High-Throughput DNA Quantification for Next Generation Sequencing on the VICTOR Nivo

Introduction
Next generation sequencing (NGS) library preparation requires accurate quantification of the genomic DNA (gDNA) starting material to begin the workflow. After library preparation, dsDNA library quantification may also be necessary prior to sequencing. There are many different methods of quantification available on the market and each can vary in precision, accuracy and throughput. Typically, DNA in NGS workflows is measured using tube-based fluorescent assays such as Qubit® or qPCR. Unlike traditional 260 nm absorbance values, these fluorescent assays can accurately quantify small amounts of dsDNA in the presence of salts, urea, ethanol, chloroform, or detergents. However, since samples have to be measured one at a time, this can create bottlenecks in a high-throughput lab. Using an automated liquid handler and plate-reader instead of manually pipetting tube-based measurements can significantly decrease measurement time and increase throughput. In this application note, we compare the results of these two dsDNA quantification methodologies.
The VICTOR Nivo™ multimode plate reader is a flexible, compact benchtop system with a dynamic filter wheel system for measurement of fluorescent wavelengths and the option of either filters or a spectrometer for absorbance measurements. It is therefore well-suited to the determination of dsDNA concentration. The VICTOR Nivo can be used in conjunction with the Zephyr® G3 NGS Workstation to perform fast measurements for high-throughput quantification of DNA samples. The VICTOR Nivo multimode plate reader has previously been shown to accurately quantify purified dsDNA using Quant-iT® dsDNA reagents. In addition, we showed previously how the Qubit® protocol could be modified and used to accurately quantify gDNA in a plate-based format with the VICTOR Nivo™ utilizing the Zephyr G3 Workstation. Here we extended these studies and obtained consistent, reproducible and high-throughput results using the VICTOR Nivo plate reader to quantify DNA with reagents from Quant-iT® assay kits with the aid of the Zephyr G3 NGS workstation.

Materials and Methods

Reagents

Quant-iT® dsDNA Assay Kit, broad range (Invitrogen #Q33130), Quant-iT® dsDNA Assay Kit, high sensitivity (Invitrogen #Q33120), Qubit® dsDNA BR Assay Kit (Invitrogen #Q32850) and Qubit® dsDNA HS Assay kit (Invitrogen # Q32851) were used for quantification of DNA samples. The genomic DNA sample used in this study was NA12878 gDNA (Coriell Institute for Medical Research). This was selected because this genome has been extensively characterized and is commonly used as a reference genome for validation studies of NGS technologies. In addition to the genomic DNA standard, two standards from each of the kits (80 ng/µL for Quant-iT® broad range and 10 ng/µL for the Quant-iT® high sensitivity dsDNA kits) were serially diluted in TE and used as unknowns for quantification.

Working solutions were made by diluting Quant-iT® reagents 1:200 into the buffer that is supplied with the kit according to the manufacturer’s protocol.

Serial dilutions of the gDNA and standard DNA samples were manually pipetted 2-fold in TE buffer into a 96-well StorPlate. Ten different dilutions of the gDNA sample and seven different dilutions of the two standards were made. Then, each sample was pipetted into 96-well Bio-Rad Hard-Shell Assay plates. gDNA samples were added to the assay plate in replicates of four while the diluted standards were performed in replicates of three. On each assay plate, a standard curve in replicates of three was plated using the DNA standards from either the Quant-iT® broad range or Quant-iT® high sensitivity dsDNA kits.

The Zephyr G3 NGS workstation was used to pipette the 158 µL of Quant-iT® reagents into the 96-well plate. Next, it added 2 µL of each sample from the assay plate containing the DNA samples into the 96-well OptiPlate containing the Quant-iT® reagents. Finally, the plate was transported from the Zephyr to the VICTOR Nivo for measurement.

Samples from the assay plate were measured on the Qubit® Fluorometer using the standard protocol from the manual for each kit.

Instrumentation

Samples were prepared on the Zephyr NGS G3. The Zephyr NGS G3 workstation is a benchtop liquid handler designed to automate the construction of NGS libraries. The simplified user-interface and integrated hardware directs users on starting the Zephyr NGS G3 workstation. The Zephyr NGS G3 workstation’s efficient liquid handling and simultaneous pipetting action is utilized to prepare Quant-iT® assay plates for 8 x 96 samples on deck prior to measurement on the VICTOR Nivo plate reader. Additionally, workbooks are packaged with the application used to guide proper assay preparation and for tracking automation steps performed by the Zephyr NGS G3 workstation.

DNA samples were read on the VICTOR Nivo 5S using 485/30 nm excitation filter and 535/30 nm emission filter with a 50/50 beam split mirror. A modification from the prewritten Fluorescein protocol was used with a 50 ms measurement time and 10 mJ flash energy to reduce background fluorescence.

The Qubit® 2.0 Fluorometer (Invitrogen, #Q32866) was used with the standard Qubit reagent kit to quantify the DNA standards as an orthogonal assay to verify DNA quantification.

Protocols

The gDNA sample and four standard DNA samples were manually diluted 2-fold in TE buffer into a 96-well StorPlate. Ten different dilutions of the gDNA sample and seven different dilutions of the two standards were made. Then, each sample was pipetted into 96-well Bio-Rad Hard-Shell Assay plates. gDNA samples were added to the assay plate in replicates of four while the diluted standards were performed in replicates of three. On each assay plate, a standard curve in replicates of three was plated using the DNA standards from either the Quant-iT® broad range or Quant-iT® high sensitivity dsDNA kits.

The Zephyr G3 NGS workstation was used to pipette the 158 µL of Quant-iT® reagents into the 96-well plate. Next, it added 2 µL of each sample from the assay plate containing the DNA samples into the 96-well OptiPlate containing the Quant-iT® reagents. Finally, the plate was transported from the Zephyr to the VICTOR Nivo for measurement.

Samples from the assay plate were measured on the Qubit® Fluorometer using the standard protocol from the manual for each kit.

Figure 1. VICTOR Nivo Plate Reader.

Figure 2. Zephyr G3 Automated Workstation.
Data Analysis

Data was analyzed using MyAssays® Desktop Software, which is included with the VICTOR Nivo instrument. The standard curves were analyzed using a four-parameter fit with a 1/Y² weighting. Data for Figures 5 and 8 were plotted in GraphPad Prism after analysis in MyAssays Desktop Software for visualization of the data.

Results and Discussion

Validation of Standard Curves Using the VICTOR Nivo

Eight standard DNA samples are included with the Quant-iT® broad range and high sensitivity dsDNA kits to be used as a reference for quantifying the unknown DNA samples. The standard protocol for these kits is to use 10 µL for the standard curve. However, to accurately quantify the DNA samples using the standard curve, we kept the same volume between the standard curve and the unknown samples. To preserve sample, we chose 2 µL for each (instead of 10 µL) and validated the assay for the standard curves. In addition, to make the assay automation friendly, the Quant-iT® reagent was lowered from 198 µL (as suggested in the manual) to 158 µL (for a total volume of 160 µL). Using MyAssays Desktop software to analyze the data, we obtained $R^2$ values of > 0.999 for both standard curves. Figures 3 and 4 show the fitted standard curves and the data output from MyAssays Desktop software. For both kits, we see low % CVs (<10%) for all samples and high accuracy for the measurements (95-105%).

Figure 3. Representative data from a standard curve using Quant-iT® broad range dsDNA kit analyzed with MyAssays Desktop software. A) Standard curve plotted in MyAssays Desktop Software and exported to Excel. B) Table of analysis from My Assays Desktop software. "Positions" refers to the position in the 96-well assay plate. "Conc. (Defined)" is the defined concentration in the standard curve. "Fl (Average)" is the average of the fluorescence units measured on the VICTOR Nivo. "Fl" refers to the relative fluorescence units. "Conc. (Calc.)" is the interpolated concentration for each replicate from the plotted standard curve. "%CVs" is the percent of coefficient variation from the replicates in the standard curve. The "% Accuracy" is determined by back-calculating the average interpolated concentration to the known concentration of the standard.

Figure 4. Representative data from a standard curve using Quant-iT® high sensitivity dsDNA kit analyzed with MyAssays Desktop software. A) Standard curve plotted in MyAssays Desktop Software and exported to Excel. B) Table of analysis from My Assays Desktop software. See Figure 3 legend for abbreviation explanation.
Quantifying Unknown Standard DNA Samples

After validation of the standard curves, we took a DNA standard from each kit (80 ng/µL for Quant-iT® dsDNA assay kit broad range and 10 ng/µL for the Quant-iT® dsDNA assay kit high sensitivity) and serially diluted them 2-fold into TE buffer. We then tested how well the VICTOR Nivo could quantify the concentrations using MyAssays Desktop software. As shown in Figure 5A, samples that had been taken from a serial dilution of the 80 ng/µL were accurately quantified using the Quant-iT® dsDNA assay kit broad range.

Figure 5B shows representative data from the serial dilution of the 10 ng/µL sample using Quant-iT® dsDNA Assay kit high sensitivity. In both cases, results were obtained with high percent accuracy (95-105%) except for samples that were below the lower limit of detection of the kit (<5 ng/µL for broad range kit and <0.5 ng/µL for the high sensitivity kit), which in both cases slightly overestimated the concentration of the samples.

Quantification of Genomic DNA Samples

Since gDNA samples are more challenging to quantify due to heterogeneity and the dynamic nature of the samples, we tested quantification of serial dilutions (2-fold) of gDNA samples (NA12878) using Quant-iT® broad range for the first seven dilutions (1-7) and Quant-iT® high sensitivity kit for dilutions 4-10. Each sample was pipetted in replicates of four. High reproducibility was obtained for the relative fluorescence unit of each replicate (“Fl” in Figures 6 and 7). Using MyAssays Desktop software, the concentrations were determined and then corrected for the dilution factor to determine the initial concentration of the gDNA sample. The reported concentration of the sample from the vial was 365 ng/µL. The average corrected concentration using the Quant-iT® broad range kit (Figure 6) was 332 ng/µL. This correlates well with the average concentration obtained for the Quant-iT® high sensitivity samples (Figure 7), which was 335 ng/µL.

Comparison with Tube-Based DNA Quantification

Standard DNA quantification is typically done with tube-based fluorescence assays such as Qubit®. Qubit® accurately measures dsDNA concentrations but is time consuming when trying to measure multiple samples in replicate. The Quant-iT® broad range and high sensitivity dsDNA kits in combination with the VICTOR Nivo is ideal for both high throughput amounts of samples and accuracy. To verify that the concentrations measured in the assays above correlate with other methods, we measured a select number of samples with Qubit® and compared them with the values measured on the VICTOR Nivo and quantified with MyAssays Desktop software (Figure 8). Due to the time-consuming workflow of the Qubit®, the select samples were only measured once without replicates. As shown in Figure 8, we see a high correlation (>75%) between all samples tested and > 85% correlation for samples within the dynamic ranges of the kits.

Figure 5. Expected DNA concentrations compared with measured DNA concentrations as quantified using the VICTOR Nivo and MyAssays Desktop Software.
**Figure 6.** Calculated values for gDNA using Quant-iT® dsDNA assay kit broad range and analyzed using MyAssays Desktop software. "Positions" refers to the position in the 96-well assay plate. "Factor" is the dilution factor from the stock gDNA. "Fl" refers to the relative fluorescence units measured on the VICTOR Nivo. "Concentration" is the interpolated concentration from the standard curve on the plate. "Corrected" is the concentration multiplied by the dilution factor to obtain the original stock concentration of gDNA.

**Figure 7.** Calculated values for gDNA using Quant-iT® dsDNA assay kit high sensitivity and analyzed using MyAssays Desktop software. "Positions" refers to the position in the 96-well assay plate. See Figure 6 legend for explanation of abbreviations.

**Figure 8.** Quantification using Quant-iT® on the VICTOR Nivo shows correlation with Tube-Based Qubit®.
Conclusions

In this application note, we show how fast and easy it is to combine fluorescent based dsDNA quantification reagents with our multimode plate reader, the VICTOR Nivo, and PerkinElmer’s liquid handler, the Zephyr G3 Workstation. By modifying the Quant-iT® protocols, we were able to show precise measurements of the standard curves and accurately quantified a dilution series of standard purified DNA and gDNA samples. We showed that the measurements we obtain are in correlation with the tube-based Qubit®. Tube-based methods are time consuming for quantifying multiple samples. The protocol recommends moving to the Quant-iT kit when more than 20 samples are being used. Using the Zephyr G3 NGS workstation, we can accurately quantify 24 samples in replicates of three in a 96-well plate with a standard curve in as little as 20 minutes, whereas tube-based Qubit is significantly more time consuming to perform the same measurements without replicates. In addition, we show how MyAssays Desktop software can easily be integrated into the DNA quantification workflow to give immediate accurate results for measured concentrations.

References