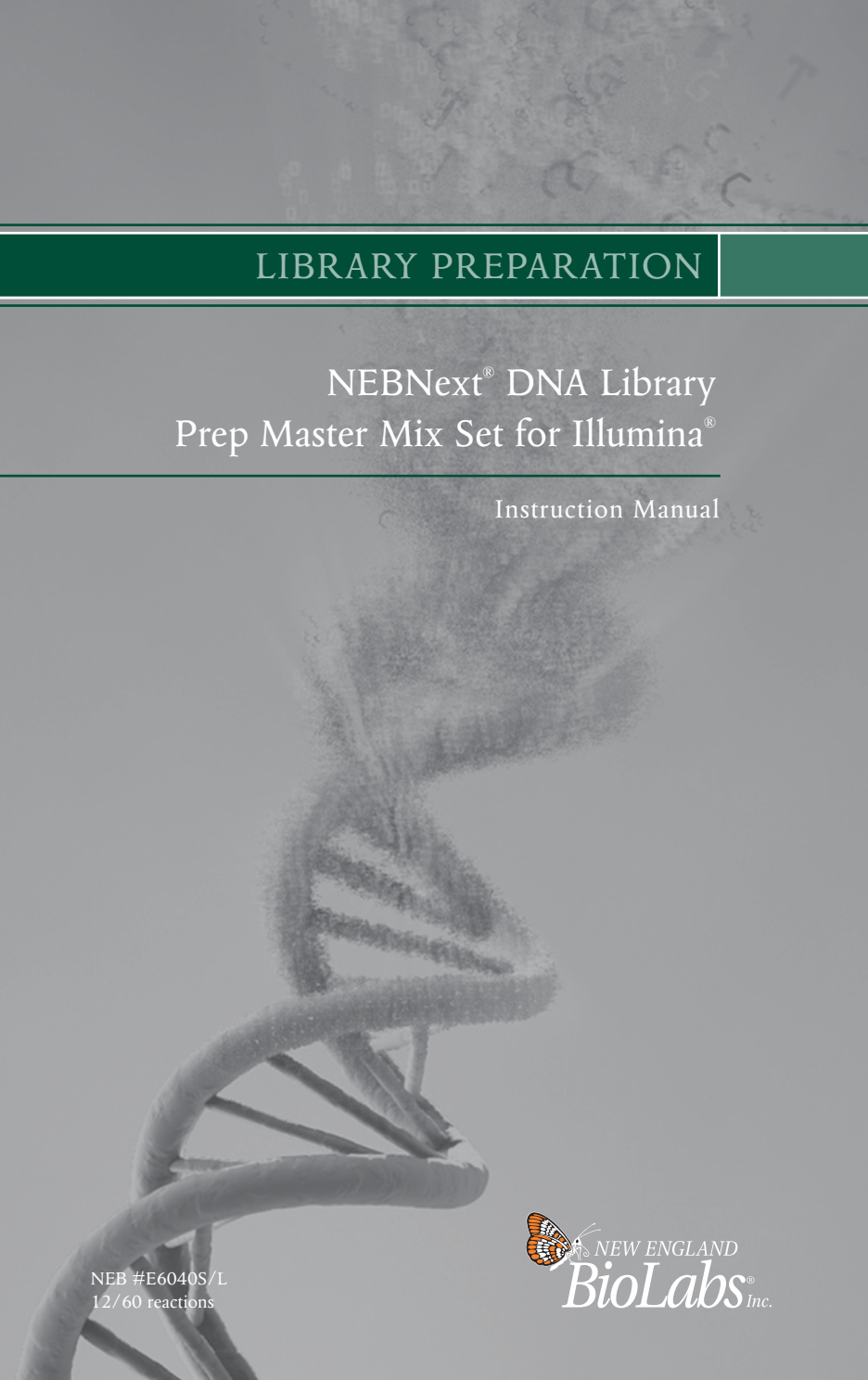
LIBRARY PREPARATION

NEBNext® DNA Library Prep Master Mix Set for Illumina®

Instruction Manual



NEB #E6040S/L
12/60 reactions

NEW ENGLAND BioLabs® Inc.

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nqa ISO 9001 Registered Quality Management

nqa ISO 14001 Registered Environmental Management

nqa ISO 13485 Registered Medical Devices

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

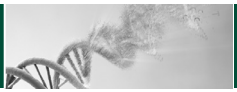


Table of Contents:

The Library Kit Includes:

The volumes provided are sufficient for preparation of up to 12 reactions (NEB #E6040S) and 60 reactions (NEB #E6040L). (All reagents should be stored at –20°C):

- (green) NEBNext End Repair Enzyme Mix
- (green) NEBNext End Repair Reaction Buffer (10X)
- (yellow) Klenow Fragment (3´→5´ exo⁻) RR
- (yellow) NEBNext dA-Tailing Reaction Buffer (10X)
- (red) Quick T4 DNA Ligase RR
- (red) NEBNext Quick Ligation Reaction Buffer (5X)
- (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix

Required Materials Not Included:

80% Ethanol (freshly prepared)

Nuclease-free Water

0.1X TE, pH 8.0

10 mM Tris-HCl, pH 7.5–8.0 (optional)

DNA LoBind Tubes (Eppendorf #022431021)

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

NEBNext Singleplex or Multiplex Oligos for Illumina (E7350, E7335, E7500 or E7600)

Magnetic rack/stand

PCR Machine

Applications:

The NEBNext DNA Library Prep Master Mix Set for Illumina contains enzymes and buffers in convenient master mix formulations 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 DNA 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.

Functional Validation: Each set of reagents is functionally validated together through construction and sequencing of a genomic DNA library on an Illumina Sequencer (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.

Protocols:

Symbols

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

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

Starting Material: 1–5 µg of Fragmented DNA

1.1 End Repair of Fragmented DNA

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

Fragmented DNA	1–85 µl
● (green) NEBNext End Repair Reaction Buffer (10X)	10 µl
● (green) NEBNext End Repair Enzyme Mix	5 µl
Sterile H_2O	variable
Total volume	100 µl

2. Incubate in a thermal cycler for 30 minutes at 20°C.

1.2 Cleanup Using AMPure XP® Beads (Beckman Coulter, Inc.)

1. Vortex AMPure XP Beads to resuspend.
2. Add 160 µl (1.6X) of resuspended AMPure XP Beads to the ligation reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Put the tube/PCR plate 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 the DNA targets.
5. Add 200 µl of 80% freshly prepared ethanol to the tube/PCR plate 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 beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

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

8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 47 µl of 10 mM Tris-HCl or 0.1X TE.

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

10. Put the tube/PCR plate in the magnetic stand until the solution is clear.Without disturbing the bead pellet, carefully transfer 42 µl of the supernatant to a fresh, sterile microfuge tube.

1.3 dA-Tailing of End Repaired DNA

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

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

2. Incubate in a thermal cycler for 30 minutes at 37°C.

1.4 Cleanup Using AMPure XP Beads

1. Vortex AMPure XP Beads to resuspend.

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

3. Incubate for 5 minutes at room temperature.

4. Put the tube/PCR plate 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 the DNA targets.

5. Add 200 µl of 80% freshly prepared ethanol to the tube/PCR plate 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 beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

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

8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 30 µl of 10 mM Tris-HCl or 0.1X TE.

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

10. Put the tube/PCR plate in the magnetic stand until the solution is clear.Without disturbing the bead pellet, carefully transfer 25 µl of the supernatant to a fresh, sterile microfuge tube.

1.5 Adaptor Ligation of dA-Tailed DNA

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

dA-Tailed DNA	25 µl
● (red) Quick Ligation Reaction Buffer (5X)	10 µl
● (red) NEBNext Adaptor* (15 µM)	10 µl
● (red) Quick T4 DNA Ligase	5 µl
Total volume	50 µl

* Adaptors can be purchased separately under NEB #E7335, #E7350, #E7500, #E7600, #E6609

2. Incubate in a thermal cycler for 15 minutes at 20°C.
3. Add 3 µl of ● (red) USER™ Enzyme Mix by pipetting up and down, and incubate at 37°C for 15 minutes.

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

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

1.6 Cleanup of Adaptor Ligated DNA

1. Vortex AMPure XP Beads to resuspend.
2. Add 90 µl of resuspended AMPure XP Beads to the ligation reaction (~53 µl). Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Put the tube/PCR plate 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 the DNA targets.
5. Add 200 µl of 80% freshly prepared ethanol to the tube/PCR plate 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 beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

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

8. Remove the tube/plate from the magnet. Elute the DNA target by adding 105 µl of 10 mM Tris-HCl or 0.1 X TE to the beads for bead-based size selection.

   Note: For size selection using E-Gel size select gels or standard 2% agarose gels, elute the DNA target at desired volume.

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

10. Put the tube/PCR plate in the magnetic stand until the solution is clear. Transfer 100 µl of supernatant (or desired volume) to a new tube/well, and proceed to bead based size selection.

1.7 Size Select Adaptor Ligated DNA Using AMPure XP Beads

Insert Size	150 bp	200 bp	250 bp	300 bp	400 bp	500 bp	700 bp
Total library size (insert + adaptor)	270 bp	320 bp	370 bp	420 bp	530 bp	660 bp	820 bp
Bead: DNA ratio* 1st bead selection	0.9X	0.8X	0.7X	0.6X	0.55X	0.5X	0.45X
Bead: DNA ratio* 2nd bead selection	0.2X	0.2X	0.2X	0.2X	0.15X	0.15X	0.15X

Table 1.1: Recommended conditions for dual bead-based size selection.

⚠ ***The following size selection protocol is for libraries with 200 bp inserts only. For libraries with different size fragment inserts, please optimize bead: DNA ratio according to Table 1.1 above.***

Note: (X) refers to the original sample volume of 100 µl

1. Add 80 µl (0.8X) resuspended AMPure XP Beads to 100 µl DNA solution. 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/PCR plate 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/well (**Caution: do not discard the supernatant**). Discard beads that contain the large fragments.

4. Add 20 µl (0.2X) resuspended AMPure XP Beads to the supernatant, mix well and incubate for 5 minutes at room temperature.

5. Put the tube/PCR plate 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/PCR plate 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. Air dry beads for 5 minutes while the tube/PCR plate Is on the magnetic stand with the lid open.

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

9. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 17 µl of 10 mM Tris-HCl or 0.1X TE.

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

11. Put the tube/PCR plate in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 15 µl of the supernatant to a clean PCR tube and proceed to enrichment.

1.8 PCR Enrichment of Adaptor Ligated DNA

⚠ **Note: NEBNext Singleplex and Multiplex Oligos for Illumina (NEB #E7350, #E7335 and #E7500) now have new primer concentrations (10 µM). Please check oligo kit lot numbers to determine how to set up your PCR reaction.**

Follow Section 1.8A if you are using the following oligos (10 µM primer):

NEBNext Singleplex Oligos for Illumina (NEB #E7350) lot 0071412

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335) lot 0091412

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500) lot 0071412

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

Follow Section 1.8B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

Follow Section 1.8C if you are using the following oligos (25 µM primer):

NEBNext Singleplex Oligos for Illumina (NEB #E7350) lots 0051402 or 0061410

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335) lots 0071402 or0081407

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500) lots 0051402 or 0061407

1.8A PCR Enrichment of Adaptor Ligated DNA

1. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	15 µl
(blue) Index Primer/i7 Primer*,**	5 µl
(blue) Universal PCR Primer/i5 Primer*,***	5 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
Total volume	50 µl

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

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

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

2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	2–4*
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* If library construction was performed with 5 µg of starting material, use 2-3 cycles of amplification. If starting material was 1 µg, use 4 cycles of amplification. However, optimization of PCR cycle number may be required to avoid over-amplification.

3. Proceed to Cleanup Using Ampure XP Beads in Section 1.9

1.8B PCR Enrichment of Adaptor Ligated DNA

1. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	15 µl
(blue) Index/ Universal Primer Mix*	10 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
Total volume	50 µl

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

2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	2–4*
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

*If library construction was performed with 5 µg of starting material, use 2-3 cycles of amplification. If starting material was 1 µg, use 4 cycles of amplification. However, optimization of PCR cycle number may be required to avoid over-amplification.

3. Proceed to Cleanup Using Ampure XP Beads in Section 1.9

1.8C PCR Enrichment of Adaptor Ligated DNA

1. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	15 µl
(blue) Index Primer*,**	2.5 µl
(blue) Universal PCR Primer*	2.5 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
Sterile H_2O	5 µl
Total volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335 or #E7500) Oligos for Illumina.

** For use with NEBNext Multiplex Oligos (NEB #E7335 or #E7500) use only one Index Primer per PCR reaction.

2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	2–4*
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

*If library construction was performed with 5 µg of starting material, use 2-3 cycles of amplification. If starting material was 1 µg, use 4 cycles of amplification. However, optimization of PCR cycle number may be required to avoid over-amplification.

3. Proceed to Cleanup Using Ampure XP Beads in Section 1.9

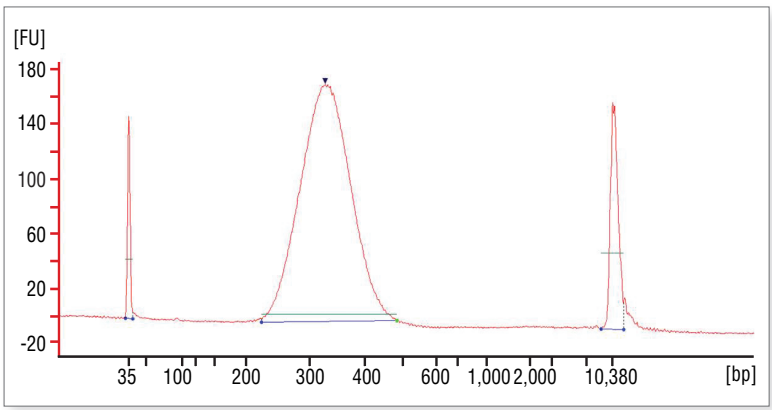
1.9 Cleanup Using AMPure XP Beads

1. Vortex AMPure XP Beads to resuspend.
2. Add 45 µl (0.9X) of resuspended AMPure XP Beads to the PCR reactions (~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. Put the tube/PCR plate 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 the DNA targets.
5. Add 200 µl of freshly prepared 80% ethanol to the tube/PCR plate 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/PCR plate is on the magnetic stand with the lid open.

   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 30 µl of 0.1X TE.
9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
10. Put the tube/PCR plate in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 25 µl of the supernatant to a clean LoBind® (Eppendorf AG) tube. Libraries can be stored at –20°C.

11. Dilute 2–3 µl of the library 20 fold with 10 mM Tris-HCl or 0.1X TE and assess the library quality on a Bioanalyzer® (Agilent Technologies, Inc.) high sensitivity chip. Check that the electropherogram shows a narrow distribution with a peak size approximately 300–320 bp.

Figure 1.1: Example of DNA library size distribution on a Bioanalyzer.



NEBNext End Repair Enzyme Mix

#E6041A: 0.06 ml
#E6041AA: 0.3 ml

Store at –20°C

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

NEBNext End Repair Enzyme Mix:
T4 Polynucleotide Kinase
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 analysis 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: 0.120 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: 0.180 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

#E6047A: 0.06 ml
#E6047AA: 0.3 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 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.

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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NEBNext Q5 Hot Start HiFi PCR Master Mix

E6630A: 0.3 ml **Concentration: 2X**
E6630AA: 1.75 ml (2 vials provided)

Store at –20°C

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

Quality Control Assays

16-Hour Incubation: A 50 µl reaction containing NEBNext Q5 Hot Start HiFi PCR Master Mix and 1 µg of HindIII digested λ DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing NEBNext Q5 Hot Start HiFi PCR Master Mix and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of NEBNext Q5 Hot Start HiFi PCR Master Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

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

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Revision History:

Revision #	Description
4.0	N/A
5.0	Include protocol for use with NEBNext Q5 Hot Start HiFi PCR Master Mix. Include protocol for changes in concentration of NEBNext Singleplex and Multiplex Oligos for Illumina. Changed all AMPure Bead drying times after ethanol washes to 5 minutes. Changed all AMPure Bead elutions to 0.1X TE or 10 mM Tris-HCl. Changed ratio of AMPure Beads to 0.9X in final cleanup after PCR reaction. Added 2 minute incubation after eluting DNA from AMPure beads. Changed PCR cycle number recommendations.
6.0	Remove protocol for use with NEBNext High-Fidelity 2X PCR Master Mix. Include protocol for use with NEBNext Multiplex Oligos (96 Index Primers, NEB #E6609).

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