Application Note

QUANTIFICATION OF DNA FOR NGS WITH THE QUBIT™ ASSAY AND TRISTAR MULTIMODE READERS

Increase your throughput and facilitate repeat measurements

Abstract

Next generation sequencing (NGS) workflows require highly accurate DNA quantification. The widely used Qubit™ assay is normally analysed with a Qubit™ fluorometer. However, they are performed in tube format. This makes the application suitable only for laboratories processing a small number of samples. In this application note the Qubit™ dsDNA BR kit is adapted to microplate format using the Tristar multimode readers. With only 4 standard points, the tested settings provide high throughput and high performance.

Introduction

Next generation sequencing (NGS) is a nucleic acid sequencing technology that enables the sequencing of thousands of genes simultaneously in multiple samples. This provides valuable information for a broad range of fields and applications, from cancer research to prenatal testing. There are many companies offering NGS solutions, but the market is currently dominated by Illumina® and Thermofisher Scientific [1].

NGS is a very powerful technology, but it has also strict requirements concerning sample quality and handling. Accurate quantification and proper quality check of next-generation sequencing libraries is key to a successful sequencing run and determines data quality and overall data yield: insufficient input will lead to under-clustered flow cells, and excess input will lead to over-clustered flow cells, compromising the quality of results. In many NGS workflows, DNA quantification is performed twice: before and after library preparation.

While DNA quantification using UV spectrophotometry is very popular for other applications, it is not recommended for NGS. Depending on the library preparation kit used, different DNA quantification methods are favoured, and fluorometric quantification using assays such as Qubit™ and Picogreen® are recommended in many cases [2]. We have already covered the use of Quant-iT™ Picogreen® with Tristar multimode readers in a previous application note [3]. In the present Application Note we are going to focus on the Qubit™ assay.

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The Qubit™ assay is intended to be performed in Qubit™ fluorometers. However, these fluorometers use tubes as sample format, and this limits throughput. Although Quant-iT™ assays are most suitable to be measured in microplates, many scientists and technicians have used Qubit™ for years and are happy to keep using it, even if sample throughput increases and requires moving to microplate format. Fortunately, the Qubit™ assay can be easily adapted to the microplate format.

In this Application Note we report that the Qubit™ dsDNA BR assay kit is easily adapted to 96-well microplate format using the Tristar 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).
- LightCompass® software from Berthold Technologies.
- Tubes (various volumes).
- Pipettes and pipette tips (various volumes).

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: 160, 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/µL DNA.

The recommended reaction volume of the Qubit™ assay is 200 µL, which fits perfectly with the working volume of most standard 96-well microplates. According to the instructions of the kit, 10 µL of each standard (1-20 for samples) have to be mixed were mixed with 190 µL of working solution (180-199 for samples). In this case, we used 10+190 for both standards and samples. To perform the assay in triplicate, triple volumes (plus 10 extra microliters, to ensure enough volume was available) were mixed in 1.5 mL microcentrifuge tubes and vortexed vigorously for 5 seconds. After an incubation of 5 minutes at room temperature protected from light, 200 µL of each standard and sample were pipetted in triplicate in the wells of the 96-well microplate. Then the microplate was measured immediately in the microplate reader using the settings described above.

One critical point of the assay is the curve fitting algorithm. According to the available documentation, Qubit™ fluorometers use a "modified Hill plot" algorithm, but no details are given about the modifications. To find out the best curve fitting algorithm, accuracy of the following fitting algorithms was compared: 4 parameter, linear, point-to-point and polynomial (2nd and 3rd degrees).

Results

Fitting algorithm

In the User Guide of the kit, it can clearly be seen that fitting algorithm used by Qubit™ fluorometers is not linear, but it produces a slightly curved line [4]. Considering this, it's not surprising that using linear regression plots a line that is relatively far from some of the standard points (Fig. 1); on the other hand, curve fitting algorithms such as 4 parameter logistic show a much better fit (Fig. 2). When expressing measurements as relative fluorescence, both the Tristar 3 and the Tristar 5 produce very similar curves (Fig. 1 and 2).

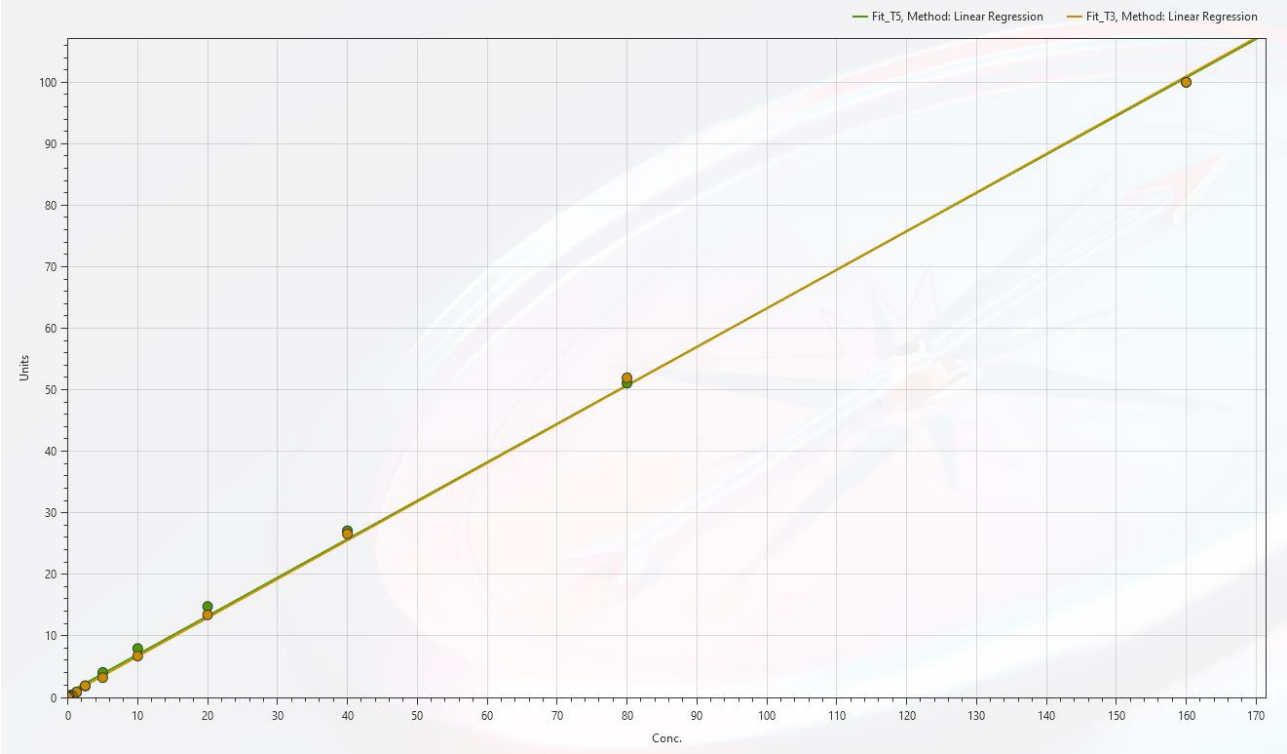

Figure 1. Standard curves generated by linear regression in the Tristar 5 and Tristar 3 instruments. Measurement values expressed as % of the maximum fluorescence, concentrations in ng/µL. All data points measured in triplicate.

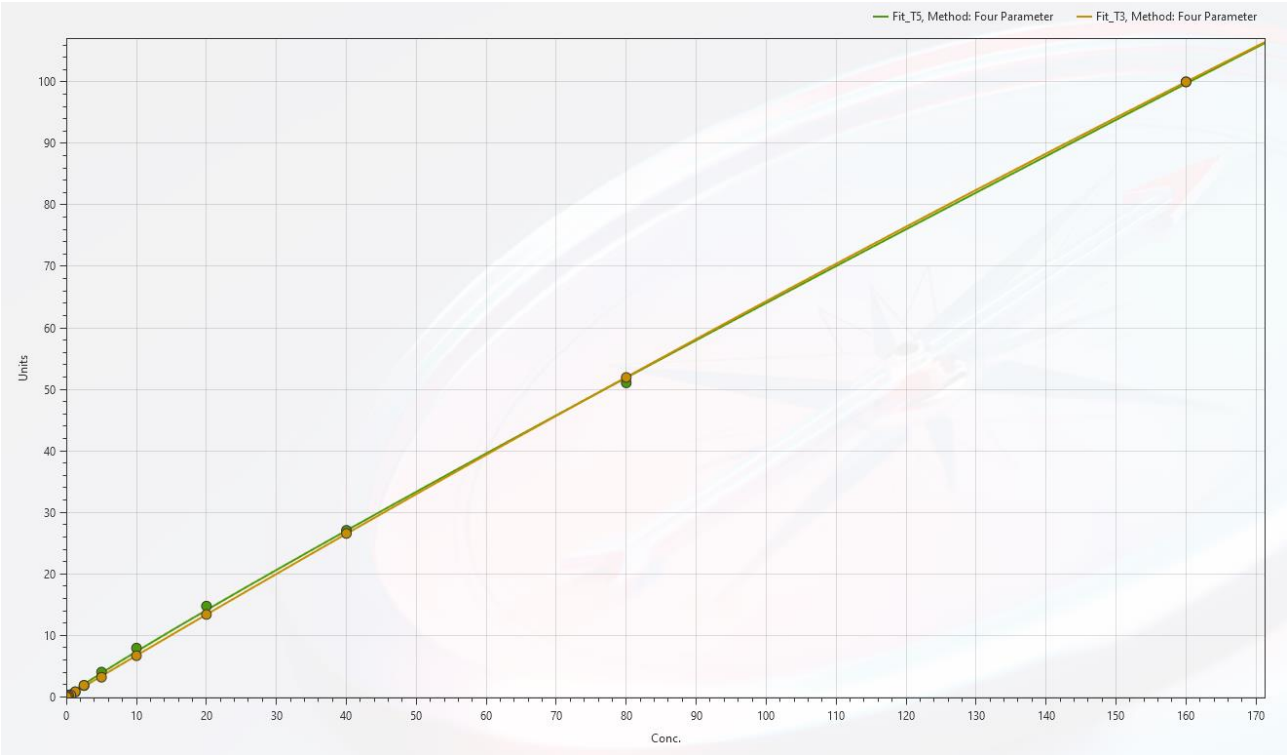

Figure 2. Standard curves generated by 4 parameter logistic regression in the Tristar 5 and Tristar 3 instruments. Measurement values expressed as % of the maximum fluorescence, concentrations in ng/µL. All data points measured in triplicate.

In the concentration ranges tested, all fitting algorithms provided good results (Table 1), with an average error <5% in all cases. Overall, the best results were obtained with 4 parameter logistic, which produced results with an average error of 2.3% and no error going above 2.8% at any data point. Considering this error comes from 3 different pipetting steps plus measurement error, this level of accuracy is remarkably good.

Tristar 5					
Theoretical concentration	4 Parameter Logistic	Linear Regression	Point-to-point	Polynomial (2nd degree)	Polynomial (3rd degree)
120	116.67	117.26	117.54	116.01	120.23
30	29.18	31.02	28.63	29.83	28.33
7.5	7.57	8.05	7.18	8.03	7.56

Tristar 3					
Theoretical concentration	4 Parameter Logistic	Linear Regression	Point to point	Polynomial (2nd degree)	Polynomial (3rd degree)
120	117.84	118.97	118.65	117.78	117.93
30	29.17	30.37	29.17	29.19	29.14
7.5	7.36	7.36	7.52	7.31	7.37

Table 1. Calculated dsDNA concentrations using different curve fitting methods. All concentrations in ng/µL. All data points measured in triplicate.

Number of standard points

Measuring the Qubit™ assay in a Qubit™ fluorometer requires only 2 standard points: 100 and 0 ng/µL, but more data points are required for accurate fitting of the curve in most methods tested above. However, using a 9-point standard curve involves considerable handling time and reagent consumption. In order to optimize the protocol, curves with 5 and 4 standard points were used and compared to results obtained with the 9-point standard curve. The standard points chosen for each reduced standard curve were:

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

Tristar 5

Theoretical concentration	9 points	5 points	4 points "high"	4 points "low"
120	116.67	116.11	116.27	114.57
30	29.18	28.86	29.06	29.58
7.5	7.57	7.53	7.59	8.49

Tristar 3

Theoretical concentration	9 points	5 points	4 points "high"	4 points "low"
120	117.84	117.78	117.03	117.76
30	29.17	29.10	29.28	29.09
7.5	7.36	7.29	7.67	7.34

Table 2. Calculated dsDNA concentrations using standard curves with different number of data points (see above). All concentrations in ng/µL. All data points measured in triplicate.

Reducing the number of concentrations for the standard curve down to 4 produces no significant loss of accuracy, with calculated concentrations very similar to the ones produced using 9 points. Between the 2 combinations of 4 points tested, the "high" one, using 5 instead of 1.25 ng/µL to draw the curve, provides the best results in the Tristar 5. In Tristar 3, both curves produce similar results.

Discussion and conclusions

Next Generation Sequencing (NGS) is a demanding method that requires very accurate quantification of dsDNA at several steps of the workflow. In many cases, fluorescent methods such as Quant-IT™ and Qubit™ are recommended for the quantification. Qubit™ has been designed to be measured in a Qubit™ fluorometer, but tube format limits throughput. Many laboratories would prefer adapting the Qubit™ assay to microplate format instead of migrating to different methods such as Quant-IT™.

In this application note we demonstrate that the Qubit™ dsDNA BR assay kit can be easily adapted to 96-well format using Tristar multimode readers and LightCompass® software The two members of the Tristar family, Tristar 5 and Tristar 3, deliver very similar results. Using a 4-parameter logistic algorithm, 4 standard points are sufficient to provide good performance in the concentration range tested: This allows rapid quantification of 44 samples in duplicate (28 samples in triplicate) in a single 96-well plate. For laboratories working with samples of lower concentration, the Qubit™ dsDNA HS assay kit is available. Our expectation is that it will also work very well in microplate format with the Tristar multimode readers.

Taking everything into account, the Tristar multimode readers are a perfect solution for measuring the Qubit™ dsDNA BR assay in microplate format. Using the curve fitting algorithms and standard points detailed in this application note, they offer a DNA quantification solution with high reliability and throughput. This makes adapting the assay protocol to the microplate format a valuable tool for laboratories that use Qubit™ assays for DNA quantification and do not want to switch to another method.. For laboratories considering other methods, the Quant-IT™ Picogreen® dsDNA assay kit also provides excellent performance in the Tristar multimode readers [3].

References

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