



CERTIFIED REFERENCE MATERIALS

BAM-F001, BAM-F002, BAM-F003, BAM-F004 and BAM-F005

Calibration Kit *SPECTRAL FLUORESCENCE STANDARDS*

for the Determination of the Relative Spectral Responsivity
of Fluorescence Instruments

Certified Properties

Normalized corrected emission spectra of BAM-F001 - BAM-F005 in ethanol for $T = 25^{\circ}\text{C}$. The emission spectra are traceable to the *spectral radiance* realized and disseminated by the Physikalisch-Technische Bundesanstalt (PTB) in Germany¹.

The complete data sets in 1nm data intervals are provided by BAM on a CD. These data sets are in a format readable by the BAM software *LINKCORR*. The certified values (according to *ISO Guide 35*) in Tables 1-5 are extracts obtained at the measurement conditions given in the tables and in the section *measurement conditions and instructions for use*. Deviation from these conditions can lead to deviations from the certified values and to increased uncertainties.

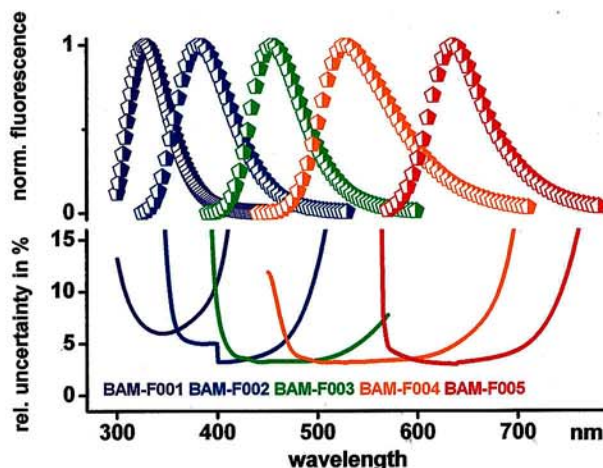


Figure 1: Normalized corrected emission spectra of BAM-F001 - BAM-F005 (top) and expanded relative uncertainties (bottom) using an expansion factor of $k = 2$ corresponding to a confidence interval of approximately 95%. The spectra were measured with three different spectral bandpasses of the emission monochromator of the spectrofluorometer SLM 8100 used for certification, see Tables 1-5. The measurement conditions are given in the headings of the tables.

¹ The *spectral radiance* is traced back to the SI unit of the temperature, Kelvin. Realization and dissemination of the *spectral radiance* is the legal obligation of the Physikalisch-Technische Bundesanstalt (PTB). The equivalence of the spectral radiance scale between PTB and the National Metrological Institutes of the other signatories of the Mutual Recognition Arrangement (MRA) was established by the *supplementary comparison* CCPR-S1.

CERTIFICATE



Table 1: Certified values (5nm-steps) of the normalized corrected emission spectra of **BAM-F001** measured in ethanol at 25°C with an excitation wavelength (λ_{ex}) of 280 nm, an absorbance at the longest wavelength absorption maximum (280 nm) of 0.04, and 3 different spectral bandpasses of the emission monochromator of the spectrofluorometer SLM 8100.

λ_{em} in nm	Spectral bandpass 1 nm		Spectral bandpass 4 nm		Spectral bandpass 8 nm	
	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty
300	0.1201	0.1319	0.1307	0.1261	0.1504	0.1119
305	0.3060	0.1094	0.3166	0.0935	0.3289	0.0931
310	0.5458	0.0935	0.5478	0.0784	0.5521	0.0800
315	0.7504	0.0822	0.7557	0.0720	0.7533	0.0712
320	0.9074	0.0742	0.9112	0.0691	0.9096	0.0653
325	0.9887	0.0685	0.9883	0.0669	0.9887	0.0615
330	0.9941	0.0645	0.9952	0.0643	0.9943	0.0590
335	0.9420	0.0619	0.9460	0.0610	0.9429	0.0573
340	0.8520	0.0603	0.8574	0.0575	0.8539	0.0560
345	0.7415	0.0598	0.7466	0.0543	0.7450	0.0549
350	0.6250	0.0602	0.6291	0.0519	0.6304	0.0538
355	0.5130	0.0615	0.5165	0.0508	0.5200	0.0528
360	0.4123	0.0635	0.4156	0.0509	0.4200	0.0518
365	0.3258	0.0662	0.3291	0.0521	0.3335	0.0510
370	0.2540	0.0694	0.2575	0.0539	0.2612	0.0505
375	0.1960	0.0731	0.1995	0.0559	0.2023	0.0503
380	0.1500	0.0775	0.1533	0.0575	0.1553	0.0505
385	0.1140	0.0828	0.1169	0.0582	0.1183	0.0511
390	0.0860	0.0895	0.0885	0.0582	0.0895	0.0520
395	0.0645	0.0986	0.0664	0.0575	0.0673	0.0530
400	0.0481	0.1116	0.0495	0.0569	0.0504	0.0415
405	0.0356	0.1305	0.0365	0.0577	0.0375	0.0406
410	0.0262	0.1582	0.0267	0.0615	0.0277	0.0418
415	0.0192	0.1988	0.0193	0.0704	0.0204	0.0447
420	0.0140	0.2573	0.0138	0.0866	0.0149	0.0481
425	0.0102	0.3403	0.0099	0.1122	0.0109	0.0534

Table 2: Certified values (5nm-steps) of the normalized corrected emission spectra of **BAM-F002** measured in ethanol at 25°C with an excitation wavelength (λ_{ex}) of 315 nm, an absorbance at the longest wavelength absorption maximum (315 nm) of 0.04, and 3 different spectral bandpasses of the emission monochromator of the spectrofluorometer SLM 8100.

λ_{em} in nm	Spectral bandpass 1 nm		Spectral bandpass 4 nm		Spectral bandpass 8 nm	
	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty
340	0.1128	0.4120	0.1169	0.4099	0.1236	0.4105
345	0.2162	0.2178	0.2199	0.2180	0.2248	0.2185
350	0.3521	0.1219	0.3532	0.1200	0.3561	0.1217
355	0.5022	0.0790	0.5029	0.0753	0.5042	0.0782
360	0.6546	0.0617	0.6527	0.0578	0.6527	0.0612
365	0.7914	0.0552	0.7870	0.0522	0.7860	0.0555
370	0.8974	0.0525	0.8933	0.0507	0.8919	0.0534
375	0.9662	0.0510	0.9639	0.0500	0.9627	0.0520
380	0.9974	0.0502	0.9968	0.0494	0.9965	0.0509
385	0.9942	0.0499	0.9949	0.0490	0.9955	0.0505
390	0.9618	0.0500	0.9633	0.0490	0.9650	0.0510
395	0.9066	0.0501	0.9086	0.0490	0.9114	0.0519
400	0.8353	0.0321	0.8377	0.0296	0.8416	0.0350
405	0.7545	0.0321	0.7572	0.0295	0.7620	0.0339
410	0.6699	0.0323	0.6728	0.0295	0.6782	0.0342
415	0.5861	0.0328	0.5891	0.0296	0.5946	0.0351
420	0.5064	0.0335	0.5093	0.0298	0.5147	0.0357
425	0.4328	0.0343	0.4357	0.0301	0.4407	0.0357
430	0.3665	0.0352	0.3692	0.0304	0.3737	0.0355
435	0.3078	0.0363	0.3103	0.0307	0.3143	0.0355
440	0.2567	0.0376	0.2590	0.0310	0.2625	0.0364
445	0.2127	0.0391	0.2147	0.0313	0.2179	0.0384
450	0.1752	0.0411	0.1770	0.0317	0.1799	0.0415
455	0.1437	0.0435	0.1452	0.0321	0.1479	0.0453
460	0.1173	0.0466	0.1186	0.0327	0.1211	0.0492
465	0.0955	0.0507	0.0965	0.0335	0.0989	0.0529
470	0.0775	0.0558	0.0783	0.0345	0.0806	0.0564
475	0.0628	0.0622	0.0634	0.0359	0.0655	0.0602
480	0.0508	0.0704	0.0513	0.0377	0.0532	0.0652
485	0.0411	0.0804	0.0414	0.0399	0.0432	0.0727
490	0.0333	0.0928	0.0335	0.0428	0.0351	0.0835
495	0.0270	0.1078	0.0271	0.0464	0.0285	0.0979
500	0.0219	0.1259	0.0220	0.0509	0.0232	0.1154
505	0.0178	0.1475	0.0178	0.0564	0.0189	0.1343
510	0.0146	0.1730	0.0145	0.0631	0.0154	0.1529
515	0.0119	0.2029	0.0119	0.0710	0.0126	0.1719
520	0.0098	0.2378	0.0098	0.0805	0.0104	0.1979
525	0.0081	0.2782	0.0080	0.0916	0.0086	0.2513
530	0.0067	0.3247	0.0067	0.1045	0.0071	0.3762

Table 3: Certified values (5nm-steps) of the normalized corrected emission spectra of **BAM-F003** measured in ethanol at 25°C with an excitation wavelength (λ_{ex}) of 380 nm, an absorbance at the longest wavelength absorption maximum (380 nm) of 0.04, and 3 different spectral bandpasses of the emission monochromator of the spectrofluorometer SLM 8100.

λ_{em} in nm	Spectral bandpass 1 nm		Spectral bandpass 4 nm		Spectral bandpass 8 nm	
	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty
390	0.0015	0.3133	0.0016	0.1854	0.0019	0.1591
395	0.0043	0.1388	0.0046	0.0942	0.0052	0.1087
400	0.0113	0.0800	0.0119	0.0581	0.0131	0.0755
405	0.0266	0.0577	0.0280	0.0449	0.0301	0.0548
410	0.0572	0.0463	0.0598	0.0394	0.0634	0.0426
415	0.1129	0.0399	0.1169	0.0359	0.1221	0.0360
420	0.2031	0.0371	0.2083	0.0332	0.2147	0.0328
425	0.3321	0.0361	0.3377	0.0314	0.3436	0.0316
430	0.4915	0.0352	0.4966	0.0305	0.5000	0.0313
435	0.6599	0.0337	0.6637	0.0303	0.6638	0.0313
440	0.8100	0.0324	0.8127	0.0302	0.8105	0.0312
445	0.9208	0.0323	0.9225	0.0297	0.9200	0.0308
450	0.9834	0.0328	0.9842	0.0290	0.9829	0.0303
455	1.0000	0.0330	0.9998	0.0285	1.0000	0.0297
460	0.9786	0.0330	0.9775	0.0285	0.9787	0.0291
465	0.9288	0.0329	0.9270	0.0292	0.9288	0.0286
470	0.8593	0.0328	0.8573	0.0292	0.8595	0.0283
475	0.7780	0.0328	0.7763	0.0292	0.7788	0.0283
480	0.6918	0.0328	0.6905	0.0293	0.6934	0.0286
485	0.6059	0.0328	0.6050	0.0295	0.6082	0.0292
490	0.5242	0.0331	0.5237	0.0297	0.5270	0.0300
495	0.4491	0.0335	0.4488	0.0299	0.4520	0.0309
500	0.3820	0.0341	0.3817	0.0302	0.3845	0.0319
505	0.3229	0.0349	0.3226	0.0305	0.3250	0.0329
510	0.2718	0.0361	0.2715	0.0309	0.2734	0.0338
515	0.2279	0.0375	0.2275	0.0314	0.2291	0.0347
520	0.1906	0.0392	0.1902	0.0320	0.1914	0.0355
525	0.1591	0.0413	0.1586	0.0328	0.1596	0.0365
530	0.1325	0.0437	0.1321	0.0338	0.1329	0.0378
535	0.1102	0.0465	0.1098	0.0352	0.1105	0.0397
540	0.0916	0.0497	0.0913	0.0370	0.0918	0.0426
545	0.0761	0.0533	0.0758	0.0393	0.0763	0.0468
550	0.0632	0.0573	0.0630	0.0421	0.0634	0.0528
555	0.0525	0.0617	0.0523	0.0457	0.0527	0.0608
560	0.0437	0.0665	0.0435	0.0500	0.0439	0.0711
565	0.0363	0.0717	0.0362	0.0553	0.0366	0.0836
570	0.0303	0.0774	0.0302	0.0617	0.0305	0.0980

Table 4: Certified values (5nm-steps) of the normalized corrected emission spectra of **BAM-F004** measured in ethanol at 25°C with an excitation wavelength (λ_{ex}) of 420 nm, an absorbance at the longest wavelength absorption maximum (420 nm) of 0.04, and 3 different spectral bandpasses of the emission monochromator of the spectrofluorometer SLM 8100.

λ_{em} in nm	Spectral bandpass 1 nm		Spectral bandpass 4 nm		Spectral bandpass 8 nm	
	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty	norm. intensity	expanded rel. uncertainty
450	0.0027	0.1189	0.0029	0.1058	0.0030	0.0979
455	0.0058	0.1065	0.0063	0.0851	0.0064	0.0814
460	0.0121	0.0843	0.0129	0.0683	0.0133	0.0680
465	0.0239	0.0639	0.0253	0.0552	0.0261	0.0574
470	0.0447	0.0497	0.0467	0.0455	0.0481	0.0491
475	0.0785	0.0416	0.0812	0.0388	0.0833	0.0427
480	0.1296	0.0378	0.1330	0.0343	0.1357	0.0378
485	0.2015	0.0360	0.2055	0.0316	0.2087	0.0343
490	0.2956	0.0348	0.3002	0.0302	0.3035	0.0317
495	0.4099	0.0336	0.4150	0.0295	0.4181	0.0300
500	0.5384	0.0325	0.5434	0.0292	0.5457	0.0289
505	0.6702	0.0319	0.6746	0.0292	0.6758	0.0282
510	0.7922	0.0319	0.7953	0.0291	0.7955	0.0279
515	0.8918	0.0321	0.8934	0.0291	0.8927	0.0279
520	0.9601	0.0321	0.9606	0.0289	0.9596	0.0280
525	0.9945	0.0317	0.9945	0.0286	0.9939	0.0282
530	0.9982	0.0321	0.9984	0.0284	0.9988	0.0285
535	0.9788	0.0321	0.9795	0.0281	0.9810	0.0287
540	0.9445	0.0322	0.9458	0.0278	0.9483	0.0290
545	0.9025	0.0324	0.9042	0.0277	0.9071	0.0291
550	0.8571	0.0326	0.8589	0.0277	0.8617	0.0293
555	0.8100	0.0328	0.8118	0.0278	0.8142	0.0294
560	0.7615	0.0331	0.7633	0.0281	0.7654	0.0294
565	0.7114	0.0334	0.7133	0.0284	0.7155	0.0294
570	0.6600	0.0337	0.6621	0.0288	0.6645	0.0294
575	0.6081	0.0339	0.6103	0.0292	0.6132	0.0294
580	0.5566	0.0342	0.5590	0.0297	0.5622	0.0295
585	0.5070	0.0344	0.5093	0.0300	0.5127	0.0296
590	0.4599	0.0347	0.4621	0.0304	0.4655	0.0297
595	0.4162	0.0352	0.4181	0.0307	0.4212	0.0300
600	0.3760	0.0358	0.3775	0.0310	0.3803	0.0304
605	0.3392	0.0366	0.3403	0.0313	0.3429	0.0310
610	0.3058	0.0376	0.3065	0.0318	0.3088	0.0318
615	0.2754	0.0390	0.2758	0.0324	0.2778	0.0328
620	0.2477	0.0406	0.2478	0.0334	0.2498	0.0341
625	0.2225	0.0426	0.2224	0.0347	0.2244	0.0356
630	0.1996	0.0449	0.1994	0.0364	0.2013	0.0375
635	0.1788	0.0476	0.1784	0.0387	0.1804	0.0398
640	0.1599	0.0505	0.1594	0.0414	0.1615	0.0426
645	0.1429	0.0539	0.1423	0.0446	0.1444	0.0458
650	0.1275	0.0576	0.1268	0.0481	0.1289	0.0495
655	0.1136	0.0619	0.1129	0.0520	0.1150	0.0539
660	0.1012	0.0667	0.1005	0.0561	0.1024	0.0589
665	0.0901	0.0723	0.0893	0.0604	0.0912	0.0647
670	0.0802	0.0792	0.0794	0.0649	0.0811	0.0713
675	0.0714	0.0877	0.0706	0.0698	0.0721	0.0788
680	0.0636	0.0985	0.0627	0.0757	0.0641	0.0874
685	0.0566	0.1127	0.0558	0.0838	0.0570	0.0972
690	0.0505	0.1314	0.0496	0.0955	0.0507	0.1082
695	0.0451	0.1562	0.0442	0.1138	0.0450	0.1207
700	0.0402	0.1891	0.0394	0.1426	0.0401	0.1348

Table 5: Certified values (5nm-steps) of the normalized corrected emission spectra of **BAM-F005** measured in ethanol at 25°C with an excitation wavelength (λ_{ex}) of 530 nm, an absorbance at the longest wavelength absorption maximum (530 nm) of 0.04, and 3 different spectral bandpasses of the emission monochromator of the spectrofluorometer SLM 8100.

λ_{em} in nm	Spectral bandpass 1 nm		Spectral bandpass 4 m		Spectral bandpass 8 nm	
	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty	normalized intensity	expanded rel. uncertainty
560	0.0049	0.1809	0.0073	0.0934	0.0060	--
565	0.0105	0.0783	0.0127	0.0498	0.0124	0.0413
570	0.0208	0.0470	0.0228	0.0459	0.0236	0.0356
575	0.0381	0.0423	0.0396	0.0444	0.0419	0.0323
580	0.0650	0.0397	0.0655	0.0420	0.0700	0.0306
585	0.1044	0.0376	0.1055	0.0411	0.1106	0.0301
590	0.1591	0.0358	0.1613	0.0415	0.1670	0.0301
595	0.2323	0.0344	0.2353	0.0417	0.2420	0.0304
600	0.3261	0.0333	0.3309	0.0412	0.3374	0.0305
605	0.4401	0.0325	0.4440	0.0413	0.4519	0.0304
610	0.5693	0.0318	0.5738	0.0426	0.5798	0.0305
615	0.7028	0.0313	0.7065	0.0415	0.7103	0.0304
620	0.8253	0.0309	0.8248	0.0409	0.8292	0.0302
625	0.9216	0.0306	0.9217	0.0409	0.9227	0.0301
630	0.9811	0.0304	0.9770	0.0412	0.9808	0.0210
635	1.0000	0.0302	1.0000	0.0416	1.0000	0.0300
640	0.9819	0.0315	0.9786	0.0418	0.9831	0.0301
645	0.9349	0.0319	0.9296	0.0419	0.9375	0.0304
650	0.8691	0.0324	0.8613	0.0418	0.8727	0.0307
655	0.7938	0.0330	0.7896	0.0417	0.7979	0.0311
660	0.7163	0.0337	0.7153	0.0418	0.7204	0.0316
665	0.6412	0.0344	0.6398	0.0422	0.6454	0.0322
670	0.5715	0.0352	0.5712	0.0428	0.5758	0.0328
675	0.5084	0.0361	0.5108	0.0436	0.5128	0.0334
680	0.4522	0.0371	0.4540	0.0444	0.4564	0.0341
685	0.4025	0.0383	0.4051	0.0452	0.4063	0.0349
690	0.3585	0.0397	0.3571	0.0459	0.3617	0.0357
695	0.3194	0.0415	0.3193	0.0464	0.3219	0.0366
700	0.2843	0.0436	0.2864	0.0468	0.2862	0.0377
705	0.2526	0.0463	0.2523	0.0474	0.2541	0.0389
710	0.2237	0.0497	0.2244	0.0484	0.2252	0.0405
715	0.1973	0.0539	0.1995	0.0501	0.1991	0.0424
720	0.1734	0.0590	0.1753	0.0526	0.1756	0.0448
725	0.1518	0.0652	0.1509	0.0561	0.1544	0.0478
730	0.1326	0.0728	0.1343	0.0603	0.1355	0.0515
735	0.1157	0.0819	0.1169	0.0649	0.1186	0.0560
740	0.1009	0.0927	0.1008	0.0692	0.1036	0.0616
745	0.0881	0.1055	0.0913	0.0729	0.0903	0.0683
750	0.0771	0.1205	0.0786	0.0764	0.0787	0.0764
755	0.0676	0.1380	0.0682	0.0815	0.0685	0.0861
760	0.0596	0.1583	0.0592	0.0930	0.0596	0.0977
765	0.0528	0.1815	0.0519	0.1209	0.0519	0.1114
770	0.0470	0.2082	0.0439	0.1827	0.0452	0.1275

Description of the Calibration Kit *Spectral Fluorescence Standards*

- Five spectral fluorescence standards BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 ready-made from Sigma-Aldrich Production GmbH (former Fluka Production GmbH), which cover the spectral region of 300 nm to 770 nm as a set. The corresponding product numbers from Sigma-Aldrich are 72594, 23924, 88418, 71237, and 73189. These dyes are ready-made in such a way that addition of aliquots of 10 ml of ethanol to each of the bottled solid dyes yields dye solution with an absorbance (A) of 0.04 ± 0.02 at the longest wavelength absorption maximum. These solutions can be measured without additional dilution steps.
- Solvent ethanol
- Corrected emission spectra of BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005, see Fig. 1, and corresponding relative expanded uncertainties ($k = 2$) for the 3 spectral bandpasses x of the emission monochromator M of the fluorometer SLM 8100 used for certification with $x = 1, 4, \text{ and } 8$ nm, see Tables 1-5. These values that were measured within the linear range of the emission detection system of fluorometer SLM 8100 are corrected for the spectral responsivity of the emission channel. The expanded relative uncertainties listed in Tables 1-5 include contributions from the relative uncertainties of the calibration of the spectral responsivity of fluorometer SLM 8100 and measurement of fluorescence spectra of the fluorescence standards as well as the homogeneity and thermal stability of the bottled dyes as determined by the propagation of uncertainties according to the *Guide to the Expression of Uncertainty (GUM)* and *ISO Guide 35*, see *certification report*. The certification was performed according to *ISO Guide 35*.
- CD with the certificate files *BAM507Mx.CTF*, the data evaluation software *LINKCORR* developed by BAM with the WINDOWS surface *LINKCORRGUI* as well as instructions for the use of BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 and for *LINKCORR*. The files *BAM507Mx.CTF* contain the certified corrected emission spectra of BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 listed in Tables 1-5 in 1nm- steps and the corresponding expanded relative uncertainties in a format readable by *LINKCORR*.

Area of Application

Calibration of fluorescence instruments and determination of the relative spectral responsivity $s(\lambda)$ of fluorescence measuring systems in the spectral region of 300 nm to 770 nm. Knowledge of this quantity is the prerequisite for the correction of emission spectra for instrument-specific effects that distort measured spectra and are time-dependent due to aging of optical and opto-electronical instrument components [2,3,4]. Consideration of $s(\lambda)$ yields corrected instrument-independent emission spectra. A correction for $s(\lambda)$ is the prerequisite for the comparability of fluorescence data across instruments and for the comparability of fluorescence data of chromophores with non-matching emission spectra as is e.g. typically the case for samples and standards used for the determination of fluorescence quantum yields. Regular determination of $s(\lambda)$ provides a tool for the evaluation of the long-term

² Resch-Genger, U.; Hoffmann, K.; Nietfeld, W.; Engel, A.; Neukammer, J.; Nitschke, R.; Ebert, B.; Macdonald, R., *J. Fluoresc.* **2005**, *15*, 337-362.

³ Hollandt, J.; Taubert, R. D.; Seidel, J.; Resch-Genger, U.; Gugg-Helminger, A.; Pfeifer, D.; Monte, C., *J. Fluoresc.* **2005**, *15*, 301-313.

⁴ Resch-Genger, U.; Pfeifer, D.; Pilz, W.; Monte, C.; Hoffmann, A.; Spieles, M.; Rurack, K.; Hollandt, J.; Taubert, D.; Schönenberger, B.; Nording, P., *J. Fluoresc.* **2005**, *15*, 315-336.

stability of the emission channel of fluorescence instruments and enables—via correction for $s(\lambda)$ —the comparability of fluorescence data measured on the same instrument but over a long period of time.

The parameter and requirements for the use of BAM-F001 to BAM-F005 are described in *Measurement Conditions and Instructions for Use*.

Application of BAM-F001 - BAM-F005 and LINKCORR

For instructions on the use of BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 as well as for *LINKCORR*, see provided CD.

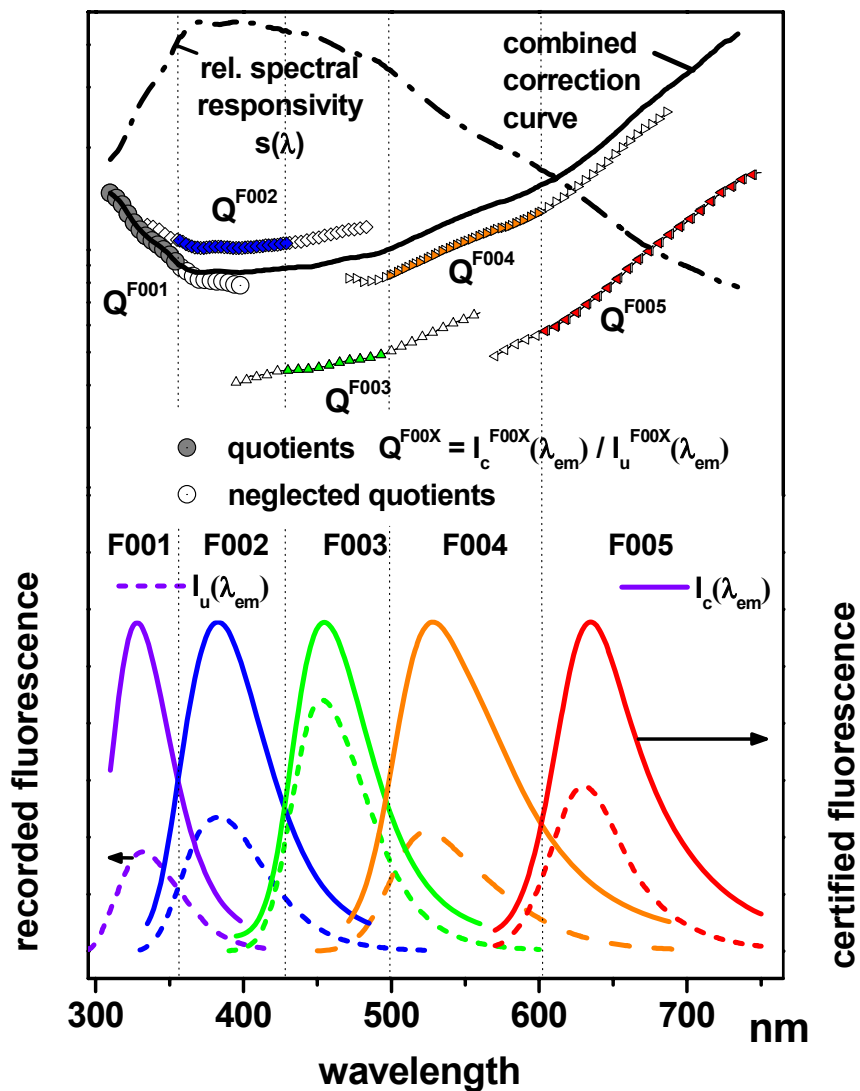


Figure 2: Determination of the relative spectral responsivity $s(\lambda)$ of a fluorescence instrument with the Calibration Kit *Spectral Fluorescence Standards* and *LINKCORR*. Bottom: Certified normalized corrected emission spectra (solid lines; $I_c(\lambda_{em})$) of the Kit components and uncorrected, i.e., instrument-dependent emission spectra (dashed lines; $I_u(\lambda_{em})$), measured with the instrument to be calibrated. Middle: Individual quotients $Q^{F00x} = I_c(\lambda_{em})/I_u(\lambda_{em})$, calculated with *LINKCORR* for the Kit components. 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 $\equiv 1/s(\lambda)$ (solid black line) calculated with *LINKCORR* from the statistically weighted Q^{F00x} as well as its reciprocal $s(\lambda)$ (black dash-dotted line).

The underlying principle of the determination of the relative spectral responsivity of fluorescence instruments with BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 and *LINKCORR* is illustrated in Figure 2. *LINKCORR* calculates the inverse relative spectral responsivity $1/s(\lambda)$ of the instrument to be calibrated (emission correction curve) from the certified corrected emission spectra ($I_C(\lambda_{em})$) and the uncorrected emission spectra of the Kit components ($I_U(\lambda_{em})$) measured with this instrument by division of the former by the latter. The resulting ratios Q^{F00x} ($Q^{F00x} = I_C(\lambda_{em})/I_U(\lambda_{em})$) of each dye are linked by a statistical weighting procedure implemented in *LINKCORR* to the combined or global emission correction curve $1/s(\lambda)$. This emission correction curve is the actual output of *LINKCORR*. Its reciprocal yields the relative spectral responsivity of the respective fluorescence instrument.

The relative uncertainty of the emission correction curve can be calculated from the relative uncertainties of the certified corrected emission spectra, (see Tables 1-5) and the relative uncertainties of repeated fluorescence measurements with these dyes according to the propagation of uncertainties, see *Guide to the Expression of Uncertainty of Measurements* (GUM) and *certification report*. The relative uncertainties of repeated fluorescence measurements with BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 have to be determined by the user.

Corrected emission spectra are obtained by multiplication of measured spectra by the emission correction curve provided by *LINKCORR*. It needs to be guaranteed that only intensity values belonging to similar wavelengths are multiplied. These corrected emission spectra are traceable to the *spectral radiance*.

Interlaboratory Comparison

The corrected emission spectra of BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 and the determination of the relative spectral responsivity of fluorescence instruments with the Kit dyes and *LINKCORR* have been tested in an interlaboratory comparison of the National Metrological Institutes active in the fluorescence area (National Institute of Standards and Technology NIST, USA; National Research Council NRC, Canada; Physikalisch-Technische Bundesanstalt PTB, Germany; Federal Institute for Materials Research and Testing BAM, Germany). In this interlaboratory comparison, the corrected emission spectra of BAM-F001 to BAM-F005 were measured at two different dye concentrations equalling absorbances of 0.04 und 0.08 at the excitation wavelength, respectively, and in two different measurement geometries ($0^\circ/90^\circ$ and $45^\circ/0^\circ$) with four different fluorescence instruments. The fluorescence instruments were characterized with physical transfer standards (PTS) employing different calibration procedures. For the control of the spectral correction with BAM-F001 to BAM-F005 and *LINKCORR*, the emission spectra of 3 test dyes (dye X, SRM 936a equalling quinine sulfate dihydrate, and dye Y) were corrected with a dye-based and a PTS-based correction curve, and the results were compared. The agreement of the corrected spectra was excellent in all cases. The data provided by NIST, NRC, and PTB are not used for certification of the corrected emission spectra of BAM-F001 to BAM-F005.

Recommended Calibration Intervals

Determination of the emission correction curve $1/s(\lambda)$ is recommended every six months. After each transport of a fluorescence instrument or interference with the emission channel like e.g. change of components such as monochromator gratings or detectors, recalibration of the emission channel is mandatory.

Storage Conditions of BAM-F001 - BAM-F005

Bottled ready-made BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 can be stored in the refrigerator at 4°C for 6 months without changes in their certified properties. Solutions of these dyes can be used for a week at maximum, if stored in the dark at 4°C in properly closed containers. Uptake of water has to be minimized to avoid spectral changes of the emission spectra of the Kit components and thus deviations from the certified property.

Tested Properties of BAM-F001 - BAM-F005

The following application-relevant properties of BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 were studied and partly included in the combined expanded relative uncertainty of the corrected emission spectra listed in Tables 1-5, see *certification report*:

- Homogeneity of the spectral profile of the emission spectra of the bottled ready-made Kit components (included)
- Thermal stability of the bottled ready-made Kit components (included)
- Photostability of the dye solutions upon excitation at the longest wavelength absorption maximum
- Influence of the temperature on the corrected emission spectra and on the anisotropy of the fluorescence in the temperature range (10-40)°C
- Influence of the excitation wavelength on the corrected emission spectra
- Influence of the dye concentration on the corrected emission spectra
- Purity of the solid dyes.

Measurement Conditions and Instructions for Use

Prerequisites for the determination of the spectral responsivity of a fluorescence instrument are the control and if necessary consideration of the wavelength accuracy of its emission channel and the operation of the detection system within its linear range.

The Kit components BAM-F001 to BAM-F005 are optimized for a measurement geometry of 0°/90°, 1cm cuvettes (quartz), an absorbance of 0.04 at the longest wavelength absorption maximum, excitation at the longest wavelength absorption maximum, and a temperature of 25°C. Under these conditions, the certification and the calculation of the expanded relative uncertainties (see Tables 1-5) were performed. The certified values are only valid for these conditions and the limits listed in Table 6.

Table 6: Recommended measurement conditions and limits.

measurement condition	region / limit
excitation wavelength λ_{ex}	$\lambda_{\text{ex}} \pm 2 \text{ nm}$
temperature T	$(25 \pm 2)^\circ\text{C}$
spectral bandpass of the emission monochromator of the instrument to be calibrated	1 nm bis 15 nm (certified emission spectra provided for spectral bandpasses of the emission monochromator of 1, 4, and 8 nm)
step width emission scan	(0.1; 0.2; 0.5; 1.0* ; 1.5; 2.0; 2.5; 5.0) nm

* value used for certification.

For the spectral correction of emission spectra with minimum uncertainty, it is necessary to determine the inverse relative spectral responsivity $1/s(\lambda)$ of the fluorescence instrument and to measure the emission spectra to be corrected under identical conditions. This implies e.g. identical optical and opto-electronical components in the emission channel as well as identical measurement geometry, spectral bandpass of the emission monochromator, detector settings, position of the emission polarizer etc. The dye solutions need to be measured in properly closed cells to be prevented from the uptake of water and solvent evaporation. Uptake of water can yield spectral deviations of the emission spectra from the certified values. To minimize the influence of the spectral bandpass of the emission monochromator on the shape of the emission spectra of BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005, it is recommended to use the corrected spectra of the Kit components, the values of which have been certified for a spectral bandpass closely resembling the spectral bandpass employed for the measurement of the dye spectra with the instrument to be calibrated. For the determination of the emission correction curve, all the Kit components need to be measured with the *same spectral bandpass*.

The corrected emission spectra that are obtained by multiplication of measured spectra by the emission correction curve provided by *LINKCORR* are traceable to the *spectral radiance*. For the *determination of fluorescence quantum yields*, the corrected emission spectra have to be multiplied by the wavelength prior to integration on a wavelength scale, as the fluorescence quantum yield is the ratio of the number of emitted to absorbed photons and not a ratio of radiated fluxes or powers [4].

If dye-based emission correction curves are used as the basis for the determination of the *anisotropy (r) of the fluorescence* of a chromophore [4], it needs to be considered that the emission of the Kit components is only nearly isotropic ($r = 0.010\text{-}0.035$ at 25°C) [4]. The influence of the anisotropy of the fluorescence of BAM-F001 to BAM-F005 on the resulting emission correction curves for emission polarizers set to 0° and 90° has not been tested by BAM. As a rough estimate, only anisotropies can be determined those exceed 0.05.

If BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 are applied under measurement conditions deviating from the conditions used for certification that are recommended for use of the Calibration Kit, e.g. different measurement geometries, excitation wavelengths, temperatures or concentrations, this could lead to spectral deviations from the corrected emission spectra listed in Tables 1-5 and/or to an increase of the relative uncertainties, see *certification report*. As an example, excitation wavelengths and excitation regimes are listed in Table 7, where the spectral deviations of the corrected emission spectra of BAM-F001 - BAM-F005 from

the certified spectra of the Kit components are at maximum 2% within the emission regimes of the dyes that are typically used for the determination of $1/s(\lambda)$ with BAM-F001 - BAM-F005 and *LINKCORR*. In principle, such effects can be taken into account by an increase of the relative uncertainties of the corrected emission spectra of the dyes.

Tabelle 7: Recommended excitation wavelength for BAM-F001 to BAM-F005 as well as excitation wavelengths and excitation wavelength regions where spectral deviations from the certified corrected emission spectra listed in Tables 1-5 of at maximum 2% are observed.

standard	recommended excitation wavelength λ_{ex} *	excitation wavelength and wavelength regions for limited use**
BAM-F001	280 nm	278 nm – 282 nm
BAM-F002	315 nm	280 nm – 335 nm
BAM-F003	380 nm	254 nm; 340 nm – 410 nm
BAM-F004	420 nm	254 nm; 265 nm; 355 nm – 488 nm
BAM-F005	550 nm	364 nm; 405 nm – 590 nm

*excitation wavelength set at the fluorescence instrument; the certification of the corrected emission spectra has been performed for this excitation wavelength; **excitation wavelength and wavelength regions differing from λ_{ex} for which the emission profiles deviate at maximum by 2% from normalized certified emission spectra abweichend. These deviations have been derived from the quotients q of the corrected emission spectra $q = I_C(\lambda_{ex} = x) / I_C(\lambda_{ex} = \text{“certification condition”})$ determined for a spectral bandpass of the emission monochromator of 4 nm. For spectral bandpasses < 4 nm, these statements cannot be automatically accepted.

The photostability studies with BAM-F001 - BAM-F005 were performed at the excitation wavelengths employed for certification, see *certification report*. If excitation wavelengths are used that differ from the certification conditions and/or if very intense excitation light sources like e.g. lasers are employed, the photostability of the dyes needs to be controlled under these conditions by the user.

The emission correction curve $1/s(\lambda)$ or its reciprocal, the relative spectral responsivity $s(\lambda)$ can be used for the *control of the long-term stability of the emission channel of a fluorescence instrument*. This implies always measurement of these quantities with identical instrument settings. Temporal changes of the wavelength-dependent correction factors determined with BAM-F001 to BAM-F005 and *LINKCORR* can be caused not only by aging-induced changes of the emission channel, but also by fluctuations of the spectral irradiance reaching the sample and by changes of the reference channel (for instance exchange of optical and/or optoelectronic components and/or aging of these components). Changes in the reference channel affect always the measured emission signal in the case of fluorescence instruments which automatically record quotients of the signals of the emission channel and the reference channel (“ratio mode”) and do not provide separate signals for both channels. For the evaluation of the long-term stability of such ratio mode-type instruments, accordingly, the comparison of the (at similar wavelengths) *normalized emission correction curves* or the *normalized relative spectral responsivities* is strongly recommended as changes in the emission channel influence the spectral shape of the emission correction curve and $s(\lambda)$ whereas changes in the reference channel do not influence the normalized emission correction curves or the normalized relative spectral responsivities.

The evaluation of *temporal changes of the wavelength-dependent sensitivity* of the emission channel by comparison of non-normalized emission correction curves or non-normalized relative spectral responsivities requires an emission channel the

signal of which is independently recorded from that of the reference channel and knowledge of the spectral irradiance at sample position. This is rarely fulfilled.

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