Protocol for use with NEBNext Small RNA Library Prep Set for Illumina (E7300, E7580, E7560, E7330)

Introduction

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but isdependent on a user variable, like the type of RNA input.

Colored bullets indicate the cap color of the reagent to be added.

Libraries prepared by this method are compatible with paired-end flow cells.

Starting Material: 100 ng–1 µg Total RNA. Small RNA fragments should have a 5´ phosphate and 3´ OH to ligate and must be free of ATP.

Protocol

1. Ligate the 3´ SR Adaptor

Note: For total RNA inputs of 100 ng, dilute the (green) 3´ SR Adaptor for Illumina 1:2 (For example: 1 µl of 3´ SR adaptor and 1 µl nuclease-free water) in nuclease-free water. For total RNA inputs closer to 1 µg, do not further dilute the adaptor. Adaptor dilutions may need to be optimized further.

1.1. Mix the following components in a sterile nuclease-free PCR tube. It is ok to premix the reagents. Use immediately.

Component	Volume
Input RNA	1–6 µl
(green) 3´ SR Adaptor for Illumina	1 µl

Nuclease-Free Water	variable
Total volume	**7 µl**

1.2. Incubate in a preheated thermal cycler for 2 minutes at 70°C. Transfer tube to ice.

1.3. Add and mix the following components. It is ok to premix the reagents. Use immediately

Component	**Volume**
(green) 3´ Ligation Reaction Buffer (2X)	10 µl
(green) 3´ Ligation Enzyme Mix	3 µl
Total volume	**20 µl**

1.4. Incubate for 1 hour at 25°C in a thermal cycler.

Note: Longer incubation times and reduced temperatures (18 hours; 16°C) increase ligation efficiency of methylated RNAs such as piwi-interacting RNAs (piRNAs) (if present in the sample). However, some concatamerization products might be formed.

2. Hybridize the Reverse Transcription Primer

This step is important to prevent adaptor-dimer formation. The SR RT Primer hybridizes to the excess of 3´ SR Adaptor (that remains free after the 3´ ligation reaction) and transforms the single stranded DNA adaptor into a double-stranded DNA molecule. dsDNAs are not substrates for ligation mediated by T4 RNA Ligase 1 and therefore do not ligate to the 5´ SR Adaptor in the subsequent ligation step.

Note: For total RNA inputs of 100 ng, dilute the (pink) SR RT Primer for Illumina 1:2 in nuclease free water. For total RNA inputs closer to 1 µg do not dilute the primer. Depending on the small RNA quantity and quality of your sample additional dilution optimization may be required.

2.1. Add and mix the following components to the ligation mixture from Step 1.4 and mix well. It is ok to premix the reagents.

Component	**Volume**
Nuclease-Free Water	4.5 µl
(pink) SR RT Primer for Illumina	1 µl
Total volume should now be	**25.5 µl**

2.2. Place in a thermocycler with heated lid set to > 85°C and run the following program:

5 minutes at 75°C
15 minutes at 37°C
15 minutes at 25°C
Hold at 4°C

3. Ligate the 5´ SR Adaptor

3.1. With 5 minutes remaining, resuspend the (yellow) 5´ SR adaptor in 120 µl of nuclease free water.

Note: For total RNA inputs closer to 100 ng, additionally dilute the (yellow) 5´ SR Adaptor for Illumina 1:2 in nuclease free water. For total RNA inputs closer to 1 µg do not dilute the adaptor further.

3.2. Aliquot the (yellow) 5´ SR Adaptor into a separate, nuclease-free 200 µl PCR tube, for the number of samples in the experiment plus an excess of 10%.

3.3. Incubate the adaptor in the thermal cycler at 70°C for 2 minutes and then immediately place the tube on ice. Keep the tube on ice and use the denatured adaptor within 30 minutes of denaturation.

Note: Store the remaining resuspended 5´ SR adaptor at –80°C. Denature aliquots before use. Please minimize freeze/thaw cycles. If only a few libraries are to be made at a time, the 5´ SR adaptor could be aliquoted.

3.4. Add and mix the following components to the ligation mixture from Step 2.2 and mix well. Do not premix reagents.

Component	Volume
(yellow) 5´ SR Adaptor for Illumina (denatured)	1 µl
(yellow) 5´ Ligation Reaction Buffer (10X)	1 µl
(yellow) 5´ Ligation Enzyme Mix	2.5 µl
Total volume	**30 µl**

3.5. Incubate for 1 hour at 25°C in a thermal cycler.

4. Perform Reverse Transcription

4.1. Mix the following components in a sterile, nuclease-free tube. It is ok to premix the reagents. Use immediately.

Reagents Supplied	Volume
Adaptor Ligated RNA from Step 3.5	30 µl
(red) First Strand Synthesis Reaction Buffer	8 µl
(red) Murine RNase Inhibitor	1 µl
(red) ProtoScript II Reverse Transcriptase	1 µl
Total volume	**40 µl**

4.2. Incubate for 60 minutes at 50°C.

4.3. Immediately proceed to PCR amplifcation.

Safe Stopping Point: If you do not plan to proceed immediately to PCR amplifcation, then heat inactivate the RT reaction at 70°C for 15 minutes. Samples can be safely stored at –15°C to –25°C.

5. Perform PCR Amplifcation

5.1. Add and mix the following components to the RT reaction mix from Step 4.2 and mix well:

Reagents Supplied	Volume
(blue) LongAmp *Taq* 2X Master Mix	50 µl
(blue) SR Primer for Illumina	2.5 µl
(blue) Index (X) Primer*	2.5 µl
Nuclease free water	5 µl
Total volume	**100 µl**

**Note: The NEBNext Multiplex Small RNA Library Prep Set for Illumina Set 1 (E7300) contains 1–12 PCR primers, each with a different index. For each reaction, only one of the 12 PCR primer; the NEBNext Multiplex Small RNA Library Prep Set for Illumina Set 2 (E7580) contains 13-24 PCR primers; the NEBNext Multiplex Small RNA Library Prep Kit for Illumina Index Primers 1-48 (E7560) contains 1–48 PCR primers, each with a different index. NEBNext Small RNA Library Prep Set for Illumina Multiplex Compatible (E7330) contains index 1. If using only index 1 from E7330 libraries cannot be pooled together. For each reaction, only one of the PCR primer indices is used during the PCR step.*

PCR Cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	94°C	30 sec	1
Denaturation	94°C	15 sec	12–15*
Annealing	62°C	30 sec	
Extension	70°C	15 sec	
Final Extension	70°C	5 min	1
Hold	4°C	∞	

*Amplifcation conditions may vary based on RNA input amount, tissue, and species. This protocol was optimized using 1 µg of total RNA from human brain and 12 PCR cycles. The number of PCR cycles may need to be adjusted if clear and distinct bands are not observed in the gel image. For 100 ng total RNA input run 15 cycles of PCR. For samples containing high amounts of small RNA, less than 12 cycles may be appropriate.

Safe Stopping Point: It is safe to store the library at -20°C after PCR. Avoid leaving the sample at 4°C overnight if possible.

6. Quality Control Check and Size Selection

*Note: There are several different methods for performing size selection. It is recommended to choose the appropriate method based on the QC check of the library using the Bioanalyzer. Size selection using AMPure XP Beads does not remove small fragments. If you perform the QC check and your sample contains Adaptor dimer (127 bp peak) or excess primers (70-80 bp) it is recommended to use gel or Pippin Prep for size selection. Please download either protocol **6A**, **6B**, or **6C** from NEB.com.*

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Links to this resource

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