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A guide for reliable nucleic acid characterization in high-throughput applications

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Abstract

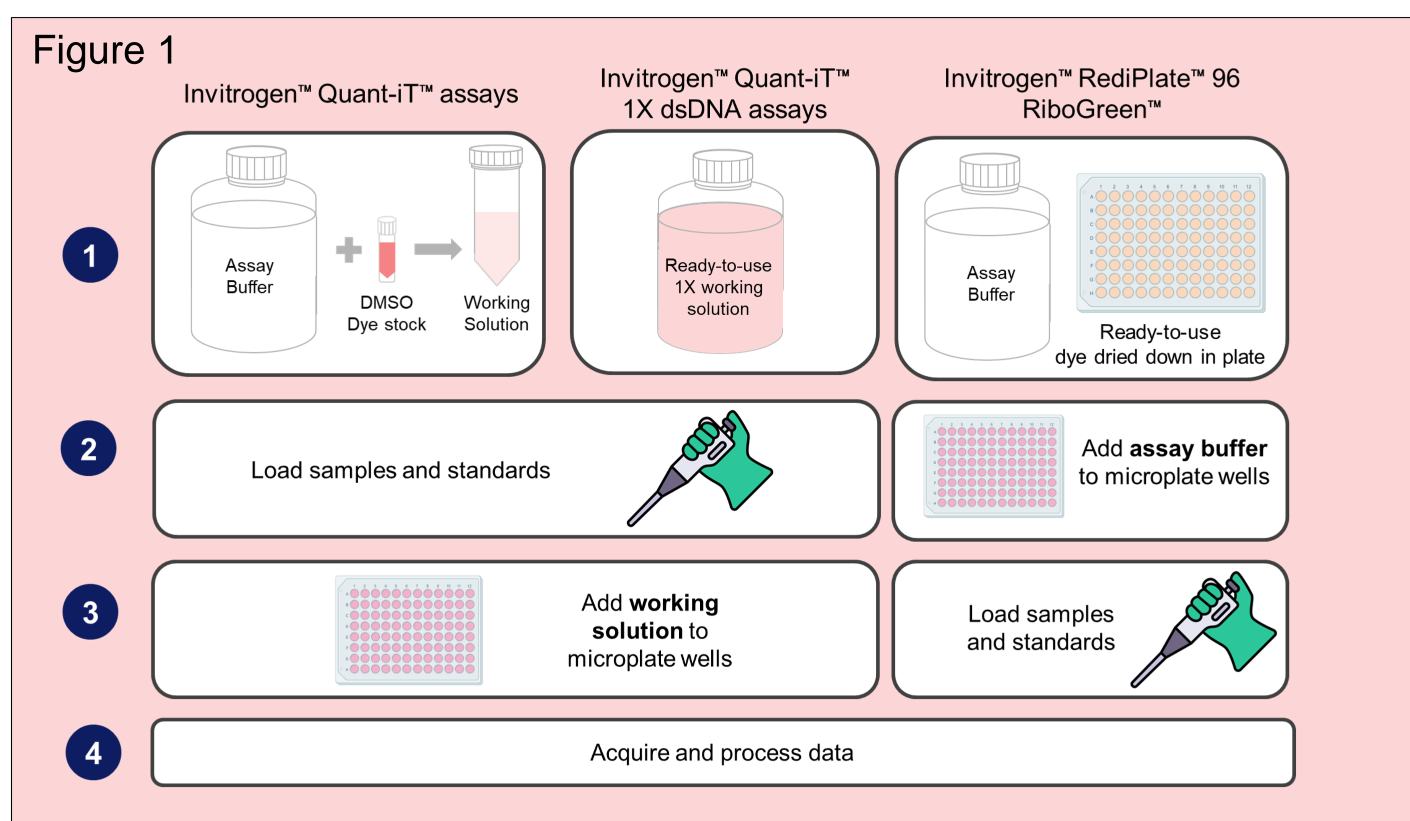
Fluorescence-based quantification of nucleic acids is an important step in many molecular biology experiments, such as next-generation sequencing (NGS) and research for drug delivery systems. This technique provides sensitive and target-specific quantification of DNA and RNA using as little as 1 µL of sample. Here, we overview the Thermo Fisher Scientific Quant-iT fluorescence-based assays for nucleic acid (DNA/RNA) characterization, share best practices for the use of these assays in automated high throughput workflows. We also detail new analysis approaches that allow for accurate and reproducible DNA and RNA measurements at low sample volume inputs. These outlined efforts will enable users to better leverage the Quant-iT reagents and assays as a toolbox for reliable nucleic acid characterization while reducing user variability and hands-on time.

Key takeaways

- Many experimental workflows rely on accurate quantification of target analytes including DNA, RNA, proteins and endotoxin. Commonly used quantification assays are available for low-medium throughput (e.g. Qubit™) and higher throughputs (e.g. Quant-iT™) settings. Along with standard formulations, some assays are also available in ready-to-use formulations (e.g. 1X) and prefilled microplates (e.g. RediPlate™).
- For unique and customized workflows (e.g. 384 well plates), microplate reader optical settings can have large impacts on otherwise properly prepared assay plates. Additionally, curve-fitting with background correction optimization and non-linear models can substantially improve accuracy at the low detection range.

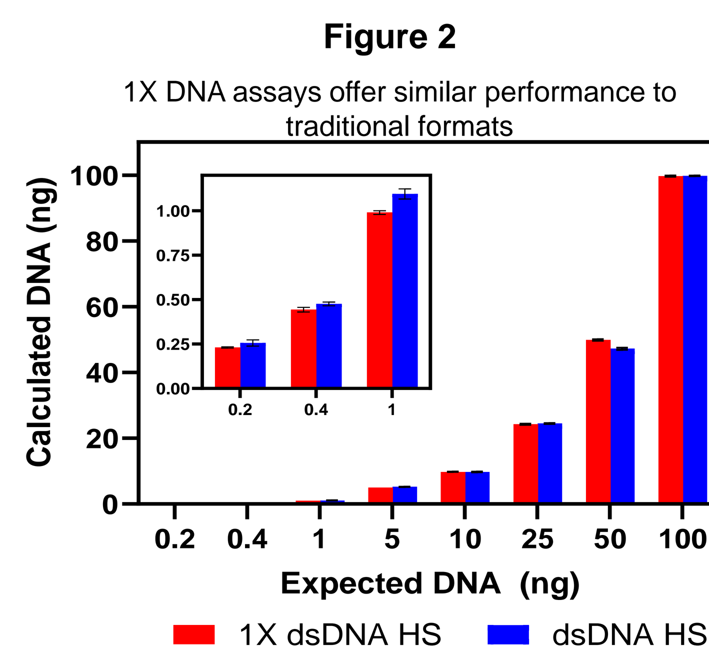
Methods and materials

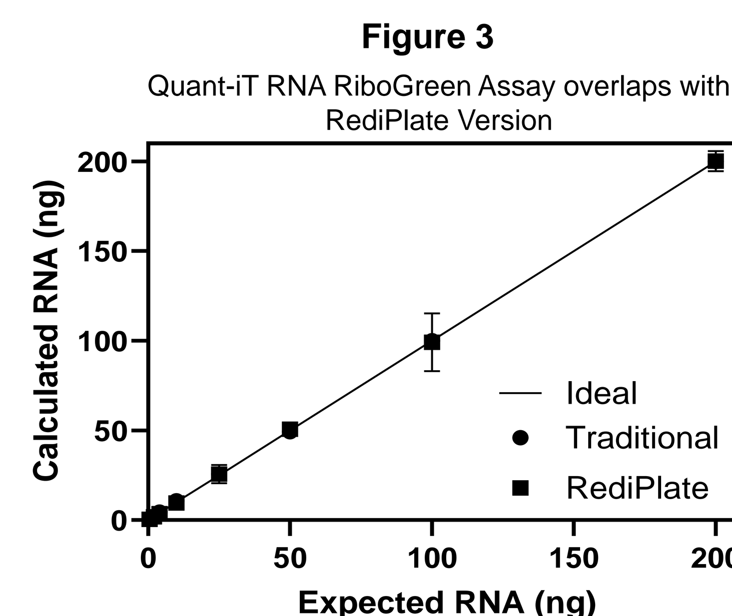
Invitrogen™ Quant-iT™ dsDNA Assays and Invitrogen™ RediPlate™ 96 RiboGreen™ were compared for performance across the core quantification ranges. Measurements were carried out using standard protocols with the key workflow steps outlined in **Figure 1**. The Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader was used for data acquisition with settings adjusted for optimal results.



Streamlining workflows without sacrificing performance

Quant-iT 1X assays (Figure 2) feature ready-to-use working solutions with a specially formulated fluorogenic dye and assay buffer. The 1X formulations enable users to combine their DNA samples with the provided 1X working solution without the need for assay preparation. These save workflow time and can be left on the bench during a full day of automation runs while matching performance to the traditional assay kit format.

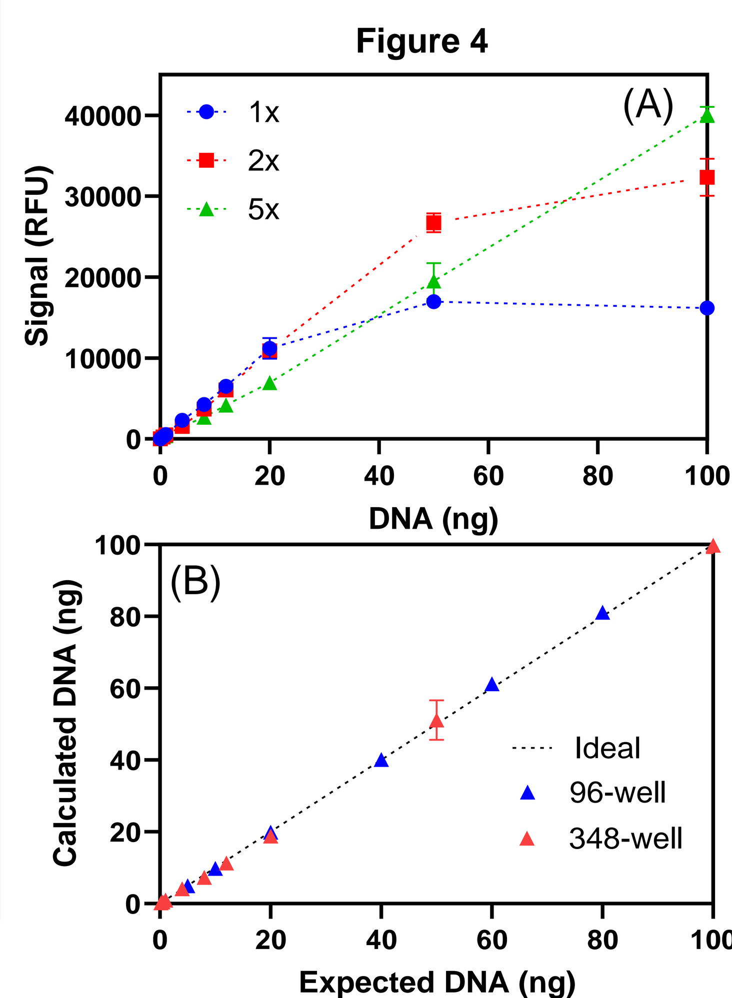




RiboGreen RediPlate (Figure 3) is a rapid, simple, and sensitive fluorescence-based RNA-quantitation assay. The microplate is pre-loaded with RiboGreen reagent, just add buffer and samples to the microplate wells and read the fluorescence in any standard fluorescence-based microplate reader. The versatile RediPlate 96 design makes it ideal for automated, high-throughput measurements of RNA concentrations.

Increasing throughput requires optimization of assay conditions

Adaptation to 384-well plates for higher throughput testing can be done for Quant-iT assays. However, to maintain the desired dynamic range, the amount of dye needed in the working solution needs to be adjusted. For example, 1 µL of dye is used for every 200 µL sample assay in a standard 96-assay format. In a 384-well using 40 µL (or 1/5th of the previous volume) 5x the dye is needed for the DNA HS assay.



Protocol for DNA HS assay in 384-well microplate

- Plate 2 µL of sample (or up to 10 µL) and 2 µL of Quant-iT standards
- Add 5x dye to buffer to prepare working solution
    - Ex: For 1X 384 well plate, add 400 µL of dye to 15.6 mL buffer for 16 mL or 400 reactions.
- Add 38 µL of working solution to all wells.
    - If more than 2 µL of sample was used, compete those samples to 10 µL using buffer only, then add 30 µL of the prepared working solution.
- For the best correlation to a 96-well preparation, plot the x-axis in ng of the target analyte.

Figure 4: (A) Increased dye is required to maintain the expected dynamic range (0-100 ng) for the DNA HS assay. (B) Once the dye concentration has been properly adjusted, the 96-well and 384-well configurations can produce comparable results.

Different instruments may require optimization of settings

Each Quant-iT assay offers a recommended excitation and emission setting for data acquisition, however, for some users and use cases that might not be specific enough. Using the Varioskan LUX, optimal measurement settings were determined based on signal-to-background ratio, sample variation, CV (%) and Z-score. The excitation wavelength was adjusted above and below the recommended value and a corresponding +30 nm emission setting was used for each measurement. Next, the read mode (top vs. bottom read) and bandwidth were fine-tuned to achieve optimal results. Shown below (Table 1) are data using the dsDNA 1X HS assay, but the general concepts are universal to other Quant-iT plate reader assays.

Table 1

	Optimizing excitation/emission settings						Fine/Tuning		
Excitation [nm]	485	490	500	505	510	515	505	505	505
Emission [nm]	520	525	530	535	540	545	535	535	535
Optics	Top	Top	Top	Top	Top	Top	Bottom	Top	Top
Excitation bandwidth [nm]	12	12	12	12	12	12	12	5	12
S/B	313	394	572	603	570	451	295	758	636
Low, S/B	1.13	1.16	1.19	1.19	1.21	1.22	1.19	1.68	1.23
Z*	0.1	0.1	0.1	0.2	0.2	0.2	1	0.2	0.1
CV Mean	4%	3%	5%	5%	5%	6%	15%	8%	4%

Data processing methods affect measurement accuracy

To demonstrate the effects of different curve fitting models, the 1X dsDNA HS assay was used with the provided eight pre-diluted standards to measure low concentration samples ranging from 5 to 0.0625 ng.

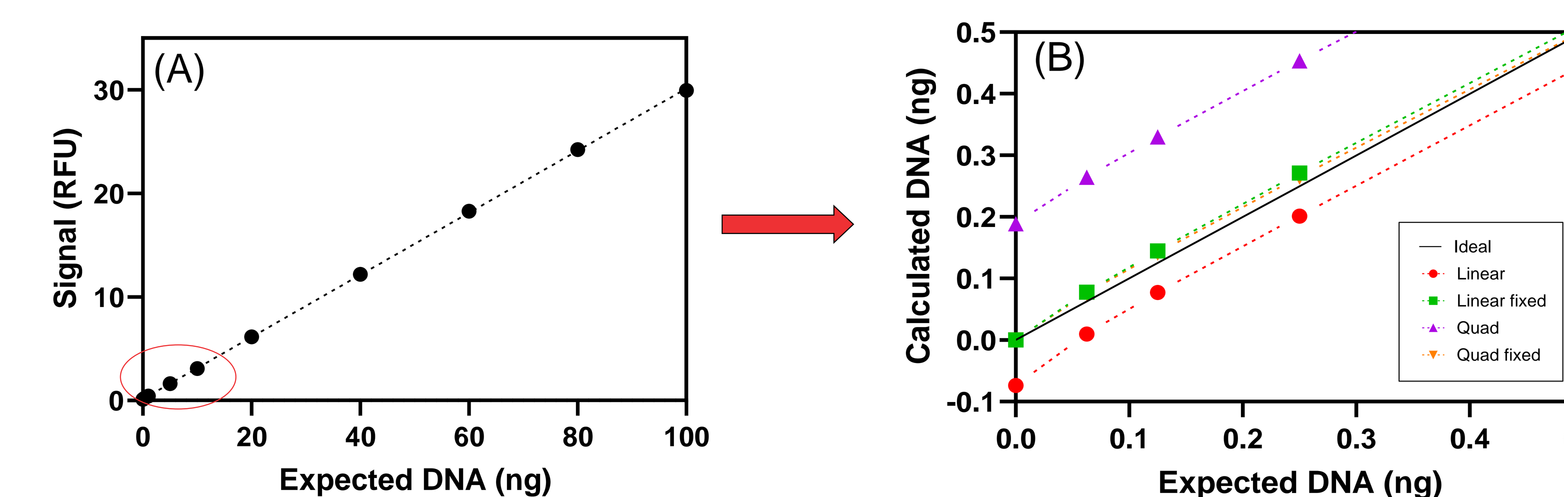
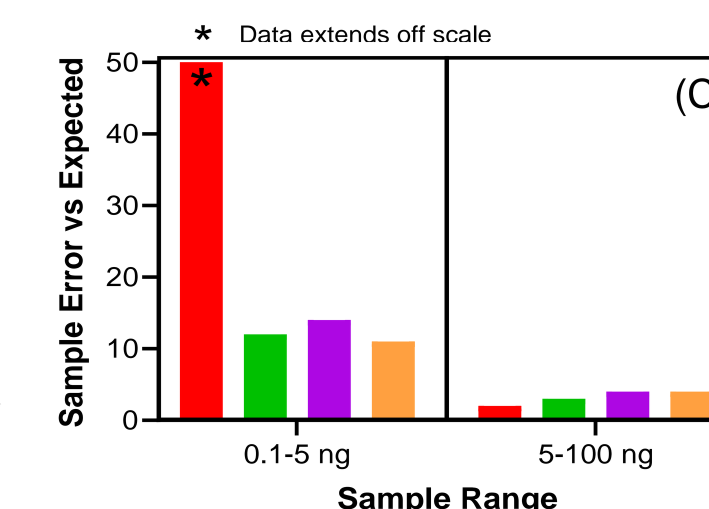


Figure 5: (A) In the core standard region, 5-100 ng, the signal response from samples is effectively linear. However, using a linear fit model for concentration measurements leads to misrepresentation of low-end data (B). This can be properly accounted for by first using background subtraction or fixing the y-intercept to equal the background test value. A non-linear model such as quadradic or 4-point logistical function may further improve results (C). Both approaches can be automated in traditional processing software.



Additional Resources at ThermoFisher.com

- Comparison of accuracy and prevision of Quant-iT and Qubit dsDNA quantification assays.
- Qubit 1X dsDNA assays: simplified workflow and improved performance
- Thermofisher.com/QuantiT

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