How pipetting choice and volume affect results of nucleic acid quantitation

Introduction

Many molecular biology workflows, including next-generation sequencing (NGS) and PCR, rely on accurate quantitation of DNA and RNA samples. Invitrogen™ Qubit™ DNA and RNA quantitation kits offer quick results that users can trust. One benefit of the Qubit assays is that only small sample volumes (1– 20 µL) are required for quantitation. However, as with any nucleic acid quantitation method, the accuracy and precision* of sample pipetting can have a profound effect on the assay results, particularly when low sample volumes are used. Here we use the Invitrogen™ Qubit™ 1X dsDNA HS Assay Kit to investigate the role of pipetting when quantitating low-volume nucleic acid samples.

Method

The Qubit 1X dsDNA HS Assay Kit (Cat. No. Q33230) was used to analyze sample solutions of DNA that spanned the dynamic range of the assay (0.2–100 ng). Three concentrations of λ DNA were prepared: 0.2, 2, and 20 ng/µL. The DNA samples were added to the 1X dsDNA HS Working Solution in volumes of 1 or 2 µL using a 2 or 10 µL pipette, for a final volume of 200 µL in a Qubit tube. Each Qubit tube was vortexed to ensure thorough mixing. The samples were then incubated for 2 minutes before being read on the Invitrogen™ Qubit™ 4 Fluorometer (Cat. No. Q33226). All data were normalized to the mean of each set of replicates, to address the precision (sample-to-sample variation) of the measurements.

The traditional pipetting method was compared to reverse pipetting—a technique that can improve pipetting accuracy and precision of small volumes—using a 10 µL pipette. For traditional pipetting, 1 or 2 µL was drawn into the pipette tip by pressing the operating button to the first stop to fill the tip, and the entire volume was displaced. For reverse pipetting, a larger volume of sample was drawn into the pipette tip by pressing the operating button to the second stop to fill the tip, and only 1 or 2 µL of the volume was displaced (Figure 1).

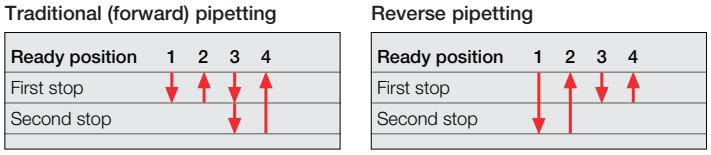

Figure 1. Traditional and reverse pipetting methods. In traditional pipetting, the operating button on the pipette is pressed to the first stop to fill the tip (ready positions 1 and 2). When the sample is dispensed, the operating button is pressed to the second stop to completely empty the tip (ready position 3). In reverse pipetting, the operating button is pressed to the second stop to fill the tip with a larger volume of sample than needed (ready positions 1 and 2). To dispense the sample, the operating button is only pressed to the first stop to deliver the desired amount (ready position 3), and the remainder is discarded with the tip.

To further demonstrate the importance of pipetting methods to precision, solutions of Invitrogen™ Alexa Fluor™ 488 dye were prepared and analyzed on the Qubit 4 Fluorometer. Three concentrations of the Alexa Fluor 488 dye were prepared to replicate the fluorescence obtained from the Qubit 1X dsDNA HS Assay experiments. Then, using either a 2 or 10 µL pipette and the traditional pipetting method, 1 µL of Alexa Fluor 488 dye solution was transferred to a Qubit tube containing 199 µL of 10 mM TE, pH 7.5. The samples were mixed thoroughly by vortexing, and 8 replicates of each sample were measured on the Qubit 4 Fluorometer.

* Accuracy refers to the degree of closeness of measurements of a quantity to that quantity's true value. Precision is the degree to which repeated measurements under unchanged conditions show the same results. (Wikipedia, "Accuracy and precision", July 12, 2018)

Results

Results are summarized in Table 1 and Figure 2. In the figure, each dot represents a single measurement, and eight replicate measurements were obtained for each of the three sample concentrations. Samples prepared with a 10 µL pipette were the most variable and contained significant outliers. Samples prepared using a 2 µL pipette were very precise by comparison. The measured sample variations (CV, calculated as the standard deviation of the sample set divided by the mean) for the two approaches was 73% for samples prepared with a 10 µL pipette, compared to 6% for samples prepared with a 2 µL pipette, when analyzing 1 µL samples using the traditional pipetting method (Table 1).

Table 1. Summary of data comparing low-volume pipetting methods.

Pipette	Volume	Method	CV (%)
10 µL	1 µL	Traditional	73
	2 µL		18
	1 µL	Reverse	11
	2 µL		13
2 µL	1 µL	Traditional	6
	2 µL		3

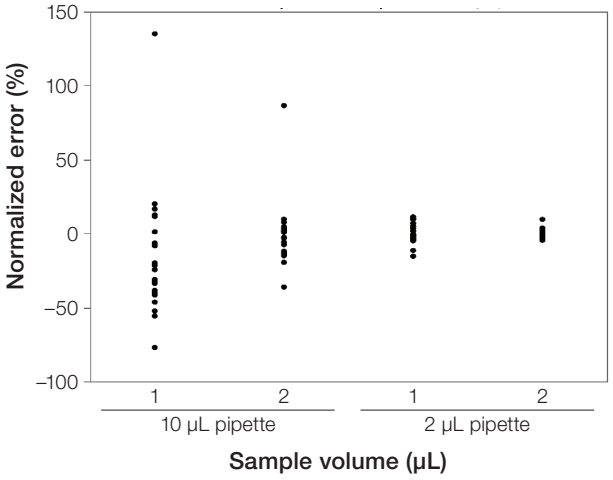

Figure 2. Precision of Qubit assay measurement when varying the sample volume and pipette using the traditional pipetting method. Precision was measured as a function of the deviation from the mean of replicates. This was performed for each concentration tested and each pipette and volume used. The data highlight that using a 10 µL pipette to dispense 1 or 2 µL samples was significantly less precise than using a 2 µL pipette for the same sample volumes. Additionally, with either pipette, using a larger sample volume increased measurement precision.

Sample volume is an important variable to consider when preparing samples for analysis, regardless of the quantitation system or type of assay being performed. When using a 10 µL pipette and traditional pipetting to prepare samples for the Qubit 1X dsDNA HS Assay, the precision increased from a CV of 73% to 18% by increasing the sample volume from 1 to 2 µL. This trend was also true for samples prepared with a 2 µL pipette; however, the difference was less pronounced, as the precision increased from a CV of 6% to 3% (Table 1).

If one does not have access to a 2 µL pipette and has limited sample, reverse pipetting offers increased precison over the traditional pipetting technique. In reverse pipetting, more sample is drawn into the pipette tip than needed, and only a fraction of that volume is dispensed. The use of larger volumes in the pipette tip increases accuracy by diminishing error due to capillary action and droplet tension. Samples were prepared using either the traditional or reverse pipetting technique with a 10 µL pipette. The reverse pipetting method provided notably increased precision and decreased the measured variation (CV) from 45% to 11%. Additionally, no significant outliers were observed with the reverse pipetting technique (Figure 3).

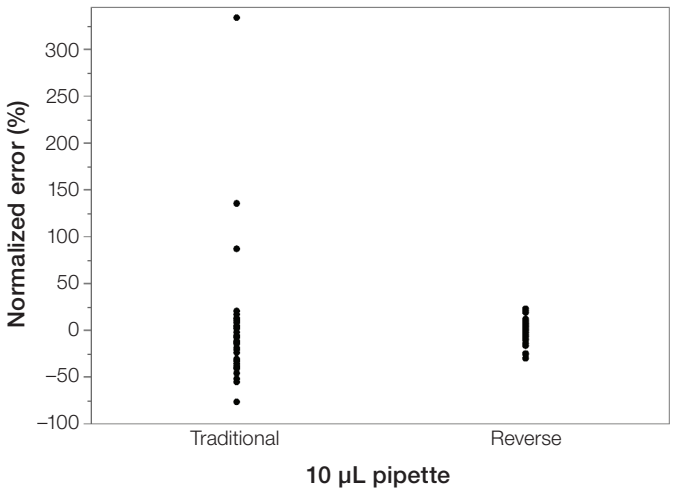

Figure 3. Precision of Qubit assay measurements with a 10 µL pipette using traditional versus reverse pipetting of 1 µL. When using a 10 µL pipette, a user's technique can have a profound effect on the precision of their measurements. Data were collected using a 10 µL pipette where samples were prepared by either traditional or reverse pipetting. For 1 µL samples, reverse pipetting increased the precision significantly (see Table 1).

If low-volume samples are required, using a 2 µL pipette will provide the greatest precision (Figure 2). Measurements of the 1 µL samples prepared with a 2 µL pipette exhibited a significantly lower CV (6%) than a 10 µL pipette (73%) using the traditional pipetting method (Table 1). Additionally, using an increased sample volume of 2 µL improved the precision of the results to a CV of 3% (Figure 4). Interestingly, the concentration of the stock solution did not play a role in the measurement's precision (Figure 5). The dispersion of the measurements appears consistent across the three sample concentrations when analyzed for samples prepared with the 2 µL pipette, regardless of whether 1 or 2 µL sample volumes were used.

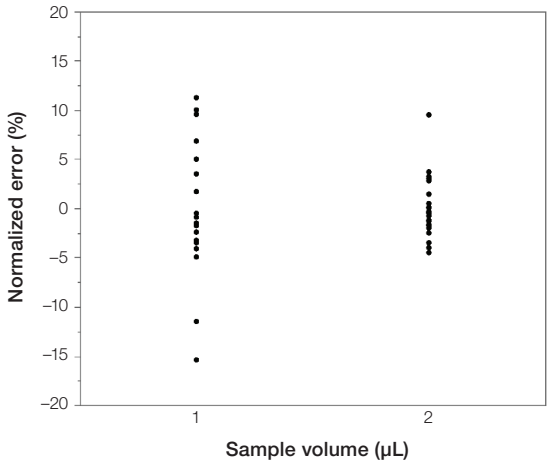

Figure 4. Precision of Qubit assay measurements when using a 2 µL pipette and different sample volumes. Using the traditional pipetting method and a 2 µL pipette, samples prepared with either 1 or 2 µL of DNA were compared. The resulting CVs were 6% for 1 µL samples and 3% for 2 µL samples, suggesting that greater precision and confidence can be obtained if larger sample volumes are used in the Qubit assay.

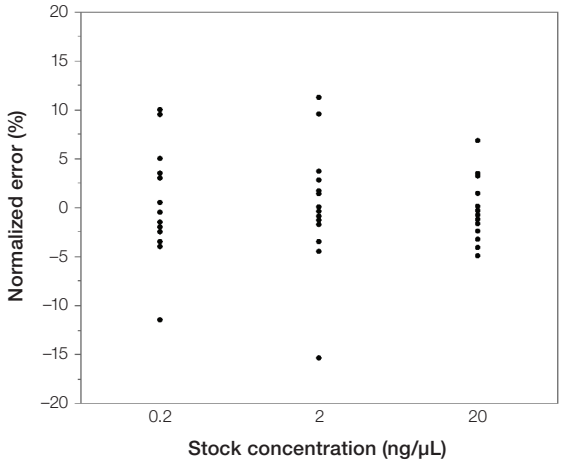

Figure 5. Precision of Qubit assay measurements with different sample concentrations when using a 2 µL pipette and traditional pipetting. Three concentrations of λ DNA were prepared, and samples of 1 or 2 µL were analyzed for deviation from the mean. The data show that sample concentration had a minimal effect on precision of the results.

To demonstrate the universality of these observations, Alexa Fluor 488 dye solutions of different concentrations were prepared and analyzed on the Qubit 4 Fluorometer. In line with the previous observations, the measurements from samples prepared using a 10 µL pipette contained significant outliers and had a CV of 61%. For samples prepared using a 2 µL pipette, the precision of the measurements significantly improved to a CV of 10% (Figure 6).

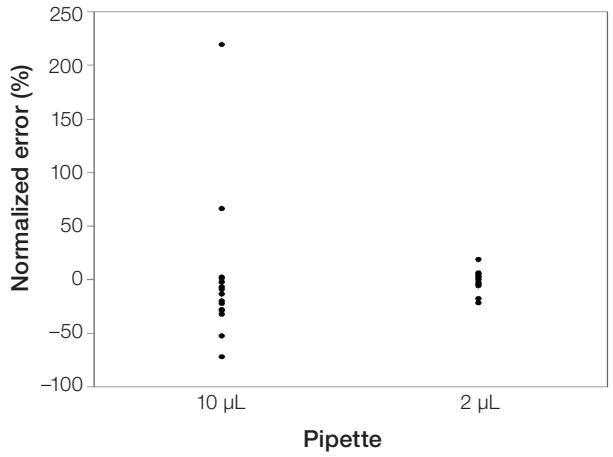

Figure 6. Precision of measurements of 1 µL of Alexa Fluor 488 dye when using a 2 µL or a 10 µL pipette. To assess whether the observations described previously are unique to the Qubit 1X dsDNA HS Assay, dilutions of a known fluorescent dye were prepared. Samples were prepared with 1 µL of each dilution using a 10 µL and 2 µL pipette and the traditional pipetting technique. In line with the Qubit 1X dsDNA HS Assay results, Alexa Fluor 488 samples prepared with a 2 µL pipette were more precise (CV 10%) than those prepared with a 10 µL pipette (CV 61%).

Conclusion

Here we used the Qubit 1X dsDNA HS Assay to demonstrate that pipetting technique can significantly affect the precision of low-volume assay measurements, a common issue in many assays that rely on accurate pipetting of small sample volumes. For the best precision with nucleic acid quantitation methods, such as the Qubit assays, use larger sample volumes and a 2 µL pipette when possible. If neither option is possible, consider using reverse pipetting to get the best precision for your measurements.

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