APPLICATION NOTE

High-throughput DNA quantification for next generation sequencing on the VICTOR Nivo.

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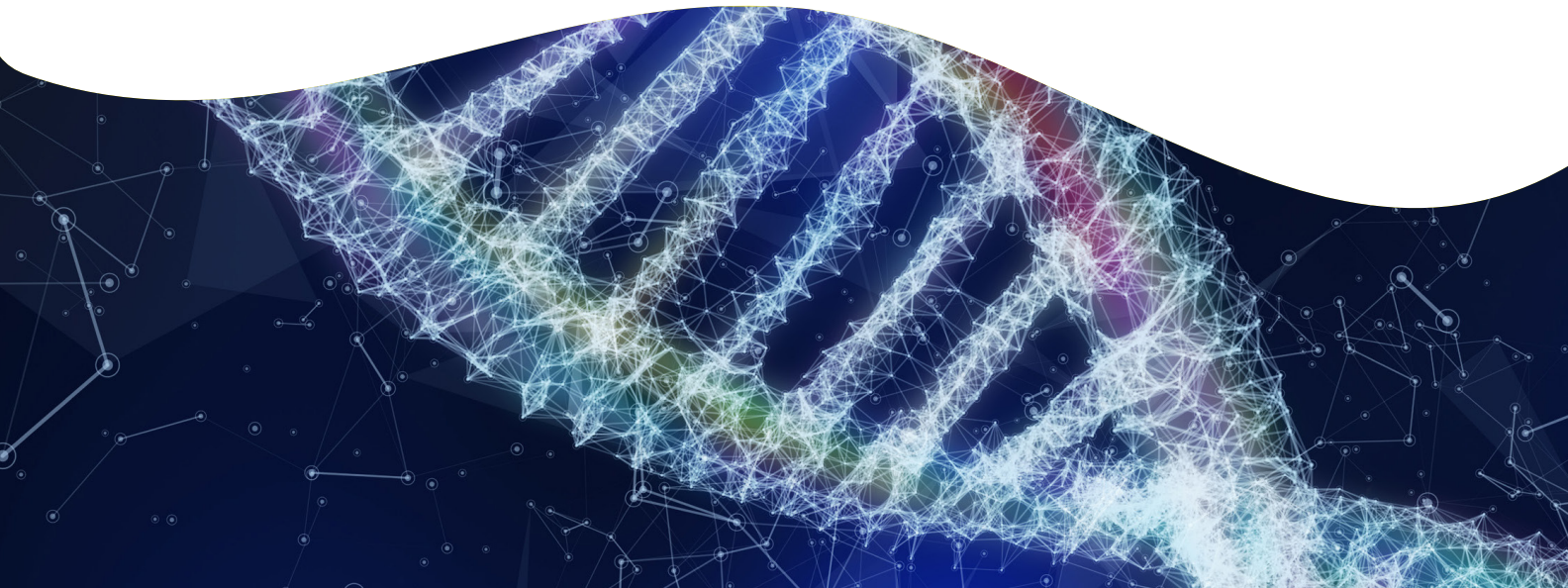
Introduction

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.[1] 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.[2] 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 in 96-well StorPlates-96V (#6008290). Then samples were transferred to a 96-well Hard-Shell Assay plate (Bio-Rad # 6008870). Finally, the Quant-iT® working solutions and samples were mixed and read in a 96-well Black OptiPlate (#6007270).

Figure 1. VICTOR Nivo Plate Reader.

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.

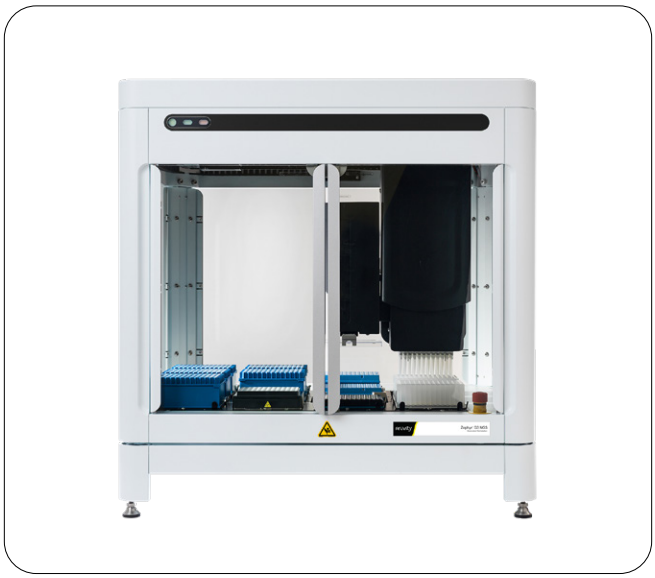


Figure 2. Zephyr G3 Automated Workstation.

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 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 uL) 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 R2 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%).

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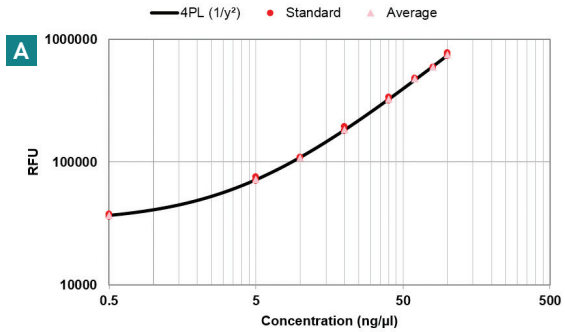

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Calibrator	Positions	Conc. (ng/ul) (Defined)	FI	FI (Average)	Conc. (ng/ul) (Calc.)	%CV (Conc. Calc.)	% Accuracy
Standard1	A1	0.5	35755	36856	0.36	24.64	98.2801
	A2		37706		0.59		
	A3		37106		0.52		
Standard2	B1	5	75607	72555	5.48	6.91	101.481
	B2		71294		4.91		
	B3		70765		4.84		
Standard3	C1	10	109959	108657	10.07	1.70	98.9635
	C2		107480		9.74		
	C3		108532		9.88		
Standard4	D1	20	193832	184229	21.58	5.76	101.224
	D2		180757		19.76		
	D3		178098		19.40		
Standard5	E1	40	~~Flagged~~	317624	(NA)	0.80	97.3901
	E2		319170		39.18		
	E3		316078		38.74		
Standard6	F1	60	477703	477061	61.85	0.85	102.931
	F2		480343		62.23		
	F3		473136		61.19		
Standard7	G1	80	596128	590495	79.00	0.94	97.7251
	G2		588972		77.96		
	G3		586384		77.58		
Standard8	H1	100	778591	749327	105.68	3.70	101.385
	H2		738169		99.75		
	H3		731220		98.73		

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. "FI" refers to the relative fluorescence units measured on the VICTOR Nivo. "FI (Average)" is the average of the fluorescence units of the three replicates. "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

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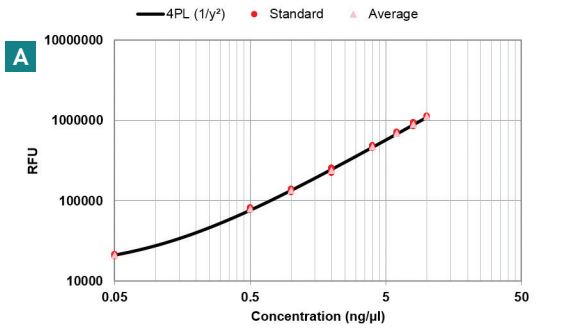

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Calibrator	Positions	Conc. (ng/ul) (Defined)	FI	FI (Average)	Conc. (ng/ul) (Calc.)	%CV (Conc. Calc.)	% Accuracy
Standard1	A1	0.05	20347	21099	0.04	10.07	98.5601
	A2		21593		0.05		
	A3		21356		0.05		
Standard2	B1	0.5	79797	79466	0.52	5.59	104.278
	B2		82767		0.55		
	B3		75834		0.49		
Standard3	C1	1	127416	134533	0.93	5.42	99.0733
	C2		138907		1.03		
	C3		137275		1.01		
Standard4	D1	2	242115	240573	1.94	8.34	96.1443
	D2		257755		2.08		
	D3		221849		1.76		
Standard5	E1	4	461464	468582	3.93	4.82	99.863
	E2		492063		4.21		
	E3		452219		3.84		
Standard6	F1	6	722926	694216	6.37	3.90	101.645
	F2		675606		5.92		
	F3		684115		6.00		
Standard7	G1	8	845137	891525	7.53	6.21	99.6117
	G2		881935		7.88		
	G3		947503		8.50		
Standard8	H1	10	1133136	1113622	10.29	1.67	100.989
	H2		1108176		10.05		
	H3		1099555		9.96		

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.

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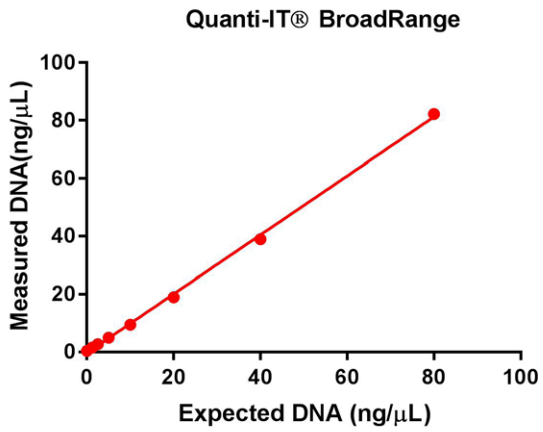

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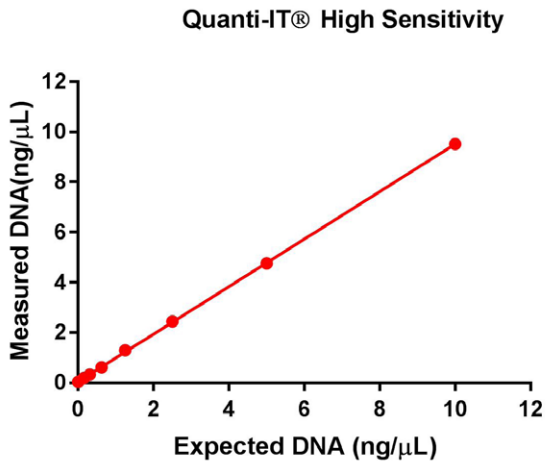

Quant-iT® dsDNA assay kit broad range		
Expected Value (ng/µL)	Measured Value (ng/µL)	% Accuracy
80	82.23	102.8
40	38.96	97.4
20	18.95	94.8
10	9.48	94.8
5	4.94	98.8
2.5	2.75	108.8
1.25	1.52	121.6
0	0.37	-

Quant-iT® dsDNA assay kit high sensitivity		
Expected value (ng/µL)	Measured value (ng/µL)	% Accuracy
10	9.51	95.1
5	4.76	95.2
2.5	2.44	97.6
1.25	1.29	103.2
0.625	0.61	97.6
0.3125	0.32	102.4
0.156	0.19	121.8
0	0.03	-

Figure 5. Expected DNA concentrations compared with measured DNA concentrations as quantified using the VICTOR Nivo and MyAssays Desktop Software.

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 seral 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 ("FI" 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.

Sample	Positions	Factor	FI	Concentration (ng/ul)	Corrected (ng/ul)
NA12878-1	A9	2	1549642	224.21	448.42
	A10		1547425		
	A11		1583992		
	A12		1608557		
NA12878-2	B9	4	584416	78.08	312.31
	B10		587351		
	B11		581649		
	B12		605716		
NA12878-3	C9	8	325962	39.58	316.63
	C10		317931		
	C11		325022		
	C12		319142		
NA12878-4	D9	16	176906	20.05	320.83
	D10		179212		
	D11		181077		
	D12		194151		
NA12878-5	E9	32	105777	9.96	318.62
	E10		107463		
	E11		110046		
	E12		113126		
NA12878-6	F9	64	71993	5.11	327.33
	F10		71786		
	F11		73643		
	F12		74033		
NA12878-7	G9	128	47988	2.17	277.79
	G10		50125		
	G11		52032		
	G12		50829		
TE Buffer	H9	1	35463	0.32	0.32
	H10		35238		
	H11		35472		
	H12		35636		

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. "FI" 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

Sample	Positions	Factor	FI	Concentration (ng/ul)	Corrected (ng/ul)
NA12878-4	A9	16	1902606	24.59	393.50
	A10		2029705		
	A11		1848033		
	A12		2149641		
NA12878-5	B9	32	917592	11.91	381.14
	B10		1027927		
	B11		1022967		
	B12		1007170		
NA12878-6	C9	64	470299	5.65	361.71
	C10		511595		
	C11		519168		
	C12		463439		
NA12878-7	D9	128	206039	2.24	286.67
	D10		208406		
	D11		202270		
	D12		215272		
NA12878-8	E9	256	116701	1.14	291.75
	E10		114805		
	E11		108728		
	E12		114445		
NA12878-9	F9	512	59992	0.57	291.41
	F10		60448		
	F11		68020		
	F12		65199		
NA12878-10	G9	1024	40971	0.33	337.74
	G10		40068		
	G11		42982		
	G12		43201		
TE Buffer	H9	1	14865	0.05	0.05
	H10		15351		
	H11		16331		
	H12		15569		

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.

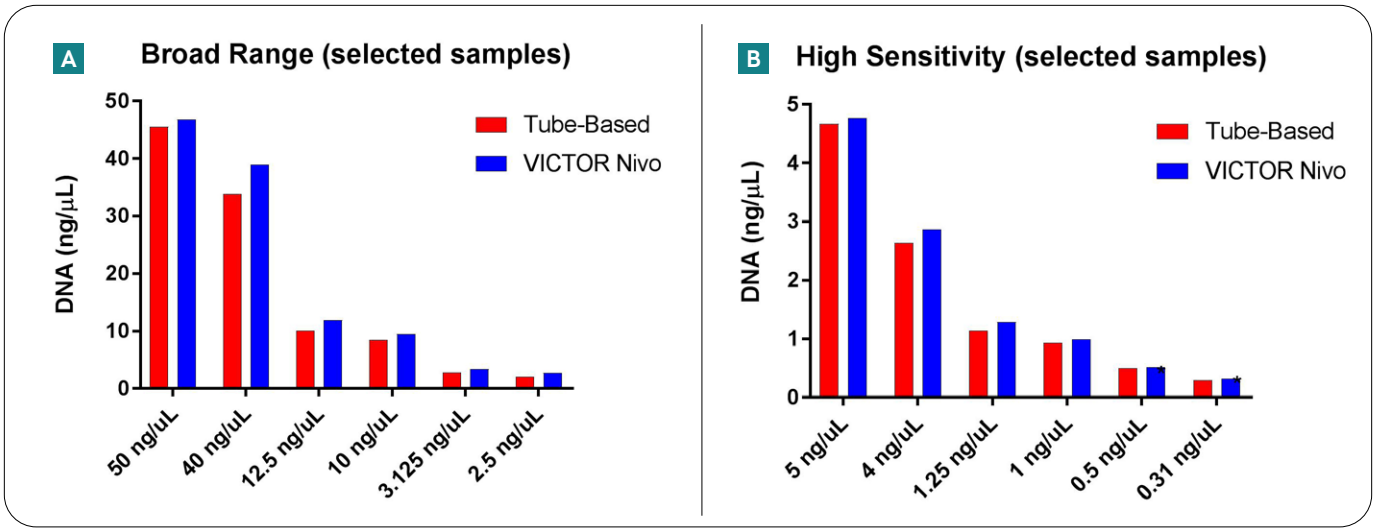

Figure 8. Quantification using Quant-iT® on the VICTOR Nivo shows correlation with Tube-Based Qubit®.

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 Revvity's liquid handler, the Zephry 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

1. Rapid and Simple Quantification of DNA and Protein Using the VICTOR Nivo Multimode Plate Reader. Revvity Application Note 2017.

2. High-throughput Quantification of DNA for NGS Library Prep with the Zephyr G3 Workstation. Revvity Application Note 2018.

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