Application Note

MINIATURISATION OF THE QUBIT™ ASSAY WITH TRISTAR MULTIMODE READERS

SMALLER SAMPLE FORMATS REDUCE COSTS AND INCREASE THROUGHPUT

Abstract

Fluorescence-based DNA quantification methods offer higher sensitivity and specificity than UV spectrophotometry. Qubit™ assays are a widely used method of this type. They are performed in tube format, being suitable only for laboratories processing low numbers of samples We have already demonstrated that they can be easily adapted to the 96-well format. Moving to even smaller formats not only enables a further increase in throughput, but also cost savings. In this application note we show that the Qubit™ dsDNA BR kit can be miniaturized to 1/10th of its volume while keeping good performance by using small volume 384-well microplates and the Tristar multimode readers.

Introduction

Most molecular biology methods require either normalizing all samples to the same quantity of starting DNA, or a specific input quantity. Thus, accurate DNA quantification is needed for techniques such as Next-Generation Sequencing (NGS), Polymerase Chain Reaction (PCR), Real-Time PCR (quantitative PCR; qPCR), cloning, transfection, and others.

Francesc Felipe
Berthold Technologies GmbH
www.berthold.com/bio

DNA quantification is performed very often using UV spectrophotometry. This method is simple, cost-effective and requires minimal sample quantity (typically 1 µL). However, it suffers from low specificity: it cannot distinguish between dsDNA and ssDNA or between DNA and RNA, or even between degraded and intact DNA. In addition, it is sensitive to the presence of contaminants that are frequent in DNA samples, such as protein, phenol or guanidine salts. Depending on the type of sample and downstream method to be used, it may be necessary to use methods of higher specificity. This is the case, for example, of NGS, which we already covered in a previous application note [1].

DNA quantification methods based on fluorescence offer a much higher specificity than UV spectrophotometry. They use intercalating dyes that are specific of the type of nucleic acid to be quantified and don't bind to free nucleotides. Qubit™ assay kits are a very popular example of fluorescence-based DNA quantification methods. While they are intended to be used by laboratories processing low numbers of samples and are performed in tube format, we have previously shown that they can be measured in 96-well plates with good performance using the Tristar multimode readers [1].

Moving from 0.5 mL tubes to 96-well microplates notably increases throughput, but moving to a microplate format with higher well count and smaller wells would allow to miniaturize the assay, providing even higher throughput and reducing reagent costs:

typical working volume are 200 µL in 96-well microplates, 75 µL in 384-well microplates and 20 µL in small volume 384-well microplates. Hence, moving from 96- to 384-well microplates would bring more than a 60% saving in reagent costs, a 90% if moving to small volume 384-well microplates.

In this Application Note we compare the performance of the Qubit™ dsDNA BR assay kit in 96-, 384- and small volume 384-well (384sv) microplates using the Tristar 3 and Tristar 5 multimode microplate readers.

Tristar Series

Application flexibility you can count on

Developed for high flexibility and equipped with the proprietary ONE-4-ALL optical system, the Tristar Serie combines the user friendliness of a multimodal optical system with the sensitivity and performance of a dedicated optical device. You can choose between the affordable Tristar 3 and the more advanced and flexible Tristar 5. The Tristar series provides you with flexibility for today, tomorrow, and beyond.

- Monochromator Technology
- High-sensitivity Luminescence
- BRET
- UV/VIS Absorbance
- UV/VIS Fluorescence
- UV/VIS FRET
- Time-Resolved Fluorescence (TRF)
- Time-Resolved FRET (TR-FRET / HTRF®)
- Fluorescence Polarization
- AlphaScreen®
- Top and Bottom Reading
- Incubation

Materials

- Tristar 3 Multimode Microplate Reader from Berthold Technologies (Id. Nr. 69173-10).
- Tristar 5 Multimode Microplate Reader from Berthold Technologies (Id. Nr. 69185-15).
- Qubit™ dsDNA BR Assay Kit from ThermoFisher (Cat. # Q32850).
- Invitrogen™ Salmon Sperm DNA, sheared (10 mg/mL) from ThermoFisher (Cat. #AM9680).
- Black 96-well microplates from Berthold Technologies (Id. Nr. 23302).
- Black 384-well microplates from Greiner (Ref. 781076).
- Black small volume 384-well microplates from Greiner (Ref. 784900).
- Tubes (various volumes).
- Pipettes and pipette tips (various volumes).
- LightCompass® software from Berthold Technologies.

Instrument settings

- Reading mode: Fluorescence Endpoint
- Excitation filter: 485/14
- Emission filter: 535/25
- Counting time: 0.1 s
- All other settings with default values

Methods

Reagents were prepared following the manufacturer's instructions. In order to further extend the concentration range of the assay, a more concentrated DNA standard was used instead of the one included with the kit. The DNA stock was diluted to a concentration of 160 ng/µL using TE buffer. To prepare the standard curve, a ½ dilution series was prepared using this diluted stock, producing the following DNA concentrations: 80, 40, 20, 10, 5, 2.5, and 1.25 ng/µL DNA. TE buffer was used as blank.

In addition, to assess the performance of the assay, 3 "unknown" samples were prepared with concentrations different to that of the standard points: 120, 30 and 7.5 ng/uL DNA.

To minimize the differences between microplate formats due to sample handling, one single tube was prepared for all microplate formats and replicates of each standard or sample. 50 µL of standard or sample were mixed with 950 µL of Qubit working reagent in 1.5 mL microcentrifuge tubes and vortexed vigorously for 5 seconds. After incubating the tubes 5 minutes at room temperature protected from light, the following volumes were pipetted in triplicate in the wells of the corresponding microplates: 200 µL for 96-well microplates, 75 µL for 384-well microplates and 20 µL for 384sv microplates. Then, the microplate was measured in the Tristar reader using the settings described above. 4-parameter logistic (4PL) regression was used to calculate concentrations, as this algorithm provided the best results in previous tests [1].

Results

The standard curves produced were slightly curved in all cases (Fig. 1), in agreement with example results displayed in the User Guide of the Qubit™ assay [2] and with our previous results [1]. Again, when expressing measurements as relative fluorescence, both the Tristar 3 and the Tristar 5 produce very similar curves (Fig. 1 and 2). In both instruments, the curved shape is more accentuated in the case of 384sv microplates.

Results from a previous application note show that using standard curves with 5 or 4 points produces very similar results to the full 9-point standard curve [1]. Hence, curves with 5 and 4 standard points were used to calculate the concentrations of the unknown samples. The standard points chosen for each standard curve were the following ones:

5 points:	160, 40, 10, 2.5, 0 ng/µL
4 points "high":	160, 40, 5, 0 ng/µL
4 points "low":	160, 40, 1.25, 0 ng/µL

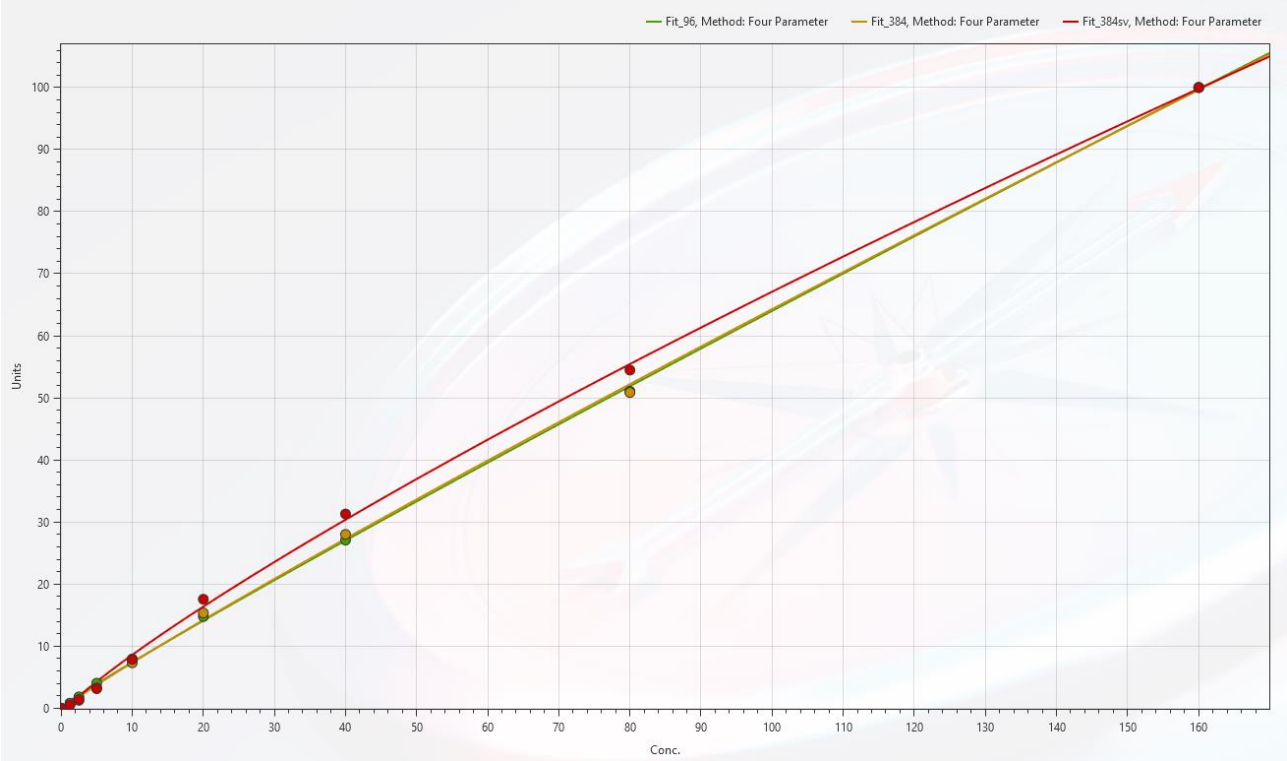

Figure 1. Standard curves generated by 4PL regression in the Tristar 5 in 96-, 384- and small volume 384-well (384sv) microplates. Measurement values expressed as % of the maximum fluorescence, concentrations in ng/µL. All data points measured in triplicat e.

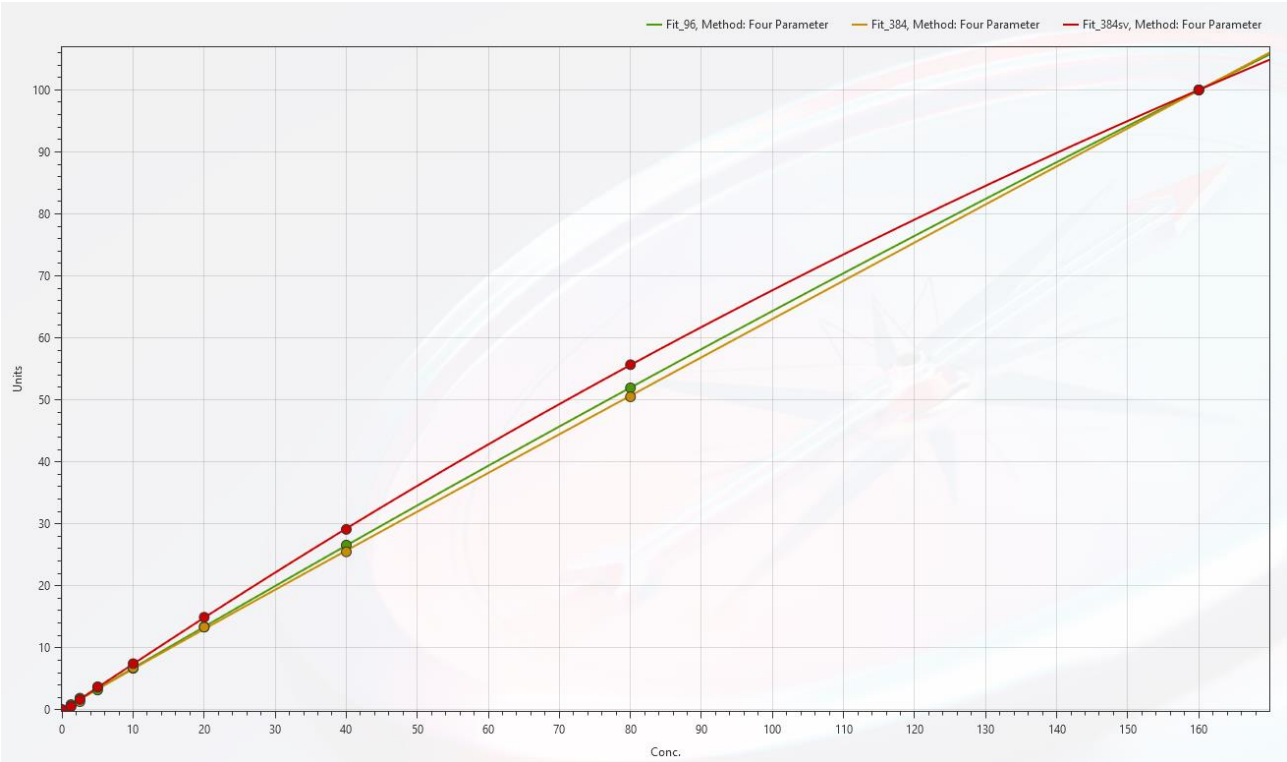

Figure 2. Standard curves generated by 4PL regression in the Tristar 3 in 96-, 384- and small volume 384-well (384sv) microplates. Measurement values expressed as % of the maximum fluorescence, concentrations in ng/µL. All data points measured in triplicat e.

The accuracy of the quantification is different depending on the microplate format and the standard curve used. In 96-well microplates, both the 5-point and 4-point "high" standard curves deliver highly accurate results in both instruments (Table 1), with an average error below 2.5%. On the other hand, the accuracy is worse when using the 'low' curve. There are differences between the instruments tested for standard 384-well microplates: With the Tristar 5, 4-point curves give slightly better results than the 5-point curve, but the differences are comparatively small. With the Tristar 3, the best results are obtained with the 5-point standard curve. Finally, in small volume 384-well microplates, the best results in both instruments are obtained with the 5-point standard curve.

If only the results between both models of 384-well microplates are compared, it is remarkable that the accuracy in small volume is better than in standard microplates: in standard 384-well microplates, the curve providing the best results produces quantifications with an average error of 6.6% in the Tristar 5 (6.3% in the Tristar 3), while in small volume 384-well microplates the 5-point standard curve delivers an average error of quantification of only 3.2% and 4.9%, respectively.

Tristar 5	**96-well**			**384-well**			**384sv**		
ng/uL	5 points	4 points "high"	4 points "low"	5 points	4 points "high"	4 points "low"	5 points	4 points "high"	4 points "low"
120	116.11	116.27	114.57	115.24	114.10	114.10	125.85	123.85	122.98
30	28.86	29.06	29.58	31.55	31.82	31.81	30.59	31.07	31.25
7.5	7.53	7.59	8.49	6.49	6.85	6.85	7.70	8.55	8.94
Tristar 3	**96-well**			**384-well**			**small volume 384-well**		
ng/uL	5 points	4 points "high"	4 points "low"	5 points	4 points "high"	4 points "low"	5 points	4 points "high"	4 points "low"
120	117.78	117.03	117.76	120.49	120.59	122.98	128.06	128.01	125.01
30	29.10	29.28	29.09	27.48	27.41	31.25	27.78	27.81	28.70
7.5	7.29	7.67	7.34	6.76	6.48	8.94	7.54	7.53	8.99

Table 1. Calculated dsDNA concentrations using 4PL algorithm and different standard curves. All concentrations in ng/µL. All data points measured in triplicate.

Discussion and conclusions

Adapting the Qubit™ dsDNA BR assay kit to 96-well microplate format increases throughput while delivering very good accuracy in both Tristar multimode readers with a standard curve of only 4 points. Switching to 384-well microplates further increases throughput and, in addition, reduces the amount of reagent needed for the quantification. Accuracy in 384-well microplates is lower than in 96-well format but, when choosing the right microplate and standard curve, accuracy loss is small: in the Tristar 5 average error increases from 2.5% in 96-well microplates to only a 3.2% in 384sv microplates using the 5-point standard curve, a 4.9% in the Tristar 3. In many cases, this loss in accuracy is more than offset by the savings and increase in throughput.

It is remarkable that performance is worse in standard 384-well microplates than in small volume ones in both instruments. A possible factor is that wells are square in standard microplates but round in small volume ones. Determining the origin of this differences is beyond the scope of this application note but, from a practical perspective, any laboratories considering moving to 384-well microplates should choose small well ones, as they provide not only the highest reagent savings, but also the best performance compared to standard 384-well microplates.

The best experimental setup for each microplate format for the concentration range tested is summarized in table 2:

	96-well	384-well	384sv
µL/well	200	75	20
Algorithm	4PL	not recommended	4PL
Standard points	4		5
Standard concentrations (ng/µL)	160, 40, 5, 0		160, 40, 10, 2.5, 0

Table 2. Optimal experimental settings for each microplate format.

A final thought on the concentration range: the specific concentrations for the standard curve used in this application note are arbitrary, and laboratories can choose different concentration ranges depending on their needs and the expected concentrations of their samples (for example, up to 100 ng/µL only, to make use of the DNA standard included with the kit).

In this application note we demonstrate that the Qubit™ dsDNA BR assay can be miniaturized, reducing total reaction volume from 200 µL down to 20 µL, using the Tristar multimode readers and small volume 384-well microplates. Using a 4-parameter logistic algorithm, 5 standard points are sufficient to provide good performance in the concentration range tested: this allows 187 samples to be quickly quantified in duplicate (123 samples in triplicate) in a single 384-well plate and using only 7.3 mL of Qubit™ working reagent instead of the 18.2 mL needed in 96-well format. For laboratories working with low-concentration samples, the Qubit™ dsDNA HS assay kit is available; we expect it to also work very well in microplate format using the Tristar multimode readers.

Taking everything into account, the Tristar multimode readers are a very good solution to miniaturize the Qubit™ dsDNA BR assay. Using the curve fitting algorithms and standard points detailed in this application note, they offer a DNA quantification solution with low running costs and high reliability and throughput. With the adjustments we have made to the protocol, it will be a valuable tool for laboratories using Qubit™ assays for DNA quantification in large quantities of samples.

References

1. Quantifying DNA for NGS with the Qubit™ dsDNA BR kit and Tristar multimode readers (2024). Berthold Technologies. Available for download at www.berthold.com.
2. User Guide of the Qubit™ dsDNA BR Assay Kit (2022). Pub. No. MAN0002325, Rev. B0. Thermo Fisher Scientific.

For Research Use Only. Not for use in diagnostic procedures.

Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

www.berthold.com