AUTOMATED PROTOCOL

Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Setup Protocol for the Tecan Freedom EVO® Workstation

Instructions for Use of Products **E2670 and E4870**

For Research Use Only. Not for use in diagnostic procedures.

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Promega

Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Setup Protocol for the Tecan Freedom EVO® Workstation

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Automated Protocol.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

This document describes the automated protocol for the QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems on the Tecan Freedom EVO® laboratory automation workstation. This automated method performs sample dilutions and prepares 96-well assay plates for double-stranded DNA (dsDNA) quantitation for up to 88 unknown DNA samples per plate.

The automated method allows the user flexibility in standard curve setup and ease of use for unknown sample dilution. Guided by either the TouchTools™ Graphical User Interface (GUI) or a series of EVOware® prompts, the user first provides information for the method. Prompts then guide the user through the physical setup of the Tecan Freedom EVO® workstation. Users are asked to specify the following:

a. **Kit to be used for assay setup.** The user selects either the QuantiFluor® dsDNA or QuantiFluor® ONE dsDNA System. Kit choice defines both the final concentration of DNA standards in the assay plate and the dilution scheme for unknown samples.

b. **Number of standard curve replicates to be transferred to the assay plate.** This selection determines the maximum unknown sample number: up to 88 unknown samples if one replicate of the standard curve is specified, up to 80 unknown samples if two replicates of the standard curve are specified or up to 72 unknown samples if three replicates of the standard curve are specified. The standard curve consists of a reagent blank and dilutions of a DNA standard at seven different concentrations covering the linear range of the selected kit.

c. **Concentration of the DNA standard.** The user has flexibility to use the DNA standard provided in the QuantiFluor® System of choice or a user-preferred DNA standard.

d. **Sample number to process.** The user inputs the number of unknown samples to be quantitated from 0 unknown samples to the maximum unknown sample number described above.

e. **Sample labware source.** The user has the flexibility to supply unknown samples in tubes or a 96-well plate.

f. **Highest estimated unknown sample DNA concentration.** The user is asked to estimate the DNA concentration in ng/µl of the most concentrated unknown sample. Based on this concentration estimate, the method automatically determines which unknown sample dilution path will be used during the run: 1) direct dilution of the unknown sample in the assay plate or 2) an extended dilution of the unknown sample in a dilution plate before the final transfer to the assay plate. Section 7 describes the unknown sample dilution paths in more detail.

Prior to implementing this method on your workstation, contact Promega Technical Services at: **techserv@promega.com**

For troubleshooting QuantiFluor® dsDNA or QuantiFluor® ONE dsDNA chemistry issues, refer to the *QuantiFluor® dsDNA System Technical Manual* #TM346 or *QuantiFluor® ONE dsDNA System Technical Manual* #TM405. All Promega Technical Manuals are available at: **www.promega.com/protocols/**

For more information about the TouchTools™ software, contact Tecan.

2. Requirements and Product Storage Conditions

PRODUCT	SIZE	CAT.#
QuantiFluor® dsDNA System	**1ml**	**E2670**

This system contains sufficient reagents for 2,000 manual assays at 200µl scale. Includes:

- 25ml 20X TE Buffer (pH 7.5)
- 1ml QuantiFluor® dsDNA Dye, 200X
- 100µg Lambda DNA Standard, 100µg/ml

Note: Due to the extra volumes required for the automated method, fewer than 2,000 automated assays are possible with the volume of 200X Dye provided in the kit.

Storage Conditions: Product may arrive frozen. Upon receipt, store the QuantiFluor® dsDNA Dye at 2–10°C, protected from light. Store the Lambda DNA Standard, 100µg/ml, at 2–10°C. Do not refreeze the Lambda DNA Standard, 100µg/ml. Store the 20X TE Buffer at –30°C to +30°C.

If the Lambda DNA Standard, 100µg/ml, arrived frozen, a concentration gradient may have formed and should be dispersed upon thawing. Store the Lambda DNA Standard, 100µg/ml, at 2–10°C overnight, then warm to room temperature and mix well before use.

For handling and disposal instructions for the QuantiFluor® dsDNA Dye, refer to the *QuantiFluor® dsDNA System Technical Manual* #TM346.

PRODUCT	SIZE	CAT.#
QuantiFluor® ONE dsDNA System	**500 reactions**	**E4870**

This system contains sufficient reagents for 500 manual assays at a reaction volume of 200µl. Includes:

- 5 × 20ml QuantiFluor® ONE dsDNA Dye
- 400µg QuantiFluor® ONE Lambda DNA (400µg/ml)
- 25ml 1X TE Buffer (pH 7.5)

Note: The automated method dilutes standards and unknown samples into the linear range of the assay based on a user-provided concentration estimate. Depending on the volumes required for unknown sample dilution, additional 1X TE Buffer may be required. 20X TE Buffer (Cat.# A2651) and Nuclease-Free Water (Cat.# P1197) may be purchased separately and used to prepare 1X TE Buffer as indicated in Section 4.A.

Storage Conditions: Store QuantiFluor® ONE dsDNA Dye and QuantiFluor® ONE Lambda DNA at –30°C to +10°C. Store the 1X TE Buffer (pH 7.5) at –30°C to +30°C.

If the QuantiFluor® ONE Lambda DNA, 400µg/ml, arrived frozen, a concentration gradient may have formed and should be dispersed upon thawing. Store the QuantiFluor® ONE Lambda DNA, 400µg/ml, at 2–10°C overnight, then warm to room temperature and mix well before use.

For handling and disposal instructions for the QuantiFluor® ONE dsDNA Dye, refer to the *QuantiFluor® ONE dsDNA System Technical Manual* #TM405.

2. Requirements and Product Storage Conditions (continued)

Items Available Separately

PRODUCT	SIZE	CAT.#
20X TE Buffer (pH 7.5)	25ml	A2651
K562 Genomic DNA, 400µg/ml	80µg	E4931
Nuclease-Free Water	500ml	P1197

3. Materials to be Supplied by the User

- fluorometer or multimode reader capable of reading 96-well plates ($504nm_{Ex}/531nm_{Em}$) (e.g., GloMax®-Multi+ Detection System with Instinct® Software, Cat.# E8032, or GloMax® Discover System, Cat.# GM3000)
- black flat-bottom 96-well plates (e.g., Costar Cat.# 3915) compatible with working volumes of 200µl
- **optional:** K562 Genomic DNA (Cat.# E4931)

See Sections 5.A and 5.B for instrumentation and labware requirements.

4. Before You Begin

4.A. Preparation of Solutions

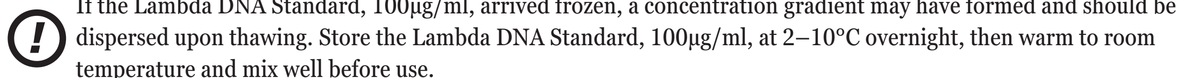
Equilibrate all assay components of the QuantiFluor® System of choice to room temperature before use. Prior to beginning the automated method, prepare the following solutions:

QuantiFluor® dsDNA System

QuantiFluor® dsDNA Dye, 200X: Thaw at room temperature, protected from light. Once completely thawed, vortex thoroughly.

Note: The automated method will guide you in the proper dilution of the 200X dye during setup. Per Section 4.C, Note 1, **do not** dilute the dye as instructed for the manual method described in the Technical Manual (#TM346).

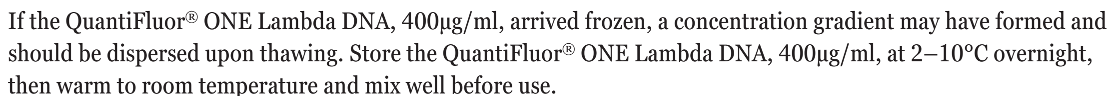
1X TE buffer: Dilute the 20X TE Buffer 20-fold with Nuclease-Free Water (Cat.# P1197). Up to ~50ml of 1X TE buffer may be required per automated run for the preparation of dye working solution and dilution of unknown samples and DNA standards. For example, add 25ml of 20X TE Buffer to 475ml of Nuclease-Free Water, and mix. Store excess 1X TE buffer at –30°C to +30°C for future use.



If the Lambda DNA Standard, 100µg/ml, arrived frozen, a concentration gradient may have formed and should be dispersed upon thawing. Store the Lambda DNA Standard, 100µg/ml, at 2–10°C overnight, then warm to room temperature and mix well before use.

QuantiFluor® ONE dsDNA System

1X TE buffer: Use the 25ml of 1X TE Buffer (pH 7.5) provided with the system for the automated dilution of unknown samples and DNA standards. If necessary, prepare additional 1X TE Buffer as follows: Add 25ml of 20X TE Buffer (pH 7.5) (Cat.# A2651) to 475ml of Nuclease-Free Water (Cat.# P1197), and mix. Store excess 1X TE buffer at –30°C to +30°C for future use.



If the QuantiFluor® ONE Lambda DNA, 400µg/ml, arrived frozen, a concentration gradient may have formed and should be dispersed upon thawing. Store the QuantiFluor® ONE Lambda DNA, 400µg/ml, at 2–10°C overnight, then warm to room temperature and mix well before use.

4.B. Sample Preparation

The QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems can be used to quantitate the amount of dsDNA in purified unknown samples. The automated method can start with unknown samples in one of two formats: tube format (e.g., 1.5ml or 2ml tubes) or 96-well plate format. A sufficient volume of each unknown sample should be present in the sample tubes or wells of the sample plate to allow a maximum of 10µl of unknown sample to be removed. This volume will vary with the specific tube or plate used.

Make sure the unknown samples are located at the bottom of the tube or plate well and do not contain bubbles.

Note: Unknown samples that are highly viscous or that pipet differently than the DNA standard may result in inaccurate or inconsistent quantitation (i.e., unknown samples that differ greatly in either DNA concentration, DNA molecular weight or storage solution composition).

4.C. QuantiFluor® Dye Working Reagents

The automated method allows you to use the QuantiFluor® dsDNA System or QuantiFluor® ONE dsDNA System. You will be prompted to prepare or add the appropriate QuantiFluor® Dye working solution to the Tecan Freedom EVO® workstation.

QuantiFluor® dsDNA System

The automated method calculates the volume of QuantiFluor® Dye working solution to be added to the reagent trough and prompts the user for the volumes of 1X TE buffer and 200X dye required to manually prepare the QuantiFluor® Dye working solution.

Notes:

1. When using the QuantiFluor® dsDNA System with the automated method, QuantiFluor® Dye working solution is prepared at a different dye concentration than that used in the manual method described in Technical Manual (#TM346). To ensure the correct final concentration of the dye in the assay plate and optimal assay performance, the QuantiFluor® Dye working solution **must be manually prepared as indicated by the method prompts** when using the automated method.
2. Immediately prior to placing on the worktable of the instrument, combine the 1X TE buffer and 200X dye as indicated by the method prompts and thoroughly vortex. Mixing of the QuantiFluor® Dye working solution by repeated inversion or shaking of the solution may not be sufficient and may adversely affect the performance of the method.

4.C. QuantiFluor® Dye Working Reagents (continued)

QuantiFluor® ONE dsDNA System

The automated method calculates the volume of QuantiFluor® ONE dsDNA Dye required for the experiment and prompts the user to add the dye directly to the reagent trough.

Note:

1. Do not dilute the QuantiFluor® ONE dsDNA Dye prior to dispensing in the reagent trough. Thoroughly vortex the QuantiFluor® ONE dsDNA Dye immediately prior to placing the dye in the trough. Mixing of the dye by repeated inversion or shaking of the reagent bottle may not be sufficient and may adversely affect the performance of the method.

4.D. DNA Standard Curve Considerations

Quantitation of unknown samples requires comparison to a standard curve. We recommend that the standard curve extends above and below the likely concentration range for the unknown samples. The final assay concentration of the DNA standards in the standard curve must be within the linear range for the assay chemistry. In addition, a reagent blank containing 1X TE Buffer and the appropriate QuantiFluor® dye should be used to assess the background level of the assay. To ensure that the standard curve dilutions are within the linear range of the assay chemistry, the automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems method is programmed to perform all necessary calculations and liquid-handling steps required to prepare an appropriate standard curve on each assay plate, irrespective of the starting DNA standard concentration. The final concentrations are listed in Table 1.

The following information provided by the user during setup determines the nature of the standard curve preparation: kit selection, number of standard curve replicates and concentration of the DNA standard. The method is programmed to prepare seven dilutions of the DNA standard and one reagent blank in column 12 of the dilution plate. The concentrations of the DNA standard dilutions are defined by the system selection so that the DNA concentrations in the assembled QuantiFluor® assays are within the linear range of the QuantiFluor® System used (Table 1). The automated method transfers 10µl of each DNA standard dilution to the final assay plate (column 12 of the assay plate for one replicate, columns 12 and 11 for two replicates, and columns 12–10 for three replicates) containing 190µl of either the diluted QuantiFluor® Dye working solution (if using QuantiFluor® dsDNA System) or QuantiFluor® ONE dsDNA Dye. Figure 6 in Section 7 shows the position of DNA standard dilutions and reagent blank in the dilution and assay plates.

Table 1. Final Assay Concentrations of DNA Standard Dilutions in the Assay Plate (200µl Assay Volume). The final concentrations of the DNA standard dilutions in the assay plate are set as part of the automated method. The concentrations depend on the QuantiFluor® System selected.

	QuantiFluor® dsDNA System (Cat.# E2670)	QuantiFluor® ONE dsDNA System (Cat.# E4870)	Row in Dilution and Assay Plates
DNA Standard 1	1ng/µl	2ng/µl	A
DNA Standard 2	0.25ng/µl	1ng/µl	B
DNA Standard 3	0.0625ng/µl	0.25ng/µl	C
DNA Standard 4	0.016ng/µl	0.0625ng/µl	D
DNA Standard 5	0.0039ng/µl	0.016ng/µl	E
DNA Standard 6	0.001ng/µl	0.0039ng/µl	F
DNA Standard 7	0.0002ng/µl	0.001ng/µl	G
Reagent Blank	0ng/µl	0ng/µl	H

Note: The minimum amount of detectable dsDNA depends on factors such as the plasticware and reader used to measure fluorescence.

The automated method allows flexibility in the choice of the DNA standard used. The user may choose to use the DNA standard supplied with the QuantiFluor® System of choice or a dsDNA of known concentration preferably with similar molecular weight to the unknown samples being quantitated. For example, if quantitating high-molecular-weight genomic DNA, we recommend using a high-molecular-weight genomic DNA of known concentration such as K562 Genomic DNA (Cat.# E4931).

5. Automated Processing Requirements for the Tecan Freedom EVO® Workstation

This section lists the instrumentation and labware requirements for the automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems method on the Tecan Freedom EVO® workstation.

5.A. Tecan Instrumentation and Labware Requirements for the Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Method on the Tecan Freedom EVO® Workstation

The following is a list of Tecan parts and their corresponding part numbers that are required for the automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems method on a Tecan Freedom EVO® workstation.

Part Description	Quantity	Tecan Part#
Tecan Freedom EVO® 100 instrument (or larger) configured with an 8-tip Disposable Tip LiHa[1] Arm and RoMa Gripper (Freedom EVOware® standard software required[2], TouchTools™ Graphical User Interface optional)	1	Contact Tecan
Wash Station, LiHa, with DiTi Waste Chute and Trough Carrier, 100ml, 3-Position	1	10650037
Tube Carrier, 1.5ml or 2.0ml Microfuge with Hinged Cap, 16-Position **Note:** If samples are supplied in 96-well plate format, this carrier is not required.	6	10613035[3]
Te-Shake™ Microplate Shaker Unit[4]	1	10760723
Microplate Nest, 1-Position, with Hold Down, Te-Shake™ Shaker Unit	1	10760724
Mounting Plate, Te-Shake™ 1-Position	1	10760722
DiTi Carrier, LiHa, 3-Position	1	10613022
Microplate Carrier, 3-Position, Landscape	1	10612604 or 10612624

[1]The method was tested with a Tecan Freedom EVO® instrument configured with a liquid displacement LiHa arm and either 500µl or 1ml syringes. The method volumes are also fully compatible with 250µl syringes. A Tecan Freedom EVO® instrument configured with an air-displacement LiHa arm also may be used. If an air-displacement LiHa arm is used, a Trough Carrier, 3-Position (10650020 or 10613020), and a DiTi Waste Chute are required instead of the Wash Station listed above.

[2]The method was tested with Tecan Freedom EVOware® standard software version 2.5 service pack 4.

[3]To use 2ml Screw Cap Tubes, the following items are required instead of the Tube Carrier for hinged capped tubes listed above: six Tube Carrier, 10mm (black inserts), 16-Position (1 × 16) (10613014), and six Insert 10mm striprack D12mm*12.7mm (16 pieces) (30015203).

[4]The method requires the Te-Shake™ Microplate Shaker Unit to be configured for a 3mm orbit.

Consumables

Part Description	Quantity	Tecan Part#
200µl LiHa Disposable Tips with Filter (24 tips per run)[1]	1 tray per run	30000629
50µl LiHa Disposable Tips with Filter[1,2]	2 trays per run	30032114
Trough, Disposable, 100ml, Polypropylene Gray[3]	3 per run	10613049
Trough, Disposable, 25ml Maximum Recovery, Polypropylene	1 per run	30055743

[1]This method requires the use of conductive 200µl and 50µl disposable tips for capacitive liquid level detection.

[2]The number of 50µl disposable tips required per run depends on the number of unknown samples, the number of DNA standard curve replicates, whether additional 1X TE buffer is required in the assay plate and whether the samples are directly transferred to the assay plate or are diluted in the dilution plate prior to transfer to the assay plate. The automated method calculates the number of tips required based on user input and prompts the user to supply the appropriate number during the workstation setup. The maximum number of 50µl disposable tips required in a single run is 192.

[3]One 100ml trough is required for 1X TE buffer, another is required to hold a 25ml Maximum Recovery Trough and a third acts a holder for a Four-Position Tube Holder (Section 5.B). The latter two 100ml troughs are used as labware holders only and may be reused.

5.B. Promega Labware Requirements for the Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Method on the Tecan Freedom EVO® Workstation

Part Description	Quantity	Promega Cat.#
Four-Position Tube Holder	1	V1601

Consumables

Part Description	Quantity	Promega Cat.#
1.1ml, Square-Well, V-Bottom Deep Well Plate[1]	1 per run	V6821

[1]Used for serial dilution of the DNA standard in all runs and for extended unknown sample dilution if required.

Note: We do not recommend use of labware different than those listed above.

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5.C. Tecan Freedom EVO® Deck Configuration

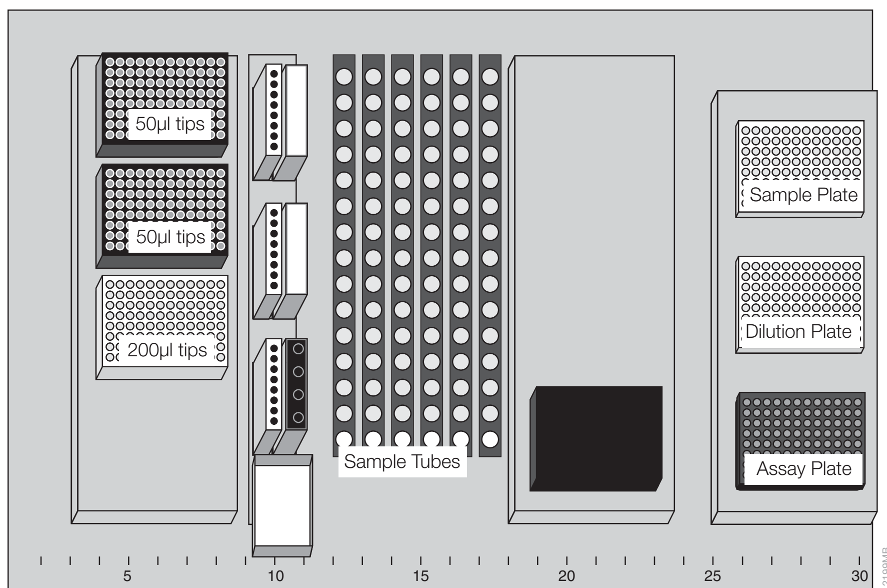

Figure 1. Tecan Freedom EVO® initial deck configuration.

Grid 3	DiTi Carrier, 3-Position	
	Position 1 (rear)	50µl LiHa disposable tips with filter
	Position 2 (middle)	50µl LiHa disposable tips with filter
	Position 3 (front)	200µl LiHa disposable tips with filter
Grid 9	LiHa Wash Station with DiTi Waste Chute and 3-Position Trough Carrier	
	Positions 1–3 (left)	LiHa Wash Station with tip disposal
	Position 4 (right, rear)	100ml trough
	Position 5 (right, middle)	100ml trough and 25ml maximum recovery trough
	Position 6 (right, front)	100ml trough and Promega Four-Position Tube Holder
Grids 12–17	Sample Tube Racks, 16-position (if unknown samples are in tubes)	
	Positions 1–16	Sample tubes compatible with Sample Tube Racks
Grid 18	Mounting Plate, Te-Shake™ 1-Position	
	Position 1 (front)	Te-Shake™ Shaker Unit
Grid 25	Microplate Carrier, 3-Position	
	Position 1 (rear)	Sample plate (if unknown samples are in a plate)
	Position 2 (middle)	1.1ml, Square-Well, V-Bottom Deep Well Plate
	Position 3 (front)	black flat-bottom 96-well assay plate

5.D. DNA Quantitation Procedure

The automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems method can be run with or without the TouchTools™ GUI. The TouchTools™ software allows control of the Tecan Freedom EVO® laboratory liquid handler via an easy-to-use touch-screen interface. For more information about the TouchTools™ software, contact Tecan. If the TouchTools™ software is not installed, the user enters run parameters via a series of Freedom EVOware® prompt windows that capture the same information as indicated in the description below for the TouchTools™ GUI.

TouchTools™ GUI

After logging into the software, select the Application Starter from the Available Operating Modes screen. Select QuantiFluor_dsDNAV1_0 from the available scripts.

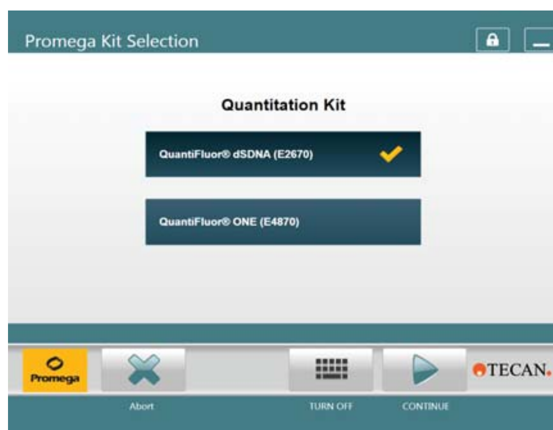

Figure 2. Promega Kit Selection window. Select the QuantiFluor® System of choice.

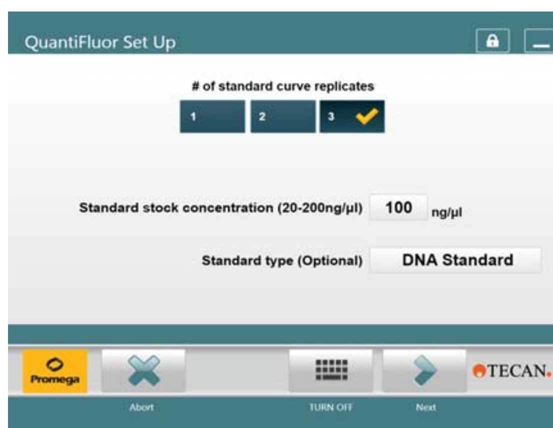

Figure 3. Standard curve window. Select the number (1, 2 or 3) of replicates of the standard curve to be added to the assay plate. Specify concentration (in ng/µl) of the DNA standard stock solution. When using the QuantiFluor® dsDNA System, the allowed concentration range is 20–200ng/µl; when using the QuantiFluor® ONE dsDNA System, the allowed concentration range is 40–400ng/µl.
Optional: Enter a name or identifier for the DNA standard used. This is an optional user input recorded in the method output file; it is not used by the automated method for any calculation.

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5.D. DNA Quantitation Procedure (continued)

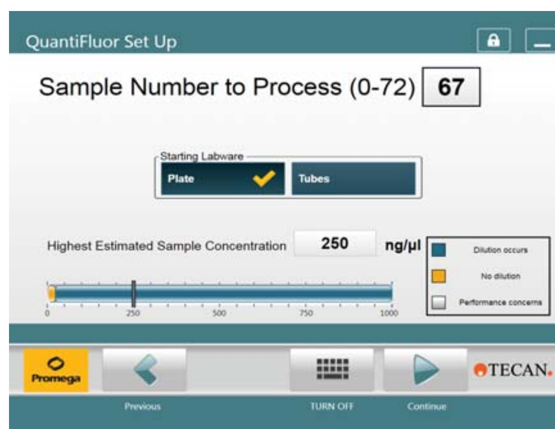

Figure 4. Sample window. Enter the number of unknown samples to be processed in the Sample Number to Process text box. The allowed range is 0 to 72 unknown samples if three replicates of the standard curve were selected on the previous screen, 0 to 80 unknown samples if two replicates of the standard curve were selected and 0 to 88 unknown samples if one replicate of the standard curve was selected. Next, specify the sample format type (i.e., plate versus tubes) by selecting the Starting Labware type. Enter the highest estimated DNA concentration (in ng/µl) of unknown samples in the text box. When the estimate is entered, the dilution indicator bar below the text box will signal whether the unknown samples will be transferred from the sample starting labware directly to the assay plate (no dilution), an extended dilution will occur (dilution occurs) in the dilution plate or if there is a possible concern that the unknown samples are either too dilute or too concentrated (performance concerns) for accurate DNA quantitation.

When all required user information is entered, the method displays the dilution factor that will be applied to all unknown samples, the concentration of the DNA standards in the assay plate and the location of a method output file (see Figure 5 and Section 8 for more details on the method output file). Finally, the method guides the user to place labware, reagents and unknown samples on the workstation.

Upon completion of the automated method, the user manually transfers the assay plate to an appropriate fluorometer or multimode reader and measures fluorescence ($504nm_{Ex}/531nm_{Em}$). For example, use the Blue Fluorescence Optical Kit ($490nm_{Ex}/510–570nm_{Em}$) with the GloMax®-Multi+ Detection System with Instinct® Software or use wavelength settings of $475nm_{Ex}/500–550nm_{Em}$ on the GloMax® Discover System.

The fluorescence data may be analyzed using either a linear regression or a power regression; DNA concentrations of the unknown samples are determined by comparison to the standard curve. See the Appendix (Section 10) for details.

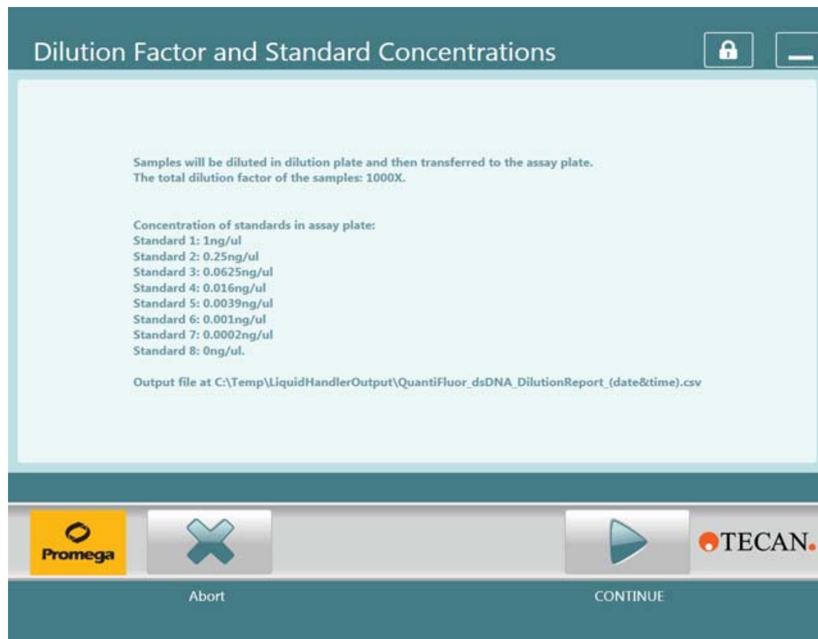

Figure 5. Total dilution factor and assay concentration of standards.

5.E. Freedom EVO® Reagent Dispense Volumes

Following the prompts presented in the TouchTools™ GUI or Freedom EVOware® prompt windows, manually dispense the reagents listed below. User-defined input variables enable the automated method to determine the minimum volume of each reagent required at the start of the method. See Sections 4.A and 4.C for solution preparation instructions.

The following locations require reagents to be dispensed manually:

Grid 9	Position 4 (right, rear)	Dispense 1X TE Buffer into the 100ml trough at the volume specified by the GUI or prompt.
Grid 9	Position 5 (right, middle)	**QuantiFluor® dsDNA System:** Prepare the QuantiFluor® dsDNA Dye working solution in a 50ml tube using the volumes of 1X TE buffer and 200X dye specified by the GUI or prompt. Dispense the prepared QuantiFluor® dsDNA Dye working solution to the 25ml maximum recovery trough. **Note:** Be sure to thoroughly vortex both the 200X dye prior to use and the working solution before dispensing into the trough. Mixing of the 200X dye and the working solution by repeated inversion or shaking may not be sufficient and may adversely affect the performance of the method. **QuantiFluor® ONE dsDNA System:** Thoroughly vortex the QuantiFluor® ONE dsDNA Dye, and dispense the volume specified by the GUI or prompt to the 25ml maximum recovery trough. **Note:** Mixing of the dye by repeated inversion or shaking of the reagent bottle may not be sufficient and may adversely affect the performance of the method.
Grid 9	Position 6 (right front)	Thoroughly mix the DNA standard. Manually pipet the volume of DNA standard specified by the GUI or prompt into a 1.5ml microcentrifuge tube. Place the tube in the **rear-most position** of the Four-Position Tube Holder. Be sure that the DNA standard is well mixed prior to placing in tube. Be sure the cap is open and secure in the holder.

6. Description of the Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Method

This section describes the general liquid-handling and incubation steps involved in the automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems method.

1. **QuantiFluor® Reagent Transfer:** The liquid handler transfers 190µl of QuantiFluor® dsDNA Dye working solution or QuantiFluor® ONE dsDNA Dye from the reagent trough to each unknown sample or DNA standard well of the assay plate.
2. **Diluent Transfer:** The liquid handler transfers 1X TE buffer to the dilution plate for unknown sample dilution if an extended dilution is required. The liquid handler transfers 1X TE buffer to the dilution plate for the serial dilution of the DNA standard. If required, the liquid handler transfers 1X TE buffer to the unknown sample wells of the assay plate.

   Note: If 1X TE buffer is added to the unknown sample wells of the assay plate, the tips come into contact with the QuantiFluor® dsDNA Dye working solution or QuantiFluor® ONE dsDNA Dye in the wells of the assay plate. Therefore, a small amount of dye may be transferred to the 1X TE buffer reagent trough during this step. We recommend discarding the 1X TE buffer remaining in the 100ml trough at the end of the run.
3. **Extended Dilution of Unknown Samples in Dilution Plate:** If required, the liquid handler transfers unknown samples from the sample tubes or sample plate to the dilution plate. The dilution plate is moved by the RoMa Gripper to the Te-Shake™ Shaker Unit.
4. **Serial Dilution of DNA Standard:** The liquid handler transfers an appropriate volume of concentrated DNA standard to well A12 of the dilution plate. After a brief tip mix, the dilution plate is thoroughly mixed using the Te-Shake™ Shaker Unit (1100rpm), mixing both the DNA standard dilution in well A12 and any unknown sample dilutions (if present in the dilution plate). Then the liquid handler transfers an appropriate volume from well A12 to well B12 and performs another tip mix. The dilution plate is again thoroughly mixed using the Te-Shake™ Shaker Unit. This process is repeated for the remaining DNA standard dilutions in the standard curve. After all DNA standard dilutions are prepared, the RoMa Gripper returns the dilution plate to the 3-position microplate carrier and transfers the assay plate to the Te-Shake™ Shaker Unit.
5. **Addition of Unknown Samples to Assay Plate:** The liquid handler transfers unknown samples to the assay plate from the dilution plate if an extended sample dilution was required or directly from the sample plate or sample tubes.
6. **Addition of DNA Standard Dilutions to Assay Plate:** The liquid handler transfers 10µl of each DNA standard dilution to the assay plate from column 12 of the dilution plate. DNA standards are added in the following order when 1, 2 or 3 standard curve replicates are specified by the user: column 12 only, columns 12 and 11 or columns 12, 11 and 10, respectively.
7. **Incubation of Assay Plate:** The assay plate is mixed and incubated on the Te-Shake™ Shaker Unit for 5 minutes (900rpm, uncovered).
8. **Method Ends.**

7. Description of Sample Dilution Schemes Performed by the Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Method

To accurately quantitate dsDNA using the QuantiFluor® dsDNA or QuantiFluor® ONE dsDNA System, the DNA concentrations of unknown samples in the assay plate must be within the linear range of the chemistry. When quantitating unknown samples, it can be difficult to decide how much or how little the unknown sample should be diluted so that the final assay concentration is within the linear range of the assay. The automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems method is designed to simplify the decision-making process.

During setup, the user provides an estimate of the highest DNA concentration expected for the unknown samples. Based on that estimate, the method follows one of two paths: 1) direct transfer of unknown sample from the sample plate or sample tube to the assay plate or 2) an extended dilution of the unknown sample through a dilution plate prior to transfer of diluted sample to the assay plate (see Figure 6). All unknown samples on the assay plate are diluted in the same way. The method provides the dilution factor for the unknown samples in an output file (see Section 8), which is required in the analysis of the data (see Section 10). As all samples are diluted in the same manner based on user-provided DNA concentration estimate, quantitation of unknown samples with greatly varying concentrations within a single run may be problematic, resulting in overdilution of highly dilute unknown samples or underdilution of unknown samples more concentrated than the estimated concentration.

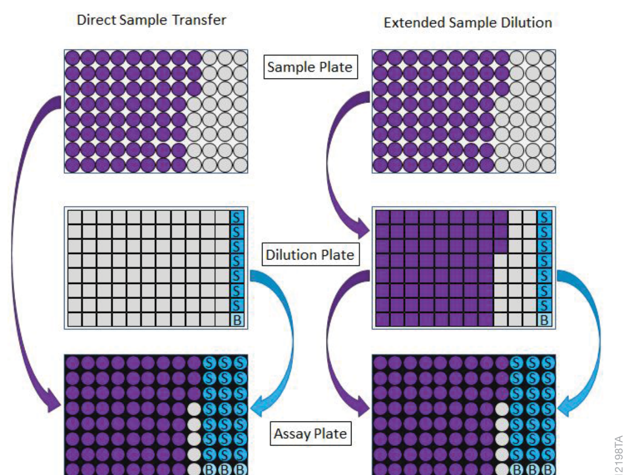

Figure 6. Sample dilution pathways. Direct transfer of unknown sample into assay plate (left). Extended dilution of unknown sample through a dilution plate prior to transfer into assay plate (right). Purple shading of a well indicates the presence of an unknown sample; purple arrows indicate the path 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 samples.

In determining which dilution scheme to follow, the method is programmed to consume the minimum volume of original unknown sample possible while maintaining a final assay concentration within the linear range of the chemistry. To accomplish this, the method is programmed with a minimum and maximum unknown sample consumption volume of 2µl and 10µl, respectively.

8. Explanation of Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Method Output File

The output file is a comma-delimited file generated by the method to record information necessary for data analysis. The file can be found at: C:\Temp\LiquidHandlerOutput\QuantiFluor_dsDNA_DilutionReport_(date&time).csv, where the date (format MMDDYYY) and time (format HHMMSS) of file generation is automatically added to the file name for archiving purposes.

The output file contains the following information:

QuantiFluor Kit: QuantiFluor® dsDNA System or QuantiFluor® ONE dsDNA System, as specified by user.

[EstimatedHighestSample](ng/µl): The highest estimated DNA concentration for the unknown samples supplied by the user.

SampleNumber: The number of unknown samples processed, as specified by the user.

Sample Wells: The location of the unknown samples in the final assay plate.

TE in Dilution (µl): The volume of 1X TE buffer used in the dilution plate for unknown sample dilution (if extended sample dilution was required).

Sample in Dilution (µl): The volume of unknown sample transferred from the sample tubes or sample plate to the dilution plate (if extended sample dilution was required).

TE in Assay (µl): The volume of 1X TE buffer used in the unknown samples wells of the assay plate to bring each assay to a final volume of 200µl (if necessary).

Sample in Assay (µl): The volume of unknown sample directly transferred from the sample tube or plate to the assay plate (if extended sample dilution was not required) or from the dilution plate to the assay plate (if extended sample dilution was required).

Assay Volume (µl): Total volume of each assay in the assay plate. **Note:** This method was designed to be used at a total assay volume of 200µl.

[EstimatedAssay](ng/µl): Calculated final DNA concentration of the unknown sample with the highest estimated DNA concentration when diluted in the assay plate.

Total Dilution Factor: Dilution factor applied to all unknown samples; accounts for the dilution of unknown samples into dilution plate (if extended dilution occurs in the dilution plate) and dilution of unknown samples into assay plate.

#StandardReplicates: User-specified number of DNA standard curve replicates transferred to the assay plate.

8. Explanation of Automated QuantiFluor® dsDNA and QuantiFluor® ONE dsDNA Systems Method Output File (continued)

[StandardStock] (ng/µl): User-specified concentration of the stock DNA standard.

StandardType: Optional user-specified name, type or identifier of the DNA standard.

[Standards 1–7] (ng/µl): Concentration and placement of the DNA standards in the assay plate as described in Table 1.

[Standard 8] (ng/µl): Concentration and placement of the reagent blank in the assay plate as described in Table 1.

QuantiFluor Kit	QuantiFluor dsDNA	
[EstimatedHighestSample](ng/ul)	250	
SampleNumber	67	
Sample Wells	A1 through C9	
TE in Dilution (ul)	98	
Sample in Dilution (ul)	2	
TE in Assay (ul)	0	
Sample in Assay (ul)	10	
Assay Volume (ul)	200	
[EstimatedAssay](ng/ul)	0.25	
Total Dilution Factor	1000	
#StandardReplicates	3	
[StandardStock] (ng/ul)	100	
StandardType	DNA Standard	
[Standard 1] (ng/ul)	1	Row A Columns 10-12
[Standard 2] (ng/ul)	0.25	Row B Columns 10-12
[Standard 3] (ng/ul)	0.0625	Row C Columns 10-12
[Standard 4] (ng/ul)	0.016	Row D Columns 10-12
[Standard 5] (ng/ul)	0.0039	Row E Columns 10-12
[Standard 6] (ng/ul)	0.001	Row F Columns 10-12
[Standard 7] (ng/ul)	0.0002	Row G Columns 10-12
[Standard 8] (ng/ul)	0	Row H Columns 10-12

Figure 7. An example of a method output file. This figure illustrates the output for an experiment conducted using the parameters as defined in Figures 2 through 6.

9. Important Considerations

1. The automated method was designed to be used with final assay volumes of 200µl. We do not recommend changing the assay volume.
2. Although one replicate of the standard curve is allowed by the method, we recommend using two or three standard curve replicates.
3. The automated method was designed and tested using a liquid-displacement LiHa arm with 500µl or 1ml syringes. The method can be adapted for the use of a liquid-displacement LiHa arm with 250µl syringes or an air-displacement LiHa arm.
4. By default, the automated method allows transfer volumes of as small as 2µl. Although testing of 2µl transfers using the liquid-displacement LiHa with 1ml syringes and 50µl disposable tips showed acceptable reproducibility, for optimal reproducibility we recommend limiting transfers to no less than 5µl when using 1ml syringes.
5. Always use aerosol-resistant tips to minimize the risk of cross-contamination.
6. To ensure homogeneity, thoroughly vortex all reagents before placing them on the worktable. Mixing by inversion or shaking of reagent bottles may not be sufficient and may adversely affect performance.
7. The reagent volumes indicated in the GUI or prompts include excess reagent to ensure that enough reagent is placed on the worktable and available for all unknown sample and standard wells.
8. Pipetting techniques used must be calibrated to ensure accurate volume handling for unknown samples and standards.
9. When defining labware, be certain to set Z-max heights carefully at the well bottom of each labware present on the worktable.
10. It is recommended that an unused 25ml maximum recovery trough be used for each automated setup.

10. Appendix

Data may be analyzed by fitting a line to an equation. Analyze the data using either a linear regression or a power regression for accurate DNA concentration determinations from 0.01ng/µl through the top portion of the DNA standard curve. We recommend performing a power regression for unknown samples that are expected to have DNA concentrations <0.01ng/µl. If the concentration range of the samples is large (greater than one order of magnitude), fitting the line to a power regression will avoid inaccuracies associated with heteroscedasticity, the condition of unequal variances. When the concentration range of DNA standard curve samples is greater than one order of magnitude, the larger samples tend to display greater variance (or deviation), which can skew the fit of a linear regression line and impair accuracy at the low end of the range. A power regression will more evenly account for dsDNA standard curve sample variances to allow for more accurate quantitation within this portion of the range.

10.A. Analysis of Data Using Linear Regression

1. Subtract the average fluorescence of the reagent blank (1X TE buffer, standard 8) from the fluorescence measurements for all unknown samples and DNA standard dilutions.
2. Plot the standard curve values, with the final DNA concentration of the DNA standard dilutions in the assay plate in ng/µl on the X axis (supplied in the method output file) and blank-subtracted average fluorescence in relative fluorescence units (RFU) on the Y axis.
3. Fit a line to the standard curve values, and display the linear regression calculation for that line. This will take the form of $y = mx + b$, or [fluorescence = (slope × dsDNA concentration) + y-intercept].
4. Calculate the concentration for each blank-subtracted unknown sample by using fluorescence as y in the equation from Step 3. Solve for x: $x = (y-b)/m$
5. Multiply the resulting assay concentration by the Total Dilution Factor supplied in the method output file to obtain the DNA concentration of the undiluted unknown sample.

10.B. Analysis of Data Using Power Regression

1. Subtract the average fluorescence of the reagent blank (1X TE buffer, standard 8) from the fluorescence measurements for all unknown samples and DNA standard dilutions.
2. Plot the standard curve values, with the final DNA concentration of the DNA standard dilutions in the assay plate in ng/µl on the X axis (supplied in the method output file) and blank-subtracted average fluorescence in relative fluorescence units (RFU) on the Y axis. Change both the X axis and Y axis to a logarithmic scale (base 10).
3. Fit a line to the standard curve values using power regression, and display the calculation for that line. This will take the form of $y = bx^m$, or [fluorescence = $(\text{y-intercept} \times \text{dsDNA concentration})^{\text{slope}}$].
4. Calculate the concentration for each blank-subtracted unknown sample by using fluorescence as y in the equation from Step 3. Solve for x: $x = (y/b)^{(1/m)}$
5. Multiply the resulting assay concentration by the Total Dilution Factor supplied in the method output file to obtain the DNA concentration of the undiluted unknown sample.

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