NEBNext® Multiplex

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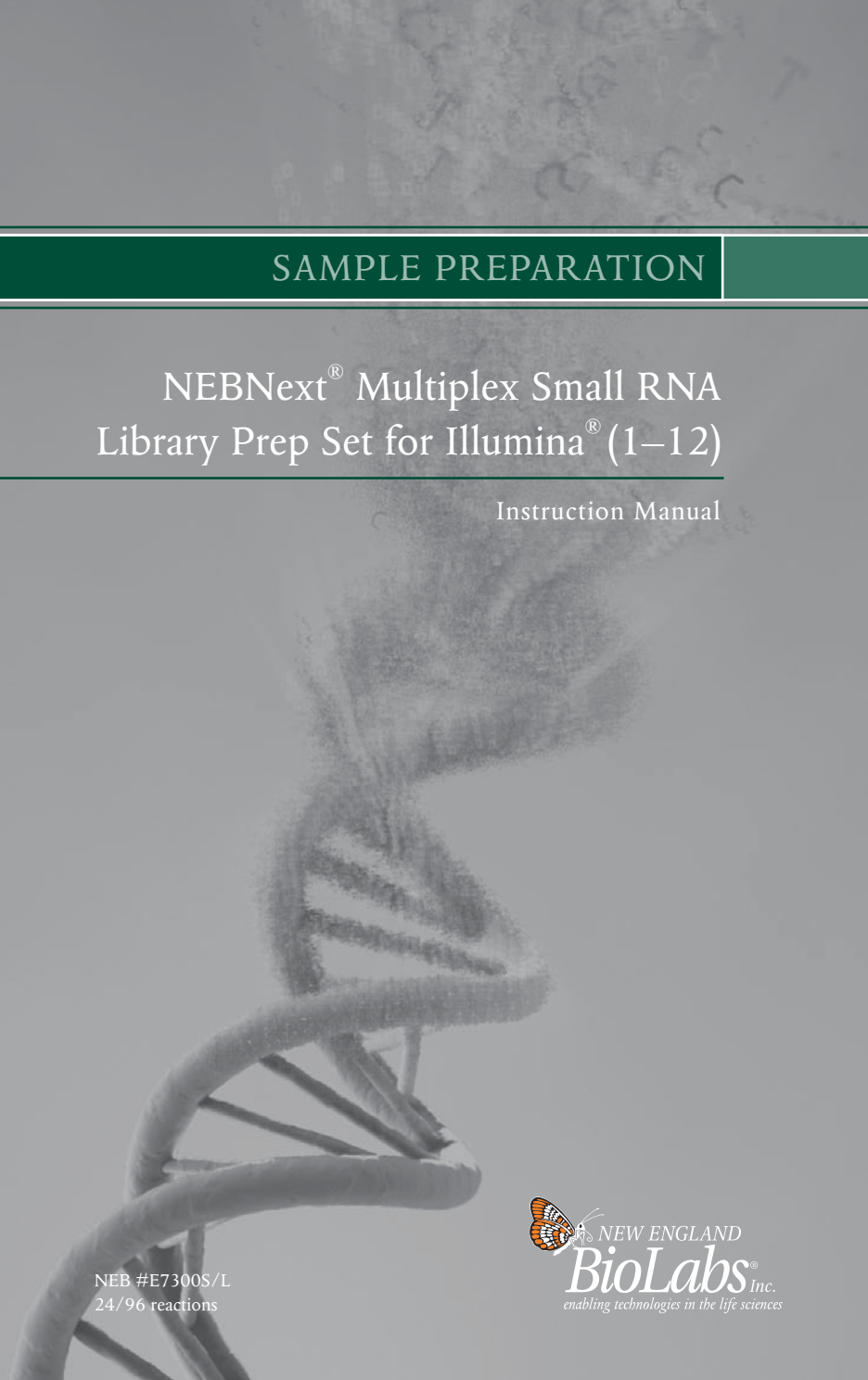
SAMPLE PREPARATION

NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (1–12)

Instruction Manual



NEB #E7300S/L
24/96 reactions

NEW ENGLAND BioLabs® Inc.
enabling technologies in the life sciences

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The NEBNext Multiplex Small RNA Library Prep Set for Illumina (1–12) Includes:

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.

NEBNext 3´ Ligation Reaction Buffer (2X)

NEBNext 3´ Ligation Enzyme Mix

NEBNext Multiplex 3´ SR Adaptor for Illumina

NEBNext Multiplex 5´ SR Adaptor for Illumina

NEBNext 5´ Ligation Reaction Buffer (10X)
NEBNext 5´ Ligation Enzyme Mix
NEBNext Multiplex SR RT Primer for Illumina
Deoxynucleotide Solution Mix (10 mM each dNTP)
Murine RNase Inhibitor
LongAmp *Taq* 2X Master Mix
NEBNext Multiplex SR Primer for Illumina
NEBNext Index 1 Primer for Illumina
NEBNext Index 2 Primer for Illumina
NEBNext Index 3 Primer for Illumina
NEBNext Index 4 Primer for Illumina
NEBNext Index 5 Primer for Illumina
NEBNext Index 6 Primer for Illumina
NEBNext Index 7 Primer for Illumina
NEBNext Index 8 Primer for Illumina
NEBNext Index 9 Primer for Illumina
NEBNext Index 10 Primer for Illumina
NEBNext Index 11 Primer for Illumina
NEBNext Index 12 Primer for Illumina
Gel Loading Dye, Blue (6X)
Quick-Load pBR322 DNA-MspI Digest
DNA Gel Elution Buffer, 1X
Linear Acrylamide (10 mg/ml)
TE Buffer
Nuclease-free Water

Required Materials Not Included:

3 M Sodium Acetate, pH 5.5
100% Ethanol
80% Ethanol
SuperScript® III Reverse Transcriptase (Life Technologies, Inc. #18080-044)
5X First Strand Buffer (Supplied with SuperScript III Reverse Transcriptase)
0.1 M DTT (Supplied with SuperScript III Reverse Transcriptase)
Corning®, Costar®, Spin-X® Centrifuge Tube Filters (Cellulose Acetate Filters) (Sigma Aldrich # CLS8162)
RNase-free Disposable Pellet Pestles® (Kimble Kontes Asset Management, Inc. #749521-1590)
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)
QIAquick PCR Purification Kit (Qiagen #28104)
Dry Ice/Methanol Bath or –80°C freezer
Bioanalyzer® (Agilent Technologies, Inc.)

Applications:

The NEBNext Multiplex Small RNA Library Prep Set for Illumina (1–12) contains adaptors, primers, enzymes and buffers that are ideally suited to convert small RNA transcripts into barcoded cDNA libraries for next-generation sequencing on the Illumina (Illumina, Inc.) platform. Each of these components must pass rigorous quality control standards and is lot controlled, both individually and as a set of reagents.

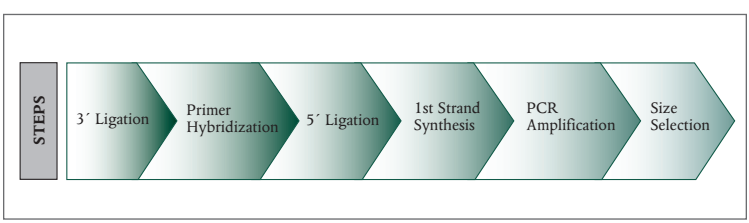
Lot Control: The lots provided in the NEBNext Multiplex Small RNA Library Prep Set for Illumina (1–12) are managed separately and are qualified by additional functional validation. Individual reagents undergo standard enzyme activity and quality control assays, and also meet stringent criteria in the additional quality controls listed on each individual component page.

Functionally Validated: Each set of reagents is functionally validated together through construction and sequencing of barcoded 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.

Multiplex Small RNA Library Prep Workflow:

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



Protocols:

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

Starting Material: 1–10 µg Total RNA. Alternatively, previously isolated small RNA from 1–10 µg Total RNA can be used as starting material.

Ligate the Multiplex 3´ SR Adaptor

Note: For total RNA inputs less than 5 µg, dilute (1:2 dilution in Nuclease-Free Water) the Multiplex 3´ SR Adaptor.

1. Mix the following components in a sterile nuclease-free PCR tube:

Input RNA	1–6 µl
Multiplex 3´ SR Adaptor	1 µl
Nuclease-Free Water	variable
Total volume	7 µl

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

3. Add the following Components:

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

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.

Hybridize the Reverse Transcription Primer

This step is important to prevent adaptor-dimer formation. The Multiplex SR RT Primer hybridizes to the excess of Multiplex 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 Multiplex 5´ SR Adaptor in the subsequent ligation step.

5. Add the following components to the ligation mixture from step 4 and mix well:

Nuclease-Free Water	4.5 µl
Multiplex SR RT Primer	1 µl
Total volume now should be	25.5 µl

   Note: For total RNA inputs less than 5 µg, dilute (1:2 dilution in Nuclease-Free Water) the Multiplex SR RT Primer.

6. Heat samples for 5 minutes at 75°C. Transfer to 37°C for 15 minutes, followed by 15 minutes at 25°C.

Ligate the Multiplex 5´ SR Adaptor

7. With 5 minutes remaining, resuspend the Multiplex 5´ SR adaptor in Nuclease-Free Water (resuspend NEB #E7328A in 60 µl Nuclease-Free Water and resuspend NEB #E7328AA in 300 µl Nuclease-Free Water).

   Note: For total RNA inputs less than 5 µg, dilute (1:2 dilution in Nuclease-Free Water) the Multiplex 5´ SR Adaptor.

8. Aliquot 1.1 N µl of the Multiplex 5´ SR Adaptor into a separate, nuclease-free 200 µl PCR tube, with N equal to the number of samples being processed for the current experiment.

9. 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 Multiplex 5´ SR adaptor at –80°C.

10. Add the following components to the ligation mixture from step 6 and mix well:

Multiplex 5´ SR Adaptor (denatured)	1 µl
5´ Ligation Reaction Buffer (10X)	1 µl
5´ Ligase Enzyme Mix	2.5 µl
Total volume	30 µl

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

Perform Reverse Transcription

12. Mix the following components in a sterile, nuclease-free tube:

Adaptor Ligated RNA from Step 11	24 µl
Deoxynucleotide Solution Mix	2 µl
5X First Strand Buffer (supplied with SuperScript III)	8 µl
0.1 M DTT (supplied with SuperScript III)	4 µl
Murine RNase Inhibitor	1 µl
SuperScript III Reverse Transcriptase	1 µl
Total volume	40 µl

13. Incubate for 60 minutes at 50°C.

14. 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.

Perform PCR Amplification

Note: For total RNA inputs less than 5 µg, dilute (1:2 dilution in Nuclease-Free Water) the Multiplex SR Primer and the Index (X) Primer.

15. Add the following components to the RT reaction mix from step 14 and mix well:

LongAmp *Taq* 2X Master Mix	50 µl
Multiplex SR Primer for Illumina	5 µl
Index (X) Primer*	5 µl
Total volume now should be	100 µl

***Note: The NEBNext Multiplex Small RNA Library Prep Set for Illumina (1–12) 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	
Annealing	62°C	30 sec	12*
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 can be adjusted if clear and distinct bands are not observed in the gel image. Run only between 12 and 15 cycles.

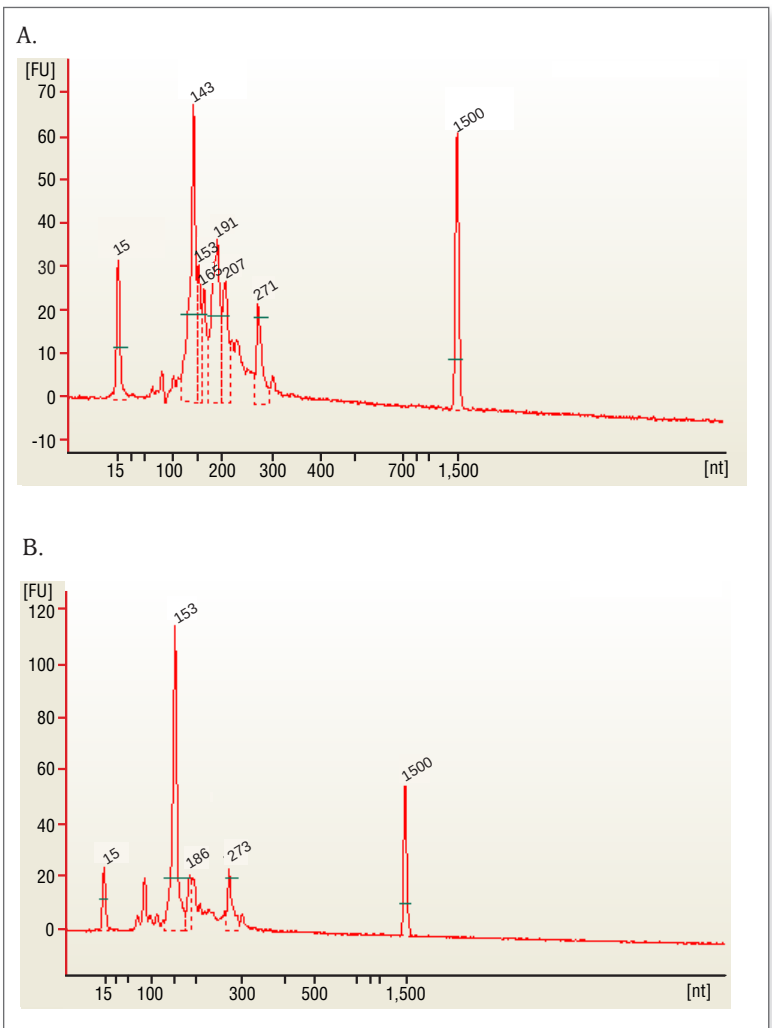
16. Purify the PCR amplified cDNA construct (100 µl) using a QIAQuick PCR Purification Kit.

IMPORTANT: Before eluting the DNA from the column, centrifuge the column with the lid of the spin column open for 5 minutes at 13,200 rpm. Centrifugation with the lid open ensures that no ethanol remains during DNA elution. It is important to dry the spin column membrane of any residual ethanol that may interfere with the correct loading of the sample on the PAGE gel.

17. Elute amplified DNA in 25 µl Nuclease-free Water.

18. Load 1 µl 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 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.

Size Select the Amplified cDNA Library

1. Mix the purified PCR product (25 µl) with 5 µl of Gel Loading Dye, Blue (6X).
2. Load 5 µl of Quick-Load pBR322 DNA-MspI Digest in one well on the 6% PAGE 10-well gel.
3. Load two wells with 15 µl each of mixed amplified cDNA construct and loading dye on the 6% PAGE 10-well gel.
4. 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.
5. 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 transiluminator (Figure 2).

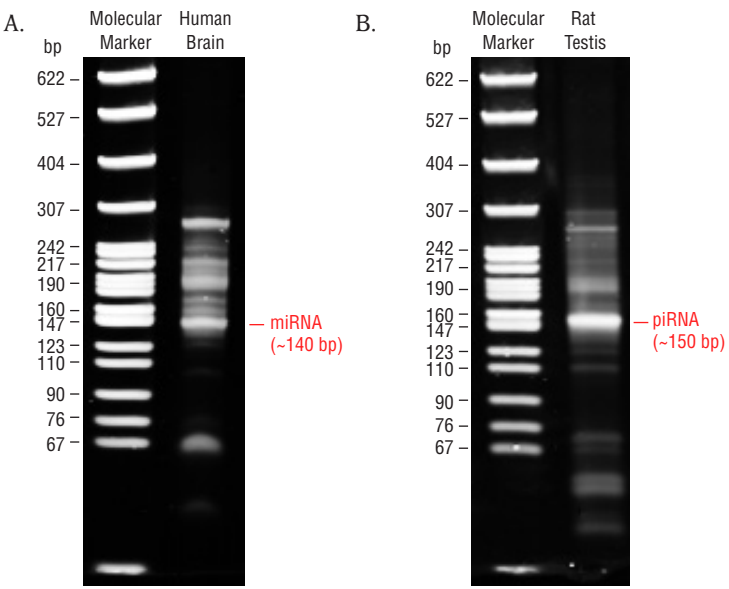


Figure 2: Shows typical results from Human Brain (A) and Rat Testis (B) Total RNA libraries. The 140 and 150 bp bands correspond to miRNAs (21 nt) and piRNAs (30 nt), respectively.

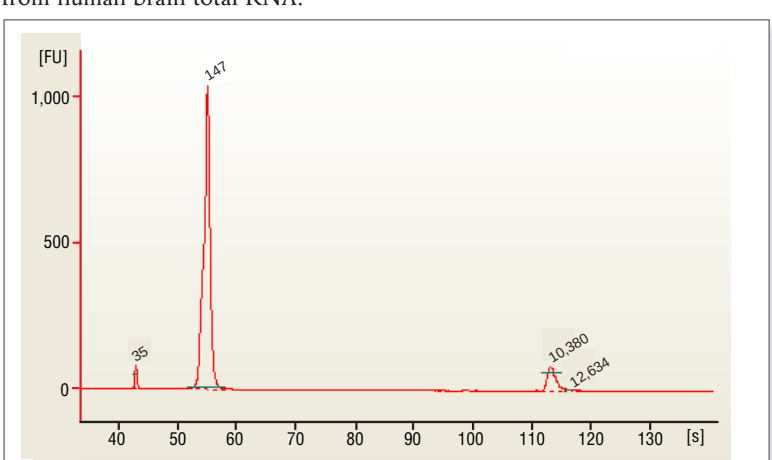
6. The 140 and 150 nucleotide bands correspond to adapter-ligated constructs derived from the 21 and 30 nucleotide RNA fragments, respectively. For miRNAs, isolate the bands corresponding to ~140 bp. For piRNAs, isolate the band corresponding to ~150 bp.
7. Place the two gel slices from the same sample 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).

8. Rotate end-to-end for at least 2 hours at room temperature.
9. Transfer the eluate and the gel debris to the top of a gel filtration column.
10. Centrifuge the filter for 2 min at > 13,200 rpm.
11. Recover eluate and add 1 µl Linear Acrylamide, 25 µl 3M sodium acetate, pH 5.5 and 750 µl of 100% ethanol.
12. Vortex well.
13. Precipitate in a dry ice/methanol bath or at –80°C for at least 30 minutes.
14. Spin in a microcentrifuge @ > 14,000 x g for 30 minutes at 4°C.
15. Remove the supernatant taking care not to disturb the pellet.
16. Wash the pellet with 80% ethanol by vortexing vigorously.
17. Spin in a microcentrifuge @ > 14,000 x g for 30 minutes at 4°C.
18. Air dry pellet for up to 10 minutes at room temperature to remove residual ethanol.
19. Resuspend pellet in 12 µl TE Buffer.

Validate the Library

1. 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 size selected purified library from human brain total RNA.



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

NEBNext 3´ Ligation Reaction Buffer

#E7301A: 0.24 ml **Concentration: 2X**
#E7301AA: 0.96 ml

Store at –20°C

1X NEBNext 3´ Ligation Reaction Buffer:
50 mM Tris-HCl
10 mM $MgCl_2$
1 mM DTT
12.5% Polyethylene glycol (PEG 8000)
pH 7.5 @ 25°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 $MgCl_2$) 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.

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

#E7302A: 0.072 ml
#E7302AA: 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.

1X NEBNext 3´ Ligation Enzyme Mix:
133,333 units/ml T4 RNA Ligase 2, truncated$_f$
13,333 units/ml Murine RNase Inhibitor

Supplied in: 10 mM Tris-HCl (pH 7.5 @ 25°C), 100 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA and 50% glycerol.

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 $MgCl_2$) 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.

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 $MgCl_2$, 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 Multiplex 3´ SR Adaptor for Illumina

#E7303A: 0.024 ml
#E7303AA: 0.096 ml

Store at –20°C

Description: 5´ adenylated, 3´ blocked oligodeoxynucleotide

Sequence: 5´-rAppAGATCGGAAGAGCACACGTCT-NH_2-3´

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µl NEBNext Multiplex 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 Multiplex 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 Multiplex 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 Multiplex 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 Multiplex 3´ SR Adaptor 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.

HPLC: NEBNext Multiplex 3´ SR Adaptor purity is determined by HPLC to be > 99%.

Lot Controlled

NEBNext Multiplex 5´ SR Adaptor for Illumina

#E7328A: 1350 pmol
#E7328AA: 6750 pmol

Store at –20°C

Sequence: 5´- rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrC-3´

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µl NEBNext Multiplex 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 Multiplex 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 Multiplex 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 Multiplex 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 Multiplex 5´ SR Adaptor 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.

HPLC: NEBNext Multiplex 5´ SR Adaptor purity is determined by HPLC to be > 99%.

Lot Controlled

NEBNext 5´ Ligation Reaction Buffer

#E7304A: 0.024 ml
#E7304AA: 0.096 ml

Store at –20°C

1X NEBNext 5´ Ligation Reaction Buffer:
50 mM Tris-HCl
10 mM $MgCl_2$
1 mM DTT
3 mM ATP
pH 7.5 @ 25°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 $MgCl_2$) 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.

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.

1X NEBNext 5´ Ligation Enzyme Mix:
2,568 units/ml T4 RNA Ligase 1
16,000 units/ml Murine RNase Inhibitor

Supplied in: 10 mM Tris-HCl (pH 7.5 @ 25°C), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA and 50% glycerol.

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 $MgCl_2$) 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.

Functional Activity: One unit of T4 RNA Ligase 1 is defined as the amount of enzyme required to convert 1 nmol of 5´-[^{32}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 Multiplex SR RT Primer for Illumina

#E7306A: 0.024 ml
#E7306AA: 0.096 ml

Store at –20°C

Sequence: 5´-AGACGTGTGCTCTTCCGATCT-3´

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µl NEBNext Multiplex SR RT Primer 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 NEBNext SR RT Primer 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 Multiplex SR RT Primer 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 Multiplex 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 µl NEBNext Multiplex 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

Deoxynucleotide Solution Mix

#E7307A: 0.048 ml 10 mM each dNTP
#E7307AA: 0.192 ml

Store at –20°C

Description: Deoxynucleotide Solution Mix is an equimolar solution of ultrapure dATP, dCTP, dGTP and dTTP, provided for the PCR enrichment reaction.

Supplied in: Milli-Q® water (Millipore Corporation) as a sodium salt @ pH 7.5.

Concentration: Each nucleotide is supplied at a concentration of 10 mM. (40 mM total nucleotide concentration).

Quality Assurance: Nucleotide solutions are certified free of nucleases and phosphatases.

Notes: Storing nucleotide triphosphates in solutions containing magnesium promotes triphosphate degradation.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing a minimum of 2 mM dNTPs 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 a minimum of 2 mM dNTPs 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.

RNase Activity: Incubation of 1 mM dNTP 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.

Phosphatase Activity: Incubation of a minimum of 5 mM dNTP 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.

HPLC: dNTP purity is determined by HPLC to be > 99%.

Functional Activity (PCR): The dNTPs are tested in 25 cycles of PCR amplification generating 0.5 kb, 2 kb, and 5kb amplicons from lambda DNA.

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 µl reactions containing a minimum of 40 units of Murine RNase Inhibitor 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 a minimum of 40 units of Murine RNase Inhibitor 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.

Exonuclease Activity: Incubation of a 50 µl reaction containing 200 units of Murine RNase Inhibitor with 1 µg of a mixture of single and double-stranded [^{3}H] *E. coli* DNA (20^5 cpm/µg) 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 µl 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 µl 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 µl reaction containing 40 units of Murine RNase Inhibitor 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.

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 $MgCl_2$) 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.

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

1X LongAmp *Taq* Master Mix:
60 mM Tris-SO_4
2 mM $MgSO_4$
0.3 mM dNTP
125 units/ml LongAmp *Taq* DNA Polymerase
20 mM ammonium sulfate
pH 9.0 @ 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 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 $MgCl_2$) 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.

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 [^{3}H]-dTTP and 200 µg/ml activated Calf Thymus DNA; in 30 minutes at 65°C.

Lot Controlled

NEBNext Multiplex SR Primer for Illumina

#E7310A: 0.12 ml
#E7310AA: 0.48 ml

Store at –20°C

Sequence: 5´-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCG-s-A-3´

Where -s- indicates phosphorothioate bond.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µl NEBNext Multiplex SR Primer 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 NEBNext Multiplex SR Primer for Illumina 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 Multiplex SR Primer 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 Multiplex SR 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 µl NEBNext Multiplex SR 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.

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 poylacrylamide 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.

1X Gel Loading Dye, Blue (6X):
2.5% Ficoll 400
11 mM EDTA
3.3 mM Tris-HCl
0.017% SDS
0.15% Bromophenol Blue
pH 8.0 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1X Gel Loading Dye 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 Gel Loading Dye 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 Gel Loading Dye with 1 µg of ϕX174 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 µl 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.

Supplied in: 3.3 mM Tris-HCl (pH 8.0 @ 25°C), 11 mM EDTA, 0.015% Bromophenol Blue, 0.017% SDS and 2.5% Ficoll 400.

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 µl reactions containing 1 µl Quick-Load pBR322 MspI DNA Digest 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 Quick-Load pBR322 MspI DNA Digest 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 Quick-Load pBR322 MspI DNA Digest with 1 µg of ϕX174 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 µl reaction containing 1 µl 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 $MgCl_2$) 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

DNA Gel Elution Buffer

#E7324A: 12 ml **Concentration: 1X**
#E7324AA: 48 ml

Store at –20°C or 4°C

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

DNA Gel Elution Buffer, 1X:
10 mM Tris-HCl
pH 8.0 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 10 µl DNA Gel Elution 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 10 µl DNA Gel Elution 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 10 µl DNA Gel Elution 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 (nicked molecules) as determined by agarose gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing 1 µl DNA Gel Elution 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 DNA Gel Elution Buffer 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

Linear Acrylamide

#E7325A: 0.048 ml **Concentration: 10 mg/ml**
#E7325AA: 0.192 ml

Store at –20°C or 4°C

1X Linear Acrylamide:
10 mg/ml Linear Acrylamide in sterile water

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µg Linear Acrylamide 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 µg Linear Acrylamide 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 µg Linear Acrylamide with 1 µg of ϕX174 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 µl reaction containing 1 µg Linear Acrylamide 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 µg Linear Acrylamide 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

TE Buffer

#E7326A: 0.48 ml
#E7326AA: 1.92 ml

Store at –20°C or 4°C

TE Buffer:
10 mM Tris-HCl
1 mM EDTA pH 8.0

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 10 µl TE 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 10 µl TE 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 10 µl TE 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 (nicked molecules) as determined by agarose gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing 1 µl TE 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 TE Buffer 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

Nuclease-free Water

#E7327A: 1.0 ml
#E7327AA: 3.0 ml

Store at –20°C or 4°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 µl reactions containing Nuclease-free Water 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 Nuclease-free Water 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 Nuclease-free Water with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C produced no nicked molecules as determined by gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing Nuclease-free Water 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 Nuclease-free Water 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.

NEBNext Index 1–12 Primers for Illumina

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

#E7311A: 0.010 ml **#E7311AA: 0.040 ml**	**NEBNext Index 1 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGATC-GTGATGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7312A: 0.010 ml **#E7312AA: 0.040 ml**	**NEBNext Index 2 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGATA-CATCGGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7313A: 0.010 ml **#E7313AA: 0.040 ml**	**NEBNext Index 3 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-GCCTAAGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7314A: 0.010 ml **#E7314AA: 0.040 ml**	**NEBNext Index 4 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGATTG-GTCAGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7315A: 0.010 ml **#E7315AA: 0.040 ml**	**NEBNext Index 5 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-CACTGTGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7316A: 0.010 ml **#E7316AA: 0.040 ml**	**NEBNext Index 6 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-TATTGGCGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7317A: 0.010 ml **#E7317AA: 0.040 ml**	**NEBNext Index 7 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-GATCTGGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7318A: 0.010 ml **#E7318AA: 0.040 ml**	**NEBNext Index 8 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-TTCAAGTGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7319A: 0.010 ml **#E7319AA: 0.040 ml**	**NEBNext Index 9 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-TCTGATCGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7320A: 0.010 ml **#E7320AA: 0.040 ml**	**NEBNext Index 10 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-AAGCTAGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7321A: 0.010 ml **#E7321AA: 0.040 ml**	**NEBNext Index 11 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-GTAGCCGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´
#E7322A: 0.010 ml **#E7322AA: 0.040 ml**	**NEBNext Index 12 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-TACAAGGTGACTGGAGTTCAGACGTGT-GCTCTTCCGATC-s-T-3´

Where -s- indicates phosphorothioate bond.

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 µl reactions containing 1 µl NEBNext Index [X] Primer 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 NEBNext Index [X] Primer for Illumina 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 Index [X] Primer with 1 µg of ϕX174 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 µl reaction containing 1 µl 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 µl 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.

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