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ERF Assignment of Supra Rainbow Quantitative Particles Using SRM® 1934 and NIST Internal RMs

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Standard Reference Material (SRM) 1934 was used to assign fluorescence intensity values to Supra Rainbow Quantitative Particles (SRQP) (Spherotech) for quantitative flow cytometry. The set of three particle suspensions have low (SRQP-L), medium (SRQP-M) and high (SRQP-H) fluorescence intensities. 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. In addition, NIST uses internal RMs (not for sale), consisting of three ampoules. Each ampoule contains a different fluorophore solution 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 SRQP microparticles (beads) under those same measurement conditions (see "Instructions for Use" in SRM certificate). 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), is found in their corresponding sections of the SRM 1934 certificate. The NIST internal reference fluorophores, Pacific Orange (PO) in dimethyl sulfoxide (DMSO) solution (referred to as RM-PO), Alexa Fluor 700 (AF700) in DMSO solution (referred to as RM-AF700) and Alexa Fluor 750 (AF750) in DMSO solution (referred to as RM-AF750) are used in the same way as SRM 1934. Additional information for the NIST internal reference fluorophores is given in Appendix A.

ERF Values of SRQP Microparticles Using SRM 1934 Part C Excited at 375 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (Coumarin 30 molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
SRQP-L	427.5 - 472.5	9.53×10^6	7.8×10^5	0.995	0.010
SRQP-L	505 - 545	2.50×10^5	2.1×10^4	0.995	0.010
SRQP-M	427.5 - 472.5	1.06×10^8	1.4×10^7	1.000	0.010
SRQP-M	505 - 545	3.81×10^6	4.6×10^5	1.000	0.010
SRQP-H	427.5 - 472.5	6.54×10^8	6.8×10^7	0.997	0.010
SRQP-H	505 - 545	7.52×10^7	7.1×10^6	0.997	0.010

ERF Values of SRQP Microparticles Using RM-PO Excited at 375 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (Pacific Orange molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
SRQP-L	660 - 690	1.21×10^5	2.6×10^4	0.997	0.010
SRQP-L	722.5 – 757.5	3.09×10^6	6.5×10^5	0.997	0.010
SRQP-M	660 - 690	2.07×10^6	2.3×10^5	1.000	0.010
SRQP-M	722.5 – 757.5	1.37×10^7	1.9×10^6	1.000	0.010
SRQP-H	660 - 690	1.03×10^8	1.6×10^7	0.997	0.010
SRQP-H	722.5 – 757.5	3.42×10^8	7.1×10^7	0.997	0.010

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ERF Values of SRQP 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
SRQP-L	425 - 475	2.75×10^5	4.1×10^4	0.997	0.010
SRQP-L	500 - 550	5.30×10^4	6.4×10^3	0.997	0.010
SRQP-L	600 - 620	1.98×10^5	3.0×10^4	0.997	0.010
SRQP-M	425 - 475	1.56×10^6	1.0×10^5	1.000	0.010
SRQP-M	500 - 550	4.53×10^5	3.1×10^4	1.000	0.010
SRQP-M	600 - 620	1.75×10^6	1.2×10^5	1.000	0.010
SRQP-H	425 - 475	1.17×10^7	7.5×10^5	0.997	0.010
SRQP-H	500 - 550	3.86×10^6	2.4×10^5	0.997	0.010
SRQP-H	600 - 620	2.59×10^7	1.9×10^6	0.997	0.010

ERF Values of SRQP Microparticles Using RM-PO Excited at 405 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (Pacific Orange molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
SRQP-L	600 - 620	1.70×10^4	2.4×10^3	0.995	0.010
SRQP-L	655 - 685	2.67×10^4	4.5×10^3	0.995	0.010
SRQP-L	685 – 735	6.98×10^4	8.6×10^3	0.995	0.010
SRQP-L	741.5 – 784.5	2.24×10^5	4.3×10^4	0.995	0.010
SRQP-L	750 – 810	6.02×10^5	2.4×10^5	0.995	0.010
SRQP-M	600 - 620	1.94×10^5	1.2×10^4	0.997	0.010
SRQP-M	655 - 685	3.91×10^5	2.3×10^4	0.997	0.010
SRQP-M	685 – 735	8.19×10^5	4.8×10^4	0.997	0.010
SRQP-M	741.5 – 784.5	1.62×10^6	9.6×10^4	0.997	0.010
SRQP-M	750 – 810	2.36×10^6	1.4×10^5	0.997	0.010
SRQP-H	600 - 620	2.93×10^6	1.8×10^5	1.000	0.010
SRQP-H	655 - 685	4.23×10^6	2.6×10^5	1.000	0.010
SRQP-H	685 – 735	1.35×10^7	9.0×10^5	1.000	0.010
SRQP-H	741.5 – 784.5	2.81×10^7	1.9×10^6	1.000	0.010
SRQP-H	750 – 810	4.52×10^7	3.5×10^6	1.000	0.010

ERF Values of SRQP 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
SRQP-L	500 - 550	2.57×10^4	3.2×10^3	0.997	0.010
SRQP-M	500 - 550	2.86×10^5	3.0×10^4	0.997	0.010
SRQP-H	500 - 550	1.91×10^6	2.7×10^5	0.997	0.010

ERF Values of SRQP 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
SRQP-L	562.5 – 587.5	5.31×10^5	6.3×10^4	0.997	0.010
SRQP-L	600 - 620	9.45×10^4	1.1×10^4	0.997	0.010
SRQP-L	660 - 690	6.11×10^4	7.4×10^3	0.997	0.010
SRQP-L	665 - 715	6.13×10^4	7.4×10^3	0.997	0.010
SRQP-L	750 - 810	9.17×10^4	1.1×10^4	0.997	0.010
SRQP-M	562.5 – 587.5	5.00×10^6	6.7×10^5	1.000	0.010
SRQP-M	600 - 620	7.56×10^5	9.6×10^4	1.000	0.010
SRQP-M	660 - 690	5.44×10^5	6.9×10^4	1.000	0.010
SRQP-M	665 - 715	5.64×10^5	7.2×10^4	1.000	0.010
SRQP-M	750 - 810	8.75×10^5	1.1×10^5	1.000	0.010
SRQP-H	562.5 – 587.5	5.11×10^7	6.5×10^6	0.998	0.010
SRQP-H	600 - 620	7.76×10^6	9.4×10^5	0.998	0.010
SRQP-H	660 - 690	6.95×10^6	8.4×10^5	0.998	0.010
SRQP-H	665 - 715	9.17×10^6	1.1×10^6	0.998	0.010
SRQP-H	750 - 810	2.85×10^7	3.5×10^6	0.998	0.010

ERF Values of SRQP Microparticles Using SRM 1934 Part B Excited at 561 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (Nile Red molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
SRQP-L	564 – 606	1.08×10^5	1.2×10^4	0.994	0.010
SRQP-L	600 - 620	4.26×10^4	4.8×10^3	0.994	0.010
SRQP-L	660 - 690	7.08×10^4	8.0×10^3	0.994	0.010
SRQP-L	685 - 735	1.18×10^5	1.3×10^4	0.994	0.010
SRQP-L	741.5 – 784.5	2.32×10^5	2.6×10^4	0.994	0.010
SRQP-L	750 - 810	2.58×10^5	2.9×10^4	0.994	0.010
SRQP-M	564 – 606	1.06×10^6	1.3×10^5	0.997	0.010
SRQP-M	600 - 620	4.24×10^5	5.0×10^4	0.997	0.010
SRQP-M	660 - 690	7.88×10^5	9.3×10^4	0.997	0.010
SRQP-M	685 - 735	1.31×10^6	1.5×10^5	0.997	0.010
SRQP-M	741.5 – 784.5	2.49×10^6	3.0×10^5	0.997	0.010
SRQP-M	750 - 810	2.66×10^6	3.2×10^5	0.997	0.010
SRQP-H	564 – 606	9.09×10^6	1.0×10^6	0.997	0.010
SRQP-H	600 - 620	3.57×10^6	4.1×10^5	0.997	0.010
SRQP-H	660 - 690	6.18×10^6	7.1×10^5	0.997	0.010
SRQP-H	685 - 735	1.50×10^7	1.7×10^6	0.997	0.010
SRQP-H	741.5 – 784.5	3.00×10^7	3.6×10^6	0.997	0.010
SRQP-H	750 - 810	3.78×10^7	4.6×10^6	0.997	0.010

ERF Values of SRQP 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
SRQP-L	655 - 685	6.84×10^3	9.1×10^2	0.997	0.010
SRQP-L	699.5 – 724.5	2.50×10^4	2.7×10^3	0.997	0.010
SRQP-L	707.5 – 752.5	2.37×10^4	2.6×10^3	0.997	0.010
SRQP-L	741.5 – 784.5	3.60×10^4	3.8×10^3	0.997	0.010
SRQP-L	750 - 810	3.94×10^4	5.3×10^3	0.997	0.010
SRQP-M	655 - 685	7.43×10^4	8.0×10^3	0.997	0.010
SRQP-M	699.5 – 724.5	2.86×10^5	3.0×10^4	0.997	0.010
SRQP-M	707.5 – 752.5	2.64×10^5	2.8×10^4	0.997	0.010
SRQP-M	741.5 – 784.5	3.98×10^5	4.3×10^4	0.997	0.010
SRQP-M	750 - 810	4.54×10^5	5.3×10^4	0.997	0.010
SRQP-H	655 - 685	2.54×10^5	2.5×10^4	0.997	0.010
SRQP-H	699.5 – 724.5	2.24×10^6	2.5×10^5	0.997	0.010
SRQP-H	707.5 – 752.5	2.04×10^6	2.4×10^5	0.997	0.010
SRQP-H	741.5 – 784.5	3.20×10^6	3.9×10^5	0.997	0.010
SRQP-H	750 - 810	4.83×10^6	7.0×10^5	0.997	0.010

ERF Values of SRQP Microparticles Using RM-AF700 Excited at 633 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (AF700 molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
SRQP-L	699.5 – 724.5	3.18×10^5	2.2×10^4	1.000	0.010
SRQP-L	707.5 – 752.5	1.22×10^5	8.6×10^3	1.000	0.010
SRQP-L	741.5 – 784.5	1.05×10^5	7.4×10^3	1.000	0.010
SRQP-L	750 - 810	1.21×10^5	8.5×10^3	1.000	0.010
SRQP-M	699.5 – 724.5	3.81×10^6	3.1×10^5	0.997	0.010
SRQP-M	707.5 – 752.5	1.46×10^6	1.1×10^5	0.997	0.010
SRQP-M	741.5 – 784.5	1.26×10^6	9.2×10^4	0.997	0.010
SRQP-M	750 - 810	1.48×10^6	1.1×10^5	0.997	0.010
SRQP-H	699.5 – 724.5	3.15×10^7	3.8×10^6	0.997	0.010
SRQP-H	707.5 – 752.5	1.21×10^7	1.3×10^6	0.997	0.010
SRQP-H	741.5 – 784.5	1.10×10^7	1.2×10^6	0.997	0.010
SRQP-H	750 - 810	1.64×10^7	1.9×10^6	0.997	0.010

ERF Values of SRQP Microparticles Using RM-AF750 Excited at 808 nm

Fluorescent label	Fluorescence Emission Wavelength Range (nm)	ERF (AF700 molecules / bead) main population		Intensity fraction main population	
		value	uncertainty	value	uncertainty
SRQP-L	830 – 850	9.56×10^4	6.6×10^3	0.994	0.010
SRQP-L	865 – 905	6.75×10^4	4.7×10^3	0.994	0.010
SRQP-M	830 – 850	3.01×10^5	2.8×10^4	0.997	0.010
SRQP-M	865 – 905	2.16×10^5	2.0×10^4	0.997	0.010
SRQP-H	830 – 850	7.27×10^7	9.7×10^6	0.997	0.010
SRQP-H	865 – 905	4.70×10^7	5.8×10^6	0.997	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 or NIST internal RM 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 for the SRQP 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, 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 are shown in the workbook spreadsheet "CS". All fluorescence measurements were taken at 21 °C ± 1.0 °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 SRQP 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 SRQP 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 SRQP microparticles, a set of three particle suspensions having low (SRQP-L), medium (SRQP-M) and high (SRQP-H) fluorescence intensities and submitted by Spherotech, was performed by P.C. DeRose, L. Tian, N. Xu and L. Wang of the NIST Biosystems and Biomaterials Division under the Flow Cytometry Quantitation Consortium CRADA with Spherotech (CRADA Identification Number: CN-20-0115). This report summarizes the assignment results for SRQP microparticles using 7 different reference fluorophores and explains the accompanying Excel workbooks, one for each of the 35 combinations of excitation wavelengths, reference fluorophores and emission wavelength ranges. This explanation includes how Spherotech can revise these workbooks based on its customer's application needs to automatically re-calculate a corresponding ERF value.

Appendix A: Summary of Purity and Concentration Determinations for NIST Internal Reference Fluorophores

The purity of three fluorescent dyes, Pacific Orange (PO), Alexa Fluor 700 (AF700) and Alexa Fluor 750 (AF750), was determined using quantitative ^{1}H-NMR (qNMR). HPLC was used as supporting evidence to help assign the identities of impurities. Each dye was dried in an oven before weighing and dissolved in dimethyl sulfoxide (DMSO). The resulting fluorescent reference solutions for internal NIST use were assigned reference values for concentration in units of mass of dye per mass of solution, using gravimetry and absolute dye purity. The dye solutions were put into flame-sealed ampoules under argon gas in 2 mL aliquots. The concentrations of dye solution determined from measured masses of dye and solvent were adjusted for purity. The corresponding uncertainties include those related with both the purity and mass measurements.

High Performance Liquid Chromatography (HPLC)

The fluorescent dyes analyzed here are expected to have impurities that are either aromatic compounds, such as starting materials and intermediates that did not fully react to become the final dye product, or solvent residues that were not removed completely during the organic syntheses that produced the dyes. The separation of mixtures of organic compounds with similar structures can often be done most effectively using reversed-phase high performance liquid chromatography (HPLC). The two fluorescent dyes and their aromatic impurities were separated and detected using HPLC with UV absorbance detection. All impurities were detected with the greatest sensitivity at an absorbance wavelength of 262 nm. Therefore, absorbance at 262 nm was used to determine purity with HPLC. Chromatograms of each solution and the solvent were collected. The solvent chromatogram was subtracted from the solution chromatogram to give a solvent-corrected chromatogram for each solution. The areas of the main and impurity peaks were integrated. The peak areas were expressed as a percentage of the total constituent areas, such that the sum of all peak areas is equal to one (100 %). Peaks were considered to be significant if their area percent was ≥ 0.1 %.

For PO, two impurity peaks were observed with retention times after the main peak and a summed impurity area of 1.4 %. For AF700, two impurity peaks were observed at retention times before the main peak and two more were observed after the main peak. The summed area of all four impurity peaks was 0.8 %. For AF750, one impurity peak was observed at a retention time before the main peak. The area of the impurity peak was 4.0 %

qNMR Purity Determination

Quantitative NMR with an internal standard was used to determine the absolute purity for the solid samples of PO, AF700 and AF750. The absolute purity of PO was determined to be 0.9390 g/g (gram of PO with counter ion per gram of PO powder) and 0.7839(78) g/g (gram of PO without counter ion per gram of PO powder). Applying the latter purity value to the concentration gives a value of 20.10(23) mg PO / kg DMSO solution (3.724(43) x 10^{-5} mol / kg). Assuming a DMSO density of 1.0984(10) g/mL at 22 °C and using a molecular weight (MW) for PO (w/o counter ion) of 539.55 gives a value in moles per liter of 4.091(47) x 10^{-5} mol PO / L DMSO solution or about 41 μM.

The absolute purity of AF700 was determined to be 0.9556 g/g (gram of AF700 with counter ion per gram of AF700 powder) and 0.7124(60) g/g (gram of AF700 without counter ion per gram of AF700 powder). Applying the latter purity value to the concentration gives a value of 20.15(17) mg AF700 / kg DMSO solution (2.044(17) x 10^{-5} mol / kg). Assuming a DMSO density of 1.0984(10) g/mL at 22 °C and using a molecular weight (MW) for AF700 (w/o counter ion) of 985.93 gives a value in moles per liter of 2.245(19) x 10^{-5} mol AF700 / L DMSO solution or about 22 μM.

The absolute purity of AF750 was determined to be 0.8762 g/g (gram of AF750 with counter ion per gram of AF750 powder) and 0.6054(81) g/g (gram of AF750 without counter ion per gram of AF750 powder). Applying the latter purity value to the concentration gives a value of 15.99(21) mg AF750 / kg DMSO solution (1.812(24) x 10^{-5} mol / kg). Assuming a DMSO density of 1.0984(10) g/mL at 22 °C and using a molecular weight (MW) for AF750 (w/o counter ion) of 882.02 gives a value in moles per liter of 1.991(27) x 10^{-5} mol AF750 / L DMSO solution or about 20 μM.

The purity by mass of each dye was reported with and without counter ion because the stoichiometry found by qNMR for each dye was not an integer for the counter ion. The stoichiometric ratio for the counter ion versus the dye was determined to be 1.05, 3.3 and 3.9 for PO, AF700 and AF750, respectively. This implies that the molecular weight of the dye with counter ion could not be determined accurately, therefore, the molecular weight of the dye without counter ion was used to determine the purity in grams of dye per gram of solid sample. These values of 0.784 g/g ± 0.018 g/g, 0.712 g/g ± 0.012 g/g and 0.605 g/g ± 0.016 g/g were determined at a 95 % confidence interval for PO, AF700 and AF750, respectively. Note that these are the purity and uncertainty reference values for the dyes.

The purity value with counter ion was used to estimate the percent impurities by weight for each dye. This gives impurity values of 6.1 %, 4.4 % and 13.4 % for PO, AF700 and AF750, respectively. HPLC suggested aromatic impurities of 1.4 %, 0.8 % and 4.0 % respectively, which did not absorb or fluoresce light in the same spectral region as the main dye. The NMR peaks from impurities agreed approximately with the HPLC values and did not account for the larger amounts of impurities determined by qNMR. These unknown impurities could be due to inorganics or solvent molecules bound to the dye that could not be driven off easily with heat. The identification of the unknown impurities will be pursued using ICP-MS and LC-MS in the future, contingent on more dye samples being obtained from the manufacturer.

The concentration and uncertainty for each ampouled reference dye solution is given below. They are given as reference, not certified, values, even though the magnitude of the uncertainties for purity and gravimetry are within acceptable limits, because the nature of the unknown impurities was not identified.

[PO]	**U_{95}**	**[AF700]**	**U_{95}**	**[AF750]**	**U_{95}**
mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
20.0951	0.462	20.1497	0.340	15.9852	0.428
mol/kg	mol/kg	mol/kg	mol/kg	mol/kg	mol/kg
3.724E-05	8.6E-07	2.044E-05	3.4E-07	1.812E-05	4.9E-07
mol/L	mol/L	mol/L	mol/L	mol/L	mol/L
4.091E-05	9.4E-07	2.245E-05	3.8E-07	1.991E-05	5.3E-07

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