National Institute of Standards & Technology

Certificate

Standard Reference Material® 2082

Pathlength Absorbance Standards for Microliter Volume Spectrophotometers

This Standard Reference Material (SRM) is a certified absorbance standard for the determination of pathlength on microliter volume spectrophotometers and fixed pathlength cuvettes. A unit of SRM 2082 consists of three 2 mL ampoules, one ampoule each of a blank buffer solution (10 mM 2-amino-2-hydroxymethyl-propane-1,3 diol (TRIS) buffer, pH 8.0); tryptophan (1.4 mM) in TRIS buffer (component A); and uracil (1.0 mM) in TRIS buffer (component B). Each ampoule contains approximately 1.8 mL of solution.

Certified Values: 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 [1]. Values obtained using equations 1 to 6 are certified within the limits given in this certificate. Equations 1 and 2 are the functions of the absorbance at 280 nm, A_{280}, and 260 nm, A_{260}, with respect to pathlength in millimeters for component A and component B, respectively, measured against the TRIS buffer, with a spectral bandwidth of 0.8 nm, and a temperature of 22.0 ºC ± 0.1 ºC. The absorbance at the respective wavelengths are calculated as the absorbance of the component solution, at 280 nm or 260 nm, minus the absorbance of the TRIS buffer at the same wavelength. For conditions other than 22 °C and 0.8 nm spectral bandwidth, there are correction factors for temperatures between 18 °C and 30 °C and for spectral bandwidths from 0.8 nm to 5.0 nm given as equations 3 and 4 with coefficients and constants given in Table 1. Equation 5 and 6 are used to calculate the pathlength in millimeters. The uncertainty is estimated from the evaluation of multiple vials of each component and from the uncertainty of the pathlengths of the calibrated cuvettes, temperature and spectral bandwidth. Uncertainties are given in the form of equation 7, and are dependent on the measurement parameters of the SRM with coefficients and constants given in Table 2. The measurand is pathlength. Metrological traceability is to the SI unit for length, expressed as millimeters.

Information Values: A NIST information value is a value that may be of interest to the SRM user, but insufficient information is available to assess the uncertainty associated with the value, therefore no uncertainty is provided [1]. Values obtained with equations 10 and 11 are for information purposes only. Information values cannot be used to establish metrological traceability.

Expiration of Certification: The certification of **SRM 2082** is valid, within the measurement uncertainty specified, until **30 October 2020**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Handling, Storage, and Use"). The 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 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 notification.

Production and certification of this SRM were performed by B.E. Lang, K.D. Cole, and A.A. Urbas of the NIST Biosystems and Biomaterials Division, and S.J. Choquette.

Statistical consultation was provided by H.K. Liu of the NIST Statistical Engineering Division.

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

Anne Plant, Chief
Biosystems and Biomaterials Division

Gaithersburg, MD 20899
Certificate Issue Date: 04 August 2016

Steven J. Choquette, Director
Office of Reference Materials

INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

Handling and Storage: SRM 2082 should be stored at –20 °C until it is ready for use. Keep the product in the dark while in storage, as long term exposure to UV light can cause photo-degradation of component A (tryptophan). It is recommended that the SRM vials be kept in the storage box, and be thawed at 4 °C overnight or thawed at room temperature for a few hours. The SRM vials should not be heated to thaw as this may promote degradation of the SRM components.

Based upon our stability studies, SRM 2082 may be stored at 4 °C for up to three months without degradation. It should be mixed thoroughly and centrifuged before using after standing for long periods of time.

SRM SOLUTIONS MUST BE PROPERLY MIXED PRIOR TO USE. After the SRM has been completely thawed, the individual vials must be mixed thoroughly since it has been observed that freezing will promote concentration gradients and/or sample adhesion to the supplied tubes. These gradients will remain in the vial after thawing without adequate mixing. Mixing should be accomplished by first inverting the vials 20 times, insuring that the air bubble has traveled all the way up the vial, then by mechanical mixing using a vortexing mixer for 30 seconds. The inversion of the vial and vortexing should be performed a total of 3 times to ensure the sample is ready for use. Vortexing the vials at a low angle (30° from horizontal) will improve the efficiency of mixing. Ensure that the vial is securely closed before mixing. Brief centrifugation before opening is recommended as well.

Use: SRM 2082 is used to calibrate the pathlength for fixed pathlength cuvettes or microvolume spectrophotometers that utilize fixed or variable pathlength sample cells and is certified for use with pathlengths between 0.1 mm and 2.0 mm. SRM 2082 should be brought up to the temperature of the spectrophotometer on which the measurements will be made. Often this is room temperature; however, this may not be the case depending on the set up of the instrument, and provisions should be made accordingly. The spectrophotometer should be started and run using the manufacturer's recommendations with sufficient time to warm up to operating temperature if necessary.

Starting with the buffer solution, pipette enough of the liquid into the measurement chamber of the spectrophotometer to meet the manufacturer's specifications for proper measurements. Measure a baseline UV absorbance at the wavelength(s) of interest, which is ideally a spectrum from 340 nm to 240 nm. The baseline may be subtracted automatically by the instrument, or it may be subtracted manually at a later time. Repeat the measurements using component A (tryptophan) and component B (uracil). Ensure that enough replicate measurements have been made using all parts of SRM 2082 to achieve the desired statistical sampling for the measurements.

The vial used for the SRM should be opened, sample removed, and closed securely to prevent evaporation, which may cause a change in the concentration of the solutions.

When using SRM 2082, the temperature of the room or instrument should be recorded along with the spectral bandwidth of the instrument. The temperature should be measured with a calibrated thermometer that is accurate to within $\pm$ 0.25 K and is traceable to the International Temperature Scale of 1990. Spectral bandwidth of the spectrophotometer should be obtained from the instrument manufacturer.

For user convenience, a spreadsheet containing the coefficients of the certified model, confidence band, and a calculator for the pathlength and uncertainty is available and may be found at https://www-s.nist.gov/srmors/view_detail.cfm?srm=2082 [2]. Utilizing estimates of absorbance uncertainty, u(Ec), below 0.001 A is not recommended unless the user has performed a careful uncertainty analysis of their instrumentation. Instrument precision alone may significantly underestimate uncertainty.

Preparation and Analysis: The SRM 2082 solutions were prepared and packaged at NIST. Uracil (#U0750, Lot No. SLBD1250V) and L-Tryptophan (#93659, Lot No. 1400132V) were obtained from Sigma-Aldrich and used as received. A stock solution of TRIS buffer (1 M, pH 8.0) was obtained from Ambion Life Technologies (#AM9856, Lot No. 1001017). Bulk solutions were prepared to attain final concentrations of nominally 1.0 mM uracil and 1.5 mM tryptophan in 10 mM TRIS buffer. The prepared solutions were dispensed in 1.7 mL aliquots into sterile 2 mL sample tubes in a sterile hood. The packaged samples were stored at –20 ºC.

Cuvettes constructed from Spectrosil® quartz were obtained from Starna with nominal pathlengths of 0.5 mm, 1 mm, and 2 mm. Additional quartz cuvettes were obtained from Precision Cell Inc., with nominal pathlengths of 0.2 mm and 0.1 mm. The pathlength of the 0.5 mm, 1.0 mm and 2.0 mm cuvettes were measured by NIST Dimensional Metrology Group using a coordinate measuring machine with a fiber probe having a tip with an ellipsoidal geometry. The uncertainty for this instrument has been shown to have a deviation of $\pm$ 0.11 µm for measurements in one dimension. Pathlengths for the 0.5 mm, 0.1 mm, and 0.2 mm cells were determined by interference fringes in the

near-infrared spectral region. Measurements across the collection of cuvettes of both uracil and tryptophan were observed to be linear with respect to pathlength and thus adhered to the Beer-Lambert law.

To test for homogeneity, 12 units were selected using a step randomization method based on filling order, with two random units selected out of every sequential hundred units. All units were measured at 22 °C, at a spectral bandwidth of 0.8 nm, using the same 0.5 mm cuvette. No significant heterogeneity or correlations with respect to filling order were observed and the lot was deemed suitably homogenous.

Certification: The buffer corrected absorbance of component A and component B have been determined in the UV region at the absorbance peaks of tryptophan and uracil, 280 nm and 260 nm, respectively. The spectra of tryptophan and uracil are shown in Figure 1. Absorbance measurements are traceable to the NIST High Accuracy Spectrophotometer [3]. Absorbance measurements were made at 22 °C and a spectral bandwidth of 0.8 nm. Correction factors have been measured to allow for the determination of the pathlength in the temperature range between 18 °C and 30 °C and for the variation in spectral bandwidth from 0.8 nm to 5.0 nm.

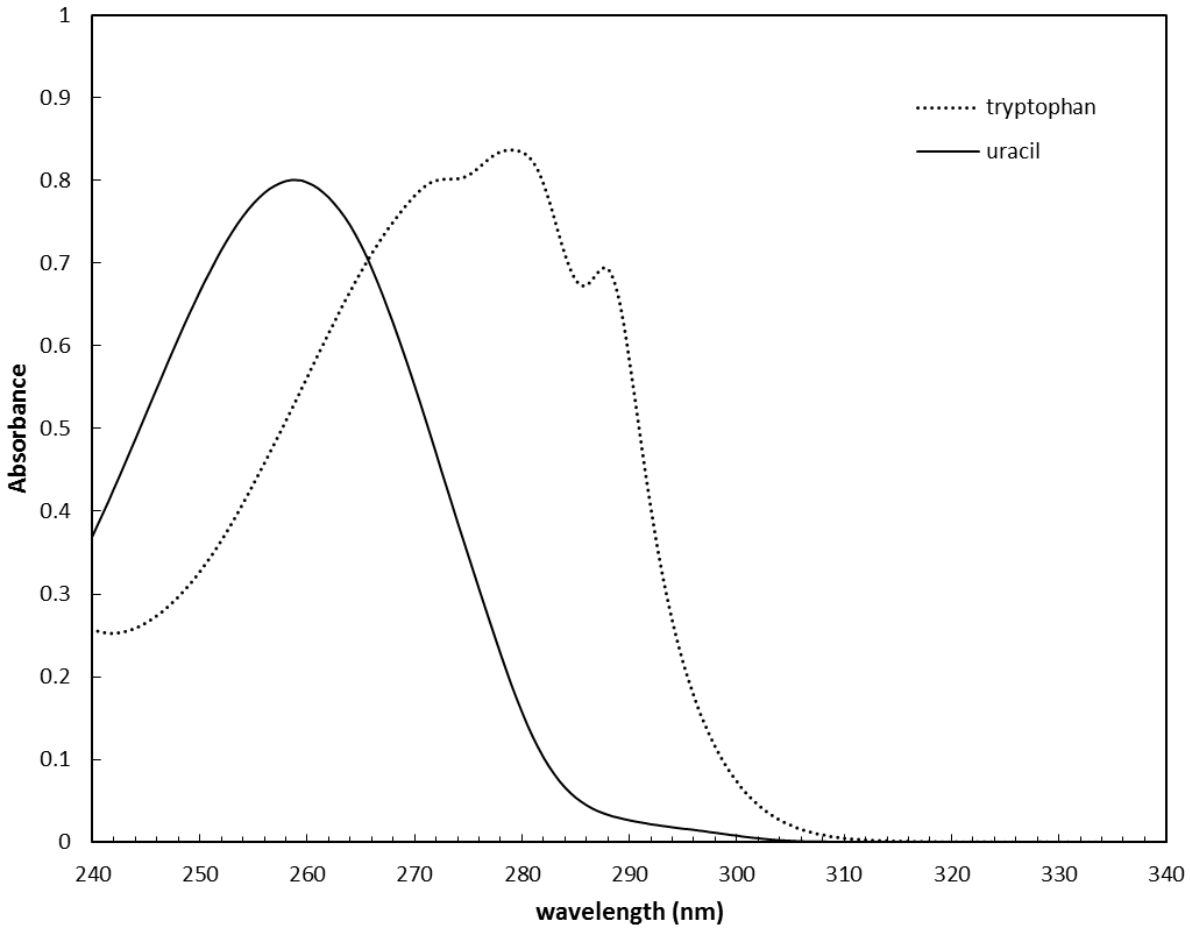

Figure 1. Absorbance spectrum (1 mm) of SRM 2082 component A (tryptophan) and component B (uracil) from 240 nm to 340 nm, at 22 °C and 0.8 nm spectral bandwidth.

The absorbance, A, of component A (tryptophan) and component B (uracil) follow the Beer-Lambert law for pathlengths used in the certification process (0.1 mm to 2.0 mm). Thus, at 22 °C and 0.8 nm spectral bandwidth the absorbance with respect to pathlength, l in millimeters, for components A and B are described by the following linear equations:

$$A_{280(\text{tryptophan})} = 0.83498(\text{mm}^{-1})\cdot l \quad (1)$$

$$A_{260(\text{uracil})} = 0.79903(\text{mm}^{-1})\cdot l \quad (2)$$

where the constants of the equations can be expressed as the generic constant E_c

Table 1. Coefficients and Constants for Pathlength Calculation Using SRM 2082

	Component A, Tryptophan		Component B, Uracil	
	A_{280}	Uncertainty	A_{260}	Uncertainty
E_c	0.83498 (mm^{-1})	0.000297	0.79903 (mm^{-1})	0.000304
a_1	4.997×10^{-4}	1.108×10^{-5}	2.266×10^{-4}	1.033×10^{-5}
a_0	-0.01097	2.648×10^{-4}	-5.066×10^{-3}	2.470×10^{-4}
b_2	6.104×10^{-4}	8.602×10^{-5}	2.374×10^{-4}	3.623×10^{-5}
b_1	-2.656×10^{-5}	5.428×10^{-4}	-9.160×10^{-6}	2.286×10^{-4}
b_0	-6.198×10^{-4}	6.558×10^{-4}	-2.210×10^{-4}	2.768×10^{-4}

Coefficients used in the calculation of the pathlength based on the TRIS buffer corrected absorbance measurements of SRM 2082 for Component A (tryptophan) and Component B (uracil) at 280 nm and 260 nm, respectively are shown in Table 1. The absorbance A, of tryptophan and uracil follow the Beer-Lambert law in the region studied. The constant E_c correlates the absorbance of the tryptophan or uracil solutions to the pathlength, l (in millimeters), through the relationship $A = E_c \cdot l$ and are the same as the constants in equations 1 and 2. Coefficients a_1 and a_0 are used in equation 3 to relate the change in absorbance to temperature from 18 °C to 30 °C. Coefficients b_2, b_1, and b_0 are used in equation 4 to relate the change in absorbance to the spectral bandwidth from 0.8 nm to 5.0 nm.

The pathlength can be determined from equations 1 and 2 by rearrangement into the form $l = A/E_c$. If the absorbance is not measured at 22 °C and 0.8 nm spectral bandwidth, correction factors to the absorbance must be applied to calculate the pathlength.

The absorbance change due to temperature is defined as $\Delta_t A = A_{22°C} - A_t$, where $A_{22°C}$ is the absorbance at 22 °C and A_t is the absorbance at temperature t, in Celsius. The change in absorbance with respect to temperature for a 1 mm pathlength is linear in the range from 18 °C to 30 °C, and can be expressed in the following form:

$$\Delta_t A = a_1 \bullet t + a_0 \tag{3}$$

where a_1 and a_0 are constants, and are listed in Table 1 for components A and B. The value of $\Delta_t A$ scales proportionately with pathlength, with equation 3 representing $\Delta_t A$ at a reference condition of 1 mm.

The second correction to the absorbance measurements arises from the effect of the spectral bandwidth on the apparent absorbance of the SRM. The change in absorbance due to the spectral bandwidth, $\Delta_B A$, is defined as $\Delta_B A = A_{0.8\ nm} - A_{B\lambda}$, where $A_{0.8\ nm}$ is the absorbance at the reference spectral bandwidth of 0.8 nm and $A_{B\lambda}$ is the measured absorbance at spectral bandwidth, B_λ, in nm. The deviation of the absorbance with respect to the spectral bandwidth is nonlinear and between 0.8 nm and 5 nm may be fit to quadratic equations of the form:

$$\Delta_B A = b_2 \bullet B_\lambda^2 + b_1 \bullet B_\lambda + b_0 \tag{4}$$

where b_2, b_1, and b_0 are constants, and B_λ is the spectral bandwidth (in nanometers). The constants for the deviation of the absorbance due to spectral bandwidth over a 1 mm pathlength are given in Table 1. The functions of $\Delta_B A$ were determined using absorbance measurements at two pathlengths, with the value of $\Delta_B A$ scaling linearly with pathlength. Equation 4 is defined at 1 mm pathlength as a convenient reference condition.

To calculate the pathlength in mm based on the absorbance, temperature, and spectral bandwidth, the following equation should be used:

$$l = \frac{A}{\left[E_c - \left(a_1 t + a_0 + b_2 B_\lambda^2 + b_2 B_\lambda + b_0\right)\right]} \tag{5}$$

Or alternatively

$$l = \frac{A}{(E_c - \Delta A)} \tag{6}$$

where $\Delta A = \Delta_t A + \Delta_B A$, from equations 3 and 4. Absorbance values measured outside of the temperature range of 18 °C to 30 °C, and/or with a spectral bandwidth greater than 5 nm will **NOT** yield certified values for a calculated pathlength.

Uncertainty Calculation: The combined standard measurement uncertainty comprises one set of components evaluated by application of statistical methods (Type A evaluation). The Type A uncertainties include contributions attributable to the uncertainties in the reference cuvette pathlength, the uncertainty in absorbance from the transfer spectrophotometer, variation in the measured spectral bandwidth, uncertainty in the absolute temperature, and the sample to sample variation.

A certified model of the derived pathlength uncertainty was obtained by propagation of error methods in accordance with Supplement 1 to the ISO/JCGM Guide [4]. The combined uncertainty is derived from uncertainties of the absorbance as well as from uncertainties given in Table 1. The uncertainty in the calculated pathlength, $u(l)$, as calculated from equation 6 takes the form:

$$u^2(l) = \frac{A^2}{(E_c - \Delta A)^4}[u^2(E_c) + u^2(\Delta_t A) + u^2(\Delta_B A)] + \frac{1}{(E_c - \Delta A)^2}u^2(A) \tag{7}$$

where $u(E_c)$ is the uncertainty of the absorbance with respect to pathlength (see equations 1 and 2), $u(\Delta_t A)$ is the uncertainty of the temperature correction, $u(\Delta_B A)$ is the uncertainty of the spectral bandwidth correction, and $u(A)$ is the uncertainty of the measured absorbance.

The uncertainty of the temperature correction derives from the uncertainty of the constants a_1 and a_0 in equation 3 (listed in Table 1). Since the constants are interrelated and dependent on the temperature, the total uncertainty from temperature is determined as a function of t using the covariance between a_1 and a_0, and is expressed as a simple quadratic equation of the form:

$$u^2(\Delta_t A) = a_{u2} \cdot t^2 + a_{u1} \cdot t + a_{u0} \tag{8}$$

where a_{u2}, a_{u1}, and a_{u0} are constants.

Similarly, the uncertainty of the spectral bandwidth correction derives from the uncertainty of the constants b_2, b_1, and b_0 from equation 4 (listed in Table 1). This correction factor is a function of the spectral bandwidth, B_λ, and the uncertainty is also calculated from the covariance of the constants b_2, b_1, and b_0. The uncertainty of the spectral bandwidth can be expressed simply as a polynomial of the form:

$$u^2(\Delta_B A) = b_{u4} \cdot B_\lambda^4 + b_{u3} \cdot B_\lambda^3 + b_{u2} \cdot B_\lambda^2 + b_{u1} \cdot B_\lambda + b_{u0} \tag{9}$$

where b_{u3}, b_{u2}, b_{u1}, and b_{u0} are constants.

All of the constants for the calculation of the uncertainty are listed in Table 2 for both tryptophan and uracil.

The last term in equation 7 relates to the customer's uncertainty in the experimental absorbance measurement. It is recommended that the user make ten replicate measurements of the absorbance A, with reloading of the sample for each measurement. The average value of A may be used to calculate l, and the standard deviation of A, σ_A, will approximate $u(A)$.

The final expanded uncertainty of the pathlength, $U_{95}(l)$, using the absorbance of SRM 2082 is calculated by $U_{95}(l) = k \cdot u(l)$, where k is the coverage factor for the 95 % confidence interval. For the recommended ten replicate absorbance measurements, $k = 2.26$ and the expanded uncertainty is $U_{95}(l) = 2.26\, u(l)$.

Table 2. Coefficients and Constants for Uncertainty Calculation Using SRM 2082

	Component A, Tryptophan	Component B, Uracil
	A_{280}	A_{260}
$u(E_c)$	0.000297	0.000304
a_{u2}	1.2271×10^{-10}	1.0671×10^{-10}
a_{u1}	-5.7849×10^{-9}	-5.0309×10^{-9}
a_{u0}	-7.0128×10^{-8}	6.0988×10^{-8}
b_{u4}	7.4002×10^{-9}	1.3126×10^{-9}
b_{u3}	-9.1491×10^{-8}	-1.6227×10^{-8}
b_{u2}	3.9443×10^{-7}	6.9996×10^{-8}
b_{u1}	-6.7305×10^{-7}	-1.1970×10^{-7}
b_{u0}	4.3012×10^{-7}	7.6621×10^{-8}

Coefficients for the determination of the uncertainty of the pathlength of SRM 2082 utilizing equation 7 for Component A (tryptophan) and Component B (uracil) at 280 nm and 260 nm, respectively, are shown above. The constant $u(E_c)$ corresponds to the uncertainty of equations 1 and 2. Coefficients a_{u2}, a_{u1}, and a_{u0} are used in equation 8 to map the uncertainty of change in absorbance to temperature, $u(\Delta_t A)$. Coefficients b_{u3}, b_{u2}, b_{u1}, and b_{u0} are from equation 9 for the uncertainty of the change in absorbance from the spectral bandwidth, $u(\Delta_B A)$.

Information Values: Some instruments may have fixed spectral bandwidths that are outside of the range measured in the preparation of the certificate for SRM 2082. To extend the useful range, we have used a convolution method outlined by Travis *et* al. to calculate spectra out to a spectral bandwidth of 10 nm using the experimental spectra obtained at 1.0 nm spectral bandwidth [5].

The change in absorbance due to spectral bandwidths larger than 5 nm for tryptophan at 280 nm is provided as an information value only:

$$\Delta_B A_{tryp} = -1.169 \times 10^{-7} B_\lambda^6 + 5.158 \times 10^{-6} B_\lambda^5 - 7.783 \times 10^{-5} B_\lambda^4 + 4.311 \times 10^{-4} B_\lambda^3 - 2.084 \times 10^{-4} B_\lambda^2 + 2.135 \times 10^{-5} B_\lambda - 7.113 \times 10^{-5} \quad (10)$$

For uracil, the change in absorbance due to spectral bandwidths larger than 5 nm at 260 nm is provided as an information value only:

$$\Delta_B A_{uracil} = 2.485 \times 10^{-4} B_\lambda^2 - 8.861 \times 10^{-4} B_\lambda - 5.040 \times 10^{-5} \quad (11)$$

where B_λ is the spectral bandwidth in nanometers for both equations. There are no uncertainty estimates for these functions for spectral bandwidths greater than 5 nm. Equations 10 and 11 do **NOT** give certified values and users must apply these functions with caution.

REFERENCES

[1] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definition of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260-136 (2000); available at http://www.nist.gov/srm/upload/SP260-136.PDF (accessed Aug 2016).

[2] NIST Standard Reference Materials, SRM 2082, https://www-s.nist.gov/srmors/view_detail.cfm?srm=2082, (accessed Aug 2016).

[3] Burke, R.W.; Mavrodineau, R.; *Accuracy in Analytical Spectroscopy*; NIST Special Publication 260-81 (1983); available at http://www.nist.gov/srm/publications.cfm (accessed Aug 2016).

[4] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Aug 2016); 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://www.nist.gov/pml/pubs/tn1297/index.cfm (accessed Aug 2016).

[5] Travis, J.C.; Acosta, J.; Andor, G.; Bastie, J.; Blattner, P.; Chunnilall, C.; Crosson, S.; Duewer, D.; Early, E.; Hengstberger, F.; Kim, C.-S.; Liedquist, L.; Manoocheri, F.; Mercader, F.; Monard, L.; Nevas, S.; Mito, A.; Nilsson, M.; Noël, M.; Rodriguez, A.; Ruíz, A.; Schirmacher, A.; Smith, M.V.; Valencia, G.; van Tonder, N.; Zwinkels, J.;*Intrinsic Wavelength Standard Absorbance Bands in Holmium Oxide Solution for UV/visible Molecular Absorption Spectroscopy*; J. Phys. Chem. Ref. Data, Vol. 34, pp. 41–56 (2005).

Users of this SRM should ensure that the Certificate in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.