ILLUMINA TRUSEQ STRANDED RNA
SAMPLE PREPARATION ON THE
SCICLONE NGS WORKSTATION

User Guide

Workflow: Maestro TruSeq™ Stranded RNA Rev1

Compatible with:
Illumina TruSeq™ Stranded Total RNA Sample Prep kits
Sciclone® NGS Workstation with Maestro 6.0
Sciclone NGSx Workstation with Maestro 6.0 or 6.3
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Introduction
Preparation of samples for stranded total RNA sequencing on the Illumina platform requires a series of manipulations to remove ribosomal RNA, fragment the RNA, convert the RNA to double-stranded cDNA, and efficiently ligate appropriate indexed adapters to produce libraries with strand orientation information. 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™ Stranded RNA Workflow from PerkinElmer provides a pre-programmed solution for Illumina TruSeq™ Stranded Total RNA Library Preparation on the PerkinElmer NGS Workstation.

Maestro TruSeq™ Stranded RNA Workflow Overview
The Maestro TruSeq™ Stranded RNA workflow is an optimized process for library preparation from total RNA samples that follows the steps outlined in the flowcharts below. 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. Alternatively, up to 96 pre-arrayed adapters may be placed on the deck at the appropriate step during the workflow to transfer a pre-determined pattern of indexes to the samples. Simple-to-follow user interfaces guide the reagent and deck setup process and prompt the user for any necessary interventions.

Maestro TruSeq Stranded RNA Workflow

Figure 1. Overview of the Three Maestro Applications in the TruSeq™ Stranded RNA Workflow.
Three independent Maestro Applications are used in the TruSeq™ Stranded RNA Workflow:

<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  TruSeq™ Stranded RNA cDNA Prep</td>
<td>1 hour</td>
<td>5 hours</td>
</tr>
<tr>
<td>2  TruSeq™ Stranded RNA Library Prep</td>
<td>40 min</td>
<td>3 hours</td>
</tr>
<tr>
<td>3  TruSeq™ Stranded RNA Post-PCR SPRI</td>
<td>10 min</td>
<td>45 min</td>
</tr>
</tbody>
</table>

The automated Maestro TruSeq™ Stranded RNA cDNA Prep application follows the Illumina protocol with a few minor modifications (see figure 2). For Ribosomal RNA removal with RiboZero, samples and reagents are set up in BioRad 96-well Hardshell PCR plates. Ribosomal RNA Binding Mix consisting of 5 µl rRNA Binding Buffer, 5 µl rRNA Removal Mix, and 2 µl water per sample is prepared prior to the run. This rRNA Binding Mix is added to the sample just prior to the denaturing step at 68 °C. The addition of 2 µl water compensates for evaporation that occurs when the lidded samples are incubated at 68 °C for 5 min on the deck. As recommended by Illumina, the samples are transferred to a plate containing the rRNA removal beads and mixed immediately. Supernatant from the rRNA removal beads is transferred off the magnet to a clean plate 2 times sequentially to ensure that no rRNA removal beads are carried into the RNA Clean bead cleanup step.

The RNA Clean beads are washed two times with 150 µl 80% EtOH (instead of one time with 200 µl 70% EtOH).

For cDNA synthesis, 8.5 µl of sample eluted from the RNA Clean beads is transferred directly to a plate containing 8.5 µl per well of Elute, Prime, Fragment High Mix. The samples are taken off-deck for incubation at 94 °C with a heated lid on a thermocycler. The Illumina protocol should be consulted for guidance on fragmentation time with regard to the desired average length of library inserts. While the samples are fragmented on the thermocycler, the Sciclone application may be continued to prepare for 1st Strand Synthesis by broadcasting the 1st Strand Synthesis ActD + SuperScript II® Mix into a 384-well staging plate. The application will pause until the user indicates that the samples are back on the deck and ready for the addition of 1st Strand Synthesis reagents. Once the reagents

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**Figure 2. Steps in the TruSeq™ Stranded RNA cDNA Prep Application**
are added and the samples have been mixed, the sample plate is again taken off-deck for a thermocycler step. Toward the end of the thermocycler step, the Sciclone application may be continued to prepare for 2nd Strand Synthesis by broadcasting the 2nd Strand Synthesis Mix into the 384-well staging plate. The application will pause until the user indicates that the samples are back on the deck and ready for the addition of 2nd Strand Synthesis reagents. The 16 °C incubation for 2nd Strand Synthesis is done on the deck. At the completion of the incubation, a SPRI cleanup is performed with two washes with 150 µl 80% EtOH (instead of two washes with 200 µl each). Beads are resuspended in 20 µl RSB, but the eluted sample is not removed from the beads. Samples/beads may be stored overnight at 4 °C or -20 °C, or the user may proceed directly to the Library Prep application.

The TruSeq™ Stranded RNA Library Prep application may be run immediately after the cDNA prep or on the following day. If running the cDNA prep and Library prep on the same day, the amplified libraries may be left on the thermocycler, held at 4 °C overnight at the end of the PCR program, and cleaned up with the Post-PCR SPRI application the following day. If running the cDNA prep and Library prep on different days, the samples on the AMPure® beads may be stored at either 4 °C or -20 °C.

The automated Maestro TruSeq™ Stranded RNA Library Prep workflow differs from the Illumina protocol in some aspects. The A-tail mix and Stop Ligase Solution are both diluted slightly with RSB to allow for the overage volume necessary to add the reagents simultaneously to all samples. The lidded A-tailing reaction is incubated on deck at 37 °C and is terminated with an on-deck incubation at 70 °C for 5 min followed by 22 °C for 5 min. At the start of the application, the user may select one of two options for presenting indexed adapters. Either the Sciclone will array indexed adapters from designated wells of a Master Mix plate into the correct pattern in a 384-well staging plate, or the application will pause and prompt the user to place a 96-well plate with pre-arrayed adapters on the deck at the appropriate point in the run. The post-ligation SPRI cleanup is executed by driving DNA onto the AMPure® beads retained in the sample from the SPRI cleanup at the end of the cDNA prep.

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**TruSeq Stranded RNA Library Prep Workflow**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broadcast 15 µl A-tail Mix to Q2 of 384-Well Plate.</td>
<td>Incubate 10 min at 22°C, Broadcast 30 µl PCR Master Mix to Clean PCR Plate.</td>
</tr>
<tr>
<td>Transfer 15 µl A-tail Mix to 15 µl cDNA Samples, Mix, Incubate 30 min at 37°C.</td>
<td>Move to Magnet, Remove SN, Wash 2X with 150 µl 80% EtOH, Dry 5 min at 37°C.</td>
</tr>
<tr>
<td>During Incubation, Broadcast 4 µl Indexed Adapters to Q1 and 8 µl Ligation Mix to Q4 of 384-Well Plate.</td>
<td>Resuspend Sample in 22 µl RSB, Mix, Incubate 3 min at 22°C.</td>
</tr>
<tr>
<td>Incubate Samples 5 min at 70°C, 5 Min at 22°C to Stop A-Tailing Reactions.</td>
<td>Move to Magnet, Incubate 5 Min, Transfer 20 µl to PCR Plate with PCR Master Mix</td>
</tr>
<tr>
<td>Transfer 2.5 µl Indexed Adapter and 5 µl Ligase Mix to Samples, Mix.</td>
<td>Amplify Libraries on Thermocycler.</td>
</tr>
<tr>
<td>Incubate 10 min at 30°C, Broadcast 7 µl Stop Ligase to Q3 of 384-Well Plate.</td>
<td></td>
</tr>
<tr>
<td>Transfer 5 µl Stop Ligase to Samples and Mix.</td>
<td></td>
</tr>
<tr>
<td>Transfer 12.5 µl 20%PEG/2.5 M NaCl Sol’n to Samples and Mix.</td>
<td></td>
</tr>
</tbody>
</table>
Illumina TruSeq™ Stranded RNA Sample Preparation on the Sciclone NGS Workstation

Introduction

The TruSeq™ Stranded RNA Post-PCR SPRI application is used after amplification of the adapter-ligated libraries to remove PCR reaction components, primers, and any amplified adapter-dimers. This is a standard 1:1 SPRI cleanup in which 50 µl fresh AMPure® XP reagent is added to the 50 µl PCR reaction. After washing 2X with 150 µl 80% EtOH the sample is resuspended in 32 µl water or elution buffer and 30 µl is transferred off the beads into a clean plate.

Figure 4. Steps in the TruSeq™ Stranded RNA Post-PCR SPRI application
Required Materials and Reagents

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Vendor and Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TruSeq™ Stranded Total RNA LT Sample Prep Kit (48 samples)</td>
<td>Illumina</td>
</tr>
<tr>
<td>Set A with Ribo-Zero Human/Mouse/Rat: RS-122-2201</td>
<td></td>
</tr>
<tr>
<td>Set B with Ribo-Zero Human/Mouse/Rat: RS-122-2202</td>
<td></td>
</tr>
<tr>
<td>Set A with Ribo-Zero Gold: RS-122-2301</td>
<td></td>
</tr>
<tr>
<td>Set B with Ribo-Zero Gold: RS-122-2302</td>
<td></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>RNA Clean XP Beads</td>
<td>Beckman Coulter — A63987 (40 mL)</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>Sigma E7023</td>
</tr>
<tr>
<td>50% PEG 8000</td>
<td>Hampton Research HR2-535 (200 mL)</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>Sigma S5150</td>
</tr>
<tr>
<td>RNase/DNase Decontaminating Spray</td>
<td>Various</td>
</tr>
</tbody>
</table>

Note: TruSeq™ Stranded Total 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 two independent runs.

Note: The Maestro TruSeq™ Stranded RNA Workflow does not 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>BioRad (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>Caliper LabChipGX or Agilent Bioanalyzer with</td>
<td></td>
</tr>
<tr>
<td>Appropriate Chips and Reagents</td>
<td></td>
</tr>
</tbody>
</table>

**The standard sample plates used in this application are fully skirted BioRad 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.**
### Sciclone NGS Workstation Accessories

<table>
<thead>
<tr>
<th>Accessory</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accessory</td>
<td>PerkinElmer Part Number</td>
</tr>
<tr>
<td>Agencourt 96-ring Magnet (2)</td>
<td>CLS 128316</td>
</tr>
<tr>
<td>spacer Assembly for Agencourt 96-ring Magnet</td>
<td>CLS 135277 (qty 4)</td>
</tr>
<tr>
<td>Inheco 384-well Plate Adapter</td>
<td>CLS 128373</td>
</tr>
<tr>
<td>Inheco 96-Well Adapters (3)</td>
<td>CLS 128372</td>
</tr>
<tr>
<td>Inheco 96-Well Adapter/Shaker</td>
<td>CLS 100852</td>
</tr>
</tbody>
</table>

### Consumables

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Description</th>
<th>PerkinElmer Part No.</th>
<th>Vendor and Part No.</th>
<th>No. used per run</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Plates</td>
<td>96 Well PCR Plate, Bio-Rad Hard-Shell® Full Skirt</td>
<td>6008870</td>
<td>BioRad HSP-9631</td>
<td>20</td>
</tr>
<tr>
<td>Boxed Tips</td>
<td>Pipet Tip-150 ul, Art, Box, 10-96 Sterile Racks</td>
<td>111426</td>
<td>Photo Lab 600630</td>
<td>28 (96 samples)</td>
</tr>
<tr>
<td></td>
<td>10-96 Sterile Racks</td>
<td></td>
<td></td>
<td>150 (24 samples)</td>
</tr>
<tr>
<td>Deep Well Plates</td>
<td>Deepwell-96 Pos, Square 2.0 ml Well, Polypro, Seahorse</td>
<td>6008880</td>
<td>Seahorse Bioscience 201379-100</td>
<td>2</td>
</tr>
<tr>
<td>Deep Well Reservoirs</td>
<td>Reservoir-Deepwell, 96 Pyramid Bottom, 287 ml, Seahorse</td>
<td>6008730</td>
<td>Seahorse Bioscience 201244-100</td>
<td>2</td>
</tr>
<tr>
<td>Lids</td>
<td>946 Lid-Universal, Robotic Friendly, Polystyrene</td>
<td>6000030</td>
<td>Seahorse Bioscience 200856-100</td>
<td>10</td>
</tr>
<tr>
<td>384 Well Plates</td>
<td>Microplate-384 Well, Round Bottom, Polypropylene</td>
<td>6008890</td>
<td>Corning 3672</td>
<td>2</td>
</tr>
</tbody>
</table>

The chart above lists the exact number of consumables needed for a single run of up to 12 sample columns through the TruSeq Stranded RNA sample preparation workflow. An appropriate set of consumables for individual runs through the workflow may be ordered from PerkinElmer as a preformatted kit: NGS Consumables Kit B. **Please note that the kit does not include tips, which must be ordered separately.**

### NGS Consumables Kit B #6060009

<table>
<thead>
<tr>
<th>Kit components</th>
<th>Pcs/k</th>
<th>Re-order number</th>
<th>Pcs per re-order number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypropylene deep-well storage plate, 96 x 2 mL square well, V-bottom</td>
<td>3</td>
<td>6008880</td>
<td>25</td>
</tr>
<tr>
<td>Polypropylene deep-well reservoir, 12 column, V-bottom, 21 mL</td>
<td>3</td>
<td>6008700</td>
<td>25</td>
</tr>
<tr>
<td>Universal lid</td>
<td>20</td>
<td>6000030</td>
<td>100</td>
</tr>
<tr>
<td>Hard-Shell thin-wall 96-well skirted PCR plate (blue)</td>
<td>30</td>
<td>6008870</td>
<td>50</td>
</tr>
<tr>
<td>Polypropylene low-volume microplate, 384 x 35 µL, round bottom</td>
<td>2</td>
<td>6008890</td>
<td>50</td>
</tr>
</tbody>
</table>

### Order Separately

<table>
<thead>
<tr>
<th>Kit components</th>
<th>Pcs/k</th>
<th>Re-order number</th>
<th>Pcs per re-order number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 150 µL barrier tips (box of 10 racks)</td>
<td>111426</td>
<td>10 racks</td>
<td></td>
</tr>
</tbody>
</table>
Running the Maestro TruSeq™ Stranded RNA Prep Applications

Please read and familiarize yourself with all steps described in this section prior to beginning the workflow. The recommended workflow requires two consecutive days. Day one includes reagent preparation/distribution, deck setup, and Maestro Run for the TruSeq™ Stranded RNA cDNA Prep application (rRNA depletion and cDNA synthesis). Samples are stored on beads overnight at 4 °C or -20 °C. Day two includes reagent preparation/distribution, deck setup, and Maestro Run for the TruSeq™ Stranded RNA Library Prep, Amplification of the adapter-ligated libraries, and Maestro Run for the TruSeq™ Stranded Post-PCR SPRI applications. Alternatively, the cDNA Prep and Library Prep applications may be run on the same day (8-9 hours total time, including setup). For this workflow, adapter-ligated libraries are placed on a thermocycler at the end of the day for PCR followed by an overnight hold at 4 °C, and the post-PCR SPRI application is run the following day.

Please note that the Illumina reagent First Strand Synthesis Mix Act D contains toxic Actinomycin D. Handle this reagent with care and dispose of tips and consumables that come into contact with this reagent appropriately. In addition, care should be taken to maintain an RNase-free environment during all steps of the cDNA Prep application. It is advisable to clean the Sciclone deck and accessories with RNase decontaminating spray prior to beginning the run.

Sample Preparation

Total RNA samples should be presented for the Maestro TruSeq™ Stranded RNA run in a BioRad Hardshell PCR plate as 0.1 to 1 µg RNA in 10 µl water. Quality of RNA samples should be checked by analysis on a LabChip GX or Agilent Bioanalyzer system: RQS or RIN scores of greater than 8.0 are required for optimal results.

The standard protocol produces library inserts averaging 150 bp in length when starting with high quality total RNA. If starting with partially degraded total RNA, or if larger insert fragments are desired, the thermocycler settings for the Elute Fragment Prime step may be modified. Please consult the Illumina protocol for details.

TruSeq™ Stranded RNA cDNA Prep Run Preparation Steps

Note: rRNA Removal beads, RNA Clean 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, Elution Buffer and RSB may be thawed at RT prior to beginning. (It is helpful to place the tubes containing Elution Buffer and RSB in water to speed the thawing process.) Do not thaw enzyme mixes or other reagents 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 TruSeq™ Stranded RNA Workbook to specify the number of samples to run

The workbook must be located in the filepath: C:\ProgramData\CaliperLS\Maestro\Workbooks and must have the name “TruSeq™ Stranded RNA 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 cell D2 of the worksheet titled “cDNA Synthesis”. The Maestro application only processes full columns of eight samples each. Running more than six columns of samples will require two TruSeq™ Stranded 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 “TruSeq™ Stranded RNA Reagent Setup” spreadsheet to use as a guide while setting up reagents.

2. Start the Maestro TruSeq™ RNA Application

Launch the Maestro software and open the TruSeq™ Stranded RNA cDNA Prep 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.*

Confirm that the Maestro software has read the correct 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.

Ensure that the Inheco units have the correct adapters for the TruSeq™ Stranded RNA application

- 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

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 22 and the Inheco unit for position D2 is set to 68 °C and is heating.

3. Thaw the reagents and enzyme mixes and place on ice

The following reagents should be thawed at this time: rRNA Removal Mix, rRNA Binding Buffer, EPF High Mix, First Strand Synthesis Act D Mix, Second Strand Marking Master Mix, and SuperScript II. Once thawed, mix each tube by inverting gently, spin briefly to bring all contents to the bottom of the tube, and place on ice. Care should be taken to avoid freeze-thaw cycles with all reagent/enzyme mixes. Reagents should be aliquoted appropriately if planning to use a kit for more than two independent runs.

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

Using a multichannel pipettor, aliquot the indicated volumes of buffers to the BioRad Hardshell PCR plates and wells specified on the spreadsheet. Plates should be made for: Elution Buffer (ELB), Elute/Prime/Fragment High Mix (EPF), and Resuspension Buffer (RSB). Label each plate, cover, and store at room temperature until ready to set up the Sciclone deck.

Ensure that RNA purification beads, RNA Clean beads, and AMPure®XP beads are warmed to room temperature and thoroughly resuspended by inverting/rotating the tube/bottle. Using a multichannel pipettor, aliquot the indicated volumes of beads to the specified wells of BioRad Hardshell PCR plates. Label each plate, 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 Deepwell reservoir, cover with a lid and store at room temperature. Depending on the number of columns of samples to be processed, an open reservoir or 12-column divided reservoir (5 mL EtOH per column) may be used.

5. Prepare the Master Mix Plate

Prepare rRNA Binding Mix, First Strand Master Mix, and Second Strand Master Mix in RNase-free tubes according to the recipes on the spreadsheet. Care should be taken to pipet accurately, as some reagents are viscous and minimal overage volumes are used. Fill the specified columns of a BioRad Hardshell PCR plate with the specified volumes of each Master Mix. 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

Step through the pictures displayed by the Maestro application, placing the indicated consumables and 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 the TruSeq™ Stranded RNA 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 TruSeq™ Stranded RNA cDNA Prep application will automatically proceed through the rRNA removal, RNA cleanup, and cDNA preparation steps indicated in the flowchart in Figure 2. 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 for a Step-by-Step guide to the TruSeq™ Stranded RNA cDNA prep-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 transferred to the thermocycler to run the appropriate program. It is advised to pre-program the thermocycler with methods for Elution2-Frag-Prime, and 1st Strand as described in the Illumina TruSeq™ Stranded total RNA protocol:

**Elution 2 – Frag – Prime**

**1st Strand**

Run with lid at 100 °C
- 94 °C for 8 min
- 4 °C hold

Run with lid at 100 °C
- 25 °C for 10 min
- 42 °C for 15 min
- 70 °C for 15 min
- 4 °C hold

**Note:** Incubations for RNA 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 remove the plate containing cDNA and AMPure® beads in 20 µl RSB. The plate should be sealed and stored at 4 °C or 20 °C overnight. If planning to run the Library Prep application immediately after the cDNA prep, the plate may be lidded and stored on ice temporarily. After removing the plate, close the dialog box to shut down the leg lights and Inheco units.

### TruSeq™ Stranded RNA 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 TruSeq™ Stranded RNA Workbook to specify the number of samples to run and the adapter index pattern you wish to apply**

The workbook must be located in the filepath: C:\ProgramData\Caliperls\MaestroWorkbooks, and must have the name “TruSeq™ Stranded RNA 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 cell D2 of the worksheet titled “On Bead Library Prep”. The Maestro application only processes full columns of eight samples each. Running more than six columns of samples will require two TruSeq™ Stranded total RNA reagent kits, as each kit contains reagents for up to 48 samples.

**Figure 6.** “On Bead Library Prep” worksheet in the TruSeq™ Stranded RNA Workbook.

![Figure 6](http://www.perkinelmer.com)
The adapter index pattern is set by designating the appropriate adapter numbers to each well ID in the spreadsheet titled “Indexing”. Up to 24 different adapters may be used. The TruSeq™ adapters are numbered from ID001 to ID027, with adapters ID017, ID024 and ID026 omitted. Enter an adapter index number in the “Index Well” column for each of the samples to be run. 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 “On Bead Library Prep” to modify the volume necessary for each adapter index mix and update the appropriate pattern of adapters to be supplied on the Master Mix plate. Ensure that adapter numbers are entered in column B for all samples to be processed during the run and that no values are entered in column B for samples not included in the run.

Note: If desired, custom primers may be used and different names for adapters may be entered in cells I4-I30 of the Indexing spreadsheet. Alternatively, indexed adapters may be supplied on the Sciclone deck pre-arrayed in a 96-well plate in the pattern appropriate for the samples processed in the run. The option to have the Sciclone array the indexed adapters in the appropriate pattern or to have the application prompt the user to place a pre-arrayed adapter plate on the deck at the appropriate time is presented at the start of the run.

After modifying entries for the number of columns to process and the adapter index pattern, the spreadsheet will update the recipes for the reagent mixes and the appropriate volumes of reagents to place in the reagent plates.

Save the modified spreadsheet with its original name in its original file path. Print the “On Bead Library Prep” spreadsheet to use as a guide while setting up reagents.

2. Start the Maestro TruSeq™ DNA Library Prep run

Launch the Maestro software and open the TruSeq™ Stranded RNA Library Prep 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 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.
The application will prompt the user to select an option for Indexed Adapter presentation.

If “Sciclone will broadcast adapters to a 384-well PCR plate on deck A3” is selected, the user must enter the appropriate indexed adapter information in the “Indexing” spreadsheet of the “TruSeq™ Stranded RNA Workbook”. During the A-tailing incubation step, the Sciclone will array the indexed adapters one at a time into the appropriate pattern in Q1 of the 384-well plate at A3. If “User Prompt for 96 well pre-broadcasted Adapter Plate” is selected, the application will pause before setting up the Ligation reaction and prompt the user to place a plate containing the pre-arrayed indexed adapters on the deck.

Confirm that the Maestro software has read the correct 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.

Ensure that the Inheco units have the correct adapters for the TruSeq™ Stranded RNA Library Prep application. Verify that the Inheco units for positions A3 and A4 are set to 4 °C and are cooling.

### 3. Thaw the TruSeq™ Library Prep reagents and place on ice

The following reagents should be thawed at this time: A-Tailing Mix, Stop Ligation Buffer, Ligation Mix, PCR Master Mix, PCR Primer Cocktail, and Indexed Adapters. Once thawed, mix each tube by inverting gently, spin briefly to bring all contents to the bottom of the tube, and place on ice. Care should be taken to avoid freeze-thaw cycles with all reagent/enzyme mixes. Reagents should be aliquoted appropriately if planning to use a kit for more than two independent runs.

### 4. Prepare the 80% EtOH reservoir, PEG plate, and RSB 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 Deepwell reservoir, cover with a lid and store at room temperature. Depending on the number of columns of samples to be processed, an open reservoir or 12-column divided reservoir (5 mL EtOH per column) may be used.

Make 10 mL fresh 20% PEG/2.5 M NaCl solution by mixing 4 mL 50% PEG + 5 mL 5M NaCl + 1 mL water.

Use the “On Bead Library Prep” spreadsheet as a guide for setting up the reagent plates. With a multichannel pipettor, aliquot 30 µl 20% PEG/2.5 M NaCl solution per well into a BioRad hardshell PCR plate for each column of samples to be run. Aliquot 60 µl Resuspension Buffer per well into a BioRad Hard-Shell® PCR plate for each column of samples to be run.

Label the plates, cover with a lid, and store at room temperature. Inspect the 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 Master Mixes

The following mixes should be prepared according to the recipes in the “On Bead Library Prep” spreadsheet: A-Tailing Mix, Ligation Mix, PCR Mix. After mixing the appropriate volumes of reagents in nuclease-free tubes, mix by inverting and spin briefly 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.

If using a TruSeq™ Library Prep kit for the first time, adjust the volume of Stop Ligation Buffer (STL). Vortex the tube to mix, then spin briefly to collect contents at the bottom of the tube. Use a P1000 to measure the volume. Add the necessary volume of RSB to the tube to bring the total volume to 410 µl. Dilution of the STL mix by up to 50% does not affect performance of the library prep. The volume adjustment ensures enough STL buffer to support three runs of 16 samples through the Library Prep workflow.

### 6. Aliquot the Master Mixes and Indexed Adapters into the Reagent Plate

The BioRad Hard-Shell® PCR plate should be filled with the specified volumes of A-Tailing Mix, Ligase Mix, Stop Ligase Buffer, Indexed Adapters (optional), and PCR Master Mix in the specified wells. Aliquot the reagent mixes in the wells indicated on the “On Bead Library Prep” spreadsheet. 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 plate.
broadly 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.

Note: Indexed adapters are only needed in the Reagent Plate if running with the selected option “Sciclone will broadcast adapters to a 384-well PCR plate on deck A3”.

7. Set up the Sciclone deck

Step through the pictures displayed by the Maestro application, placing the indicated consumables and 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™ Stranded RNA Library Prep 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.

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 TruSeq™ Stranded RNA Library Prep application will automatically proceed through A-tailing, Ligation, and PCR setup steps as indicated in the flowchart in Figure 3. 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 for a Step-by-Step guide to the Sciclone steps of the TruSeq™ Stranded RNA 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.

PCR Enrichment

Illumina recommends the following program for amplification of libraries prepared from 0.1 to 1 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**
  - Use heated lid set to 100 °C
  - 98 °C for 30 seconds
  - 15 cycles of:
    - 98 °C for 10 seconds
    - 60 °C for 30 seconds
    - 72 °C for 30 seconds
  - 72 °C for 5 minutes
  - Hold at 4 °C

TruSeq™ Stranded RNA Post-PCR Run Preparation Steps

If desired, the user may designate a different liquid handler for post-PCR sample processing to avoid any possible 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™ Stranded RNA Post-PCR SPRI application on the Sciclone.

Note: AMPure® 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 BioRad Hardshell 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 library prep kit.

1. Modify the Maestro TruSeq™ Stranded RNA Workbook to specify the number of samples to run

The sample number must be set by indicating the number of columns to run in cell D2 of the worksheet titled “PostPCRSPRI”. The workbook must be located in the filepath: C:\ProgramData\Caliper\Maestro\Workbooks\ and must have the name “TruSeq™ Stranded RNA 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.
Running the Maestro NimbleGen® SeqCapEZ Workflow on the Sciclone NGS

2. Start the TruSeq™ Stranded RNA Post-PCR SPRI Run

Launch the Maestro software and open the TruSeq™ Stranded RNA 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.

Confirm that the 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.

Ensure that the Inheco units have the correct adapters for the TruSeq™ Stranded RNA Post-PCR SPRI application.

Position A3 Not used
Position A4 96-well PCR Plate Adapter
Position D2 96-well PCR Plate Adapter
Position D4 96-well PCR Plate Shaker Adapter

3. Prepare the SPRI Beads Plate and RSB Plate

Aliquot 60 µl of AMPure® XP beads per well in a BioRad Hard-Shell® PCR plate as indicated in the PostPCRSPRI worksheet. Ensure that the beads are at room temperature and well mixed prior to pipeting.

If the 96-well BioRad Hard-Shell® PCR plate containing RSB used in the TruSeq™ Stranded RNA Library Prep 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 new plate as indicated in the workbook.

4. Prepare 80% Ethanol Reservoir

If the 80% EtOH reservoir used in the TruSeq™ Stranded RNA 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 Deepwell reservoir and cover with a lid. Depending on the number of columns of samples to be processed, an open reservoir or 12-column divided reservoir (5 mL EtOH per column) may be used.

5. Setup the Sciclone Deck

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™ Stranded RNA 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™ Stranded RNA 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™ Stranded RNA 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 seven 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 libraries 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 BioRad Hard-Shell® skirted 96-PCR plate. Mix well by pipeting 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.

Expected Results

Figure 11. Representative LabChip GX data from a 1:25 dilution of a TruSeq™ Stranded RNA library prepared from 800 ng total RNA (UHR: Universal Human Reference RNA, Agilent PN 740000) and amplified with 15 cycles of PCR.

Figure 12. LabChip GX data from 1:25 dilutions of eight replicate libraries prepared from 800 ng total RNA (UHR). Libraries were stored on beads overnight at either 4 °C or -20 °C between the cDNA prep and the library prep and were amplified with 15 cycles of PCR.

Figure 13. Data based on LabChip GX smear analysis of the libraries shown in Figure 12.