National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 1934

Fluorescent Dyes for Quantitative Flow Cytometry (Visible Spectral Range)

This Standard Reference Material (SRM) is intended for use in assigning fluorescence intensity values to calibration standards for quantitative flow cytometry in the visible spectral range. A unit of SRM 1934 consists of four amber ampoules, each containing 2 mL of a different fluorophore solution or suspension. The solutions include Part A: Fluorescein Solution (60 µM aqueous borate buffer solution); Part B: Nile Red Solution (60 µM acetonitrile solution); Part C: Coumarin 30 Solution (acetonitrile solution); and Part D: APC Suspension (Allophycocyanin fluorescent protein in aqueous buffer solution, 100 µL).

This reference scale for fluorescence intensity is based upon Equivalent Reference Fluorophore (ERF) units [1–2]. The ERF scale is established for a particular set of experimental conditions by measuring the fluorescence intensity of known concentrations of each ampoule under those experimental conditions (see "Instructions for Handling, Storage, and Use").

Certified Concentration Values: The certified fluorophore concentration and purity values for Parts A, B, and C listed in Tables 1A, 2A, and 3A respectively, are provided based on gravimetric preparation and analysis of purity by proton nuclear magnetic resonance spectroscopy. 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: A reference value for the allophycocyanin (APC) suspension concentration for Part D is provided in Table 4A. The reference values for the molar extinction coefficients are provided for each solution corrected for purity and based on gravimetric preparation and analysis of the absorbance of visible light at the peak wavelength. A NIST reference value is a noncertified value, which represents the best estimate of the true value based on available data; however, the value does not meet the NIST criteria for certification and is 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 [3].

Information Values: Information values are given for pH, buffer concentration and mass density for Parts A and D, and for mass density for the solvents used in Parts B and C; corrected fluorescence emission spectrum of Parts A through D at their recommended excitation wavelength; and APC purity for Part D. A NIST information value is a value that may be of use to the SRM user, but insufficient information is available to assess adequately the uncertainty associated with the value [3]. An information value cannot be used to establish metrological traceability.

Expiration of Certification: The certification of **SRM 1934** is valid, within the measurement uncertainty specified, until **01 July 2021**, provided the SRM is handled and stored in accordance with instructions given in this certificate (see "Instructions for Handling, Storage, and Use"). This certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate this notification.

Production and certification of this SRM were performed by P.C. DeRose, L. Wang, A.K. Gaigalas, K.D. Cole, A. Urbas, B. Coxon, G.W. Kramer of the NIST Biosystems and Biomaterials Division; with assistance from B.A. Benner, D.L. Duewer, B. Lang, M. Nelson, J.R. Sieber, T.W. Vetter, and L.L. Yu of the NIST Chemical Sciences Division; D.M. Bunk, and E. White V. of the NIST Biomolecular Measurement Division; M.B. Satterfield of the NIST Material Measurement Laboratory; and S.A. Margolis, J.B. Smeller, Y. Tewari, M.J. Welch formerly of NIST.

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Statistical consultation was provided by B. Toman and J. Lu of the NIST Statistical Engineering Division.

Ancillary measurements were provided by G.E. Marti, F. Abbasi, and J. Weaver of the U.S. Food and Drug Administration, R.F. Vogt of the Centers for Disease Control and Prevention, and Y-z. Zhang of Molecular Probes, Inc.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

Handling: This SRM is comprised of a suspension in a plastic ampoule (Part D) and three solutions (Parts A-C), each contained in a tip-sealed glass ampoule with pre-scored stem. Therefore, all appropriate safety precautions, including the use of gloves during handling, should be taken to avoid accidental breakage or spillage.

Storage: Unopened ampoules should be stored in the dark between 2 °C and 6 °C in an upright position. The ampoules should **NOT** be frozen because of the possibility of breakage during freezing and thawing. Once the ampoule is opened, the solution should be used promptly with minimal exposure to any light (exposure to incandescent lighting is preferable to illumination from daylight or fluorescent lighting). Any unused solution in the ampoule should be discarded according to local regulations.

NOTICE: NIST monitored the stability of SRM 1934. Prior to certification, SRM 1934 parts were monitored as follows: Part A for over 24 months, Part B and Part C for five months, and Part D for approximately 9 months. Within the error of the measurements, the absorbance spectrum and the fluorescence spectral radiance (as indicated by the fluorescence signal spectrum) of the solution did not change. Therefore, the ampouled solution, if stored as indicated above, is likely to maintain its original optical properties for the duration of its certification period.

Opening an Ampoule: When an ampoule is opened, that area of the stem where the pre-scored band is located should be wiped with a clean, damp cloth and the body of the ampoule wrapped in absorbent material. Then holding the ampoule steady and with the thumb and forefinger grasping the stem above the scoring, **minimal** thumb pressure should be applied to the stem to snap it. Correctly done, the stem should break easily where pre-scored. The use of a metal file to break the stem is **NOT** recommended.

PREPARATION AND ANALYSIS[(1)]

SRM 1934 Part A Fluorescein

Certified Values: The certified concentration of fluorescein provided in Table 1A is based on gravimetric preparation and analysis of purity by proton nuclear magnetic resonance spectroscopy that was independently verified by analysis of impurities. The fluorescein purity determination provided below is based on proton nuclear magnetic resonance spectroscopy. The measurands are the concentration and purity of fluorescein. Metrological traceability is to the SI derived unit for amount-of-substance concentration and mass fraction (expressed as micromoles per kilogram and percent, respectively).

Table 1A. Certified Amount-of-Substance Concentration and Mass Fraction for SRM 1934 Part A

Fluorescein (concentration)	60.97 μmol/kg	±	0.4 μmol/kg
Fluorescein (purity)	97.55 %	±	0.64 %

Reference Values: Table 1B provides reference values for the molar extinction coefficient of SRM 1934 Part A corrected for purity and fluorescence bias. The measurand is the molar extinction coefficient at the peak wavelengths listed based on the method indicated. Metrological traceability is the SI units for mass, length, and amount-of-substance (expressed as $kg \cdot cm^{-1} \cdot mol^{-1}$).

Table 1B. Reference Values for Molar Extinction Coefficient of SRM 1934 Part A at 22.4 °C ± 0.5 °C

Wavelength (nm)	Molar Extinction Coefficient ($kg \cdot cm^{-1} \cdot mol^{-1}$)	Uncertainty ($kg \cdot cm^{-1} \cdot mol^{-1}$)
488.0	8.50×10^4	0.07×10^4
490.0	8.70×10^4	0.07×10^4
490.5	8.71×10^4	0.07×10^4
491.0	8.70×10^4	0.07×10^4

[(1)]Certain commercial instruments, materials, or processes are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the instruments, materials, or processes identified are necessarily the best available for the purpose.

Determination of Fluorescein Purity: Table 1C summarizes the purity determinations of the fluorescein material used to prepare SRM 1934 Part A. The measurands are the constituents listed in Table 1C as determined by the indicated methods. Metrological traceability is to the SI derived unit for mass fraction (expressed as a percent).

Table 1C. Reference Values for Fluorescein Purity Determinations and Associated Uncertainties

Constituent	Technique	Mass Fraction (%)	Uncertainty (%)
DHBBA[(b)]	^{1}H NMR[(a)] & HPLC[(c)]	1.04	0.20
Ethanol	^{1}H NMR[(a)]	0.21	0.04
Methyl Isobutyl Ketone	^{1}H NMR[(a)]	0.02	0.02
HOAc/Acetate	^{1}H NMR[(a)]	0.02	0.01
Ethyl Acetate	^{1}H NMR[(a)]	0.02	0.01
Water	Karl Fisher	0.25	0.02
Total Organics	CHO Analysis[(d)]	99.19	0.82
Potassium	FAES[(e)]	0.50	0.14
Sodium	FAES[(e)]	0.03	0.03
Chloride	Argentimetry[(f)]	0.37	0.03

[(a)] 500 MHz Proton Nuclear Magnetic Resonance Spectroscopy

[(b)] 2-(2',4'Dihydroxybenzoyl) benzoic acid

[(c)] High performance Liquid Chromatography and High Performance Liquid Chromatography-Mass Spectrometry, both normal and reversed phase

[(d)] Elemental analyses carried out by Schwartzkopf Microanalytical Laboratory, Inc. (Woodside, NY), Atlantic Microlab, Inc. (Norcross, GA), and Galbraith Laboratories, Inc. (Knoxville, TN)

[(e)] Flame Atomic Emission Spectroscopy

[(f)] Halide content identified solely as chloride by X-ray Fluorescence and Inductively Coupled Plasma-Mass Spectrometry

Information Values: Table 1D provides information values for SRM 1934 Part A. The buffer concentration is the concentration of boric acid in the buffer solution into which the fluorescein was dissolved. Figure 1 provides the fluorescence emission spectrum of fluorescein. Additional information values for relative signal in power units and emission wavelength in nanometers is located on the Material Details web page under the Data Files icon for SRM 1934 (https://www-s.nist.gov/srmors/view_detail.cfm?srm=1934).

Table 1D. Information Values for SRM 1934 Part A at 22 °C

pH	Buffer Concentration	Mass Density
9.48	0.10 mol/L	1.003 g/mL

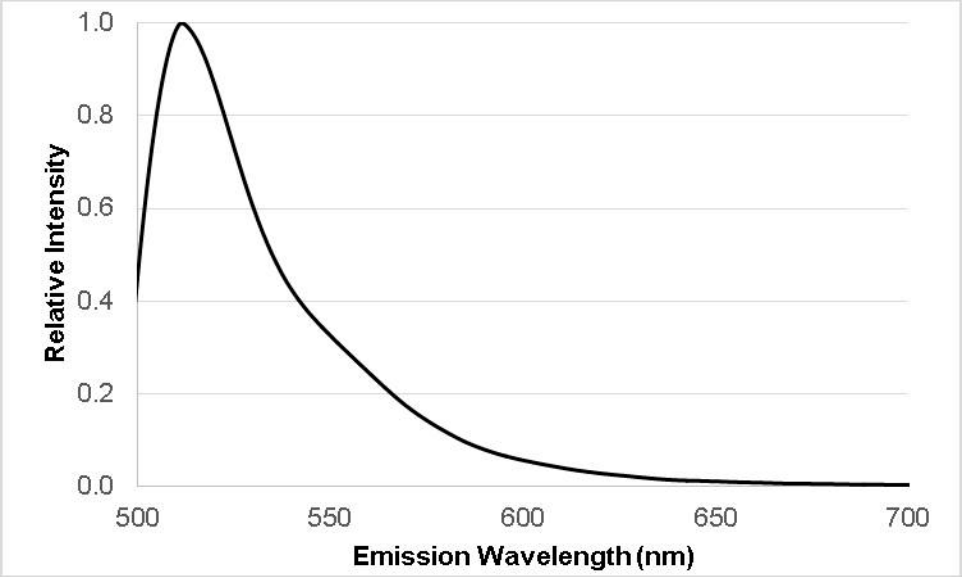

Figure 1. The fluorescence emission spectrum of fluorescein is a corrected fluorescence emission spectrum for SRM 1934 Part A, diluted 100 fold using borate buffer, at an excitation wavelength of 488 nm. A 488 nm Ar ion laser was used for excitation. The spectral bandpass of the detection system was set at 2 nm.

Source of Material: The fluorescein [2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid, $C_{20}H_{12}O_5$, relative molecular mass 332.311, CAS No. 2321-07-5] powder used in SRM 1934 was prepared especially for this purpose by Molecular Probes, Inc. (Eugene, OR; Leiden, The Netherlands), 71358, MPR, Lot W018073. Boric acid granules were obtained from Mallinckrodt (St. Louis, MO), Lot 2549 KVTK, relative molecular mass 61.83. Sodium hydroxide pellets were from Mallinckrodt, Catalog No. 7708, Lot 7708M484721, relative molecular mass 40.00. All water used was NIST deionized water that was then subsequently passed through a second purification system (Millipore Mill-Q A10) to produce water having a resistivity ≥18 MΩ·cm.

Preparation of Part A: The borate buffer solution was prepared by dissolving 18.56 g of boric acid granules in slightly less than 3 L of water. The pH was adjusted to above 9.1 using 40 mL of an approximately 3.0 mol/L NaOH solution made by dissolving 29.87 g of sodium hydroxide pellets in 0.250 L of water. The mixture volume was increased to 3.00 L with water to give a borate buffer with a boric acid concentration of about 0.10 mol/L.

The SRM solution was prepared in a darkened room by dissolving 0.05636 g ± 0.00004 g of fluorescein (buoyancy corrected) in 2.7134 kg ± 0.0006 kg of borate buffer solution (buoyancy corrected). The mass of fluorescein used was corrected for the fluorescein purity yielding a final fluorescein concentration of 60.97 μmol/ kg ± 0.40 μmol/kg. The fluorescein solution contained in an amber bottle was immediately aliquotted into ampoules that were subsequently flame-sealed. The pH and mass density of the SRM were determined from measurements on four ampoules selected randomly from the lot.

Preparation of Part A for Analysis: The SRM 1934 Part A solution should always be diluted gravimetrically at least one-hundred fold with the same buffer system used for the analyte of interest. Subsequent gravimetric dilutions can then be employed to generate a calibration curve. The calibration curve describes the relationship between fluorescence intensity, as determined by the fluorometer and the concentration of fluorescein, as determined by the gravimetric dilution. Care must be taken in making the gravimetric dilutions because uncertainties expand during this serial process. An error at one level adversely affects determinations at all subsequent levels. The calibration curve measurements should be generated starting with the lowest concentration. For best results, the conditions used for determining the calibration curve such as solution degassing, temperature, ionic strength, pH, etc., should closely match those used in the measurement of the analyte. Because fluorescence is a highly sensitive technique, great attention must be paid to the cleanliness of glassware and any other apparatus that contacts the solutions. Many plastics and gloves can contaminate samples with small amounts of highly fluorescent materials such as release agents and plasticizers. The running of blanks to check for such contamination is highly recommended.

Depending on the solution pH, aqueous fluorescein solutions are complex, rapidly equilibrating mixtures of its several forms (cation, neutral species, monoanion, and dianion). Each species has unique absorbance and fluorescence spectra. Above pH 9, aqueous fluorescein exists almost exclusively as the highly fluorescent dianion. However, as the pH of the solution is reduced, the concentration of the dianion decreases, and the concentrations of the much less fluorescent monoanion and neutral forms increase [4]. Accordingly, the sensitivity of the fluorometric assay for fluorescein also decreases, and quantitation becomes very dependent on knowing or maintaining the precise pH of the solutions during calibration as well as during the assay itself. While the calibration strategy described above can work with solutions below pH 9, the uncertainties of such measurements inevitably grow larger as the solution pH is lowered.

Assignment of Uncertainties: Standard uncertainty components equivalent to the estimated standard deviation were assigned for sample inhomogeneity and measurement uncertainties. These values were then combined with balance accuracy and estimated instrument method uncertainties using the root-sum-of-squares method. An expansion factor of $k = 2$ was applied so that the expanded uncertainties given in this certificate express an interval within which the true value is expected to fall with a level of confidence of approximately 95 % for a normal distribution [5].

Part B: Nile Red Solution

Certified Value: The certified concentration of Nile Red provided in Table 2A is based on gravimetric preparation and analysis of purity by proton nuclear magnetic resonance spectroscopy that was quantified using an internal standard. The measurands are the concentration and purity of Nile Red. Metrological traceability is to the SI derived unit for amount-of-substance concentration and mass fraction (expressed as micromoles per kilogram and percent, respectively).

Table 2A. Certified Amount-of-Substance Concentration and Mass Fraction for SRM 1934 Part B

Nile Red (concentration)	118.7 µmol/kg	±	2.3 µmol/kg
Nile Red (purity)	97.74 %	±	1.02 %

Reference Values: Table 2B provides reference values for the molar extinction coefficient of the SRM 1934 Part B corrected for purity. Fluorescence bias was found to be statistically insignificant. The measurand is the molar extinction coefficient at the peak wavelengths listed based on the method indicated. Metrological traceability is the SI units for mass, length, and amount-of-substance (expressed as $kg \cdot cm^{-1} \cdot mol^{-1}$).

Table 2B. Reference Values for Molar Extinction Coefficient of SRM 1934 Part B at 22.0 °C ± 0.5 °C

Wavelength (nm)	Molar Extinction Coefficient ($kg \cdot cm^{-1} \cdot mol^{-1}$)	Uncertainty ($kg \cdot cm^{-1} \cdot mol^{-1}$)
488.0	1.83×10^4	0.04×10^4
532.0	3.47×10^4	0.08×10^4

Determination of Nile Red Purity: Table 2C summarizes the purity determinations of the Nile Red material used to prepare SRM 1934 Part B. The measurands are the constituents listed in Table 2C as determined by the indicated methods. Metrological traceability is to the SI derived unit for mass fraction (expressed as a percent).

Table 2C. Reference Values for Nile Red Purity Determinations and Associated Uncertainties

Constituent	Technique	Mass Fraction (%)	Uncertainty (%)
Dichloromethane	^{1}H NMR[(a)] & HPLC[(b)]	0.62	0.20
Similar MW to NR[(c)]	^{1}H NMR[(a)]	<0.3	0.15
Water	TGA[(d)]	<0.09	0.04

(a) 600 MHz Proton Nuclear Magnetic Resonance Spectroscopy
(b) High Performance Liquid Chromatography, reversed phase
(c) Constituents with a molecular weight similar to Nile Red
(d) Thermogravimetric analysis

Information Value: Table 2D provides an information value for the mass density of acetonitrile (ACN) in SRM 1934 Part B. Figure 2 provides the fluorescence emission spectrum of Nile Red. Additional information values for relative signal in power units and emission wavelength in nanometers is located on the Material Details web page under the Data Files icon for SRM 1934 (https://www-s.nist.gov/srmors/view_detail.cfm?srm=1934).

Table 2D. Mass Density Information Value for SRM 1934 Part B at 22 °C [6]

ACN	0.7799 g/mL

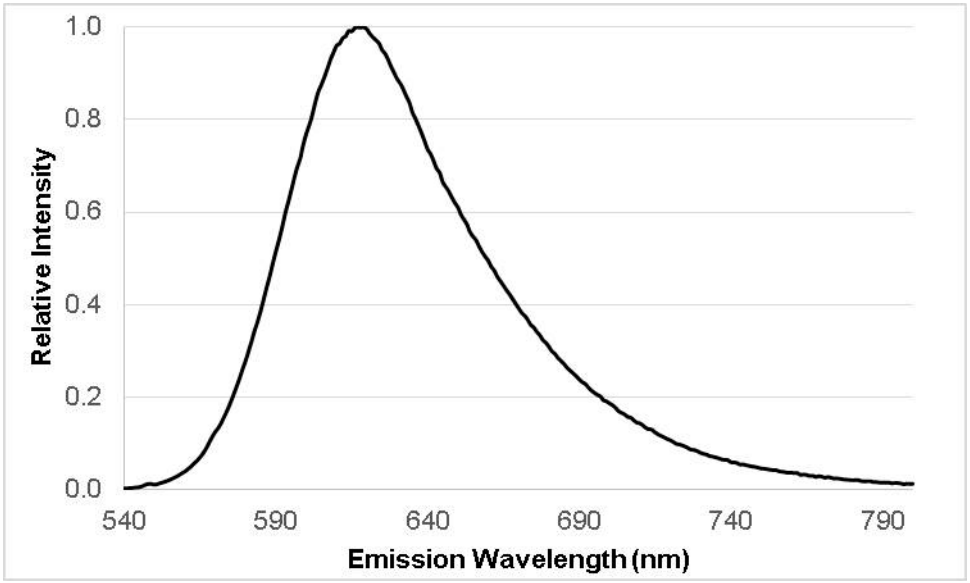

Figure 2. Corrected fluorescence emission spectrum for SRM 1934 Part B, diluted 100 fold using ACN, at an excitation wavelength of 488 nm. A 488 nm diode laser was used for excitation. The spectral bandpass of the detection system was set at 2.5 nm.

Source of Material: The Nile Red (NR) (9-diethylamino-5-benzo[α]phenoxazinone, $C_{20}H_{18}N_2O_2$, relative molecular mass 318.369, CAS No. 7385-67-3) was produced by Molecular Probes® (Life Technologies/Thermo Fisher Scientific) as Cat. # N1142, Lot # 1189983, and determined by the manufacturer to be 99 % pure using HPLC with UV absorption detection at 254 nm. Dye solutions were dissolved in acetonitrile (ACN) and concentrations were determined gravimetrically. The ACN used was Mallinckrodt UltimAR®, (HPLC/Spectrphotometric grade) Lot # H454 T37465. The balance used was a Mettler AT20, which was internally calibrated. All gravimetric measurements were taken at temperature T = 21.0 °C, 25 % relative humidity (rh) and 995 mbar atmospheric pressure.

Preparation of Part B: The SRM solution was prepared by dissolving 0.030039 g ± 0.000005 g of Nile Red in 776.7 g ± 0.2 g of ACN. The certified Nile Red concentration was corrected for the mass of air displaced by the dye and ACN during weighing and the purity of the Nile Red, yielding a value of 118.7 μmol/kg ± 2.3 μmol/kg. The Nile Red solution contained in an amber bottle was aliquotted into ampoules that were subsequently flame-sealed.

Preparation of Part B for Analysis: The SRM 1934 Part B solution should always be diluted gravimetrically at least one-hundred fold with ACN. Subsequent gravimetric dilutions can then be employed to generate a calibration curve. The calibration curve describes the relationship between fluorescence intensity, as determined by the fluorometer and the concentration of Nile Red, as determined by the gravimetric dilution. Care must be taken in making the gravimetric dilutions because uncertainties expand during this serial process. An error at one level adversely affects determinations at all subsequent levels. The calibration curve measurements should be generated starting with the lowest concentration. For best results, the conditions used for determining the calibration curve should closely match those used in the measurement of the analyte. Because fluorescence is a highly sensitive technique, great attention must be paid to the cleanliness of glassware and any other apparatus that contacts the solutions. Many plastics and gloves can contaminate samples with small amounts of highly fluorescent materials such as release agents and plasticizers. The running of blanks to check for such contamination is highly recommended.

Assignment of Uncertainties: A standard uncertainty component equivalent to the estimated standard deviation was assigned for sample inhomogeneity. This value was then combined with gravimetric and purity uncertainties using the root-sum-of-squares method. An expansion factor of $k = 2$ was applied so that the expanded uncertainties given in this certificate express an interval within which the true value is expected to fall with a level of confidence of approximately 95 % for a normal distribution [5]. The upper limit of the bias due to evaporation of solvent during ampouling was also estimated and added to the expanded uncertainty.

Part C: Coumarin 30 Solution

Certified Value: The certified concentration of Coumarin 30 (C30) provided in Table 3A is based on gravimetric preparation and analysis of purity by proton nuclear magnetic resonance spectroscopy that was quantified using an internal standard. The C30 purity determinations provided below is based on proton nuclear magnetic resonance spectroscopy. The measurands are the concentration and purity of C30. Metrological traceability is to the SI derived unit for amount-of-substance concentration and mass fraction (expressed as micromoles per kilogram and percent, respectively).

Table 3A. Certified Amount-of-Substance Concentration and Mass Fraction for SRM 1934 Part C

Coumarin 30 (concentration)	130.5 μmol/kg	±	1.7 μmol/kg
Coumarin 30 (purity)	97.35 %	±	0.46 %

Reference Values: Table 3B provides reference values for the molar extinction coefficient of the SRM 1934 Part C corrected for purity. Fluorescence bias was found to be statistically insignificant. The measurand is the molar extinction coefficient at the peak wavelength listed based on the method indicated. Metrological traceability is the SI units for mass, length, and amount-of-substance (expressed as $kg \cdot cm^{-1} \cdot mol^{-1}$).

Table 3B. Reference Values for Molar Extinction Coefficient of SRM 1934 Part C at 22.0 °C ± 0.5 °C

Wavelength (nm)	Molar Extinction Coefficient ($kg \cdot cm^{-1} \cdot mol^{-1}$)	Uncertainty ($kg \cdot cm^{-1} \cdot mol^{-1}$)
405.0	3.28×10^4	0.05×10^4

Determination of Coumarin 30 Purity: Table 3C summarizes the purity determinations of the C30 material used to prepare SRM 1934 Part C. The measurands are the constituents listed in Table 3C as determined by the indicated methods. Metrological traceability is to the SI derived unit for mass fraction (expressed as a percent).

Table 3C. Reference Values for C30 Purity Determinations and Associated Uncertainties

Constituent	Technique	Mass Fraction (%)	Uncertainty (%)
Diethyl ether	^{1}H NMR[(a)]	0.16	0.05
Similar MW to C30[(b)]	^{1}H NMR[(a)]	<0.3	0.15
Water	TGA[(c)]	<0.04	0.02

[(a)] 600 MHz Proton Nuclear Magnetic Resonance Spectroscopy
[(b)] Constituents with a molecular weight similar to C30
[(c)] Thermogravimetric analysis

Information Values: Table 3D provides information values for SRM 1934 Part C. Figure 3 provides the fluorescence emission spectrum of C30. Additional information values for relative signal in power units and emission wavelength in nanometers is located on the Material Details web page under the Data Files icon for SRM 1934 (https://www-s.nist.gov/srmors/view_detail.cfm?srm=1934).

Table 3D. Information Values for SRM 1934 Part C

solvent	Mass Density (g/mL)
ACN	0.7799
IPA	0.7837

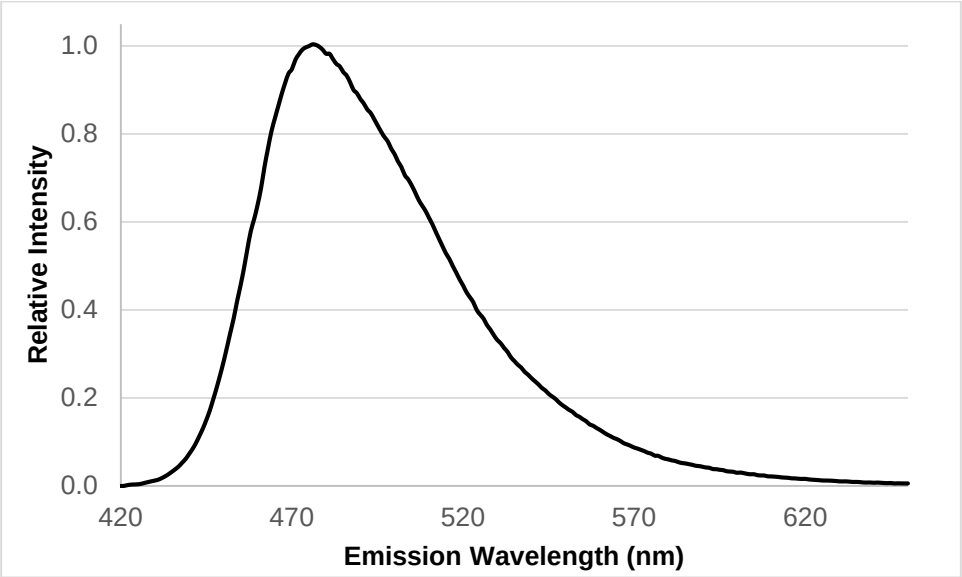

Figure 3. Corrected fluorescence emission spectrum for SRM 1934 Part C, diluted 100 fold using IPA, at an excitation wavelength of 405 nm. A 405 nm diode laser was used for excitation. The spectral bandpass of the detection system was set at 2.5 nm.

Source of Material: The C30 [3-(2-*N*-methylbenzimidazolyl)-7-*N*,*N*-diethylaminocoumarin, $C_{21}H_{21}N_3O_2$, relative molecular mass 347.410, CAS No. 41044-12-6], also known as Coumarin 515, was produced by Aldrich (Sigma-Aldrich) as Cat. # 546127, Lot # BCBH1078V, and determined by the manufacturer to be 99 % pure with unspecified analysis techniques. Dye solutions were dissolved in acetonitrile (ACN) and concentrations were determined gravimetrically. The ACN used was Mallinckrodt UltimAR®, (HPLC/Spectrphotometric grade) Lot # H454 T37465. The balance used was a Mettler AT20, which was internally calibrated. All gravimetric measurements were taken at temperature T = 21.0 °C, 25 % relative humidity (rh), and 995 mbar atmospheric pressure.

Preparation of Part C: The SRM solution was prepared by dissolving 0.036239 g ± 0.000006 g of C30 in 778.0 g ± 0.2 g of ACN. The certified C30 concentration was corrected for the mass of air displaced by the dye and ACN during weighing and the purity of the C30, yielding a value of 130.5 μmol/kg ± 1.7 μmol/kg. The C30 solution, contained in an amber bottle, was aliquotted into ampoules that were subsequently flame-sealed.

Preparation of Part C for Analysis: The SRM 1934 Part C solution should always be diluted gravimetrically at least one-hundred fold with isopropanol. Subsequent gravimetric dilutions can then be employed to generate a calibration curve. The calibration curve describes the relationship between fluorescence intensity, as determined by the fluorometer and the concentration of C30, as determined by the gravimetric dilution. Care must be taken in making the gravimetric dilutions because uncertainties expand during this serial process. An error at one level adversely affects determinations at all subsequent levels. The calibration curve measurements should be generated starting with the lowest concentration. For best results, the conditions used for determining the calibration curve should closely match those used in the measurement of the analyte. Because fluorescence is a highly sensitive technique, great attention must be paid to the cleanliness of glassware and any other apparatus that contacts the solutions. Many plastics and gloves can contaminate samples with small amounts of highly fluorescent materials such as release agents and plasticizers. The running of blanks to check for such contamination is highly recommended.

Assignment of Uncertainties: A standard uncertainty component equivalent to the estimated standard deviation was assigned for sample inhomogeneity. This value was then combined with gravimetric and purity uncertainties using the root-sum-of-squares method. An expansion factor of $k = 2$ was applied so that the expanded uncertainties given in this certificate express an interval within which the true value is expected to fall with a level of confidence of approximately 95 % for a normal distribution [5]. The upper limit of the bias due to evaporation of solvent during ampouling was also estimated and added to the expanded uncertainty.

Part D: Allophycocyanin Suspension

Reference Values: The purity of the allophycocyanin (APC) was estimated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC) for material qualification. The protein concentration was determined using amino acid analysis [7,8]. The measurand is the concentration of APC provided in Table 4A based on the indicated method. An information APC purity value is provided in Table 4C.

Table 4A. Reference Amount-of-Substance Concentration for SRM 1934 Part D

APC (concentration)	30.0 μmol/L	±	2.5 μmol/L

Table 4B provides reference values for the molar extinction coefficient of the SRM 1934 Part D corrected for purity and fluorescence bias was found be statistically insignificant. The molar extinction coefficient at the wavelength of 652 nm in PBS, pH 7.4 with 0.02 % (w/w) Tween 20 was calculated using the absorbance value divided by the concentration and the path length, and is reported for reference value. The measurand is the molar extinction coefficient at the peak wavelengths listed based on the method indicated.

Table 4B. Reference Values for Molar Extinction Coefficient of SRM 1934 Part D at 22.4 °C ± 0.5 °C

Wavelength (nm)	Molar Extinction Coefficient ($L \cdot cm^{-1} \cdot mol^{-1}$)	Uncertainty ($L \cdot cm^{-1} \cdot mol^{-1}$)
652.0	8.81×10^5	0.74×10^5

Information Values: Table 4C provides information values for SRM 1934 Part D. Figure 4 provides the fluorescence emission spectrum of APC. Additional information values for relative signal in power units and emission wavelength in nanometers is located on the Material Details web page under the Data Files icon for SRM 1934 (https://www-s.nist.gov/srmors/view_detail.cfm?srm=1934).

Table 4C. Information Values for SRM 1934 Part D at 22 °C

	Mass Fraction	Mass Fraction[(a)]
APC	>95 % (purity)	<5 % (impurity)

pH	Buffer Concentration	Mass Density
7.0	0.05 mol/L potassium phosphate 60 % saturated ammonium sulfate	1.1684 g/mL

[(a)] Value provided based on impurity bands of the total intensity.

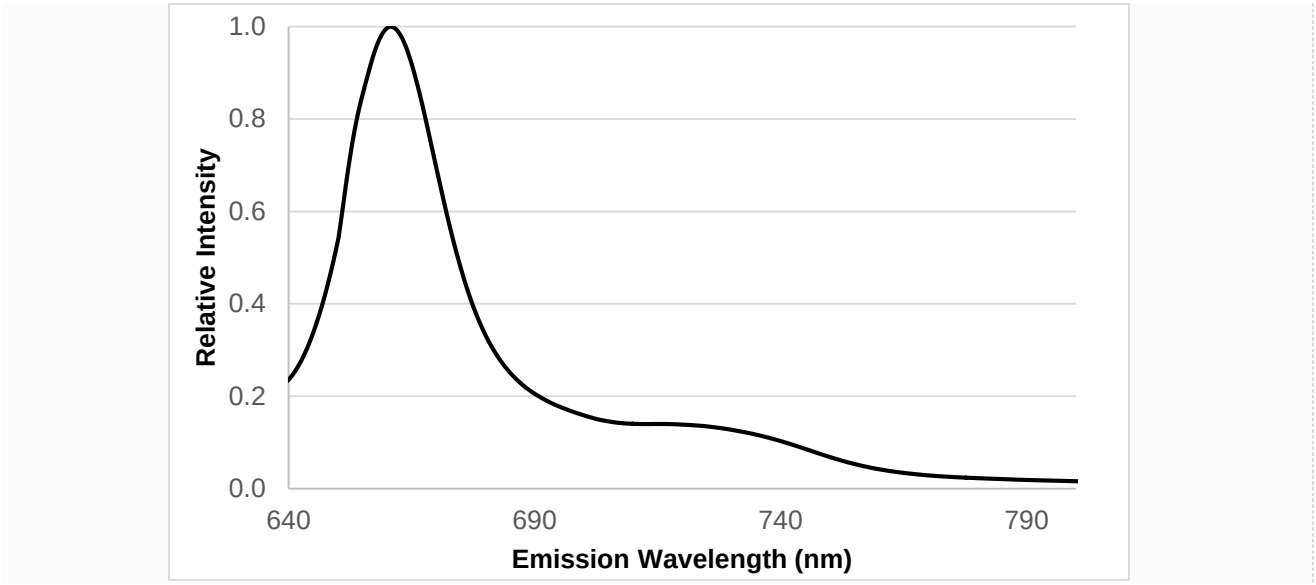

Figure 4. Corrected fluorescence emission spectrum for SRM 1934 Part D, diluted 100 fold using phosphate buffer, at an excitation wavelength of 633 nm. A 633 nm HeNe laser was used for excitation. The spectral bandpass of the detection system was set at 2 nm.

Source of Material: The APC suspension was supplied by Life Technologies (Catalog #: A803, Lot #: 1575860, Product information: ~4 mg/mL, M.W. ~104,000, medium, 60 % saturated ammonium sulfate, 50 mM potassium phosphate, pH 7.0).

Preparation of Part D: Twenty-eight vials of APC suspension were pooled after vortex to a single 50 mL centrifuge tube. A small stir bar was added to the tube and the tube was placed on a stir plate with gentle stirring to ensure the suspension was mono-dispensed. A calibrated pipettor was used to allocate a 105 µL suspension into a 0.5 mL polypropylene Sarstedt tubes (#72.730.105). After tightening a cap on the tube and placing the SRM Part D label on the side of tube and a fill order indicator label on the top of the tube. The tubes were stored in 4 °C refrigerator.

Preparation of Part D for Analysis: The SRM 1934 Part D is a suspension that should be resuspended using vigorous vortexing immediately before sampling, and it should always be diluted gravimetrically at least one-hundred fold with PBS, pH 7.4 with 0.02 % Tween 20. Subsequent gravimetric dilutions can then be employed to generate a calibration curve. The calibration curve describes the relationship between fluorescence intensity, as determined by the fluorometer and the concentration of APC, as determined by the gravimetric dilution. Care must be taken in making the gravimetric dilutions because uncertainties expand during this serial process. An error at one level adversely affects determinations at all subsequent levels. The calibration curve measurements should be generated starting with the lowest concentration. For best results, the conditions used for determining the calibration curve should closely match those used in the measurement of the analyte. Because fluorescence is a highly sensitive technique, great attention must be paid to the cleanliness of glassware and any other apparatus that contacts the solutions. Many plastics and gloves can contaminate samples with small amounts of highly fluorescent materials such as release agents and plasticizers. The running of buffer blanks to check for such contamination is highly recommended.

Assignment of Uncertainties: The uncertainty includes all Type A evaluated elements, that is, between amino acid, within as well as between vial variability, calibration and pipetting [9,10]. A 3 % additional Type B uncertainty was also included based on further expert evaluation for the APC concentration determination. The standard and expanded uncertainty for the determination of the extinction coefficient includes all Type A sources from APC concentration analysis and those from the absorbance coefficient analysis as well as 3 % Type B uncertainty in the APC concentration.

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