USER’S GUIDE ILLUMINA TRUSEQ RNA LIBRARY PREPARATION ON THE SCICLONE NGS LIQUID HANDLER

Compatible with:
Illumina TruSeq™ RNA Sample Prep v2 kits
Sciclone® NGS Hardware with Maestro™ 6.0
Sciclone NGSx Hardware with Maestro 6.2
Introduction

Preparation of samples for mRNA sequencing on the Illumina, Inc. platform requires a series of manipulations to isolate and fragment mRNA, convert the RNA to double-stranded cDNA, and efficiently ligate appropriate indexed adapters to produce paired-end libraries. Automating the process has the advantage of avoiding sample tracking errors and reducing sample-to-sample variability while dramatically increasing throughput. The Maestro-based TruSeq™ RNA Application from PerkinElmer provides a pre-programmed solution for Illumina TruSeq™ RNA Library Preparation on the Sciclone NGS from PerkinElmer.

Maestro TruSeq™ RNA Workflow

![Diagram of TruSeqRNA workflow]

*Figure 1. Overview of the steps for TruSeq™ RNA library preparation on the Sciclone NGS.*
**Sciclone NGS: Illumina TruSeq™ RNA**

**Maestro TruSeq™ RNA Workflow Overview**

<table>
<thead>
<tr>
<th>Maestro Application</th>
<th>Set-up Time</th>
<th>Run Time (Including Thermocycler Steps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 “TruSeqRNA”</td>
<td>1 hour</td>
<td>5 hours</td>
</tr>
<tr>
<td>2 “TruSeqDNA Library Prep”</td>
<td>1 hour</td>
<td>4.5 hours</td>
</tr>
<tr>
<td>3 “TruSeq Post-PCR SPRI”</td>
<td>10 min</td>
<td>45 min</td>
</tr>
</tbody>
</table>

Three Independent Maestro Applications are Used in the 2-day TruSeq™ RNA Workflow

The Maestro Application for TruSeq™ RNA sample preparation is a validated process for library preparation from total RNA samples that follows the steps outlined in the flowcharts on Page 5. Samples are processed in 96-well PCR plates, and the number of samples to process (1 to 12 columns of 8 samples each) is selected at the start of each run. Pre-set tip-tracking utilities written into the application guide the instrument to pick up appropriate numbers of tips and refill/replace tip boxes as needed. INHECO temperature blocks installed on the Sciclone deck allow for appropriate 4 °C storage of reagents and heated incubations of reactions. Reaction mixes are pre-arrayed prior to addition to sample to ensure equal incubation times across the sample plate. Adapter indexing patterns may be executed during the run by arraying 1-24 different adapters to appropriate well locations as specified by the user in an excel workbook. Easy-to-follow user interfaces guide the reagent and deck setup process and prompt the user for any necessary interventions.

The “TruSeqRNA” application method (Day 1) includes mRNA isolation and cDNA synthesis steps and follows the Illumina, Inc. protocol closely. All reactions are set up in Bio-Rad Hard-Shell® PCR plates. Washes for mRNA purification are done with 150 µL Bead Washing Buffer (instead of the 200 µL used in the manual process). Incubations at 65 °C and 16 °C, for mRNA denaturation and 2nd strand synthesis, are done on the Sciclone deck. However, the 80 °C and 94 °C incubations for mRNA elution and fragmentation and the first-strand synthesis reactions are done in a thermocycler with a heated lid to avoid excessive evaporation. Two magnets are used in the protocol, one with a spacer to lift the plate and allow beads to pellet closer to the bottom of the wells where they are more easily resuspended by pipetting.

The Maestro “TruSeqDNA Library Prep” Application steps (Day 2) differ from the Illumina, Inc. protocol in several important aspects. First, a single aliquot of AMPure® XP beads is used per sample throughout the post-End Repair, A-tailing, and Ligation steps. The DNA in the sample is driven on and off the beads via changes in PEG and NaCl concentrations. This strategy increases yield by limiting the number of times samples are transferred to new wells/plates. Second, the volumes/concentrations of some reagent additions have been reduced from the Illumina-recommended volumes/concentrations. These reductions allow for the necessary overage volume during automated pipeting steps without significantly affecting yields. Reducing the concentration of adapters in the ligation step helps prevent excess adapter-dimer formation. Third, purification and size selection of ligation products is done with a single AMPure® XP bead cleanup, rather than gel-based size selection. The AMPure® XP cleanup is amenable to automation and high throughput sample processing, and has proved effective at reducing the adapter-dimer concentration prior to PCR enrichment of the library fragments. The modifications summarized above have led to the development of a robust automated procedure (mRNA isolation through post-PCR cleanup) for up to 96 samples that can be completed in two 6-7 hour days.
Sciclone NGS: Illumina TruSeq™ RNA

Figure 2. Day 1 “TruSeqRNA” Application (mRNA isolation and cDNA synthesis).

Figure 3. Day 2 “TruSeqDNA Library Prep” and “TruSeq Post-PCR SPRI” Applications.
Required Materials and Reagents

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Vendor and Part No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TruSeq™ RNA Sample</td>
<td>Illumina, Inc.</td>
</tr>
<tr>
<td>Preparation Kit (48 samples)</td>
<td>With Set A adapters – RS-122-2001</td>
</tr>
<tr>
<td></td>
<td>With Set B adapters – RS-122-2002</td>
</tr>
<tr>
<td>SuperScript II Reverse Transcriptase</td>
<td>Invitrogen 18064-014</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>Various</td>
</tr>
<tr>
<td>AMPure® XP Beads</td>
<td>Beckman Coulter – A63881 (60 mL)</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>Sigma-Aldrich E7023</td>
</tr>
<tr>
<td>50% PEG 8000</td>
<td>Sigma-Aldrich 83271</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>Sigma-Aldrich S5150</td>
</tr>
<tr>
<td>RNase/DNase decontaminating spray</td>
<td>Various</td>
</tr>
</tbody>
</table>

**Note:** TruSeq™ RNA kits supply enough reagents to process 48 samples. If kits are used in multiple runs with <48 samples per run, volumes of specific reagents may become limiting. Care should be taken to avoid freeze-thaw cycles with all TruSeq™ reagents. Reagents should be aliquoted appropriately if planning to use the kit for more than 2 independent runs.

**Note:** The Maestro "TruSeqDNA Library Prep" Application is not currently written to process samples with the Illumina-supplied End Repair Control, A-Tailing Control, or Ligase Control reagents. These kit components will not be used.

General Laboratory Equipment and Supplies

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microfuge</td>
<td>Various</td>
</tr>
<tr>
<td>Vortexer</td>
<td>Various</td>
</tr>
<tr>
<td>200 µL Multichannel pipettor and appropriate barrier tips</td>
<td>Various</td>
</tr>
<tr>
<td>1000 µL Multichannel pipettor and appropriate barrier tips</td>
<td>Various</td>
</tr>
<tr>
<td>Microplate centrifuge</td>
<td>Various</td>
</tr>
<tr>
<td>Thermocycler**</td>
<td>Bio-Rad Laboratories (MJ Research) DNA Engine PTC-200, or equivalent</td>
</tr>
<tr>
<td>Plate Seals</td>
<td>Various – compatible with Thermocycler and freezer storage</td>
</tr>
<tr>
<td>PerkinElmer LabChip® GX or Agilent Technologies Bioanalyzer with appropriate chips and reagents</td>
<td><strong>The standard sample plates used in this application are fully skirted Bio-Rad Hard-Shell® 96-PCR plates. Please check if your thermocycler is compatible with this plate type. If it is not, please contact your PerkinElmer Field Application Scientist to discuss modifications to the application to support semi-skirted PCR plates.</strong></td>
</tr>
</tbody>
</table>

Sciclone NGS Accessories

<table>
<thead>
<tr>
<th>Accessory</th>
<th>PerkinElmer Part No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agencourt 96-ring Magnet (2)</td>
<td>CLS128316</td>
</tr>
<tr>
<td>Spacer Assembly for Agencourt 96-ring Magnet</td>
<td>CLS135277 (Qty. 4) and CLS133314 (1)</td>
</tr>
<tr>
<td>INHECO 384-well plate adapter</td>
<td>CLS128373</td>
</tr>
<tr>
<td>INHECO 96-well adapters (3)</td>
<td>CLS128372</td>
</tr>
<tr>
<td>INHECO 96-well adapter/shaker</td>
<td>CLS100852</td>
</tr>
</tbody>
</table>
Running the Maestro TruSeq™ RNA Applications

Please read and familiarize yourself with all steps described in this section prior to beginning the run. For best results, the entire process should be completed in two 6-7 hour days. The first will include reagent preparation/distribution, deck setup, and Maestro Run for the “TruSeqRNA” application (mRNA isolation and cDNA synthesis). The second will include reagent preparation/distribution, deck setup, and Maestro Runs for the “TruSeqDNA Library Prep” and “TruSeq Post-PCR SPRI” applications.

Care should be taken to maintain an RNase-free environment during all steps of the Day 1 application. It is advisable to clean the Sciclone deck and accessories with RNase decontaminating spray prior to beginning.

Sample Preparation

Total RNA samples should be presented for the Maestro “TruSeqRNA” run in a Bio-Rad Hard-Shell® PCR plate as 0.1 to 4 µg RNA in 50 µL water. Quality of RNA samples should be checked by analysis on a LabChip GX or Agilent Technologies Bioanalyzer system: RQS or RIN scores of greater than 8.0 are required for optimal results.

The “TruSeqDNA Library Prep” application (Day 2) is optimized for 300-400 bp cDNA fragments. Samples fragmented to a smaller size will require alternative processing (see Step 4 in “TruSeqDNA Library Prep” application steps for instructions on how to exchange AMPure® XP bead buffer to favor retention of smaller fragments in post-End Repair SPRI® cleanup).

Day 1: “TruSeqRNA” Run Preparation Steps

Note: mRNA Purification beads and AMPure® XP beads should be warmed at Room Temperature for about 30 min before use. They may be taken out of 4 °C storage before beginning. If frozen, TruSeq™ RNA buffers may be thawed at RT prior to beginning. (It is helpful to place the tubes containing RSB and Bead Washing Buffer in water to speed the thawing process.) Do not thaw enzyme mixes until steps 1 and 2 have been completed.

If necessary, boot up the system by first starting the Sciclone and the INHECO units, then starting the PC controller.

1. Modify the Maestro “TruSeqRNA Workbook” to Specify the Number of Samples to Run

The workbook must be located in the filepath: C:\Program Data\CaliperLS\MaestroWorkbooks\ and must have the name “TruSeqRNA Workbook.xls”. If the file is moved or the name is changed, Maestro will not be able to find the information necessary to begin the run.
The sample number must be set by indicating the number of columns to run in the worksheet titled “TruSeqRNA Reagent Setup”. The Maestro application only processes full columns of 8 samples each. Running more than 6 columns of samples will require 2 TruSeq™ RNA reagent kits, as each kit contains reagents for up to 48 samples.

After modifying the entry for the number of columns to process, the spreadsheet will update the appropriate volumes for the reagent plates. Save the modified spreadsheet with its original name in its original file path. Print the “TruSeqRNA Reagent Setup” spreadsheet to use as a guide while setting up reagents.

2. Start the Maestro “TruSeqRNA” Application

Launch the Maestro software and open the “TruSeqRNA” Application. Start the run by selecting the play button. If running in Editor mode, be sure to start the Main Method.

Note: When the run is started, the instrument will complete all initialization steps for the hardware and the specific application. The run will automatically pause and prompt the user to set up the deck and confirm proper setup prior to proceeding with the run. Starting the application prior to thawing and diluting reagents ensures that the cold blocks are pre-chilled and ready for on-deck reagent storage.

Ensure that the INHECO units have the correct adapters for the “TruSeqRNA” Application

- Position A3 96-well PCR Plate Adapter
- Position A4 96-well PCR Plate Adapter
- Position D2 96-well PCR Plate Adapter
- Position D4 96-well PCR Plate Shaker Adapter

Verify that the INHECO units for positions A3 and A4 are set to 4 °C and are cooling. Verify that the INHECO unit for position D4 is set to 25 °C and the INHECO unit for position D2 is set to 65 °C and is heating.

3. Thaw the Enzyme Mixes and Place on Ice

First Strand Master Mix, Second Strand Master Mix, and SuperScript II should be thawed at this time. Care should be taken to avoid freeze-thaw cycles with all enzyme mixes. Reagents should be aliquoted appropriately if planning to use a kit for more than 2 independent runs.

4. Prepare the Buffer, Bead and 80% EtOH Plates

Using a multichannel pipettor, aliquot the indicated volumes of buffers to the Bio-Rad Hard-Shell® PCR plates and wells specified on the spreadsheet. Plates should be made for: Bead Binding Buffer, Bead Washing Buffer (2), Elution Buffer, Resuspension Buffer, and Elute/Prime/Fragment Mix. Label the plates, cover, and store at room temperature until ready to set up the Sciclone deck.

Thoroughly resuspend mRNA purification beads and AMPure® XP beads (warmed to room temperature) by inverting/rotating the tube/bottle. Using a multichannel pipettor, aliquot the indicated volumes of beads to the specified wells of Bio-Rad Hard-Shell® PCR plates. Label the plates, cover, and store at room temperature until ready to set up the Sciclone deck.

Inspect all plates to ensure that air has not been trapped in the wells. If necessary, spin the plates briefly to bring reagents to the bottom of the wells.

Make 100 mL fresh 80% EtOH solution by diluting 80 mL 100% Ethanol with 20 mL nuclease-free molecular biology grade water. Pour 100 mL fresh 80% EtOH into a Seahorse Bioscience deepwell reservoir, cover with a lid and store at room temperature.

5. Prepare the Enzyme Reagent Plate and Sample Plate

Prepare First Strand Master Mix by adding 50 µL SuperScript II to the Illumina supplied tube. Mix gently by inverting and spin down. If running fewer than 48 samples, label the tube to indicate that the SuperScript II has been added prior to storing the tube at -20 °C.

Gently mix and spin the Second Strand Master Mix tube. Fill the specified columns of a Bio-Rad Hard-Shell® PCR plate with the specified volumes of First Strand and Second Strand Master Mixes. Keep the plate on ice while pipetting to keep the reagents cold. Pipet carefully into the bottom of the wells and avoid trapping air or creating bubbles. If necessary, spin the plates briefly in a plate centrifuge to ensure all reagents are at the bottom of the wells. Label the plate, cover with a lid and store on ice or at 4 °C.
6. **Set up the Sciclone Deck**

Confirm that the Maestro software has correctly read the workbook and is set to run the correct number of columns. If the incorrect number of columns is indicated, modify and save the excel workbook as described in Step 1, then start the application again from the Main Method.

Step through the pictures, placing the indicated consumables/prepared plates in the indicated locations. Be sure to check whether a lid is needed for each plate or reservoir. Place new tip boxes in the indicated locations.

7. **Run mRNA Isolation and cDNA Preparation Steps**

Confirm that the deck setup matches the final picture in the setup window. Selecting “Finished” will prompt the application to begin the protocol.

The Maestro “TruSeqRNA” Application will automatically proceed through the steps indicated in the flowchart in Figure 2 (Page 5). While the application is running, the green light at the top of the instrument will blink. If there is a problem with the run, the light will change to yellow and an alarm will sound to indicate that user intervention is necessary. See Appendix A (Page 12) for a Step-by-Step guide to the Sciclone steps of the Maestro “TruSeqRNA” Application.

When it is necessary to transfer the samples to a thermocycler, the application will pause and show a message indicating that the PCR plate should be sealed and run on the thermocycler with the appropriate program. It is advised to pre-program the thermocycler with methods for mRNA Elution, Elution/Frag/Prime, and 1st Strand as described in the Illumina, Inc. TruSeq™ RNA protocol:

- **mRNA Elution 1**
  - 80 °C for 2 min (lid at 100 °C)
  - 25 °C hold

- **Elution 2 – Frag – Prime**
  - 94 °C for 8 min (lid at 100 °C)
  - 4 °C hold

- **1st Strand**
  - Run with lid at 100 °C
  - 25 °C for 10 min
  - 42 °C for 50 min
  - 70 °C for 15 min
  - 4 °C hold

*Note: Incubations for mRNA Denaturation and Second Strand synthesis will be done on the Sciclone deck.*

*Note: Please ensure that your thermocycler is compatible with fully skirted PCR plates.*

8. **Store cDNA samples**

When the application is complete, you will be prompted to seal the plate containing cDNA in 50 µL RSB for storage at -20 °C overnight. After storing the plate, close the dialog box. The Sciclone will shut off the leg lights and inactivate the INHECO temperature control.

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**Day 2: “TruSeqDNA Library Prep” Run Preparation Steps**

*Note: AMPure® XP beads should be warmed at Room Temperature for about 30 min before use. They may be taken out of 4 °C storage before beginning. If frozen, RSB buffer may be thawed at RT prior to beginning. (It is helpful to place the tube containing RSB in water to speed the thawing process.) Do not thaw other reagent mixes until Steps 1 and 2 have been completed.*

If necessary, boot up the system by first starting the Sciclone and the INHECO units, then starting the PC controller.

1. **Modify the Maestro “TruSeqDNA Library Prep Workbook” to Specify the Number of Samples to Run and the Adapter Index Pattern**

The workbook must be located in the filepath: C:\Program Data\Caliper\Maestro\Workbooks\ and must have the name “TruSeqDNA Library Prep Workbook.xls”. If the file is moved or the name is changed, Maestro will not be able to find the information necessary to begin the run.

The sample number must be set by indicating the number of columns to run in the worksheet titled “TruSeq DNA Reagent Plates”. The Maestro application only processes full columns of 8 samples each. Running more than 6 columns of samples will require 2 TruSeq™ DNA reagent kits, as each kit contains reagents for up to 48 samples.

![Figure 5. “TruSeqDNA Reagent Plates” Spreadsheet in the TruSeq DNA Library Prep Workbook.](image)

The adapter index pattern is set by designating the appropriate adapter numbers to each well ID in spreadsheet titled “Indexing”. Up to 24 different adapters may be used. Illumina, Inc. supplies 20 µL per tube of each adapter, allowing up to 8 samples per adapter when diluting adapter 1:2 with RSB.
Enter a number from 1-27 in the “Index Well” column for each of the samples to be run. The TruSeq™ adapters are numbered from ID001 to ID027, with adapters ID017, ID024 and ID026 omitted. If desired, custom primers can be used and their names inputted in cells I4-I30 of the indexing spreadsheet. The chart on the right will automatically update the number of samples using each adapter index. This information will be passed into the worksheet for “TruSeqDNA Reagent Plates” to modify the volume necessary for each adapter index mix.

Note: when the run is started, the instrument will complete all initialization steps for the hardware and the specific application. The run will automatically pause and prompt the user to set up the deck and confirm proper setup prior to beginning the library preparation steps. Starting the application prior to thawing and diluting reagents ensures that the cold blocks are pre-chilled and ready for on-deck reagent storage.

Ensure that the INHECO units have the correct adapters for the “TruSeqDNA Library Prep” Application. Verify that the INHECO units for positions A3, A4 are set to 4C and are cooling.

Position A3 384-well Plate Adapter
Position A4 96-well PCR Plate Adapter
Position D2 96-well PCR Plate Adapter
Position D4 96-well PCR Plate Shaker Adapter

3. Thaw the TruSeq™ DNA Reagents and Place on Ice
Care should be taken to avoid freeze-thaw cycles with all TruSeq™ DNA reagents. Reagents should be aliquoted appropriately if planning to use a kit for more than 2 independent runs. It is helpful to place the tubes containing RSB and End Repair Mix in water to speed the thawing process.

4. Prepare the 80% EtOH Reservoir, PEG Plate, RSB Plate, and AMPure® XP Bead Plate
Make 100 mL fresh 80% EtOH solution by diluting 80 mL 100% Ethanol with 20 mL nuclease-free molecular biology grade water. Pour 100 mL fresh 80% EtOH into a Seahorse Bioscience deepwell reservoir, cover with a lid and store at room temperature.

Use the “TruSeqDNA Reagent Plates” spreadsheet as a guide for setting up the plates.

If necessary, make a 20% PEG/2.5M NaCl solution and 20% PEG/3M NaCl solution. These solutions may be made in advance and stored at room temperature.

Using a multichannel pipettor, aliquot 30 µL 20% PEG/2.5M NaCl solution per well into a Bio-Rad Hard-Shell® PCR plate for each column of samples to be run. Label the plate, cover with a lid, and store at room temperature.

Using a multichannel pipettor, aliquot 120 µL Resuspension Buffer per well into a Bio-Rad Hard-Shell® PCR plate for each column of samples to be run.

Note: If it is desired to retain smaller fragments (150 to 250 bp) in the post-ER SPRI cleanup, then AMPure® XP beads will need to undergo a bead buffer exchange step to substitute the existing buffer on the AMPure® XP beads with an equal volume of 20% PEG/3M NaCl solution. The higher salt content in the bead buffer will allow recovery of shorter fragments (<250 bp) in the post End Repair SPRI® cleanup step.

Save the modified spreadsheet with its original name in its original file path. Print the “TruSeqDNA Reagent Plates” spreadsheet to use as a guide while setting up reagents.

2. Start the Maestro “TruSeqDNA Library Prep” Application
Launch the Maestro software and open the “TruSeqDNA Library Prep” Application. Start the run by selecting the play button. If running in Editor mode, be sure to start the Main Method.
To exchange the bead buffer, thoroughly resuspend AMPure® XP beads (warmed to room temperature) by inverting/rotating the bottle. Transfer the total volume of beads needed (plus 10% overage volume) to one or more 2 mL eppendorf tubes or to a 15 mL falcon tube. Pellet the beads using a magnet. Remove the buffer from the beads and replace it with an equal volume of 20% PEG/3M NaCl. Thoroughly resuspend the beads by inverting/rotating the tube(s).

Using a multichannel pipettor, aliquot 105 µL AMPure® XP beads per well into a Bio-Rad Hard-Shell® PCR plate for each column of samples to be run. Cover the plate with a lid and store at room temperature.

Inspect all plates to ensure that air has not been trapped in the wells. If necessary, spin the plates briefly to bring reagents to the bottom of the wells.

5. Make the Reaction Master Mixes and Adapter Mixes

The following mixes should be prepared according to the recipes in the “TruSeqDNA Reagent Plates” spreadsheet: End-Repair Mix, A-Tailing Mix, Ligation Mix, Adapter Mixes (up to 24), PCR Mix.

Gently mix and centrifuge each Illumina supplied reagent tube prior to use. After mixing the appropriate volumes of reagents in nuclease-free tubes, mix by inverting and centrifuge again to collect all liquid at the bottoms of the tubes.

Care should be taken to pipet accurately, as some reagents are viscous and minimal overage volumes are used. Keep the reaction mixes on ice.

6. Aliquot the Reaction Mixes into Plates

The Bio-Rad Hard-Shell® PCR plate should be filled with the specified volumes of A-tailing Mix, Ligase Mix, Stop Ligase Buffer, Adapter Mixes, End Repair Mix and PCR Master Mix in the specified wells. Aliquot the reaction mixes to the reagent plate in the wells indicated on the “TruSeqDNA Reagent Plates” spreadsheet. Keep the plates on ice while pipetting to keep the reagents cold. Pipet carefully into the bottom of the wells and avoid trapping air or creating bubbles. If necessary, spin the plate briefly in a plate centrifuge to ensure all reagents are at the bottom of the wells. Label the plate, cover with a lid and store on ice or at 4 ºC.

7. Set up the Sciclone Deck

Confirm that the Maestro software has correctly read the workbook and is set to run the correct number of columns. If the incorrect number of columns is indicated, modify and save the excel workbook as described in Step 1, then start the application again from the Main Method.

Step through the pictures, placing the indicated consumables/prepared plates in the indicated locations. Be sure to check whether a lid is needed for each plate or reservoir. Place new tip boxes in the indicated locations.

8. Run Library Preparation Steps

Confirm that the deck setup matches the final picture in the setup window. Selecting “Finished” will prompt the application to begin the library preparation protocol.

The Maestro “TruSeqDNA Library Prep” application will automatically proceed through End Repair, A-tailing, Ligation, and PCR setup steps as indicated in the flowchart (Figure 2, Page 5). While the application is running, the green light at the top of the instrument will blink. If there is a problem with the run, the light will change to yellow and an alarm will sound to indicate that user intervention is necessary. See Appendix B (Page 13) for a Step-by-Step guide to the Sciclone steps of the “TruSeqDNA Library Prep” application.

When the PCR setup is complete, the application will pause and show a message indicating that the PCR plate should be sealed and placed on a thermocycler for the amplification step. Close the dialog box to complete the run and shut down the leg lights and INHECO temperature controls.

**Note:** The RSB and 80% EtOH plates may be retained for use in the Post-PCR SPRI® cleanup.

**Day 2: PCR Enrichment**

Illumina, Inc. recommends the following program for amplification of libraries prepared from 0.1 to 4 µg total RNA. The number of cycles may need to be modified according to the amount and type of input RNA in the sample. Use a heated lid to prevent condensation.

**PCR:**

98 ºC for 30 secs.
15 cycles of:

- 98 ºC for 10 secs.
- 60 ºC for 30 secs.
- 72 ºC for 30 secs.
- 72 ºC for 5 mins.
- Hold at 10 ºC

**Day 2: “TruSeq Post-PCR SPRI” Run Preparation Steps**

The “TruSeq Post-PCR SPRI” cleanup step is provided as a separate Maestro application. If desired, the user may designate a different liquid handler for post-PCR sample processing to avoid any possible cross-contamination of pre-amplification samples. “TruSeq Post-PCR SPRI” cleanup applications are available for both the Sciclone NGS and the Zephyr workstations. The instructions here are for running the “TruSeq Post-PCR SPRI” application on the Sciclone.
Note: AMPure® XP beads should be warmed at Room Temperature for about 30 min before use. They may be taken out of 4 °C storage before beginning. If the RSB remaining in the Bio-Rad Hard-Shell® 96-well plate has not been saved at the completion of the library prep, it will also be necessary to thaw the RSB buffer from the Illumina, Inc. library prep kit.

1. Open the “TruSeq Post-PCR SPRI Workbook”
Enter the number of columns to be processed and save the workbook. The workbook must be located in the filepath: C:\ProgramData\Caliper\Maestro\Workbooks\ and must have the name “TruSeq Post-PCR SPRI Workbook.xls”. If the file is moved or the name is changed, Maestro will not be able to find the information necessary to begin the run.

2. Start the Maestro “TruSeq Post-PCR SPRI” Application
Launch the Maestro software and open the “TruSeq Post-PCR SPRI” application. Start the run by selecting the play button. If running in Edit mode, be sure to start the Main Method.

Note: When the run is started, the instrument will complete all initialization steps for the hardware and the specific application. The run will automatically pause and prompt the user to set up the deck and confirm proper setup prior to beginning the library preparation steps.

3. Prepare the SPRI® Beads Plate and RSB Plate
Aliquot 55 µL of AMPure® XP beads per well in a Bio-Rad Hard-Shell® PCR plate as indicated in the “TruSeq Post-PCR SPRI Workbook”. Ensure that the beads are at room temperature and well mixed prior to pipetting.

If the 96-well Bio-Rad Hard-Shell® PCR plate containing RSB used in the “TruSeqDNA Library Prep” application is saved at the completion of the library prep steps, the same plate may be used for RSB during the post-PCR cleanup. Confirm that enough RSB remains in the wells (30 µL plus >5 µL overage volume in each well), or set up a new plate as indicated in the workbook.

4. Prepare 80% Ethanol Reservoir
If the 80% EtOH reservoir used in the “TruSeqDNA Library Prep” run has been retained, it can be used for this run. Otherwise, make 50 mL fresh 80% EtOH solution by diluting 40 mL 100% Ethanol with 10 mL nuclease-free molecular biology grade water. Pour 50 mL fresh 80% EtOH into a Seahorse Bioscience deepwell reservoir and cover with a lid.

5. Setup the Sciclone Deck
Confirm that the software has correctly read the workbook and is set to run the correct number of columns. Step through the pictures, placing the indicated consumables/prepared plates in the indicated locations. Be sure to check whether a lid is needed for each plate or reservoir. Place new tip boxes in the indicated locations.

Note: when the “TruSeq Post-PCR SPRI” application is started, the variables used for tip tracking are reset. The run must be started with new, full tip boxes in the indicated positions, as Maestro will not retain tip tracking information from the library prep run.

6. Run the “TruSeq Post-PCR SPRI” Steps
Confirm that the deck setup matches the final picture in the setup window. Selecting “Finished” will prompt the application to begin the library prep steps. See Appendix C for a Step-by-Step guide to the Sciclone steps of the “TruSeq Post-PCR SPRI” application.

When the run is complete, the application will pause and show a message indicating that the PCR plate should be sealed and stored appropriately. Close the dialog box to complete the run and shut down the leg lights and INHECO temperature controls.

Library QC and Storage
Seal the library plate and store at -20 °C for up to 7 days, or proceed directly into library validation prior to storing. The LabChip GX may be used to check the size distribution of fragments in the amplified library and estimate the concentration of fragments in the appropriate size range. Make a 1:25 dilution of library into molecular biology grade water in a Bio-Rad Hard-Shell® skirted 96-PCR plate. Mix well by pipetting up and down, and spin the plate to remove any bubbles. Run the samples on the GX using a High Sensitivity DNA chip and kit according to the standard LabChip protocol.

Additional/alternative validation and quantification, including qPCR quantification, should be done according to the user’s standard practices.