

TECHNICAL MANUAL

# PowerSeq™ Quant MS System

Instructions for Use of Product  
**PS5000**



# PowerSeq™ Quant MS System

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 Visit the web site to verify that you are using the most current version of this Technical Manual.  
 E-mail Promega Technical Services if you have questions on use of this system: [techserv@promega.com](mailto:techserv@promega.com)

1.	Description.....	1
2.	Product Components and Storage Conditions .....	3
3.	General Considerations .....	3
4.	Reaction Plate Setup .....	4
4.A.	Serial Dilution of PowerSeq™ Quant MS DNA Standard .....	5
4.B.	Dilution of PowerSeq™ System DNA Libraries.....	6
4.C.	Reaction Setup .....	6
4.D.	Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Real-Time PCR System and Applied Biosystems® 7500 Software, Version 2.0.6 .....	8
4.E.	Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Real-Time PCR System for Human Identification and HID Real-Time PCR Analysis Software, Version 1.1 or 1.2 .....	11
4.F.	Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Fast Real-Time PCR System and 7500 Software for 7500 and 7500 Fast Real-Time PCR Systems v2.3 .....	14
5.	Data Analysis .....	16
6.	Determine Library Concentration.....	17
7.	Troubleshooting.....	17
8.	Reference .....	19
9.	Related Products.....	20
10.	Summary of Changes .....	20

## 1. Description

The PowerSeq™ Quant MS System<sup>(a)</sup> contains reagents to determine the concentration of massively parallel sequencing (MPS) libraries compatible with Illumina MiSeq® platforms using qPCR. Data generated using the PowerSeq™ Quant MS System can be used to normalize the DNA concentration for MPS libraries to the desired concentration. This Technical Manual describes the PowerSeq™ Quant MS System and provides instructions for use with the Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems® 7500 Software, Version 2.0.6), the Applied Biosystems® 7500 Real-Time PCR System for Human Identification (HID Real-Time PCR Analysis Software, Version 1.1 or 1.2) and the Applied Biosystems® 7500 Fast Real-Time PCR System (7500 Software For 7500 and 7500 Fast Real-Time PCR Systems v2.3).

## 1. Description (continued)

### **PowerSeq™ Quant MS 10X Primer Mix**

The PowerSeq™ Quant MS 10X Primer Mix includes primers that target the P5 and P7 Illumina adapter sequences for quantification of Illumina libraries (1).

### **PowerSeq™ Quant 2X Master Mix**

The PowerSeq™ Quant 2X Master Mix formulation includes BRYT Green® dsDNA-binding dye, passive reference dye, hot start polymerase, MgCl<sub>2</sub>, dNTPs and a proprietary reaction buffer. The BRYT Green® dsDNA-binding dye is detected using the same filters and settings as the SYBR® Green I dye. The passive reference is a low level of the carboxy-X-rhodamine (CXR) reference dye (identical to ROX™ dye). Data from the other dye channels are normalized to the signal from the passive reference.

### **PowerSeq™ Quant MS DNA Standard**

A standard curve is required to determine the DNA concentration of the sample libraries. The PowerSeq™ Quant MS DNA Standard is supplied with the PowerSeq™ Quant MS System. Serial dilutions of this DNA standard are amplified in the same plate as the sample libraries, and the results are used to generate a standard curve. The standard curve is used to determine the DNA concentration of the sample libraries. We recommend performing triplicate amplification reactions with each dilution of DNA standard and using a dedicated set of pipettes to increase run-to-run consistency. Use the same pipettes to dispense DNA standard and sample libraries to minimize variability. Use only the DNA standard supplied with the system.

### **PowerSeq™ Quant MS 1X Dilution Buffer**

The PowerSeq™ Quant MS 1X Dilution Buffer is supplied as the diluent for serial dilution of the PowerSeq™ Quant MS DNA Standard and sample libraries. The dilution buffer is also the recommended input for the no-template controls.

## **Instrumentation**

The PowerSeq™ Quant MS System is designed and optimized for use with the Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems® 7500 Software, Version 2.0.6), the Applied Biosystems® 7500 Real-Time PCR System for Human Identification (HID Real-Time PCR Analysis Software, Version 1.1 or 1.2) and the Applied Biosystems® 7500 Fast Real-Time PCR System (7500 Software For 7500 and 7500 Fast Real-Time PCR Systems v2.3). Calibration with the FAM™/SYBR® Green I and ROX™ dyes is required before DNA quantification. The dyes used in the system are compatible with real time instruments capable of detecting SYBR® Green I or FAM™ dye. Additional testing in your laboratory may be required to show instrument compatibility.

## 2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
<b>PowerSeq™ Quant MS System</b>	<b>500 reactions</b>	<b>PS5000</b>

For Research Use Only. Not for use in Diagnostic Procedures.

Includes sufficient reagents for 500 reactions at 20µl per reaction. Components are shipped on dry ice.

- 5 × 1ml PowerSeq™ Quant 2X Master Mix
- 1 × 1ml PowerSeq™ Quant MS 10X Primer Mix
- 1 × 200µl PowerSeq™ Quant MS DNA Standard (200pM)
- 2 × 1,250µl Water, Amplification Grade
- 2 × 150ml PowerSeq™ Quant MS 1X Dilution Buffer

**Storage Conditions:** Store the PowerSeq™ Quant MS System at –30°C to –10°C in a nonfrost-free freezer protected from light. For immediate use, components may be stored at 2–10°C, protected from light, for up to 3 months. Dilutions of the PowerSeq™ Quant MS Standard in PowerSeq™ Dilution Buffer can be stored at 4°C for up to 3 months. After the initial thaw, store PowerSeq™ Quant MS DNA Standard and PowerSeq™ Quant MS 1X Dilution Buffer at 2–10°C for up to 3 months.

## 3. General Considerations

The BRYT Green® dye in the PowerSeq™ Quant 2X Master Mix has spectral properties similar to those of SYBR® Green I: excitation at 493nm and emission at 530nm. Instrument optical settings established for SYBR® Green I assays should be used with the PowerSeq™ Quant MS System. The dyes used in the system are compatible with real time instruments capable of detecting SYBR® Green I or FAM™ dye. Additional testing in your laboratory may be required to show instrument compatibility. The CXR reference dye has the same spectral properties as ROX™ dye: Excitation at 580nm and emission at 602nm. Use the instrument settings for ROX™ dye for reactions containing PowerSeq™ Quant 2X Master Mix.

The dynamic range of this assay is 20pM to 0.02pM. Sample libraries must be diluted to fall in the dynamic range. If the concentration of a diluted library falls out of this range, the library should be retested with a dilution that falls in the dynamic range of the assay.

We recommend performing triplicate amplification reactions with each dilution of the DNA standard and using a dedicated set of pipettes to increase run-to-run consistency. Use the same pipettes to dispense the DNA standard and the sample libraries to minimize variability. Do not use any other DNA as the DNA standard.

The PowerSeq™ Quant MS 1X Dilution Buffer may take several hours to thaw at room temperature. Alternatively, thaw the PowerSeq™ Quant MS 1X Dilution Buffer at 2–10°C overnight. Store the PowerSeq™ Quant MS 1X Dilution Buffer at 2–10°C after thawing.

The PowerSeq™ Quant MS System is extremely sensitive; take precautions to minimize cross contamination. We recommend storing the PowerSeq™ Quant MS reagents separately from library samples. We also recommend using clean designated work areas and separate pipettes for pre- and post-amplification steps to minimize the potential for cross-contamination between library samples and prevent carryover of nucleic acid from one run to the next. Wear a lab coat and protective eyewear. Wear gloves, and change them often. Prevent contamination by using aerosol-resistant pipette tips. Always include a no-template control (NTC) reaction to detect contamination. We recommend performing NTC reactions in triplicate.

Do not unseal reaction plates after amplification is complete. Unsealing the plates increases the risk of contaminating subsequent reactions with amplified product.

Always check to ensure the analysis settings are correct on the instrument software (see Section 4).

#### 4. Reaction Plate Setup

Before using the PowerSeq™ Quant MS System, check that the instrument was calibrated for FAM™/SYBR® Green I and ROX™ dyes. For information on performing instrument calibration, please refer to the *Applied Biosystems® 7500/7500 Fast Real-Time PCR Systems Maintenance Guide*.

##### Materials to Be Supplied by the User

- 7500 Real Time PCR Systems Spectral Calibration Kit 1 (ABI Cat.# 4349180) (includes 96-well optical reaction plate preloaded with dye [for calibration with FAM™/SYBR® Green I and ROX™ dyes])
- Applied Biosystems® 7500 Real-Time PCR System, Applied Biosystems® 7500 Real-Time PCR System for Human Identification or Applied Biosystems® 7500 Fast Real-Time PCR System
- Applied Biosystems® 7500 instrument-related consumables
  - MicroAmp® Optical 96-Well Reaction Plate (ABI Cat.# N8010560, 4316813, 4306737 or 4326659 for the Applied Biosystems® 7500 Real-Time PCR System) or
  - MicroAmp® Fast Optical 96-Well Reaction Plate, 0.1ml (ABI Cat.# 4346907, 4346906 or 4366932 for the Applied Biosystems® 7500 Fast Real-Time PCR System)
  - MicroAmp® Optical Adhesive Film (ABI Cat.# 4311971)
- sterile, aerosol-resistant pipette tips
- low-retention or siliconized microcentrifuge tubes

**Note:** The instruments described in the manual do not require additional CXR passive reference dye. However, a tube of CXR Reference Dye can be purchased separately (Cat.# C5411) for instruments that require a higher level of reference dye than that in the PowerSeq™ Quant 2X Master Mix.

#### 4.A. Serial Dilution of PowerSeq™ Quant MS DNA Standard

Vortex the PowerSeq™ Quant MS DNA Standard thoroughly before each use. We recommend long-term storage at 2–10°C. Perform serial tenfold dilutions of the PowerSeq™ Quant MS DNA Standard, and then amplify these dilutions to create four-point standard curves to determine the concentration of targets in the unknown DNA samples. Accurate serial dilution of PowerSeq™ Quant MS DNA Standard is essential to precisely quantify unknown DNA samples; carefully mix and pipet each DNA standard dilution. Use the PowerSeq™ Quant MS 1X Dilution Buffer as the diluent for the PowerSeq™ Quant MS DNA Standard. Serial dilutions of the PowerSeq™ Quant MS DNA Standard prepared with PowerSeq™ Quant MS 1X Dilution Buffer can be stored for up to 3 months at 2°–10°C.

The four-point serial dilutions prepared in this section include DNA standards in the range of 20pM to 0.02pM. When diluting the PowerSeq™ Quant MS DNA Standard, use PowerSeq™ Quant MS 1X Dilution Buffer; do not use water as a diluent. Use low-retention or siliconized tubes to prepare serial dilutions.

1. Ensure that the PowerSeq™ Quant MS 1X Dilution Buffer is completely thawed before use. Vortex the PowerSeq™ Quant MS DNA Standard three times at high speed for 10 seconds each time.  
**Note:** After the initial thaw, store PowerSeq™ Quant MS DNA Standard and PowerSeq™ Quant MS 1X Dilution Buffer at 2°–10°C.
2. Label four tubes with the following concentrations: 20pM, 2pM, 0.2pM and 0.02pM.
3. Prepare fresh serial dilutions of PowerSeq™ Quant MS DNA Standard as indicated in Table 1. Vortex each dilution for 10 seconds before removing an aliquot for the next dilution. Change pipette tips between dilutions.

**Table 1. Serial Dilution of PowerSeq™ Quant MS DNA Standard (Tenfold Dilutions).**

DNA Concentration	Volume of PowerSeq™ Quant MS DNA Standard	Volume of PowerSeq™ Quant MS 1X Dilution Buffer
20pM	Use 5µl of PowerSeq™ Quant MS DNA Standard (200pM)	45µl
2pM	5µl of 20pM dilution	45µl
0.2pM	5µl of 2pM dilution	45µl
0.02pM	5µl of 0.2pM dilution	45µl

**Notes:**

1. We recommend performing amplification reactions of each DNA standard in triplicate.
2. Change gloves after handling the high-copy number DNA standard.
3. Serial dilutions of PowerSeq™ Quant MS DNA Standard prepared with PowerSeq™ Quant MS 1X Dilution Buffer can be stored for up to 3 months at 4°C.

#### 4.B. Dilution of PowerSeq™ System DNA Libraries

1. Dilute the sample libraries to concentrations within the dynamic range of the assay (20pM to 0.02pM).

**Note:** If microcentrifuge tubes will be used for the dilution of sample libraries, use low-retention or silicionized tubes.

2. Pipette a minimum volume of 2µl of the undiluted library and an appropriate volume of PowerSeq™ Quant MS 1X Dilution Buffer to prepare the dilution.

**Note:** Libraries may require dilutions that fall in the range of 1:1,000 to 1:100,000. Serial dilutions of the libraries may be used to determine a dilution factor that falls in the dynamic range of the assay. Please refer to the appropriate Promega System Technical Manual for specific dilution recommendations.

For example:

- a. Dilute sample libraries 1:100 by carefully adding 2µl of undiluted library to 198µl of PowerSeq™ Quant MS 1X Dilution Buffer.
- b. Mix the dilution thoroughly by gently pipetting 10 times using a 200µl single-channel or multichannel pipette set to 198µl.
- c. Dilute the sample library 1:10,000 by carefully adding 2µl of the 1:100 dilution (from Step 2.b) to 198µl of PowerSeq™ Quant MS 1X Dilution Buffer.

#### 4.C. Reaction Setup

Include triplicate no-template control (NTC) reactions for each set of reactions. Add 4µl of PowerSeq™ Quant MS 1X Dilution Buffer to these reactions instead of template DNA.

1. Thaw PowerSeq™ Quant 2X Master Mix, PowerSeq™ Quant MS 10X Primer Mix and Water, Amplification Grade, completely at room temperature.
2. Vortex PowerSeq™ Quant 2X Master Mix and PowerSeq™ Quant MS 10X Primer Mix for 10 seconds to mix.
3. Determine the number of reactions to set up, including NTC reactions. Increase this number by 10–15% to compensate for pipetting error and reagent loss on sides of pipette tips. While this approach requires using a small amount of extra reagent, it ensures that enough reaction mix is prepared for all amplification reactions. Amplification of the unknown DNA samples and DNA standards using the same reaction mix is critical.
4. Use Table 2 to calculate the volume of each component required to prepare sufficient reaction mix for the number of reactions determined in Step 3.

**Table 2. Preparation of Reaction Mix for PowerSeq™ Library Quantification Using the PowerSeq™ Quant MS System.**

<b>Component</b>	<b>Volume Per Reaction</b>	<b>Number of Reactions</b>	<b>Final Volume</b>
Water, Amplification Grade	4µl		
PowerSeq™ Quant 2X Master Mix	10µl		
PowerSeq™ Quant MS 10X Primer Mix	2µl		
<b>Total Volume</b>	<b>16µl</b>		

5. Prepare the reaction mix by combining the volumes of PowerSeq™ Quant 2X Master Mix, PowerSeq™ Quant MS 10X Primer Mix and Water, Amplification Grade, calculated in Step 4.
6. Vortex for 10 seconds to mix.
7. Add 16µl of reaction mix per well to the reaction wells of a MicroAmp® optical 96-well reaction plate.  
**Note:** Wear gloves at all times when handling the plate, and take care to avoid touching the plate wells and MicroAmp® optical adhesive film unnecessarily. Handle the plate by the edges, and avoid touching the bottom of the plate.
8. Add 4µl of the PowerSeq™ Quant MS DNA Standards prepared in Section 4.A or the diluted PowerSeq™ System libraries prepared in Section 4.B for a final reaction volume of 20µl.
9. Add 4µl of PowerSeq™ Quant MS 1X Dilution Buffer to the NTC reactions for a final reaction volume of 20µl.
10. Seal the plate with MicroAmp® optical adhesive film.  
**Note:** Ensure that all wells are adequately sealed to prevent evaporation during thermal cycling.
11. Centrifuge the plate briefly to collect contents of each well at the bottom. The plate is ready for thermal cycling. Protect the plate from extended light exposure or elevated temperatures before thermal cycling.
12. Program the real-time thermal cycler using the following cycling parameters. (See Sections 4.D–4.F for specific instructions.)  
 95°C for 2 minutes, then:  
 30 cycles of  
     95°C for 15 seconds,  
     60°C for 15 seconds,  
     72°C for 45 seconds (perform fluorescence data collection during this step)



#### 4.D. Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Real-Time PCR System and Applied Biosystems® 7500 Software, Version 2.0.6

The following instructions are for the Applied Biosystems® 7500 Real-Time PCR System instrument using the Applied Biosystems® 7500 Software, Version 2.0.6. This instrument is also compatible with HID Real-Time PCR Analysis Software, Version 1.1 or 1.2; to use this software, use the instructions provided in Section 4.E.

A template file (.edt) can be used to store the dye information; target names and tasks; well locations and DNA concentrations for the DNA standards; run method and analysis settings.

Amplification is complete in approximately one hour.

##### Creating a Run Template

1. Turn on the computer associated with the Applied Biosystems® 7500 Real-Time PCR System.
2. Turn on the Applied Biosystems® 7500 Real-Time PCR System.
3. Open the Applied Biosystems® 7500 Software, Version 2.0.6.
4. Before using the PowerSeq™ Quant MS System, check that the instrument was calibrated for FAM™/SYBR® Green I and ROX™ dyes.
5. On the 'Home' screen under 'Setup', select the **Advanced Setup** icon. Alternatively, select the **New Experiment** button, and choose **Advanced Setup** from the drop-down menu.
6. In the Experiment Properties form, specify the following parameters:
  - Enter a name for the template in the 'Experiment Name' field.
  - Select the instrument you are using to run the experiment by selecting **7500 (96 wells)**.
  - Select the type of experiment you want to set up by selecting the **Quantitation-Standard Curve**.
  - Select the reagents you want to use to detect the target sequence by selecting **SYBR® Green Reagents**.
  - **Uncheck** the box for 'Include Melt Curve'.
  - Select the ramp speed you want to use in the instrument run by selecting **Standard (~2 hours to complete run)**.
7. Select **Plate Setup** from the 'Setup' menu in the left panel.
8. Select the 'Assign Targets and Samples' tab, and highlight the wells in use on the 'View Plate Layout' tab by dragging the pointer over the plate wells. Assign the target to the selected wells by selecting the box under 'Assign.'

9. Highlight the wells containing the DNA standards on the 'View Plate Layout' tab, and select **S** as the 'Task' for the target.
10. Enter the concentration for each DNA standard in the 'Quantity' field without the unit of measure (e.g., 20 for 20pM).  
**Note:** Multiple wells with DNA standards at the same DNA concentration can be highlighted at the same time so that values need to be entered only once.
11. Select **ROX** from the 'Select the dye to use as the passive reference' drop-down menu.
12. From the 'Analysis' menu at the top of the screen, select **Analysis Settings**.
13. On the 'CT Settings' tab in the 'Select a Target' panel, select the target.
14. In the 'CT Settings' section, *uncheck* the **Use Default Settings** box, and *uncheck* the **Automatic Threshold** box. Enter **1** for the threshold. Leave the **Automatic Baseline** box *checked*.
15. Select **Apply Analysis Settings**.
16. Select **Run Method** under 'Setup' in the left panel. Modify the default run method as directed below:
  - Enter **20** for 'Reaction Volume per Well.'
  - Select the **first Holding Stage**, and **right-click** to bring up a menu. Choose **Delete Selected** to delete this stage.
  - Change the 'second Holding Stage' to **2 minutes at 95°C**.
  - Enter **30** for the 'Number of Cycles' under 'Cycling Stage.'
  - Change 'Step 1' to **95° for 15 seconds**.
  - Select **Step 2**, and right-click to bring up a menu. Choose **Add Step**, then choose **Before**.
  - Change 'Step 2' to **60°C for 15 seconds**, and change 'Step 3' to **72°C for 45 seconds**.
  - Ensure that the 'Data Collection On' icon is active for 'Step 3' only of the 'Cycling Stage'.**Note:** The 'Data Collection On' icon is shown in the legend at the bottom of the screen.
17. From the 'File' menu at the top of the screen, select **Save as Template**, and choose a location to save the file. The instrument setup and thermal cycling program can be saved as a template for future use. Use this .edt file as a template to create the experiment document as described in Starting a Run.

### Starting a Run

1. To create the experiment document, open the template file created in the section on Creating a Run Template by selecting **Open** from the 'File' menu at the top of the screen. Navigate to the .edt file and select **Open**.  
**Note:** If the template file was just created, the .edt file will still be open.
2. Save the .edt template as an .eds file by selecting **Save As...** from the 'File' menu at the top of the screen. Choose a location to save the file.
3. Highlight the unused wells, and deselect the target.

#### 4.D. Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Real-Time PCR System and Applied Biosystems® 7500 Software, Version 2.0.6 (continued)

4. To add sample names, select **Plate Setup** from the 'Setup' menu in the left panel, and select the 'Define Targets and Samples' tab. Select **Add New Sample**, and enter the sample name in the section provided. Repeat for all samples.
5. When all sample names are entered, navigate to the 'Assign Targets and Samples' tab. Highlight the well or wells that contain replicates of the same sample, and **check** the 'Assign' box adjacent to the corresponding sample name in the 'Assign sample(s) to the selected wells' section of the 'Assign Targets and Sample' tab. Repeat until all samples are assigned.
6. If a sample was assigned to the incorrect well, select the well and uncheck the sample under 'Assign sample(s) to the selected wells' section, and then assign the correct sample name.
7. Check that the correct passive reference is selected.
8. Save the .eds file.
9. Press the tray door.
10. Place the plate prepared in Section 4.C in the open tray door, and ensure that the plate sits correctly in the plate holder, with well A1 in the top left corner. Push the tray door to close, and immediately press, **Start Run**. Run time is approximately one hour.

#### Evaluating Standard Curves Using the Applied Biosystems® 7500 Software, Version 2.0.6

1. Select **Analysis** in the left side panel, and ensure that all wells to be analyzed are highlighted on the 'View Plate Layout' tab and that no targets are selected for unused wells.
2. Select **Analyze**.
3. To display the standard curve, select **Standard Curve** from the 'Analysis' menu in the left side panel.

#### Exporting Analyzed Data from the Applied Biosystems® 7500 Software, Version 2.0.6

1. Ensure that all wells with data for export are highlighted in the plate map. Select **Export** from the toolbar.
2. On the 'Export Properties' tab of the 'Export Data' window, select the following Export Properties:
  - Choose **Results** in the 'Select data to export' section.
  - Choose **One File** from the 'Select one file or separate files' drop-down menu.
  - Choose **.xls** as the File Type.
  - Specify the appropriate export file name.
  - Use the **Browse** button to select the file location.
  - Select **Start Export**.
  - In the 'Export Completed' window that appears, select **Close Export Tool**.

#### 4.E. Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Real-Time PCR System for Human Identification and HID Real-Time PCR Analysis Software, Version 1.1 or 1.2

The following instructions are for the Applied Biosystems® 7500 Real-Time PCR System for Human Identification with HID Real-Time PCR Analysis Software, Version 1.1. or 1.2.

A template file (.edt) can be used to store the dye information target names and tasks, well locations and concentrations for the DNA standards, and run method and analysis settings.

Amplification is complete in approximately one hour.

##### Creating a Run Template

1. Turn on the computer associated with the Applied Biosystems® 7500 Real-Time PCR System for Human Identification.
2. Turn on the Applied Biosystems® 7500 Real-Time PCR System for Human Identification.
3. Open the HID Real-Time PCR Analysis Software, Version 1.1 or 1.2.
4. Before using the PowerSeq™ Quant MS System, check that the instrument was calibrated for FAM™/SYBR® Green I and ROX™ dyes.
5. On the 'Home' screen, select the **Custom Assays** button, or from the 'Assays' menu select **Custom Assays**.
6. Select **New Experiment** from the toolbar. Select **Advanced Setup** from the drop-down menu.
7. In the Experiment Properties form, specify the following parameters:
  - Enter a name for the template in the 'Experiment Name' field.
  - Select the instrument you are using to run the experiment by selecting **7500 (96 wells)**.
  - Select the type of experiment you want to set up by selecting the **Quantitation-Standard Curve**.
  - Select the reagents you want to use to detect the target sequence by selecting **SYBR® Green Reagents**.
  - **Uncheck** the box for 'Include Melt Curve.'
  - Select the ramp speed you want to use in the instrument run by selecting **Standard (~2 hours to complete run)**.
8. Select **Plate Setup** from the 'Setup' menu in the left panel.
9. Select the **Assign Targets and Samples** tab, and highlight the wells in use on the 'View Plate Layout' tab by dragging the pointer over the plate wells. Assign the target to the selected wells by selecting the box under 'Assign.'
10. Highlight the wells containing the DNA standards on the 'View Plate Layout' tab, and select **S** as the 'Task' for the target.
11. Enter the concentration for each DNA standard in the Quantity field without the unit of measure (e.g., 20 for 20pM).

**Note:** Multiple wells with DNA standards at the same DNA concentration can be highlighted at the same time so that values need to be entered only once.



#### 4.E. Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Real-Time PCR System for Human Identification and HID Real-Time PCR Analysis Software, Version 1.1 or 1.2 (continued)

12. Select **ROX** from the 'Select the dye to use as the passive reference' drop-down menu.
13. From the 'Analysis' menu at the top of the screen, select **Analysis Settings**.
14. On the 'CT Settings' tab in the 'Select a Target' panel, select the target.
15. In the 'CT Settings' section, *uncheck* the **Use Default Settings** box, and *uncheck* the **Automatic Threshold** box. Enter **1** for the threshold. Leave the **Automatic Baseline** box *checked*.
16. Select **Apply Analysis Settings**.
17. Select **Run Method** under 'Setup' in the left panel. Modify the default run method as directed below:
  - Enter **20** for 'Reaction Volume per Well.'
  - Select the **first Holding Stage**, and **right-click** to bring up a menu. Choose **Delete Selected** to delete this stage.
  - Change the 'second Holding Stage' to **2 minutes at 95°C**.
  - Enter **30** for the 'Number of Cycles' under 'Cycling Stage.'
  - Change 'Step 1' to **95° for 15 seconds**.
  - Select 'Step 2', and right-click to bring up a menu. Choose **Add Step**, then choose **Before**.
  - Change 'Step 2' to **60°C for 15 seconds**, and change 'Step 3' to **72°C for 45 seconds**.
  - Ensure that the 'Data Collection On' icon is active for 'Step 3' only of the 'Cycling Stage'.  
**Note:** The 'Data Collection On' icon is shown in the legend at the bottom of the screen.
18. From the 'File' menu at the top of the screen, select **Save as Template**, and choose a location to save the file. The instrument setup and thermal cycling program can be saved as a template for future use. Use this .edt file as a template to create the experiment document as described in Starting a Run.

#### Starting a Run

1. To create the experiment document, open the template file created in the section on Creating a Run Template by selecting **Open** from the 'File' menu at the top of the screen. Navigate to the .edt file and select **Open**.  
**Note:** If the template file was just created, the .edt file will still be open.
2. Save the .edt template as an .eds file by selecting **Save As...** from the 'File' menu at the top of the screen. Choose a location to save the file.
3. Highlight the unused wells, and deselect the target.
4. To add sample names, select **Plate Setup** from the 'Setup' menu in the left panel, and select the 'Define Targets and Samples' tab. Select **Add New Sample**, and enter the sample name in the section provided. Repeat for all samples.

5. When all sample names are entered, navigate to the 'Assign Targets and Samples' tab. Highlight the well or wells that contain replicates of the same sample, and **check** the 'Assign' box adjacent to the corresponding sample name in the 'Assign sample(s) to the selected wells' section of the 'Assign Targets and Sample' tab. Repeat until all samples are assigned.
6. If a sample was assigned to the incorrect well, select the well and uncheck the sample under 'Assign sample(s) to the selected wells,' and then assign the correct sample name.
7. Check that the correct passive reference is selected.
8. Save the .eds file.
9. Press the tray door.
10. Place the plate prepared in Section 4.C in the open tray door, and ensure that the plate sits correctly in the plate holder, with well A1 in the top left corner. Push the tray door to close, and immediately press, **Start Run**. Run time is approximately one hour.

#### Evaluating Standard Curves Using the HID Real-Time PCR Analysis Software, Version 1.1 or 1.2

1. Select **Analysis** in the left side panel, and ensure that all wells to be analyzed are highlighted on the 'View Plate Layout' tab and that no targets are selected for unused wells.
2. Select **Analyze**.
3. To display the standard curve, select **Standard Curve** from the 'Analysis' menu in the left side panel.

#### Exporting Analyzed Data from the HID Real-Time PCR Analysis Software, Version 1.1 or 1.2

1. Ensure that all wells with data for export are highlighted in the plate map. Select **Export** from the toolbar.
2. On the 'Export Properties' tab of the 'Export Data' window, select the following Export Properties:
  - Choose **Results** in the 'Select data to export' section.
  - Choose **One File** from the 'Select one file or separate files' drop-down menu.
  - Choose **".xls"** as the File Type.
  - Specify the appropriate export file name.
  - Use the **Browse** button to select the file location.
  - Select **Start Export**.
  - In the 'Export Completed' window that appears, select **Close Export Tool**.

#### 4.F. Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Fast Real-Time PCR System and 7500 Software for 7500 and 7500 Fast Real-Time PCR Systems v2.3

The following instructions are for the Applied Biosystems® 7500 Fast Real-Time PCR System instrument using the 7500 Software for 7500 and 7500 Fast Real-Time PCR Systems v2.3.

A template file (.edt) can be used to store the dye information; target names and tasks; well locations and DNA concentrations for the DNA standards; run method and analysis settings.

Amplification is complete in approximately one hour.

##### Creating a Run Template

1. Turn on the computer associated with the Applied Biosystems® 7500 Fast Real-Time PCR System.
2. Turn on the Applied Biosystems® 7500 Fast Real-Time PCR System.
3. Open the 7500 Software for 7500 and 7500 Fast Real-Time PCR Systems v2.3.
4. Before using the PowerSeq™ Quant MS System, check that the instrument was calibrated for FAM™/SYBR® Green I and ROX™ dyes.
5. On the 'Home' screen under 'Setup', select the **Advanced Setup** icon. Alternatively, select the **New Experiment** button, and choose **Advanced Setup** from the drop-down menu.
6. In the Experiment Properties form, specify the following parameters:
  - Enter a name for the template in the 'Experiment Name' field.
  - Select the instrument you are using to run the experiment by selecting **7500 Fast (96 wells)**.
  - Select the type of experiment you want to set up by selecting **Quantitation-Standard Curve**.
  - Select the reagents you want to use to detect the target sequence by selecting **SYBR® Green Reagents**.
  - **Uncheck** the box for 'Include Melt Curve.'
  - Select the ramp speed you want to use in the instrument run by selecting **Fast (~40 minutes to complete a run)**.
7. Select **Plate Setup** from the 'Setup' menu in the left panel.
8. Select the 'Assign Targets and Samples' tab, and highlight the wells in use on the 'View Plate Layout' tab by dragging the pointer over the plate wells. Assign the target to the selected wells by selecting the box under 'Assign'.
9. Highlight the wells containing the DNA standards on the 'View Plate Layout' tab, and select "S" as the Task for the target.
10. Enter the concentration for each DNA standard in the 'Quantity' field without the unit of measure (e.g., 20 for 20pM).

**Note:** Multiple wells with DNA standards at the same DNA concentration can be highlighted at the same time so that values need to be entered only once.

11. Select **ROX** from the 'Select the dye to use as the passive reference' drop-down menu.
12. From the 'Analysis' menu at the top of the screen, select **Analysis Settings**.
13. On the 'CT Settings' tab in the 'Select a Target' panel, select the target.
14. In the 'CT Settings' section, *uncheck* the **Use Default Settings** box, and *uncheck* the **Automatic Threshold** box. Enter **1** for the threshold. Leave the **Automatic Baseline** box *checked*.
15. Select **Apply Analysis Settings**.
16. Select **Run Method** under 'Setup' in the left panel. Modify the default run method as directed below:
  - Enter **20** for 'Reaction Volume per Well.'
  - Change the 'Holding Stage' to **2 minutes at 95°C**.
  - Enter **30** for the 'Number of Cycles' under 'Cycling Stage.'
  - Change 'Step 1' to **95° for 15 seconds**, and change 'Step 2' to **60°C for 15 seconds**.
  - While **Step 2** is selected, **right-click** to bring up a menu. Choose **Add Step**, and select **Before**.
  - Change 'Step 2' to **60°C for 15 seconds**, and change 'Step 3' to **72°C for 45 seconds**.
  - Ensure that the 'Data Collection On' icon is active for 'Step 3' only of the 'Cycling Stage'.

**Note:** The 'Data Collection On' icon is shown in the legend at the bottom of the screen.
17. From the 'File' menu at the top of the screen, select **Save as Template**, and choose a location to save the file. The instrument setup and thermal cycling program can be saved as a template for future use. Use this .edt file as a template to create the experiment document as described in Starting a Run.

### Starting a Run

1. To create the experiment document, open the template file created in the section on Creating a Run Template by selecting **Open** from the 'File' menu at the top of the screen. Navigate to the .edt file and select **Open**.

**Note:** If the template file was just created, the .edt file will still be open.
2. Save the .edt template as an .eds file by selecting **Save As...** from the 'File' menu at the top of the screen. Choose a location to save the file.
3. Highlight the unused wells, and deselect the target.
4. To add sample names, select **Plate Setup** from the 'Setup' menu in the left panel, and select the 'Define Targets and Samples' tab. Select **Add New Sample**, and enter the sample name in the section provided. Repeat for all samples.
5. When all sample names are entered, navigate to the 'Assign Targets and Samples' tab. Highlight the well or wells that contain replicates of the same sample, and **check** the 'Assign' box adjacent to the corresponding sample name in the 'Assign sample(s) to the selected wells' section of the 'Assign Targets and Sample' tab. Repeat until all samples are assigned.
6. If a sample was assigned to the incorrect well, select the well and uncheck the sample under 'Assign sample(s) to the selected wells', and the assign the correct sample name.



#### 4.F. Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Fast Real-Time PCR System and 7500 Software for 7500 and 7500 Fast Real-Time PCR Systems v2.3 (continued)

7. Check that the correct passive reference is selected.
8. Save the .eds file.
9. Press the tray door.
10. Place the plate prepared in Section 4.C in the open tray door, and ensure that the plate sits correctly in the plate holder, with well A1 in the top left corner. Push the tray door to close and immediately press **Start Run**. Run time is approximately one hour.

#### Evaluating Standard Curves Using the 7500 Software for 7500 and 7500 Fast Real-Time PCR Systems v2.3

1. Select **Analysis** in the left side panel, and ensure that all wells to be analyzed are highlighted on the 'View Plate Layout' tab and that no targets are selected for unused wells.
2. Select **Analyze**.
3. To display the standard curve, select **Standard Curve** from the 'Analysis' menu in the left side panel.

#### Exporting Analyzed Data from the 7500 Software for 7500 and 7500 Fast Real-Time PCR Systems v2.3

1. Ensure that all wells with data for export are highlighted in the plate map. Select **Export** from the toolbar.
2. On the 'Export Properties' tab of the 'Export Data' window, select the following Export Properties:
  - Choose **Results** in the 'Select data to export' section.
  - Choose **One File** from the 'Select one file or separate files' drop-down menu.
  - Choose **.xls** as the File Type.
  - Specify the appropriate export file name.
  - Use the **Browse** button to select the file location.
  - Select **Start Export**.
  - In the 'Export Completed' window that appears, select **Close Export Tool**.

#### 5. Data Analysis

1. Review standard curve.
2. In general, using the default settings, we see slopes for the standard curves in the range of  $-3.30$  to  $-3.70$ . The  $R^2$  values for all standard curves should be greater than 0.990. Labs should perform internal testing to determine slopes and Y-intercept ranges.

## 6. Determine Library Concentration

To determine the library concentration, average the sample replicates and multiply by the dilution factor used in Section 4.B. The DNA standard provided is the optimal size for quantifying PowerSeq™ libraries; no size correction is needed.

**Note:** This will give the concentration in pM. Convert to nM for the library concentration to be compatible with Illumina sequencing manuals.

Use the concentration to normalize the library concentration. The optimal loading concentration may vary for different sample types or library construction methods. Refer to the library preparation kit and Illumina instrument manuals for guidance on loading concentrations and library pooling.

## 7. Troubleshooting

### Symptoms

Flat amplification curves and no  $C_q$  values detected for a subset of samples in the 96-well plate

### Causes and Comments

Verify that reaction mix was added to the failed amplification reactions.

Examine the volume of liquid in each well of the reaction plate to verify that evaporation did not occur during cycling. Ensure that all wells were adequately sealed to prevent evaporation during thermal cycling.

Flat amplification curves and no  $C_q$  values detected for all samples and standards in the 96-well plate

Verify that the thermal cycler was programmed correctly (see Section 4).

The PowerSeq™ Quant 2X Master Mix lost activity or was degraded. Minimize the number of freeze-thaw cycles. Store the PowerSeq™ Quant 2X Master Mix protected from light.

Verify that PowerSeq™ Quant MS 10X Primer Mix and/or DNA libraries were added to the amplification reactions.

From the Experiment menu in the Applied Biosystems® 7500 software, select the Analysis section, and then select the Multicomponent Plot to determine if fluorescence was detected for any of the system dyes, including the passive reference dye. If no fluorescence was detected, check to be sure that the PowerSeq™ Quant 2X Master Mix was added to the amplification reactions.

**7. Troubleshooting (continued)**

<b>Symptoms</b>	<b>Causes and Comments</b>
Late or no $C_q$ value for DNA libraries and standard curve is normal (linear with slope in specified range)	<p>Adapter sequences were not ligated to the DNA libraries. Prepare the DNA libraries again.</p> <p>The library dilution is significantly outside the dynamic range of the assay. Retest the library with correct dilution.</p> <p>Quantity and/or quality of amplified product was insufficient for library preparation. Please refer to your laboratory's internal validation manual for guidance.</p>
Non-linear standard curve ( $R^2$ values less than 0.98) and/or slope outside of specified range	<p>Be sure that the DNA standard is completely thawed and well mixed before use.</p> <p>Be sure that each dilution of the DNA standard is well mixed before removing an aliquot for the next serial dilution. Use the same pipette for each aliquot. Change pipette tip between each addition of DNA standard to the 96-well plate.</p> <p>Calibrate pipettes to minimize variability when pipetting.</p> <p>Amplify the standard curve in triplicate to minimize the effects of variation.</p> <p>An error was made during dilution of the DNA standard. Verify all calculations and repeat the dilution of the DNA standard. Avoid pipetting volumes less than 2<math>\mu</math>l.</p> <p>Incorrect concentration values were entered into the Applied Biosystems® 7500 Real-Time PCR System software. Verify that all of the standard DNA concentrations were entered correctly.</p> <p>Ensure that the correct passive reference was selected (ROX™ dye).</p> <p>Examine the volume of liquid in each well of the reaction plate to verify that evaporation did not occur during cycling. Ensure that all wells were adequately sealed to prevent evaporation during thermal cycling.</p> <p>Repeat the DNA standard dilution if the standards have been stored for longer than recommended.</p> <p>Analysis Settings in the Applied Biosystems® 7500 Real-Time PCR System software were not set correctly. Verify that the analysis settings were set as described in Section 4.</p> <p>Check that low-retention or siliconized tubes were used to prepare the serial dilutions of the DNA standard.</p>

<b>Symptoms</b>	<b>Causes and Comments</b>
Inconsistency between replicates of the same DNA sample	Examine the volume of liquid in each well of the reaction plate to verify that evaporation did not occur during cycling. Evaporation of the reaction mix can affect $C_q$ values. Check that library dilution falls into dynamic range of standard curve (20pM to 0.02pM). Retest library with corrected dilution.
DNA detected in no-template controls (NTCs)	An amplification curve that crosses the amplification threshold could indicate the presence of contaminating DNA.
Noisy amplification plots with no distinct amplification curve	Wells with no reaction mix were analyzed. Be sure that only wells that contain amplification reactions are highlighted and analyzed in Section 4.
Irregular amplification curve for the 20pM standard	Instrument software subtracted early amplification as background. Review the baseline and threshold values, and manually adjust as necessary.

## 8. Reference

1. *Illumina Adapter Sequences* (2018) Illumina, Inc. ([https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry\\_documentation/experiment-design/illumina-adapter-sequences-1000000002694-06.pdf](https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences-1000000002694-06.pdf)) [Accessed 03/27/2018]



## 9. Related Products

Product	Size	Cat.#
CXR Reference Dye	100µl	C5411
2800M Control DNA (10ng/µl)*	25µl	DD7101
2800M Control DNA (0.25ng/µl)*	500µl	DD7251

\*Not For Medical Diagnostic Use.

## 10. Summary of Changes

The following changes were made to the 5/20 revision of this Technical Manual:

1. In Section 2, the volume of PowerSeq™ Quant MS DNA Standard was changed from 25µl to 200µl.
2. An expired disclaimer was removed.

<sup>(a)</sup>U.S. Pat. Nos. 8,598,198 and 9,206,474 and other patents and patents pending.

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