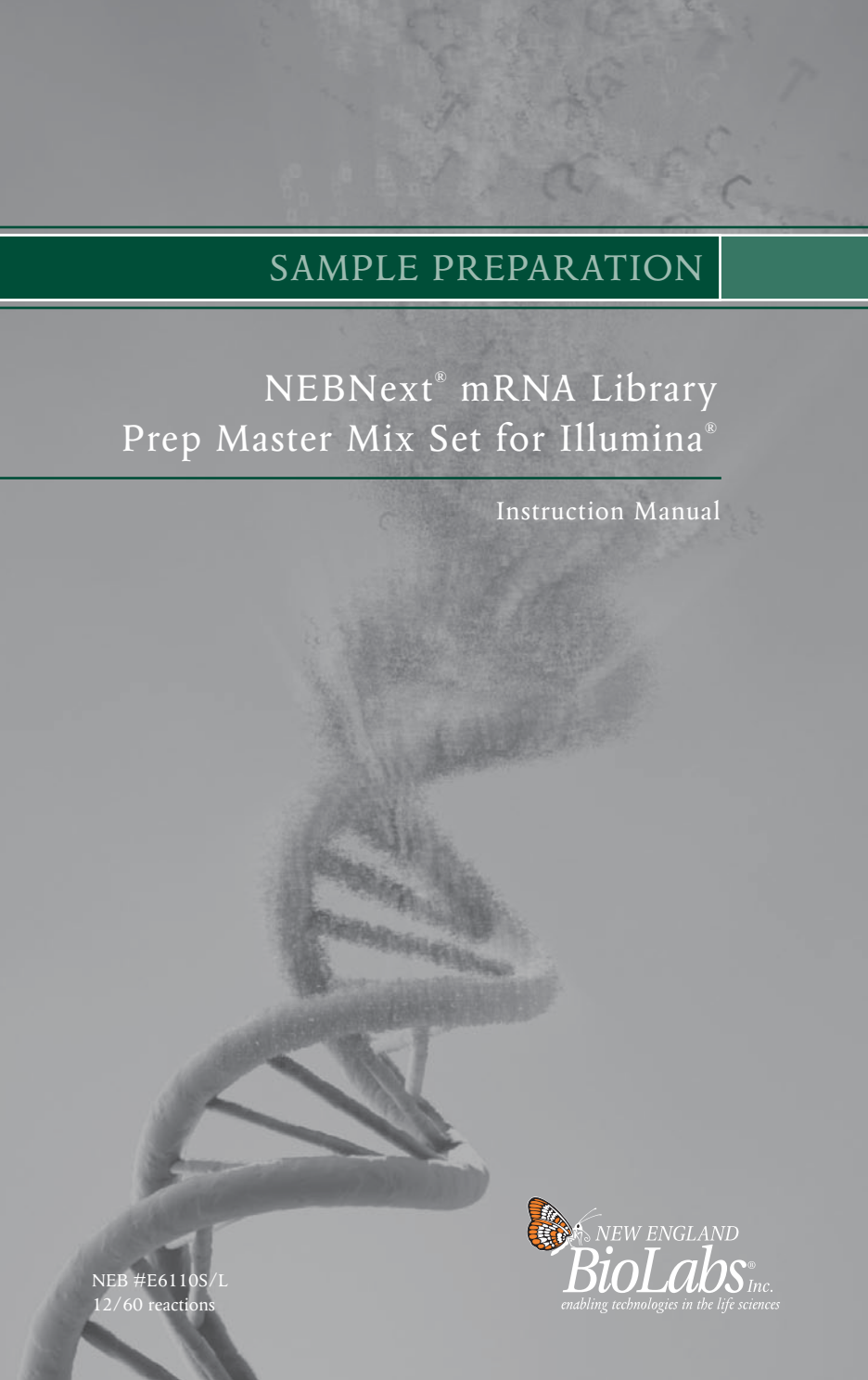
SAMPLE PREPARATION

NEBNext® mRNA Library Prep Master Mix Set for Illumina®

Instruction Manual



NEB #E6110S/L
12/60 reactions

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enabling technologies in the life sciences

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nqa ISO 9001 Registered Quality Management

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nqa ISO 13485 Registered Medical Devices

Cloned at NEBiolabs | Recombinant Enzyme | NEB2 Optimum Buffer | BSA Requires BSA | Yes Heat Inactivation

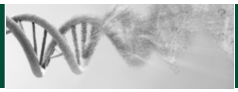


Table of Contents:

The Master Mix Set Includes:

The volumes provided are sufficient for preparation of up to 12 reactions (NEB #E6110S) and 60 reactions (NEB #E6110L). (All reagents should be stored at –20°C).

NEBNext RNA Fragmentation Buffer (10X)

NEBNext RNA Fragmentation Stop Solution (10X)

Linear Acrylamide (10 mg/ml)

Random Primers (3 µg/µl)

Murine RNase Inhibitor

NEBNext First Strand Synthesis Reaction Buffer (5X)

NEBNext Second Strand Synthesis Enzyme Mix

NEBNext Second Strand Synthesis Reaction Buffer (10X)

NEBNext End Repair Enzyme Mix

NEBNext End Repair Reaction Buffer (10X)

Klenow Fragment (3´→5´ exo$^-$)

NEBNext dA-Tailing Reaction Buffer (10X)

Quick T4 DNA Ligase

M-MuLV Reverse Transcriptase (RNase H$^-$)

NEBNext Quick Ligation Reaction Buffer (5X)

Nuclease-free water

Phusion High-Fidelity PCR Master Mix with HF Buffer

NEBNext High-Fidelity 2X PCR Master Mix (SAMPLE)

Required Materials Not Included:

3 M Sodium Acetate, pH 5.5

100% Ethanol

70% Ethanol

PCR Column Purification Kit (Qiagen or other)

DNA Gel Extraction Column Purification Kit

DNA Adaptors and Primers or Vector

Size Selection Materials [E-Gel® (Life Technologies, Inc.), Agarose Gel or AMPure® (Beckman Coulter, Inc.) XP Beads]

Applications:

The NEBNext mRNA Library Prep Master Mix Set for Illumina 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 Master Mix Set for Illumina 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 Genome Analyzer IIx (Illumina, Inc.).

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.

Protocol for Use with NEBNext Singleplex (#E7350) or Multiplex (#E7335, #E7500) Oligos for Illumina

Starting Material: Purified mRNA (50–250 ng)

mRNA Fragmentation Protocol

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

Purified mRNA	1–18 µl
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). Other types of mRNA may require optimizing incubation time to obtain desired fragment size distribution.
3. Transfer tube to ice.
4. Add 2 µl 10X RNA Fragmentation Stop Solution.

Clean Up Fragmented RNA Using RNeasy MinElute Spin Columns (Strongly Recommended)

1. Add 78 µl of the nuclease-free water to the 22 µl fragmented RNA from step 4. Purify sample using RNeasy® MinElute® Cleanup Kit (Qiagen #74204) following manufacturer instructions. Elute in 15.5 µl nuclease-free water. The recovered volume should be ~14.5 µl.

Note: Column purification removes short RNA Fragments and enriches the sample for RNA fragments longer than 200 nucleotides.

Alternatively, Clean Up Fragmented RNA Using Ethanol Precipitation

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

	Volume (µl)
Fragmented RNA from Step 4	22
3M Sodium Acetate, pH 5.5	2
Linear Acrylamide, 10 mg/ml	1–2
100% Ethanol	60
total volume	85–86

2. Incubate at –80°C or in a dry ice/methanol bath for 1 hour.

3. Centrifuge at 14,000 rpm for 25 minutes at 4°C in a microcentrifuge.
4. Carefully remove ethanol.
5. Wash pellet with 300 µl of freshly prepared 70% ethanol. Carefully pipette up and down the pellet. Make sure the pellet does not get stuck in the tip.
6. Centrifuge at 14,000 rpm for 5 minutes at 4°C in a microcentrifuge. Carefully remove all 70% ethanol.
7. Repeat steps 5 and 6 twice.

Note: Insufficient pellet washing results in inhibition of the first strand cDNA synthesis due to carryover of magnesium and EDTA. If the Bioanalyzer traces of the mRNA fragments show a noisy baseline (Figure 2) repeat the ethanol precipitation step and pellet washing steps.

8. Air dry pellet for up to 10 minutes at room temperature (or longer if necessary) to remove residual ethanol.
9. Resuspend in 14.5 µl Nuclease-free Water.

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 with sufficient washing on the Bioanalyzer show a distinct band (Figure 1).

Figure 1: Relative size distribution of eukaryotic mRNA fragments as seen using the Bioanalyzer 2100.

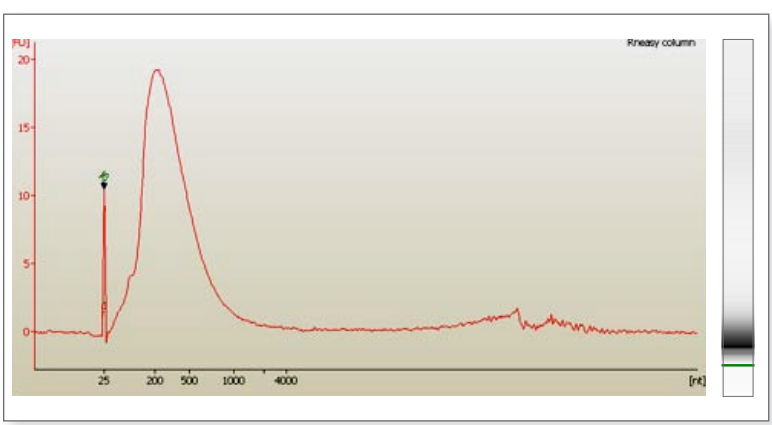
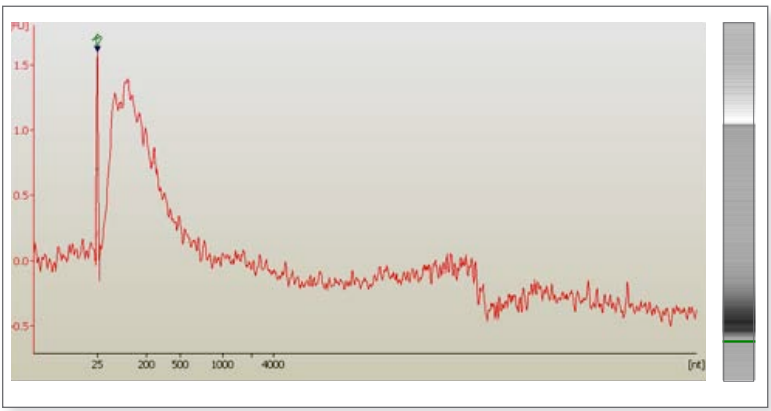


Figure 2: Bioanalyzer traces of mRNA Fragments with insufficient washing show a noisy baseline.



First Strand cDNA Synthesis

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

Fragmented mRNA	13.5 µl
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:

5X First Strand Synthesis Reaction Buffer	4 µl
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 M-MuLV Reverse Transcriptase (RNase H^-) to the reaction.
7. Incubate sample 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.

Second Strand cDNA Synthesis

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

Nuclease-free Water	48 µl
10X Second Strand Synthesis Reaction Buffer	8 µl
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.

Purify the double-stranded cDNA using 1.8X Agencourt AMPure XP Beads (Recommended).

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 10 minutes while the tube is on the magnetic stand with lid open.
8. Elute the DNA target from the beads into 52 µl water. Mix well on a vortex mixer or by pipetting up and down, and 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.

Alternatively, purify the double-stranded cDNA using QIAquick PCR Column Purification Kit and elute in 52 µl nuclease-free water.

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	35 µl
NEBNext End Repair Reaction Buffer	10 µl
NEBNext End Repair Enzyme Mix	5 µl
Total volume	100 µl

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

Purify the end-repaired cDNA using 1.8X Agencourt AMPure XP Beads (Recommended). 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 10 minutes while the tube is on the magnetic stand with lid open.
8. Elute the DNA target from the beads into 45 µl water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear. Remove supernatant (42 µl) and transfer to a clean 1.5 ml LoBind tube.

Alternatively, purify the double-stranded cDNA using QIAquick PCR Column Purification Kit and elute in 42 µl nuclease free water.

dA-Tailing of cDNA Library

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

Purified, End Repaired cDNA	42 µl
10X NEBNext dA-Tailing Reaction Buffer	5 µl
Klenow Fragment (3´→5´ exo⁻)	3 µl
Total volume	50 µl

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

Purify the dA-Tailed DNA using 1.8X Agencourt AMPure XP Beads (Recommended).

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 10 minutes while the tube is on the magnetic stand with the lid open.
8. Elute the DNA target from the beads into 40 µl water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear. Remove supernatant (38 µl) and transfer to a clean 1.5 ml LoBind tube.

Alternatively, purify the double-stranded cDNA using QIAquick PCR Column Purification Kit and elute in 40 µl nuclease free water.

Adaptor Ligation of cDNA Library

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

Purified, dA-Tailed cDNA	38 µl
5X NEBNext Quick Ligation Reaction Buffer	10 µl
NEBNext Adaptor (15 µM)	1 µl
Quick T4 DNA Ligase	1 µl
Total volume	50 µl

2. Incubate 15 minutes at room temperature.
3. Add 3 µl of USER™ enzyme, mix by pipetting up and down, and incubate at 37°C for 15 minutes.

Purify using AMPure XP Beads (strongly recommended).

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 10 minutes while the tube is on the magnetic stand with lid open.
8. Elute the DNA target from the beads into 150 µl water for bead-based size selection or into 20 µl water for size selection using E-Gel size select gels. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear.
9. Remove supernatant and transfer to a clean 1.5 ml LoBind tube.

Size Selection of Adaptor-ligated DNA using Agencourt AMPure XP Beads or Invitrogen E-Gels (Strongly Recommended)

Caution: 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 10 minutes while the tube is on the magnetic stand with lid open.
10. Elute DNA target from beads into 25 µl nuclease free water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear.

11. Transfer 23 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 25 µl of nuclease free water.

Alternatively, Size Selection of Adaptor-ligated DNA can be Performed Using a 2% Agarose Gel

1. Purify the adaptor-ligated DNA using QIAGEN MinElute PCR Purification Kit and elute in 12 µl of nuclease free water.

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 is carried over during DNA elution. Residual ethanol interferes with the correct loading of the sample on the gel.

2. Pour a 5 mm thick 2% agarose gel with a 1mm thick gel space and allow to cool.
3. Run adaptor ligated cDNA for 2 hours at 150 V or until the dye front has traveled 10 cm.
4. Isolate the desired cDNA fragment from the gel.
5. Purify DNA from the gel slice using a QIAquick Gel Extraction Purification Kit, purifying the sample on one column and elute in 25 µl of nuclease free water.

PCR Enrich Adaptor Ligated cDNA Library

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

Size Selected cDNA	23 µl
NEBNext High-Fidelity 2X PCR Master Mix**	25 µl
Universal PCR Primer (25 µM)	1 µl
Index Primer (X)* (25 µM)	1 µl
Total volume	50 µl

* If you are using the NEBNext Multiplex Oligos for Illumina (#E7335, #E7500), for each reaction only one of the 12 PCR primer Indices Is used during the PCR step.

** NEBNext High-Fidelity 2X PCR Master Mix will be replacing Phusion High-Fidelity PCR Master Mix. Both vials will be supplied for a limited time only.

2. PCR cycling conditions:

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	10 seconds	1
Denaturation Annealing Extension	98°C 65°C 72°C	10 seconds 30 seconds 30 seconds	10–12*
Final Extension	72°C 4°C	5 minutes hold	1

*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.

Purify using AMPure XP Beads (strongly recommended)

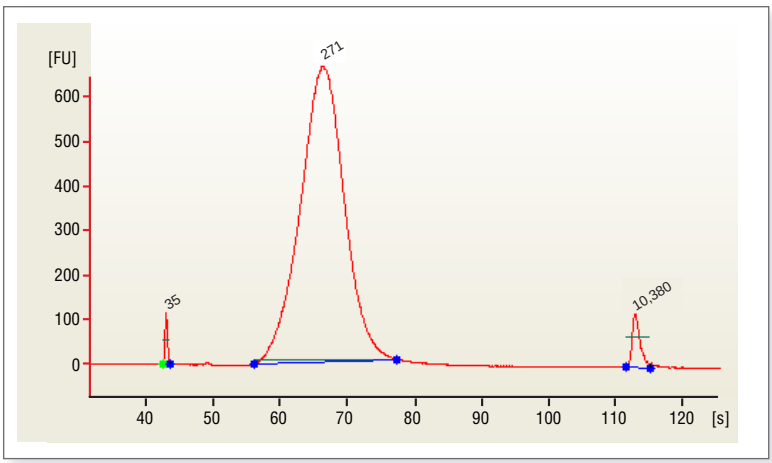
1. Vortex AMPure XP beads to resuspend.
2. Add 60 µl (1.2X) of resuspended AMPure XP beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Place the tube on an appropriate magnetic stand to separate 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.
8. Elute the DNA target from the beads into 22.5 µl 0.1X TE Buffer. Mix well on a vortex mixer or by pipetting up and down, and 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, and store at –20°C.

Alternatively, purify the PCR enriched cDNA using a QIAquick PCR Column Purification Kit and elute in 20 µl of sterile water or elution buffer.

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 3: Example of mRNA Library size distribution on a Bioanalyzer.



Protocol for use with End User Supplied Adaptors & Primers:

Starting Material: Purified mRNA (50–250 ng)

mRNA Fragmentation Protocol

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

Purified mRNA	1–18 µl
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). Other types of mRNA may require optimizing incubation time to obtain desired fragment size distribution.
3. Transfer tube to ice.
4. Add 2 µl 10X RNA Fragmentation Stop Solution.

Clean Up Fragmented RNA Using RNeasy MinElute Spin Columns (Strongly Recommended)

1. Add 78 µl of the nuclease-free water to the 22 µl fragmented RNA from step 4. Purify sample using RNeasy MinElute Cleanup Kit (Qiagen #74204) following manufacturer instructions. Elute in 15.5 µl nuclease-free water. The recovered volume should be ~14.5 µl.

Note: column purification removes short RNA Fragments and enriches the sample for RNA fragments longer than 200 nucleotides.

Alternatively, Clean Up Fragmented RNA Using Ethanol Precipitation

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

	Volume (µl)
Fragmented RNA from Step 4	22
3M Sodium Acetate, pH 5.5	2
Linear Acrylamide, 10 mg/ml	1–2
100% Ethanol	60
total volume	85–86

2. Incubate at –80°C or in a dry ice/methanol bath for 1 hour.
3. Centrifuge at 14,000 rpm for 25 minutes at 4°C in a microcentrifuge.

4. Carefully remove ethanol.
5. Wash pellet with 300 µl of freshly prepared 70% ethanol. Carefully pipette up and down the pellet. Make sure the pellet does not get stuck in the tip.
6. Centrifuge at 14,000 rpm for 5 minutes at 4°C in a microcentrifuge. Carefully remove all 70% ethanol.
7. Repeat steps 5 and 6 twice.

Note: Insufficient pellet washing results in inhibition of the first strand cDNA synthesis due to carryover of magnesium and EDTA. If the Bioanalyzer traces of the mRNA fragments show a noisy baseline (Figure 2) repeat the ethanol precipitation step and pellet washing steps

8. Air dry pellet for up to 10 minutes at room temperature (or longer if necessary) to remove residual ethanol.
9. Resuspend in 14.5 µl Nuclease-free Water.

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

Run 1 µl in the Agilent Bioanalyzer 2100 using a RNA Pico chip.

Note: Clean mRNA fragments with sufficient washing on the Bioanalyzer show a distinct band (Figure 1).

Figure 1: Bioanalyzer traces of clean mRNA Fragments. The mRNA fragments should have a normal distribution with a peak at 200 nucleotides.

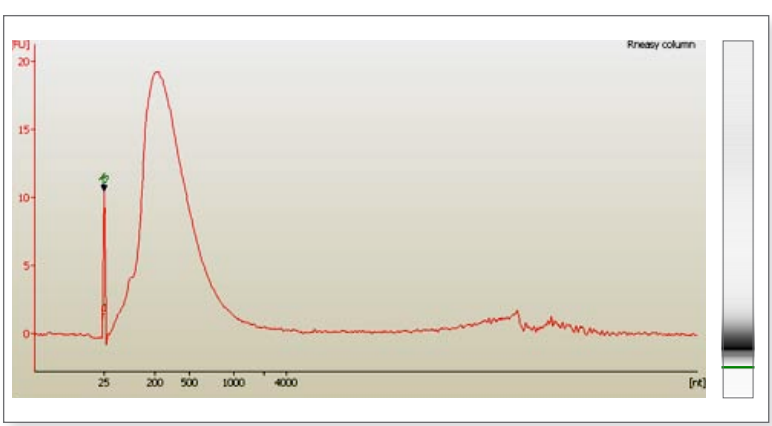
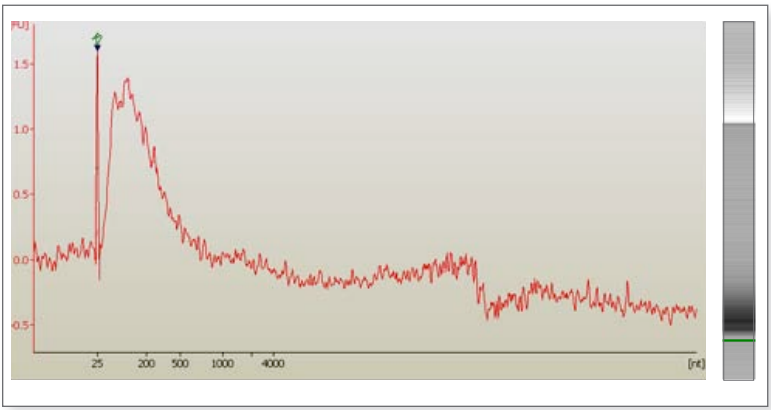


Figure 2: Bioanalyzer traces of clean mRNA fragments with insufficient washing show a noisy baseline.



First Strand cDNA Synthesis

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

Fragmented mRNA	13.5 µl
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:

5X First Strand Synthesis Reaction Buffer	4 µl
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 M-MuLV Reverse Transcriptase (RNase H^-) to the reaction.
7. Incubate sample 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.

Second Strand cDNA Synthesis

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

Nuclease-free Water	48 µl
10X Second Strand Synthesis Reaction Buffer	8 µl
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.

Purify the double-stranded cDNA using 1.8X Agencourt AMPure XP Beads (Recommended). Note: X refers to original sample volume.

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 10 minutes while the tube is on the magnetic stand with lid open.
8. Elute the DNA target from the beads into 52 µl water. Mix well on a vortex mixer or by pipetting up and down, and 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 tube.

Alternatively, purify the double-stranded cDNA using QIAquick PCR Column Purification Kit and elute in 52 µl nuclease-free water.

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	35 µl
NEBNext End Repair Reaction Buffer	10 µl
NEBNext End Repair Enzyme Mix	5 µl
Total volume	100 µl

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

Purify the end-repaired cDNA using 1.8X Agencourt AMPure XP Beads (Recommended).

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 10 minutes while the tube is on the magnetic stand with lid open.
8. Elute the DNA target from the beads into 45 µl water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear. Remove supernatant (42 µl) and transfer to a clean 1.5 ml LoBind tube.

Alternatively, purify the double-stranded cDNA using QIAquick PCR Column Purification Kit and elute in 42 µl nuclease free water.

dA-Tailing of cDNA Library

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

Purified, End Repaired cDNA	42 µl
10X NEBNext dA-Tailing Reaction Buffer	5 µl
Klenow Fragment (3´→5´ exo⁻)	3 µl
Total volume	50 µl

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

Purify the dA-Tailed DNA using 1.8X Agencourt AMPure XP Beads (Recommended).

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 10 minutes while the tube is on the magnetic stand with lid open.
8. Elute the DNA target from the beads into 40 µl water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear. Remove supernatant (38 µl) and transfer to a clean 1.5 ml LoBind tube.

Alternatively, purify the double-stranded cDNA using QIAquick PCR Column Purification Kit and elute in 40 µl nuclease free water.

Adaptor or Vector Ligation of cDNA Library

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

Purified, dA-Tailed cDNA	38 µl
5X NEBNext Quick Ligation Reaction Buffer	10 µl
DNA Adaptors (15 µM) or vector	1 µl
Quick T4 DNA Ligase	1 µl
Total volume	50 µl

2. Incubate 15 minutes at room temperature.

Purify using AMPure XP Beads (strongly recommended).

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 10 minutes while the tube is on the magnetic stand with lid open.

8. Elute the DNA target from the beads into 150 µl nuclease free water for bead-based size selection or into 20 µl nuclease free water for size selection using E-Gel size select gels. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear.

9. Remove supernatant and transfer to a clean 1.5 ml LoBind tube.

Alternatively, purify adaptor ligated DNA on a MinElute column and elute in the appropriate volume for desired method of size selection.

Size Selection of Adaptor-ligated DNA using Agencourt AMPure XP Beads or Invitrogen E-Gels (Strongly Recommended)

Caution: 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 10 minutes while the tube is on the magnetic stand with lid open.

10. Elute DNA target from beads into 25 µl nuclease free water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear.

11. Transfer 23 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 25 µl of nuclease free water.

Alternatively, Size Selection of Adaptor-ligated DNA can be Performed Using a 2% Agarose Gel

1. Purify the adaptor-ligated DNA using QIAGEN MinElute PCR Purification Kit and elute in 12 µl of nuclease free water.

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 is carried over during DNA elution. Residual ethanol interferes with the correct loading of the sample on the gel.

2. Pour a 5 mm thick 2% agarose gel with a 1 mm thick gel space and allow to cool.
3. Run adaptor ligated cDNA for 2 hours at 150 V or until the dye front has traveled 10 cm.
4. Isolate the desired cDNA fragment from the gel.
5. Purify DNA from the gel slice using a QIAquick Gel Extraction Purification Kit, purifying the sample on one column and elute In 25 µl of nuclease free water.

PCR Enrich Adaptor Ligated cDNA Library

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

Size Selected cDNA	23 µl
NEBNext High-Fidelity 2X PCR Master Mix*	25 µl
Primer 1 (25 µM)	1 µl
Primer 2 (25 µM)	1 µl
Total volume	50 µl

* NEBNext High-Fidelity 2X PCR Master Mix will be replacing Phusion High-Fidelity PCR Master Mix. Both vials will be supplied for a limited time only.

2. PCR cycling conditions:

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	10 seconds	1
Denaturation Annealing Extension	98°C 65°C 72°C	10 seconds 30 seconds 30 seconds	10–12*
Final Extension	72°C 4°C	5 minutes hold	1

*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.

Purify using AMPure XP Beads (Recommended)

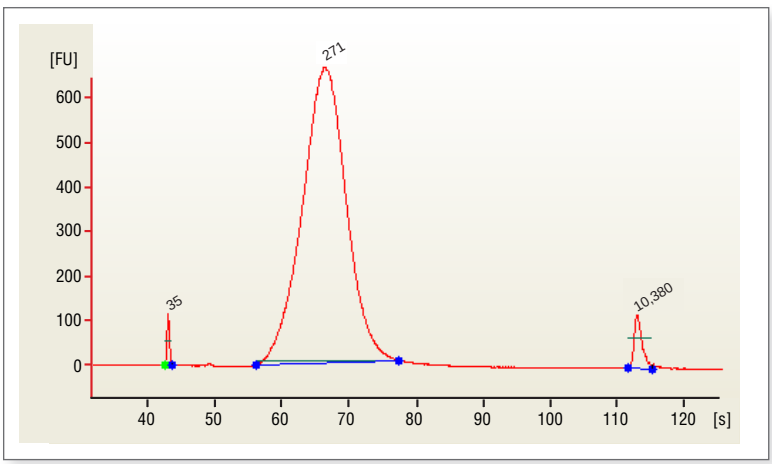
1. Vortex AMPure XP beads to resuspend.
2. Add 60 µl (1.2X) of resuspended AMPure XP beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Place the tube on an appropriate magnetic stand to separate 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.
8. Elute the DNA target from the beads into 22.5 µl 0.1X TE Buffer. Mix well on a vortex mixer or by pipetting up and down, and 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, and store at –20°C.

Alternatively, purify the PCR enriched cDNA using a QIAquick PCR Column Purification Kit and elute in 20 µl of sterile water or elution buffer.

Assess Library Quality on a Bioanalyzer

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

Figure 3: Example of RNA 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 degradation of RNA 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

Linear Acrylamide

#E6103A: 0.024 ml **Concentration: 10 mg/ml**
#E6103AA: 0.120 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 10 µl reaction containing 1 µg Linear Acrylamide 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 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

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 degradation of RNA 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

NEBNext End Repair Enzyme Mix

#E6041A: 0.06 ml
#E6041AA: 0.300 ml

Store at –20°C

Description: NEBNext End Repair Enzyme Mix is optimized to convert 1 to 5 µg of fragmented to repaired DNA having 5´-phosphorylated, blunt ends.

NEBNext End Repair Enzyme Mix:
10,000 units/ml T4 Polynucleotide Kinase
3,000 units/ml T4 DNA Polymerase

Storage Conditions:
10 mM Tris-HCl
100 mM KCl
1 mM DTT
0.1 mM EDTA
50% Glycerol
0.1% Triton X-100
pH 7.4 @ 25°C

Quality Control Assays

SDS-PAGE Purity: SDS-PAGE analyses of each individual enzyme indicates > 95% enzyme purity.

Endonuclease Activity: Incubation of a minimum of 10 µl of this enzyme mix 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 10 µl of this 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 (Nucleotide Incorporation): 0.2 µl of this enzyme mix 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.

Functional Activity (Nucleotide Incorporation and Phosphorylation): 5 µl of this enzyme mix repairs and phosphorylates the ends of > 95% of 10 µg of DNA fragments containing both 3´ and 5´ overhangs within 30 minutes at 20°C in 1X End Repair Buffer, as determined by capillary electrophoresis.

Lot Controlled

NEBNext End Repair Reaction Buffer

#E6042A: 0.12 ml **Concentration: 10X**
#E6042AA: 0.6 ml

Store at –20°C

1X NEBNext End Repair Reaction Buffer:
50 mM Tris-HCl
10 mM $MgCl_2$
10 mM DTT
1 mM ATP
0.4 mM dATP
0.4 mM dCTP
0.4 mM dGTP
0.4 mM dTTP
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

Klenow Fragment (3´→5´ exo⁻)

#E6044A: 0.036 ml
#E6044AA: 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 5´→3´ exonuclease activity. Mutations (D355A, E357A) abolish the 3´→5´ exonuclease activity (1). Klenow Fragment (3´→5´ exo⁻) with dA-Tailing buffer can be used to add a dAMP to the 3´ end at a blunt DNA fragment (2).

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.
2. Clark, J.M. et al. (1987) *J. Mol. Biol.* 198(1); 123–127.

NEBNext dA-Tailing Reaction Buffer

#E6045A: 0.06 ml **Concentration: 10X**
#E6045AA: 0.3 ml

Store at –20°C

1X NEBNext dA-Tailing Reaction Buffer:
10 mM Tris-HCl
10 mM $MgCl_2$
50 mM NaCl
1 mM DTT
0.2 mM dATP
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.

M-MuLV Reverse Transcriptase (RNase H$^-$)

#E6014A: 0.015 ml **Concentration: 200,000 U/ml**
#E6014AA: 0.06 ml

Store at –20°C

Description: M-MuLV Reverse Transcriptase (RNase H$^-$) 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 M-MuLV Reverse Transcriptase (RNase H$^-$) 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 M-MuLV Reverse Transcriptase (RNase H$^-$) 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 M-MuLV Reverse Transcriptase (RNase H$^-$) 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 M-MuLV Reverse Transcriptase (RNase H$^-$) 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): M-MuLV Reverse Transcriptase (RNase H$^-$) is > 95% pure as determined by SDS PAGE analysis using Coomassie blue detection.

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NEBNext Quick Ligation Reaction Buffer

#E6048A: 0.12 ml **Concentration: 5X**
#E6048AA: 0.6 ml

Store at –20°C

1X NEBNext Quick Ligation Reaction Buffer:
66 mM Tris-HCl
10 mM $MgCl_2$
1 mM dithiothreitol
1 mM ATP
6% 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 (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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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

Phusion High-Fidelity PCR Master Mix with HF Buffer

E6012A: **0.3 ml** **Concentration: 2X**
E6012AA: **1.5 ml**

Store at –20°C

Storage and Stability: Store at –20°C. After thawing the mix can be refrozen or optionally stored at 4°C for three months.

Component Specifications: 2X Phusion High-Fidelity Master Mix with HF Buffer contains 0.04 units/µl Phusion DNA Polymerase, 2X Phusion HF Buffer (provides 1.5 mM $MgCl_2$ in final reaction concentration), and 400 µM of each dNTP.

Manufactured and quality controlled by New England Biolabs, Inc., Thermo Scientific Phusion® High-Fidelity DNA Polymerase is purified from an *E. coli* strain expressing the cloned Phusion DNA Polymerase gene. This DNA polymerase possesses the following activities: 5´→3´ DNA polymerase activity and 3´→5´ exonuclease activity. Phusion DNA Polymerase is purified free of contaminating endo- and exonucleases.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1X Phusion Master Mix and 1 µg of HaeIII digested ϕX174 RF I 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 Phusion Master 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.

Phosphatase Activity: Incubation of 1X Phusion 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.

7.5 kb Genomic and 20 kb Lambda PCR: 30 cycles of PCR amplification in a 50 µl reaction containing 50 ng genomic DNA with 1X Phusion High-Fidelity PCR Master Mix with HF Buffer and 1.0 µM primers results in the expected 7.5 kb product.

20 kb Lambda DNA PCR: 22 cycles of PCR amplification in a 50 µl reaction containing 10 ng Lambda DNA with 1X Phusion High-Fidelity PCR Master Mix with HF Buffer and 1.0 µM primers results in the expected 20 kb product.

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Notice to Customer: Phusion® DNA Polymerase was developed by Finnzymes Oy, now a part of Thermo Fisher Scientific.

NEBNext High-Fidelity 2X PCR Master Mix

E6013A: 0.3 ml **Concentration: 2X**
E6013AA: 0.75 ml

Store at –20°C

Description: The NEBNext High-Fidelity 2X 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 High-Fidelity DNA Polymerase also has an ultra-low error rate (> 50-fold lower than that of *Taq* DNA Polymerase and 6-fold lower than that of *Pyrococcus furiosus* (Pfu) DNA Polymerase)

Quality Control Assays

16-Hour Incubation: A 50 µl reactions containing NEBNext High-Fidelity 2X PCR 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 100 units of NEBNext High-Fidelity 2X 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 High-Fidelity 2X 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.

GC-Rich Genomic PCR: 30 cycles of PCR amplification of 20 ng genomic DNA in a 50 µl reaction containing 0.5 µM primers and 1X NEBNext High-Fidelity PCR Master Mix result in the expected 737 bp product.

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