National Institute of Standards & Technology

ERF Assignment of URB Microparticles
Using SRM® 1934

Standard Reference Material (SRM) 1934 was used to assign fluorescence intensity values to URB calibration microparticles (Spherotech) for quantitative flow cytometry. This reference scale for fluorescence intensity is based upon Equivalent Reference Fluorophore (ERF) units [1–2]. SRM 1934 consists of four ampoules, each containing a different fluorophore (e.g., fluorescent dye) solution or suspension. The SRM solutions, three of the four ampoules, are certified for concentration of fluorophore with a certified purity. The fourth ampoule (part D) contains a Reference Material (RM), i.e., a fluorophore suspension suppled with reference values for purity and concentration. The ERF scale is established for a particular set of experimental conditions by measuring the fluorescence intensity of known concentrations of each SRM/RM fluorophore and the URB calibration microparticles (beads) under those same measurement conditions (see "Instructions for Use"). Specific information for reference fluorophores, fluorescein in buffer solution (part A), Nile Red (NR) in acetonitrile solution (part B), Coumarin 30 (C30) in acetonitrile solution (part C), and allophycocyanin (APC) in buffer suspension (part D), are found in their corresponding sections of the SRM 1934 certificate.

ERF Values of URB Microparticles Using SRM 1934 Part C Excited at 405 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (Coumarin 30 molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
URB2	425 - 475	5.24×10^4	2.7×10^3	0.879	0.010
URB3	425 - 475	3.23×10^5	1.6×10^4	0.887	0.010
URB4	425 - 475	9.95×10^5	5.4×10^4	0.885	0.010
URB5	425 - 475	3.83×10^6	2.5×10^5	0.890	0.010
URB6	425 - 475	7.17×10^6	5.1×10^5	0.898	0.010

ERF Values of URB Microparticles Using SRM 1934 Part A Excited at 488 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (Fluorescein molecules / bead) main population		Intensity fraction main population	
		value	Uncertainty	value	uncertainty
URB2	510 - 550	8.02×10^3	1.7×10^3	0.890	0.010
URB3	510 - 550	7.10×10^4	4.8×10^3	0.889	0.010
URB4	510 - 550	2.09×10^5	1.1×10^4	0.891	0.010
URB5	510 - 550	4.99×10^5	4.3×10^4	0.892	0.010
URB6	510 - 550	1.03×10^6	1.3×10^5	0.899	0.010

ERF Values of URB Microparticles Using SRM 1934 Part B Excited at 488 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (Nile Red molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
URB2	565 - 605	6.49×10^4	4.2×10^3	0.893	0.010
URB3	565 - 605	6.53×10^5	4.3×10^4	0.896	0.010
URB4	565 - 605	1.71×10^6	1.2×10^5	0.893	0.010
URB5	565 - 605	4.41×10^6	4.0×10^5	0.894	0.010
URB6	565 - 605	8.93×10^6	9.6×10^5	0.903	0.010

ERF Values of URB Microparticles Using SRM 1934 Part D Excited at 633 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (APC molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
URB2	655 - 675	1.11×10^3	5.3×10^2	0.921	0.010
URB3	655 - 675	6.48×10^3	2.1×10^3	0.905	0.010
URB4	655 - 675	1.80×10^4	4.3×10^3	0.896	0.010
URB5	655 - 675	7.33×10^4	1.0×10^4	0.897	0.010
URB6	655 - 675	3.31×10^5	2.9×10^4	0.903	0.010

Certified Value: A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [3].

Reference Values: NIST reference values are noncertified values, which represent the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [3] and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

An EXCEL workbook is supplied for each ERF bead determination listed in the Tables above. The following sections explain how the fluorescence intensities and bead concentrations were measured and how they are presented in the workbook. The ERF calculation is also explained in enough detail to allow the user to recalculate ERF values and corresponding uncertainties for different fluorescence emission wavelength ranges using the workbook; for example, when a different emission filter is used.

Fluorescence Intensity Measurements: The fluorescence intensity of the microparticles was measured in units of equivalent reference fluorophores (ERF). This was achieved by first determining plots of fluorescence intensity versus reference fluorophore concentration using serial dilutions of the appropriate SRM 1934 fluorophore. A straight line was fitted to the plot. The fluorescence intensity of a microsphere suspension was then measured and its location on the fitted straight line was determined, giving the number of reference fluorophores needed to produce a fluorescence intensity equal to that of the microsphere suspension.

Fluorescence intensity was measured by integrating a fluorescence spectrum (fluorescence intensity versus emission wavelength) over the fluorescence emission wavelength range determined by the emission filter corresponding to a particular fluorescence channel of a flow cytometer. Each fluorescence spectrum was measured using a fluorescence spectrometer with a CCD detector and laser excitation. These spectra are shown in the workbook spreadsheets with the fluorescent label name for the URB microparticles and "…soln2", "…soln3", etc. for the reference solutions.

The relative radiometric accuracy as a function of wavelength of the signal (emission) detection system was corrected using a calibrated light source with calibrated diffuse reflector, traceable to the NIST realization of the International System of Units (SI) [4-8]. The correction factors for the relative spectral responsivity of our fluorescence spectrometer is shown in the workbook spreadsheet "CS". All fluorescence measurements were taken at 21 °C ± 0.5 °C using a 90° transmitting geometry with the excitation beam incident on and normal to one of the polished surfaces of the sample cuvette. All emission spectra were corrected for the responsivity of the detection system and normalized to the mean laser intensity measured over the same time period as each spectrum was taken.

Bead Concentration Measurements: A light obscuration-based, liquid particle counter was used to determine the bead concentration of the suspension. Particle concentration is obtained by dividing a particle count by the sample volume. Traceability to the SI is assured by determining the confidence that all particles within the sample volume are counted [9] and by determining the actual sample volume. Qualification of the particle counter for high accuracy measurements and determination of uncertainties includes gravimetric calibration of volume, pump volume dependence of particle counts to determine timing error, and concentration dependence of particle counts to determine the linear range, correct for coincidence and determine sampling error due to bead adsorption to surfaces.

A flow cytometer was also used to confirm the light-obscuration-based bead concentration. This was done by using TruCount beads as an internal standard in the URB bead suspension. These measurement values are also given in the workbook, but the light obscuration measurement is used to calculate the ERF values given in the Table. This is because the uncertainties in the light obscuration measurement are more thoroughly understood, such that the resulting bead concentration is traceable to the SI.

ERF Calculation Using EXCEL Workbook: An EXCEL workbook for each labeled bead was produced to calculate the ERF value for that bead. The workbook includes several spreadsheets. The first spreadsheet is always named "Int vs conc" and contains the log-log plot of fluorescence intensity versus reference fluorophore concentration (blue diamonds). The black line on the graph is the linear fit to the plotted data points, which defines the ERF scale. The green triangle is the ERF value for the bead suspension, which falls on the fitted line, and is reported in column M. Note that all spreadsheet locations referred to here pertain to spreadsheet "Int vs conc" unless specified otherwise.

The bead concentrations determined using both light obscuration and flow cytometry are reported in column L. The ERF value for the entire microparticle population and corresponding expanded uncertainty [10] are reported in columns N and O, respectively. The intensity fraction of the main population, determined through population gating using flow cytometry, is reported in column P. The ERF value for the main bead population and corresponding expanded uncertainty from both uncertainty of the entire microparticle population and uncertainty of the intensity fraction of the main population are reported in columns Q and R, respectively.

The fluorescence emission wavelength range, typically defined by the bandpass filter for the fluorescence channel, is given in column T. The manufacturer and/or user can change this range by inputting its lower and upper bounds in

cells T3 and T4, respectively. The workbooks for all of the URB microparticles use a fluorescence emission wavelength range (FEWR) that is the same for both the reference solutions and the bead suspension. If the user wants to specify different FEWR values for the microparticles and the reference solutions, this can be done using the "local integration" wavelength range specified in cells X2 and X3 of the corresponding bead and reference solution spreadsheets, but the NIST technical contact should be consulted for details.

Summary: ERF assignment of URB microparticles submitted by Spherotech was performed by P.C. DeRose and L. Wang of the NIST Biosystems and Biomaterials Division under the Flow Cytometry Quantitation Consortium CRADA with Spherotech (CRADA Identification Number: CN-16-0074). This report summarizes the assignment results for 5 different fluorescent URB microparticles using 4 different reference fluorophores and explains the accompanying Excel workbooks, one for each of the 20 pairs of microparticles and reference fluorophores. This explanation includes how Spherotech can revise these workbooks based on its customer's application needs to automatically re-calculate a corresponding ERF value.

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