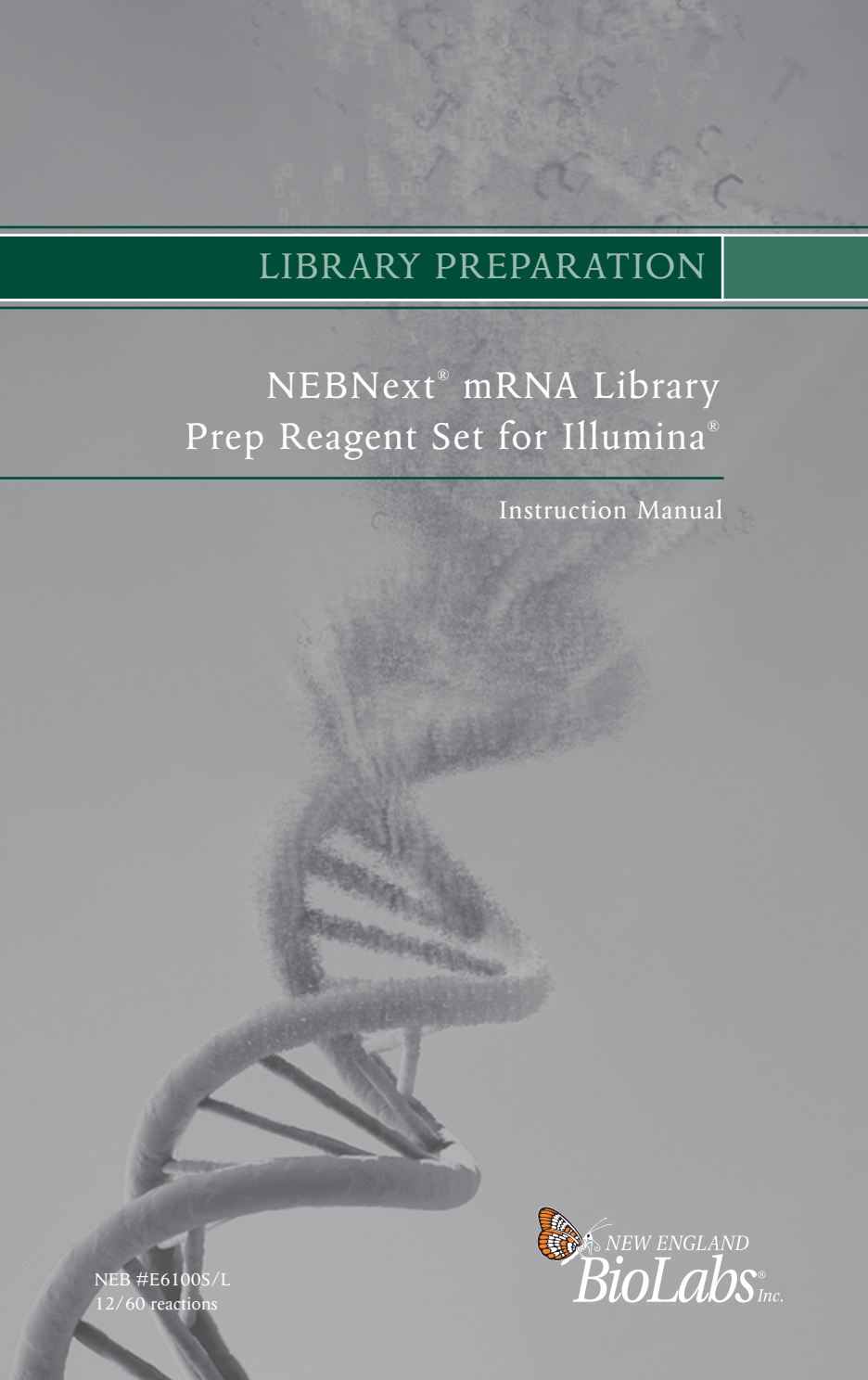
LIBRARY PREPARATION

NEBNext® mRNA Library Prep Reagent Set for Illumina®

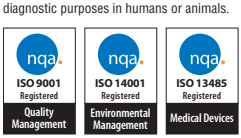
Instruction Manual



NEB #E6100S/L
12/60 reactions

NEW ENGLAND BioLabs® Inc.

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.



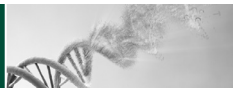


Table of Contents:

The Reagent Set Includes:

The volumes provided are sufficient for preparation of up to 12 reactions (NEB #E6100S) and 60 reactions (NEB #E6100L). (All reagents should be stored at –20°C).

- (white) NEBNext RNA Fragmentation Buffer (10X)
- (white) NEBNext RNA Fragmentation Stop Solution (10X)
- (pink) Random Primers (3 µg/µl)
- (pink) Murine RNase Inhibitor RRI
- (pink) NEBNext First Strand Synthesis Reaction Buffer (5X)
- (pink) ProtoScript II Reverse Transcriptase
- (orange) NEBNext Second Strand Synthesis Enzyme Mix
- (orange) NEBNext Second Strand Synthesis Reaction Buffer (10X)
- (green) Phosphorylation Reaction Buffer (10X)
- (green) Deoxynucleotide Solution Mix (10 mM each dNTP)
- (green) T4 DNA Polymerase RRI
- (green) DNA Polymerase I, Large (Klenow) Fragment RRI
- (green) T4 Polynucleotide Kinase RRI
- (yellow) Deoxyadenosine 5´- Triphosphate (dATP) (1.0 mM)
- (yellow) Klenow Fragment (3´→5´ exo⁻) RRI
- (yellow) NEBuffer 2 for Klenow Fragment (3´→5´ exo⁻) (10X)
- (red) Quick T4 DNA Ligase RRI
- (red) NEBNext Quick Ligation Reaction Buffer (2X)

Nuclease-free water

- (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix

Required Materials Not Included:

80% Ethanol (freshly prepared)

0.1X TE, pH 8.0

10 mM Tris-HCl, pH 7.5–8.0 (optional)

NEBNext Singleplex or Multiplex Oligos for Illumina (NEB #E7350, #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600)

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

Agencourt RNAClean XP Beads (Beckman Coulter, Inc., #A63987)

Agilent Bioanalyzer

Applications:

The NEBNext mRNA Library Prep Reagent Set for Illuimna contains enzymes and buffers that are ideally suited for sample preparation for next-generation sequencing, and for preparation of expression libraries. Each of these components must pass rigorous quality control standards and are lot controlled, both individually and as a set of reagents.

Lot Control: The lots provided in the NEBNext mRNA Library Prep Reagent Set for Illuimna 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 a transcriptome library on an 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.

Protocols:

Symbols

⚠ *This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.*

● *Colored bullets indicate the cap color of the reagent to be added to a reaction.*

Starting Material: Purified mRNA (50–250 ng)

1.1 mRNA Fragmentation Protocol

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

Purified mRNA	1–18 µl
○ (white) 10X RNA Fragmentation Reaction Buffer	2 µl
Nuclease-free Water	variable
Total volume	20 µl

2. Incubate in a preheated thermal cycler for 5 minutes at 94°C. This is the optimal condition for eukaryotic mRNA to generate 200 nucleotide RNA fragments (see Figure 1.1). Other types of mRNA may require optimizing incubation time to obtain desired fragment size distribution.
3. Transfer tube to ice.
4. Add 2 µl ○ (white) 10X RNA Fragmentation Stop Solution.

1.2 RNA Purification after RNA Fragmentation using Agencourt RNAClean XP

1. Add 28 µl of the nuclease-free water to the 22 µl fragmented RNA from Step 4 of the mRNA Fragmentation Protocol.
2. Add 2.2X (110 µl) Agencourt RNAClean XP Beads and mix by pipetting up and down.
3. Incubate samples on ice for 15 minutes.
4. Place the tube on an appropriate magnetic rack to separate beads from the supernatant.
5. When the solution is clear (about 5 minutes), discard the supernatant.
6. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant

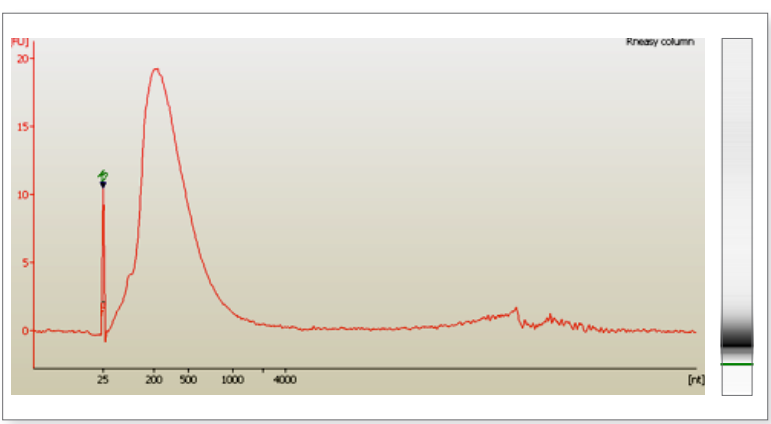
7. Repeat Step 6 once for a total of 2 washes.
8. Briefly spin the tube, and put the tube back in the magnetic rack.
9. Completely remove the residual ethanol, and air dry the beads.
10. Remove the tube from the magnetic rack. Elute RNA from the beads with 15 µl nuclease-free water.
11. Mix well by pipetting up and down, and put the tube in the magnetic rack until the solution is clear.
12. Use 1 µl of the supernatant to QC the fragment size distribution on an Agilent Bioanalyzer RNA pico chip (Section 1.3).
13. Transfer 13.5 µl of the supernatant to a clean PCR tube.
14. Place the sample on ice and proceed with first strand cDNA synthesis in Section 1.4.

1.3 Assess the Yield and the Size Distribution of the Fragmented mRNA.

Run 1 µl in the Agilent Bioanalyzer® 2100 (Agilent Technologies, Inc.) using a RNA Pico chip.

Note: Clean mRNA fragments show a distinct band on the Bioanalyzer (Figure 1.1).

Figure 1.1: Bioanalyzer traces of clean mRNA Fragments. The mRNA fragments should have a normal distribution with a peak at 200 nucleotides.



1.4 First Strand cDNA Synthesis

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

Fragmented mRNA	13.5 µl
(pink) Random Primers	1 µl
Total volume	14.5 µl

2. Incubate in a preheated thermal cycler for 5 minutes at 65°C.
3. Spin tube briefly and place on ice.
4. To the fragmented mRNA and Random Primers add:

(pink) 5X First Strand Synthesis Reaction Buffer	4 µl
(pink) Murine RNase Inhibitor	0.5 µl
Total volume	19 µl

5. Incubate in a preheated thermal cycler for 2 minutes at 25°C.
6. Add 1 µl (pink) ProtoScript II Reverse Transcriptase to the reaction.
7. Incubate sample with the heated lid set to 105°C as follows:
   10 minutes at 25°C
   50 minutes at 42°C
   15 minutes at 70°C
   Hold at 4°C
8. Place the tube on ice.

1.5 Second Strand cDNA Synthesis

1. Add the following reagents to the First Strand Synthesis reaction:

Nuclease-free Water	48 µl
(orange) 10X Second Strand Synthesis Reaction Buffer	8 µl
(orange) Second Strand Synthesis Enzyme Mix	4 µl
Total volume	80 µl

2. Mix thoroughly by gentle pipetting.
3. Incubate in a thermal cycler for 2.5 hours at 16°C.

   Note: If you need to stop at this point in the protocol after the 2.5 hours incubation at 16°C, samples can be left in the thermal cycler overnight at 4°C.

1.6 Purify the double-stranded cDNA using 1.8X Agencourt AMPure XP Beads.

1. Vortex AMPure XP Beads to resuspend.
2. Add 1.8X (144 µl) of resuspended AMPure XP Beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. 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.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Mix by pipetting up and down. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with lid open.

   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
8. Remove the tube from the magnet. Elute the DNA target from the beads into 55 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature.
9. Put the tube in the magnetic stand until the solution is clear. Remove supernatant (50 µl) and transfer to a clean 1.5 ml LoBind® (Eppendorf AG) tube.

1.7 End Repair of cDNA Library

1. Mix the following components in a sterile 1.5 ml microcentrifuge tube:

Purified double-stranded cDNA	50 µl
Nuclease-free Water	25 µl
● (green) 10X Phosphorylation Reaction Buffer	10 µl
● (green) Deoxynucleotide Solution Mix	4 µl
● (green) T4 DNA Polymerase	5 µl
● (green) *E. coli* DNA Polymerase I, Large (Klenow) Fragment	1 µl
● (green) T4 Polynucleotide Kinase	5 µl
Total volume	100 µl

2. Incubate in a heat block for 30 minutes at 20°C.

1.8 Purify the end-repaired cDNA using 1.8X Agencourt AMPure XP Beads. Note: X refers to original sample volume

1. Vortex AMPure XP Beads to resuspend.
2. Add 1.8X (180 µl) of resuspended AMPure XP Beads to the end-repaired DNA (~100 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. 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.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Mix by pipetting up and down. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with lid open.

   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
8. Remove the tube from the magnet. Elute the DNA target from the beads into 40 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature.
9. Put the tube in the magnetic stand until the solution is clear. Remove supernatant (32 µl) and transfer to a clean 1.5 ml LoBind® (Eppendorf AG) tube.

1.9 dA-Tailing of cDNA Library

1. Mix the following components in a sterile 1.5 ml microcentrifuge tube:

Purified, End Repaired cDNA	32 µl
○ (yellow) NEBuffer2	5 µl
○ (yellow) Deoxyadenosine 5´-Triphosphate (1 mM)	10 µl
○ (yellow) Klenow Fragment (3´→5´ exo⁻)	3 µl
Total volume	50 µl

2. Incubate in a heat block for 30 minutes at 37°C.

1.10 Purify the dA-Tailed DNA using 1.8X Agencourt AMPure XP Beads

1. Vortex AMPure XP Beads to resuspend.
2. Add 1.8X (90 µl) of resuspended AMPure XP Beads to the dA tailed DNA (~50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. 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.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Mix by pipetting up and down. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open.

   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
8. Remove the tube from the magnet. Elute the DNA target from the beads into 30 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature.
9. Put the tube in the magnetic stand until the solution is clear. Remove supernatant (23 µl) and transfer to a clean 1.5 ml LoBind® (Eppendorf AG) tube.

1.11 Adaptor Ligation of cDNA Library

1. Mix the following components in a sterile 1.5 ml microcentrifuge tube:

Purified, dA-Tailed cDNA	23 µl
● (red) 2X Quick Ligation Reaction Buffer	25 µl
● (red) NEBNext Adaptors (15 µM)*	1 µl
● (red) Quick T4 DNA Ligase	1 µl
Total volume	50 µl

*The NEBNext adaptor is provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina.

2. Incubate 15 minutes at room temperature.

3. Add 3 µl of ● (red) USER™ enzyme, mix by pipetting up and down, and incubate at 37°C for 15 minutes.

   NOTE: This step is only for use with NEBNext adaptors only. USER enzyme can be found in the NEBNext Singleplex ((NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina.

⚠ ***A precipitate can form upon thawing of the NEBNext Q5 Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while performing cleanup of adaptor-ligated DNA. Once thawed, gently mix by inverting the tube several times.***

1.12 Cleanup of Adaptor-Ligated DNA

1. Vortex AMPure XP Beads to resuspend.

2. Add 1.8X (90 µl) of resuspended AMPure XP Beads to the ligation reaction (~50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

3. Incubate for 5 minutes at room temperature.

4. 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.

5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Mix by pipetting up and down. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

6. Repeat Step 5 once.

7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with lid open.

   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8. Remove the tube/plate from the magnet. Elute the DNA target by adding 155 µl of 10 mM Tris-HCl or 0.1 X TE to the beads for bead-based size selection.

   Note: For size selection using E-Gel size select gels, elute the DNA into 20 µl 0.1X TE or 10 mM Tris-HCl.

9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.

10. Put the tube/PCR plate in the magnetic stand until the solution is clear. Remove supernatant (150 µl) and transfer to a clean 1.5 ml LoBind tube.

1.13 Size Selection of Adaptor-ligated DNA using Agencourt AMPure XP Beads

⚠ ***The following size selection protocol is for libraries with 200 bp inserts only. For libraries with larger fragment inserts, please optimize bead: DNA ratio accordingly.***

Note: X refers to the original sample volume of 150 µl.

1. Add 135 µl (0.9X) resuspended AMPure XP Beads to 150 µl eluted DNA from step 9. Mix well on a vortex mixer or by pipetting up and down at least 10 times.
2. Incubate for 5 minutes at room temperature.
3. 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 to a new tube (**Caution: do not discard the supernatant**). Discard beads that contain the large fragments.
4. Add 30 µl (0.2X) resuspended AMPure XP beads to the supernatant, mix well and incubate for 5 minutes at room temperature.
5. Put 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**).
6. Add 200 µl 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.
7. Repeat Step 6 once.
8. Briefly spin the tube, and put the tube back in the magnetic stand.
9. Completely remove the residual ethanol, and air dry beads for 5 minutes while the tube is on the magnetic stand with lid open.

   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

10. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 22 µl of 10 mM Tris-HCl or 0.1X TE.
11. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.

12. Put the tube/PCR plate in the magnetic stand until the solution is clear. Transfer 20 µl of the supernatant to a clean PCR tube, and proceed to PCR enrichment.

Adaptor ligated DNA can also be size selected using a E-Gel size select gel. After size selection, purify DNA sample on one QIAQuick column and elute in 22 µl of nuclease free water.

1.14 PCR Enrichment of Adaptor Ligated DNA

⚠ **Follow Section 1.14A if you are using the following oligos (10 µM primer):**

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow Section 1.14B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

1.14A PCR Enrich Adaptor Ligated cDNA Library

1. Mix the following components in sterile strip tubes:

Size Selected cDNA	20 µl
(blue) Index Primer/i7 Primer*,**	2.5 µl
(blue) Universal PCR Primer/i5 Primer*,***	2.5 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
Total volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up the PCR reaction.

** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one index primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

*** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	10 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	10–12*
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on mRNA input. If 50 ng of purified mRNA is the starting input, it is recommended to perform 12 cycles of PCR.

3. Proceed to Purify using Ampure XP Beads in Section 1.15

1.14B PCR Enrich Adaptor Ligated cDNA Library

1. Mix the following components in sterile strip tubes:

Size Selected cDNA	20 µl
(blue) Index/Universal Primer Mix*	5 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
Total volume	50 µl

* The primers are provided in NEBNext Multiplex Oligos for Illumina, NEB #E6609. Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	10 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	10–12*
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on mRNA input. If 50 ng of purified mRNA is the starting input, it is recommended to perform 12 cycles of PCR.

3. Proceed to Purify using Ampure XP Beads in Section 1.15

1.15 Purify using AMPure XP Beads

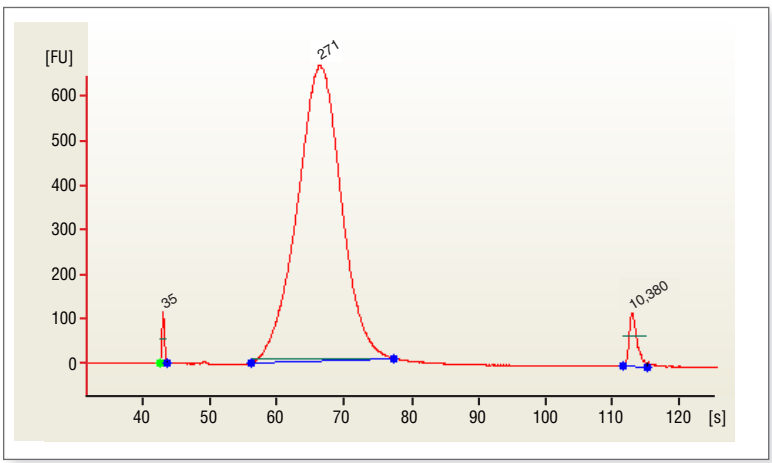
1. Vortex AMPure XP Beads to resuspend.
2. Add 60 µl (1.2X) of resuspended AMPure XP Beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Place the tube on an appropriate magnetic stand to separate 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.
5. Add 200 µl 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.
6. Repeat Step 5 once.
7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open.

   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
8. Remove the tube from the magnet. Elute the DNA target from the beads into 23 µl 0.1X TE Buffer. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature. Put the tube in the magnetic stand until the solution is clear.
9. Transfer 20 µl of the supernatant to a clean 1.5 ml LoBind tube. Libraries can be stored at –20°C.

1.16 Assess Library Quality on a Bioanalyzer (High Sensitivity Chip)

Check that the electropherogram shows a narrow distribution with a peak size approximately 270 bp.

Figure 1.2: Example of mRNA Library size distribution on a Bioanalyzer.



NEBNext RNA Fragmentation Buffer

#E6101A: 0.048 ml **Concentration: 10X**
#E6101AA: 0.240 ml

Store at –20°C or 4°C

1X NEBNext RNA Fragmentation Buffer:
40 mM Tris-OAc
100 mM KOAc
30 mM $Mg(OAc)_2$
pH 8.3 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1X RNA Fragmentation 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 RNA Fragmentation 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 RNA Fragmentation 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.

Phosphatase Activity: Incubation of 1X RNA Fragmentation 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

NEBNext RNA Fragmentation Stop Solution

#E6102A: 0.048 ml **Concentration: 10X**
#E6102AA: 0.240 ml

Store at –20°C

1X NEBNext RNA Fragmentation Stop Solution:
50 mM EDTA

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1X RNA Fragmentation Stop Solution 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 RNA Fragmentation Stop Solution 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 RNA Fragmentation Stop Solution 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 RNA Fragmentation Stop Solution with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable RNase activity as determined by gel electrophoresis.

Phosphatase Activity: Incubation of 1X RNA Fragmentation Stop Solution 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

Random Primers

#E6104A: 15 µl
#E6104AA: 60 µl

Store at –20°C

Description: This mixture of random hexanucleotides is used to prime DNA synthesis *in vitro* along multiple sites of template RNA.

Sequence: 5´ d(N^6) 3´ [N=A,C,G,T]

Phosphorylated: No.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µl Random Primers 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 Random Primers 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 1 µl Random Primers 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 1 µl Random Primers with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable RNase activity as determined by gel electrophoresis.

Phosphatase Activity: Incubation of 1 µl Random Primers 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

Murine RNase Inhibitor

#E6105A: 15 µl
#E6105AA: 30 µl

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.

Lot Controlled

References:

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

NEBNext First Strand Synthesis Reaction Buffer

#E6106A: 0.048 ml **Concentration: 5X**
#E6106AA: 0.240 ml

Store at –20°C

1X NEBNext First Strand Synthesis Reaction Buffer:
50 mM Tris-Acetate
75 mM KOAc
3.1 mM $Mg(OAc)_2$
0.5 mM dNTPs each
pH 8.3 @ 25°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 $MgCl_2$) 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 Second Strand Synthesis Enzyme Mix

#E6107A: 0.048 ml
#E6107AA: 0.240 ml

Store at –20°C

Description: NEBNext Second Strand Synthesis Enzyme Mix is optimized to conver 10–100 ng of short single-stranded cDNAs to double-stranded cDNAs with Random Primers and NEBNext Second Strand Synthesis Reaction Buffer.

NEBNext Second Strand Synthesis Enzyme Mix:
6,000 units/ml DNA Polymerase I (*E. coli*)
5,000 units/ml RNase H
25,000 units/ml *E. coli* DNA Ligase

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.

Endonuclease Activity: Incubation of a 10 µl reaction containing 1 µl Second Strand Synthesis Enzyme Mix 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 1 µl Second Strand Synthesis 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 the *E. coli* DNA Ligase ligated 50% of HindIII fragments of λ DNA (5´ DNA termini concentration of 0.12 µM, 300 µg/ml) in a total reaction volume of 20 µl in 30 minutes at 16°C in 1X *E. coli* DNA Ligase Reaction Buffer. One unit of *E. coli* DNA Polymerase I incorporated 10 nmol of dNTP into acid-insoluble material in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X EcoPol Reaction Buffer with 33 µM dNTPs including [^{3}H]-dTTP and 70 µg/ml denatured herring sperm DNA. Incubation of 50 units of RNase H with 1 µg sonicated and denatured [^{3}H]-DNA (10^5 cpm/µg) for 30 minutes at 37°C in 50 µl reaction buffer released < 0.1% radioactivity.

Lot Controlled

NEBNext Second Strand Synthesis Reaction Buffer

#E6108A: 0.096 ml **Concentration: 10X**
#E6108AA: 0.480 ml

Store at –20°C

1X NEBNext Second Strand Synthesis Reaction Buffer:
20 mM Tris-HCl
12 mM $(NH_4)_2SO_4$
5 mM $MgCl_2$
0.16 mM β-NAD
0.19 mM dNTPs each
pH 7.4 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1X NEBNext Second 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 Second 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 NEBNext Second 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 NEBNext Second 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 NEBNext Second Strand Synthesis 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.

Lot Controlled

Phosphorylation Reaction Buffer

#E6001A: 0.12 ml **Concentration: 10X**
#E6001AA: 0.6 ml

Store at –20°C

1X Phosphorylation Reaction Buffer:
50 mM Tris-HCl
10 mM $MgCl_2$
10 mM DTT
1 mM ATP
pH 7.5 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this 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 this reaction buffer at a 1X concentration with 1 µg of ϕX174 RF I DNA for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

RNase Activity: Incubation of this 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.

Phosphatase Activity: Incubation of this reaction buffer at a 1X concentration 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

#E6002A: 0.048 ml **10 mM each dNTP**
#E6002AA: 0.24 ml

Store at –20°C

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

Supplied in: Milli-Q® water (Millipore Corporation) as a sodium salt at 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 dNTPs 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 dNTPs 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

T4 DNA Polymerase

#E6003A: 0.06 ml
#E6003AA: 0.3 ml

RR BSA Yes

Store at –20°C

Description: T4 DNA Polymerase catalyzes the synthesis of DNA in the 5´→ 3´ direction and requires the presence of template and primer. This enzyme has a 3´→ 5´ exonuclease activity which is much more active than that found in DNA Polymerase I. Unlike *E. coli* DNA Polymerase I, T4 DNA Polymerase does not have a 5´→ 3´ exonuclease function.

Source: Purified from a strain of *E. coli* that carries a T4 DNA Polymerase overproducing plasmid.

Supplied in: 100 mM $KP0_4$ (pH 6.5), 1 mM DTT and 50% glycerol.

Quality Control Assays

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

Endonuclease Activity: Incubation of a minimum of 50 units of this enzyme with 1 µg of ϕX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 30 units of this enzyme 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 (Nucleotide Incorporation): One unit of this enzyme incorporates 10 nmol of dNTP into acid-precipitable material in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X T4 DNA Polymerase Reaction Buffer with 33 µM dNTPs including [^{3}H]-dTTP, 70 µg/ml denatured herring sperm DNA and 50 µg/ml BSA.

Lot Controlled

References:

1. Tabor, S. and Struhl, K. (1989). DNA-Dependent DNA Polymerases. In F. M. Ausebel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl (Eds.), *Current Protocols in Molecular Biology* (pp. 3.5.10–3.5.12). New York: John Wiley & Sons Inc.
2. Sambrook, J. et al. (1989). *Molecular Cloning: A Laboratory Manual,* (2nd ed.), (pp. 5.44–5.47). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

DNA Polymerase I, Large (Klenow) Fragment

#E6004A: 0.015 ml
#E6004AA: 0.06 ml

Store at –20°C

Description: DNA Polymerase I, Large (Klenow) Fragment is a proteolytic product of *E. coli* DNA Polymerase I which retains polymerization and 3´→ 5´ exonuclease activity, but has lost 5´→ 3´ exonuclease activity. Klenow retains the polymerization fidelity of the holoenzyme without degrading 5´ termini.

Source: A genetic fusion of the *E. coli pol*A gene, that has its 5´→ 3´ exonuclease domain genetically replaced by maltose binding protein (MBP). Klenow Fragment is cleaved from the fusion and purified away from MBP. The resulting Klenow fragment has the identical amino and carboxy termini as the conventionally prepared Klenow fragment.

Supplied in: 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol 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 a minimum of 5 units of this enzyme 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 5 units of this enzyme 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 minimum of 50 units of this enzyme with 1 µg of ϕX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 50 units of this enzyme 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 minimum of 5 units of this enzyme 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 (Nucleotide Incorporation): One unit of this enzyme incorporates 10 nmol of dNTP into acid-precipitable material in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X NEBuffer 2 with 33 µM dNTPs including [^{3}H]-dTTP, 70 µg/ml denatured herring sperm DNA and 50 µg/ml BSA.

Lot Controlled

References:

1. Sambrook, J. et al. (1989). *Molecular Cloning: A Laboratory Manual,* (2nd ed.), (pp. 5.40–5.43). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

T4 Polynucleotide Kinase

#E6005A: 0.06 ml
#E6005AA: 0.3 ml

Store at –20°C

Description: Catalyzes the transfer and exchange of P_i from the γ position of ATP to the 5´-hydroxyl terminus of polynucleotides (double- and single-stranded DNA and RNA) and nucleoside 3´-monophosphates. Polynucleotide Kinase also catalyzes the removal of 3´-phosphoryl groups from 3´-phosphoryl polynucleotides, deoxynucleoside 3´-monophosphates and deoxynucleoside 3´-diphosphates (1).

Source: An *E. coli* strain that carries the cloned T4 Polynucleotide Kinase gene. T4 Polynucleotide Kinase is purified by a modification of the method of Richardson (1).

Supplied in: 50 mM KCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 µM ATP and 50% glycerol.

Quality Assurance: Free of exonuclease, phosphatase, endonuclease and RNase activities. Each lot is tested under 5´-end-labeling conditions to assure maximal transfer of [^{32}P].

Quality Control Assays

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

16-Hour Incubation: 50 µl reactions containing a minimum of 10 units of this enzyme 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 10 units of this enzyme 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 minimum of 200 units of this enzyme with 1 µg of ϕX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 100 units of this enzyme 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 minimum of 100 units of this enzyme with 2 µg MS2 phage RNA for 1 hour at 37°C in 50 µl 1X T4 Polynucleotide Kinase Reaction Buffer followed by agarose gel electrophoresis shows no degradation. Incubation of 10 units of this enzyme 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.

Exonuclease Activity: Incubation of 300 units of enzyme with 1 µg sonicated [^{3}H] DNA (10^5 cpm/µg) for 4 hours at 37°C in 50 µl reaction buffer released < 0.1% radioactivity.

Functional Activity (Labeling): ^{32}P end labeling of 5´-hydroxyl terminated $d(T)_8$ with a minimum of 50 units of this enzyme for 30 minutes at 37°C in 50 µl 1X T4 Polynucleotide Kinase Buffer followed by 20% acrylamide gel electrophoresis reveals that less than 1% of the product has been degraded by exonuclease or phosphatase activities.

Lot Controlled

References:

1. Richardson, C.C. (1981). In P.D. Boyer (Ed.), *The Enzymes* Vol. 14, (pp. 299–314). San Diego: Academic Press.
2. Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual*, (2nd ed.), (pp. 10.59–10.67, 11.31–11.33). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Deoxyadenosine 5´- Triphosphate (dATP)

#E6006A: 0.12 ml **Concentration: 1.0 mM**
#E6006AA: 0.6 ml

Store at –20°C

Supplied in: Milli-Q water as a sodium salt at pH 7.5.

Concentration: dATP is supplied at a concentration of 1mM.

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

Notes: Storing nucleotide triphosphates in solutions containing magnesium promotes triphosphate degradation. Nucleotide concentrations are determined by measurements of absorbance.

Quality Control Assays

Phosphatase Activity: Incubation of a minimum of 1 mM dATP in protein phosphatase assay buffer (1M 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.

16-Hour Incubation: 50 µl reactions containing a minimum of 0.2 mM dATP and 1 µg of HindIII digested Lambda DNA incubated for 16 hours results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 0.2 mM dATP and 1 µg T3 DNA incubated for 16 hours also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

RNase Activity: Incubation of a minimum of 0.1 mM dATP 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.

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

Functional Activity (PCR): This dATP in a pool of dNTPs is tested in 25 cycles of PCR amplification generating 0.5 kb, 2 kb, and 5kb amplicons from lambda DNA.

Lot Controlled

Klenow Fragment (3´→ 5´ exo⁻)

#E6007A: 0.036 ml
#E6007AA: 0.18 ml

Store at –20°C

Description: Klenow Fragment (3´→ 5´ exo⁻) is an N-terminal truncation of DNA Polymerase I which retains polymerase activity, but lacks the 5´→ 3´ exonuclease activity and contains mutations (D355A, E357A), which abolish the 3´→ 5´ exonuclease activity (1).

Source: An *E. coli* strain containing a plasmid with a fragment of the *E.coli* polA (D355A, E357A) gene starting at codon 324.

Supplied in: 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT 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 a minimum of 5 units of this enzyme 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 5 units of this enzyme 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 minimum of 50 units of this enzyme with 1 µg of ϕX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 50 units of this enzyme 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 minimum of 5 units of this enzyme 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.

Exonuclease Activity: Incubation of a minimum of 200 units of this enzyme with 1 µg sonicated [^{3}H]DNA (10^5 cpm/µg) for 4 hours at 37°C in 50 µl reaction buffer releases < 0.1% radioactivity.

3´→5´ Exonuclease Activity: Incubation of a minimum of 50 units of enzyme in 20 µl of a 10 nM solution of a fluorescent 5´-FAM labeled oligonucleotide for 30 minutes at 37°C yields no detectable 3´→5´ degradation as determined by capillary electrophoresis.

Functional Activity (Nucleotide Incorporation): One unit of this enzyme incorporates 10 nmol of dNTP into acid-precipitable material in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X NEBuffer 2 with 33 µM dNTPs including [^{3}H]-dTTP, 70 µg/ml denatured herring sperm DNA and 50 µg/ml BSA.

References:

1. Derbyshire, V. et al. (1988) *Science* 240, 199–201.

NEBuffer 2 for Klenow Fragment (3´→5´ exo⁻)

#E6008A: 0.06 ml **Concentration: 10X**
#E6008AA: 0.3 ml

Store at –20°C

1X NEBuffer 2:
50 mM NaCl
10 mM Tris-HCl
10 mM $MgCl_2$
1 mM DTT
pH 7.9 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this 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 this reaction buffer at a 1X concentration with 1 µg of ϕX174 RF I DNA for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

RNase Activity: Incubation of this 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.

Phosphatase Activity: Incubation of this reaction buffer at a 1X concentration 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 T4 DNA Ligase

#E6011A: 0.015 ml
#E6011AA: 0.06 ml

Store at –20°C

Source: Purified from *E. coli* C600 pcl857 pPLc28 lig8 (2).

Quality Control Assays

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

16-Hour Incubation: 50 µl reactions containing a minimum of 2,000 units of this enzyme 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 2,000 units of this enzyme 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 minimum of 3,200 units of this enzyme with 1 µg of ϕX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 20,000 units of this enzyme 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 minimum of 2,000 units of this enzyme 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.

Exonuclease Activity: Incubation of a minimum of 3,200 units of this enzyme with 1 µg sonicated [^{3}H] DNA (10^5 cpm/µg) for 4 hours at 37°C in 50 µl reaction buffer releases < 0.1% radioactivity.

Functional Activity (Blunt End Ligation): 50 µl reactions containing a 0.5 µl Quick T4 DNA Ligase, 18 µg HaeIII digested ϕX174 and 1X T4 DNA Ligase Buffer incubated at 16°C for 7.5 min results in > 95% of fragments ligated as determined by agarose gel electrophoresis.

Functional Activity (Cohesive End Ligation): 20 µl reactions containing 0.5 µl Quick T4 DNA Ligase, 12 µg HindIII digested lambda DNA and 1X T4 DNA Ligase Buffer incubated at 37°C overnight results in > 95% of fragments ligated as determined by agarose gel electrophoresis. Redigestion of the ligated products, 50 µl reactions containing 6 µg of the ligated fragments, 40 units HindIII, and 1X NEBuffer 2 incubated at 37°C for 2 hours, results in no detectable undigested fragments as determined by agarose gel electrophoresis.

Functional Activity (Adaptor Ligation): 50 µl reactions containing 0.125 µl Quick T4 DNA Ligase, 8 nmol 12 bp adaptor, and 1X T4 DNA Ligase Buffer incubated at 16°C overnight results in no detectable unligated adaptor as determined by agarose gel electrophoresis.

Functional Activity (Transformation): After a five-minute ligation of linearized, dephosphorylated LITMUS™ 28 (containing either blunt [EcoRV] or cohesive [HindIII] ends) and a mixture of compatible insert fragments, transformation into chemically competent *E. coli* DH-5 alpha cells yields a minimum of 1 x 10^6 recombinant transformants per µg plasmid DNA.

Lot Controlled

References:

1. Engler, M. J. and Richardson, C. C. (1982). In P. D. Boyer (Ed.), *The Enzymes* Vol. 5, (p. 3). San Diego: Academic Press.
2. Remaut, E., Tsao, H. and Fiers, W. (1983) *Gene* 22, 103–113.

ProtoScript II Reverse Transcriptase

#E6014A: 0.015 ml **Concentration: 200,000 U/ml**
#E6014AA: 0.06 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^-) 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 [^{3}H] *E. coli* DNA (10^5 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

Quick Ligation Reaction Buffer

#E6010A: 0.3 ml **Concentration: 2X**
#E6010AA: 0.75 ml

Store at –20°C

1X Quick Ligation Reaction Buffer:
66 mM Tris-HCl
10 mM $MgCl_2$
1 mM dithiothreitol
1 mM ATP
7.5% Polyethylene glycol (PEG 6000)
pH 7.6 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this 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 this reaction buffer at a 1X concentration with 1 µg of ϕX174 RF I DNA for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

RNase Activity: Incubation of this 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.

Phosphatase Activity: Incubation of this reaction buffer at a 1X concentration in protein phosphatase assay buffer (1M 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

#E6109A: 4 ml
#E6109AA: 20 ml

Store at –20°C or 4°C

Description: Nuclease-free Water is free of detectable DNA and RNA nucleases and phosphatases and 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 results in < 10% conversion to RF II (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 1X Second Strand Synthesis 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.

Lot Controlled

NEBNext Q5 Hot Start HiFi PCR Master Mix

E6630A: 0.3 ml **Concentration: 2X**
E6630AA: 0.75 ml (2 vials provided)

Store at –20°C

Description: The NEBNext Q5 Hot Start HiFi PCR Master Mix is specifically optimized for robust, high-fidelity amplification of next-generation sequencing (NGS) libraries, regardless of GC content. The polymerase component of the master mix, Q5 High-Fidelity DNA Polymerase, is a novel thermostable DNA polymerase that possesses 3´→5´ exonuclease activity, and is fused to a processivity-enhancing Sso7d domain. Q5 also has an ultra-low error rate (> 100-fold lower than that of *Taq* DNA Polymerase and ~12-fold lower than that of *Pyrococcus furiosus* (Pfu) DNA Polymerase). The buffer component of the master mix has been optimized for robust amplification, even with GC-rich amplicons and offers enhanced compatibility with a variety of beads used in typical NGS workflows. These features make the NEBNext Q5 Hot Start HiFi PCR Master Mix ideal for NGS library construction. This convenient 2X master mix contains dNTPs, Mg^{++} and a proprietary buffer, and requires only the addition of primers and DNA template for robust amplification. The inclusion of the hot start aptamer allows convenient room temperature reaction set up.

Quality Control Assays

16-Hour Incubation: A 50 µl reaction containing NEBNext Q5 Hot Start HiFi PCR Master Mix and 1 µg of HindIII digested λ 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 Q5 Hot Start HiFi PCR 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.

Phosphatase Activity: Incubation of NEBNext Q5 Hot Start HiFi PCR 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 (Multiplex PCR, Bead Inhibition): 30 cycles of PCR amplification of 20 ng genomic DNA with and without carboxylated magnetic beads in a 50 µl reaction containing 0.5 µM 4-plex primer mix and 1X NEBNext Q5 Hot Start HiFi PCR Master Mix result in the four expected amplicons and no inhibition of amplification in the presence of the beads.

Lot Controlled

Revision History:

Revision #	Description
4.0	Include protocol for use with NEBNext Q5 Hot Start HiFi PCR Master Mix. Include protocol for changes in concentration of NEBNext Singleplex and Multiplex Oligos for Illumina. Changed all AMPure Bead drying times after ethanol washes to 5 minutes. Changed all AMPure Bead elutions to 0.1X TE or 10 mM Tris-HCl. Added 2 minute incubation after eluting DNA from AMPure beads. Removed protocol for clean up of fragmented RNA using ethanol precipitation. Removed linear acrylamide from kit.
5.0	Remove protocol for use with NEBNext High-Fidelity 2X PCR Master Mix. Include protocol for use with NEBNext Multiplex Oligos (96 Index Primers, NEB #E6609).
6.0	Protocol updated to include NEB #E7710 and NEB #E7730. Section C in the PCR setup step was removed because all of the 25 µM primers are now expired.

USA
New England Biolabs, Inc.
240 County Road
Ipswich, MA 01938-2723
Telephone: (978) 927-5054
Toll Free: (USA Orders) 1-800-632-5227
Toll Free: (USA Tech) 1-800-632-7799
Fax: (978) 921-1350
e-mail: info@neb.com
www.neb.com

CANADA
New England Biolabs, Ltd.
Telephone: (905) 665-4632
Toll Free: 1-800-387-1095
Fax: (905) 665-4635
Fax Toll Free: 1-800-563-3789
e-mail: info.ca@neb.com
www.neb.ca

CHINA, PEOPLE'S REPUBLIC
New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com
www.neb-china.com

FRANCE
New England Biolabs France
Free Call: 0800-100-632
Free Fax: 0800-100-610
e-mail: info.fr@neb.com
www.neb-online.fr

GERMANY & AUSTRIA
New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Free Call: 00800/246 52277 (Austria)
Fax: +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info.de@neb.com
www.neb-online.de

JAPAN
New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
Fax: +81 (0)3 5669 6192
e-mail: info@neb-japan.com
www.nebj.jp

SINGAPORE
New England Biolabs Pte. Ltd.
Telephone: +65 6776 0903
Fax: +65 6778 9228
e-mail: sales.sg@neb.com
www.neb.sg

UNITED KINGDOM
New England Biolabs (UK) Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 0800 435682
e-mail: info.uk@neb.com
www.neb.uk.com

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