. Screenshot of the online survey

2. Results and Discussion

2.1 Respondents number, geographical distribution, and background

In total, 22 responses were received that included incomplete and duplicate forms as well. After filtering out incomplete and duplicate responses, 17 independent and complete responses were analysed, 6 from North America, 10 from Europe, and 1 from Asia. Regarding institutional background, 12 responses were from academic researchers, 3 from non-profit research institutes, and 2 from national institutes.

2.2 Desired physical properties of EV reference materials

The first 6 questions of the survey were related to the desired size, concentration and refractive index ranges of EV reference materials. The majority of respondents (10 out of 17) indicated 50 nm as the desired smallest particle size, 4 respondents indicated 30 nm, and 2 respondents indicated 100 nm. Responses for the largest particle diameter were not as unequivocal as for the smallest size, because an equal number of respondents (7) indicated 500 nm and 1000 nm. The most desired size range according to the survey shows a good overlap with the size range indicated in the first objective of the 18HLT01 METVES II JRP Protocol (Table 1. first row). Besides the size, the refractive index of the particles also greatly determines their light scattering properties. Eight respondents indicated the desired lowest RI of the reference particles to be 1.35, whereas 7 respondents indicated the value of 1.37. The majority of respondents (12 out of 17) indicated 1.45 as the desired highest RI of the particles. This range shows good overlap with the values indicated in the JRP Protocol, though it is a slightly larger range in both limits (Table 1. second raw).

	Survey (most desired)	**JRP Protocol**
Diameter (nm)	50 to 500/1000	50 to 1000
RI	1.35 to 1.45	1.37 to 1.42
Concentration (mL^{-1})	10^7 to 10^{10}	10^9 to 10^{12}

Table 1. Most desired physical properties of EV reference materials, and the properties as indicated in the first objective of the JRP Protocol.

According to the result of the survey, the diameter and RI of the most desired smallest reference particle is 50 nm and 1.35, respectively. In Part B of the survey (A3.1.1), we asked the diameter of the smallest polystyrene (PS) particle detectable by the respondent's FCM instrument. Using this information and Mie-theory to calculate the diameter of particles that represent the same scattering cross-section with RI=1.35 and RI=1.37, we can estimate if the respondents could detect the desired smallest particles or not. The results of these calculations are shown in Figure 2. According to these results, none of the respondents would be able to detect 50 nm particles with RI=1.35 with their current instruments, and only two respondent's FCM is capable to detect 50 nm particles with RI=1.37. The average detection limit of the respondents' FCM instruments corresponds to 80 nm PS particles, which equals in scattering cross-section to approximately 180 nm RI=1.37 and 250 nm RI=1.35 reference particles at 405 nm wavelength. One respondent indicated the disagreement between the current detection limit and the needs for EV measurements: "*Objectively, the smallest particle size would be ~50 nm. However, we can only measure ~100 nm particles at present*". Of note, a recently developed high sensitivity flow cytometer (Zhu et al. ACS Nano, 2014, 8, 10, 10998-11006) is capable of detecting 24 nm silica nanoparticles, which approximately corresponds to the desired lowest limit for EV reference materials.

The most desired highest RI of the reference particles is 1.45, which is close to the RI of silica. Monodisperse silica reference particles are already available and therefore new reference material development should focus on lower RI particles.

The desired particle concentration range of EV reference materials was also questioned in the survey. The most desired lowest concentration is 10^7 particles/mL (12 out of 17 responses), while 8 respondents indicated 10^{10} particles/mL and 6 respondents indicated 10^{12} particles/mL for the desired highest particle concentration. This range is lower than the one indicated in the JRP Protocol (Table 1, third row), which confirms that the targeted concentration range will be suitable for flow cytometry investigation of EVs because samples can be easily diluted for FCM measurements.

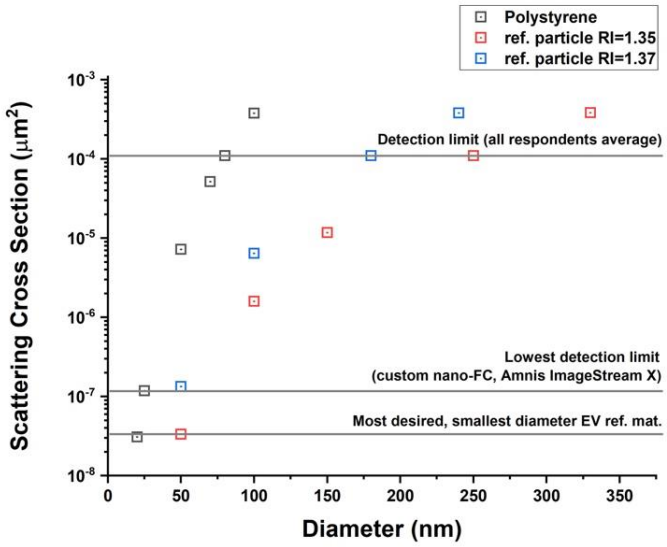


Figure 2. Total scattering cross-section as a function of particle diameter for polystyrene (PS) particles (RI=1.63 at 405 nm) and for the desired reference particles with RI=1.35 (ref. particle RI=1.35) and RI=1.37 (ref. particle RI=1.37). Average and lowest detection limit of the respondents' FCM instruments (obtained from Part B of the survey) together with the scattering cross-section value of the most desired smallest diameter reference particles (50 nm, RI=1.35) are indicated with horizontal lines.

2.3 Desired fluorescent properties of EV reference materials

The next 4 questions of the survey were related to the desired fluorescent properties of EV reference materials. First, the desired type of fluorochromes was questioned. Most of the respondents (13 out of 17) indicated FITC (fluorescein isothiocyanate), which was followed by Alexa Dyes and PE (phycoerythrin), both indicated by 11 out of 17 respondents. APC (allophycocyanin), another protein-based fluorochrome was indicated by 9 respondents. It should be highlighted that FITC and Alexa Dyes are small molecular weight organic compounds, while PE and APC are both phycobiliproteins with molecular weights of 250 000 g/mol and 105 000 g/mol. Cyanine dyes (which are also small molecular weight compounds) were indicated by 2 respondents, but many other suggestions were also received. Besides suggesting tandem dyes (e.g. PE-Cy7/5), Brilliant Violet dye was indicated by 3 respondents. Brilliant Violet dye is a π-conjugated polymer, which has a molecular weight of approx. 290 000 g/mol. Spectral properties and molecular masses of the desired fluorochromes are summarized in Table 2.

Detection channel (bandpass filter)	Fluorescent dye	Abbr.	Dye class	Mol. mass (g/mol)	Exc. max (nm)	Em. max (nm)	Ext. Coeff. L/(mol*cm)	QY
450/50	Brilliant Violet 421	Bv421	Polymer-based	not available (>200 000)	405	420	2 500 000	0,65
530/30	Fluorescein (isothiocyanate)	FITC	Xanthene dye	400	490	520	73 000	
	5(6)-Carboxyfluorescein (N-succinimidyl ester)	CFSE			500	515	83 000	
	Alexa Fluor 488	AF488	Xanthene dye	600	495	520	73 000	0,92
	Green fluorescent protein	GFP	Fluorescent protein	26 900	490	510	56 000	
	PKH67 ("green")	PKH67	Polymethine dye	600 to 1000	490	500	not available	
585/40	Phycoerythrin	PE	Phycobiliprotein	250 000	565	575	1 960 000	0,82
	Nitrobenzofurazan	NBD	Benzofurazan dye	200	465	535	22 000	
	PKH26 ("red")	PKH26	Polymethine dye	600 to 1000	550	565	not available	
630/30	PE-Dazzle594	PE-Dazzle594	Tandem dye	>250 000	565	610	1 960 000	
	Di-8-ANEPPS	Di-8-ANEPPS	ANEP dye	600	465	635	32 000	
670/40	Allophycocyanin	APC	Phycobiliprotein	105 000	650	660	700 000	0,68
	Alexa Fluor 647	AF647	Polymethine dye	1 100	650	670	270 000	0,33
	PE-Cy5	PE-Cy5	Tandem dye	>250 000	565	670	1 960 000	
720/40	PE-Cy7	PE-Cy7	Tandem dye	>250 000	565	770	1 960 000	
	Brilliant Blue 700	Bb700	Polymer-based	not available (>200 000)	485	690	not available	
780/60	Brilliant Violet 785	Bv785	Polymer-based	not available (>200 000)	405	785	2 500 000	0,04

Table 2. Spectral properties and molecular masses of the desired fluorescent dyes for EV reference materials

Regarding fluorescence intensity, the most desired range according to the responses is from 10 MESF (molecules of equivalent soluble fluorochromes) to 10 000 MESF, which shows a good overlap of the values indicated in the JRP Protocol (from 100 MESF to 100 000 MESF). It should be noted that the MESF values are connected to the type of the fluorochrome, in a way that the brighter the fluorochrome the lower the detection limit in MESF. In the last 25 years, PE and APC were the brightest probes in immunofluorescence experiments. Brilliant Violet represents a novel alternative to PE and APC. All of these fluorochromes are high molecular weight molecules, which limits the maximal number of molecules that can be coupled to the surface of a nanoparticle. For example, a PE molecule with 250 kDa molecular weight corresponds to an equivalent sphere with approximately 4 nm diameter, which means that 100 – 150 PE molecules already cover the surface of a 50 nm diameter reference particle.

Data or estimates on the antigen densities on EVs are rare. To obtain an estimate about the typical numbers, one can consider erythrocyte derived EVs and glycophorin A, which is one of the major sialoglycoproteins of the human erythrocyte membrane with a density of 0.5 million copies per erythrocyte (Francis J. Alenghat, David E. Golan, in Current Topics in Membranes, 2013). The average surface area of an erythrocyte is 140 μm^2 (Lux SE. Hemolytic

anemias III. Membrane disorders. In: Beck WS. ed. Hematology, 3rd ed. Cambridge: MIT Press, 1981 197-214.). Assuming that the glycophorin A density is the same in EVs as in the erythrocyte membrane, an EV with 50 nm diameter contains approximately 30 copies of glycophorin A. The same calculation results 250 copies of glycophorin A for an EV with a diameter of 150 nm.

The same estimation can be also calculated for platelets with glycoprotein IIb, which is a major membrane protein on the platelet surface, with an average of 80 000 GPIIb/IIIa copies per platelet (Overview of the Coagulation System, Morayma Reyes Gil, in Transfusion Medicine and Hemostasis (Third Edition), 2019). Assuming that the surface area of an activated platelet is 50 μm^2 (Natalia V. Zakharova et al. PLOS ONE, 2015, DOI:10.1371/journal.pone.0116665) and the density of GPIIb in EVs is the same as in the platelet membrane, one can obtain 13 and 110 copies for EVs with 50 nm and 150 nm diameter, respectively.

These calculations have two main consequences: (1) the maximal MESF that can be obtained for EV reference particles with 50 nm diameter and high molecular mass fluorescent dyes is in the range of several hundreds, and (2) the typical antigen densities on EVs fall below 100 copies per EV with 50 nm diameter, i.e. MESF values above 100 are probably not physiologically relevant in this size range. Of note, Part B of the survey contained a question about the fluorescent detection limit of the respondents' FCM instruments, for which the answers indicated that majority of respondents can detect particles in the 100 MESF range.

Taking into account these considerations, and the fact that the most desired fluorochromes for reference particles are classical small molecular weight compounds, xanthene dyes (FITC and Alexa Dyes belong to this family) will be attempted to label EV reference materials in the first line, but the coupling of phycobiliprotein and polymer dyes will be also investigated.

The last question regarding the desired fluorescence properties of EV reference materials was an open-ended question about the simultaneously used fluorochromes. The general trend in the answers is that in most EV measurements a xanthene dye is combined with a phycobiliprotein dye. The possibility of producing dual-labelled EV reference materials will be assessed when the labelling strategies for the two types of fluorochromes are established.

2.4 Desired stability of EV reference materials

The last question of Part A of the survey was about the desired stability of EV reference materials. The majority of respondents indicated that the reference materials to be developed should be stable for at least one year (12 out of 17). For all the three candidate materials (HOBs, liposomes, low-RI particles) this is feasible.

3. Conclusions

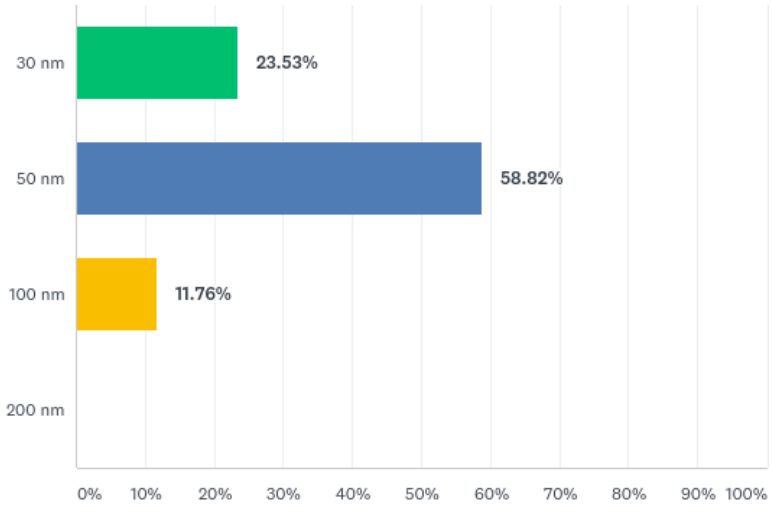
This report summarizes the findings of the survey on the requirements of clinically relevant synthetic reference materials to standardise concentration measurements of EVs, which was sent out to the members of the stakeholder committee of METVES II and the EV Flow Cytometry Working Group. The results confirm the targeted ranges for the physical properties of the EV reference materials initially formulated in the JRP Protocol of 18HLT01 METVES II, therefore those ranges will be targeted in the development of EV reference particles in WP1. Responses regarding the choice of fluorochromes will be used in WP1 for the preparation of fluorescent reference materials.

Appendix: Survey questions and responses

1. What is the desired **smallest particle size** to calibrate EV size measurements?

ANSWER CHOICES	RESPONSES	
30 nm	23.53%	4
50 nm	58.82%	10
100 nm	11.76%	2
200 nm	0.00%	0
TOTAL		17

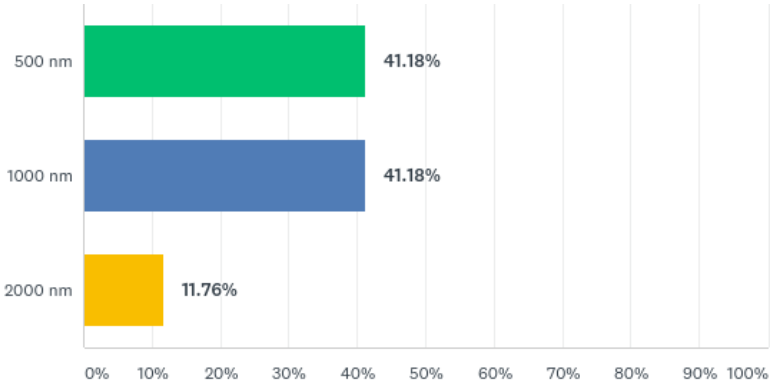
(including 1 other response: “Objectively, the smallest particle size would be ~50 nm. However, we can only measure ~100 nm particles at present”)



2. What is the desired **largest particle size** to calibrate EV size measurements?

ANSWER CHOICES	RESPONSES	
500 nm	41.18%	7
1000 nm	41.18%	7
2000 nm	11.76%	2
TOTAL		17

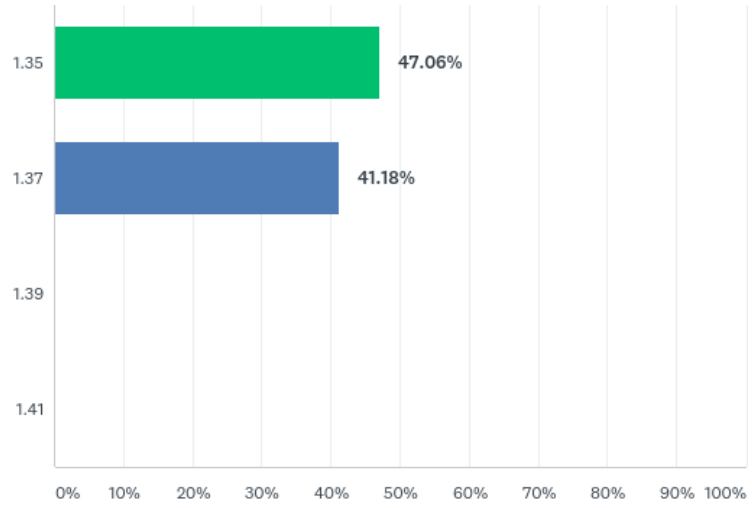
(including 1 other response: 300 nm)



3. What is the desired **lowest RI** (refractive index) of the reference particles to calibrate EV size measurements?

ANSWER CHOICES	RESPONSES	
1.35	47.06%	8
1.37	41.18%	7
1.39	0.00%	0
1.41	0.00%	0
TOTAL		17

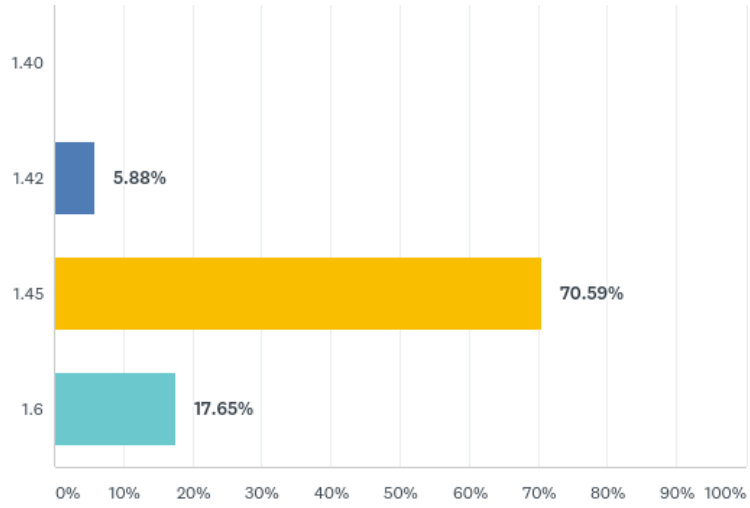
(including 2 other responses: "Not sure", "a range that includes all values given due to dependence of RI on source from which EVs are generated")



4. What is the desired **highest RI** (refractive index) of the reference particles to calibrate EV size measurements?

ANSWER CHOICES	RESPONSES	
1.40	0.00%	0
1.42	5.88%	1
1.45	70.59%	12
1.6	17.65%	3
TOTAL		17

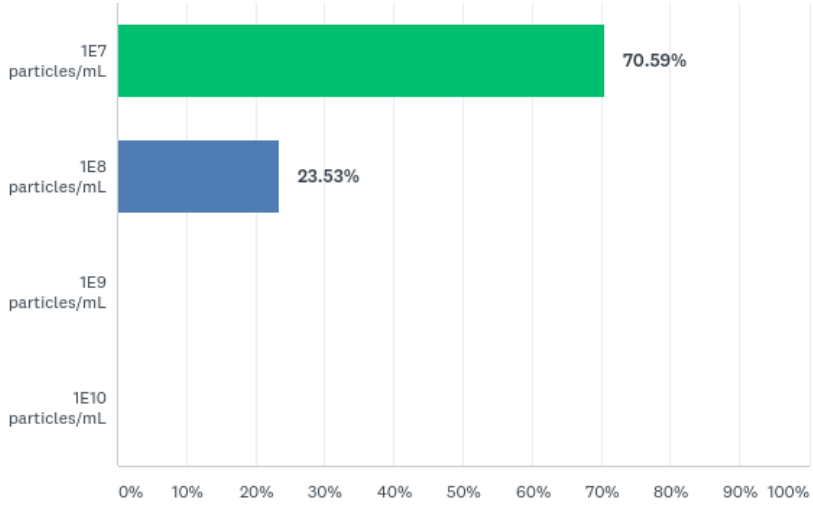
(including 1 other response: "Not sure")



5. What is the **lowest concentration** of particles that would be needed to calibrate EV concentration measurements?

ANSWER CHOICES	RESPONSES	
1E7 particles/mL	70.59%	12
1E8 particles/mL	23.53%	4
1E9 particles/mL	0.00%	0
1E10 particles/mL	0.00%	0
TOTAL		17

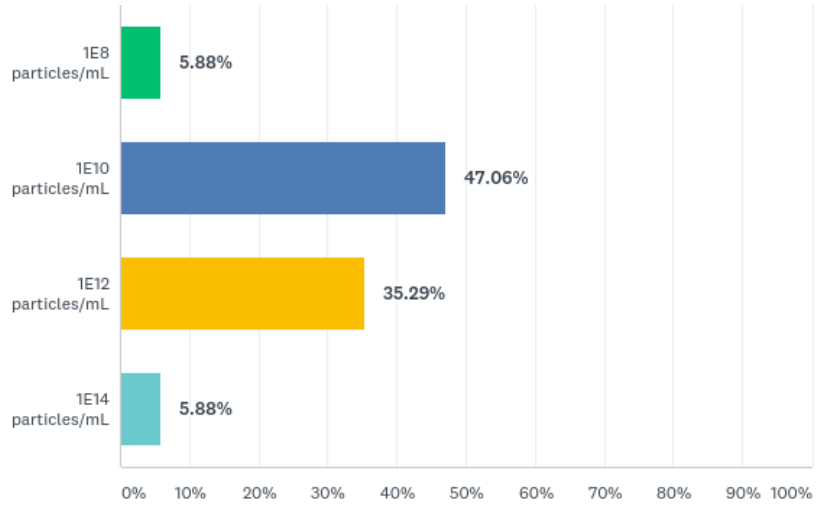
(including 2 other response: "But maybe lower for our biobanking samples" and "1E5 particles/mL")



6. What is the **highest concentration** of particles that would be needed to calibrate EV concentration measurements?

ANSWER CHOICES	RESPONSES	
1E8 particles/mL	5.88%	1
1E10 particles/mL	47.06%	8
1E12 particles/mL	35.29%	6
1E14 particles/mL	5.88%	1
TOTAL		17

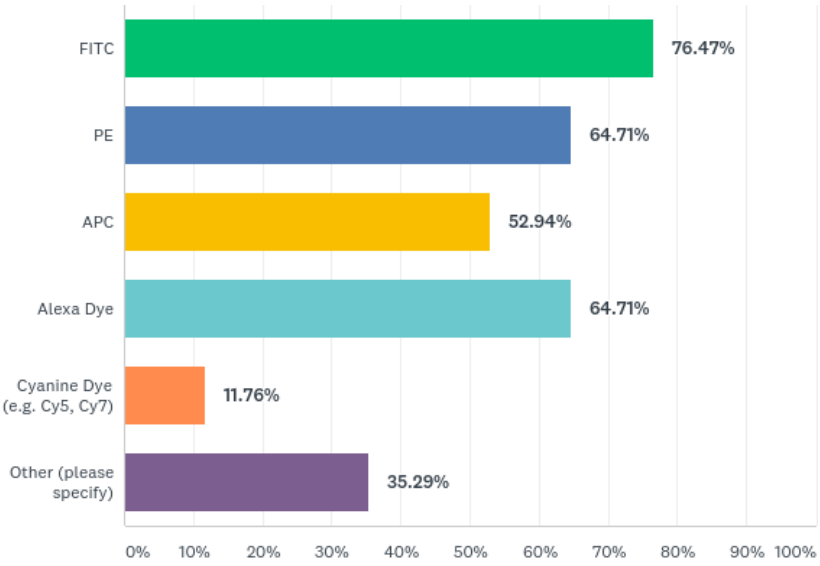
(including 1 other response: "For flow cytoemtry concentration is not really an issue, as samples can easily be diluted.")



7. What **fluorochromes** are desired for fluorescent reference particles?

ANSWER CHOICES	RESPONSES	
FITC	76.47%	13
PE	64.71%	11
APC	52.94%	9
Alexa Dye	64.71%	11
Cyanine Dye (e.g. Cy5, Cy7)	11.76%	2
Other (please specify)	35.29%	6
Total Respondents: 17		

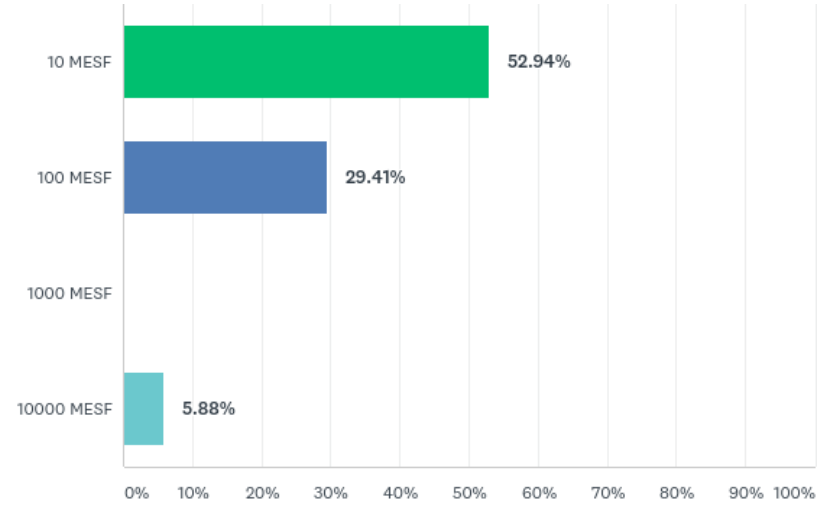
(including 6 other responses: "BV421", „No preference", „May include a few choices, e.g. FITC, PE, APC etc.", „brilliant blue/violet", „Brillant Violet 421; Pe-Cy7, Pe-Cy5", „There are dozens of dyes that might be used for immunofluorescence measurements. Suggest you coordinate with researchers active in the NIST-ISAC fluorescence standardization efforts to develop a strategy that builds on and extends the substantial amount of work that has already been done.")



8. What is the desired **lowest fluorescence intensity** for fluorescent reference particles? (in 'molecules of equivalent soluble fluorochromes' or MESF)

ANSWER CHOICES	RESPONSES	
10 MESF	52.94%	9
100 MESF	29.41%	5
1000 MESF	0.00%	0
10000 MESF	5.88%	1
TOTAL		17

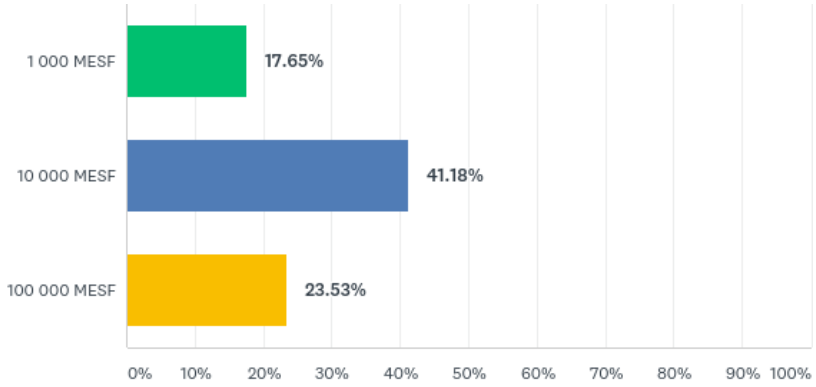
(including 2 other responses: “Not sure”, “This would depend on the type of fluorophores used”)



9. What is the desired **highest fluorescence intensity** for fluorescent reference particles? (in 'molecules of equivalent soluble fluorochromes' or MESF)

ANSWER CHOICES	RESPONSES	
1 000 MESF	17.65%	3
10 000 MESF	41.18%	7
100 000 MESF	23.53%	4
TOTAL		17

(including 3 other responses: “Not sure”, “Depending on the type of fluorophores used”, “50000 MESF”)



10. Which fluorochromes do you use simultaneously in EV measurements?

- Different combinations of generic dyes (PKH, CFSE, etc) and antibodies labeled with PE, APC or Alexa dyes
- PE, PECy7, BV421, APC, PE Dazzle 594
- CFSE, PKH67, FITC, PE, APC, BV421
- Bv421, FITC, PE, APC
- FITC/GFP, PE, APC/AF647.
- FITC/PE and APC
- Only PE and FITC so far.
- APC or Alexa647 and PE or FITC/A488
- FITC, PE, APC
- FITC/AF488, PE, APC
- FITC combined with PE-Cy5, PE-Cy7, Brilliant blue 700
- FITC/AF488 PE APC
- AF488, PE, BV421, APC
- BrillantViolet 421, Brillant Violet 786, FITC, PE, Pe-Cy5, PE-Cy7, APC
- Alexa Fluor 488, APC
- BrilliantViolet421, CFSE, PE, PECy7, APC, di-8-ANEPPS, NBD, BODIPY

11. What is the desired time period for the stability of refence particles for your EV size- and concentration measurements?

ANSWER CHOICES	RESPONSES	
1 month	0.00%	0
6 months	29.41%	5
1 year	35.29%	6
more than 1 year	35.29%	6
TOTAL		17