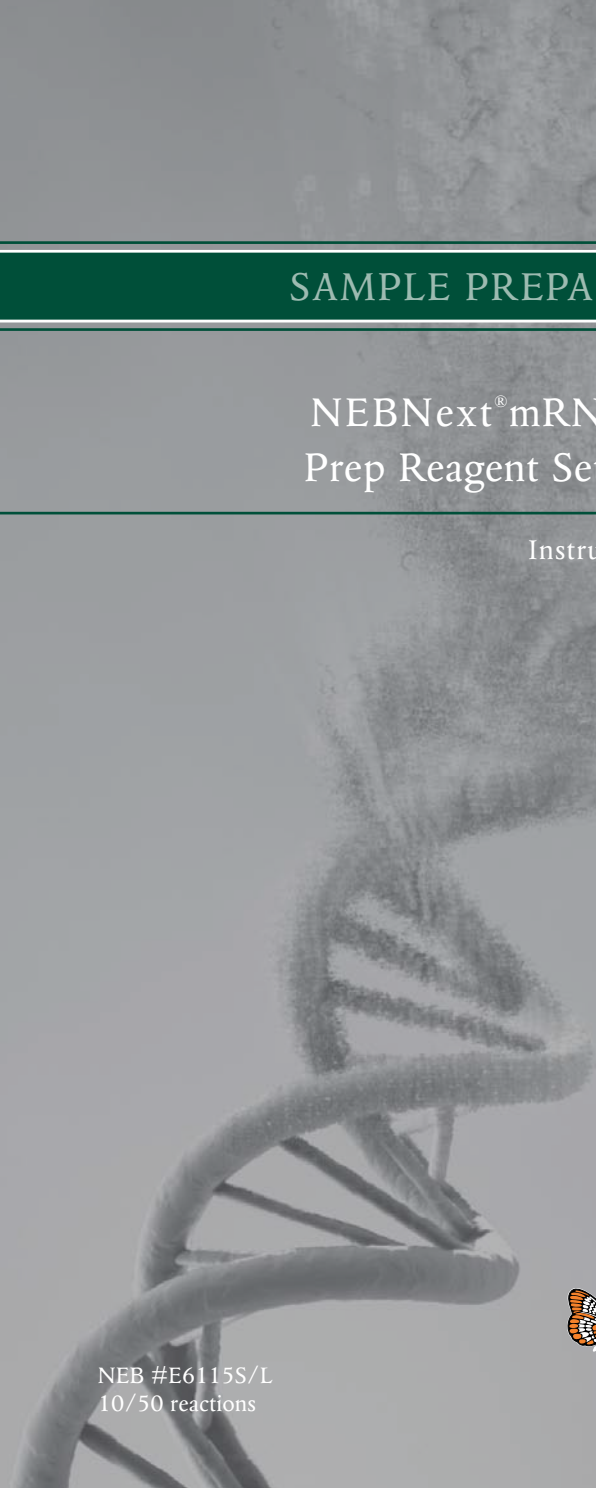
SAMPLE PREPARATION

NEBNext® mRNA Library Prep Reagent Set for 454™

Instruction Manual



NEB #E6115S/L
10/50 reactions

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

Table of Contents:

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

The Reagent Set Includes:

The volumes provided are sufficient for preparation of up to 10 reactions (NEB #E6115S) and 50 reactions (NEB #E6115L). (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 RR
NEBNext First Strand Synthesis Reaction Buffer (5X)
NEBNext Second Strand Synthesis Enzyme Mix
NEBNext Second Strand Synthesis Reaction Buffer (10X)
NEBuffer 2 (10X)
Deoxynucleotide Solution Mix (10 mM each dNTP)
T4 DNA Polymerase RR
Adenosine 5´- Triphosphate (10 mM)
T4 Polynucleotide Kinase RR
Taq DNA Polymerase RR
Quick T4 DNA Ligase RR
NEBNext Sizing Buffer
TE Buffer

Required Materials Not Included:

Agencourt AMPure® Beads (Beckman Coulter, Inc.)
Magnetic Separation Rack (NEB #S1510)
3 M Sodium Acetate, pH 5.5
100% Ethanol
70% Ethanol
SuperScript® II Reverse Transcriptase (Life Technologies, Inc.)
PCR Column Purification Kit (Qiagen or other)
RNeasy® MinElute® Columns (Qiagen or other)
DNA Adaptors and Primers or Vector

Applications:

The NEBNext mRNA Library Prep Reagent Set for 454 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 454 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 a 454 GS FLX Titanium™ System (Roche).

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:

Starting Material: 100–200 ng purified mRNA, 200 ng recommended.

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 1–5 minutes at 94°C. Fragmentation time should be adjusted depending on type of RNA and desired sizes of fragments (see Figure 1).
3. Transfer tube to ice.
4. Add 2 µl 10X RNA Fragmentation Stop Solution.

Clean Up Fragmented RNA Using RNeasy MinElute Spin Columns

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 manufacture 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

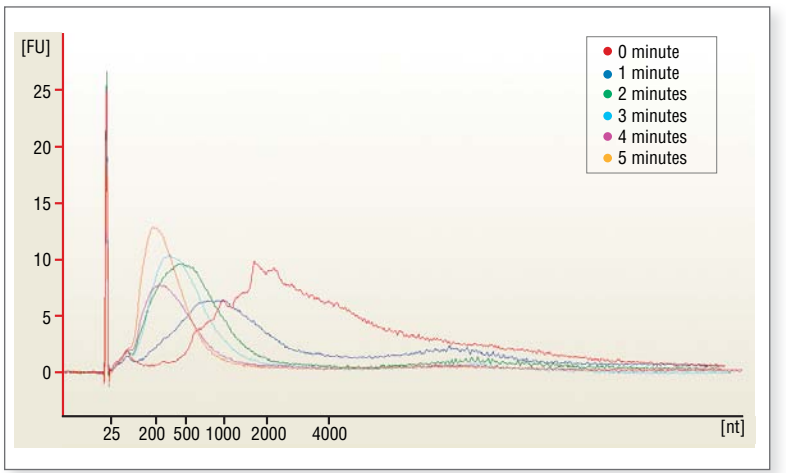
2. Incubate at –80°C for 30 minutes.
3. Centrifuge at 14,000 rpm for 25 minutes at 4°C in a microcentrifuge.
4. Carefully remove ethanol.
5. Wash pellet with 300 µl of 70% ethanol.

6. Centrifuge and carefully remove 70% ethanol.
7. Air dry pellet for up to 10 minutes at room temperature to remove residual ethanol.
8. Resuspend in 14.5 µl Nuclease-Free Water.

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

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

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



Poly (A)+ mRNA (40 ng) purified from the Universal Human Reference Total RNA (Stratagene) was fragmented in 1X NEBNext Magnesium RNA Fragmentation Buffer for 1–5 minutes at 94°C. Fragmentation Reaction was stopped in 1X NEBNext Fragmentation Stop Solution. Samples were diluted 1:10 in Nuclease-Free Water and analyzed in the Bioanalyzer 2100.

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 SuperScript II Reverse Transcriptase 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 48 µl Nuclease-Free Water to the First Strand Synthesis reaction.
2. To the reaction then add:

10X Second Strand Synthesis Reaction Buffer	8 µl
Second Strand Synthesis Enzyme Mix	4 µl
Total volume	80 µl

3. Mix thoroughly by gentle pipetting.
4. Incubate in a thermal cycler for 2.5 hours at 16°C.
5. Purify cDNA using a PCR column purification kit, purifying the sample on one column and eluting in 16 µl sterile water or elution buffer.

NEBNext End Repair and dA-Tailing

1. In a 1.7 ml micro-centrifuge tube add:

NEBuffer 2 (10X)	2.5 µl
ATP	2.5 µl
dNTP Mix	1.0 µl
T4 DNA Polymerase	1.0 µl
PNK	1.0 µl
Taq DNA Polymerase	1.0 µl
	9.0 µl

2. Mix by pipetting and add to the 16 µl purified, double stranded cDNA.
3. Vortex briefly to mix, followed by a quick spin to collect all liquid from the sides of the tube.
4. In a thermocycler, with the heated lid on, run the following program:
   20 minutes at 25°C
   20 minutes at 72°C
   Hold at 4°C

Agencourt AMPure Bead Preparation

1. Vortex AMPure beads to re-suspend.
2. Transfer 125 µl of AMPure beads to a 1.7 ml micro-centrifuge tube.
3. Place the tube on a Magnetic Separation Rack. After the beads have collected to the side of the tube and the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads.
4. Add 73 µl of TE to the beads and vortex until the beads are completely re-suspended.
5. Add 500 µl of NEBNext Sizing Buffer to the beads, vortex to mix and spin briefly to collect all liquid from the sides of the tube.

NEBNext Adaptor Ligation

1. After completion of the End Repair/dA-Tailing steps, add 1.0 µl of adaptor (or vector if generating an expression library), to the reaction tube. (Adaptors/vectors are not provided. Please use adaptors/vectors appropriate to specific application).
2. Add 1.0 µl of Quick T4 DNA Ligase. Vortex briefly to mix, followed by a quick spin.
3. Incubate for 10 minutes at 25°C.

NEBNext Small Fragment Removal

1. Add the End Repaired/dA-Tailed/Adaptor ligated DNA sample directly to the previously prepared AMPure beads. Vortex briefly to mix, followed by a quick spin to collect liquid from the sides of the tube.
2. Incubate at room temperature for 5 minutes.
3. Place the tube on a Magnetic Separator.
4. When the beads have collected to the wall of the tube and the solution is clear, remove and discard the supernatant. Be careful not to disturb the beads.
5. Add 100 µl of TE and vortex until the beads are completely re-suspended.
6. Add 500 µl of NEBNext Sizing Buffer and briefly vortex to mix.
7. Incubate at room temperature for 5 minutes.
8. Place the tube on a Magnetic Separator.
9. When the beads are collected to the wall of the tube and the solution is clear, remove and discard the supernatant. Be careful not to disturb the beads.

10. Repeat steps 5-9 one time.

11. Keep the tube on the magnet and wash the beads twice with 1 ml of 70% ethanol.

12. Keep the tube on the magnet, uncapped, and let the pellet air dry until there is no visible liquid on the sides of the tube. This typically takes 5 minutes.

13. Remove the tube from the magnet, add 53 µl of TE, vortex to re-suspend the beads and spin briefly.

14. Place the tube on the magnet, when the beads are collected to the wall of the tube, transfer 50 µl of the supernatant (library), to a new 1.7 ml micro-centrifuge tube. Be careful not to transfer any beads.

NEBNext RNA Fragmentation Buffer

#E6101A: 40 µl **Concentration: 10X**
#E6101AA: 200 µl

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: 40 µl **Concentration: 10X**
#E6102AA: 200 µl

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: 20 µl **Concentration: 10 mg/ml**
#E6103AA: 100 µl

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: 50 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: 25 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: 40 µl **Concentration: 5X**
#E6106AA: 200 µl

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: 40 µl
#E6107AA: 200 µl

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: 80 µl **Concentration: 10X**
#E6108AA: 400 µl

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

NEBuffer 2

#E6081A: 0.025 ml **Concentration: 10X**
#E6081AA: 0.125 ml

Store at –20°C

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

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1X NEBuffer 2 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 NEBuffer 2 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 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 a 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

Adenosine 5´-Triphosphate (ATP)

#E6082A: 0.025 ml **Concentration: 10 mM**
#E6082AA: 0.125 ml

Store at –20°C

Description: Adenosine 5´-Triphosphate (ATP) is a substrate for ATP-dependent enzyme systems.

Supplied in: Sterile purified water adjusted to pH 7.0 with NaOH

Molecular Weight: 551.2 daltons (disodium salt)

Quality Control Assays

Phosphatase Activity: Incubation of a minimum of 1 mM ATP 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.

16-Hour Incubation: 50 µl reactions containing a minimum of 0.2 mM ATP 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 0.2 mM ATP 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 a minimum of 0.1 mM ATP with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Lot Controlled

Deoxynucleotide Solution Mix

#E6083A: 0.015 ml **10 mM each dNTP**
#E6083AA: 0.075 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 5 kb amplicons from lambda DNA.

Lot Controlled

T4 DNA Polymerase

#E6084A: 0.015 ml
#E6084AA: 0.075 ml

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.

T4 Polynucleotide Kinase

#E6085A: 0.015 ml
#E6085AA: 0.075 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.

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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.

Taq DNA Polymerase

#E6086A: 0.015 ml
#E6086AA: 0.075 ml

Store at –20°C

Description: *Taq* DNA Polymerase is a thermostable DNA polymerase that possesses a 5´→3´ polymerase activity and a double-strand specific 5´→3´ exonuclease activity.

Source: Purified from an *E. coli* strain that carries the *Taq* DNA polymerase gene from *Thermus aquaticus* YT-1.

Supplied in: 10 mM Tris-HCl, 100 mM KCl, 1 mM Dithiothreitol, 0.1 mM EDTA, 50% Glycerol, 0.5% Tween-20 and 0.5% NP-40, pH 7.4 @ 25°C.

Quality Control Assays

Endonuclease Activity: Incubation of a 50 µl reaction in ThermoPol Reaction Buffer containing a minimum of 20 units of *Taq* DNA Polymerase with 1 µg of supercoiled ϕX174 DNA for 4 hours at 75°C results in < 10% conversion to the nicked form as determined by agarose gel electrophoresis.

3´→5´ Exonuclease Activity: Incubation of a 20 µl reaction in ThermoPol Reaction Buffer containing a minimum of 20 units of *Taq* DNA Polymerase with 10 nM fluorescent internally labeled oligonucleotide for 30 minutes at either 37°C or 75°C yields no detectable 3´→5´ degradation as determined by capillary electrophoresis.

Functional Activity (Nucleotide Incorporation): 25 cycles of PCR amplification of 5 ng Lambda DNA with 1.25 units of *Taq* DNA Polymerase, in the presence of 200 µM dNTPS and 0.2 µM primers in ThermoPol Reaction Buffer, results in the expected 5 kb product.

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Quick T4 DNA Ligase

#E6087A: 0.015 ml
#E6087AA: 0.075 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 (Adapter Ligation): 50 µl reactions containing 0.125 µl Quick T4 DNA Ligase, 8 nmol 12 bp adapter, and 1X T4 DNA Ligase Buffer incubated at 16°C overnight results in no detectable unligated adapter 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.

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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.

NEBNext Sizing Buffer

#E6088A: 15 mls **Concentration: 1.2X**
#E6088AA: 75 mls

Store at –20°C

1X NEBNext Sizing Buffer:
7% Polyethylene Glycol 8000
1 M NaCl

Description: NEBNext Sizing Buffer has been optimized for use with AMPure beads to select DNA fragments > 300 bp from a sample of mixed sized DNA fragments.

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.

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.

Functional Assay (Size Selection): Two rounds of selection, using 500 µl of this buffer and 125 µl of Agencourt AMPure beads, results in the selection of DNA Fragments > 300 bp from 0.5 µg of 100 kb DNA ladder (NEB# N3231), as determined by Bioanalyzer analysis.

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TE Buffer

#E6089A: 5 ml **Concentration: 1X**
#E6089AA: 25 ml

Store at –20°C

Description: TE Buffer is free of detectable DNA and RNA nucleases and is suitable for use in molecular biology applications.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing TE, 1X NEBuffer 2 (500 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9) 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 TE, 1X NEBuffer 2 (500 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9) 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 TE, 1X NEBuffer 2 (500 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9) and 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 TE, 1X NEBuffer 2 (500 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9) and 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 TE 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.

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