Internally stained multiple fluorescent microsphere intensity reference with NIST-assigned ERF values for quantitative flow cytometry analysis

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Introduction

Multicolor flow cytometry assays are routinely used in biomedical and clinical laboratories where instrument calibration is critical. Reliable instrument performance, assay standardization and measurement traceability can be achieved by using fluorescence intensity calibrators. These calibrators are typically micron sized particles that have been internally stained, or surface reacted to small molecule fluorescent dyes. Until recently, it has been recommended for flow cytometry users to use fluorescent particles with assigned Molecules of Equivalent Soluble Fluorophore (MESF) values. However, MESF values are currently measured by individual manufacturers using their own developed methods which leads to lot-to-lot and manufacturer-to-manufacturer variability. To address the inconsistencies of MESF values, the National Institute of Standards and Technology (NIST) has proposed a new fluorescence intensity unit known as the Equivalent number of Reference Fluorophores (ERF).[1]

Through collaborative research with NIST, a set of multi-color fluorescent microparticles, the **AccuCheck™ ERF Reference Particles**, were developed by Thermo Fisher Scientific. These multiple fluorescent, internally dye-stained particles were characterized using NIST developed measurement methods and ERF values were assigned to 26 different commonly used flow cytometry filter sets. One key advantage is that these particles fluoresce across the entire visible spectrum at three varying intensity levels, thus, allowing simultaneous fluorescence intensity measurements across multiple filter sets. Using a single set of particles is more practical than having to run multiple different single-dye-stained particle calibrators, like MESF based products, to achieve calibration of the entire fluorescence intensity scale. Furthermore, calibrators that have fluorescent dyes internally loaded within the polymer matrix have excellent shelf-life as the dye molecules are protected against environmental degradation and experience less photobleaching relative to dyes conjugated to the particle's surface.

The multiple fluorescent, internally dye-stained AccuCheck reference particles with NIST-assigned ERF values will ensure consistency and traceability of the sample's intensity measurements, make the intra- and inter-laboratory data comparison possible, and allow the performance characteristics of flow cytometers to be tracked. Perhaps most important of all will be the use of ERF particles in conjunction with a biological standard in order to quantify the expression level of surface and intracellular biomarkers.[2]

Materials

AccuCheck ERF Reference Particles Information

- Laser lines: 405 nm (Violet), 488 nm (blue), 561 nm(yellow) and 633 nm (red)
- NIST-assigned ERF intensity values for 26 emission channels, three levels of intensity (high, medium and low) for each channel
- Particle size: 3.1 µm • Concentration: 2 x10^6 particles/mL • Volume: 3 mL

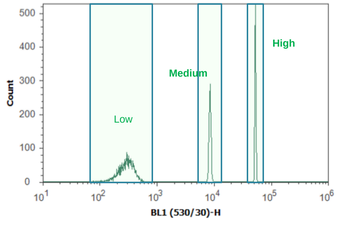


Figure 1. Example of a histogram plot of AccuCheck ERF reference particles in Attune NxT BL1 channel (488 nm blue laser excitation, 530/30 bandpass emission filter). Attune NxT cytometer was used following manufacturer's recommended protocols.

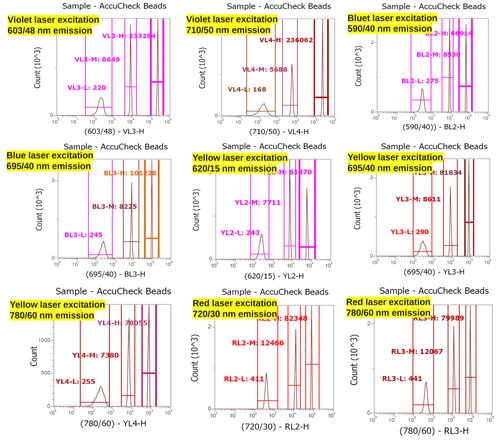
Product Features and Highlights

Table 1. NIST ERF Value Channel Assignment for the AccuCheck ERF Particles*

Laser Line	Em. Filter Set	Common Fluorophore(s)	NIST Ref. Fluorophore
405 nm Violet	440/50	Alexa Fluor 405 /BV421/Pacific Blue	Coumarin 30
	512/25	BV510 / AmCyan	Coumarin 30
	603/48	eFluor 625 / BV605 /QDot 605	Pacific Orange
	615/24	eFluor 625 / BV605 /QDot 605	Pacific Orange
	670/30	BV650/ QDot 655	Pacific Orange
	710/50	eFluor 710/ BV711	Pacific Orange
	720/60	eFluor 710/ BV711	Pacific Orange
488 nm Blue	525/35	Alexa Fluor 488/ FITC/ GFP/ Oregon Green	Fluorescein
	530/30	FITC / Alexa Fluor 488/ GFP	Fluorescein
	574/26	PE / RFP	Nile Red
	593/52	PE-Texas Red	Nile Red
	590/40	PE-Texas Red	Nile Red
	695/40	PerCP-Cy5.5	Nile Red
	780/60	PE-Cy7	Nile Red
561 nm Yellow	585/16	PE / RPE	Nile Red
	620/15	PE-Texas Red / mCherry	Nile Red
	670/30	PE-Cy5, PE-Alexa Fluor 647	Nile Red
	695/40	PE-Cy5.5	Nile Red
	720/60	PE-Cy5.5, PE-Alexa Fluor 700	Nile Red
	780/60	PE-Cy7, PE-Alexa Fluor 750	Nile Red
	789/78	PE-Cy7, PE-Alexa Fluor 750	Nile Red
633 nm Red	660/20	APC / Alexa Fluor 647 / eFluor 660	APC
	670/14	Alexa Fluor 647	APC
	670/30	APC	APC
	720/30	Alexa Fluor 700	Alexa Fluor 700
	780/60	APC-Cy7, Alexa Fluor 750	Alexa Fluor 700

* ERF values for filter sets not listed on the table can be calculated and provided

Figure 2. AccuCheck ERF reference particles fluoresce across the entire visible spectrum at three varying intensity levels, thus, allowing simultaneous fluorescence intensity measurements across multiple filter sets of a flow cytometer. Nine histogram plots are shown as examples. Attune NxT cytometer was used.

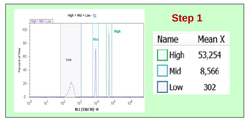


Method of Use

1. Calculate the ERF value of a sample - Method #1 (preferred method)

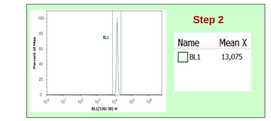
Step 1. Measure the mean fluorescence intensity (MFI) values of AccuCheck ERF Particles

- Add one drop AccuCheck ERF beads to **tube A**
- Add 1 mL of buffer and mix
- Set up the instrument and select the BL1 channel (530/30) since the sample is FITC labeled,
- Obtain the MFI values (mean X) of the tree intensity peaks (High: 53,254, Medium: 8,566 and Low: 302).



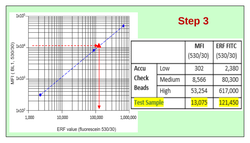
Step 2. Measure the MFI of test sample

- Add 1 mL of FITC-labeled test sample in **tube B**
- Use the same instrument MPT settings as for running **tube A** AccuCheck Beads
- Data requisition → MFI of the sample: 13,075



Step 3. Create a calibration curve

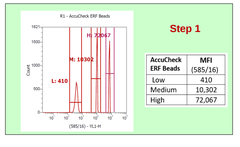
- Obtain the ERF values of AccuCheck Particles from Product Insert (for this assay, check 488 nm blue laser excitation, emission filter set: 530/30).
- Plot the assigned ERF values for each peak of AccuCheck Particles vs. the MFI of each peak (from Step 1) to create a calibration curve (**blue dots** and dotted line in the figure).
- Calculate the ERF value of the test sample by cross-calibrating its MFI (13,075) against calibration curve. The ERF value of the test sample can now be determined (121,450 ERF unit, FITC)



2. Alternative method of calculating sample ERF values – Method #2 (nearest neighbor reference)

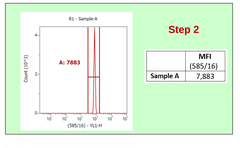
Step 1. Measure the mean fluorescence intensity (MFI) values of AccuCheck ERF Particles

- Add one drop AccuCheck beads to **tube C**
- Add 1 mL of buffer and mix
- Set up the instrument and select the YL1 channel (585/16) for intensity measurement
- Obtain the MFI values of tree intensity peaks (High: 72,067, Medium: 10,302 and Low: 410)



Step 2. Measure the MFI of test sample

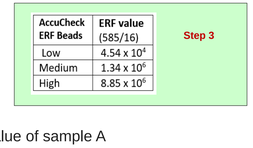
- Put 1 mL of RPE-labeled test sample A in **tube D**
- Run sample A using the same instrument MPT settings as for running **tube C**
- Data requisition → MFI of sample A: 7,883



Step 3. Calculate ERF value of test sample

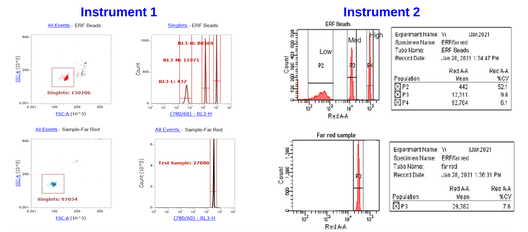
- The intensity value of sample A (MFI: 7,883) is close to the AccuCheck medium intensity beads (MFI: 10,302）
- Obtain the ERF value of medium beads from the table in Product Insert (561 nm yellow laser excitation, emission filter set: 585/16).
- Based on the ratio of MFI values of the sample A and medium intensity AccuCheck beads, calculate the ERF value of sample A

1.34x10^6 x (7,883/10,302) = 1.025x10^6 (ERF unit, RPE)



3. Example of cross instrument data comparison from different manufacturers. A) Fluorescence intensity of a sample with far red emission was measured using two instruments. B)The ERF values ware calculated and compared.

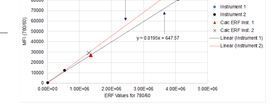
A)



B)

	NIST-assigned ERF values Filter set 780/60	Instrument 1 MFI value	Instrument 2 MFI value
Low MFI bead	1.82E+04	437	442
Medium MFI bead	5.47E+05	11971	12311
High MFI bead	4.09E+06	80369	92764
Sample		27080	29382

1. Calculate sample ERF value for Instrument 1: $\frac{27080-448}{0.0195} = 1.36 \times 10^6$
2. Calculate sample ERF value for Instrument 2: $\frac{29382+58}{0.0227} = 1.30 \times 10^6$



The test sample has 1.36 million and 1.30 million equivalent Alexa Fluor 700 fluorophores as calculated from Instrument 1 and Instrument 2, respectively. The ERF values are comparable between instruments regardless of settings (~4 % difference for this example).

Conclusions

- AccuCheck ERF reference particles with NIST-assigned ERF values will ensure consistency and traceability of the sample's intensity measurements and allow simultaneous fluorescence intensity measurements across multiple filter sets.
- AccuCheck ERF particles allows customers with multiple instruments and/or research sites to quantitate and accurately compare fluorescence intensity data between instruments and laboratories.

References

1. Wang, L.; Gaigalas A.K. *J. Res. Natl. Inst. Stand. Technol.* **2011,** 116: 671–683.
2. DeRose et. al *Material* **2020,** 13: 4111-4117.

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