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

New England Biolabs[1]

[1]New England Biolabs

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New England Biolabs (NEB)

Tech. support phone: +1(800)632-7799 email: info@neb.com

ABSTRACT

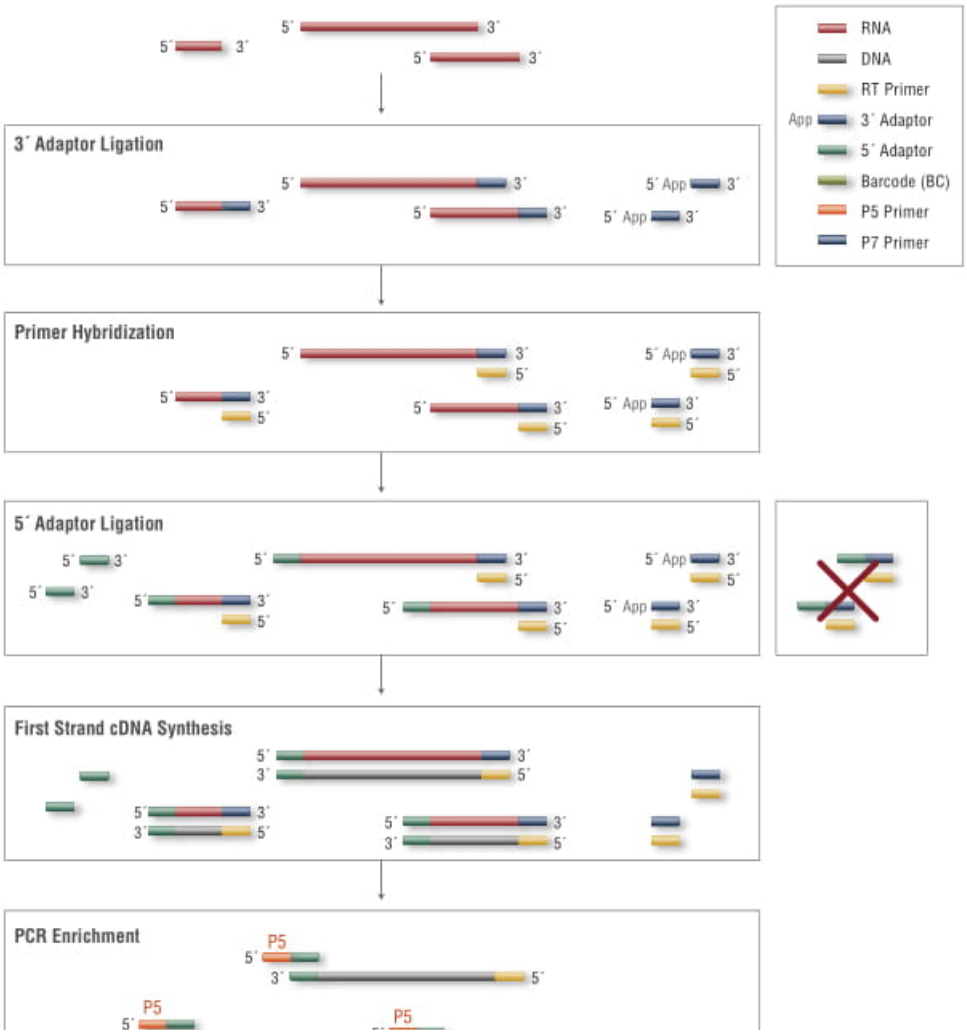
The NEBNext Multiplex Small RNA Library Prep Set for Illumina contains the adaptors, primers, enzymes and buffers required to convert small RNAs into indexed libraries for next generation sequencing on the Illumina platform. The novel workflow has been optimized to minimized adaptor dimers, while producing high-yield, high-diversity libraries.

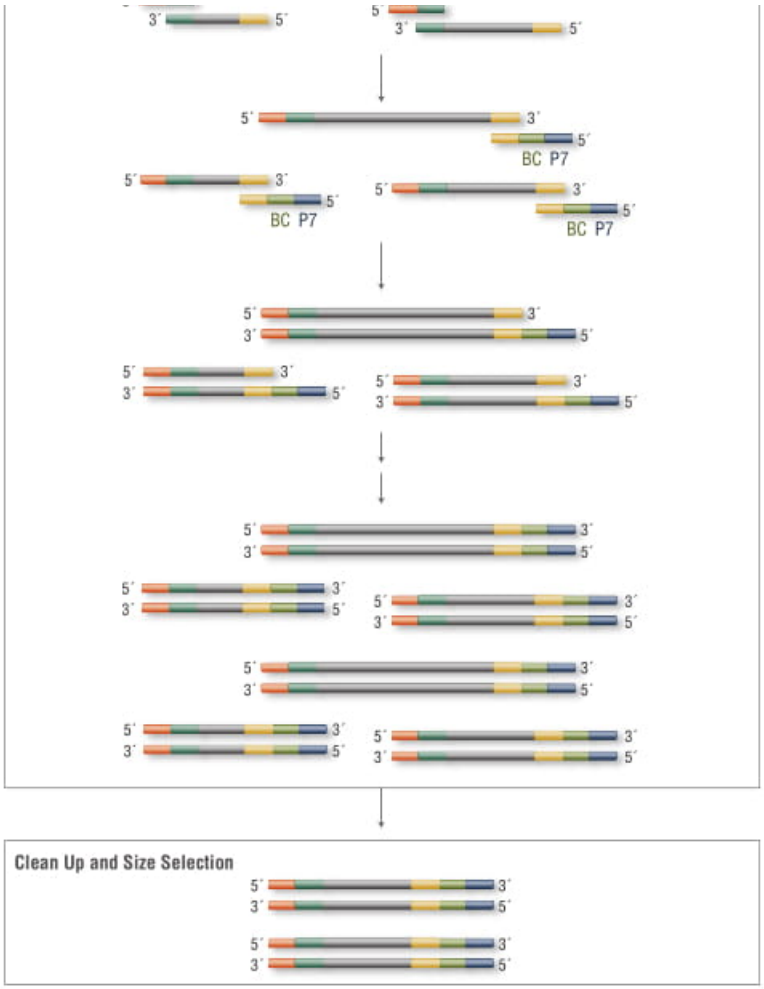
The unique workflow of the NEBNext® Small RNA library prep kits addresses the challenge of minimization of adaptor-dimers while achieving production of high-yield, diverse multiplex libraries in a simple protocol.

- Minimized adaptor-dimer contamination
- High yields
- Input RNA can be Total RNA
- Suitable for methylated small RNAs (e.g., piRNAs) as well as unmethylated small RNAs

## Multiplex Small RNA Library Prep Workflow

This kit includes a novel protocol that results in higher yields and lower adaptor-dimer contamination.






EXTERNAL LINK

https://www.neb.com/products/e7300-nebnext-multiplex-small-rna-library-prep-set-for-illumina-set-1#Protocols%20&%20Manuals_Protocols

PDF

manualE7300.pdf

PROTOCOL STATUS

**Working**

GUIDELINES

**RNA Sample Quality:** This kit was optimized using high quality human RNA (First Choice® Human Brain Reference RNA from Life Technologies, Inc. #AM7962). High Quality total RNA (RNA Integrity Number (RIN) > 7) should be used as starting material whenever possible. The quality and quantity of your sample should be assessed, for example by use of the Agilent 2100 Bioanalyzer, using an Agilent RNA 6000 Nano Chip.

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| NEBNext Multiplex Small RNA Library Prep Set for Illumina (1-12) - 96 rxns | E7300L | New England Biolabs |

| NAME | CATALOG # | VENDOR |
|---|---|---|
| NEBNext Multiplex Small RNA Library Prep Set for Illumina (1-12) - 24 rxns | E7300S | New England Biolabs |
| NEBNext Small RNA Library Prep Set for Illumina (Multiplex Compatible) - 96 rxns | E7330L | New England Biolabs |
| NEBNext Small RNA Library Prep Set for Illumina (Multiplex Compatible) - 24 rxns | E7330S | New England Biolabs |
| NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 2) - 96 rxns | E7580L | New England Biolabs |
| NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 2) - 24 rxns | E7580S | New England Biolabs |
| NEBNext Multiplex Small RNA Library Prep Set for Illumina (1-48) - 96 rxns | E7560S | New England Biolabs |

MATERIALS TEXT

**Materials Included:**

Materials for Small RNA.pdf

**Required Materials Not Included:**

- 3 M Sodium Acetate, pH 5.5
- 100% Ethanol
- 80% Ethanol
- Corning®, Costar®, Spin-X® Centrifuge Tube Filters (Cellulose Acetate Filters) (Sigma Aldrich # CLS8162)
- Monarch PCR & DNA Cleanup Kit (5 µg) (NEB #T1030)

**Size Selection Materials:**

**For gel size selection:**

- 6% Novex® TBE PAGE gel 1.0 mM 10-well (Life Technologies, Inc. #EC6265BOX)
- SYBR® Gold Nucleic Acid Gel Stain (Life Technologies, Inc. #S-11494)
- RNase-free Disposable Pellet Pestles® (Kimble Kontes Asset Management, Inc. #749521-1590)
- Dry Ice/Methanol Bath or –80°C freezer

**For bead selection:**

- Agencourt® AMPure® XP Beads (Beckman Coulter, Inc. #A63881)

**For Pippin Prep™ selection:**

- 3% Agarose Dye Free Gel (Sage Science #CDP 3010)

Bioanalyzer® (Agilent® Technologies, Inc.)

SAFETY WARNINGS

Please refer to the SDS (Safety Data Sheet) for safety warnings and hazard information.

BEFORE STARTING

**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.

## Ligate the 3´ SR Adaptor

1

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.*

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

| | |
|---|---|
| Input RNA | 1-6 µl |
| (green) 3′ SR Adaptor for Illumina | 1 µl |
| Nuclease-Free Water | variable |
| Total volume | 7 µl |

2 Incubate in a preheated thermal cycler for 00:02:00 at 70 °C . Transfer tube to ice.

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

| | |
|---|---|
| (green) 3′ Ligation Reaction Buffer (2X) | 10 µl |
| (green) 3′ Ligation Enzyme Mix | 3 µl |
| Total volume | 20 µl |

4 Incubate for 01:00:00 at 25 °C in a thermal cycler.

NOTE

*Longer incubation times and reduced temperatures ( 18:00:00 ; 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.*

## Hybridize the Reverse Transcription Primer

5 This section 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.*

6 Add and mix the following components to the ligation mixture from Step 4 and mix well. It is ok to premix the reagents.

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

7 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

## Ligate the 5′ SR Adaptor

8 With 00:05:00 remaining, resuspend the (yellow) 5´ SR adaptor in 120 µl **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.*

9 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%.

10 Incubate the adaptor in the thermal cycler at 70 °C for 00:02:00 and then immediately place the tube on ice. Keep the tube on ice and use the denatured adaptor within 00:30:00 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.**

11 Add and mix the following components to the ligation mixture from Step 7 and mix well. Do not premix reagents.

| | |
|---|---|
| (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 |

12 Incubate for 01:00:00 at 25 °C in a thermal cycler.

## Perform Reverse Transcription

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

| | |
|---|---|
| Adaptor Ligated RNA from Step 12 | 30 µl |
| (red) First Strand Synthesis Reaction Buffer | 8 µl |
| (red) Murine RNase Inhibitor | 1 µl |
| (red) ProtoScript IIReverse Transcriptase | 1 µl |
| Total volume | 40 µl |

14 Incubate for 01:00:00 at 50 °C .

15 Immediately proceed to PCR amplifcation.

NOTE

**Safe Stopping Point: If you do not plan to proceed immediately to PCR amplifcation, then heat inactivate the RT reaction at 70 °C for 00:15:00 . Samples can be safely stored at -15 °C to -25 °C**

## Perform PCR Amplifcation

16 Add and mix the following components to the RT reaction mix from Step 14 and mix well:

| | |
|---|---|
| (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 now should be | 100 µl |

NOTE

** Note: The NEBNext Multiplex Small RNA Library Prep Set for Illumina Set 1 contains 1–12 PCR primers, set 2 contains 23-24 PCR primers, kit index primers 1-48 PCR primer, each with a different index. For each reaction, only one of the 12 PCR primer indices is used during the PCR step.*

17 **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.

NOTE

**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.**

## Quality Control Check and Size Selection

18 *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 selcet either case Option A, Option B, or Option C below.*

**Option A: QC Check and Size Selection using 6% PolyAcrylamide Gel**

**Option B: QC Check and Size Selection Using Pippin Prep**

Size selection of the Small RNA library (147 bp) can done on Pippin Prep instrument using the 3% Agarose, dye free gel with internal standards (Sage Science # CDP3010).

**Option C: QC Check and Size Selection using AMPure XP Beads**

Bead size selection is only recommended for samples showing no primer dimer and no adaptor dimer on Bioanalyzer. It will be suitable to remove peaks > 150 bp. If fragments larger than 150 bp are abundant, two rounds of bead size selection may be necessary to completely eliminate the high molecular weight fragments.

Option A

QC Check and Size Selection using 6% PolyAcrylamide Gel

## QC Check and Size Selection using 6% PolyAcrylamide Gel

19 Purify the PCR amplified cDNA construct ( 100 µl ) using a Monarch PCR & DNA Kit.

**IMPORTANT: Use the 7:1 ratio of binding buffer:sample.Discard the flow through after each centrifugation step.**

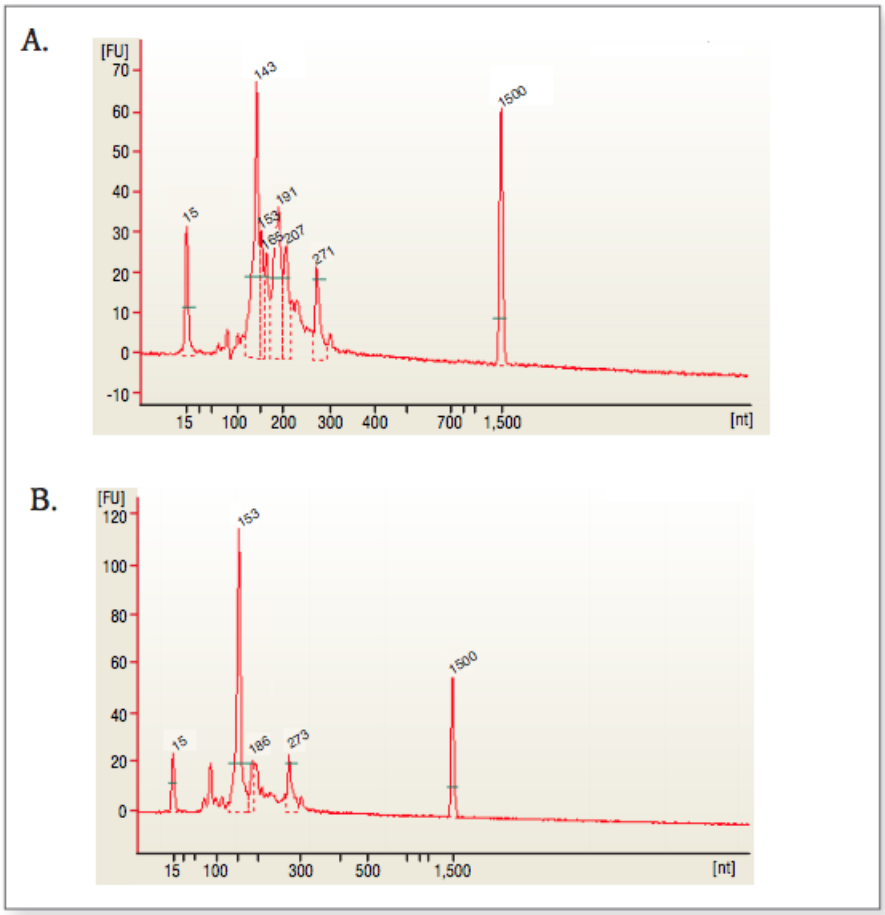
20 Elute amplified DNA in 27.5 µl **Nuclease-free Water** .

NOTE

**Safe Stopping Point: It is safe to store the library at -20 °C .**

21 Load 1 µl **purified PCR reaction** on the Bioanalyzer using a DNA 1000 chip according to the manufacturer's instructions (Figure 1).

Figure 1: Typical results from (A) human brain and (B) rat testis total RNA libraries before size selection.




*The 143 and 153 bp bands correspond to miRNAs and piRNAs, respectively. The bands on the Bionalyzer electropherograms resolve in sizes ~ 6-8 nucleotides larger than sizes observed on PAGE gels and can shift from sample to sample due to an incorrect identification of the marker by the bioanalyzer software. miRNA peak should be ~ 143-146 bp.*

22 Mix the purified PCR product ( **25 µl** ) with **5 µl Gel Loading Dye, Blue (6X)** .

NOTE
*Vortex the Gel Loading Dye, Blue throughly to mix well before using.*

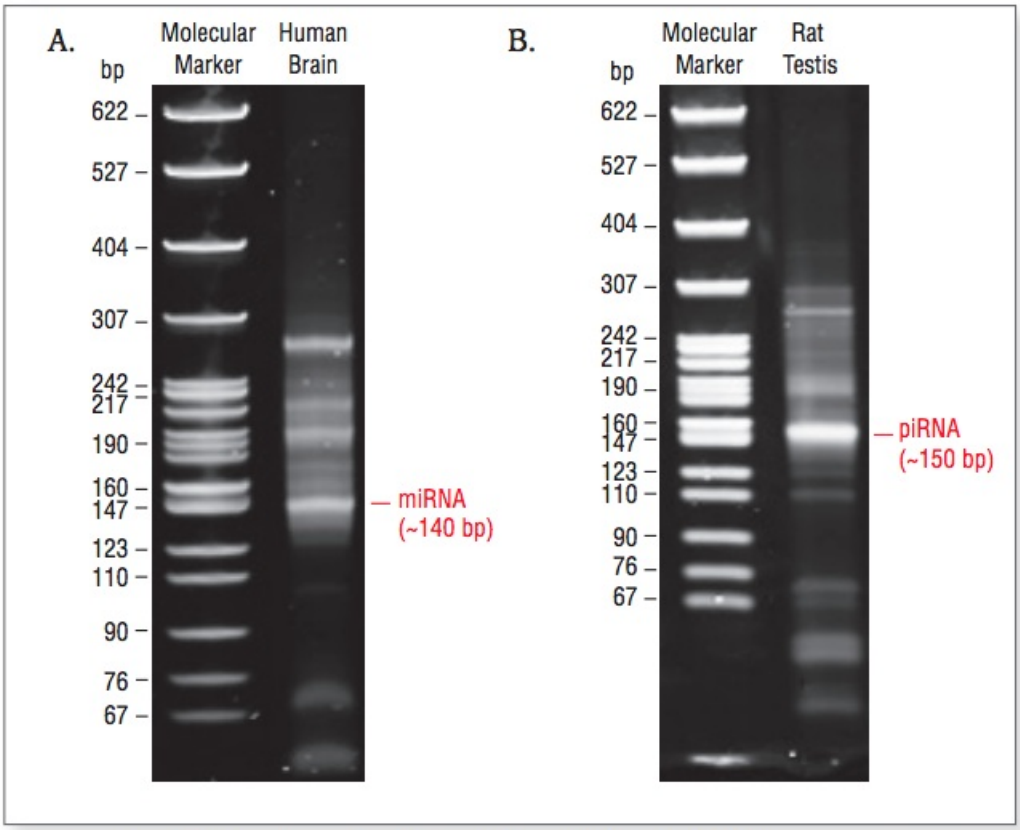
23 Load **5 µl Quick-Load pBR322 DNA-MspI Digest** in one well on the 6% PAGE 10-well gel.

24 Load two wells with **15 µl** each of mixed amplified cDNA construct and loading dye on the 6% PAGE 10-well gel.

25 Run the gel for **01:00:00** at 120 V or until the blue dye reaches the bottom of the gel. Do not let the blue dye exit the gel.

26 Remove the gel from the apparatus and stain the gel with SYBR Gold nucleic acid gel stain in a clean container for **00:02:00** – **00:03:00** minutes and view the gel on a UV transiluminator (Figure 2).

Figure 2:




*Shows typical results from Human Brain (A) and Rat Testis (B) Total RNA libraries.*
*The 140 and 150 bp bands correspond to miRNAs (21 nt) and piRNAs (30 nt), respectively.*

27 The 140 and 150 nucleotide bands correspond to adapter-ligated constructs derived from the 21 and 30 nucleotide RNA fragments, respectively. For miRNAs, isolate the bands corresponding to ~140 bp. For piRNAs, isolate the band corresponding to ~150 bp. For other small RNA, the band size may be different.

28 Place the two gel slices from the same sample in one 1.5 ml tube and crush the gel slices with the RNase-free Disposable Pellet Pestles and then soak in **250 µl DNA Gel Elution buffer (1X)** .

29 Rotate end-to-end for at least **02:00:00** at room temperature.

30 Transfer the eluate and the gel debris to the top of a gel filtration column (for example: Corning®, Costar®, Spin-X® Centrifuge Tube Filters (Cellulose Acetate Filters) (Sigma Aldrich #CLS8162).

31 Centrifuge the filter for **00:02:00** at > 13,200 rpm.

32 Recover eluate and add **1 µl Linear Acrylamide** , **25 µl 3M sodium acetate, pH 5.5** and **750 µl 100% ethanol** .

33 Vortex well.

34 Precipitate in a dry ice/methanol bath or at -80 °C for at least 00:30:00 .

35 Spin in a microcentrifuge @ > 14,000 x g for 00:30:00 at 4 °C .

36 Remove the supernatant taking care not to disturb the pellet.

37 Wash the pellet with 80% ethanol by vortexing vigorously.

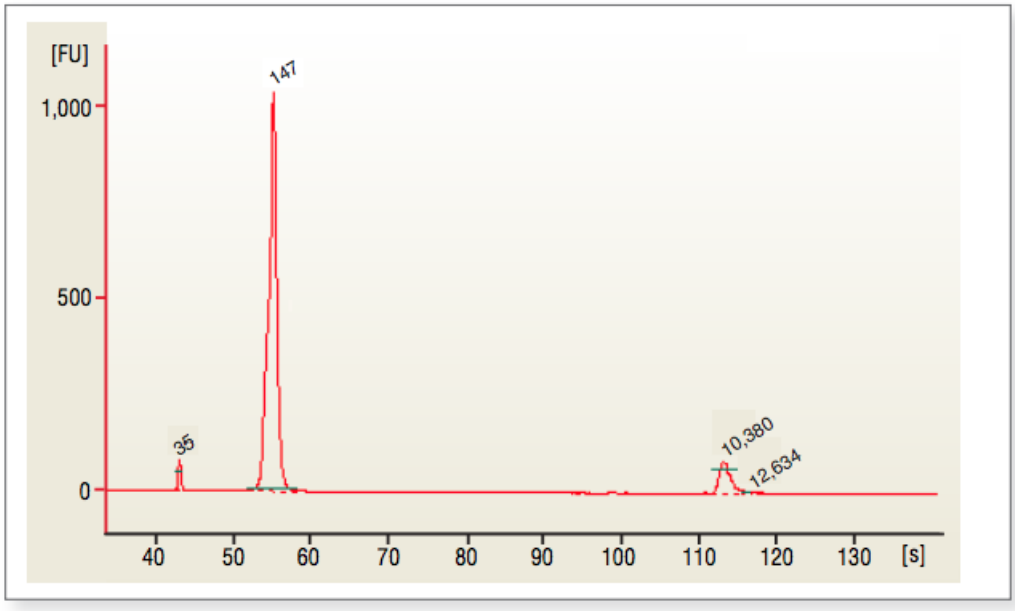
38 Spin in a microcentrifuge @ > 14,000 x g for 00:30:00 at 4 °C .

39 Air dry pellet for up to 00:10:00 at room temperature to remove residual ethanol.

40 Resuspend pellet in 12 µl TE Buffer .

41 Load 1 µl of the size selected purified library on a 2100 Bioanalyzer using a DNA 1000 or High Sensitivity DNA chip according to the manufacturer's instructions (Figure 3).

Figure 3: Electropherogram trace of the gel size selected purified library from human brain total RNA.




42 Check the size, purity, and concentration of the sample.

QC Check and Size Selection Using Pippin Prep

step case

Option B

QC Check and Size Selection Using Pippin Prep

19 Purify the PCR amplified cDNA construct (100 µl) using a Monarch PCR & DNA Cleanup Kit.

**IMPORTANT: Use the 7:1 ratio of binding buffer:sample.Discard the flow through after each centrifugation step.**

20 Elute amplified DNA in **32 µl nuclease-free water** .

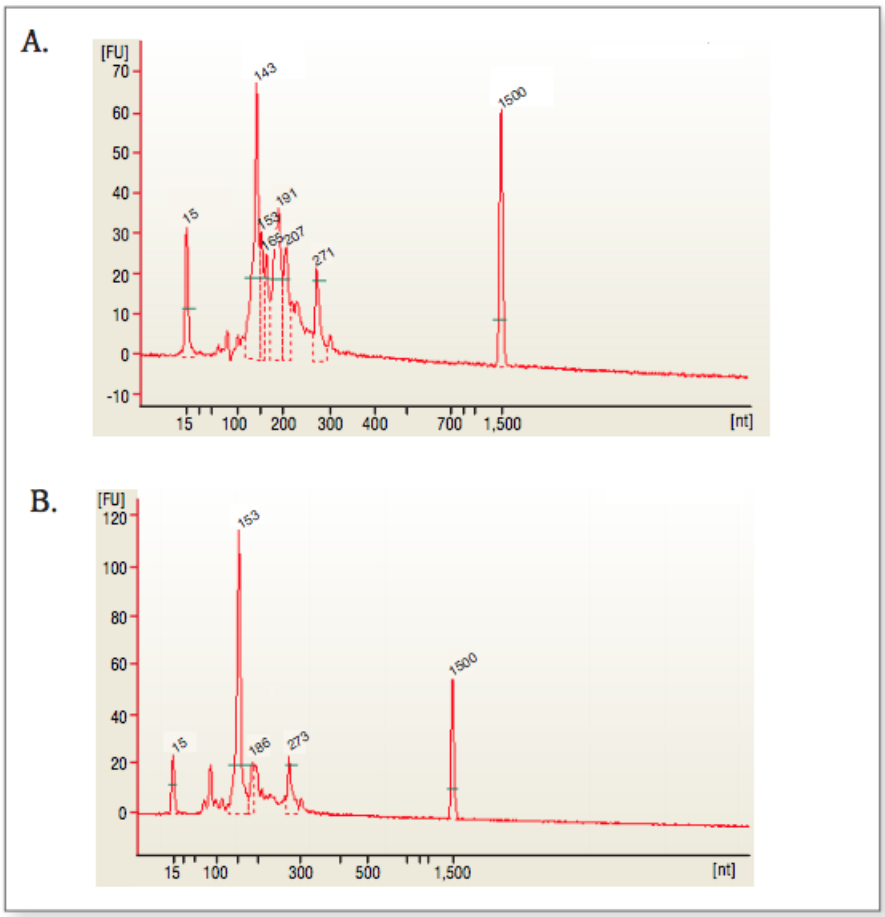
NOTE

**Safe Stopping Point: It is safe to store the library at -20 °C after PCR cleanup.**

21 **It is recommended to QC your library before performing size selection:**

Load **1 µl** of the purified PCR reaction on the Bioanalyzer using a DNA 1000 chip according to the manufacturer's instructions (Figure 1). . miRNA library should appear as a peak at 147 bp peak (that correspond for 21 nucleotide insert).

Figure 1: Typical results from (A) human brain and (B) rat testis total RNA libraries before size selection.




*The 143 and 153 bp bands correspond to miRNAs and piRNAs, respectively. The bands on the Bionalyzer electropherograms resolve in sizes ~ 6-8 nucleotides larger than sizes observed on PAGE gels and can shift from sample to sample due to an incorrect identification of the marker by the bioanalyzer software. miRNA peak should be ~ 143-146 bp.*

## QC Check and Size Selection using AMPure XP Beads

step case

Option C

QC Check and Size Selection using AMPure XP Beads

19 Purify the PCR amplified cDNA construct ( **100 µl** ) using a Monarch PCR & DNA Kit.

**IMPORTANT: Use the 7:1 ratio of binding buffer:sample. Discard the flow through after each centrifugation step.**

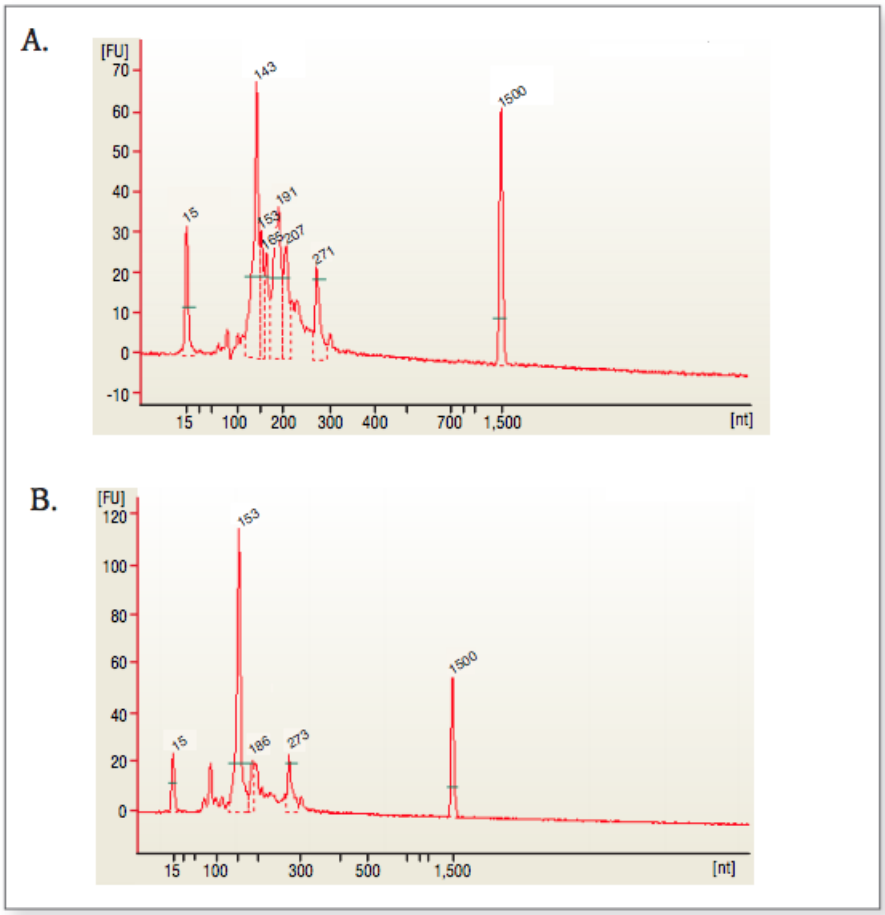
20 Elute amplified DNA in **27.5 µl Nuclease-free Water** .

NOTE

**Safe Stopping Point: It is safe to store the library at -20 °C after PCR cleanup.**

21 Load **1 µl purified PCR reaction** on the Bioanalyzer using a DNA 1000 chip according to the manufacturer's instructions (Figure 1).

Figure 1: Typical results from (A) human brain and (B) rat testis total RNA libraries before size selection.

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