Bundesanstalt für Materialforschung und -prüfung (BAM)
in Cooperation with *SIGMA-ALDRICH GmbH (now part of Merck KGaA)*

Certified Reference Materials

Calibration Kit – BAM-FCalKit

BAM-F001b, BAM-F002b, BAM-F003b, BAM-F004b and BAM-F005b

for the Determination of the Relative Spectral Responsivity of Fluorescence Instruments

Certified Properties

The certified properties are the *normalized corrected emission spectra* $I_C(\lambda_{em})$ of BAM-F001b - BAM-F005b in ethanol measured at T = 25 °C, referenced to the *spectral photon radiance* scale (Tables 1 to 5).
These emission spectra are obtained with a reference fluorometer calibrated with physical standards calibrated by Physikalisch-Technische Bundesanstalt (PTB) Berlin, thereby establishing traceability to the spectral photon radiance scale and considering the photonic nature of the emitted light. [1]

Certification was performed according to *ISO Guide 35* and according to the *Guidelines for the Development and Production of BAM Reference Materials*. [2]

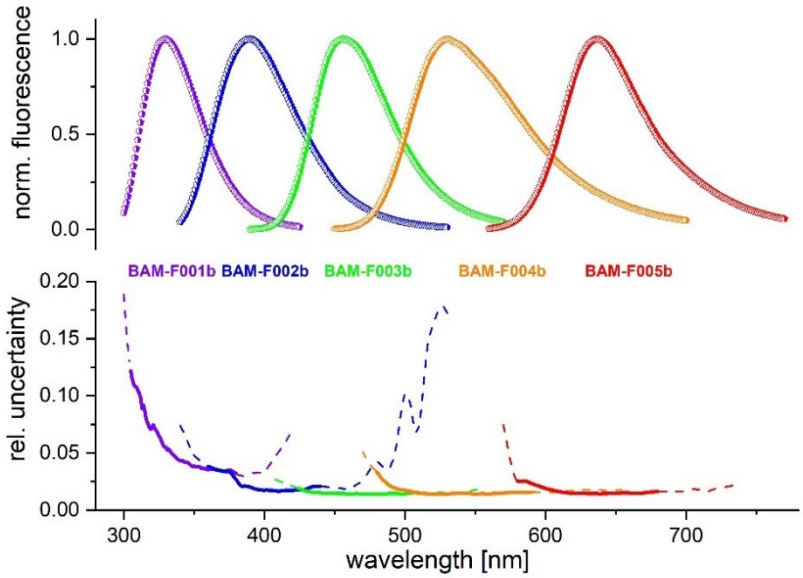

Fig. 1. Top: Normalized corrected emission spectra of BAM-F001b - BAM-F005b Bottom: Corresponding expanded relative uncertainties (k = 2, corresponding to a confidence interval of approximately 95 %). The dashed lines equal relative uncertainty values which are not considered by *LINKCORRWin1.3*.

Bottled ready-made BAM-F001b - BAM-F005b stored in the dark at 2 - 8 °C will maintain their certified properties up to 6 months after date of delivery.

Prepared working solutions must be used within 1 week and stored in the dark at 2 – 8 °C.

Determination of the certified properties

The determination of the certified properties *normalized corrected fluorescence emission spectra* of the Calibration Kit was done with the calibrated fluorescence spectrometer FLS920 using a photon counting detector.

The calibration of the fluorescence spectrometer included the determination of the device-specific spectral characteristics resulting in a calibration curve equaling the relative spectral responsivity **s(λ_{em})** as described in the *IUPAC Technical Reports* [3,4]). These characteristics were determined with physical standards (*Spectral Radiance Standard* – "SDS"; *"Reflectivity Standard*") traced to the National Measurement Standard via calibration by PTB Berlin (See *Metrological Traceability).* The calibration curve was determined at identical measurement settings as used for the certification measurements of the Calibration Kit.

The calibration curve of the emission detection system **s(λ_{em})** corresponds to the quotient of the measured uncorrected emission spectrum of the SDS (**$I_U(\lambda_{em})$**) and the certified corrected emission spectrum of the SDS (**$I_{C\text{-}SDS}(\lambda_{em})$**) given in its Calibration Certificate from PTB in *spectral radiance "SD"* [W m^{-2} sr^{-1}]. Eq. 1

$$s(\lambda_{em}) = I_U(\lambda_{em})/I_{C-SDS}(\lambda_{em}) \qquad (1)$$

The resulting calibration curve given in *spectral radiance "SD"* [W m^{-2} sr^{-1}] is then converted into *spectral photon radiance* ("PhotSD") according to Eq. 2, thereby considering the photonic nature of the emitted light. Finally, the calibration curve is normalized. [1]

$$s_{PhotSD}(\lambda_{em}) = s_{SD}(\lambda_{em}) * \lambda \qquad (2)$$

Note that the measured uncorrected emission spectra of the Calibration Kit equal the wavelength-dependent signals (photons per unit time) recorded by the fluorometer´s emission channel (**EM**) divided by the signal of the instrument´s reference detector (**Ref**) recorded at the chosen excitation wavelength. This accounts for fluctuations of the excitation light source during fluorescence measurements. This signal ratio results in uncorrected emission spectra ***$I_{EM/Ref}(\lambda)$*** without units.
These uncorrected emission spectra are then divided by the calibration curve and normalized resulting in the certified properties *normalized corrected emission spectra* ***$I_C(\lambda_{em})$***.

All instrument calibrations performed with the recertified 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 fluorometer calibrated with the Calibration Kit BAM-F001b - BAM-F005b. [1]

The following measurement settings were used for the determination of the calibration curve as well as for the certified properties of the Calibration Kit:

Sample holder thermostat: **25 °C**.
Polarizers: **ex**citation channel **0°**/**em**ission channel **54.7°** ("magic angle" conditions).
Spectral **bandpass** of the **em**ission monochromator: **6 nm**.
The spectral bandpass of the excitation monochromator used for the recertification measurements was selected considering the range of linearity of the photon counting detector used.

For the Handling of the Calibration Kit see the *Standard Operating Procedure* SOP provided by BAM.

Changes in the reference quantity of the certified properties and its consequences

In contrast to the certification of the Calibration Kit in 2006 (BAM-F001 – BAM-F005) and the recertification in 2013 (BAM-F001a – BAM-F005a), where the normalized corrected emission spectra were referenced to the *spectral radiance scale*, for the recertification of BAM-F001b – BAM-F005b, now the photonic nature of the emitted light was considered. This was done because the reference quantity *spectral photon radiance* is increasingly used by many instrument manufacturers for the calibration of their fluorometers [5], particularly for instruments with photon counting detection.
Referencing the normalized corrected emission spectra of the Calibration Kit to the *spectral photon radiance* leads to slight spectral deviations from the previously certified normalized corrected emission spectra referenced to the spectral radiance. This is shown in Figure 2.

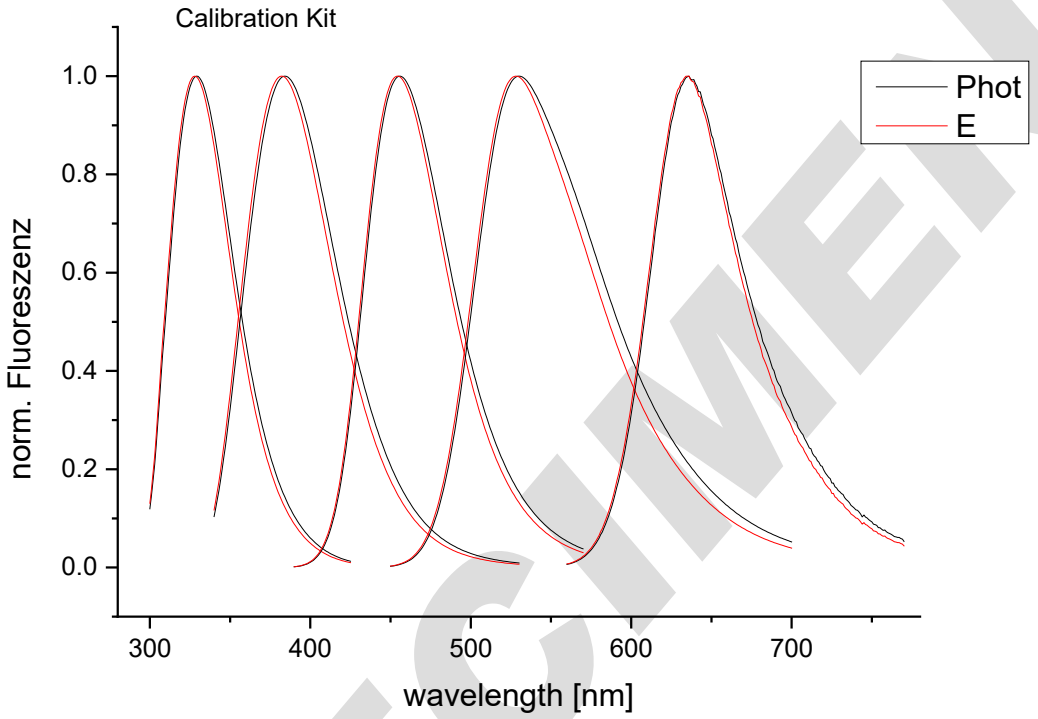

Fig. 2: Comparison of the normalized corrected emission spectra of the Calibration Kit referenced to the *spectral radiance* ("E") and to the *spectral photon radiance* ("Phot").

The slight deviation between the normalized corrected emission spectra referenced to the *spectral radiance* and to the *spectral photon radiance* underlines the importance of providing the calibration procedure and thereby indirectly the respective reference quantity.

CONTROL of (independently determined) correction curves with the Calibration Kit

Care has to be taken when the Calibration Kit is applied to control the reliability of correction factors supplied by instrument manufacturers as part of the spectrofluorometer software (e.g., as "correction file" or "correction curve"). Moreover, the reference quantity used by the instrument manufacturer must not be obvious. In the case of BAM-F001b – BAM-F005b, only correction factors obtained relative to the *spectral photon radiance* scale can be expected to yield values matching the certified properties by BAM.

Please note, this application is not the main scope of application of the Calibration Kit.

Table 1: Certified properties $I_C(\lambda_{em})$ of the normalized corrected emission spectrum of *BAM-F001b* (5 nm-steps) measured in ethanol at 25 °C with an excitation wavelength (λ_{ex}) of **280 nm**.

Wavelength λ_{em} in nm	Normalized intensity $I_C(\lambda_{em})$	Expanded relative uncertainty factor* U_{rel} (k=2)
300	0.0895	0.1890
305	0.2478	0.1215
310	0.4701	0.1045
315	0.6899	0.0865
320	0.8634	0.0721
325	0.9716	0.0648
330	0.9989	0.0547
335	0.9649	0.0485
340	0.8916	0.0431
345	0.7977	0.0419
350	0.6906	0.0384
355	0.5830	0.0368
360	0.4793	0.0353
365	0.3881	0.0353
370	0.3106	0.0343
375	0.2443	0.0351
380	0.1896	0.0320
385	0.1460	0.0298
390	0.1112	0.0323
395	0.0843	0.0325
400	0.0631	0.0343
405	0.0473	0.0418
410	0.0352	0.0506
415	0.0264	0.0598
420	0.0197	0.0700
425	0.0147	0.0810

The certified normalized corrected emission spectra of BAM-F001b are exemplary shown for the BAM Spectral Fluorescence Standards BAM-F001b to BAM-F005b.

Material Description – Content of Calibration Kit

- Five spectral fluorescence standards BAM-F001b - BAM-F005b ready-made by Sigma-Aldrich GmbH (now part of Merck KGaA) with emission spectra that cover the wavelength region of 300 nm to 770 nm are provided as a set. Addition of aliquots of 10 mL of ethanol to each of the bottled fluorescence standards yields a solution with an absorbance (E) of 0.04 ± 0.02 at each fluorescence standard`s longest wavelength absorption maximum. These solutions can be measured in 1 cm cells without additional dilution steps.
- The solvent ethanol used for the preparation of the standard solutions must be of high purity and should contain only a minimum amount of water like absolute ethanol, spectrophotometric grade, ≥ 98 % (GC). For the determination of the certified properties ethanol from Merck, product number 1.00980 and ethanol from Sigma-Aldrich (product number 34923 - which is no longer available) was used.
- Normalized corrected emission spectra of BAM-F001b - BAM-F005b, see Figure 1, and corresponding wavelength-dependent relative expanded uncertainty factors (k = 2), see Tables 1 to 5.

  The wavelength-dependent expanded relative uncertainty factors include contributions from the relative uncertainties of the calibration of the spectrofluorometer FLS920 used for certification and the measurement of the fluorescence spectra of the spectral fluorescence standards as well as from homogeneity and thermal stability studies of the Calibration Kit. These values were determined according to the *Guide to the Expression of Uncertainty* (GUM) and *ISO Guide* 35 by propagation of uncertainties.

  Note: Rounding the numbers of the certified properties to four digits is not significant for the use of the Calibration Kit, since the Software *LINKCORRWin1.3* uses up to 12 digits for all calculations.
- SOP for use of the BAM Calibration Kit and the software *LINKCORRWin1.3*
- CD containing the certificates and the certificate file *BAM1808M6.CTF*, the data evaluation software *LINKCORRWin1.3* developed by BAM, and the SOP for use of BAM-F001b - BAM-F005b. The certificate file *BAM1808M6.CTF* contains the certified properties of BAM-F001b - BAM-F005b referenced to *spectral photon radiance* in 1 nm-steps, and the corresponding wavelength-dependent expanded relative uncertainties in a format readable exclusively by *LINKCORRWin1.3*.

Recommended Use

Calibration of fluorescence instruments: determination of the wavelength-dependent relative spectral responsivity $s(\lambda)$ of the emission channel/detection system in the wavelength region of 300 nm to 770 nm under conditions used for routine fluorescence measurements.

The parameters and requirements for use of BAM-F001b - BAM-F005b are described in the SOP.

The Calibration Kit is to be used only in combination with LINKCORRWin1.3 and the SOP.

Recommended Calibration Interval

Determination of the emission correction curve $1/s(\lambda)$ is recommended every six months for frequently used fluorescence instruments. After each transport of a fluorescence instrument or modification of the emission channel like the exchange of optical or opto-electrical components such as monochromator gratings or detectors, a recalibration of the emission channel is mandatory.

Application of BAM-F001b - BAM-F005b and *LINKCORRWin1.3*

The principle of the determination of the relative spectral responsivity of fluorescence instruments with BAM-F001b - BAM-F005b and *LINKCORRWin1.3* is illustrated in Figure 3 and described in detail in the SOP.

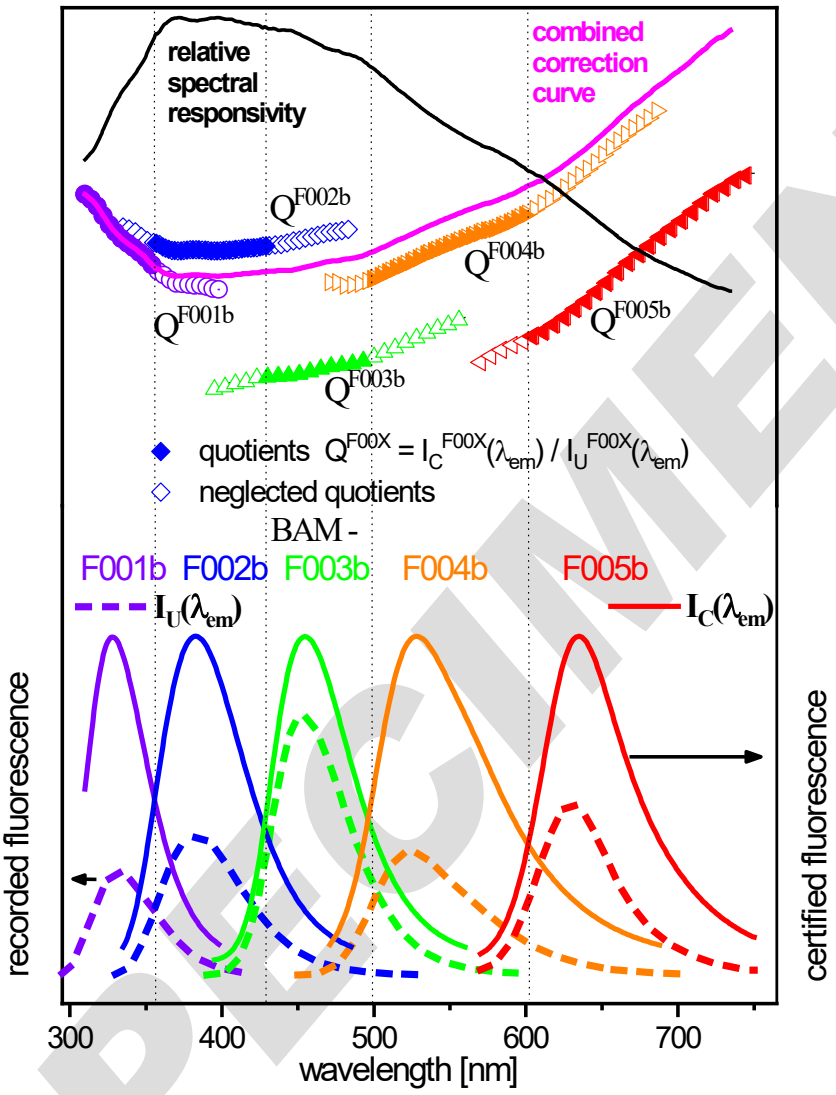

Fig. 3: Scheme of the determination of the relative spectral responsivity $s(\lambda)$ of a fluorescence instrument with the Calibration Kit and *LINKCORRWin1.3*.
Bottom: Certified normalized corrected emission spectra (solid lines; ($I_C(\lambda_{em})$) of the Calibration Kit and uncorrected, i.e., instrument-dependent emission spectra (dashed lines; $I_U(\lambda_{em})$), as measured with the instrument to be calibrated.
Middle: Individual quotients $Q^{F00x} = I_C(\lambda_{em}) / I_U(\lambda_{em})$, calculated with *LINKCORRWin1.3* for the Calibration Kit. These quotients equal the inverse relative spectral responsivity of the instrument to be calibrated within the spectral region of the respective fluorescence standard or the individual emission correction curve. Top: Combined emission correction curve ≡ $1/s(\lambda)$ (solid pink line) calculated with *LINKCORRWin1.3* from the statistically weighted Q^{F00x} as well as its reciprocal $s(\lambda)$ (black dash-dotted line).

Transport and Storage

The CRM Calibration Kit can be shipped at ambient temperature. The transport temperature should not exceed 40 °C.
After receipt, the CRM, provided in tightly closed brown glass vials, must be stored in a refrigerator at 2 - 8 °C. Under these conditions, it will preserve its certified properties for 6 months after delivery.
When handling the Calibration Kit at room temperature, care must be taken to avoid moisture uptake once the vials are opened, and the fluorescence standards are dissolved in ethanol.
The dissolved fluorescence standards, the so-called "working solutions", must be stored in the dark at 2 – 8 °C when not used for measurements.
They can be preserved under these conditions for 1 week.

BAM is not responsible for changes in the certified properties that occur during storage of the material at the customer's premises, especially not in the case of opened vials and Calibration Kit solutions.

Metrological Traceability

The metrological traceability of the Calibration Kit BAM-F001b - BAM-F005b is highlighted in Figure 4.

The normalized corrected fluorescence emission spectra of the Calibration Kit were obtained with a calibrated fluorescence spectrometer as described in the section Determination of the certified properties. The calibration of the BAM fluorescence spectrometer FLS920 was done with physical standards calibrated by PTB Berlin as documented in the Calibration Certificates 73114 PTB 17 (Oct. 2017) "Spectral Radiance Standard 10 W" and PTB-4.52-0507 (Jun. 2007) "Reflectivity Standard in Geometry 45°/0°".

The calibration certificates issued by PTB confirm that this calibration is traceable to national measurement standards. 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).

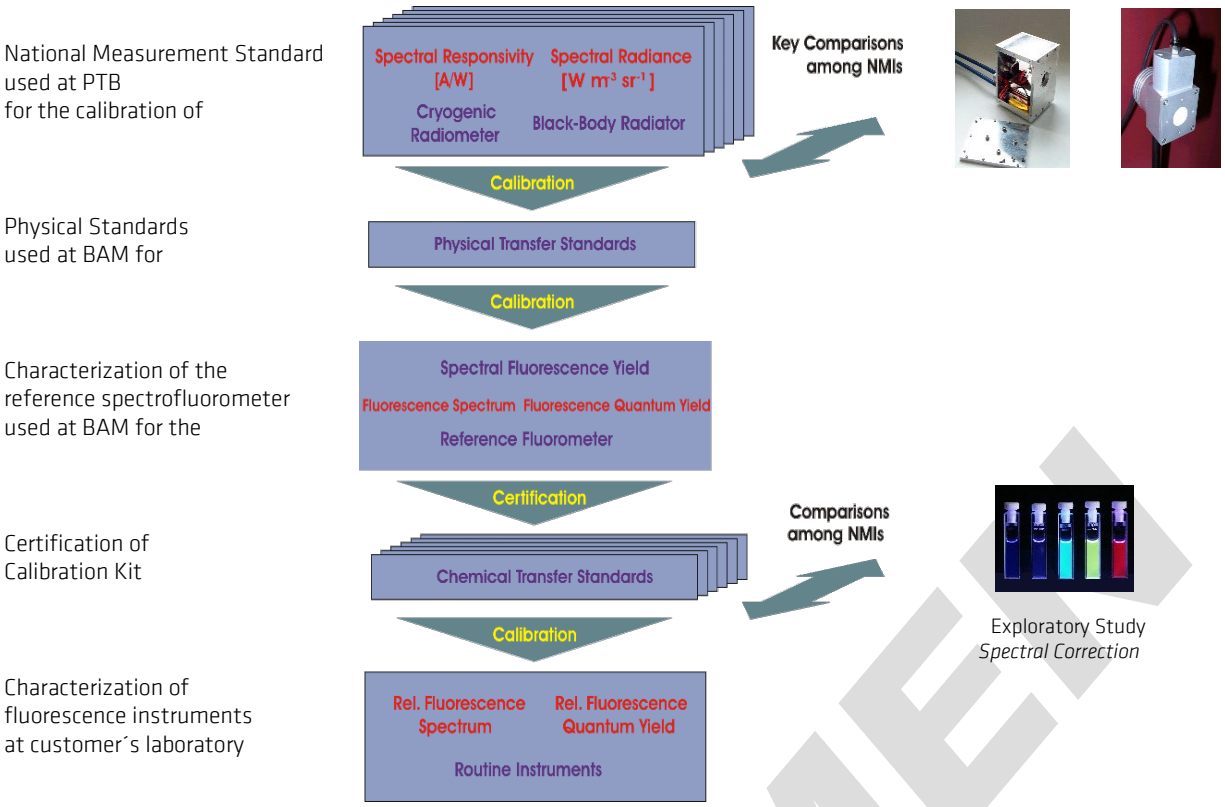

Fig. 4: Metrological traceability of fluorescence measurements and the certified normalized corrected emission spectra of BAM-F001 - BAM-F005 (and accordingly recertified Calibration Kits). The reliability of the normalized corrected emission spectra of the Calibration Kit and its suitability for the determination of the relative spectral responsivity of fluorescence instruments were assessed in an interlaboratory comparison of different National Metrology Institutes (NMIs) active in the area of high precision fluorometry [6]. The applicability of the Calibration Kit was also assessed by selected field laboratories from academia and industry [7].

The reliability of the normalized corrected emission spectra of the Calibration Kit and the determination of the relative spectral responsivity of fluorescence instruments with the BAM Calibration Kit and *LINKCORR* were assessed for the first batch of the BAM-certified Calibration Kit in an interlaboratory comparison of the National Metrological Institutes active in the area of high precision fluorometry (National Institute of Standards and Technology NIST, USA; National Research Council NRC, Canada; Physikalisch-Technische Bundesanstalt PTB, Germany; Bundesanstalt für Materialforschung und -prüfung (BAM), Germany) [6]. This comparison resulted in a set of reference values (mean normalized corrected emission spectra) for each spectral fluorescence standard, equalling the true instrument-independent emission spectra, obtained by different independent calibration procedures with different independently calibrated physical standards like calibrated light sources, white standards, and detectors [6]. All subsequently issued batches of the calibration Kit of identical composition, and thus also the recertified properties of BAM-F001b – BAM-F005b, can be compared and related to these reference values within the respective uncertainties.
In addition, the characterization of different fluorescence instruments with BAM-F001 - BAM-F005 and LINKCORR was assessed in a comparison of ten field laboratories selected from academia and industry using different types of common commercial fluorescence instruments [7].
Please note, that for these previous comparisons, all corrected emission spectra were referenced to the spectral radiance scale and not to the spectral photon radiance scale as used now.

Literature

[1] Würth, C.; Grabolle, M.; Pauli, J.; Spieles, M.; Resch-Genger, U., *Relative and absolute determination of fluorescence quantum yields of transparent samples*, Nature Protocols **2013**,8, 1535.
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[6] Resch-Genger, U.; DeRose, P.; Bremser, W.; Ebert, B.; Zwinkels, J.; Pfeifer, D.; Voigt, J.; Taubert, D.; Monte, C.; MacDonald, R.; Hollandt, J.; Gauthier, F.; Spieles, M.; Hoffmann, A., "*State-of-the Art Comparability of Corrected Emission Spectra – 1. Spectral Correction with Physical Transfer Standards and Spectral Fluorescence Standards by Expert Laboratories*", Anal. Chem. **2012**, 84, 3889.

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