Instructions For Use

# SPRIworks HT (For Illumina Next Generation Sequencers)

Generates fragment libraries for sequencing on the Illumina Next Generation Sequencers


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

B09855AA
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BECKMAN COULTER

**SPRIworks HT**
**(For Illumina Next Generation Sequencers)**
PN B09855AA (January 2012)



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# Revision History

*This document applies to the latest software listed and higher versions. When a subsequent software version changes the information in this document, a new issue will be released.*

**Initial Issue, 01/12**
Software version 1.0.0.17

# Contents

# Illustrations

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## Tables

CHAPTER 1

# Safety

Read all product manuals and consult with Beckman Coulter-trained personnel before attempting to operate instrument. Do not attempt to perform any procedure before carefully reading all instructions. Always follow product labeling and manufacturer's recommendations. If in doubt as to how to proceed in any situation, contact your Beckman Coulter Representative.

## Alerts for Danger, Warning, Caution, Important, and Note

**DANGER**

**DANGER indicates an imminently hazardous situation which, if not avoided, will result in death or serious injury.**

**WARNING**

**WARNING indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.**

**CAUTION**

**CAUTION indicates a potentially hazardous situation, which, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.**

**IMPORTANT** IMPORTANT is used for comments that add value to the step or procedure being performed. Following the advice in the Important adds benefit to the performance of a piece of equipment or to a process.

**NOTE** NOTE is used to call attention to notable information that should be followed during installation, use, or servicing of this equipment.

CHAPTER 2

# Introduction

## Product Components

**NOTE** This product is For Research Use Only. Not for use in diagnostic procedures.

SPRIworks High Throughput (SWHT) Biomek method for FX$^P$ Span8/MC hybrid product components are:

- SWHT reagent kit — 48 rxns (Part Number: B06938)
- 11 bottles/tubes of enzymes and buffers as follows. These 11 bottles of reagent are what is contained in the SWHT reagent kit.

  All the following enzymes and buffers should be stored at -20° C.

**Table 2.1** Reagent Kit Enzymes and Buffers

| Contents | Volume | Cap color |
|---|---|---|
| End Repair Enzyme | 1.35 mL | Blue |
| End Repair Buffer | 1.35 mL | Green |
| A-Tailing Enzyme | 1.35 mL | Red |
| A-Tailing Buffer | 1.35 mL | Purple |
| Ligase Enzyme | 1.36 mL | Black |
| Ligase Buffer | (3) x 1.18 mL ea. | Brown |
| PCR Master Mix | 1.35 mL | Orange |
| Agencourt AMPure XP | 36.0 mL | white/clear |
| Sizing Solution | 19.0 mL | white/clear |

## Summary and Explanation

The SWHT system uses the patented Agencourt SPRI paramagnetic-bead technology, combined with enzyme chemistry, to generate fragment libraries for sequencing on the Illumina Next Generation Sequencers.

The SWHT automated protocol is performed on the Biomek FX$^P$ Dual Arm Multi-96 and Span 8 Workstation. It processes up to 96 samples per run in a 96 well microtiter plate format, and enables the construction of four types of sized libraries:

- no size selection
- 150 to 350 bp size selection
- 250 to 450 bp size selection
- 350 to 700 bp size selection

**NOTE** Size ranges for the three selections listed above refer to insert only. Adapter size is about 100bp so actual library size ranges would be the selected range plus 100bp.

The reagents required to construct 48 libraries are included in each kit and are to be aliquotted onto the Biomek deck according to the method's instructions, along with reagents not included in the kit, such as ethanol, water, adapters and primers.

The operating temperature range for this high-throughput system is 15-30° C.

## Principles and Procedure

The SWHT uses enzymatic reactions, SPRI-based reaction purifications, and (optionally) SPRI-based size-selection to automatically generate 1 to 96 fragment libraries simultaneously per each run of the Biomek method.

The libraries are then further enriched and used as templates for sequencing on the Illumina Next Generation Sequencers.

Prior to running on SWHT, DNA is initially fragmented or sheared either by nebulization or by use of the Covaris instrument.

**NOTE** If DNA is fragmented by nebulization, a reaction clean-up step, such as the standard AMPure XP protocol, must be performed prior to the start of the method. Extra reagent volumes for this conditional step are not included in the kit.

## Hardware

The following hardware is manufactured by Beckman Coulter.

**Table 2.2** Hardware

| Part number | Description |
|---|---|
| A31844 | Biomek® FX$^P$ Dual Arm System with 96 Multichannel Pod and Span-8 Pod with 1 mL Syringes |
| 719368 | Biomek FX$^P$, 200uL Multichannel Head |
| 719356 | Biomek FX$^P$, Tip Loader |

**Table 2.2** Hardware

| Part number | Description |
|---|---|
| 719654 | Biomek FX$^P$, Span-8 Wash |
| 719662 | Tip Wash Station, 96-Channel, ALP, 220V |
| 719948 | 4x3 ALP, High-Density, 12-position |
| 719357 | 1X1 ALP (3 of these) |
| 719590 | Span-8 Disposal ALP |
| A40377 | Monitor, Flat Panel 22" |
| 987820 | Automation controller, XP |
| 379448 | Orbital Shaker ALP |
| 372795 | Frame for Reservoir (1 each) |
| A32782 | Agencourt® SPRIPlate®, 96R-Ring Super Magnet Plate |
| 373661 | 24 Position Tube Rack with White Insert |
| 373696 | White inserts for tube rack (case of 25) |

## Other Materials Used but Not Provided in the Reagent Kit

**Table 2.3** Other Materials Used but Not Provided

| Material | Description | Vendor |
|---|---|---|
| DNA Fragmentation Equipment (choose one) | Nebulizer Kit (1000541) & Nebulization Buffer (1000466) | Illumina, Inc. |
| Adapters and PCR Primers (choose one) | TruSeq DNA sample prep kit 48, and TruSeq sample prep kit - PCR 48 | Illumina, Inc. |
| | User-supplied Adapter Oligo Mix (2.5 µL at 100 µM) | Many vendors |

## Input DNA and QC Assessment

This procedure recommends an input amount of 1ug of sample DNA for sequencing.

Quality control should be performed to ensure that:

- Input DNA is from the correct target organism and that it is not contaminated with DNA from other organisms.
- Input DNA is double-stranded.
- DNA is free of particulate matter.
- Input DNA has an OD260/280 ratio of approximately 1.8.

- Input DNA sample has a minimum concentration of 20 ng/µL in water (or 10 mM Tris, pH 7.4 to 8.0).
- DNA is not otherwise contaminated. Any contamination in the starting material will directly carry over through the fragmented DNA and into the library construction process and may inhibit the library construction process.

## This guide

This guide explains how to set up the Biomek® FX$^P$ instrument and perform these methods:

- Library Construction, Size Selection, and PCR Setup
- PCR Clean-Up and qPCR Setup
- Normalization and Pooling

CHAPTER 3

# Library Construction, Size Selection, and PCR Setup

This chapter explains how to prepare up to 96 libraries, with or without size selection, from fragmented gDNA using the Biomek® FX$^P$ Dual Hybrid Workstation and the reagents provided by Beckman Coulter Genomics SPRIworks HT (SWHT) Illumina Kit.

For optimal yields with the size selection options, be sure your shear is centered appropriately:

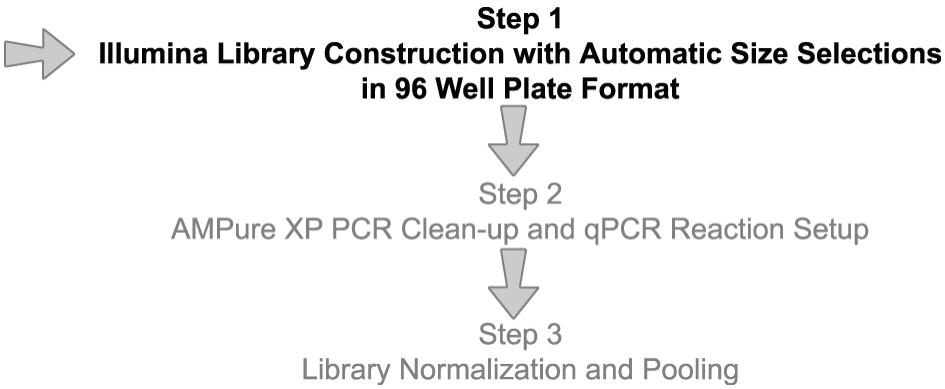
- for 150-350bp, center at 250bp
- for 250-450bp, center at 350bp
- for 350-700bp, center at 525bp

Once the method begins, if both library construction and size selection are selected, the method performs the size selection steps during the library clean-up and construction steps.

When analyzing sample chip data for a size range, keep in mind that the actual size is the size range selected plus 100bp, as adapters are annealed to your insert. The selection ranges available in the user interface reference the size of your insert only.

## Workflow

**Figure 3.1** Workflow



# System Configuration

## Labware and Consumables

The following products are recommended and have been validated with the product.

**Table 3.1** Labware and Consumables

| Part number | Manufacturer | Description |
|---|---|---|
| 379501 | Beckman Coulter | Biomek® Tips, P250, Span-8, NonSterile, 220 µL |
| 717252 | Beckman Coulter | Biomek® Tips, P250, AP96, Sterile, 240/220 µL |
| 987925 | Beckman Coulter | Biomek® Tips, Barrier, P1000, Span-8, Conductive, 1000 µL |
| 89004-298 | VWR | VWR® Screw-Cap Microcentrifuge Tubes (2mL Conical Bottom) |
| 372790 | Beckman Coulter | Quarter Reservoir |
| 372786 | Beckman Coulter | Half Reservoir |
| 50-995-860 | Fisher Scientific | Seahorse 96-well pyramid bottom reservoirs (300mL) |
| HSP-9601 | Bio-Rad | Hard-Shell® Thin-Wall 96-Well Skirted PCR Plate |
| AB-1127 | Thermo Scientific | ABgene 1.2 mL Square Well Storage Plate, Low Profile |

## Auxiliary Equipment

**Table 3.2** Auxiliary Equipment

| Part number | Manufacturer | Description |
|---|---|---|
| G2940CA | Agilent Technology | Agilent 2100 Bioanalyzer |
| | | 96-Well Thermal cycler (or equivalent) |
| 392932 | Beckman Coulter | Allegra® X-15R Centrifuge (or equivalent) |

# Kit Contents and User-Supplied Consumables

The SWHT Illumina Library Construction Kit contains:

- End Repair Buffer
- End Repair Enzyme
- A-Tailing Buffer
- A-Tailing Enzyme
- Ligation Buffer
- Ligation Enzyme
- PCR Master Mix

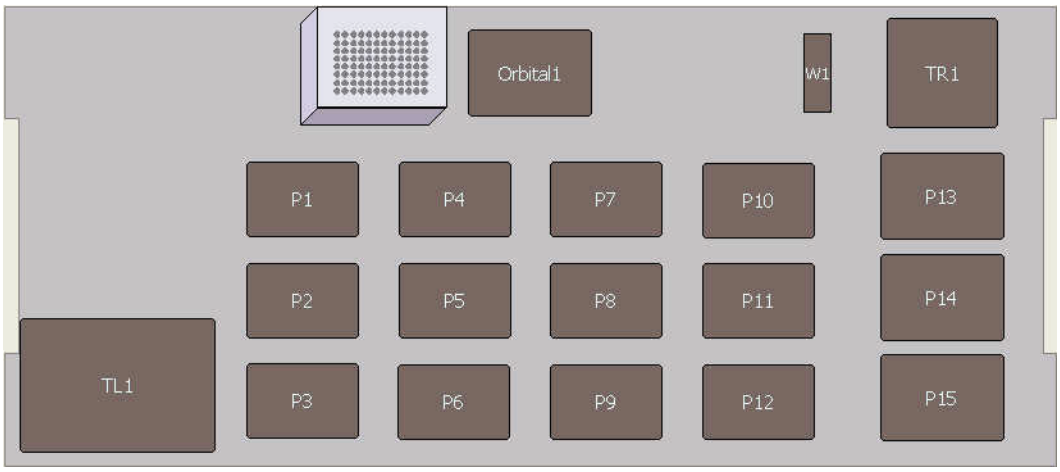
- AMPure XP
- Sizing Solution

You are responsible to supply:

- Adapters
- PCR Primers
- Ethanol (Alcohol, 81% denatured, American Bioanalytical AB04018 or equivalent 85% non-denatured alcohol)
- Water

## Set up the Deck

**Figure 3.2** SWHT Deck Layout for Biomek® FX$^P$ Laboratory Automation Workstations




## Set up the Workstation

1 Turn on the Biomek® FX$^P$ Workstation.

2 Open the Biomek Software.

3 From the **Instrument** menu, click **Home All Axes**.

4 Purge the system until there are no visible bubbles in the syringes or within the tubing.

5 Allow any frozen reagents to thaw completely on ice.

## Start the Biomek Method

This method uses SWHT Chemistry for Illumina to prepare samples for the Illumina Next Generation Sequencers. It consists of three modules: Library Construction, Size Selection, and PCR Setup.

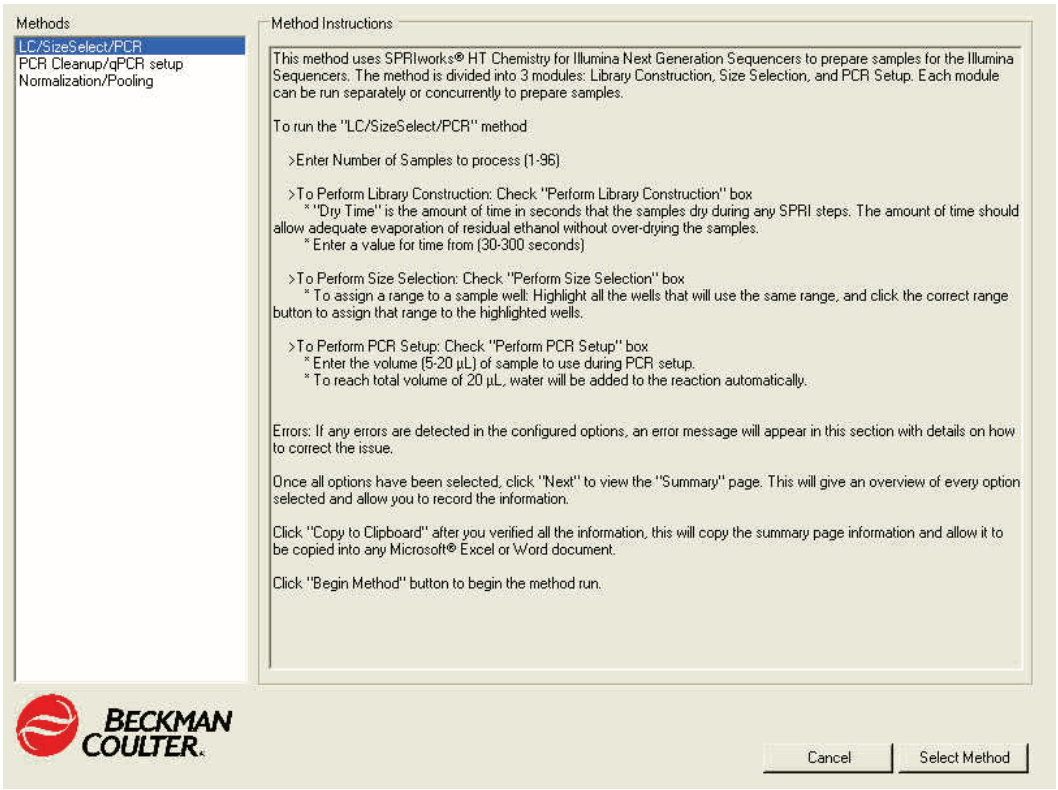
1 To open the project file, click **Project > Open Project > FX_SPRIWorks_HT**.

2 To open the Biomek method, click **File > Open > SWHT1**.

3 To start the method running, click the play button ( ).

The **Methods** page appears.

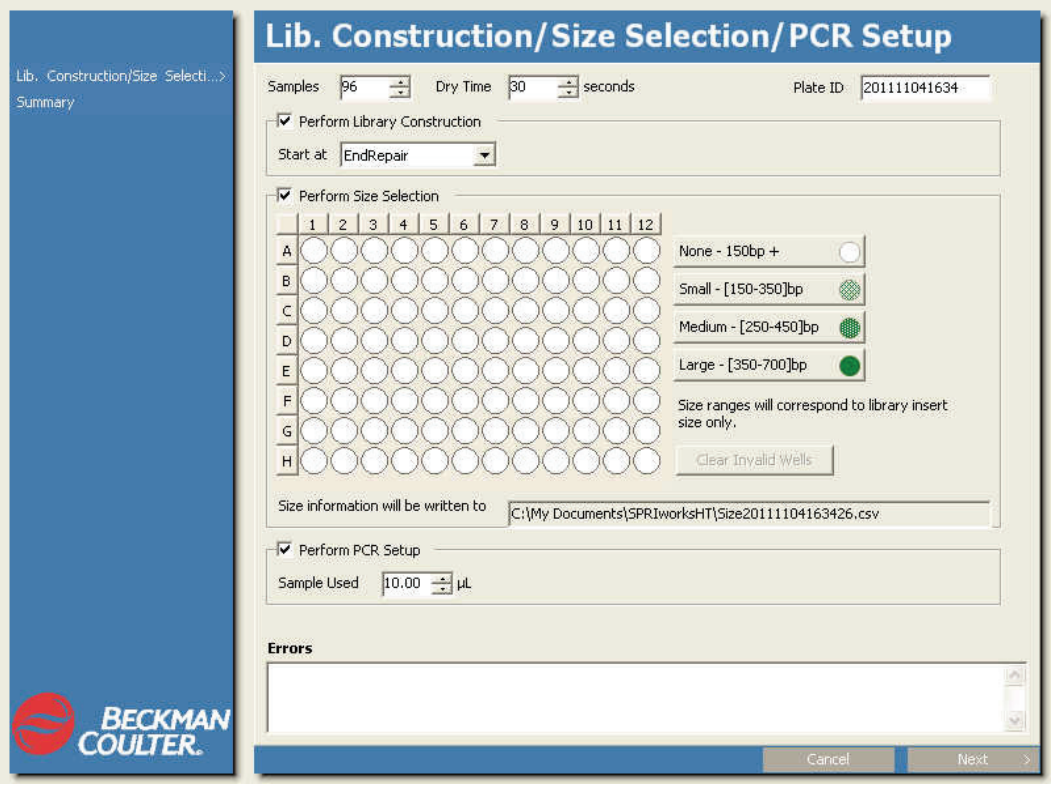
**Figure 3.3** Methods Page with LC/SizeSelect/PCR Selected



**4** In the left pane, select **LC/SizeSelect/PCR**, and click the **Select Method** button in the lower right corner of the page.

The **Lib. Construction/Size Selection/PCR** Setup page appears.

**Figure 3.4** Lib. Construction/Size Selection/PCR Setup page




**5** Either type or use the up and down arrows to select the number of samples to process (1-96), then specify the **Dry Time** (from 30-300 seconds).

**Dry Time** is the amount of time in seconds that the samples dry during any SPRI steps. Allow adequate evaporation of residual ethanol without over drying the samples.

**TIP** To skip any of the following three functions, leave the corresponding check box unchecked.

**6** Click the **Perform Library Construction** check box and specify the **Start at Option** from the drop-down list.

**7** Click **Perform Size Selection**, select all wells that will use the same range, and click the correct range button to assign that range to the highlighted wells.

**8** Click **Perform PCR Setup** and enter the volume (1-20 µL) of the sample to use during PCR setup.

To reach a total volume of 20 µL, the appropriate amount of water will be added to the reaction automatically.

If any errors are detected in the configured variables, an error message appears in the error box at the bottom of this section with details on how to correct the problem.

**9** To view a summary of the information you entered, click **Next**.

**10** After verifying the information, if desired, click **Copy to Clipboard**.

Enabling **Copy to Clipboard** copies the summary page information so that you can later paste it into Microsoft® Excel or a Word document for further processing.

**11** Click **Start**.

## Library Construction Reagent Calculator Guide

The library construction reagent calculator gives the appropriate volumes to fill the half-quarter-quarter AMPure reagent reservoir and 24-position tube block based on the inputs made in the user interface, and includes all minimum dead volumes.

The 24-position tube block guide is set up in the same orientation as the tube block itself. The positions marked "empty" will never hold a reagent tube. If running a partial plate, the positions that indicate "0 µL" will not need a reagent tube for that run (these positions will have a volume indicated when running a full plate).

**Table 3.3** Color-Coded Master Mix Tubes

| Master Mix Reagent | Provided? | Color of capped tube |
|---|---|---|
| **End Repair Master Mix** | | |
| End Repair Buffer | Yes | green |
| End Repair Enzyme | Yes | blue |
| **A-Tailing Master Mix** | | |
| A-Tailing Buffer | Yes | purple |
| A-Tailing Enzyme | Yes | red |
| **Ligation Master Mix** | | |
| Ligation Buffer | Yes | brown |
| Ligation Enzyme | Yes | black |
| **PCR Master Mix** | | |
| PCR Master Mix | Yes | orange |
| Primer Cocktail (see How to mix the primer cocktail below) | User-supplied | |

### How to mix the primer cocktail

1 Calculate the amount of primer recommended per reaction by the primer manufacturer.

2 Multiply the result by the number of samples in the run.

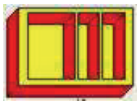
3 Bring to total volume with diH2O.

For example, if the calculator indicates 250 µL of Primer Cocktail when running 48 samples, and 2 µL of primer are to be added per sample, the calculation would be as follows:

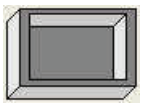
1. (48 samples) x (2 µL) = 96 µL
2. (96 µL) + (154 µL of diH2O) = 250 µL of primer cocktail

## Run the Method

1 As detailed in the previous section, follow the volumes and master mix recipes listed in the library construction reagent calculator to fill the tubes in the 24-position tube rack, and the half-quarter-quarter reservoir.



Fill the pyramid bottom ethanol reservoir with 160mL of 85% ethanol.



2 Click **OK**.

3 Place full boxes of tips, labware and reagents on the deck as indicated in the Biomek deck setup (layout) screen.

The **Swap** and **uStack - nametag** positions are only place holders and not actual labware.

Depending on run selections, some labware may be stacked as many as three pieces deep. For example, elution plate 2 may be stacked on elution plates 3 and 4. Elution plate 5 may be stacked on elution plate 6, and the adapter plate may be stacked on the final PCR plate.

To confirm any stacking, before clicking OK on the Biomek deck setup (layout) screen, click Get Method Information (highlighted in green on the left hand side).

The deck setup appears in the lower right corner of the Biomek software. To view a list of all stacked labware, pass your pointer over these positions.

4 Fill the sample plate with 1 ug of sheared DNA per well:

- If **Perform library construction** is selected, use 50 µL of volume per well.
- If **Perform library construction** is not selected, and the method is starting with **Size Selection**, use 100µL of volume per well.

5 Fill the adapter plate with 2.5µL of the desired adapter at 100uM, in the same orientation as the wells of the sample plate.

6 Click **OK** to begin the method.

## Suggested PCR Cycling Conditions

Perform the initial denature step at 98° C for 30 seconds. For each step:

1 Denature at 98° C for 10 seconds.

2 Anneal primers at 65° C for 60 seconds.

**IMPORTANT** For this step, use the annealing temperature that is recommended for your specific primers.

3 Perform the extension at 72° C for 60 seconds.

4 Repeat these steps for a total of 10 cycles.

CHAPTER 4

# PCR Clean-Up and qPCR Setup

In this chapter, AMPure XP reagent is used to capture amplicons leaving behind any unused PCR reagents and primer dimers that may have formed during the amplification process.

The KAPA Library Quantification Kit (KAPABIOSYSTEMS) is used for the setup of the qPCR quantification reaction. qPCR amplification occurs offline from automation on the 7900HT Fast Real-Time PCR System (Applied Biosystems), where it generates Ct values from each library.

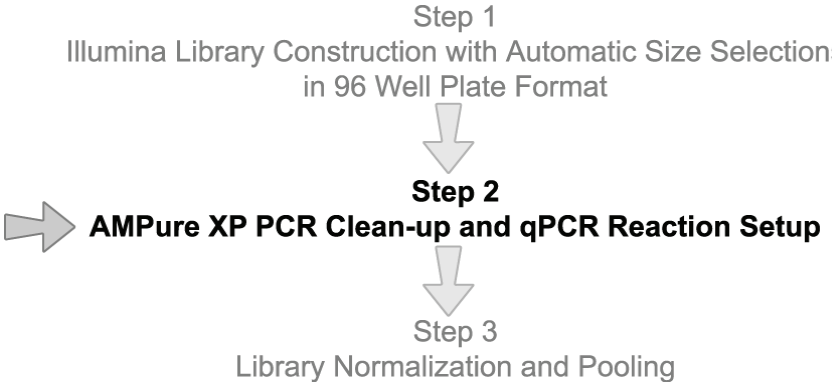
The qPCR method processes 24 samples at a time in a 96 well plate. To complete a full plate of 96 qPCR setup samples, the method must be run four times, selecting a new quadrant each time. By default, **Perform PCR cleanup** is not checked off, as this can happen for all 96 samples at once in a full plate.

PCR cleanup only needs to be run one time per full plate, only the first time a plate of more than 24 samples is processed.

Automated PCR clean-up and qPCR reaction setup can be run separately or, to streamline and simplify the operation, they may be combined. The Normalization and Pooling methods can also be run separately or combined for seamless processing.

## Workflow

**Figure 4.1** Workflow



# System Configuration

The hardware for the instrument is the same for all methods. The following table summarizes the labware, consumables and auxiliary equipment required.

**Table 4.1** Labware and Consumables

| Part number | Manufacturer | Description |
|---|---|---|
| A21578 | Beckman Coulter | Biomek® Tips, P50, Non-Conductive |
| 987925 | Beckman Coulter | Biomek® Tips, Barrier, P1000, Span-8, Conductive, 1000 µL |
| 717252 | Beckman Coulter | Biomek® Tips, P250, AP96, Sterile, 240/220 µL |
| 372790 | Beckman Coulter | Quarter Reservoir |
| 89004-298 | VWR | VWR® Screw-Cap Microcentrifuge Tubes (2mL Conical Bottom) |
| HSP-9601 | Bio-Rad | Hard-Shell® Thin-Wall 96-Well Skirted PCR Plate |
| AB-1127 | Thermo Scientific | ABgene 1.2 mL Square Well Storage Plate, Low Profile |
| 4312063 | Applied Biosystems | MicroAmp® Optical 96-Well MicroAmp Base |
| 4306736 | Applied Biosystem | MicroAmp® Optical 96-Well Reaction Plate |
| 4311971 | Applied Biosystem | MicroAmp® Optical Adhesive Film |
| 4333183 | Applied Biosystem | MicroAmp® Adhesive Seal Applicator (or equivalent) |

**Table 4.2** Auxiliary Equipment

| Part number | Manufacturer | Description |
|---|---|---|
| G2940CA | Agilent Technology | Agilent 2100 Bioanalyer |
| 7900HT | Applied Biosystems | 7900HT Fast Real-Time PCR System (or equivalent) |
| 392932 | Beckman Coulter | Allegra® X-15R Centrifuge (or equivalent ) |

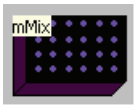
# Prepare Reagents

## PCR Clean-Up and qPCR Reagents

- KAPA Library Quantification Kits - For Illumina Genome Analyzer platform (KAPA Biosystems, KK4835).
- Tris 1M Solution, pH8.0 (American Bioanalytical, AB 14043-01000 or equivalent)
- Tween -20 (American Bioanalytical, AB 02038-01000 or equivalent)
- UltraPureTM Distilled Water (GIBCO, #10977 or equivalent)
- Ethanol (alcohol, 81% denatured from American Bioanalytical AB04018 or equivalent 85% non-denatured alcohol)

## mMix Setup

All KAPA reagents sit on a 24-position tube rack in uncapped 2 mL tubes.




1. Upon receipt of the KAPA library quantification kit, premix the following two reagents:
   - 1 mL of Illumina GA Primer Premix (10X)
   - 5 mL of KAPA SYBR FAST qPCR Master Mix (2X)

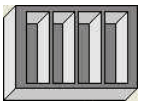
2. For each reaction, add
   - 12 µL of KAPA SYBR FAST qPCR Master Mix with Primer Premix
   - 4 µL of distilled water

3. Pipette 80 µL of each of the six KAPA standards, one into each of the 2 ml centrifuge tubes in positions A2-std 1, B2- std 2, C2- std 3, D2-std 4, A3-std 5, and B3-std 6 of the 24-position tube rack.

4. Add 130 µL distilled water to the tube in position **C3**.

## Reagent Reservoir Requirements

This reservoir includes three (3) quarter-reservoirs for PCR cleanup and one (1) quarter reservoir for performing qPCR setup. Each of the sections can hold enough reagents to process a full plate.



A reagent calculator, which is built in to the Biomek method, automatically calculates the total volumes required for each reagent based on the variables entered in the user interface. The results are displayed in the reagent calculator after clicking **Start Method**.

A full plate PCR clean-up and qPCR reaction setup needs:

- AMPure XP (Section #1: 6,300 µL)
- Distilled Water (Section #2: 4,380 µL)
- Ethanol (alcohol, 85% non-denatured) (Section #3: 20,700 µL)
- Library Dilution Buffer (LDB) (Section # 4): 25,020 µL

# Biomek Software Setup and Operation Procedure

PCR clean-up and qPCR reaction setup can run either in sequential order or separately as individual modules.

## Prepare the Instrument

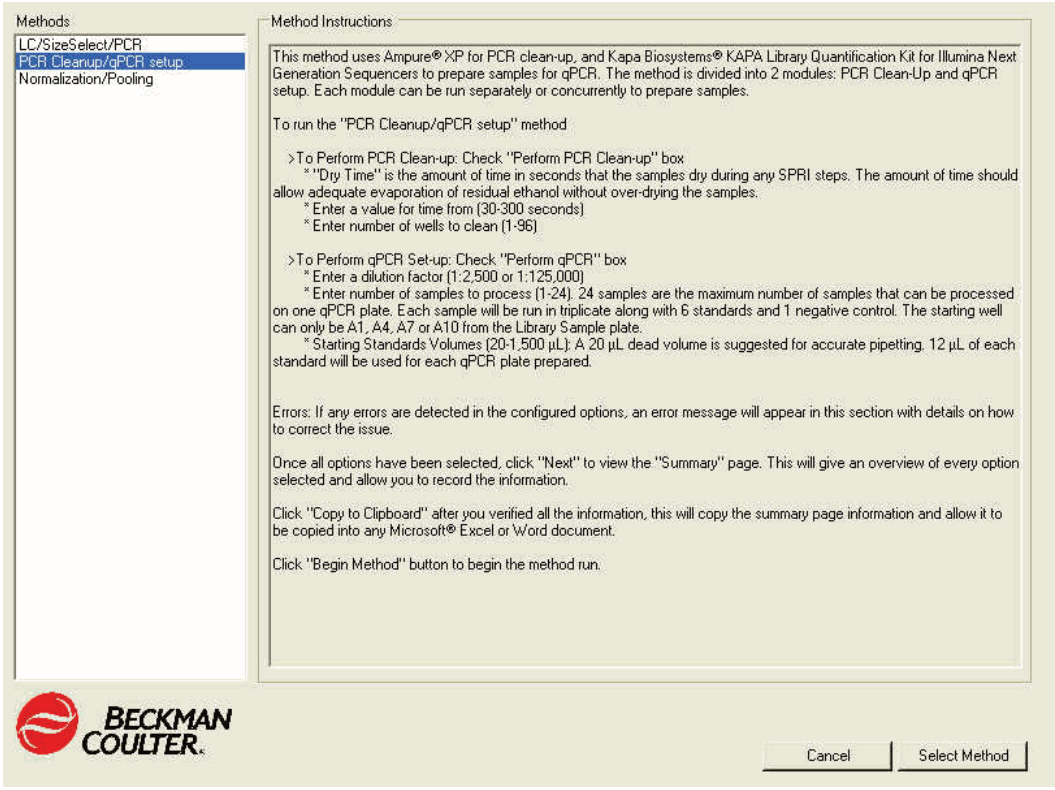
1. Turn on the Biomek® FX$^P$ instrument and click **Biomek Software > Open**.
2. From the **Instrument** menu, click **Home All Axes**.
3. Purge the system until there are no visible bubbles in the syringes or probe tubing.
4. Remove any required reagents from the freezer and thaw them completely on ice.

## Run the Biomek Method Software

1. To open the project, click **Project > Open Project > SPRIWorks_HT**.
2. To run the methods, open the SWHT1 file by clicking **File > Open > SWHT1**.
3. To start the run, click the play button ().

The **Methods** page appears.

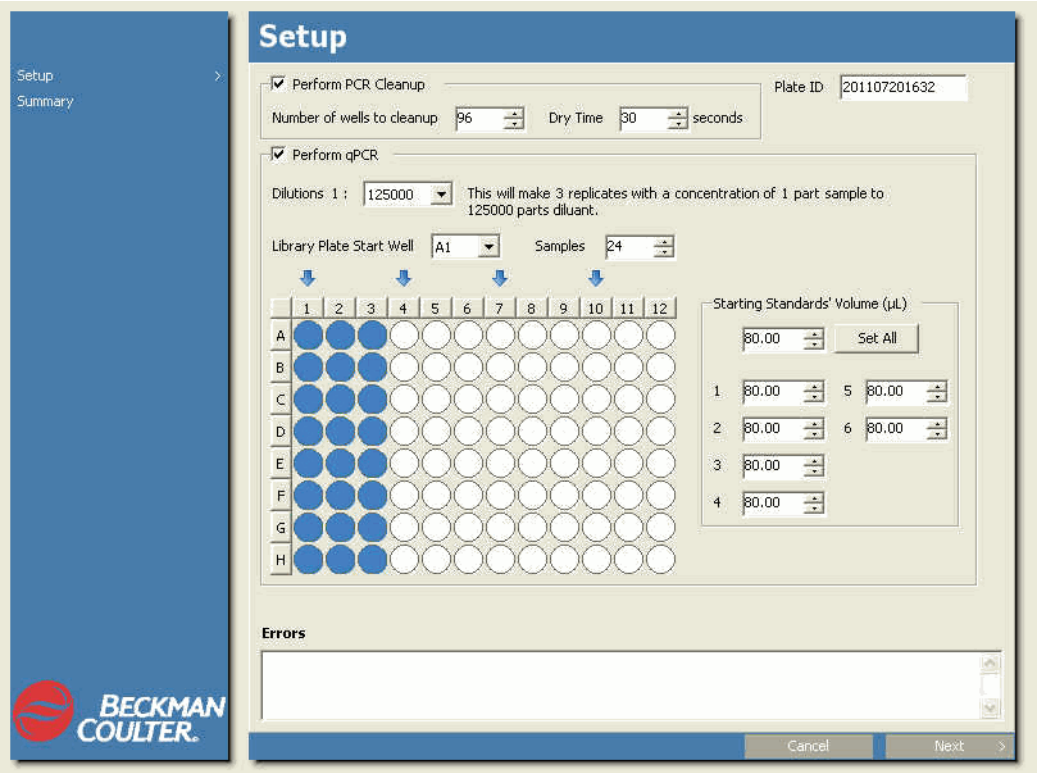
**Figure 4.2** Methods Page with PCR Cleanup/qPCR Setup Selected




**4** In the left pane, select **PCR Cleanup/qPCR setup**, then click the **Select Method** button in the bottom right corner of the page.

The **Setup** dialog box appears.

**Figure 4.3** Setup Dialog Box



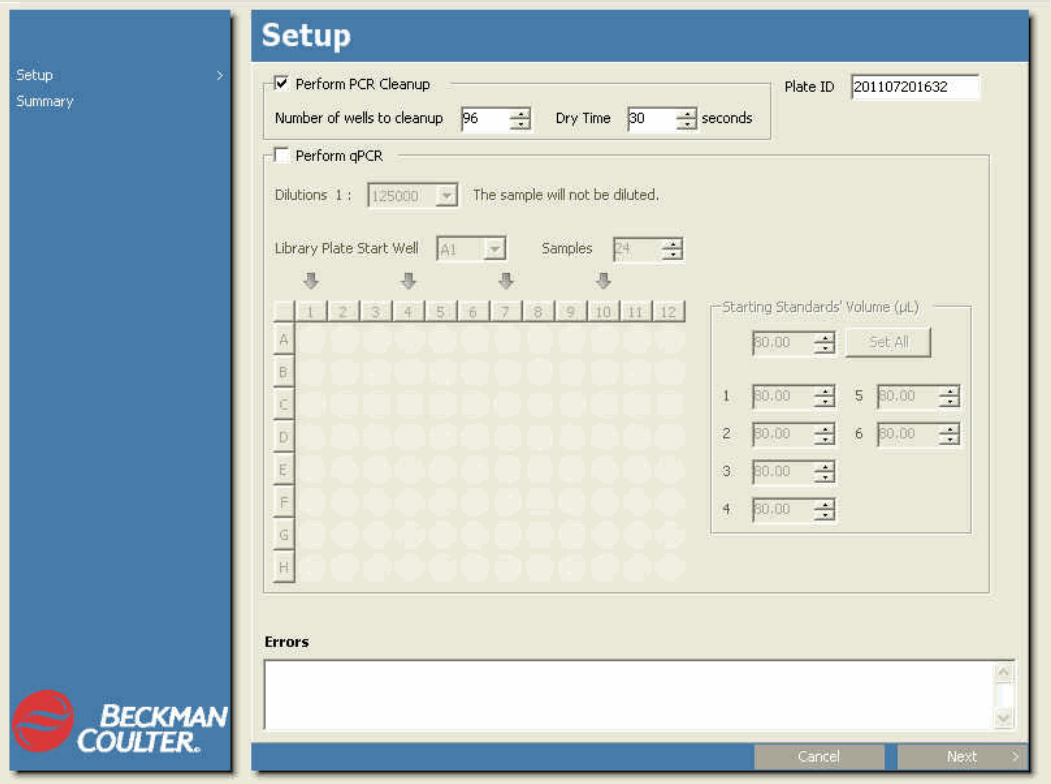

To run both methods sequentially, verify that the check box is checked for both methods, and then follow the steps in the next two sections.

## To Configure the PCR Clean-Up Method

1 Select the **Perform PCR Cleanup** check box.

The **Setup** dialog box enables the **Dry Time** variables.

**Figure 4.4** Setup Dialog Box with Perform PCR Cleanup Selected




2 Enter values for the following variables:

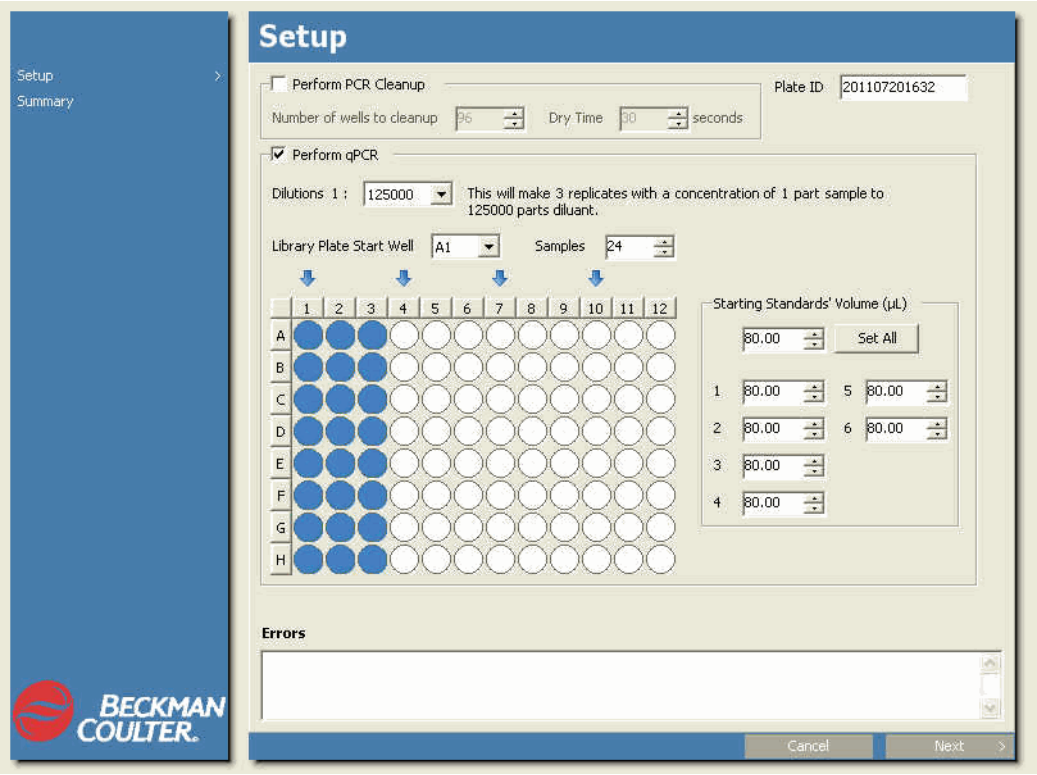
- **Wells to Clean** (1-96)
- **Dry Time** (30-300 seconds). This refers to the dry time after final ethanol wash removal and prior to elution.

## To Configure the qPCR Method

1 Select the **Perform qPCR** check box.

The **Setup** dialog box enables additional variables.

**Figure 4.5** Setup Dialog Box with Perform qPCR Selected




**2** Enter values for the following variables:

- **Dilutions** (2 = 1:2,500 or 3= 1:125,000)

  The method will dilute each sample using one of two dilution factors. The default dilution factor is **3** (1:125,000).

- **Library Plate Start Well**

  Up to 24 Illumina Libraries can be processed on one qPCR plate. Each library runs in triplicate along with six standards and one negative control. The starting well can only be: **A1**, **A4**, **A7** or **A10**.

- **Samples** (1-24)

  You may enter the number of samples or click to select the wells where the samples are located on the library plate graphic.

- **Starting Standards** (20-1,500 µL)

  20 µL dead volume is suggested for accurate pipetting. 12 µL of each standard (4 µL per rep) is used for each qPCR run.

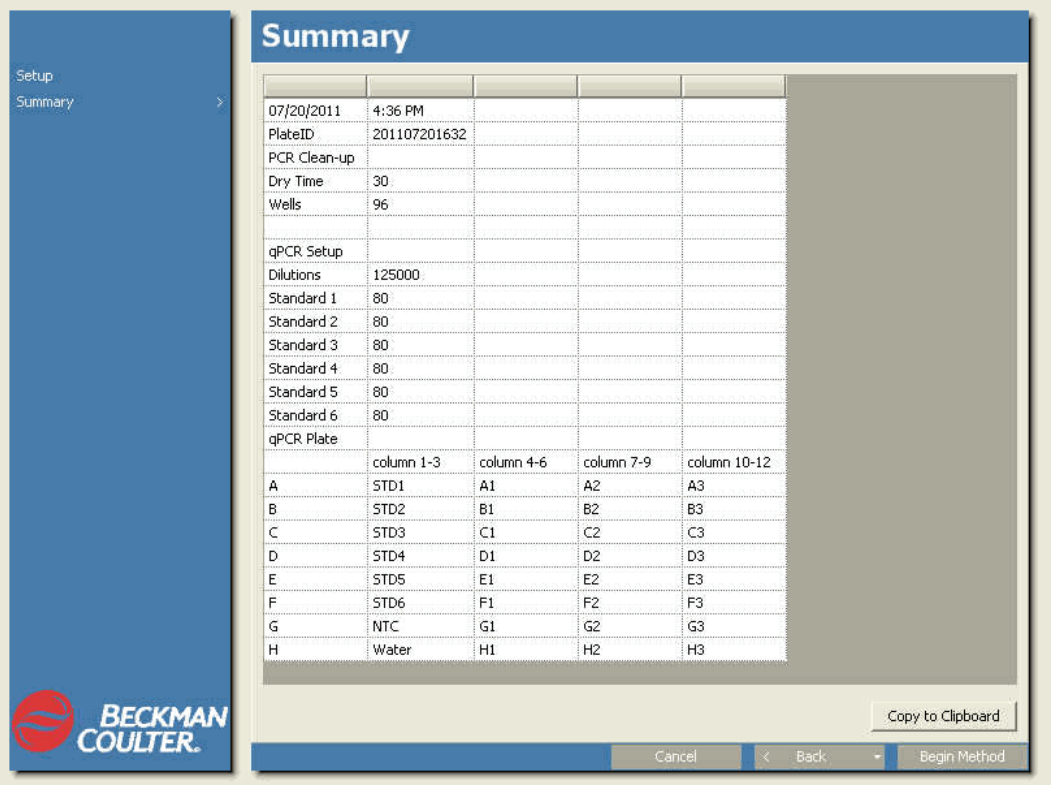
## Finish the Configuration and Final Check

If any errors occur at the configuration step, an error message appears in the error box with an explanation of how to correct the error. The **Next** button will not work until you clear all errors.

1 Once you have entered all variables, click the **Next button**.

The **Summary** page of all variables for one or both methods that you selected appears.

**Figure 4.6** Example of a Summary page for both PCR Clean-Up and qPCR Reaction Setup

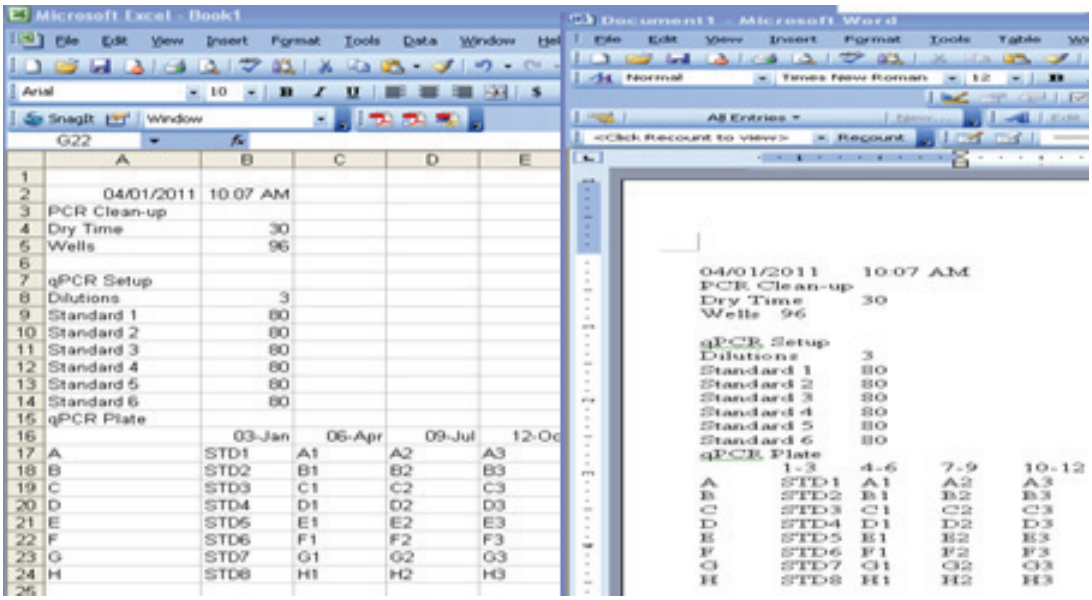



2 Click the **Copy to Clipboard** button.

This copies the summary page information, which you can paste into any Microsoft® Excel spreadsheet or Word document for tracking purposes.

Below is a sample of a Microsoft® Excel spreadsheet and a Word document containing Summary information.

**Figure 4.7** Example of Summary Information in Excel and Word

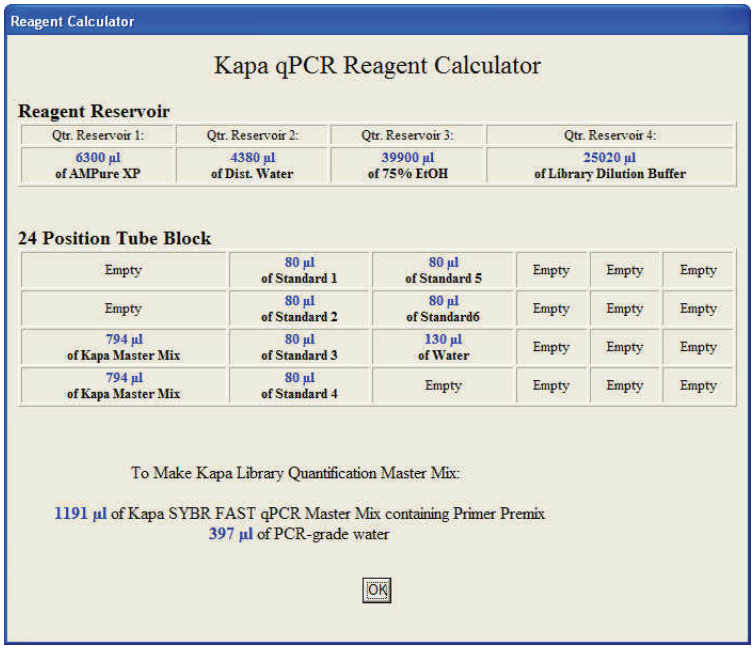



**3** To begin the method run, click the **Begin Method button** on the **Summary** page.

## Perform the Final Check before Beginning the Method

A series of pop-ups appear before the method starts. The first is the reagent calculator. The second is the deck layout according to the selected run parameters.

**Figure 4.8** PCR Cleanup and qPCR Reagent Calculator




1 Fill in each reagent volume and click **OK**.

Be sure to reference the KAPA library quantification master mix recipe at the bottom of the reagent calculator.

2 Follow the deck layout for the proper labware placement.

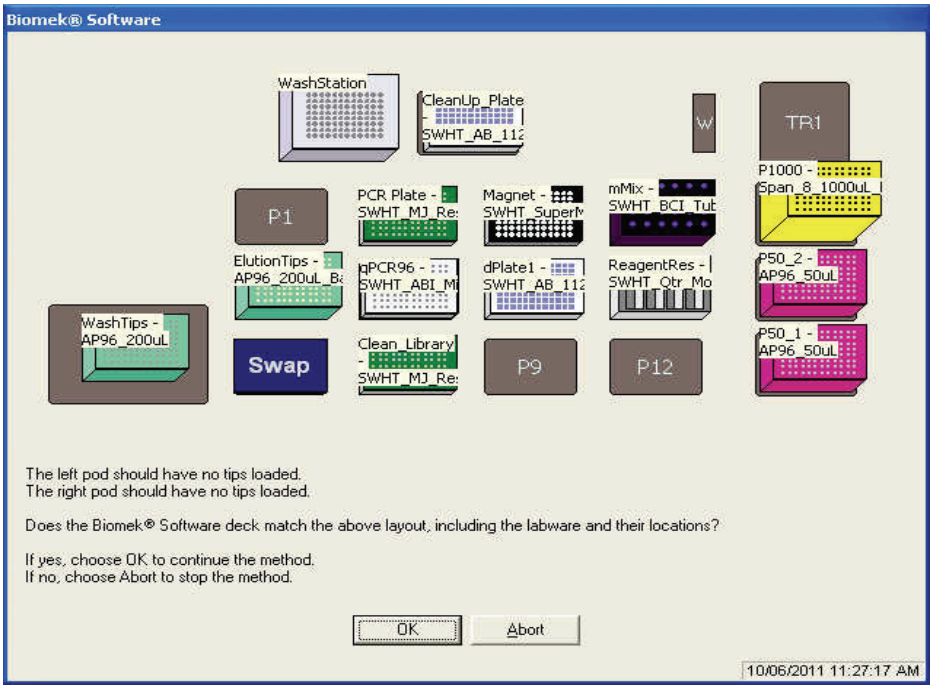
## Setting up the Deck

This section illustrates one of the possible deck configurations for this part of the method, as it would be displayed after variables are entered in the user interface. Make sure that the actual deck and the software layout match, including the labware types and their locations. If the layouts match, click **OK** to begin the Method Run, otherwise click **Abort**.

**NOTE** Always check the Instrument Setup Confirmation page at run time.

### Setup for PCR Clean-Up and qPCR Reaction Setup

**Figure 4.9** Biomek® FX$^P$ Deck Setup for PCR Clean-Up and qPCR Reaction Setup




## Prepare qPCR Plate for Amplification

1 After the run has completed, seal the plate with MicroAmp optical adhesive film using the adhesive film applicator.

2 Centrifuge the qPCR plate briefly to collect all reagents in the bottom of the wells. Use 1000 rpm for 30 seconds.

The qPCR plate is now ready to load into the 7900HT Fast Real-Time PCR system (Applied Biosystems) or equivalent.

# Real-Time PCR Amplification

This section describes real-time PCR amplification using the 7900HT real-time PCR instrument.

Create a New Document with the following attributes. For how to use the Applied Biosystems 7900HT Fast Real-Time PCR System, refer to the instruction manual.

- **Assay:** Standard Curve (AQ)
- **Container:** 96 wells Clear Plate
- **Template:** Blank Template

To use a predefined template, click **User Pre-defined Template > Browse...**, locate the predefined qPCR template (.sdt format) and click **OK**.

## Define a New Template

1 To use a blank template, click **Blank Template > OK**.

The template page appears.

2 If it is not already selected, click the **Setup** tab in the right pane.

3 You may optionally fill in each well manually using the following table.

**Table 4.3** 96-Well qPCR Plate Layout

| Columns 1-3 | Columns 4-6 | Columns 7-9 | Columns 10-12 |
|---|---|---|---|
| **Standard 1** (20 nM) | Sample #1 | Sample #9 | Sample #17 |
| **Standard 2** (2 nM) | Sample #2 | Sample #10 | Sample #18 |
| **Standard 3** (0.2 nM) | Sample #3 | Sample #11 | Sample #19 |
| **Standard 4** (0.02 nM) | Sample #4 | Sample #12 | Sample #20 |
| **Standard 5** (0.002 nM) | Sample #5 | Sample #13 | Sample #21 |
| **Standard 6** (0.0002 nM) | Sample #6 | Sample #14 | Sample #22 |
| No Template Control (**NTC**) | Sample #7 | Sample #15 | Sample #23 |
| Distilled Water (20 µL) | Sample #8 | Sample #16 | Sample #24 |

4 To add the detector, select the **A1** well and click the **Add Detector** button at the bottom of the page.

5 Select the **Kapa_spri** row and click **Copy to Plate Document**.

If the Kapa-spri is not on the detector list, click **New** and add this assay, choosing **SYBR** as the reporter and N**on Fluorescent** as the quencher.

**6** Click **Set up Standard**.

**7** Select the detector as the Kapa_Spri.

- # of points = 6
- # of replicates = 3
- Start Quantity = 20ng
- Serial Factor = 1:10
- Select **Let me select**

**8** Highlight columns 1 to 3 and click **OK.**

**9** To choose No Template Control (NTC) wells, select wells G1-H3 on the top plate format panel, and click **Task > Unknown**.

**10** To choose the unknown wells, select the rest of the wells on the top plate format pane and click **Task > NTC**.

**11** Select the **Instrument tab** and set the instrument to run the following:

- Mode: Standard
- 100% Ramp Rate
- Sample volume (µL) - 20
- 95C(5:00)
- 95C(0:30)
- 60C(0:45)
- Repeat 95/60C 35X

**12** To save the template, click **File > Save As**.

This file is saved in the .sdt template format and can be re-used later.

## Connect to the Instrument

1. Click the **Instrument** tab, and then the **Real-Time** tab.
2. Click the **Connect to Instrument** button half-way down the right pane.
3. To load the qPCR plate, click the **Open/Close** button.
   The door on the right side of the instrument opens.
4. Load the qPCR plate, lay the cover on top of the plate, and click the **Open/Close** button again.
   The instrument door closes.
5. To start the qPCR amplification, click the **Start Run** button.
   It takes approximately one hour to complete the qPCR amplification run.

## Analyze the Results

1. When the amplification run is finished, click the upper left corner of the top left pane to select the entire qPCR plate.
2. From the **Analysis** menu, click **Analyze**
3. From the **Analysis** menu, click **Analysis Settings** and change from the default setting (**Automatic Ct**) to **Manual Ct.**
4. Enter a **Threshold** of **0.30**.
5. To export the data for normalization, click **File > Export > Save As** and save the file in the .txt file format.
   These Ct values can be directly imported into the Biomek Normalization Method.

CHAPTER 5

# Normalization and Pooling

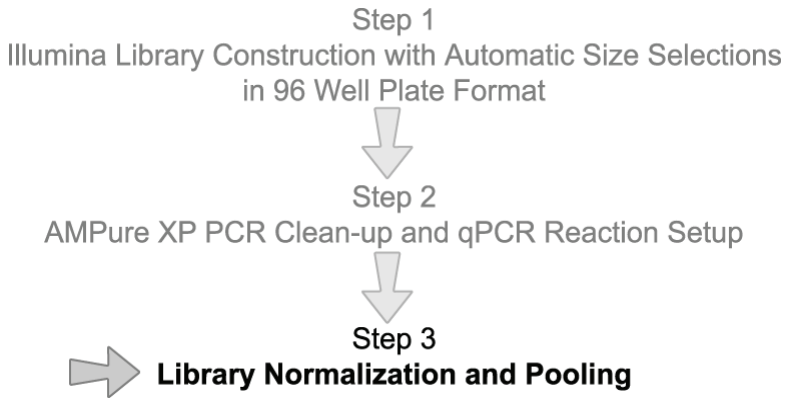
This chapter covers normalization and pooling of samples after qPCR.

To see and approve the library concentrations (making adjustments for size selected fragments) before normalization and pooling, the user imports the Ct values into the supplied Microsoft® Excel qPCR Quantification and Normalization spreadsheet. The flexible user interface allows for simple identification and selection of wells to pool for downstream applications.

**NOTE** You may need to adjust your Microsoft® Excel security settings and accept macros.

## Workflow

**Figure 5.1** Workflow




## System Configuration

The following table summarizes the labware, consumables and auxiliary equipment required.

**Table 5.1** Labware and Consumables

| Part number | Manufacturer | Description |
|---|---|---|
| A21578 | Beckman Coulter | Biomek® Tips, P50, Non-Conductive |

**Table 5.1** Labware and Consumables

| Part number | Manufacturer | Description |
|---|---|---|
| 372790 | Beckman Coulter | Quarter Reservoir |
| HSP-9601 | Bio-Rad | Hard-Shell® Thin-Wall 96-Well Skirted PCR Plate |

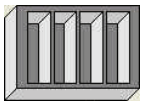
## Normalization and Pooling Reagents Requirements

You need a library dilution buffer:

- Tris 1M Solution, pH8.0 (American Bioanalytical, AB 14043-01000)

  OR
- UltraPureTM Distilled Water (GIBCO, #10977) or equivalent substitution

This reservoir includes four (4) quarter-reservoirs. Each of the four reservoirs can hold enough reagents to process a full plate.



A full plate of normalization needs:

- Empty (Section #1: 0 µL)
- Empty (Section #2: 0 µL)
- Empty (Section #3: 0 µL)
- Library Dilution Buffer (LDB) (Section # 4): 25,020 µL

## Run the Normalization Method

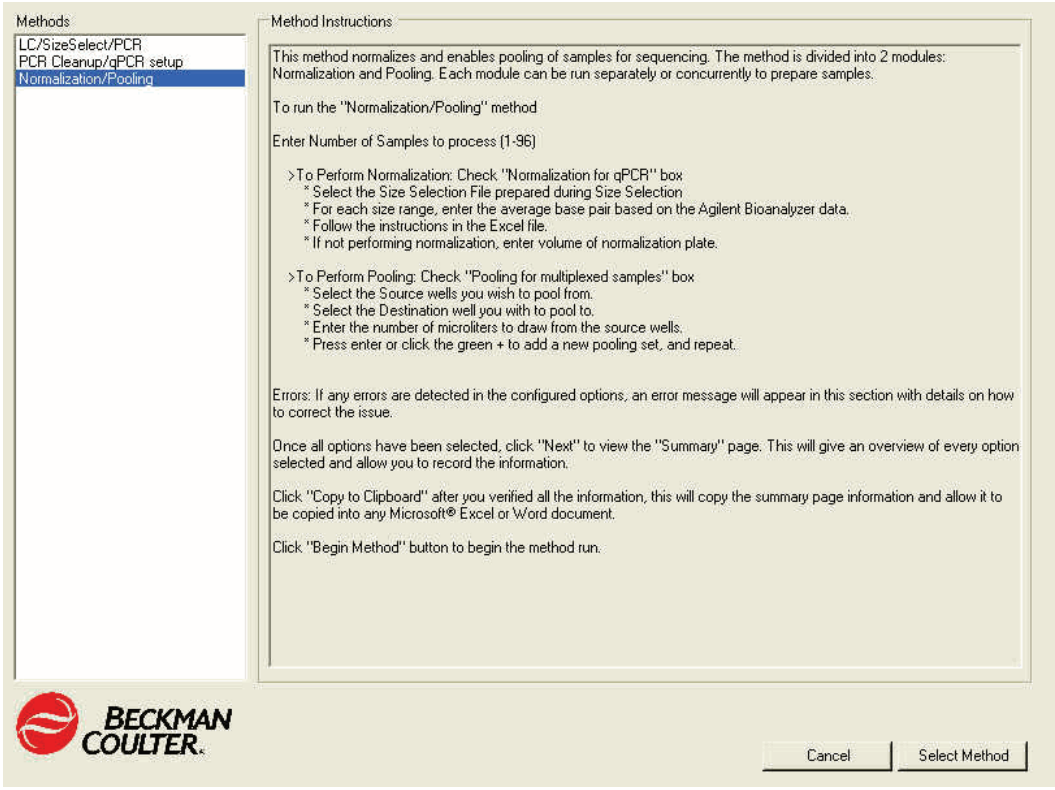
1 To open the project, click **Project > Open Project > SPRIWorks_HT**.

2 To run the methods, open the SWHT1 file by clicking **File > Open > SWHT1**.

3 To start the run, click the play button ( ).

The **Methods** page appears.

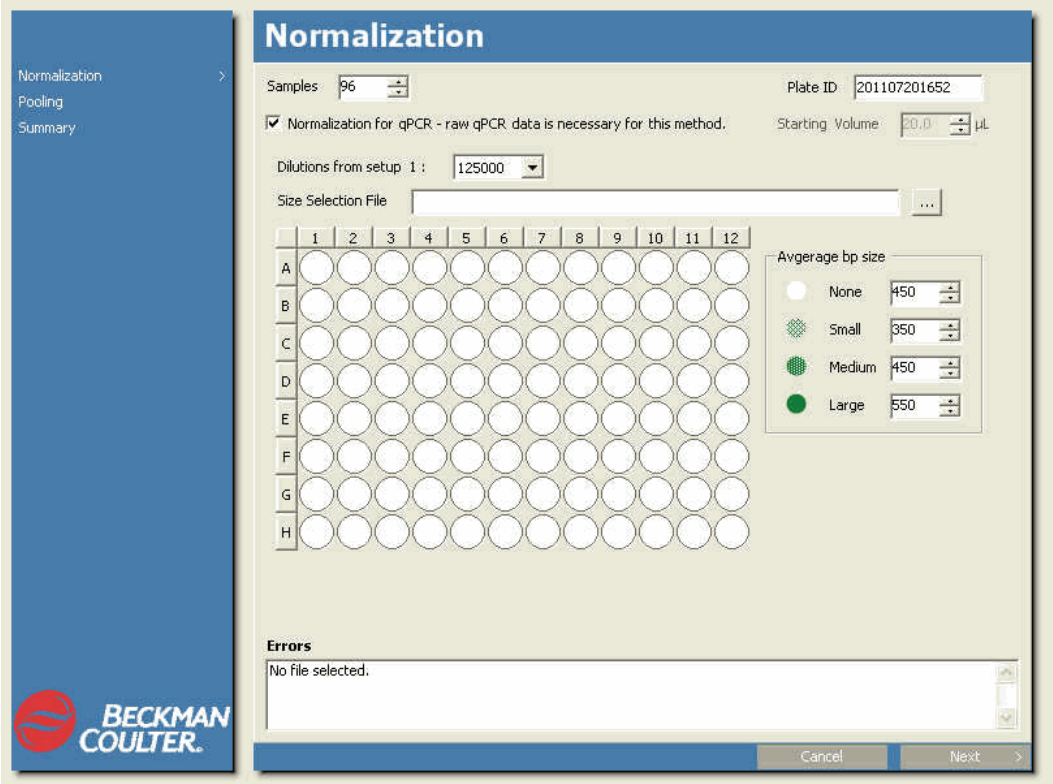
**Figure 5.2** Methods Page with Normalization/Pooling Selected




**4** Select **Normalization/Pooling** in the left pane and click the **Select Method** button in the bottom right corner.

The **Setup** page for normalization appears.

**Figure 5.3** Normalization Page




**5** Select the **Normalization for qPCR** check box.

**6** Fill in the following variables:

- **Samples** (1-96)
- **Dilutions used in setup** (2 = 1:2,500 or 3= 1:125,000)

**7** Browse for the **Size Selection File** saved from the Library Construction run.

This file is generated when the Library Construction method is run, and contains the size selections applied to each well in a plate. The default location is C:\My Documents\SPRIworks HT and the file name has a specific format:

Size*YYYYMMDDHHmmss*.csv

where:

- *YYYMMDD* is the date the library construction method was run.
- *HHmmss* is the time the library construction method was run.

**NOTE** If you scanned in a barcode for the plate, the barcode number will be saved in the file name instead of the date and time.

**8** Adjust the sizes as needed based on bioanalyzer chip data.

**NOTE** You can have sample-specific size selection in the Quant and Norm Spreadsheet. These will open when you finish entering these variables.

Any errors appear in the errors box at the bottom of the page. The **Next** button will not work until you resolve all errors.

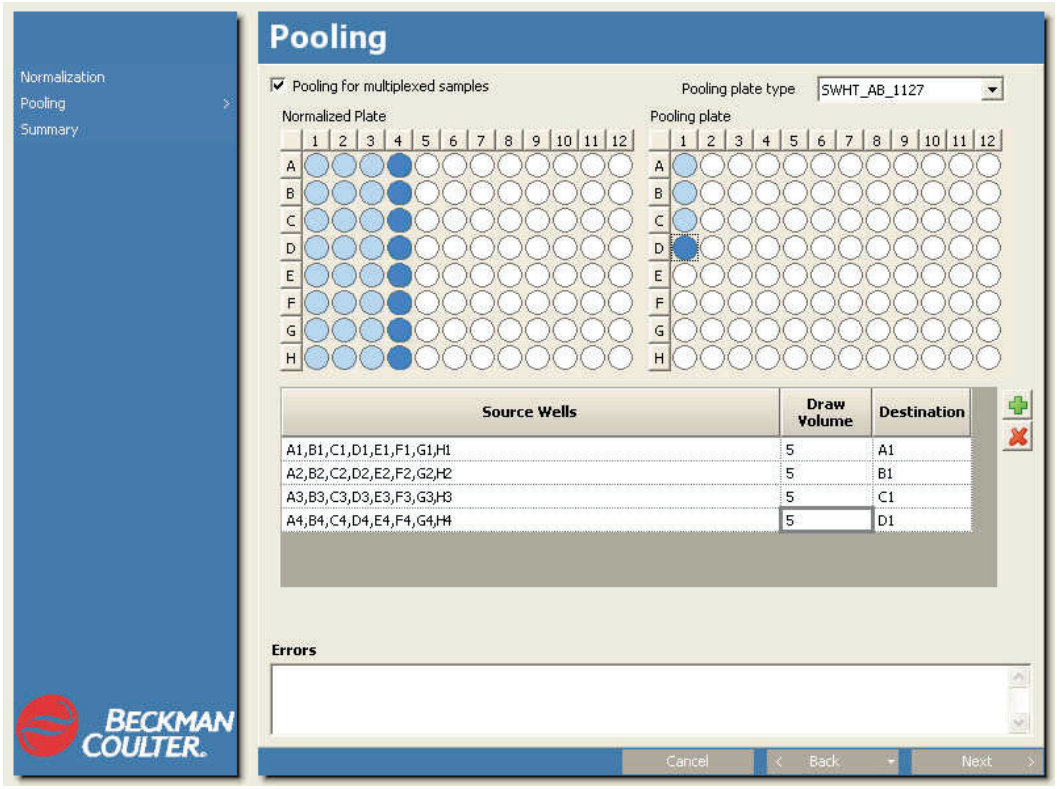
**9** Click **Next** to continue.

## Run the Pooling Method

**1** Select **Pooling** in the left pane of the **Normalization** page.

The **Pooling** page appears.

**Figure 5.4** Pooling Page




**2** Select the source wells in the **Source** (left) table and the destination wells in the **Destination** (right) table.

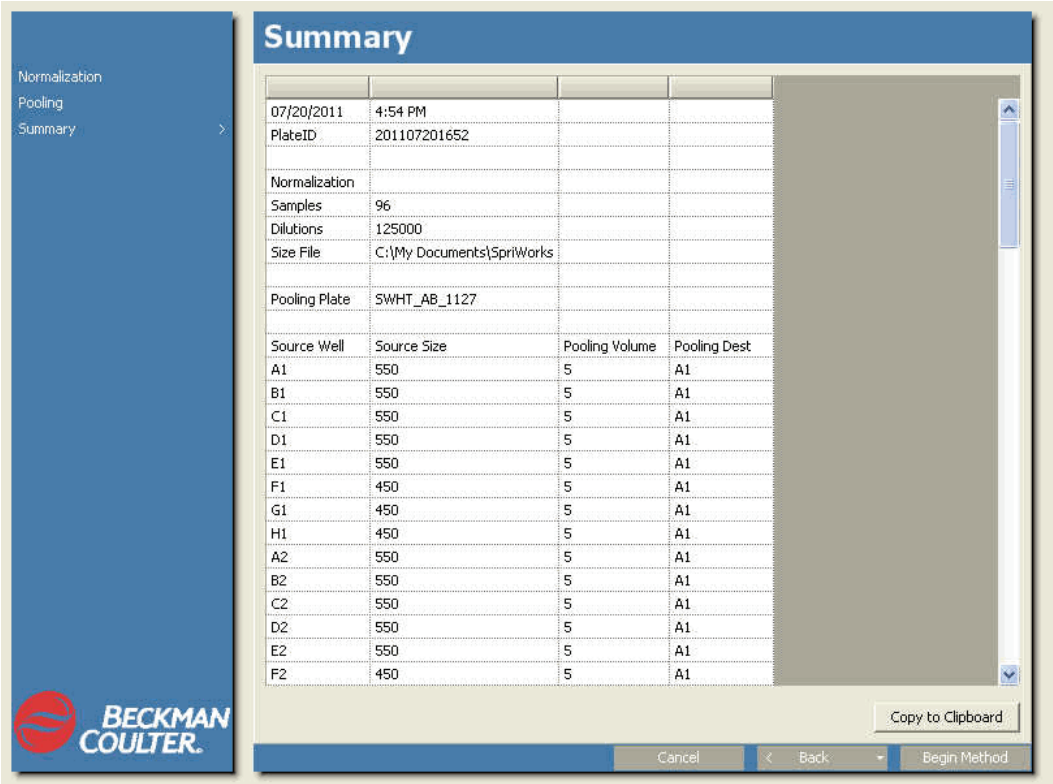
**3** Enter the volume of the sample to dispense to the destination well for pooling (1-50 µL).

Any errors appear in the error box at the bottom of the page. The **Next** button will not work until you resolve all errors.

**4** Click **Next** to continue.

The **Summary** page appears.

**Figure 5.5** Summary of Pooling Variables




**5** You may optionally click the **Copy to the Clipboard** button.

The system copies the summary page information to the clipboard for later pasting into Microsoft® Excel or Word.

**6** To begin the run, click the **Begin Method** button.

The run begins.

# Import Quantification Plate Data

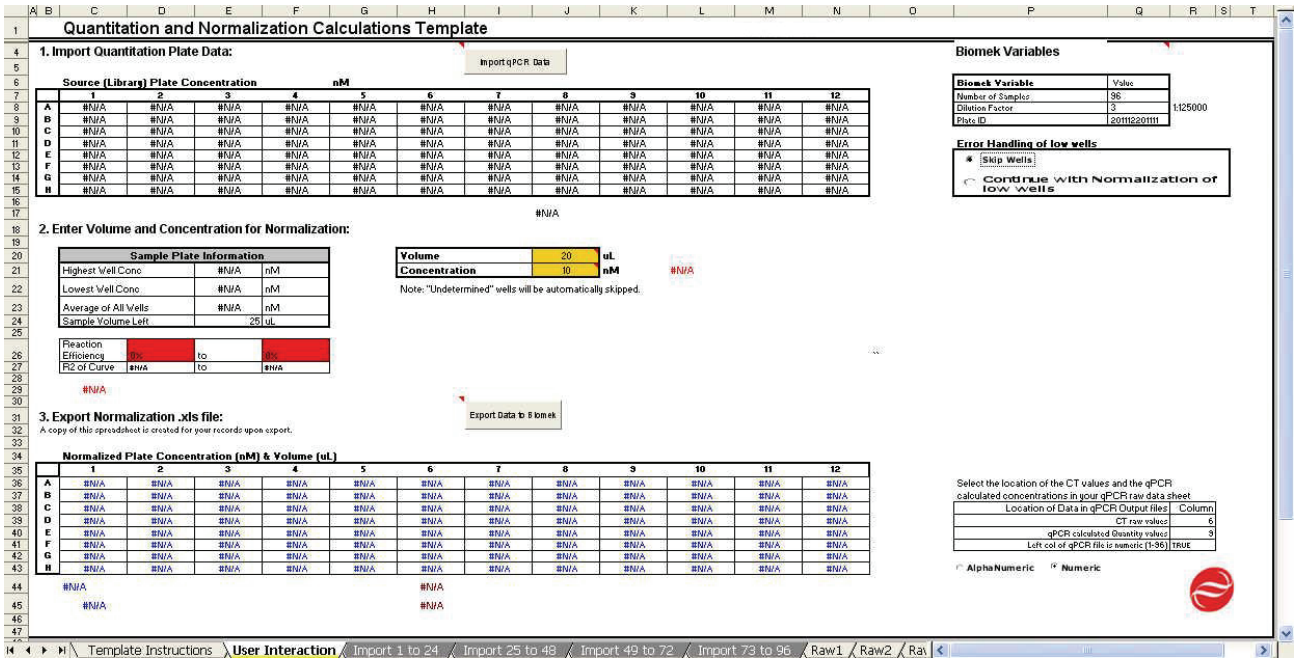
When the run is finished, the Biomek software opens the **Quant and Norm qPCR.xls** spreadsheet.

## Set up the Spreadsheet

1 Click **Enable Macros**.

The spreadsheet opens.

**Figure 5.6** Microsoft® Excel Quant and Norm Spreadsheet




2 To import the quantification plate data from the .txt file(s) generated by the Real-Time PCR system, click the **Import qPCR Data** button at the top of the spreadsheet.

3 Select the number of files to import.

Each qPCR Plate can include up to 24 samples.

4 Select the .txt files in the order in which the samples are located on the original library plate. For example, file 1 should contain samples 1-24; file 2 should contain samples 25-48; and so on.

Each data file is imported into a Raw worksheet, that is, information from file 1 is stored in the 'Raw1' worksheet; information from file 2 is stored in the 'Raw2' worksheet; and so on.

5 Make the following additional selections:

- In the lower right-hand section of the quant and norm qPCR spreadsheet, there is a table called “calculated concentrations in your qPCR raw data sheet.” Select the column where the CT values are stored and the column where the qPCR calculated quantity is stored.
- Select if the well location is alphanumeric or by number.

## Enter Volume and Concentration for Normalization

1 Enter the volume for the normalization plate in the yellow cell labeled **Volume**.

Take into account the volume left from the sample plate. If the sample plate volume is lower than the volume for normalization, you can select between the following options on the top right of the spreadsheet:

- Skip the affected wells, or
- Normalize to a lower volume (with the same concentration) for the affected wells.

2 Enter the concentration for the normalization plate in the yellow cell labeled **Concentration**.

Take into account the wells with concentrations lower than your normalization concentration. You are again able to select between the following options in the same location as above:

- Skip the affected wells, or
- Transfer the sample as-is to the normalization plate with no dilution.

## Biomek Variables

The variables for number of samples and dilution factors are automatically imported. Do not adjust these values unless you are using the spreadsheet outside of a running method.

If you made a mistake in the user interface, it is best to abort the method and restart since, at this point, you have not put any reagents or labware on the deck.

## Error Handling for Wells with Low Concentrations or Volumes

You can skip low wells or continue with the normalization including the low wells.

- If you choose to **Skip Wells**, no sample or water is transferred to the wells with low concentrations or low volumes.
- If you choose to **Continue with Normalization of Low Wells**, the concentration of the normalized well will be below the desired concentration for the low-concentration wells. The entire volume in the sample plate is transferred if it is less than the desired volume of the normalized well.

Explanations of actions automatically taken for cases where the appropriate concentration or volume is not available:

- *If the average concentration of a well is too high to make the desired concentration, the well is skipped.*
- *If the volume to be pipetted is less than 1 µL, the well is skipped. Any discrepancies between the desired concentration and the predicted concentration are indicated on the page.*
- *If there is insufficient volume to make a transfer at a specific concentration, the maximum amount of the correct concentration possible is made with the entire sample volume that is left in the library plate.*

## Evaluating the Data

Imported worksheets:

- Used to calculate the values for each of the 24 sample files. For example, file 1, which has its raw values stored in the Raw1 worksheet, has its calculations stored in the Import 1 to 24 worksheet, etc.
- Each plate has a standard curve, raw Ct values and calculated pM concentrations that are taken from the qPCR instrument's output file.
- The import worksheet allows you to further adjust the size of the fragments and provides summaries of sample properties and volumes to be transferred.
- The import worksheet alerts you when there is insufficient volume for pooling, although it does not allow you to change the pooling volumes. Wells with insufficient volume for pooling are skipped when the method begins, so be sure volumes are accurate before starting the method.

## Warnings

These warnings indicate that you need to look at the sample import worksheets.

- Reaction Efficiency

  This warning indicates the efficiency of the doubling of the standard curve and should be between 90 - 110%. If the value is out of range, the sample concentrations may not be accurate.
- R2 of the Standard Curve

  This warning indicates how closely the data points fit to the calculated slope and should be above 98%. If the value is out of range, the sample concentrations may not be accurate.

## Errors

**Table 5.2** Error Messages

| Message | Discussion |
|---|---|
| "Please Adjust Concentration to be between 5 and 20" | This error occurs when the specified concentration is out of range. This error must be corrected before you can export the spreadsheet. |
| "Please Adjust Volume to between 10 and 200uL" | This error occurs when the specified volume is out of range. This error must be corrected before you can export the spreadsheet. |

## Notification

**Table 5.3** Notification Messages

| Message | Discussion |
|---|---|
| "This Value is higher than the concentration of some wells. Some wells may be skipped due to low concentration." or "This value is higher than the concentration of some wells. Concentration of some wells may be lower than expected." | These notifications occur when a well has a concentration lower than the concentration you specified to skip wells, or to continue normalization of low wells. |
| "Some wells have been skipped." | This notification occurs when the volume to be transferred is too low to transfer. |
| "The volume of some wells may be lower than expected." | This notification occurs when there is insufficient volume to create the fully-specified volume. |

## Tips

- The spreadsheet is designed to always start with A1. To start at well A4, A7 or A10, import a blank qPCR Data Template.txt file for the samples that need to be skipped.
- The spreadsheet is designed to prevent inaccurate transfers of less than 1 µL. If a sample is close to the desired concentration, but does not transfer, adjust the target volume and/or concentration to allow the well to be transferred.

  For example, if the sample concentration is so high that 0.9 µL needs to be transferred, adjust the concentration and/or volume to allow the well to be transferred.

## Export the File

1 Click the **Export Data to Biomek** button.

The following errors must be corrected before you can export the spreadsheet:

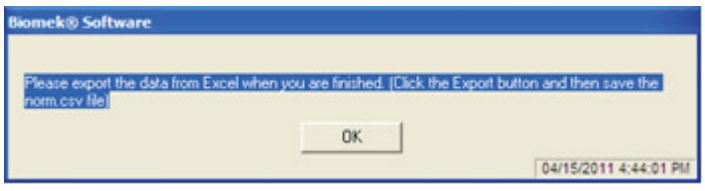
**Table 5.4** Export Notification Messages

| Message | Discussion |
|---|---|
| "qPCR data must be imported before continuing" | This error occurs when no usable files have been imported. |
| "A value must be entered for Normalization Volume" | This error occurs if no value has been entered for normalization volume. |
| "The volume of some wells may be lower than expected." | This notification occurs when there is insufficient volume to create the fully-specified volume. |
| "A value must be entered for Normalization Concentration" | This error occurs if no value has been entered for normalization concentration. |
| "Norm Concentration must be less than source concentration" | This error occurs when the entered normalization concentration is higher than the highest sample concentration. Lower the desired concentration or redo your qPCR. |
| "Please Adjust/set Concentration to be between 5 and 20" | This error occurs when the specified concentration is out of range. You must correct this error before you export the spreadsheet. |
| "Please adjust/set the Volume to be between 10 and 45uL" | This error occurs when the specified volume is out of range. This error must be corrected before you export the spreadsheet. |

2 Choose a location to save a copy of the data and click **Save**.

If you take longer than Biomek allows before exporting the data, the following message appears.

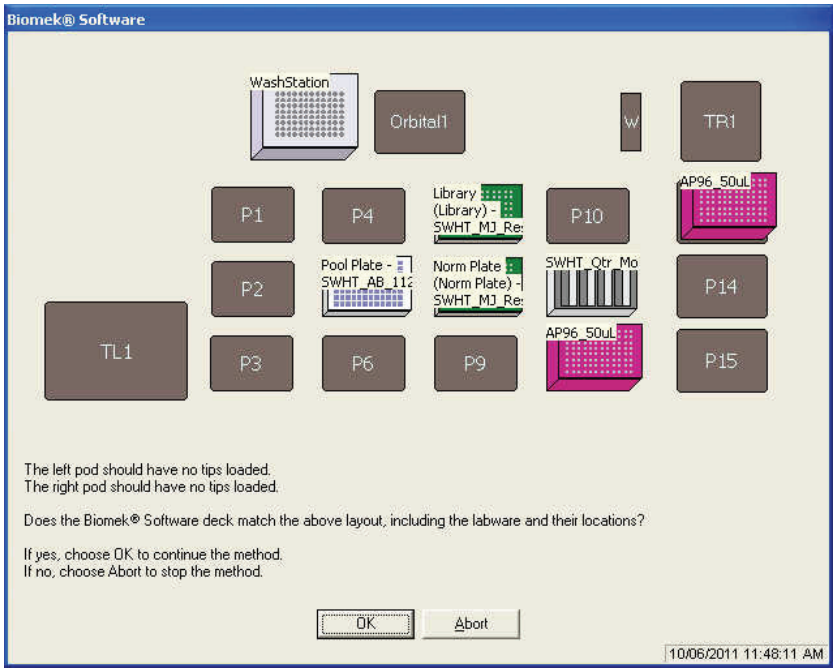
**Figure 5.7** Message that Appears if You Take a Long Time to Export




**NOTE** Biomek will not continue a method until you have corrected all errors and exported the data.

After exporting, the deck layout appears.

**Figure 5.8** Example of a Deck Layout




**3** Verify that the actual deck setup matches the deck layout and click **OK**.

**4** When the normalization and pooling method finishes, remove the plates for processing or storage.

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