Promega

Automating dsDNA Quantitation Using Promega QuantiFluor® Chemistries and Tecan Freedom EVO® System

Promega Corporation

Protocols: *QuantiFluor® dsDNA System Technical Manual* #TM346, *QuantiFluor®* ONE *dsDNA System Technical Manual* #TM405, *Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Setup Protocol for the Tecan Freedom EVO® Workstation* #EP055 and *GloMax® Discover System Technical Manual* #TM397 are available at: **www.promega.com/protocols/**

Introduction

DNA quantitation is a critical step in a number of downstream molecular biology applications such as next-generation sequencing, quantitative PCR and transfection. Achieving selective and sensitive quantitation of double-stranded DNA (dsDNA) using spectrophotometric methods can be problematic. Recognizing these difficulties, researchers have incorporated fluorescence-based quantitation techniques in their workflow to obtain the required sensitivity and target selectivity. To meet the needs of laboratories using high-throughput automated systems to extract and quantitate nucleic acids, we have developed an automated method for dsDNA quantitation using Promega QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems with the Tecan Freedom EVO® liquid handler. Automation with the Tecan Freedom EVO® platform provides walkaway quantitation assay setup for up to 88 unknown samples in approximately 45 minutes.

Here we describe an automated quantitation method using the QuantiFluor® dsDNA Dye Systems with the Tecan Freedom EVO® liquid handler and the Promega GloMax® Discover Detection System.

Materials and Methods

- QuantiFluor® dsDNA System (Cat.# E2670) or QuantiFluor® ONE dsDNA System (Cat.# E4870)
- GloMax® Discover System (Cat.# GM3000)
- Nuclease-Free Water (Cat.# P1197)
- K562 Genomic DNA (Cat.# E4931; recommended if quantitating human genomic DNA samples)
- 20X TE Buffer (pH 7.5) (Cat.#A2651; required if quantitating samples that require dilution when using the QuantiFluor® ONE dsDNA System)
- Four-Position Tube Holder (Cat.#V1601)

The optimal base configuration to implement the QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems is the Freedom EVO® 100 (or larger) platform with an 8-channel disposable tip LiHa Arm, a RoMa Arm and a Te-Shake™ shaker. Additional hardware required for performing the chemistry includes:

- LiHa wash station (if configuration includes a liquid-displacement LiHa Arm)
- disposable tip waste chute
- 1 × 100ml trough carrier (3-position)
- 3-position disposable tip carrier
- 6 microcentrifuge tube racks (if samples are presented in 1.5 or 2ml tubes)
- Te-Shake™ microplate shaker unit and Te-Shake™ mounting plate
- 3-position microplate carrier

The following consumables are required for each automated quantitation:

- 1.1ml, Square-Well, V-Bottom Deep Well Plate (Cat.# V6821)
- 3 × 100ml gray polypropylene disposable troughs
- 1 × 25ml maximum recovery gray polypropylene trough
- 1 × 96 well black flat-bottom polystyrene microplate
- 200µl LiHa Disposable Tips (filtered)
- 50µl LiHa Disposable Tips (filtered)

Tecan part numbers are available in the *Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Setup Protocol for the Tecan Freedom EVO® Workstation* #EP055.

Caution: We recommend the use of gloves, lab coats and eye protection when working with these and any chemical reagents.

The automated method was developed and tested on a Tecan Freedom EVO® 200 configured with a Te-Shake™ shaker, RoMa Arm and liquid-displacement LiHa Arm with either eight 500µl syringes or eight 1ml syringes. The method is adaptable for Tecan Freedom EVO® instruments configured with a liquid-displacement LiHa Arm with 250µl syringes, an air-displacement LiHa Arm or other compatible orbital shakers. Contact Promega for more information.

Workflow

Guided by either the TouchTools™ graphical user interface (Figure 1) or a series of prompts, the user is asked to specify the following:

- QuantiFluor® System (QuantiFluor® dsDNA System or QuantiFluor® ONE dsDNA System)
- number of standard curve replicates (1, 2 or 3) transferred to the assay plate
- concentration of the stock DNA standard
- standard type (optional)
- number of unknown samples
- sample source labware format (tubes or 96-well plate)
- highest estimated DNA concentration of the unknown samples to be quantitated

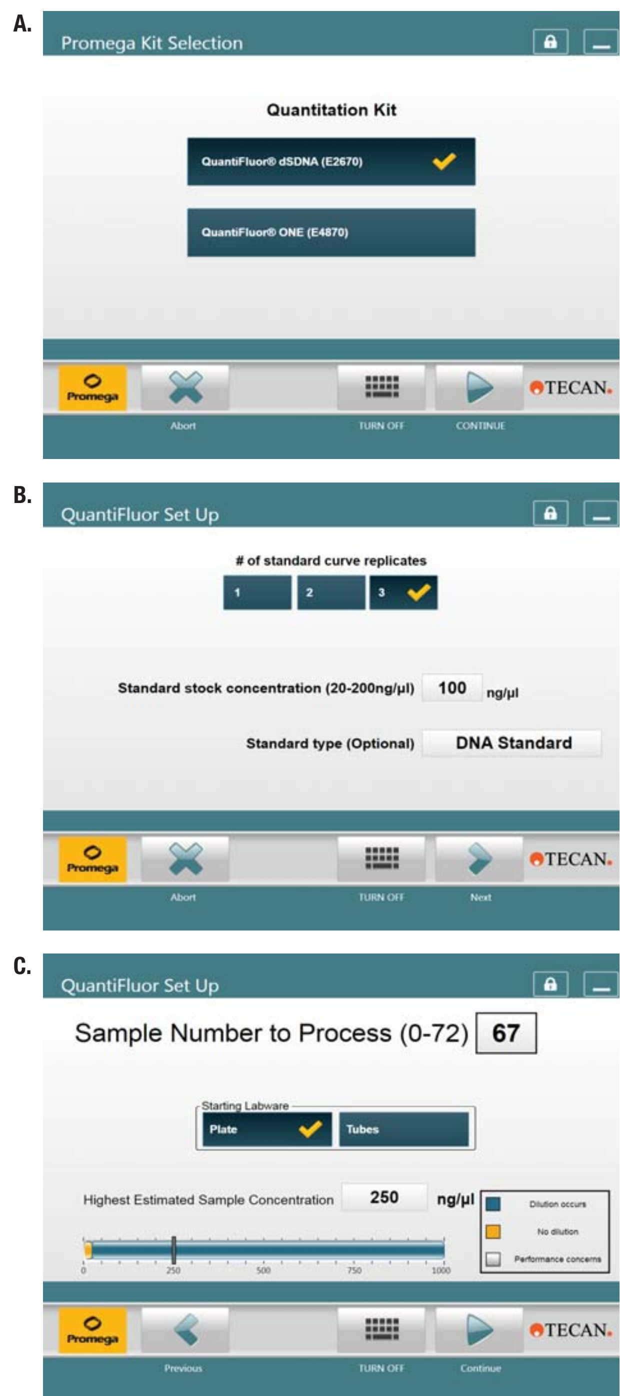

Figure 1. TouchTools™ graphical user interface. Panel A. Promega Kit Selection screen. **Panel B.** DNA Standard Selection screen. **Panel C.** Unknown Sample Selection screen.

Workflow (continued)

Using the user-provided information, the method determines the correct quantitation range based on the QuantiFluor® System and calculates the volumes required for serial dilution of the DNA standard. In addition, the method uses the provided estimated highest unknown sample DNA concentration to design a single dilution scheme to be used uniformly across the unknown samples. As all unknown samples are diluted in the same manner based on provided concentration estimate, quantitation of unknown samples with greatly varying concentrations within a single run may be problematic, resulting in overdilution of already-dilute unknown samples or underdilution of unknown samples more concentrated than the estimated concentration. Depending on the concentration estimate supplied by the user, all unknown samples will be directly transferred to the assay plate (Direct Sample Transfer) or diluted through a dilution plate before being transferred to the assay plate (Extended Sample Dilution; Figure 2). Depending on the dilution scheme required to bring the concentration of the unknown samples within the dynamic range of the assay, the method will consume as little as 2µl or as much as 10µl of the starting unknown samples.

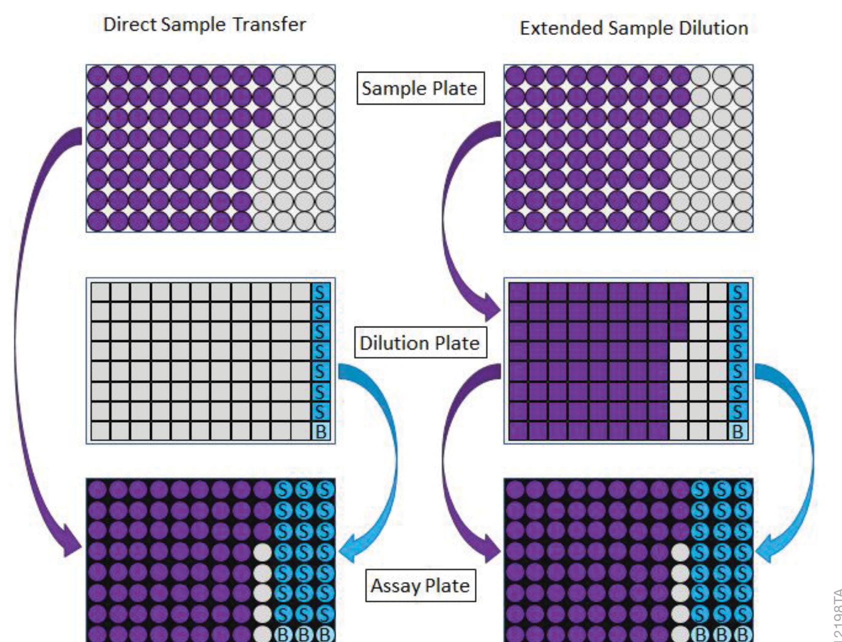

Figure 2. Sample dilution pathways. Direct transfer of unknown samples into assay plate (left). Extended dilution of unknown samples through a dilution plate prior to transfer into assay plate (right). Purple shading of a well indicates the presence of an unknown sample; purple arrows indicate the path of sample dilution. Blue shading of wells indicates the presence of a DNA standard (labeled with an "S") or reagent blank (labeled with a "B"); blue arrows indicate the path of standard curve preparation. Wells shown in shades of gray are empty. In this example, the user-specified values during setup are: plate as the starting sample labware, three replicates of the standard curve and 67 unknown samples.

After prompting for required setup information, the automated method indicates the placement of unknown samples, reagents and consumables on the deck of the Tecan Freedom EVO® Workstation (Figure 3). In the case of QuantiFluor® dsDNA System, the method provides detailed instructions for combining the 200X QuantiFluor® Dye and 1X TE Buffer to prepare a working solution of the dye. This dilution is not necessary with the QuantiFluor® ONE dsDNA System, as the dye is provided at the working concentration. With the deck setup complete, the method requires no further user intervention. The robot dilutes unknown samples (if necessary), prepares a serial dilution of the DNA standard, transfers all reagents, unknown samples and DNA standard dilutions to the assay plate and mixes the assay plate. Upon completion of the method, the assay plate is moved to a 96-well plate fluorescence reader capable of measuring excitation at 504nm and emission at 531nm. For the experiments discussed in this Application Note, the assay plate was manually transferred to the GloMax® Discover System with an excitation filter at 475nm and an emission filter at 500–550nm. The GloMax® Discover System may be integrated with the Tecan Freedom EVO® workstation and controlled using EVOware® software, if desired. Contact Promega for more information. Figure 4 shows representative DNA standard curves for each QuantiFluor® System, highlighting the ability of the Tecan instrument to pipet accurately and generate a linear standard curve.

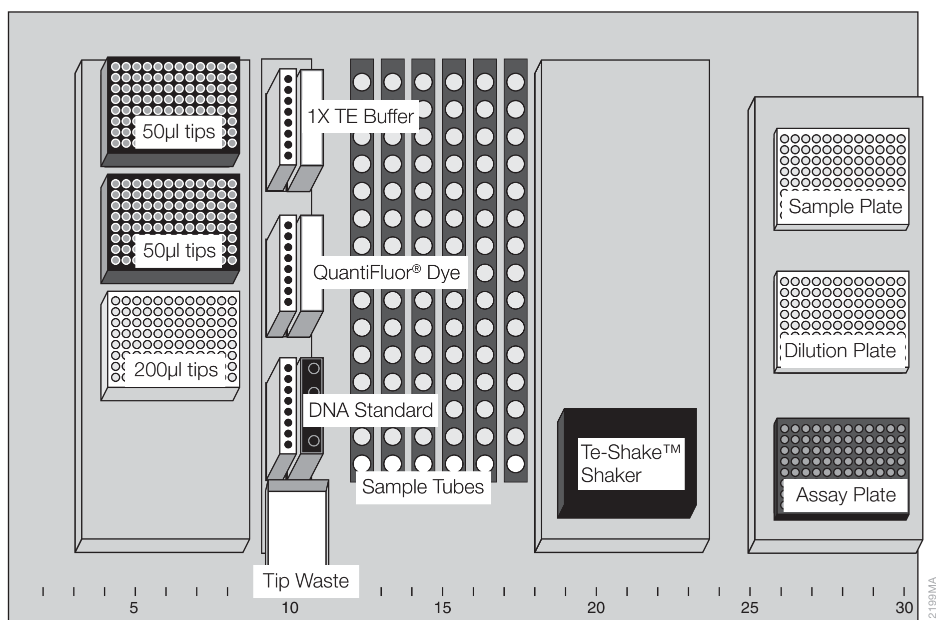

Figure 3. Example of a deck layout.

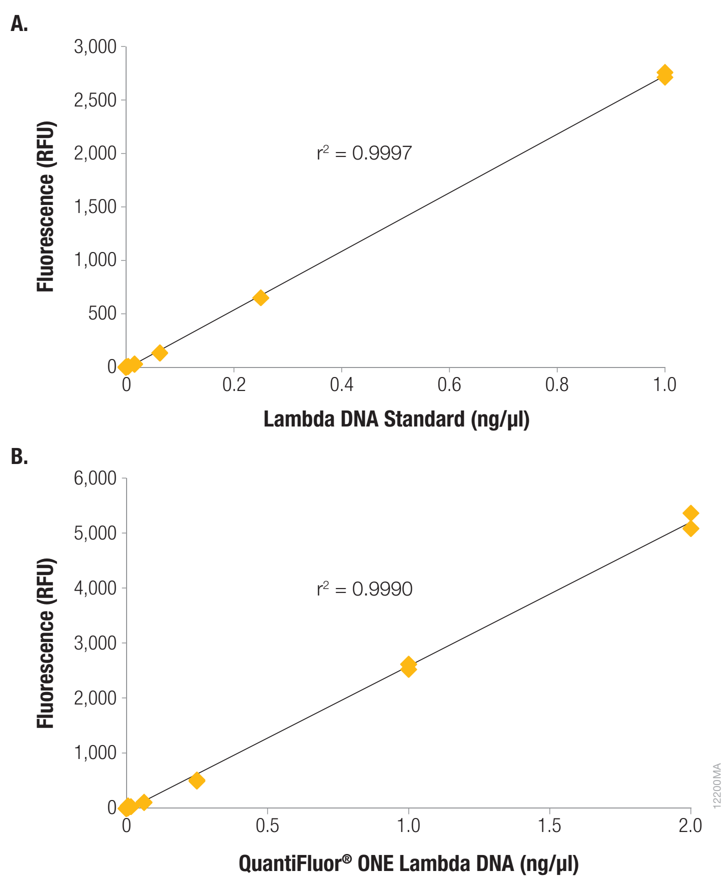

Figure 4. Representative DNA standard curves in a 96-well-plate format. Representative DNA standard curves in a 96-well-plate format generated using the QuantiFluor® dsDNA System (**Panel A**) or QuantiFluor® ONE dsDNA System (**Panel B**). Plotted on the X axis is the final concentration of the Lambda DNA Standard (Panel A) and the QuantiFluor® ONE Lambda DNA (Panel B) in the 96-well, 200µl assay format. Plotted on the Y axis is fluorescence in relative fluorescence units (RFU). Background fluorescence was subtracted from each data point. The curves shown each represent two replicates of the standard curve.

Results

Consistency of Serial Dilution of DNA Standard in Standard Curve Preparation

To assess the consistency of standard curve preparation, DNA quantitation results for a single DNA sample were compared across six identical automated runs. This DNA sample was prepared by manually diluting the Lambda DNA Standard approximately 20-fold in Nuclease-Free Water to a final concentration of ~5ng/µl. The Lambda DNA Standard also was used as the DNA standard at the supplied concentration of 100ng/µl. For each run (using the Tecan liquid handler equipped with 1ml syringes), the DNA sample was placed in each well of column 1 of the sample plate (n = 8). The following selections were used during setup of each run: QuantiFluor® dsDNA System, two replicates of standard curve, stock DNA standard concentration of 100ng/µl, eight samples to be processed and an estimated highest unknown sample DNA concentration of 5ng/µl. Based on the estimated concentration provided, the method directed the transfer of 10µl of each replicate of the DNA sample to the assay plate. Upon completion of each run, the concentration of each replicate of the DNA sample was calculated using linear regression (Table 1) as recommended in the *QuantiFluor® dsDNA System Technical Manual* #TM346. The calculated concentration of the DNA sample replicates was consistent (<15% CV) both within a single run and across the six runs. An analogous series of experiments was conducted with the QuantiFluor® ONE dsDNA System using the QuantiFluor® ONE Lambda DNA as both the DNA standard and the source of the single DNA sample. Comparable results were achieved (data not shown).

Table 1. Consistency of Standard Curve Preparation. Based on calculations performed during the method, the liquid handler transferred 10µl of each replicate of the DNA sample directly from the sample plate to the assay plate (total dilution factor of 20). Concentrations (ng/µl) of the DNA sample replicates were calculated based on linear regression analyses of the standard curves prepared in each run.

	Run Number						Inter-Run Average DNA Concentration (ng/µl; n = 6)	% CV (n = 6)
	1	2	3	4	5	6		
Sample Well	DNA Concentration (ng/µl)							
A1	5.92	6.34	6.48	6.56	6.25	6.87	6.41	5.0
B1	6.13	6.62	6.43	6.54	6.36	6.84	6.49	3.8
C1	5.99	6.32	6.53	6.53	6.45	6.87	6.45	4.5
D1	5.77	6.68	6.73	6.46	6.61	6.65	6.48	5.6
E1	6.05	6.25	6.40	6.44	6.35	6.75	6.37	3.6
F1	6.12	6.36	6.53	6.53	6.59	6.86	6.50	3.8
G1	6.15	6.43	6.63	6.60	6.69	7.02	6.59	4.4
H1	5.57	6.01	6.14	6.40	6.13	6.33	6.10	4.8
Intra-Run Average DNA Concentration (ng/µl; n = 8)	5.96	6.38	6.48	6.51	6.43	6.77	**Average DNA Concentration (ng/µl; n = 48)**	6.42
% CV (n = 8)	3.4	3.3	2.7	1.0	3.0	3.1	**% CV (n = 48)**	4.7

No Cross-Contamination During Automated Assay Setup

Potential well-to-well cross-contamination from the liquid-handling and mixing steps of the method was monitored using the Plexor® HY System (Cat.# DC1001). The Plexor® HY System is a sensitive real-time PCR assay that can be used to determine the amount of total human DNA in a reaction; it routinely detects approximately 6.4pg of DNA. In this experiment, sample blanks (TE^{-4} buffer; 10mM Tris-HCl [pH 8.0], 0.1mM EDTA) and human genomic DNA purified using ReliaPrep™ Large Volume HT gDNA Isolation System (Cat.# A2751) were manually dispensed to a 96-well sample plate in a checkerboard pattern as indicated in Figure 5, Panel A. The automated QuantiFluor® dsDNA method was conducted using the Tecan liquid handler equipped with 1ml syringes. When prompted during the method, TE^{-4} buffer was used in place of both the QuantiFluor® Dye and 1X TE buffer on the deck of the robot; TE^{-4} buffer is compatible with the Plexor® HY chemistry. The following selections were used during setup: QuantiFluor® ONE dsDNA System, two replicates of DNA standard curve, stock DNA standard concentration of 400ng/µl, 80 unknown samples to be processed and estimated highest unknown sample DNA concentration of 350ng/µl. Based on calculations performed during the method, the liquid handler transferred 2µl of each unknown sample to the dilution plate containing 48µl of TE^{-4} buffer per well, then 10µl of each diluted unknown sample to the assay plate (total dilution factor of 500). Upon completion of the run, the presence or absence of human genomic DNA in the final assay plate was assessed using Plexor® HY System (Cat.# DC1001) as described in the *Plexor® HY System for the Stratagene Mx3000P® and Mx3005P® Quantitative PCR Systems Technical Manual* #TM294. The quantification cycle (C_q) was used to analyze the qPCR results. The C_q value is the number of qPCR amplification cycles required for the fluorescence signal to cross a threshold level. The resulting C_q values are shown in Figure 5, Panel B, where "No C_q" indicates that the fluorescence signal did not cross the amplification threshold for 38 cycles, evidence that the wells contain no or too little DNA to detect using the Plexor® HY System. As seen in Figure 5, Panel B, each well to which DNA sample was added (Figure 5, Panel A) has a C_q value, whereas no C_q value was determined for the blank wells. This demonstrates that the automated procedure does not introduce sample-to-sample cross-contamination.

A.

	1	2	3	4	5	6	7	8	9	10
A	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank
B	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA
C	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank
D	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA
E	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank
F	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA
G	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank
H	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA	Blank	DNA

B.

	1	2	3	4	5	6	7	8	9	10
A	25.32	No C_q	24.98	No C_q	23.56	No C_q	23.10	No C_q	24.98	No C_q
B	No C_q	24.11	No C_q	25.01	No C_q	23.95	No C_q	23.83	No C_q	25.57
C	23.56	No C_q	24.63	No C_q	23.74	No C_q	23.27	No C_q	24.46	No C_q
D	No C_q	24.35	No C_q	25.03	No C_q	24.01	No C_q	24.12	No C_q	25.34
E	25.23	No C_q	23.77	No C_q	24.58	No C_q	22.93	No C_q	23.89	No C_q
F	No C_q	23.56	No C_q	24.32	No C_q	24.41	No C_q	23.01	No C_q	25.05
G	23.26	No C_q	23.09	No C_q	24.02	No C_q	24.04	No C_q	24.04	No C_q
H	No C_q	23.79	No C_q	25.28	No C_q	24.56	No C_q	24.69	No C_q	23.73

Figure 5. Assessing for cross-contamination during automated method. Panel A. Representation of sample plate used. Wells to which purified genomic DNA was added are labeled "DNA". Wells to which only TE^{-4} buffer was added are labeled "Blank". **Panel B.** Quantification cycle (C_q) results.

Consistency and Accuracy of Unknown Sample Dilution Process

To assess consistency and accuracy of unknown sample transfer and dilution by the Tecan Freedom EVO® workstation, we used the liquid handler with 500µl syringes to dilute and quantify several unknown samples with the QuantiFluor® dsDNA System. The following selections were used during setup: QuantiFluor® dsDNA System, three replicates of DNA standard curve, stock DNA standard concentration of 200ng/µl and an estimated highest unknown sample DNA concentration of 400ng/µl. Setting the highest estimated concentration to 400ng/µl necessitated extended dilution of the unknown samples: 2µl of each unknown sample was diluted with 158µl of 1X TE buffer in the dilution plate, then 10µl of the diluted unknown samples was added to 190µl of prepared QuantiFluor® Dye in the assay plate (for a total dilution factor of 1600). Each unknown sample was quantitated as eight replicates. The K562 Genomic DNA (Cat.# E4931) was manually diluted to 200ng/µl with 1X TE buffer and used as the stock DNA standard to generate the standard curve. The same standard curve was used to quantify all unknown samples. The average calculated DNA concentrations for the replicates of each unknown DNA sample and % CV values are shown in Table 2. Excellent consistency is seen for all unknown samples, with % CV values <10%, illustrating that, even with a dilution factor of 1600 and two transfer steps, the Tecan liquid handler consistently pipettes unknown samples. Quantitation results for the undiluted (400ng/µl) K562 Genomic DNA were within 10% of the expected result, highlighting the accuracy of the liquid handler.

Table 2. Consistency of Unknown Sample Dilution Process.

Sample	Average Calculated DNA Concentration (ng/µl; n = 8)	% CV (n = 8)
human genomic DNA purified from blood using ReliaPrep™ Large Volume HT gDNA Isolation System (Cat.# A2751)	465	4.0
bacterial genomic DNA purified from the JM109 strain using the Wizard® Genomic DNA Purification Kit (Cat.# A1125)	529	5.1
human genomic DNA purified from blood using Maxwell® 16 LEV Blood DNA Kit (Cat.# AS1290)	326	4.7
K562 Genomic DNA (Cat.# E4931; 400ng/µl[1])	436	7.0

[1]Concentration provided on the Promega product label and information sheet.

Conclusion

The data presented demonstrate that using the QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems with the Tecan Freedom EVO® Workstation offers the following advantages:

- verified performance with Tecan Freedom EVO® system equipped with 500µl and 1ml syringe configurations, adaptable to 250µl syringe configuration and the use of an air-displacement LiHa Arm
- excellent reproducibility of assay plate preparation
- user-friendly graphical user interface using Tecan TouchTools™ software
- flexibility to use either the QuantiFluor® dsDNA or QuantiFluor® ONE dsDNA System
- automated serial dilution of DNA standard
- choice of 1–3 replicates of the standard curve
- ease of automatically adjusting the unknown sample dilution scheme based on user-estimated highest unknown sample DNA concentration
- flexibility to use user-provided concentrated DNA standard or DNA standard provided with the system

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