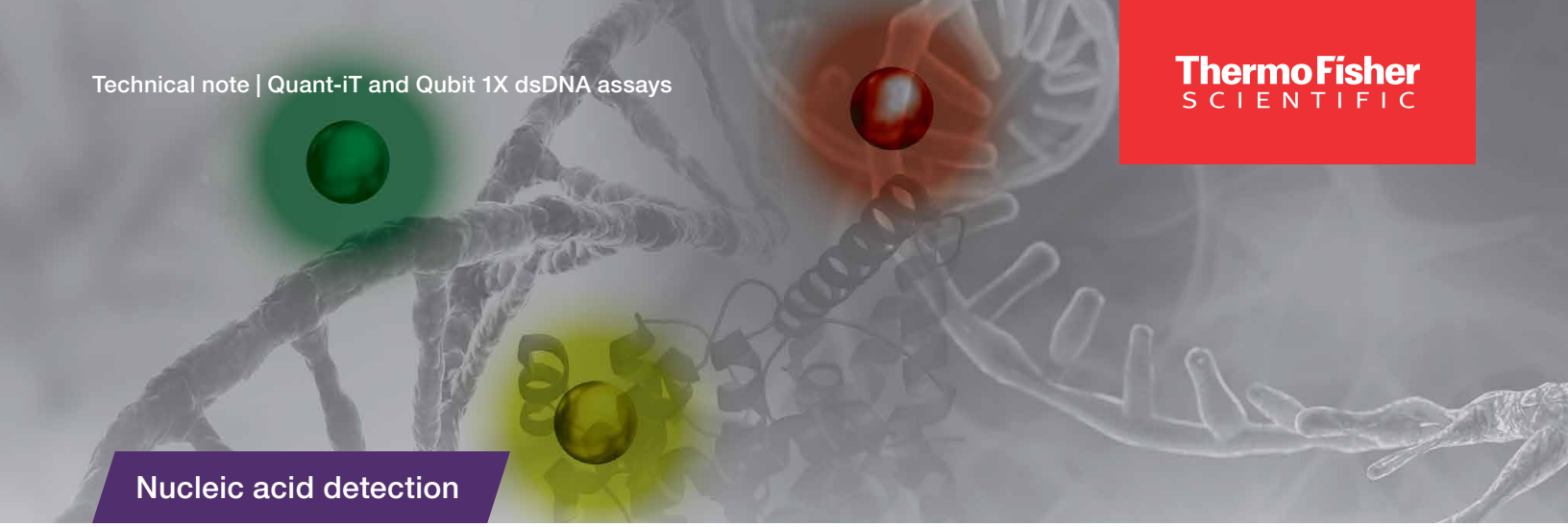

Nucleic acid detection

Comparison of accuracy and precision of Quant-iT and Qubit dsDNA quantification assays

Summary

- Quant-iT and Qubit assays offer best-in-class quantification of nucleic acids, with solutions for low, medium, and high throughputs, and automated laboratory settings.
- Choices in microplate reader settings can have large impacts on otherwise properly prepared assay plates. Signal-to-background ratio, Z-score, and coefficient of variation (CV) are helpful parameters to establish ideal settings.
- Key recommendations include using a top-read and a 12 nm bandwidth with an excitation wavelength of 495–505 nm for Quant-iT 1X dsDNA HS Assays and 505–515 nm for Quant-iT 1X dsDNA BR Assays, with a corresponding +30 nm emission setting.
- Quant-iT assays require optimization of data analysis to ensure optimal results; curve-fitting with background correction substantially improves accuracy.

Introduction

Fluorescence-based quantification of nucleic acids is an important step in many molecular biology experiments, such as next-generation sequencing (NGS). This technique provides sensitive and target-specific quantification of DNA and RNA using as little as 1 µL of sample. Invitrogen™ Qubit™ fluorometers and assays enable rapid and reliable fluorescence-based quantification, with easy-to-use touchscreen menus, automated calculations, and flexible export options.

While Qubit instruments and assays provide simple nucleic acid quantification for low and medium throughputs, Invitrogen™ Quant-iT™ assays are formulated to provide accurate and precise quantification of nucleic acids for higher-throughput laboratories using a fluorescence microplate reader and standard microplates. Because of differences in assay kit format, instrumentation, and data processing, results can vary between Qubit and Quant-iT assays. Here we demonstrate the performance of the Invitrogen™ Quant-iT 1X dsDNA High Sensitivity (HS) and 1X dsDNA Broad Range (BR) Assays and define methods to achieve results that are comparable with performance from Qubit fluorometers.

Materials and methods

Qubit and Quant-iT 1X dsDNA HS and BR Assays were compared for performance across the core quantification ranges. DNA sample was added to wells of a 96-well plate in quadruplicate at 2 µL and 10 µL volumes (Figure 1). Then, the provided ready-to-use working solution was added for a final reaction volume of 200 µL; the plates were then thoroughly mixed using a plate mixer. Plating the small-volume DNA sample first, followed by the larger-volume working solution may help to effectively and adequately mix the sample.

	1	2	3	4	5	6	7	8	9	10	11	12
2 µL samples												
A	0 ng	0 ng	0.1 ng	0.2 ng	0.5 ng	1 ng	4 ng	8 ng	12 ng	20 ng	50 ng	100 ng
B	0 ng	0 ng	0.1 ng	0.2 ng	0.5 ng	1 ng	4 ng	8 ng	12 ng	20 ng	50 ng	100 ng
C	0 ng	0 ng	0.1 ng	0.2 ng	0.5 ng	1 ng	4 ng	8 ng	12 ng	20 ng	50 ng	100 ng
D	0 ng	0 ng	0.1 ng	0.2 ng	0.5 ng	1 ng	4 ng	8 ng	12 ng	20 ng	50 ng	100 ng
10 µL samples												
E	0 ng (std. 1)	0 ng (std. 1)	0.125 ng	0.25 ng	1.0 ng	5 ng (std. 2)	10 ng (std. 3)	20 ng (std. 4)	40 ng (std. 5)	60 ng (std. 6)	80 ng (std. 7)	100 ng (std. 8)
F	0 ng (std. 1)	0 ng (std. 1)	0.125 ng	0.25 ng	1.0 ng	5 ng (std. 2)	10 ng (std. 3)	20 ng (std. 4)	40 ng (std. 5)	60 ng (std. 6)	80 ng (std. 7)	100 ng (std. 8)
G	0 ng (std. 1)	0 ng (std. 1)	0.125 ng	0.25 ng	1.0 ng	5 ng (std. 2)	10 ng (std. 3)	20 ng (std. 4)	40 ng (std. 5)	60 ng (std. 6)	80 ng (std. 7)	100 ng (std. 8)
H	0 ng (std. 1)	0 ng (std. 1)	0.125 ng	0.25 ng	1.0 ng	5 ng (std. 2)	10 ng (std. 3)	20 ng (std. 4)	40 ng (std. 5)	60 ng (std. 6)	80 ng (std. 7)	100 ng (std. 8)

Figure 1. Microplate reader setup for a Quant-iT 1X dsDNA HS Assay. Replicates of four were added to a 96-well plate using 2 µL volumes for rows A through D (beige) and 10 µL volumes for rows E through H (blue). Prediluted standards provided with the Quant-iT assay were used to generate a standard curve.

invitrogen

Quant-iT assay analysis was performed at room temperature using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader (Cat. No. VLB000GD0). Using the setup parameters on the Varioskan LUX reader, plate settings (excitation, emission, bandwidth, and top vs. bottom readings) were adjusted to find the optimal values. Both assays were also analyzed using the Thermo Scientific™ Fluoroskan™ Microplate Fluorometer (Cat. No. 5200111) with a top-read at a 485/538 nm filter.

A similar methodology was used to prepare samples for the Invitrogen™ Qubit™ Flex Fluorometer (Cat. No. Q33327), where 10 µL or 2 µL samples in replicates of four were combined with 190 or 198 µL of working solution, respectively, in Invitrogen™ Qubit™ Flex Assay Tube Strips (Cat. No. Q33252), and then were mixed thoroughly using a vortex mixer. A two-point standard curve was generated on the Qubit Flex Fluorometer using the provided standards for both the Qubit 1X dsDNA HS and BR assays. The quantification results using Quant-iT assays were then compared to those using Qubit assays.

Results

Optimizing ex/em settings for monochromator-style plate readers

Using the Varioskan LUX reader, optimal measurement settings were determined based on:

- Signal-to-background ratio
- Sample variation (CV (%))
- Z-score (lowest signal separation with 95% confidence)

The excitation wavelength was adjusted in 5 nm increments above and below the recommended value, and a corresponding +30 nm emission setting was used for each measurement. Once the ex/em parameters were set, the optics (top vs. bottom read) and bandwidth were fine-tuned to achieve optimal results (Tables 1 and 2).

Table 1. Plate reader settings for the Quant-iT 1X dsDNA HS Assay. Excitation settings at 505 nm show the highest signal-to-background (S/B) ratio. Exploration of this setting shows a top-read with a 12 nm bandwidth maintains a high S/B ratio while demonstrating a CV of <5%. Samples were measured at the standard 100 ms measurement time as well as at 200 ms (*). The increased measurement time showed little to no improvement.

	Establishing excitation/emission settings						Additional settings		
Excitation (nm)	485	490	495	500	505	510	505	505	505*
Emission (nm)	515	520	525	530	535	540	535	535	535*
Bandwidth (nm)	12	12	12	12	12	12	12	5	12
Orientation	Top	Top	Top	Top	Top	Top	Bottom	Top	Top
S/B	249	360	464	519	561	533	246	645	563
Z-score at 0.125 ng	0.05	0.26	0.42	0.40	0.23	0.18	<0	<0	0.14
CV mean	2%	2%	2%	2%	3%	3%	6%	7%	3%

Table 2. Plate reader settings for the Quant-iT 1X dsDNA BR Assay. Excitation settings at 515 nm show the highest signal-to-background (S/B) ratio. Exploration of this setting shows a top-read with a 12 nm bandwidth maintains a high S/B ratio while demonstrating a CV of <5%. Samples were measured at the standard 100 ms measurement time as well as at 200 ms (*). The increased measurement time showed modest improvement in background separation (Z-score).

	Establishing excitation/emission settings						Additional settings		
Excitation (nm)	495	500	505	510	515	520	515	515	515*
Emission (nm)	525	530	535	540	545	550	545	545	545*
Bandwidth (nm)	12	12	12	12	12	12	12	5	12
Orientation	Top	Top	Top	Top	Top	Top	Bottom	Top	Top
S/B	105	127	149	154	175	168	124	154	169
Z-score at 1.25 ng	0.82	0.84	0.85	0.70	0.44	0.56	<0	0.27	0.80
CV mean	5%	5%	4%	5%	4%	3%	6%	6%	3%

For the Quant-iT 1X dsDNA HS Assay, the user guide specifies excitation and emission values at 502/523 nm. Starting at 485/520 nm, the optical settings were increased in 5 nm increments to a final setting of 515 nm where a stark decrease in performance began to emerge. Optimal excitation appears in the 495–505 nm range with corresponding emission at 525–535 nm. The top-read setting, at the highest sample concentration, offers nearly two times the signal-to-background ratio over the bottom-read setting in addition to a lower detection limit (Z-score). Comparing a 5 nm bandwidth to the standard 12 nm instrument setting shows an increase in signal-to-background ratio at the expense of both the detection limit and coefficient of variation (CV).

The same analysis was performed for the Quant-iT 1X dsDNA BR Assay, which has recommended excitation and emission values of 510/527 nm. Optimal signal-to-background ratio and detection limits were observed with excitation at 505–515 nm with corresponding emission at 535–545 nm. Similar to results with the Quant-iT 1X dsDNA HS Assay, decreased bandwidth settings increased the sample-to-sample variation without an observed benefit to signal.

This experimental framework could be used to optimize plate reader settings for other Quant-iT assays and is recommended for validation of any new assay or instrument. Once optimal read settings are defined, they should be held constant for the duration of an intended application.

Effect of data processing on quantification accuracy

Having established optimal settings to read the Quant-iT assay samples, the standard curve data were treated with various fitting models (Figures 2 and 3). The primary models tested were linear and quadratic with and without background correction, as they can easily be performed on Thermo Scientific™ SkanIt™ Software, Microsoft™ Excel™ platform, or other data processing software. Logistic fittings (e.g., 4PL and 5PL) and higher ordered polynomials were determined to be less ideal for this application because they do not readily extrapolate beyond the exact values used to build the standard curve and often require specialized software, limiting their general applicability. Data were also evaluated for outliers that may reduce the accuracy of the regression. Outliers can stem from a variety of sources, including well contamination, pipetting inaccuracy, or mixing inconsistencies. The data from Quant-iT assays using various processing methods were compared to results obtained from the Qubit Flex Fluorometer.

In general, sample concentrations measured along the standard curve behaved similarly with low error and low sample variations, independent of the curve fitting model. However, sample concentrations measured below the concentration of the lowest standard demonstrated differences. Using the Quant-iT 1X dsDNA HS Assay with low-concentration samples (<5 ng in mass or <2.5 ng/µL using a 2 µL sample), a standard linear fit was shown to have error from the expected value of greater than 50% (Figure 2). When a linear or quadratic background-corrected model was applied to those same measurements, the observed error was lowered to less than 15%. This was observed for both 2 µL and 10 µL sample volumes on both the Varioskan LUX reader and Fluoroskan fluorometer. Background-subtracted curve fitting similarly decreased measurement error from the Quant-iT 1X dsDNA BR Assay (Figure 3). For both assays, the background-corrected processed data provided a much closer reflection of the results obtained using the Qubit Flex Fluorometer. When using the Quant-iT 1X dsDNA BR Assay, the background-corrected linear regression model is preferable to properly model higher-concentration samples (greater than 1,000 ng in mass or 500 ng/µL using a 2 µL sample). Notably, changing the concentration of standards to better encompass the low concentration range did not improve performance when a background-subtracted model was used.

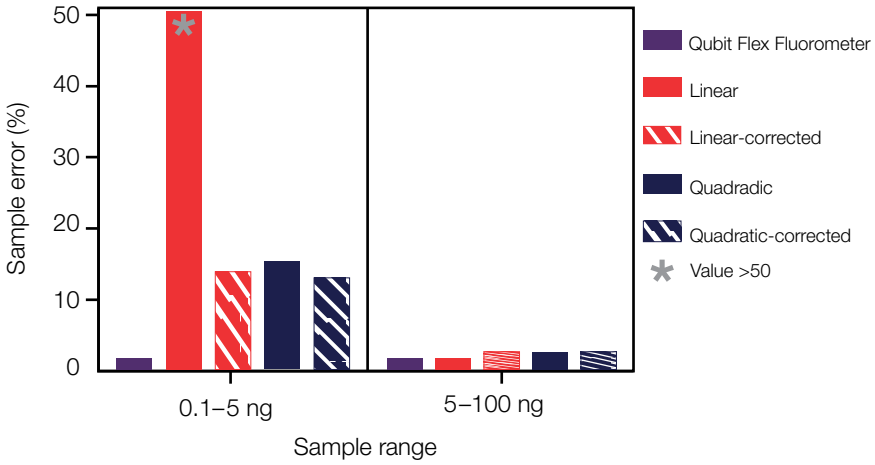

Figure 2. Quant-iT 1X dsDNA HS Assay performance using 2 µL samples. Measurements of sample concentrations <2.5 ng/µL (<5 ng of mass) using a standard linear regression had an error greater than 50% from the expected value. When a linear or quadratic background-corrected model is applied to those same measurements, the observed error is less than 15%. For samples in the 5–100 ng range, the curve fitting model had minimal effect on the calculated results.

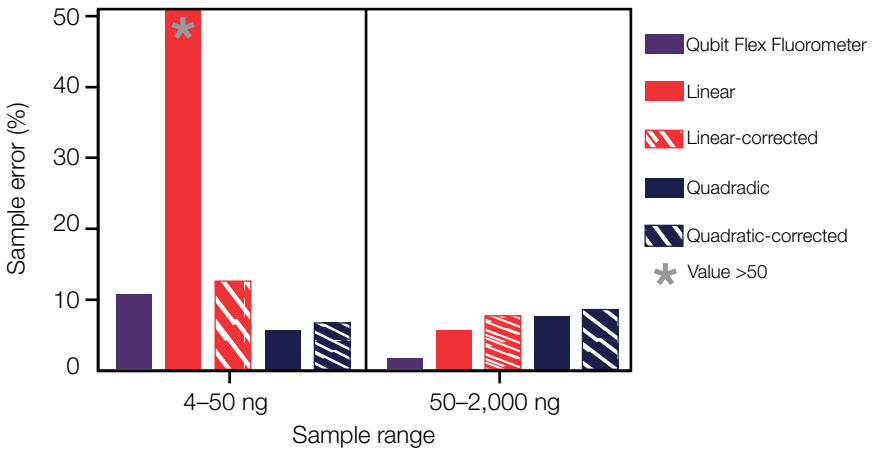

Figure 3. Quant-iT 1X dsDNA BR Assay performance using 2 µL samples. Measurements using a standard linear regression for concentrations <25 ng/µL (50 ng of mass) had an error greater than 50% from the expected value. When a linear or quadratic background-corrected model is applied to those same measurements, the observed error is less than 15%. For samples in the 50–2,000 ng range, the curve fitting model had minimal effect on the calculated results.

Conclusions

Qubit assays, designed for low- to medium-throughput, offer best-in-class quantification of nucleic acids while Quant-iT assays offer comparable performance for higher throughput settings. While Qubit instruments provide convenient, automated data processing, Quant-iT assays require optimization of plate reader settings and data analysis to ensure optimal results. As demonstrated, choices in optical settings and data processing can have large impacts on otherwise properly prepared assay plates. Each plate reader type and model may offer subtle differences, so individual optimizations may be required. Helpful parameters to establish ideal settings include signal-to-background ratio, Z-score, and coefficient of variation.

In general, it is recommended to use top read with 12 nm bandwidth settings and a background-corrected data processing method. For monochromator-based instruments (e.g., the Varioskan LUX reader), excitation settings of 495–505 nm for the Quant-iT 1X dsDNA HS Assay and 505–515 nm for the Quant-iT 1X dsDNA BR DNA Assay demonstrate accurate measurements across the dynamic range. For filter-based instruments (e.g., the Fluoroskan Microplate Fluorometer), matching these settings as closely as possible will offer ideal results, but a FITC-like filter set will be sufficient. Furthermore, processing data using background-corrected linear or quadratic curve fitting substantially improves accuracy. When these recommendations are followed, Qubit and Quant-iT assays provide valid and reliable quantification of dsDNA samples, with comparable results.

Ordering information

Product	Quantity	Cat. No.
Qubit Flex Fluorometer	1 each	Q33327
Qubit Flex Assay Tube Strips	125 tube strips	Q33252
Qubit 1X dsDNA HS Assay Kit	100 assays	Q33230
	500 assays	Q33231
Qubit 1X dsDNA BR Assay Kit	100 assays	Q33265
	500 assays	Q33266
Quant-iT 1X dsDNA HS Assay Kit	1 kit	Q33232
Quant-iT 1X dsDNA BR Assay Kit	1 kit	Q33267
Varioskan LUX Multimode Microplate Reader	1 each	VLB000GD0
Microplates for Fluorescence-Based Assays, 96-well	10 plates	M33089

Learn more at **thermofisher.com/qubit**