

## LIBRARY PREPARATION

# NEBNext<sup>®</sup> Multiplex Small RNA Library Prep Set for Illumina<sup>®</sup> (Set 1)

Instruction Manual

NEB #E7300S/L  
24/96 reactions  
Version 4.0 9/17

 NEW ENGLAND  
**BioLabs**<sup>®</sup> inc.

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drive DISCOVERY  
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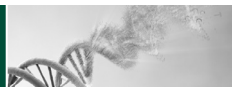
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# The NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 1) Includes:

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*The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7300S) and 96 reactions (NEB #E7300L). All reagents should be stored at -20°C.*

- (green) NEBNext 3' Ligation Reaction Buffer (2X)
- (green) NEBNext 3' Ligation Enzyme Mix
- (green) NEBNext 3' SR Adaptor for Illumina
- (yellow) NEBNext 5' SR Adaptor for Illumina
- (yellow) NEBNext 5' Ligation Reaction Buffer (10X)
- (yellow) NEBNext 5' Ligation Enzyme Mix
- (pink) NEBNext SR RT Primer for Illumina
- (red) NEBNext First Strand Synthesis Reaction Buffer
- (red) ProtoScript II Reverse Transcriptase
- (red) Murine RNase Inhibitor
- (blue) LongAmp *Taq* 2X Master Mix
- (blue) NEBNext SR Primer for Illumina
- (blue) NEBNext Index 1 Primer for Illumina
- (blue) NEBNext Index 2 Primer for Illumina
- (blue) NEBNext Index 3 Primer for Illumina
- (blue) NEBNext Index 4 Primer for Illumina
- (blue) NEBNext Index 5 Primer for Illumina
- (blue) NEBNext Index 6 Primer for Illumina
- (blue) NEBNext Index 7 Primer for Illumina
- (blue) NEBNext Index 8 Primer for Illumina
- (blue) NEBNext Index 9 Primer for Illumina
- (blue) NEBNext Index 10 Primer for Illumina
- (blue) NEBNext Index 11 Primer for Illumina
- (blue) NEBNext Index 12 Primer for Illumina
- (orange) Gel Loading Dye, Blue (6X)
- (orange) Quick-Load pBR322 DNA-MspI Digest
- (white) DNA Gel Elution Buffer, 1X
- (white) Linear Acrylamide (10 mg/ml)
- (white) TE Buffer
- (white) Nuclease-free Water

## Required Materials Not Included:

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3 M Sodium Acetate, pH 5.5

100% Ethanol

80% Ethanol

Corning<sup>®</sup>, Costar<sup>®</sup>, Spin-X<sup>®</sup> 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<sup>®</sup> TBE PAGE gel 1.0 mM

10-well (Life Technologies, Inc. #EC6265BOX)

SYBR<sup>®</sup> Gold Nucleic Acid Gel Stain (Life Technologies, Inc. #S-11494)

RNase-free Disposable Pellet Pestles<sup>®</sup> (Kimble Kontes Asset Management, Inc.  
#749521-1590)

Dry Ice/Methanol Bath or -80°C freezer

#### for bead selection:

Agencourt<sup>®</sup> AMPure<sup>®</sup> XP Beads (Beckman Coulter, Inc. #A63881)

#### for Pippin Prep<sup>™</sup> selection:

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

Bioanalyzer<sup>®</sup> (Agilent<sup>®</sup> Technologies, Inc.)

## Overview

The NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 1) 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.

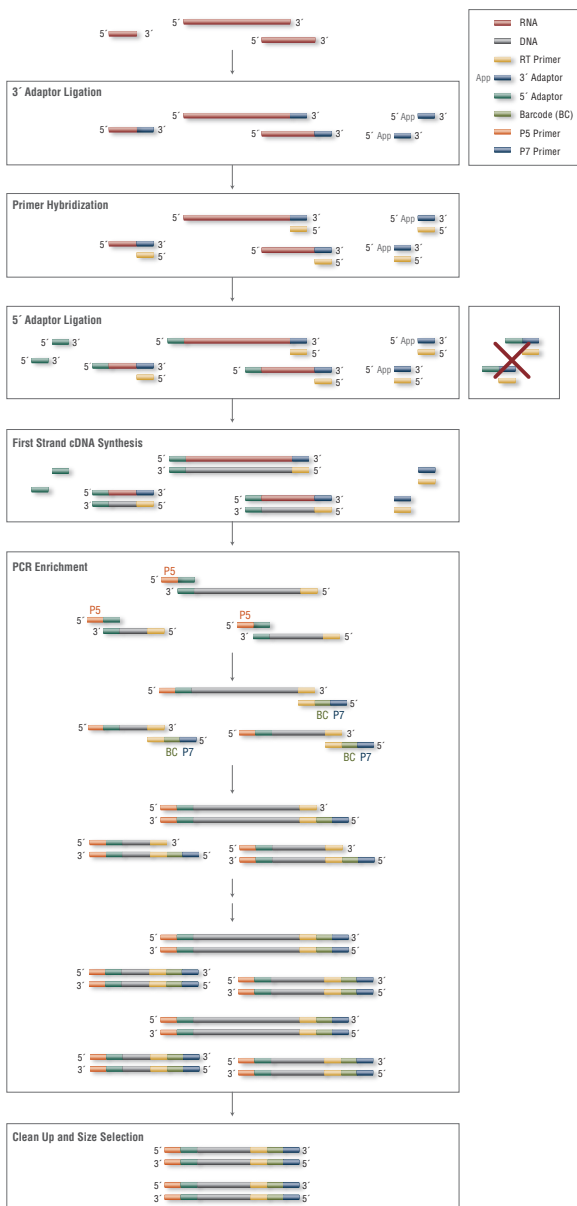
Each kit component must pass rigorous quality control standards, and each set of reagents is functionally validated together by construction and sequencing of indexed small RNA libraries on the Illumina sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

**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.

# Multiplex Small RNA Library Prep Workflow

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



# Protocols

Please refer to revision history for a summary of protocol updates

## 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 is dependent 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 Illumina 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.

### 1. Ligate the 3′ SR Adaptor

*Note: For total RNA inputs closer to 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.

Input RNA	1–6 µl
● (green) 3′ SR Adaptor for Illumina	1 µl
Nuclease-Free Water	variable
<hr/>	
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.

● (green) 3′ Ligation Reaction Buffer (2X)	10 µl
● (green) 3′ Ligation Enzyme Mix	3 µl
<hr/>	
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 closer to 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.

Nuclease-Free Water	4.5 µl
● (pink) SR RT Primer for Illumina	1 µl
<hr/>	
Total volume now should 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 pre-mix reagents.

● (yellow) 5' SR Adaptor for Illumina (denatured)	1 $\mu$ l
● (yellow) 5' Ligation Reaction Buffer (10X)	1 $\mu$ l
● (yellow) 5' Ligation Enzyme Mix	2.5 $\mu$ l
<hr/>	
Total volume	30 $\mu$ l

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 pre-mix the reagents. Use immediately.

Adaptor Ligated RNA from Step 3.5	30 $\mu$ l
● (red) First Strand Synthesis Reaction Buffer	8 $\mu$ l
● (red) Murine RNase Inhibitor	1 $\mu$ l
● (red) ProtoScript II Reverse Transcriptase	1 $\mu$ l
<hr/>	
Total volume	40 $\mu$ l

4.2. Incubate for 60 minutes at 50°C.

4.3. Immediately proceed to PCR amplification.



**Safe Stopping Point: If you do not plan to proceed immediately to PCR amplification, 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 Amplification

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

● (blue) LongAmp Taq 2X Master Mix	50 $\mu$ l
● (blue) SR Primer for Illumina	2.5 $\mu$ l
● (blue) Index (X) Primer*	2.5 $\mu$ l
Nuclease free water	5 $\mu$ l
<hr/>	
Total volume now should be	100 $\mu$ l

*\*Note: The NEBNext Multiplex Small RNA Library Prep Set for Illumina Set 1 contains 1–12 PCR primers, each with a different index. For each reaction, only one of the 12 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	∞	

\*Amplification 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.*

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

- 6A.1. Purify the PCR amplified cDNA construct (100  $\mu$ l) using a Monarch PCR & DNA Kit.

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

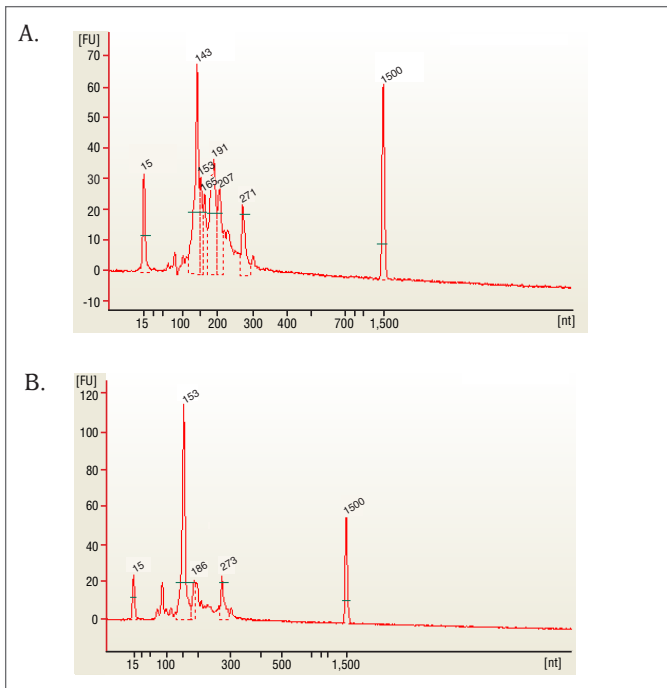
- 6A.2. Elute amplified DNA in 27.5  $\mu$ l Nuclease-free Water.



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

- 6A.3. Load 1  $\mu$ l of the 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 Bioanalyzer 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.

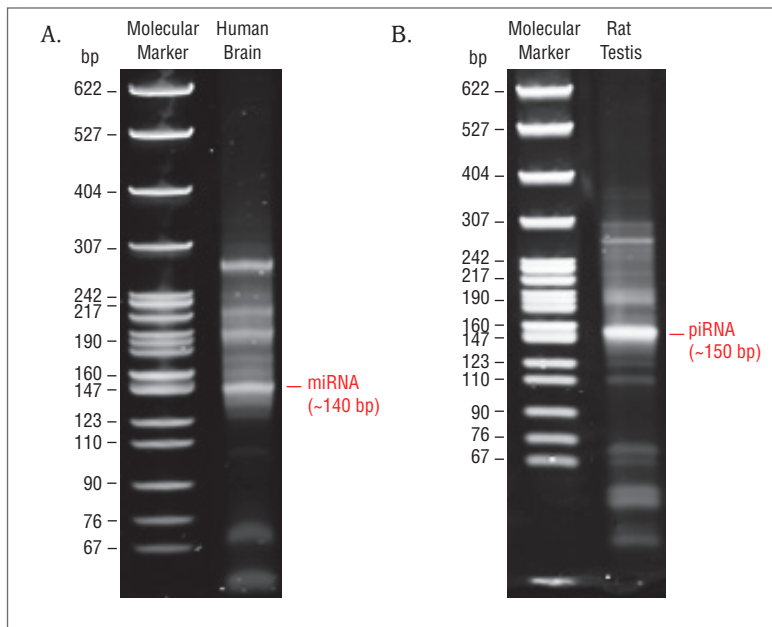
- 6A.4. Mix the purified PCR product (25  $\mu$ l) with 5  $\mu$ l of Gel Loading Dye, Blue (6X).

*Note: Vortex the Gel Loading Dye, Blue thoroughly to mix well before using.*

- 6A.5. Load 5  $\mu$ l of Quick-Load pBR322 DNA-MspI Digest in one well on the 6% PAGE 10-well gel.
- 6A.6. Load two wells with 15  $\mu$ l each of mixed amplified cDNA construct and loading dye on the 6% PAGE 10-well gel.
- 6A.7. Run the gel for 1 hour at 120 V or until the blue dye reaches the bottom of the gel. Do not let the blue dye exit the gel.

- 6A.8. Remove the gel from the apparatus and stain the gel with SYBR Gold nucleic acid gel stain in a clean container for 2–3 minutes and view the gel on a UV transilluminator (Figure 2).

Figure 2:

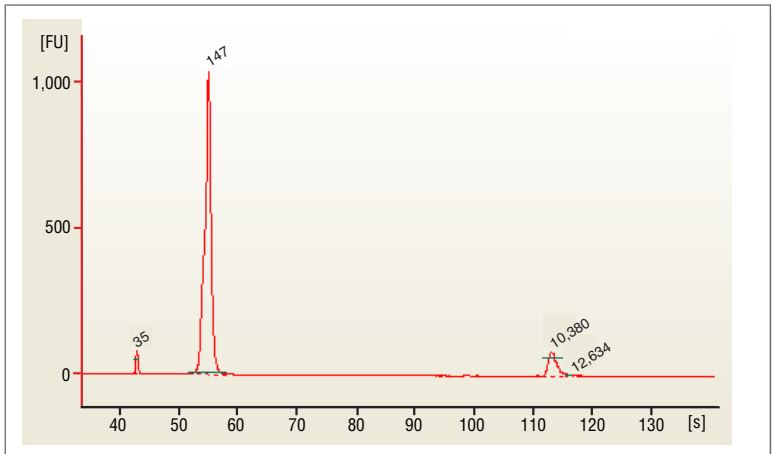


Shows typical results from Human Brain (A) and Rat Testis (B) Total RNA libraries. The 140 and 150 bp bands correspond to miRNAs (21 nt) and piRNAs (30 nt), respectively.

- 6A.9. 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.
- 6A.10. 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  $\mu$ l DNA Gel Elution buffer (1X).
- 6A.11. Rotate end-to-end for at least 2 hours at room temperature.
- 6A.12. Transfer the eluate and the gel debris to the top of a gel filtration column.
- 6A.13. Centrifuge the filter for 2 min at > 13,200 rpm.
- 6A.14. Recover eluate and add 1  $\mu$ l Linear Acrylamide, 25  $\mu$ l 3M sodium acetate, pH 5.5 and 750  $\mu$ l of 100% ethanol.

- 6A.15. Vortex well.
- 6A.16. Precipitate in a dry ice/methanol bath or at  $-80^{\circ}\text{C}$  for at least 30 minutes.
- 6A.17. Spin in a microcentrifuge @  $> 14,000 \times g$  for 30 minutes at  $4^{\circ}\text{C}$ .
- 6A.18. Remove the supernatant taking care not to disturb the pellet.
- 6A.19. Wash the pellet with 80% ethanol by vortexing vigorously.
- 6A.20. Spin in a microcentrifuge @  $> 14,000 \times g$  for 30 minutes at  $4^{\circ}\text{C}$ .
- 6A.21. Air dry pellet for up to 10 minutes at room temperature to remove residual ethanol.
- 6A.22. Resuspend pellet in  $12 \mu\text{l}$  TE Buffer.
- 6A.23. Load  $1 \mu\text{l}$  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).
- 6A.24. Check the size, purity, and concentration of the sample.

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



## 6B. QC Check and Size Selection Using Pippin Prep

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

- 6B.1. Purify the PCR amplified cDNA construct (100  $\mu$ l) 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.**

- 6B.2. Elute amplified DNA in 32  $\mu$ l nuclease-free water.



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

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

- 6B.3. Load 1  $\mu$ l 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 corresponds for 21 nucleotide insert).

**Program the protocol for size selection on Pippin Prep Instrument as follows:**

- 6B.4. In the Pippin Prep software, go to the Protocol Editor Tab.
- 6B.5. Click "Cassette" folder, and select "3% DF Marker P".
- 6B.6. Select the collection mode as "Range" and enter the size selection parameters as follow: BP start (105) and the BP end (155). BP Range Flag should indicate "broad". *Note: This protocol is optimized to select for 147–149 bp peak. When targeting other small RNA these settings may have to be adjusted.*
- 6B.7. Click the "Use of Internal Standards" button.
- 6B.8. Make sure the "Ref Lane" values match the lane numbers.
- 6B.9. Press "Save As" and name and save the protocol.

**Prepare sample for size selection as follows:**

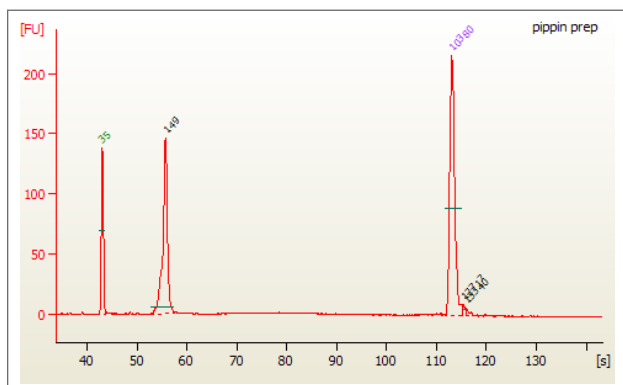
- 6B.10. Bring loading solution to room temperature
- 6B.11. For each sample, combine 30  $\mu$ l sample with 10  $\mu$ l of DNA marker P (labeled P).
- 6B.12. Mix samples thoroughly (vortex mixer). Briefly centrifuge to collect.
- 6B.13. Load 40  $\mu$ l (DNA plus marker) on one well of the 3% agarose cassette.
- 6B.14. Run the program with the settings indicated above.



6B.15. After sample has been eluted, collect 40  $\mu$ l sample from elution well. Run 1  $\mu$ l in a Bioanalyzer using the high sensitivity chip.

**Note: If the Ethidium Bromide free cassettes was used, no purification is required before running sample on the bioanalyzer.**

Figure 4: Electropherogram trace of Pippin Prep size selected library from human brain total RNA.



## 6C. QC Check and Size Selection using AMPure XP Beads

**Note:** 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.

- 6C.1. Purify the PCR amplified cDNA construct (100  $\mu$ l) using a Monarch PCR & DNA Kit.

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

- 6C.2. Elute amplified DNA in 27.5  $\mu$ l Nuclease-free Water.



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

- 6C.3. Load 1  $\mu$ l of the purified PCR reaction on the Bioanalyzer using a DNA 1000 chip according to the manufacturer's instructions (Figure 1).

- 6C.4. To the purified PCR reaction (25  $\mu$ l), add 32.5  $\mu$ l (1.3X) of resuspended AMPure XP beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

- 6C.5. Incubate for 5 minutes at room temperature.

- 6C.6. Place the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant (57.5  $\mu$ l) to a new tube (**Caution: do not discard the supernatant**). Discard beads that contain the large DNA fragments.

- 6C.7. Add 92.5  $\mu$ l (3.7X) of resuspended AMPure XP beads to the supernatant (57.5  $\mu$ l), mix well and incubate for 5 minutes at room temperature.

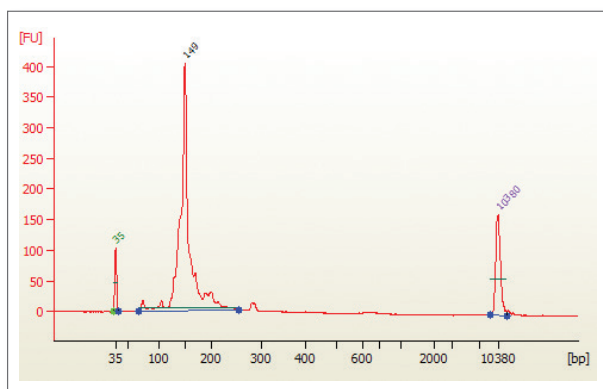
- 6C.8. Place the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard beads**).

- 6C.9. Add 200  $\mu$ l of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

- 6C.10. Repeat Step 6C.9. once.

- 6C.11. Briefly spin the tube, and put the tube back in the magnetic stand.
- 6C.12. Completely remove the residual ethanol, and air dry beads for up to 10 minutes while the tube is on the magnetic stand with lid open.
- Caution: Do not overdry the beads, which may result in lower recovery of the DNA target. Elute the sample when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.**
- 6C.13. Elute the DNA target from the beads with 15  $\mu$ l nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes and put the tube in the magnetic stand until the solution is clear.
- 6C.14. Transfer the supernatant to a clean PCR tube.
- 6C.15. Run 1  $\mu$ l on the Bioanalyzer High Sensitivity chip. Check peak distribution and concentration of the small RNA library.

Figure 5: Electropherogram trace of the bead size selected purified library from human brain total RNA.



## Frequently Asked Questions (FAQs)

Q: *How should my NEBNext Small RNA Library be trimmed?*

A. Use the following:

Single Ends Reads (Read 1): AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC

Paired End Reads (Read 2): GATCGTCGGACTGTAGAACTCTGAAC

Q: *Can I use Total RNA to make small RNA libraries or do I have to isolate or enrich the sample for small RNA?*

A. Small RNA libraries can be done using Total RNA. It is not necessary to enrich the sample for small RNA if small RNA species are in a concentration higher than 0.5%. Total RNA with a RIN (RNA Integrity Number) higher than 7 is recommended.

Q: *Why does the RT primer hybridization occur before 5' adaptor ligation?*

A. The small RNA library prep protocol has been improved to prevent adaptor-dimer formation. The RT primer is added to anneal with the un-ligated 3' adaptor and transform a single-stranded DNA adaptor in to a double-stranded DNA molecule that is no longer a substrate for T4 RNA ligase 1.

Q: *Do I have to hybridize the RT primer again after 5' ligation?*

A. No, RT primer hybridization occurs before the 5' adaptor ligation. After the 5' ligation reaction, keep the sample on ice and do not heat the sample to avoid denaturation of the RT annealed primer.

Q: *How are the barcodes introduced in the Multiplex libraries?*

A. A six-base indices are introduced during the PCR step. This design allows for the indexes to be read using a second read and significantly reduces bias compared to designs which include the index within the first read.

Q: *During size selection on 6% PAGE gel, which bands should I cut out of the gel?*

A. The 140 and 150 bp bands correspond to adaptor-ligated constructs derived from the 21 and 30 nucleotide RNA fragments, respectively. The miRNA libraries run at 140 bp corresponding to 21 nucleotides and piRNA libraries run at 150 bp corresponding to 30 nucleotides.

Q: *Are libraries prepared by this method compatible with paired-end flowcells for cluster generation?*

A. Yes, libraries prepared by this method can be loaded on paired-end flowcells.

## Frequently Asked Questions (FAQs) (continued)

Q: *Can I use the small RNA sample preparation kit for Directional-RNA sequencing?*

A. Yes, the Multiplex small RNA library preparation kit can be used for Directional RNA-Seq. For this application use purified and fragmented messenger RNA as input. Depending on the amount of mRNA used as input, dilution of the adaptors and reverse transcription primer and PCR primers might be required.

Q: *What should I do if my small RNA may not have 5' phosphate and 3' OH groups?*

A. Please contact NEB Techsupport for an end repair protocol using T4 PNK.

Q: *Can I use enriched small RNA instead of total RNA for the NEBNext Small RNA Library Prep for Illumina?*

A. Yes, enriched small RNA can be used. If using closer to 10 ng of enriched small RNA, follow the adaptor dilution recommendations for 100 ng total RNA. If using closer to 100 ng enriched small RNA, follow the adaptor dilution recommendations for 1 µg of total RNA.

## Checklist:

### 1. Ligate the 3' SR Adaptor

- 1.0. Adaptor Dilution if < 100 ng total RNA input 1:2
- 1.1. Add Reagents to 1-6  $\mu$ l sample:
  - 1  $\mu$ l SR Adaptor
  - X  $\mu$ l nuclease free water
- 1.2. Mix and incubate at 70°C for 2 min. Transfer to ice.
- 1.3. Add Reagents
  - 10  $\mu$ l 3' Ligation Reaction Buffer (2X)
  - 3  $\mu$ l 3' Ligation Enzyme Mix
- 1.4. Mix and incubate at 25°C for 1 hour

### 2. Hybridize the Reverse Transcription Primer

- 2.0. Dilute adaptor if necessary
- 2.1. Add reagents to sample:
  - 4.5  $\mu$ l water
  - 1  $\mu$ l SR RT Primer
- 2.2. Mix and incubate 75°C for 5 min, 37°C for 15 min, 25°C for 15 min.

### 3. Ligate the 5' SR Adaptor

- 3.1. Resuspend 5' SR adaptor in 120  $\mu$ l nuclease free water; dilute?
- 3.2. Aliquot.
- 3.3. Denature one aliquot 70°C for 2 min., then immediately put on ice
- 3.4. Add Reagents to Sample
  - 1  $\mu$ l 5' SR adaptor
  - 1  $\mu$ l 5' Ligation Reaction Buffer (10X)
  - 2.5  $\mu$ l 5' Ligation Enzyme
- 3.5. Mix and incubate at 25°C for 1hr.

#### **4. Perform Reverse Transcription**

- [ \_ ] 4.1. Add Reagents to Sample
  - [ \_ ] 8  $\mu$ l First Strand Synthesis Reaction Buffer
  - [ \_ ] 1  $\mu$ l Murine RNase Inhibitor
  - [ \_ ] 1  $\mu$ l ProtoScript II Reverse Transcriptase
- [ \_ ] 4.2. Mix and incubate at 50°C for 1 hour.
- [ \_ ] 4.3. Immediately proceed to PCR or heat inactivate at 70°C for 15 min

#### **5. Perform PCR Amplification**

- [ \_ ] 5.1. Add Reagents to Sample
  - [ \_ ] 50  $\mu$ l LongAmp *Taq* 2X Master Mix
  - [ \_ ] 2.5  $\mu$ l SR primer for Illumina
  - [ \_ ] 2.5  $\mu$ l Index Primer
  - [ \_ ] 5  $\mu$ l Nuclease-free water
- [ \_ ] 5.2. Mix and thermal cycle (94°C 30 Sec, 12-15 cycles of 94°C 15 sec, 62°C 30 sec, 70°C 15 sec; 70°C for 5 min, 4°C hold)

#### **6. Quality Control Check and Size Selection**

##### **6A. QC Check and Size Selection using 6% Poly Acrylamide Gel**

- [ \_ ] 6A.1 Purify the PCR using Monarch PCR & DNA Cleanup Kit
- [ \_ ] 6A.2 Elute in 27.5  $\mu$ l Nuclease-free Water
- [ \_ ] 6A.3 Load 1  $\mu$ l on a Bioanalyzer DNA 1000 Chip
- [ \_ ] 6A.4 Vortex Gel Loading Dye well and mix 25  $\mu$ l PCR product with 5  $\mu$ l Gel Loading Dye
- [ \_ ] 6A.5 Load 5  $\mu$ l Quick-Load pBR322 DNA-MspI on one well
- [ \_ ] 6A.6 Load two wells with each sample
- [ \_ ] 6A.7 Run 1 hour 120V
- [ \_ ] 6A.8 Stain with SYBR Gold 2-3 min and view
- [ \_ ] 6A.9 Cut out appropriate bands
- [ \_ ] 6A.10 Place gel in 1.5 ml tubes and soak in 250  $\mu$ l gel elution buffer
- [ \_ ] 6A.11 Rotate for at least 2 hours at RT

- 6A.12. Transfer eluate and gel to filter column
- 6A.13. Spin 2 min > 13,200 rpm
- 6A.14. Recover eluate, add
  - 1  $\mu$ l linear acrylamide
  - 25  $\mu$ l 3 M Sodium Acetate pH 5.5
  - 750  $\mu$ l 100% ethanol
- 6A.15. Vortex
- 6A.16. Precipitate for > 30 min
- 6A.17. Spin > 14,000 x g for 30 min at 4°C
- 6A.18. Remove supernatant
- 6A.19. Add 80% ethanol and vortex
- 6A.20. Spin > 14,000 x g for 30 min at 4°C
- 6A.21. Air dry pellet 10 min
- 6A.22. Resuspend pellet in 12  $\mu$ l TE buffer
- 6A.23. Load 1  $\mu$ l on Bioanalyzer
- 6A.24. Check size, concentration and purity

## **6B. QC Check and Size Selection Using Pippin Prep**

- 6B.1. Purify the PCR using Monarch PCR and DNA Cleanup Kit
- 6B.2. Elute in 32  $\mu$ l Nuclease-free Water
- 6B.3. Load 1  $\mu$ l on a Bioanalyzer DNA 1000 Chip
- 6B.4. Go to protocol editor tab
- 6B.5. Click cassette folder and select 3% DF marker P
- 6B.6. Select collection mode as "Range"; BP Start (105) and BP End (155), range flag broad
- 6B.7. Use internal standards
- 6B.8. Check ref lane values match lane numbers
- 6B.9. Save protocol
- 6B.10. Warm loading solution to RT
- 6B.11. Combine 30  $\mu$ l sample and 10  $\mu$ l DNA Marker P



- [ \_ ] 6B.12. Vortex and quick spin
- [ \_ ] 6B.13. Load 40  $\mu$ l sample in agarose cassette
- [ \_ ] 6B.14. Run program
- [ \_ ] 6B.15. Collect sample from elution well and load 1  $\mu$ l on Bioanalyzer High Sensitivity Chip

**6C. QC Check and Size Selection using AMPure XP or SPRIselect Beads**

- [ \_ ] 6C.1. Purify the PCR using Monarch PCR & DNA Cleanup Kit
- [ \_ ] 6C.2. Elute in 27.5  $\mu$ l Nuclease-free Water
- [ \_ ] 6C.3. Load 1  $\mu$ l on a Bioanalyzer DNA 1000 Chip
- [ \_ ] 6C.4. Add 32.5  $\mu$ l of beads to 25  $\mu$ l sample and mix by pipetting 10 times
- [ \_ ] 6C.5. Incubate 5 min
- [ \_ ] 6C.6. Place tubes on magnet, separate, and transfer supernatant to a new tube (keep supernatant!)
- [ \_ ] 6C.7. Add 92.5  $\mu$ l of beads to the supernatant and mix by pipetting 10 times. Incubate 5 min
- [ \_ ] 6C.8. Place tubes on magnet. Wait 5 min then remove the supernatant (keep the beads)
- [ \_ ] 6C.9. On magnet add 200  $\mu$ l 80% ethanol, wait 30 seconds and remove
- [ \_ ] 6C.10. Repeat Step 6C.9 once
- [ \_ ] 6C.11. Briefly spin tube and return to magnet
- [ \_ ] 6C.12. Remove residual ethanol, air dry beads, do not overdry
- [ \_ ] 6C.13. Off magnet add 15  $\mu$ l Nuclease-free Water; mix by pipetting 10 times. Incubate 2 min; place tubes on magnet. Wait 5 min
- [ \_ ] 6C.14. Transfer supernatant to a new tube
- [ \_ ] 6C.15. Run 1  $\mu$ l on a Bioanalyzer High Sensitivity Chip

# NEBNext 3' Ligation Reaction Buffer

#E7301A: 0.24 ml

Concentration: 2X

#E7301AA: 0.96 ml

Store at -20°C

## Quality Control Assays

**16-Hour Incubation:** 50 µl reactions containing 3' Ligation Reaction Buffer at 1X concentration and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50 µl reaction containing 1X 3' Ligation Reaction Buffer with 1 µg of φX174 RF I DNA in assay buffer for 4 hours at 37°C results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**Phosphatase Activity:** Incubation of 1X 3' Ligation Reaction Buffer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**RNase Activity:** Incubation of a 10 µl reaction of 3' Ligation Reaction Buffer at 1X concentration with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

**Lot Controlled**

# NEBNext 3' Ligation Enzyme Mix

#E7288A: 0.072 ml

#E7288AA: 0.288 ml

**Store at -20°C**

**Description:** NEBNext 3' Ligation Enzyme Mix has been optimized to ligate short single-stranded RNAs (17–30 nucleotide length) to a 5'-adenylated, 3'-blocked single-stranded DNA adaptor in 1X NEBNext 3' Ligation Reaction Buffer at 25°C.

## Quality Control Assays

**SDS-PAGE Purity:** SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

**16-Hour Incubation:** 50 µl reactions containing 1 µl of 3' Ligation Enzyme Mix and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1 µl of 3' Ligation Enzyme Mix and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50 µl reaction containing 1 µl 3' Ligation Enzyme Mix with 1 µg of φX174 RF I DNA for 4 hours at 37°C results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**Phosphatase Activity:** Incubation of a minimum of 1 µl 3' Ligation Enzyme Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**RNase Activity:** Incubation of a 10 µl reaction containing 1 µl 3' Ligation Enzyme Mix with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase Activity as determined by polyacrylamide gel electrophoresis.

**Functional Activity:** 200 units of T4 RNA Ligase2, truncated, ligates 80% of a 31-mer RNA to the pre-adenylated end of a 17-mer DNA [Universal miRNA Cloning Linker (NEB #S1315)] in a total reaction volume of 10 µl in 1 hour at 25°C. Unit assay conditions: 1X Reaction Buffer (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, pH 7.5 @ 25°C) supplemented to 10% (w/v) PEG MW 4000, 5 pmol of 5'-FAM labeled RNA, and 10 pmol preadenylated DNA linker. After incubation at 25°C for 1 hour, the ligated product is detected on a 15% denaturing polyacrylamide gel.

One unit of Murine RNase Inhibitor inhibits the activity of the 5 ng of RNase A by 50%. Activity is measured by the inhibition of hydrolysis of cytidine 2, 3' - cyclic monophosphate by RNase A.

**Lot Controlled**

# NEBNext 3' SR Adaptor for Illumina

#E7332A: 0.024 ml

#E7332AA: 0.096 ml

**Store at -20°C**

**Description:** 5' adenylated, 3' blocked oligodeoxynucleotide

**Sequence:** 5'-rAppAGATCGGAAGAGCACACGTCT-NH<sub>2</sub>-3'

## Quality Control Assays

**16-Hour Incubation:** 50 µl reactions containing 1 µl NEBNext 3' SR Adaptor and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1 µl NEBNext 3' SR Adaptor and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50 µl reaction containing 1 µl NEBNext 3' SR Adaptor with 1 µg of φX174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10 µl reaction containing 1 µl NEBNext 3' SR Adaptor with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 1 µl NEBNext 3' SR Adaptor in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**

# NEBNext 5' SR Adaptor for Illumina

#E7328A: 1350 pmol

Store at -20°C

**Sequence:** 5'- rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrC-3'

## Quality Control Assays

**16-Hour Incubation:** 50 µl reactions containing 1 µl NEBNext 5' SR Adaptor and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1 µl NEBNext 5' SR Adaptor and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50 µl reaction containing 1 µl NEBNext 5' SR Adaptor with 1 µg of φX174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10 µl reaction containing 1 µl NEBNext 5' SR Adaptor with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 1 µl NEBNext 5' SR Adaptor in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**

## NEBNext 5' Ligation Reaction Buffer

#E7304A: 0.024 ml

#E7304AA: 0.096 ml

Store at -20°C

### Quality Control Assays

**16-Hour Incubation:** 50 µl reactions containing 1X 5' Ligation Reaction Buffer and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1X 5' Ligation Reaction Buffer and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50 µl reaction containing 1X 5' Ligation Reaction Buffer with 1 µg of φX174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**Phosphatase Activity:** Incubation of 1X 5' Ligation Reaction Buffer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**RNase Activity:** Incubation of a 10 µl reaction containing 1X 5' Ligation Reaction Buffer with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by polyacrylamide gel electrophoresis.

**Lot Controlled**

## NEBNext 5' Ligation Enzyme Mix

#E7305A: 0.06 ml

#E7305AA: 0.24 ml

**Store at -20°C**

**Description:** NEBNext 5' Ligation Enzyme Mix has been optimized to ligate short single-stranded RNAs (17–30 nucleotide length) in 1X NEBNext 5' Ligation Reaction Buffer at 25°C.

### Quality Control Assays

**SDS-PAGE Purity:** SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

**16-Hour Incubation:** 50 µl reactions containing 1 µl of 5' Ligation Enzyme Mix and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1 µl of 5' Ligation Enzyme Mix and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50 µl reaction containing 1 µl 5' Ligation Enzyme Mix with 1 µg of φX174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**Phosphatase Activity:** Incubation of a minimum of 1 µl 5' Ligation Enzyme Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Functional Activity:** One unit of T4 RNA Ligase 1 is defined as the amount of enzyme required to convert 1 nmol of 5'-[<sup>32</sup>P] rA16 into a phosphatase-resistant form in 30 minutes at 37°C.

One unit of Murine Inhibitor inhibits the activity of 5 ng of RNase A by 50%. Activity is measured by the inhibition of hydrolysis of cytidine 2, 3'-cyclic monophosphate by RNase A.

**Lot Controlled**

## NEBNext SR RT Primer for Illumina

#E7333A: 0.024 ml

#E7333AA: 0.096 ml

Store at -20°C

**Sequence:** 5'-AGACGTGTGCTCTTCCGATCT-3'

### Quality Control Assays

**16-Hour Incubation:** 50  $\mu$ l reactions containing 1  $\mu$ l NEBNext SR RT Primer and 1  $\mu$ g of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu$ l reactions containing NEBNext SR RT Primer and 1  $\mu$ g of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50  $\mu$ l reaction containing 1  $\mu$ l NEBNext SR RT Primer with 1  $\mu$ g of  $\phi$ X174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu$ l reaction containing 1  $\mu$ l NEBNext SR RT Primer with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 1  $\mu$ l NEBNext SR RT Primer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM  $MgCl_2$ ) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**



# NEBNext First Strand Synthesis Reaction Buffer

#E7334A: 0.192 ml

Concentration: 5X

#E7334AA: 0.768 ml

Store at -20°C

## Quality Control Assays

**16-Hour Incubation:** 50 µl reactions containing 1X First Strand Synthesis Reaction Buffer and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1X First Strand Synthesis Reaction Buffer and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 10 µl reaction containing 1X First Strand Synthesis Reaction Buffer with 1 µg of φX174 RF I supercoiled DNA for 4 hours at 37°C results in < 10% conversion to RF II (nicked molecules) as determined by gel electrophoresis.

**RNase Activity:** Incubation of a 10 µl reaction containing 1X First Strand Synthesis Reaction Buffer with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 1X First Strand Synthesis Reaction Buffer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**

# ProtoScript II Reverse Transcriptase

#E7355A: 0.024 ml

Concentration: 200,000 U/ml

#E7355AA: 0.096 ml

**Store at -20°C**

**Description:** ProtoScript II Reverse Transcriptase is a recombinant M-MuLV reverse transcriptase with reduced RNase H activity and increased thermostability. It can be used to synthesize first strand cDNA at higher temperatures than the wild type M-MuLV. The enzyme is active up to 50°C, providing higher specificity, higher yield of cDNA and more full-length cDNA product up to 12 kb.

**Source:** The gene encoding a mutant M-MuLV Reverse Transcriptase (RNase H<sup>-</sup>) is expressed in *E. coli* and purified to near homogeneity.

**Supplied in:** 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% (v/v) IGEPAL® CA-630, 50% (v/v) glycerol

## Quality Control Assays

**16-Hour Incubation:** A 50 µl reaction containing 1 µg of φX174 DNA and 100 units of ProtoScript II Reverse Transcriptase incubated for 16 hours at 37°C resulted in a DNA pattern free of detectable nuclease degradation as determined by agarose gel electrophoresis.

**Exonuclease Activity:** Incubation of a 50 µl reaction containing 100 units of ProtoScript II Reverse Transcriptase with 1 µg of a mixture of single and double-stranded [<sup>3</sup>H] *E. coli* DNA (10<sup>5</sup> cpm/µg) for 4 hours at 37°C released < 0.2% of the total radioactivity.

**RNase Activity:** Incubation of a 10 µl reaction containing 100 units of ProtoScript II Reverse Transcriptase with 40 ng of RNA transcripts for 2 hours at 37°C resulted in no detectable degradation of the RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 100 units of ProtoScript II Reverse Transcriptase in protein phosphatase assay buffer containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Protein Purity (SDS-PAGE):** ProtoScript II Reverse Transcriptase is > 95% pure as determined by SDS PAGE analysis using Coomassie blue detection.

**Lot Controlled**

# Murine RNase Inhibitor

#E7308A: 0.024 ml

#E7308AA: 0.096 ml

Store at -20°C

**Description:** Murine RNase inhibitor is a 50 kDa recombinant protein of murine origin. The inhibitor specifically inhibits RNases A, B and C. It inhibits RNases by binding noncovalently in a 1:1 ratio with high affinity. It is not effective against RNase 1, RNase T1, S1 Nuclease, RNase H or RNase from *Aspergillus*. In addition, no inhibition of polymerase activity is observed when RNase Inhibitor is used with *Taq* DNA Polymerase, AMV or M-MuLV Reverse Transcriptases, or Phage RNA Polymerases (SP6, T7, or T3).

Recombinant murine RNase inhibitor does not contain the pair of cysteines identified in the human version that is very sensitive to oxidation, which causes inactivation of the inhibitor (1). As a result, Murine RNase Inhibitor has significantly improved resistance to oxidation compared to the human/porcine RNase inhibitors, even under conditions where the DTT concentration is low. Therefore, it is advantageous to use murine RNase inhibitor in reactions where high concentration DTT is adverse to the reaction (eg. Real-time RT-PCR).

**Source:** An *E. coli* strain that carries the ribonuclease inhibitor gene from mouse

**Supplied in:** 20 mM HEPES-KOH, 50 mM KCl, 8 mM DTT and 50% glycerol

## Quality Control Assays

**16-Hour Incubation:** 50  $\mu$ l reactions containing a minimum of 40 units of Murine RNase Inhibitor and 1  $\mu$ g of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu$ l reactions containing a minimum of 40 units of Murine RNase Inhibitor and 1  $\mu$ g of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Exonuclease Activity:** Incubation of a 50  $\mu$ l reaction containing 200 units of Murine RNase Inhibitor with 1  $\mu$ g of a mixture of single and double-stranded [<sup>3</sup>H] *E. coli* DNA (20<sup>5</sup> cpm/ $\mu$ g) for 4 hours at 37°C released < 0.5% of the total radioactivity.

**Latent RNase Assay:** Heating the Murine RNase Inhibitor for 20 minutes at 65°C, followed by incubation of a 10  $\mu$ l reaction containing 40 units of RNase Inhibitor with 40 ng of RNA transcript for 4 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu$ l reaction containing 40 units of Murine RNase Inhibitor with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Endonuclease Activity:** Incubation of a 10  $\mu$ l reaction containing 40 units of Murine RNase Inhibitor with 1  $\mu$ g of  $\phi$ X174 RF I supercoiled DNA for 4 hours at 37°C results in < 10% conversion to RF II (nicked molecules) as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of a minimum of 40 units of Murine RNase Inhibitor in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Functional Activity:** One unit of Murine RNase Inhibitor inhibits the activity of 5 ng of RNase A by 50%. Activity is measured by the inhibition of hydrolysis of cytidine 2, 3'-cyclic monophosphate by RNase A.

**Lot Controlled**

**References:**

1. Kim, B.M. et al. (1999). *Protein Science*, 8, 430-434.

# LongAmp *Taq* 2X Master Mix

#E7309A: 1.2 ml

Concentration: 2X

#E7309AA: 4.8 ml

Store at -20°C

## Quality Control Assays

**SDS-PAGE Purity:** SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

**16-Hour Incubation:** 50 µl reactions containing 1 µl of LongAmp *Taq* 2X Master Mix and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1 µl of LongAmp *Taq* 2X Master Mix and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a minimum of 10 µl of LongAmp *Taq* 2X Master Mix with 1 µg of φX174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10 µl reaction containing 1 µl of LongAmp *Taq* 2X Master Mix with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by polyacrylamide gel electrophoresis.

**Phosphatase Activity:** Incubation of a minimum of 10 µl of LongAmp *Taq* 2X Master Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Functional Activity:** One unit of LongAmp *Taq* DNA Polymerase is defined as the amount of enzyme that will incorporate 10 nmol of dNTP into acid-insoluble material in 1X ThermoPol Reaction Buffer, 200 µM dNTPs including [<sup>3</sup>H]-dTTP and 200 µg/ml activated Calf Thymus DNA; in 30 minutes at 65°C.

**Lot Controlled**

# NEBNext SR Primer for Illumina

#E7310A: 0.060 ml

#E7310AA: 0.240 ml

Store at  $-20^{\circ}\text{C}$

**Sequence:** 5'-AATGATACGGCGACCACCGAGATCTACAGTTCAGAGTTCTACAGTCCG-s-A-3'

Where -s- indicates phosphorothioate bond.

## Quality Control Assays

**16-Hour Incubation:** 50  $\mu\text{l}$  reactions containing 1  $\mu\text{l}$  NEBNext SR Primer and 1  $\mu\text{g}$  of HindIII digested Lambda DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu\text{l}$  reactions containing NEBNext SR Primer for Illumina and 1  $\mu\text{g}$  of T3 DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50  $\mu\text{l}$  reaction containing 1  $\mu\text{l}$  NEBNext SR Primer with 1  $\mu\text{g}$  of  $\phi\text{X174}$  RF I supercoiled DNA for 4 hours at  $37^{\circ}\text{C}$  results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu\text{l}$  reaction containing 1  $\mu\text{l}$  NEBNext SR Primer with 40 ng of RNA transcript for 16 hours at  $37^{\circ}\text{C}$  resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 1  $\mu\text{l}$  NEBNext SR Primer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM  $\text{MgCl}_2$ ) containing 2.5 mM *p*-nitrophenyl phosphate at  $37^{\circ}\text{C}$  for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

## Gel Loading Dye, Blue

#E6138A: 0.2 ml

Concentration: 6X

#E6138AA: 1 ml

Store at 25°C

**Description:** Gel Loading Dye, Blue (6X) is a pre-mixed loading buffer with a tracking dye for agarose and non-denaturing polyacrylamide gel electrophoresis. This solution contains SDS, which often results in sharper bands, as some enzymes are known to remain bound to their DNA substrates following cleavage. EDTA is also included to chelate magnesium (up to 10 mM) in enzymatic reactions, thereby stopping the reaction. Bromophenol Blue migrates at approximately 300 bp on a standard 1% TBE agarose gel.

### Quality Control Assays

**16-Hour Incubation:** 50  $\mu$ l reactions containing 1X Gel Loading Dye and 1  $\mu$ g of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu$ l reactions containing 1X Gel Loading Dye and 1  $\mu$ g of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50  $\mu$ l reaction containing 1X Gel Loading Dye with 1  $\mu$ g of  $\phi$ X174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu$ l reaction containing 1X Gel Loading Dye with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 1X Gel Loading Dye in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM  $MgCl_2$ ) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**

## Quick-Load pBR322 MspI-DNA Digest

#E7323A: 0.24 ml

Concentration: 50 µg/ml

#E7323AA: 0.96 ml

Store at -20°C or 4°C

**Description:** Quick-Load pBR322 DNA-MspI Digest is a pre-mixed, ready to load molecular weight marker containing Bromophenol Blue as a tracking dye. The MspI Digest of pBR322 DNA yields 26 fragments. The double-stranded DNA is digested to completion with MspI, phenol extracted, and equilibrated to 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

Fragment	Size (bp)
1	622
2	527
3	404
4	307
5	242
6	238
7	217
8	201
9	190
10	180
11,12	160
13,14	147
15	123
16	110
17	90
18	76
19	67
20,21	34
22,23	26
24	15
25,26	9

## Quick-Load pBR322 MspI-DNA Digest (Cont.)

### Quality Control Assays

**16-Hour Incubation:** 50  $\mu$ l reactions containing 1  $\mu$ l Quick-Load pBR322 MspI DNA Digest and 1  $\mu$ g of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu$ l reactions containing 1  $\mu$ l Quick-Load pBR322 MspI DNA Digest and 1  $\mu$ g of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50  $\mu$ l reaction containing 1  $\mu$ l Quick-Load pBR322 MspI DNA Digest with 1  $\mu$ g of  $\phi$ X174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu$ l reaction containing 1  $\mu$ l Quick-Load pBR322 MspI DNA Digest with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of Quick-Load pBR322 MspI DNA Digest in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**



## DNA Gel Elution Buffer

#E7324A: 12 ml

Concentration: 1X

#E7324AA: 48 ml

Store at  $-20^{\circ}\text{C}$  or  $4^{\circ}\text{C}$

**Description:** DNA Gel Elution Buffer is provided for the extraction of the size selected amplified cDNA library from the polyacrylamide gel.

### Quality Control Assays

**16-Hour Incubation:** 50  $\mu\text{l}$  reactions containing 10  $\mu\text{l}$  DNA Gel Elution Buffer and 1  $\mu\text{g}$  of HindIII digested Lambda DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu\text{l}$  reactions containing 10  $\mu\text{l}$  DNA Gel Elution Buffer and 1  $\mu\text{g}$  of T3 DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50  $\mu\text{l}$  reaction containing 10  $\mu\text{l}$  DNA Gel Elution Buffer with 1  $\mu\text{g}$  of  $\phi\text{X174}$  RF I supercoiled DNA for 4 hours at  $37^{\circ}\text{C}$  results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu\text{l}$  reaction containing 1  $\mu\text{l}$  DNA Gel Elution Buffer with 40 ng of RNA transcript for 16 hours at  $37^{\circ}\text{C}$  resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of DNA Gel Elution Buffer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM  $\text{MgCl}_2$ ) containing 2.5 mM *p*-nitrophenyl phosphate at  $37^{\circ}\text{C}$  for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**

# Linear Acrylamide

#E7325A: 0.048 ml

Concentration: 10 mg/ml

#E7325AA: 0.192 ml

Store at  $-20^{\circ}\text{C}$  or  $4^{\circ}\text{C}$

## Quality Control Assays

**16-Hour Incubation:** 50  $\mu\text{l}$  reactions containing 1  $\mu\text{g}$  Linear Acrylamide and 1  $\mu\text{g}$  of HindIII digested Lambda DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu\text{l}$  reactions containing 1  $\mu\text{g}$  Linear Acrylamide and 1  $\mu\text{g}$  of T3 DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50  $\mu\text{l}$  reaction containing 1  $\mu\text{g}$  Linear Acrylamide with 1  $\mu\text{g}$  of  $\phi\text{X174}$  RF I supercoiled DNA for 4 hours at  $37^{\circ}\text{C}$  results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu\text{l}$  reaction containing 1  $\mu\text{g}$  Linear Acrylamide with 40 ng of RNA transcript for 16 hours at  $37^{\circ}\text{C}$  resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 1  $\mu\text{g}$  Linear Acrylamide in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM  $\text{MgCl}_2$ ) containing 2.5 mM *p*-nitrophenyl phosphate at  $37^{\circ}\text{C}$  for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**

## TE Buffer

#E7326A: 0.48 ml

#E7326AA: 1.92 ml

Store at  $-20^{\circ}\text{C}$  or  $4^{\circ}\text{C}$

### Quality Control Assays

**16-Hour Incubation:** 50  $\mu\text{l}$  reactions containing 10  $\mu\text{l}$  TE Buffer and 1  $\mu\text{g}$  of HindIII digested Lambda DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu\text{l}$  reactions containing 10  $\mu\text{l}$  TE Buffer and 1  $\mu\text{g}$  of T3 DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50  $\mu\text{l}$  reaction containing 10  $\mu\text{l}$  TE Buffer with 1  $\mu\text{g}$  of  $\phi\text{X174}$  RF I supercoiled DNA for 4 hours at  $37^{\circ}\text{C}$  results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu\text{l}$  reaction containing 1  $\mu\text{l}$  TE Buffer with 40 ng of RNA transcript for 16 hours at  $37^{\circ}\text{C}$  resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of TE Buffer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM  $\text{MgCl}_2$ ) containing 2.5 mM *p*-nitrophenyl phosphate at  $37^{\circ}\text{C}$  for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

**Lot Controlled**

## Nuclease-free Water

#E7327A: 5.0 ml

#E7327AA: 20.0 ml

Store at  $-20^{\circ}\text{C}$  or  $4^{\circ}\text{C}$

**Description:** Nuclease-free Water is free of detectable DNA and RNA nucleases and phosphatases and is suitable for use in DNA and RNA applications.

### Quality Control Assays

**16-Hour Incubation:** 50  $\mu\text{l}$  reactions containing Nuclease-free Water and 1  $\mu\text{g}$  of HindIII digested Lambda DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu\text{l}$  reactions containing Nuclease-free Water and 1  $\mu\text{g}$  of T3 DNA incubated for 16 hours at  $37^{\circ}\text{C}$  results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 10  $\mu\text{l}$  reaction containing Nuclease-free Water with 1  $\mu\text{g}$  of  $\phi\text{X174}$  RF I supercoiled DNA for 4 hours at  $37^{\circ}\text{C}$  produced no nicked molecules as determined by gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu\text{l}$  reaction containing Nuclease-free Water with 40 ng of RNA transcript for 16 hours at  $37^{\circ}\text{C}$  resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of Nuclease-free Water in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM  $\text{MgCl}_2$ ) containing 2.5 mM *p*-nitrophenyl phosphate at  $37^{\circ}\text{C}$  for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

## NEBNext Index 1–12 Primers for Illumina

**Description:** 12 Index Primers are included for producing barcoded libraries.

**Concentration:** 10  $\mu$ M

#E7311A: 0.010 ml #E7311AA: 0.040 ml	NEBNext Index 1 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGATC-GTGATGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7312A: 0.010 ml #E7312AA: 0.040 ml	NEBNext Index 2 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGATC-CATCGGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7313A: 0.010 ml #E7313AA: 0.040 ml	NEBNext Index 3 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGATC-GCCTAAGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7314A: 0.010 ml #E7314AA: 0.040 ml	NEBNext Index 4 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGATTG-GTCAGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7315A: 0.010 ml #E7315AA: 0.040 ml	NEBNext Index 5 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGATC-CACTGTGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7316A: 0.010 ml #E7316AA: 0.040 ml	NEBNext Index 6 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGAT-TATTGGCGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7317A: 0.010 ml #E7317AA: 0.040 ml	NEBNext Index 7 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGAT-GATCTGGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7318A: 0.010 ml #E7318AA: 0.040 ml	NEBNext Index 8 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGAT-TTCAAGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7319A: 0.010 ml #E7319AA: 0.040 ml	NEBNext Index 9 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGAT-TCTGATCGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7320A: 0.010 ml #E7320AA: 0.040 ml	NEBNext Index 10 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGAT-AAGCTAGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7321A: 0.010 ml #E7321AA: 0.040 ml	NEBNext Index 11 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGAT-GTAGCCGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'
#E7322A: 0.010 ml #E7322AA: 0.040 ml	NEBNext Index 12 Primer for Illumina	5'-CAAGCAGAAGACGGCATAACGAGAT-TACAAGGTGACTGGAGTTCAGACGTGT-GCTCTCCGATC-s-T-3'

Where -s- indicates phosphorothioate bond.

Note: If fewer than 12 indexes are used in a lane for sequencing, it is recommended to use the following combinations:

Pool of 2 Samples: Index# 6 and 12

Pool of 3 Samples: Index# 4, 6 and 12

Pool of 6 Samples: Index# 2, 4, 5, 6, 7 and 12

## NEBNext Index 1–12 Primers for Illumina (Cont.)

Table 1: Index Sequences

Index	Sequence
Index 1	ATCACG
Index 2	CGATGT
Index 3	TTAGGC
Index 4	TGACCA
Index 5	ACAGTG
Index 6	GCCAAT
Index 7	CAGATC
Index 8	ACTTGA
Index 9	GATCAG
Index 10	TAGCTT
Index 11	GGCTAC
Index 12	CTTGTA

### Quality Control Assays

**16-Hour Incubation:** 50  $\mu$ l reactions containing 1  $\mu$ l NEBNext Index [X] Primer and 1  $\mu$ g of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50  $\mu$ l reactions containing NEBNext Index [X] Primer for Illumina and 1  $\mu$ g of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

**Endonuclease Activity:** Incubation of a 50  $\mu$ l reaction containing 1  $\mu$ l NEBNext Index [X] Primer with 1  $\mu$ g of  $\phi$ X174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.

**RNase Activity:** Incubation of a 10  $\mu$ l reaction containing 1  $\mu$ l NEBNext Index [X] Primer with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

**Phosphatase Activity:** Incubation of 1  $\mu$ l NEBNext Index [X] Primer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM  $MgCl_2$ ) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

### Lot Controlled

## Revision History

REVISION #	DESCRIPTION	DATE
2.0	Replaced M-MuLV Reverse Transcriptase RNase H <sup>-</sup> with ProtoScript II Reverse Transcriptase.	
2.1	Formatted components with cap color information. Added Pippin Prep as an alternative method for size selection. Adding more recommendations on starting material and how to choose the method for size selection. Removed AMPure Bead Protocol that did not require the Qiagen column cleanup.  Provided more clarification on how to choose size selection method based on Library QC.  Added note to vortex loading dye prior to use.  Added FAQ section to the manual.	
2.2	Updated marker settings for size selection using the Pippin Prep. Marker P is replacing Marker M.	
3.0	Change catalog number for NEBNext 3' Ligation Enzyme Mix to NEB #E7288.	
4.0	Workflow diagram added. Protocol new numbering applied and text edits applied. New Checklist and FAQs applied. Note added to NEBNext Index 1-12 Primers for Illumina. In Step 2.2 added heated lid temperature. Added where it is ok to premix reagents. Added Safe Stop after PCR and after PCR cleanup. Clarification of when it's ok to use AMPure XP Size Selection. Changed Pippin Prep Cassette Type and Marker to newly released product. Replaced Qiagen Cleanup kit with Monarch Cleanup Kit.	9/17



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