National Institute of Standards & Technology

Report of Investigation

Reference Material 8640

Microspheres With Immobilized Fluorescein Isothiocyanate

This Reference Material (RM) is intended for use in establishing a reference scale for fluorescence intensity based upon molecules of equivalent soluble fluorophore (MESF) units [1,2,3]. This RM provides reference values for the number of equivalent soluble fluorescein molecules on a microsphere (see Table 1). The MESF scale is established for a particular set of experimental conditions by measuring the fluorescence intensity of known amounts of this RM under the specified set of conditions, as described under the "Instructions for Use" section of this certificate.

Each unit of RM 8640 consists of six sealed plastic bottles containing a suspension that has a nominal microsphere concentration of 10^6 particles/mL. One bottle (Blank) contains microspheres without immobilized fluorescein isothiocyanate (FITC). The other five bottles contain microspheres with different amounts of immobilized FITC. Approximately 2.0 mL of the suspension is sealed into each individual plastic bottle.

Expiration of Reference Values: The reference values of this RM are valid until **31 January 2006**, within the measurement uncertainties specified, provided the RM is handled and stored in accordance with the instructions given here, see "Instructions for Use". The certification is valid only for unopened ampoules that have been stored in the dark at 4 °C ± 2 °C.

Maintenance of RM Reference Value: NIST will monitor this RM. If substantive changes occur that affect the reference value before the expiration date, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The overall direction and coordination of technical measurements for this RM were performed by A.K. Gaigalas of the NIST Biotechnology Division.

Production of RM 8640 was performed by L. Wang and A.K. Gaigalas of the NIST Biotechnology Division.

Packaging of RM 8640 was coordinated through the NIST Standard Reference Materials Program by M.P. Cronise of the NIST Measurement Services Division.

Statistical consultation was provided by J. Lu of the NIST Statistical Engineering Division. A reference light source was provided by G.W. Kramer of the NIST Analytical Chemistry Division.

The support aspects involved in the issuance of this RM were coordinated through the NIST Standard Reference Materials Program by B.S. MacDonald of the NIST Measurement Services Division.

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Certificate Issue Date: 28 January 2005
See Certificate Revision History on Last Page

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Reference Values: A NIST *reference value* [4] is a noncertified value that is the best estimate of the true value; however, the value does not meet NIST criteria for certification and is provided with associated uncertainties that may reflect only measurement precision and may not include all sources of uncertainty. Table 1 gives reference values for the MESF of the RM 8640 as determined by averaging four independent measurements obtained with the fluorometer. The uncertainties, u_c, were calculated from the standard deviation of these four independent determinations of MESF values [5].

Assignment of Uncertainties: Standard uncertainty components equivalent to the estimated standard deviation for a normal distribution were assigned for measurement repeatability and were combined with balance accuracy uncertainties and estimated instrument method uncertainties using the root-sum-of-squares method to produce the combined uncertainty, u_c. An expansion factor of $k = 2$ has to be applied such that the expanded uncertainties express an interval within which the true value is expected to fall with a level of confidence of approximately 95 %.

Table 1. Average MESF Values of RM 8640 Microsphere Suspensions Assigned with Fluorometer

Bottle Number	MESF[a]		
1	1 700	±	300
2	5 200	±	600
3	15 400	±	3 200
4	102 000	±	19 400
5	250 000	±	44 000

[a] Molecules of equivalent soluble fluorophore

Table 2 gives the average linearized MESF values. These were obtained by requiring that the MESF values correlate linearly with mean channels determined in a cytometer. The values are obtained from the best linear fit of the MESF values and the corresponding mean channel in a cytometer measurement. The slope of the fit is constrained to that characterizing the cytometer. These are the reference MESF values on the RM bottles.

Table 2. Average Linearized MESF Values of RM 8640 Microsphere Suspensions

Bottle Number	MESF[a]		
1	1 100	±	300
2	4 700	±	1 000
3	19 800	±	4 300
4	113 700	±	25 300
5	283 400	±	65 000

[a] Molecules of equivalent soluble fluorophore

Source of Material: The microsphere suspensions were provided by Bangs[1] laboratory. All water used was NIST deionized water that was then subsequently passed through a second purification system (Millipore Mill-Q A10[1]) to produce water having a resistivity ≥ 18 MΩ·cm.

Preparation of the RM: The microspheres were manufactured using a swollen emulsion polymerization process. The composition of the polymer is 70 % methyl methacrylate, 20 % glycidyl methacrylate and 10 % ethylene dimethacrylate by mass. The size is 7.56 µm, and the density is approximately 1.14 g/cm^3. The fluorescein isothiocyanate (FITC) molecules are covalently attached to the surface of the microspheres via a linker molecule. The chosen linker is optimized to provide a controlled distance between the microsphere surface and the fluorochrome (tether length). By controlling the tether length of the FITC molecule, the RM exhibits excitation and emission spectra very similar to that of FITC-labeled cell samples.

[1]Certain commercial equipment, instruments, or materials are identified in this certificate in order 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 materials or equipment identified are necessarily the best available for the purpose.

INSTRUCTIONS FOR USE

Stability and Storage: This RM is a suspension contained in opaque plastic bottles. **CAUTION:** Unopened and opened bottles should be stored in the dark at 4 °C ± 2 °C in an upright position. The bottles should **NOT** be frozen because of the possibility of degrading the suspension. Once the bottle is opened, the suspension should be used with minimal exposure to any light (exposure to incandescent lighting is preferable to illumination from daylight or fluorescent lighting). Any unused suspension in the bottle should be discarded properly.

Use: The RM 8640 suspension is intended for use in flow cytometers. The bottles are designed for repeated use of the suspension. Two drops of the suspension from each bottle may be placed in a solution identical to that used for the analyte. The resulting suspension containing all the microsphere populations should be run through the cytometer. The resulting calibration curve describes the relationship between mean channels of the microsphere populations, as determined by the cytometer, and the MESF values assigned to the five populations of microspheres. 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. The construction and use of a calibration curve is discussed in detail in reference 6.

Since fluorescence is a highly sensitive technique, great attention must be paid to the cleanliness of glassware and any other apparatus that contacts the suspension. 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 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. Accordingly, the sensitivity of the fluorometric assay for fluorescein also decreases, and quantitation becomes very dependent on the knowing or maintaining the precise pH of the solutions during calibration as well during the assay itself. While the calibration strategy described above can work with solutions below pH 9, the uncertainties of such measurements will inevitably grow larger as the solution pH is lowered.

REFERENCES

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[2] Schwartz, A.; Wang, L.; Early, E.; Gaigalas, A.K.; Zhang, Y–Z.; Marti, G.E.; Vogt, R.F.; *Quantitating Fluorescence Intensity from Fluorophores: The Definition of MESF Assignment;* J. Res. Natl. Inst. Stds. & Tech.; U.S. Department of Commerce: Gaithersburg, MD; Vol. 107, pp. 83-91 (2002).

[3] Wang, L.; Gaigalas, A.K.; Abbasi, F.; Marti, G.E.; Vogt, R.F.; Schwartz, A.; *Quantitating Fluorescence Intensity From Fluorophores: Practical Use of MESF Values;* J. Res. Natl. Inst. Stds. & Tech.; U.S. Department of Commerce: Gaithersburg, MD; Vol. 107, pp. 339-353 (2002).

[4] May, W.; Parris, R.; Beck, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S; Gills, T; Colbert, J.; Gettings, R; MacDonald, B.; *Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*, NIST Special Publication 260-136; U.S. Government Printing Office: Washington, DC (2000).

[5] ISO *Guide to the Expression of Uncertainty in Measurement*; ISBN 92-67-10188-9, 1st Ed., ISO: Geneva, Switzerland (1993): see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297, U.S. Government Printing Office: Washington, DC (1994); available at http://physics.nist.gov/Pubs.

[6] NCCLS ILA24-P; *Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity; Proposed Guideline*; ISBN 1-56238-000-0; NCCLS: Wayne, PA (2003).

Certificate Revision History: 28 January 2005 (This revision reflects an extension in the certification period); 15 March 2004 (Original certificate date).

Users of this RM should ensure that the report in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.