

PowerSeq™ Quant MS System

Instructions for Use of Product PS5000



Quick Protocol

This document is for experienced users of the PowerSeq™ Quant MS System to determine the concentration of massively parallel sequencing (MPS) libraries compatible with Illumina MiSeq® platforms using qPCR. Data generated can be used to normalize the DNA concentration for MPS libraries. For complete protocol information and troubleshooting tips, see the *PowerSeq™ Quant MS System Technical Manual #TM511*. The Technical Manual provides instructions for use with the Applied Biosystems® 7500 Real-Time PCR System, Applied Biosystems® 7500 Real-Time PCR System for Human Identification and Applied Biosystems® 7500 Fast Real-Time PCR System.

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*.

Serial Dilution of PowerSeq™ Quant MS DNA Standard

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.
2. Prepare fresh serial dilutions of PowerSeq™ Quant MS DNA Standard as indicated in the following table. Vortex each dilution for 10 seconds before removing an aliquot for the next dilution. Change pipette tips between dilutions.

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

Dilution of PowerSeq™ System DNA Libraries

1. Dilute sample libraries to concentrations within the dynamic range of the assay (20pM to 0.02pM).
2. Pipet a minimum volume of 2µl of the undiluted library and an appropriate volume of PowerSeq™ Quant MS 1X Dilution Buffer to prepare the dilution.

PowerSeq™ System Reaction Setup

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 no-template control (NTC) reactions. Increase this number by 10–15% to compensate for pipetting error and reagent loss on sides of pipette tips.
4. Use the following table to calculate the volume of each component required to prepare sufficient reaction mix for the number of reactions determined in Step 3.

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

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.
8. Add 4µl of the PowerSeq™ Quant MS DNA Standard prepared in “Serial Dilution of PowerSeq™ Quant MS DNA Standard” or the diluted PowerSeq™ System libraries prepared in “Dilution of PowerSeq™ System DNA Libraries” 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.
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 of the *PowerSeq™ Quant MS System Technical Manual* #TM511 for specific instructions. Instructions for the Applied Biosystems® 7500 Real-Time PCR System for Human Identification (HID Real-Time PCR Analysis Software, Version 1.1 or 1.2) follow in “Run Setup and Thermal Cycling”.
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)

Run Setup and Thermal Cycling

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. To see instructions for the Applied Biosystems® 7500 Real-Time PCR System and Applied Biosystems® 7500 Fast Real-Time PCR System, see the *PowerSeq™ Quant MS System Technical Manual* #TM511.

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.

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. 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).
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 follows:
 - 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**, and 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'.
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 "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**.
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.
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 “Reaction Setup” 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 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 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 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**.

Data Analysis

1. Review standard curve.
2. Using the default settings, standard curve slopes are generally in the range of -3.30 to -3.70 . R^2 values for all standard curves should be >0.990 . Perform internal testing to determine slopes and Y-intercept ranges for your laboratory.

Library Concentration Determination

1. To determine the library concentration, average the sample replicates and multiply by the dilution factor used in “Dilution of PowerSeq™ System DNA Libraries”. The DNA standard provided is the optimal size for quantifying PowerSeq™ libraries; no size correction is needed. Convert from pM to nM for the library concentration to be compatible with Illumina sequencing manuals.
2. 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.

Additional protocol information is in Technical Manual #TM511, available online at: www.promega.com