BAM
Bundesanstalt für Materialforschung und -prüfung

SPECIMEN - CERTIFICATE

CERTIFIED REFERENCE MATERIALS OF BAM-FCalKit2

The customized *Calibration Kit's* certified spectral fluorescence standards:

BAM-F001c - BAM-F005c, BAM-F007, BAM-F009

for the determination of the relative spectral responsivity $s(\lambda)$ of fluorescence instruments.

Certified Properties - Overview

The *normalized corrected fluorescence emission spectra* $I_C(\lambda_{em})$ of the components of **BAM-FCalKit2** in absolute ethanol at T = 25 ± 2 °C referenced to the spectral photon radiance scale. (Table 1).

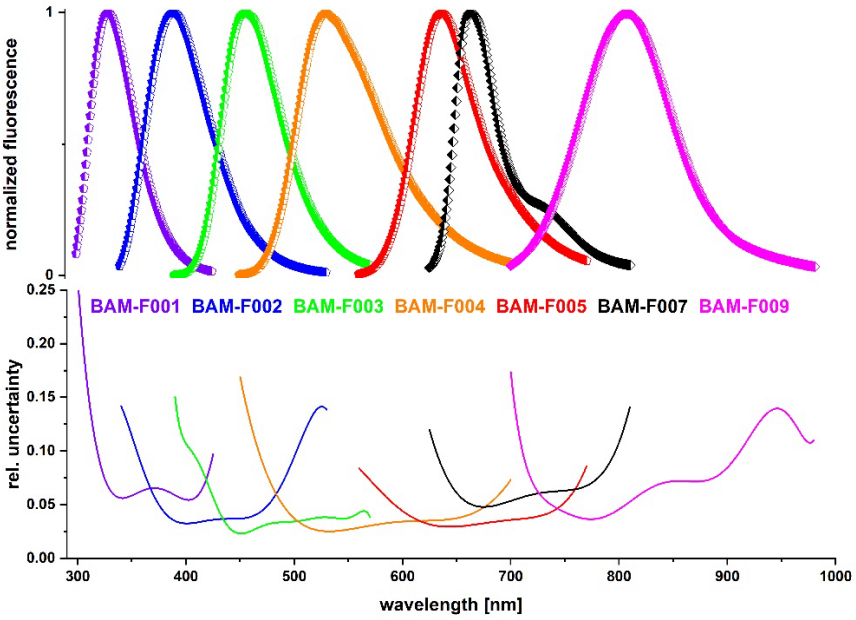

Fig. 1. Top: *Normalized corrected fluorescence emission spectra* $I_C(\lambda_{em})$ of the components of **BAM-FCalKit2** in absolute ethanol at T = 25 ± 2 °C. Bottom: Corresponding expanded uncertainties U_{rel} with a coverage factor of k = 2 corresponding to a level of confidence of 95 %, as defined in the Guide to the expression of uncertainty.[1]

End of Validity

The individual certificates are valid for 6 months after delivery if the solid CRM is stored at 2 - 8 °C in the dark. **BAM-FCalKit2** can be shipped at ambient temperature.

Date of delivery:

e.g. Table 1: Certified *normalized corrected fluorescence emission spectrum* $I_C(\lambda_{em})$ of the individual certificates of **BAM-F001c – BAM-F005c, BAM-F007, BAM-F009** in 5 nm steps, measured in absolute ethanol at 25 °C using individual excitation wavelengths (λ_{exc}). The absorbance at the excitation wavelength $A(\lambda_{exc})$ is stated in each certificate.

Wavelength λ_{em} in nm	Normalized intensity $I_C(\lambda_{em})$	Expanded rel. uncertainty * U_{rel} (k=2)	Wavelength λ_{em} in nm	Normalized intensity $I_C(\lambda_{em})$	Expanded rel. uncertainty * U_{rel} (k=2)
390	0.0013	0.1502	520	0.2263	0.0376

Material Description

The certified spectral fluorescence standards are supplied as a solid in a tightly closed glass bottle. **BAM-F001c – BAM-F005c** were ready-made by Sigma-Aldrich GmbH (now part of Merck KGaA), **BAM-F007, BAM-F009** were produced by BAM.

The supplied bottles of the solid CRMs must be dissolved by adding 10 mL absolute ethanol (spectrophotometric grade $\geq$ 99.8 % (GC)).
Generally, only a freshly opened bottle of absolute ethanol should be used and uptake of water from air by the solvent and the ethanolic solutions of **BAM-FCalKit2** should be avoided by properly closing bottles and cuvettes. The uptake of water could lead to changes of the certified *normalized corrected fluorescence emission spectra* $I_C(\lambda_{em})$.

The CRMs are provided with a certificate file of the certified *normalized corrected fluorescence emission spectra* $I_C(\lambda_{em})$ and the corresponding expanded relative uncertainties U_{rel}. For the intended use of the *Calibration Kit*, the respective certificate file **BAM2406M6.CTF** is electronically supplied together with the BAM software ***LinkCorrWin***. This file can be readout exclusively by ***LinkCorrWin***.

Recommended Use

The *Calibration Kit* **BAM-FCalKit2** dissolved in absolute ethanol is certified for use as spectral fluorescence standards. The intended application includes:

- The calibration of fluorescence instruments: determination of the wavelength-dependent relative spectral responsivity $s(\lambda)$ of the emission channel/detection system of fluorescence instruments using the uncorrected (instrument-specific) fluorescence emission spectra of the spectrally overlapping CRMs of **BAM-FCalKit2**, obtained under conditions used for routine fluorescence measurements, and the evaluation software ***LinkCorrWin***.

For the correct determination of the relative spectral responsivity $s(\lambda)$ using the **BAM-FCalKit2** and ***LinkCorrWin*** follow the instructions provided in the SOP "Standard Operating Procedure (SOP) for Use of *LINKCORRWin* in combination with the Certified Reference Materials (CRMs) '*Calibration Kit*' - certified spectral fluorescence standards" provided by BAM via ***LinkCorrWin***.

Please note that applying the measured uncorrected data of the *Calibration Kit* in ***LinkCorrWin*** will lead to a reduction of the wavelength region covered by the resulting relative spectral responsivity $s(\lambda)$ compared to the certified properties listed in the respective certificates. This is due to the data evaluation procedure used by ***LinkCorrWin*** as described e.g., in Chapter 3 of the SOP.[2]

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The knowledge if the instrument-specific relative spectral responsivity $s(\lambda)$ of fluorescence instruments is mandatory for the determination of corrected (instrument-independent) fluorescence emission spectra that are comparable across instruments and for the determination of fluorescence quantum yields.
Recommended instrument prerequisites and characterizations for the reliable determination of the relative spectral responsivity $s(\lambda)$ of fluorescence instruments with the *Calibration Kit* include:

a.) Calibration of the wavelength scale of the fluorescence spectrometer
b.) Determination of the range of linearity of the detectors of the fluorescence spectrometer

Recommended Calibration Interval

The determination of the relative spectral responsivity $s(\lambda)$ is recommended every six months for frequently used fluorescence instruments. After a modification of the emission channel like the exchange of optical or opto-electrical components such as monochromator gratings or detectors, or transport of a fluorescence instrument, a recalibration of the emission channel is mandatory.

Extended Application

The individual CRMS of **BAM-FCalKit2** can also be used for:

- Determination of a corrected (instrument-independent) fluorescence emission spectrum:
  The CRMs can be used without the spectrally neighbouring CRMs of the *Calibration Kit* and the evaluation software *LinkCorrWin* for the determination of a corrected fluorescence emission spectrum.
- Determination of the relative spectral responsivity $s(\lambda)$ without the other components of the *Calibration Kit* and *LinkCorrWin*:
  The CRMs can also be used for the determination of the relative spectral responsivity of fluorescence instruments $s(\lambda)$ in the wavelength region covered by its emission band by adapting the procedure described in the SOP in chapter 3.
- Control of instrument calibration, (independently determined) correction curves:
  Care must be taken when The CRMs are applied to control the reliability of correction factors supplied by instrument manufacturers as part of the fluorescence spectrometer`s software (e.g., as "correction file" or "correction curve"), as the reference quantity used by the instrument manufacturer may not be obvious. For The CRMs only correction factors obtained relative to the spectral photon radiance scale can be expected to yield values matching the properties certified by BAM.

Handling

For the preparation of the measurement solution, the solid CRMs of **BAM-FCalKit2** must be dissolved in 10 mL absolute ethanol (spectrophotometric grade $\geq$ 99.8 % (GC)). Shake each bottle containing the dissolved solution several times and wait ~15 min for complete dissolution.

For further details see the respective certificate.

The recommended measurement conditions to determine the fluorescence emission of the solutions of **BAM-FCalKit2** are summarized in Table 2.

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Table 2 Recommended measurement conditions.

Measurement conditions	**Region / Limit**
Excitation wavelength (λ_{exc})	(See respective CRM certificate)
Polarizers set to (if available)	**Exc**itation channel **0°** / **Em**ission channel **54.7°** ("magic angle" conditions)
Absorbance at λ_{exc}	(See respective CRM certificate)
Temperature	**25 °C** ± 2°C
Spectral bandpass excitation monochromator	Selected considering the range of linearity of the detector used
Spectral bandpass of the emission mono-chromator of the instrument to be calibrated	2 nm to 10 nm (value used for certification: **6 nm**)
Step width emission scan	0.1 up to 5.0 nm (value used for certification: **1.0 nm**)
Emission range (at least)	(See respective CRM certificate)

If **BAM-FCalKit2** is applied under measurement conditions deviating from the conditions recommended in Table 2, e.g., at different measurement geometries, excitation wavelengths, temperatures, or concentrations, this could lead to spectral deviations from the certified property and/or to an increase of the relative measurement uncertainty. In principle, such effects can be considered by an increase of the relative uncertainties of the certified property.

The excitation wavelength regions differing from λ_{exc} for which the obtained emission profiles deviate at maximum by 2 % from the certified property are listed in Table 3.

Table 3 Excitation wavelengths for limited use.

Measurement conditions	**Region / Limit**
Excitation wavelength region for limited use**	(See respective CRM certificate)

**excitation wavelength region differing from λ_{exc} for which the obtained emission profiles deviate at maximum by 2 % from the certified property.

Transport and Storage

BAM-FCalKit2 should be shipped at ambient temperature. The transport temperature should not exceed 40 °C.
After receipt, the CRM must be stored at 2 - 8 °C. Under these conditions, its certified property is preserved for **6 months after the date of delivery**.

The diluted ethanolic measurement solutions of **BAM-FCalKit2** must be stored in the dark at 2 – 8 °C in a tightly closed container to prevent water uptake from air and must be used within 1 week.

BAM is not responsible for any changes in the certified property that may occur during storage of the CRM at the customer's premises, especially not in the case of opened bottles and prepared solutions.

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Analytical Method(s)

The certification of **BAM-FCalKit2** was performed according to *ISO 33405:2024* and according to *ISO 17034:2016 - General requirements for the competence of reference material producers.*[3,4]

The determination of the certified property *normalized corrected fluorescence emission spectra* $I_C(\lambda_{em})$ of **BAM-FCalKit2** at T = (25°± 2) °C were done using a calibrated fluorescence spectrometer equipped with a photon counting detection system and Glan-Thompson-Polarizers in the excitation and emission channel set to 0°$_{exc}$/54.7°$_{em}$ using 1 nm data intervals. For the detection of the fluorescence emission spectra a PMT detector and ruled monochromator gratings were used. A reference detector (Si-diode) was employed for monitoring the fluctuation of the continuous Xe-light source.
The certification measurements involving **BAM-FCalKit2** were carried out using a temperature-controlled sample holder (set to T = 25 °C).
The fluorescence spectrometer calibration was performed with identical instrument settings as used for the certification measurements of **BAM-FCalKit2**. (See chapter Metrological Traceability.) The instrument settings are listed in the respective CRM certificates.

The measured uncorrected emission spectra of the solutions of **BAM-FCalKit2** equal the wavelength-dependent signals (photons per unit time) recorded by the fluorescence spectrometer´s emission channel (EM) divided by the signal of the instrument´s reference detector (Ref) recorded at the chosen excitation wavelength. This accounts for the consideration of fluctuations in the spectral radiance of the used excitation light source during fluorescence measurements. This signal ratio results in the uncorrected emission spectra $I_{EM/Ref}(\lambda)$.
The uncorrected emission spectrum is blank-corrected and then divided by the correction curve $s(\lambda_{em})$ according to Equation 1 and normalized. This provides the certified property *normalized corrected fluorescence emission spectrum* $I_C(\lambda_{em})$ referenced to the spectral photon radiance scale via the instrument calibration. Thereby the photonic nature of the emitted light is considered.

$$I_C(\lambda_{em}) = I_{EM/Ref}(\lambda) / s(\lambda_{em}) \qquad \text{Eq. 1}$$

Photostability studies with **BAM-FCalKit2** have been performed at the excitation wavelengths employed for certification. If excitation wavelengths are used that differ from the certification conditions and/or if very intense excitation light sources like lasers are employed, it is strongly recommended to control the photostability of the CRMs under these conditions prior to using the applied excitation source.

Assignment of Uncertainties

Standard uncertainty components equivalent to the relative standard deviation of the mean were assigned for measurement uncertainties, and sample inhomogeneity. The resulting experimental and sample related uncertainties were combined with the systematic uncertainty due to wavelength accuracy, and the uncertainty of the determination of the relative spectral responsivity $s(\lambda)$ of the spectrofluorometer´s detection channel (due to e.g., uncertainty in the certified spectral radiance scale and reflectance values of the calibrated light source and reflector). A coverage factor of k = 2 was applied so that the expanded uncertainties given in this certificate express an interval ($I_C \pm U_{95}$) within which the true value is expected to fall with a level of confidence of approximately 95 % for a normal distribution.

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Metrological Traceability

The certified property *normalized corrected fluorescence emission spectra* $I_C(\lambda_{em})$ of **BAM-FCalKit2** were obtained with a calibrated fluorescence spectrometer.

The calibration of the fluorescence spectrometer included the determination of the wavelength accuracy and the device-specific spectral characteristics resulting in an emission correction curve equaling the relative spectral responsivity $s(\lambda)$ of the fluorescence spectrometer´s detection channel as described in the *IUPAC Technical Reports.*[5,6] These characteristics were determined using calibrated physical transfer standards as documented in the respective Calibration Certificates. The physical transfer standards used were a *Spectral Radiance Standard "SDS" 10 W* calibrated by Physikalisch-Technische Bundesanstalt (PTB Berlin) and a *White Standard in Geometry 45°/0°* calibrated by National Physical Laboratory (NPL). The PTB certificates are consistent with the Calibration and Measurement Capabilities (CMC) that are included in Annex C of the Mutual Recognition Arrangement (MRA) drawn up by the International Committee for Weights and Measures (CIPM). The NPL certificate also provides traceability to the SI system.

For the calibration of the fluorescence spectrometer´s detection channel, the traceable light source *"SDS"* is diffusely reflected into the emission detection channel of the instrument via the reflective *White Standard* at the sample position illuminated at a 45° angle. This procedure, performed with identical instrument settings as the certification measurement of the corrected fluorescence emission spectra of **BAM-FCalKit2** together with calibration of the physical transfer standards assures traceability to the SI system.

The subsequently determined emission correction curve $s(\lambda_{em})$ of the emission detection system of the fluorescence spectrometer corresponds to the quotient of the measured uncorrected emission spectrum of the "SDS" $I_U(\lambda_{em})$ corrected for the determined dark current of the detector, and possible inaccuracies of the wavelength scale. The corrected spectrum $I_{U_LC}(\lambda_{em})$ is then divided by the certified wavelength-dependent spectral radiance factor of the *White Standard* $I_{White}(\lambda_{em})$ in *radiance factor [%]* given in the calibration certificate and the certified emission spectrum of the SDS $I_{SDS}(\lambda_{em})$ given in the calibration certificate in *spectral radiance* [W m^{-3} sr^{-1}]. (see Equation 2)

$$s(\lambda_{em}) = I_{U_LC}(\lambda_{em}) / (I_{White}(\lambda_{em}) * I_{SDS}(\lambda_{em})) \quad \text{Eq. 2}$$

The resulting calibration curve is normalized and given in *spectral photon radiance* [s^{-1} m^{-3} sr^{-1}]. Thereby establishing traceability to the spectral photon radiance scale and considering the photonic nature of the emitted light.

In general, the *spectral radiance* $L_{e,\lambda}$ [W m^{-3} sr^{-1}] is converted into *spectral photon radiance* $L_{p,\lambda}$ [s^{-1} m^{-3} sr^{-1}] according to Equation 3.

$$L_{p,\lambda} = L_{e,\lambda} / h_c / \lambda \quad \text{Eq. 3}$$

All instrument calibrations performed with the components of the *Calibration Kit* are automatically referenced to the spectral photon radiance scale. This enables e.g., the straightforward calculation of fluorescence quantum yields from corrected emission spectra obtained with a fluorescence spectrometer calibrated with the *Calibration Kit*.

Technical report

A more detailed description of the analysis procedures and of the treatment of the analytical data used for certification is presented in Richter, M. et al., "*Extending certified spectral fluorescence standards for the calibration and performance validation of fluorescence instruments to the NIR–closing the gap from 750 to 940 nm with two novel NIR dyes"* Anal. Bioanal. Chem. **2025**[8] including the published supporting Information.

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Literature

[1] GUM, *Evaluation of measurement data – Guide to the expression of uncertainty in measurement*. Joint Committee for Guides in Metrology, Working Group 1 **2008**.

[2] Pfeifer, D.; Hoffmann, K.; Hoffmann, A.; Monte, C.; Resch-Genger, U., *The Calibration Kit Spectral Fluorescence Standards: A Simple Tool for the Standardization of the Spectral Characteristics of Fluorescence Instruments*, *J. Fluoresc*. **2006**, *16*, 581.

[3] ISO 33405:2024, *Reference materials – Approaches for characterization and assessment of homogeneity and stability*, **2024**

[4] ISO 17034:2016, *General requirements for the competence of reference material producers*, **2016**

[5] Resch-Genger, U.; DeRose, P., *Characterization of photoluminescence measuring systems (IUPAC Technical Report)*, Pure and Applied Chemistry **2012**, 84, 1815.

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[8] Richter, M.; Güttler, A.; Pauli, J; Vogel, K.; Homann, C.; Würth, C.; Resch-Genger, U., *Extending certified spectral fluorescence standards for the calibration and performance validation of fluorescence instruments to the NIR–closing the gap from 750 to 940 nm with two novel NIR dyes;* Anal. Bioanal. Chem. **2025**, https://doi.org/10.1007/s00216-024-05723-w

Specimen

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