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Evaluation of kits for RNA determination using the Eppendorf BioSpectrometer® fluorescence

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Abstract

Different kits for RNA quantification, by the vendors Promega® (QuantiFluor®) and Life Technologies® (Quant-iT™), are evaluated. For the purpose of optimizing the measurement process, various regression analysis methods are employed which are pre-programmed on the Eppendorf BioSpectrometer fluorescence.
A further aspect to be demonstrated in this context is the optimization of sample quantification by simple adjustment of the regression analysis.
Use of the UVette® offers the possibility of reducing the final volume required for measurement from 2000 µL (Quant-iT™) or 1000 µL (QuantiFluor®), respectively, to 100 µL, thus saving considerable amounts of reagent in both kits.

Introduction

Nucleic acid concentrations are typically determined by measuring light absorption in UV-VIS spectrometers. The absorbance is measured at 260 nm and the concentration is determined using a sample-specific conversion factor. Qualitative analysis of the sample is conducted by additional measurements at 230, 280 and 320 nm, which are able to detect contamination by proteins and other organic materials, as well as interfering particles [1]. However, one major disadvantage of photometric quantification is the fact that this method is not very sensitive and relatively large amounts of sample are required for this method. Distinctly smaller nucleic acid concentrations can be measured using fluorescence detection [2]. Here, the target molecule is not detected directly but rather indirectly via a fluorescent dye. Determination of sample concentration is conducted using a previously generated standard curve. In addition to the increased sensitivity, this measurement technique is usually also more specific due to the fact that the fluorophores are only able to emit fluorescent light once they have come into contact with their target molecule (figure 1). The fluorophores bind to certain biomolecules with high specificity; e.g. one fluorophore binds exclusively to dsDNA whereas another will bind only to RNA.

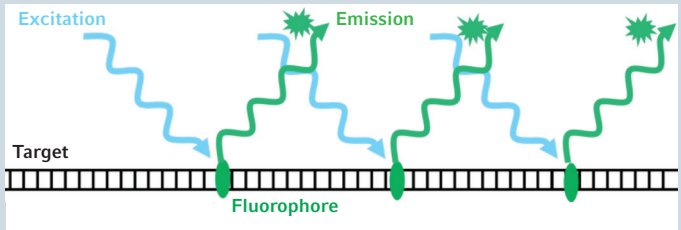

Figure 1: Principle of fluorescence measurement of nucleic acids

The present article will evaluate the applicability of detection kits by the companies Promega and Life Technologies using the Eppendorf BioSpectrometer fluorescence. Detection methods for RNA quantification are selected. Both kits are evaluated with respect to accuracy and detection limit, thereby explaining in detail how the respective method parameters are programmed on the Eppendorf BioSpectrometer fluorescence, as well as the actual measurement process.

Materials and Methods

Materials:

Kits: QuantiFluor® RNA System (Promega), Quant-iT™ RiboGreen® RNA Assay Kit (Life Technologies); 1 x TE buffer, total RNA from human liver (Ambion®, # 7960), water (molecular biology grade).

Instruments: Eppendorf BioSpectrometer® fluorescence, Eppendorf Research® pipettes

Cuvettes, vessels: UVette®, Eppendorf Safe-Lock tubes.

Process:

All standard concentrations are programmed into the Eppendorf BioSpectrometer fluorescence in accordance with the protocols provided by the respective manufacturers. The nucleic acid standards used are also provided in the kits.

Three replicates are prepared and measured for each standard concentration. All measurements are performed in the UVette in a final volume of 100 µL. Standards are measured starting from the lowest concentration, continuing to the highest concentration. One series of measurements is consistently inserted into the cuvette shaft facing the same direction. The cuvette is rinsed with 200 µL 1 x TE buffer between measurements.

Detailed measurement protocol:

Prior to measurement all components should be warmed to room temperature. For both kits the "high concentration" area is selected.

Preparation of the 1 x TE working buffer: The kits contain a 20 x TE buffer which is diluted in water accordingly.

For preparation of the reagent solution, the fluorescent dye provided in the kit needs to be diluted 1:200 with 1 x TE working buffer.

Preparation of RNA stock solution: The instructions provided in the kit manual are followed step by step.

It is recommended to validate the concentration of the RNA stock solution prepared for the standard curve by photometric means prior to generating the individual standards. To this end, the respective concentrations for the kits by both vendors are shown in table 1. The measurements are performed in a UVette with a 10 mm light path and a measurement volume of 100 µL.

Table 1: Concentrations of the stock solutions

Kit	Absorbance of the stock solution at 260 nm	Concentration of the stock solution [µg/mL]
Life Technologies: Quant-iT	0.05	2
Promega: Quantifluor	0.125	5

In addition to the fluorimetric measurement methods, the Eppendorf BioSpectrometer fluorescence is equipped with a complete spectrometer module which is able to perform the control measurements (figure 2).

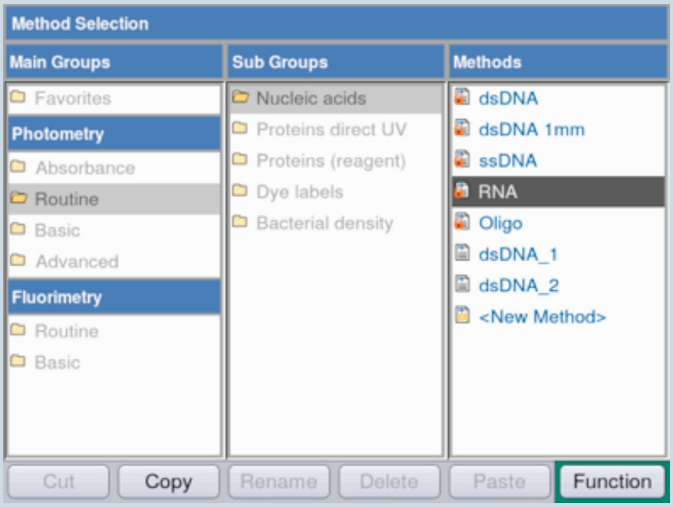

Figure 2: Selection of the RNA method for the purpose of verifying the RNA stock solution via absorbance measurements. Photometric as well as fluorimetric methods are available on the Eppendorf BioSpectrometer fluorescence.

Following method selection, the parameters may be verified and/or changed if required (figure 3).

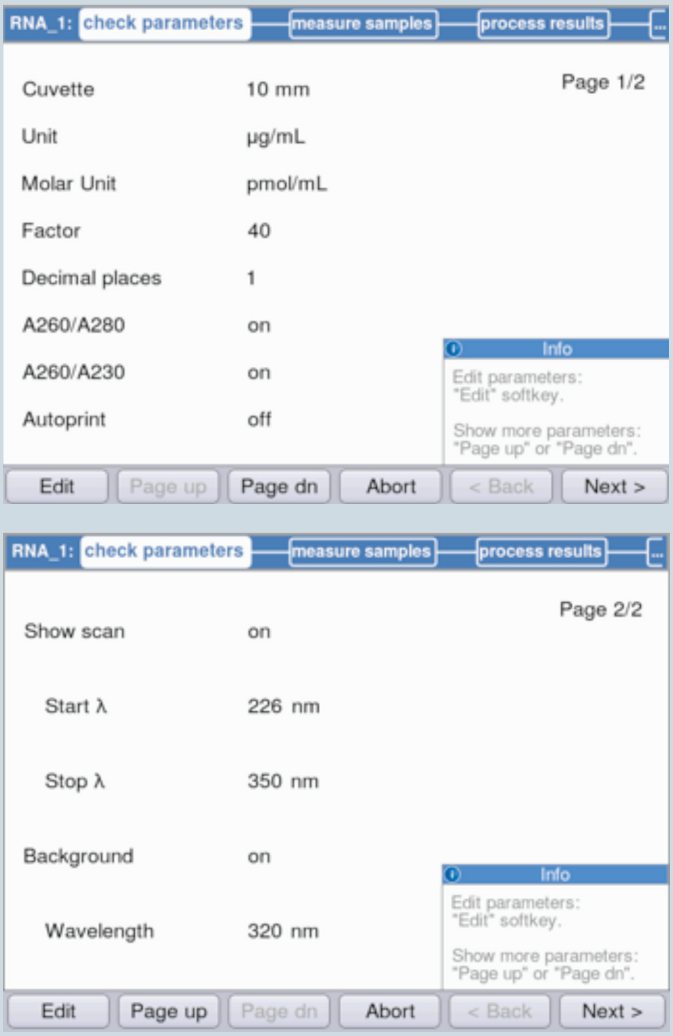

Figure 3: Verification of parameters prior to absorbance measurement.

The measured RNA curve was to display an absorbance maximum at a wavelength of 260 nm. An example of the result is shown in figure 4.

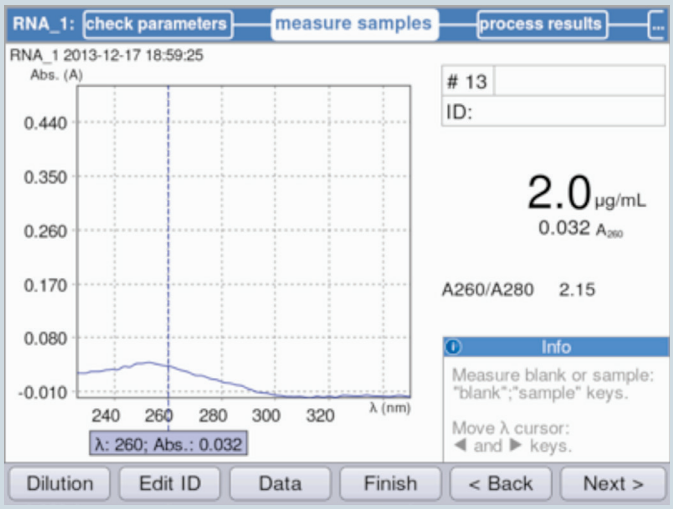

Figure 4: Calculation of the RNA concentration of the stock solution for the purpose of calculating the individual standards (example: Quant-iT Kit by Life Technologies).

Standards and samples are prepared in parallel: 50 µL of each standard and sample solution are pipetted into each of three Eppendorf Safe-Lock tubes. 50 µL of reagent solution are added to each tube, mixed well, and incubated for 5 minutes at room temperature. Following transfer of the reactions to an UVette, the fluorescence of the individual standards, followed by the samples, is measured in the Eppendorf BioSpectrometer fluorescence (excitation: 470 nm/emission: 520 nm).
Table 2 lists all standard and sample concentrations for both kits, by Life Technologies and Promega, respectively.

Table 2: Standards and samples to be measured with the respective RNA detection kits

RNA standards Quant-iT/Life Technologies RNA detection kit [ng/mL]	Sample concentration (liver RNA) [ng/mL]	RNA-standards Quantifluor/Promega RNA detection kit [ng/mL]	Sample concentration (liver RNA) [ng/mL]
0	20	0	20
20	40	39	40
100	60	78	60
500	200	156	200
1000	400	313	400
	800	625	800
	1000	1250	1000
		2500	

The standard concentrations are selected in accordance with the instructions provided by the respective kit manuals. The same sample concentrations are selected for both kits in order to ensure identical conditions with respect to accuracy, recovery and lower limit of detection.

Programming of standards on the instrument:
For the measurements, the method RiboGreen is selected under Fluorimetry/Routine/Nucleic acids/RiboGreen (figure 5).
The RNA samples (see table 2) are to be analyzed by linear regression as well as quadratic regression.

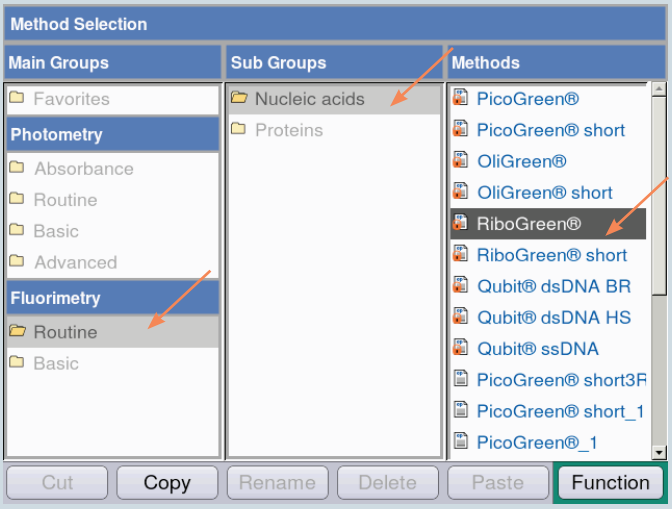

Figure 5: Method selection

Following the initiation of the method, the parameters for the measurement of the standards need to be determined. Depending on the kit, either 5 or 8 standards, with 3 replicates each, are programmed.

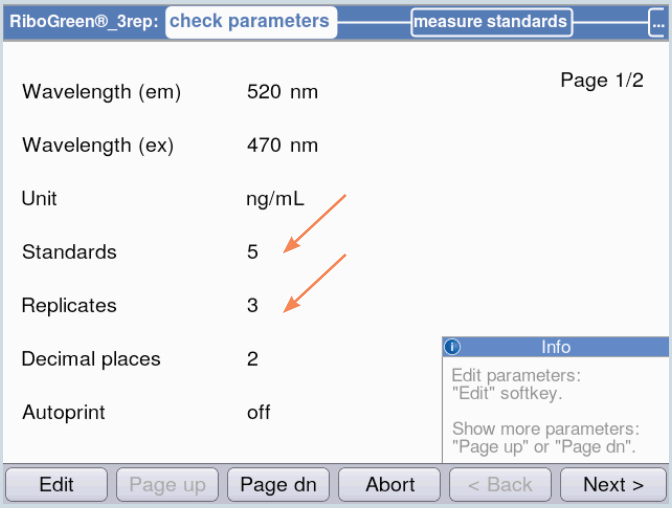

Figure 6: Establishing standards and replicates

"Next >" will guide the user to the area "measure standards" (figure 6) where the standards are measured in succession from lowest to highest concentration (figure 7). The standard curve is first analyzed via linear regression, followed by quadratical regression.
Generally, the Eppendorf BioSpectrometer fluorescence offers the option of adjusting the regression analysis during generation of the standard curve through "curve fit". Five possible regression analysis options are available: "linear interpolation", "linear regression", "quadratic regression", "cubic regression" and "spline interpolation".

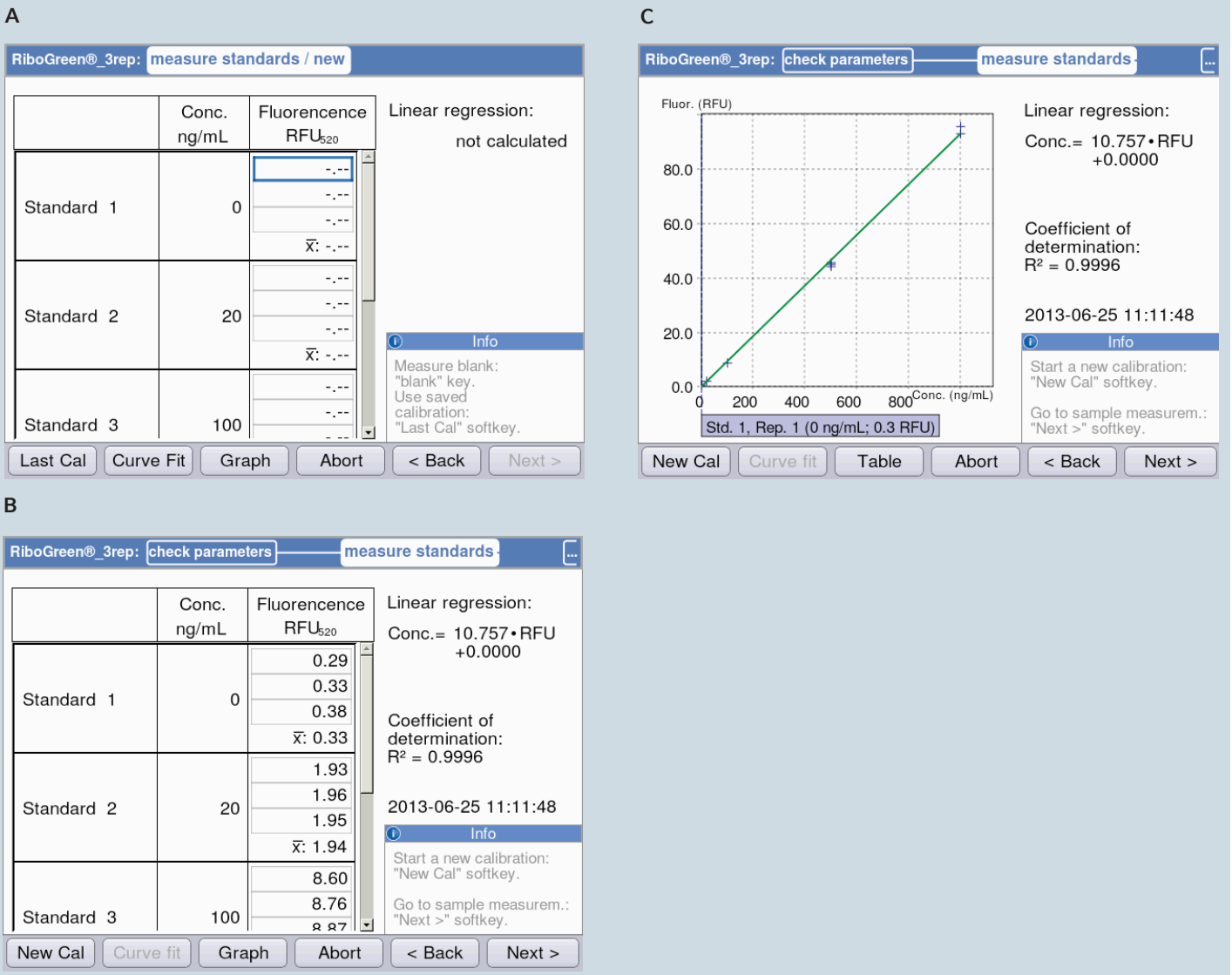

Figure 7: Measurement of the standard curve with the example of the RNA quantification kit "Quant-iT" (Life Technologies). Regression accuracy is displayed during measurement (R^2 value).
A) Beginning of measurement of the standard curve.
B) Table format display of the standard curve.
C) Graphic plot of the standard curve.

Results and discussion

First, accuracies of the standard curves were evaluated for both kits. Curve analysis was carried out *via* linear as well as quadratical regression. Table 3 lists the relative fluorescence units (RFU) measured for the RNA standards from the kit by Life Technologies. Figure 8 shows the relative curves.

Table 3: RNA standard curves Life Technologies

Concentration (ng/mL)	0	20	100	500	1000
	0.29	1.93	8.60	44.85	92.88
	0.33	1.96	8.76	44,07	93.05
RFU	0.38	1.95	8.87	45.48	95.52
Average (RFU)	0.33	1.94	8.74	44.80	93.82
SD (RFU)	0.05	0.01	0.14	0.71	1.48
CV (%)	/	0.75	1.60	1.58	1.58

As evident from table 3, all respective measured replicates are in good agreement.

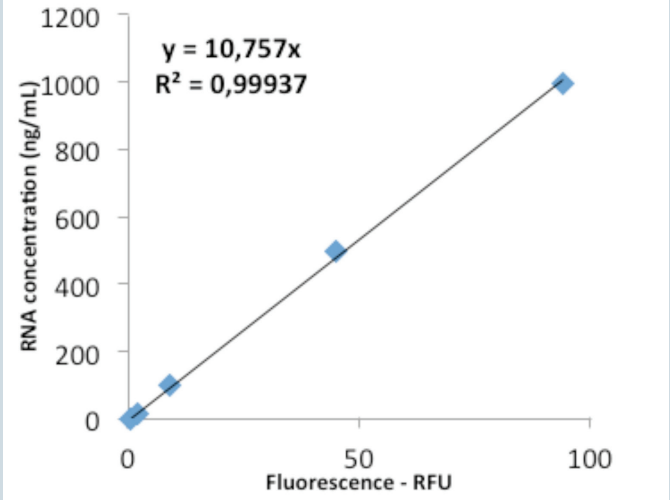

Figure 8a: Standard curve "Life Technologies" – evaluation via linear regression.

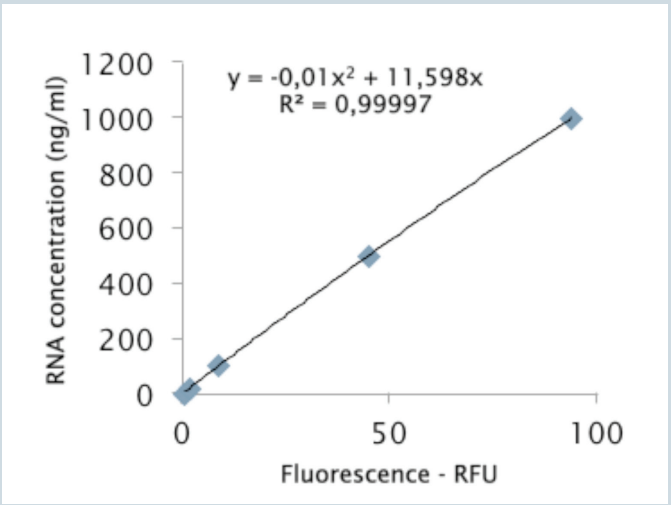

Figure 8b: Analysis via quadratic regression.

The R^2 value in figure 8a highlights the fact that the standard curve clearly follows a linear regression. A slightly improved agreement can be obtained with quadratic regression as shown in figure 8b. It was subsequently tested whether quadratic regression would indeed yield more precise sample determination. The RNA samples (see table 2) were analyzed via linear regression as well as quadratic regression, and their relative deviations from the target value were compared. The results are shown in tables 4a and 4b, respectively.

Table 4a: Analysis of the RNA samples via linear regression – Life Technologies

Nominal concentration (ng/mL)	20	40	200	400	800	1000
Calculated concentration (ng/mL)	21	41	194	395	806	1011
	22	43	193	397	800	992
	22	40	192	390	803	982
Average	22	41	193	394	803	995
SD	0	1	1	3	3	15
CV (%)	1.89	3.01	0.68	0.87	0.35	1.49

Table 4b: Analysis of the RNA samples vial quadratic regression – Life Technologies

Nominal concentration (ng/mL)	20	40	200	400	800	1000
Calculated concentration (ng/mL)	23	44	206	413	813	1002
	24	46	205	414	808	984
	24	43	203	408	810	975
Average	24	45	205	412	810	987
SD	0	1	1	3	3	13
CV (%)	1.88	3.00	0.67	0.84	0.32	1.36

According to tables 4a and 4b, the results obtained with quadratic and linear regression analyses are similar. For the lower concentration range (20–40 ng/mL) values determined by linear regression are in fact closer to the nominal value. Table 5 shows the concentrations calculated with the help of both evaluation methods. The percent deviation of the measured concentrations in relation to their respective nominal values is shown.

Table 5: Deviation of target concentration from nominal concentration – Life Technologies

Nominal concentration [ng/mL]	Measured concentration – linear regression [ng/mL]	Deviation [%]	Measured concentration – quadratic regression [ng/mL]	Deviation [%]
20	22	10	24	20
40	41	2.5	45	12.5
200	193	3.5	205	2.5
400	394	1.5	412	3
800	803	0.4	810	1.3
1000	995	0.5	987	1.3

The RNA concentrations shown in table 2 were also measured using the Promega RNA kit (QuantiFluor™).

Table 6 shows the results obtained for the RNA standards provided in the Promega kit.

Table 6: RNA standard curve Promega

Concentration (ng/mL)	0	39	78	156	313	625	1250	2500
	0.83	1.54	2.48	4.59	10.08	21.97	47.21	107.47
	0.85	1.55	2.62	4.66	9.99	20.64	48.24	104.32
RFU	0.78	1.55	2.52	4.77	10.13	22.35	47.83	101.13
Average	0.82	1.55	2.54	4.67	10.07	21.65	47.76	104.31
SD (RFU)	0.04	0.01	0.07	0.09	0.07	0.90	0.52	3.17
CV (%)	/	0.36	2.89	1.96	0.73	4.16	1.09	3.04

Similar to the Life Technologies kit, the individual repeats are in good agreement. The respective graphs with linear and quadratical regression are depicted in figures 9a and 9b, respectively.

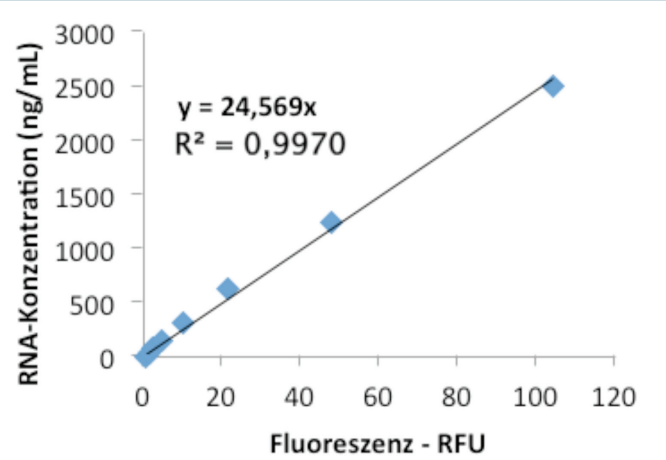

Figure 9a: Standard curve "Promega" – evaluation via linear regression.

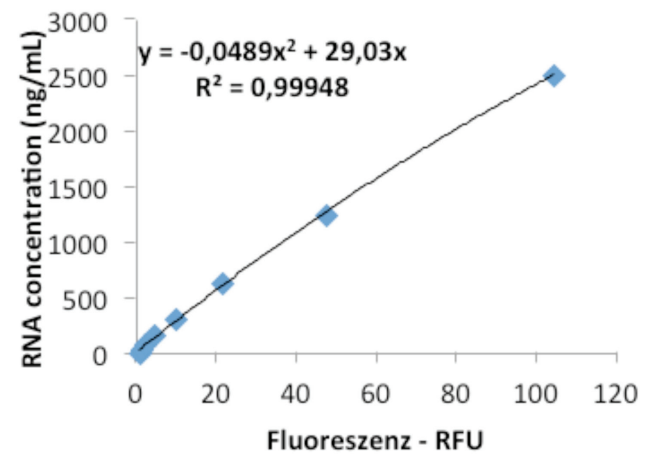

Figure 9b: Evaluation via quadratic regression.

The Promega kit showed considerably higher precision (R^2) of the standard curve when quadratic regression was employed as compared to linear regression. Subsequent investigations were initiated in order to test whether a change in standard curve regression could yield improved precision during sample analysis. Tables 7a and 7b show analysis via linear and quadratic regression, respectively.

Table 7a: Analysis of the RNA samples via linear regression – Promega

Nominal concentration (ng/mL)	20	40	200	400	800	1000
Calculated concentration (ng/mL)	28	41	153	328	723	914
	29	41	157	324	703	933
	29	39	158	337	720	925
Average	29	40	156	330	715	924
SD	1	1	3	6	11	10
CV (%)	3.42	3.04	1.80	1.94	1.51	1.06

Table 7b: Analysis of the RNA samples via quadratic regression – Promega

Nominal concentration (ng/mL)	20	40	200	400	800	1000
Calculated concentration (ng/mL)	33	49	181	383	820	1022
	35	49	186	378	798	1043
	35	46	187	392	816	1034
Average	34	48	184	384	812	1033
SD	1	1	3	7	12	10
CV (%)	3.42	3.03	1.78	1.90	1.43	0.99

As evident from tables 7a and 7b, more accurate sample determination for the Promega kit can be achieved when the standard curve is analyzed via quadratic regression in the Eppendorf BioSpectrometer fluorescence. Whereas the measured concentration in the lower concentration range (20–40 ng/mL) is closer to the nominal value when analyzed with linear regression, the values across the entire range of concentrations (> 1000 ng/mL) are more precise when quadratic regression is employed. Table 8 shows an overview of measured RNA concentrations determined by both evaluation methods, as well as their respective percent deviation from the nominal value.

Table 8: Deviation of target concentration from nominal concentration – Promega Kit

Nominal concentration [ng/mL]	Measured concentration – linear regression [ng/mL]	Deviation [%]	Measured concentration – quadratic regression [ng/mL]	Deviation [%]
20	29	45	33	65
40	40	0	49	22.5
200	156	22	184	8
400	330	17.5	384	4
800	715	10.6	812	1.5
1000	924	7.6	1033	3.3

For the Promega kit, sample replicates also show good agreement. Therefore, optimization for sample quantification needs to occur at the level of analysis rather than at the level of sample preparation.

Conclusion

Evaluation of results

Both RNA detection kits, by Promega as well as by Life Technologies, are suitable for use on the Eppendorf BioSpectrometer fluorescence. It is to be noted that the standard curve prepared from the kit by Life Technologies may be generated by linear as well as by quadratic regression, as evident from subsequent sample analyses. However, when analysis was carried out via linear regression or the standard curve, rather than by quadratic regression, for the RNA kit by Promega, the sample values deviated considerably from their nominal value. This observation highlighted the advantage of the Eppendorf BioSpectrometer, which is capable of adjusting the regression analysis to the curve in order to capture the actual shape of the standard curve accurately.

Furthermore, the kit by Life Technologies showed higher precision than the kit by Promega in the concentration range between 20 and 40 ng/mL. Table 9 shows an overview of RNA sample quantification performed with both kits. Representative examples for both kits show the evaluations obtained by quadratic regression.

Table 9: Evaluation of RNA samples via Quant-iT™ and QuantiFluor RNA determination kits (standard analysis via quadratic regression).

Nominal concentration [ng/mL]	Measured concentration – Life Technologies [ng/mL]	Deviation [%]	Measured concentration – Promega [ng/mL]	Deviation [%]
20	24	20	33	65
40	45	12.5	49	22.5
200	205	2.5	184	8
400	412	3	384	4
800	810	1.3	812	1.5
1000	987	1.3	1033	3.3

Operation on the Eppendorf BioSpectrometer fluorescence
The kit by Life Technologies is somewhat easier to program due to the smaller number of standards. In theory, fewer standards would most likely suffice for evaluation purposes. As previously shown for the kit for fluorimetric determination of dsDNA by Life Technologies, a reduction of the number of standards to 2 is generally possible [2]. As always, care should be taken to limit exposure of the samples and standards to light, as light exposure may cause strong measurement deviations (photo bleaching effect). For this reason, amber tubes may be useful during the preparation of standards and samples. In addition to the choice between different regression analysis methods for standard curves, the Eppendorf BioSpectrometer fluorescence offers the additional advantage that all pertinent measurement parameters are pre-programmed for the respective methods. Sample concentrations are thus directly converted and displayed in accordance with the programmed standard curve. Furthermore, the Eppendorf BioSpectrometer fluorescence is simultaneously a fully functional UV-VIS spectrometer. Since the stock solution for the standard curve is verified photometrically, all measurements may be comfortably carried out on one instrument.

Reduction of measurement volume:
Use of the UVette allowed reductions of the volume required for measurement of samples and standards to 100 µL. As a result, up to 4000 measurements could be performed at the cuvette scale for both kits. The standard number of quantifications for the Quant-iT kit and the QuantiFluor assay are 200 and 400, respectively. Thus, the combination of the Eppendorf BioSpectrometer and the UVette helps save money, as considerably lower amounts of reagents are needed than with the use of standard cuvettes.

Literature

[1] Gallagher, Sean R. (2001), Quantification of DNA and RNA with Absorption and Fluorescence Spectroscopy, Current Protocols in Cell Biology, Appendix 3D
[2] Armbrecht M, Gloe J, Goemann W (2013) – Determination of nucleic acid concentrations using fluorescent dyes in the Eppendorf BioSpectrometer® fluorescence, Eppendorf Application Note 271

Ordering information

Name	Order no. international	Order no. North America
Eppendorf BioSpectrometer® fluorescence – 230 V/50–60 Hz, electrical plug Europe, additional electrical connection variants available – 120 V/50–60 Hz, electrical plug North America	 6137 000.006 6137 000.014	 6137000014
Eppendorf BioSpectrometer® fluorescence reference filter set Filter set for verification of photometric accuracy and wavelength accuracy (in accordance with NIST®) as well as for verification of fluorimetric precision (accidental measurement deviation) and linearity.	6137 928.009	6137928009
UVette® 220 nm–1600 nm Original Eppendorf plastic cuvette, individually packaged, certified RNase-, DNA- and protein free, 80 pcs.	0030 106.300	952010051
UVette® routine pack 220 nm–1600 nm Eppendorf Quality purity, re-sealable box, 200 pcs.	0030 106.318	952010069
Cuvette rack for for 16 cuvettes	4308 078.006	940001102

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