LIBRARY PREPARATION

NEBNext® Ultra™ DNA Library Prep Kit for Illumina®

Instruction Manual

NEB #E7370S/L
24/96 reactions

NEW ENGLAND BioLabs® Inc.

Sign up for the NEBNext e-newsletter

Scan this code or visit www.neb.com/NEBNextnews2 to sign up for the NEBNext bimonthly e-newsletter to learn about new NEBNext products, recent publications and advances in library prep for next gen sequencing.

NEBNEXT®, NEW ENGLAND BIOLABS® and Q5® are registered trademarks of New England Biolabs, Inc.
LITMUS™, USER™ and ULTRA™ are trademarks of New England Biolabs, Inc.
AMPURE® is a registered trademark of Beckman Coulter, Inc.
ILLUMINA® is a registered trademark of Illumina, Inc.

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

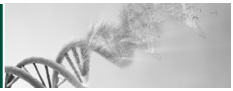


Table of Contents:

Applications....2
Protocol....3
NEBNext End Prep Enzyme Mix....12
NEBNext End Repair Reaction Buffer....13
Blunt/TA Ligase Master Mix....14
NEBNext Ligation Enhancer....15
NEBNext Q5 Hot Start HiFi PCR Master Mix....16
Revision History....17

The Library Kit Includes:

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7370S) and 96 reactions (NEB #E7370L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

- (green) NEBNext End Repair Reaction Buffer (10X)
- (green) NEBNext End Prep Enzyme Mix
- (red) Blunt/TA Ligase Master Mix
- (red) NEBNext Ligation Enhancer
- (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 (required for DNA input <100 ng)

10 mM NaCl (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 Ultra DNA Library Prep Kit for Illumina contains enzymes and buffers that are ideal to convert a small amount of DNA input into indexed libraries for next-generation sequencing on the Illumina platform (Illumina, Inc). The workflow of NEBNext Ultra DNA Library Prep Kit for Illumina is very user-friendly and fast with minimal hands-on time. 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 Ultra DNA Library Prep Kit for Illumina are managed separately and 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 an indexed DNA library on the Illumina sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

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: 5 ng–1 µg fragmented DNA.

1.1 NEBNext End Prep

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

● (green) End Prep Enzyme Mix	3.0 µl
● (green) End Repair Reaction Buffer (10X)	6.5 µl
Fragmented DNA	55.5 µl
Total volume	65 µl

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

3. Place in a thermocycler, with the heated lid on, and run the follwing program:

   30 minutes @ 20°C

   30 minutes @ 65°C

   Hold at 4°C

1.2 Adaptor Ligation

⚠ *If DNA input is < 100 ng, dilute the NEBNext Adaptor for Illumina (provided at 15 µM) 10-fold in 10 mM Tris-HCl or 10 mM Tris-HCl with 10 mM NaCl to a final concentration of 1.5 µM, use immediately.*

1. Add the following components directly to the End Prep reaction mixture and mix well:

● (red) Blunt/TA Ligase Master Mix	15 µl
● (red) NEBNext Adaptor for Illumina*	⚠ 2.5 µl
● (red) Ligation Enhancer	1 µl
Total volume	83.5 µl

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

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

3. Incubate at 20°C for 15 minutes in a thermal cycler.

4. Add 3 µl of ● (red) USER™ Enzyme to the ligation mixture from Step 3.

   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, #E7600 and #E6609) Oligos for Illumina.

5. Mix well and incubate at 37°C for 15 minutes.

⚠ ***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 size selection/cleanup of adaptor-ligated DNA. Once thawed, gently mix by inverting the tube several times.***

1.3 Size Selection or Cleanup of Adaptor-ligated DNA

⚠ ***Size selection is optional. If the starting material is greater than 50 ng, follow the protocol for size selection in Section 1.3A. For input less than 50 ng, size selection is not recommended. Follow the protocol for cleanup without size selection in Section 1.3B.***

1.3A Size Selection of Adaptor Ligated DNA

⚠ ***The following size selection protocol is for libraries with 200 bp inserts only. For libraries with different size fragment inserts, refer to Table 1.1 for the appropriate volume of beads to be added. The size selection protocol is based on a starting volume of 100 µl.***

1. Vortex AMPure XP Beads to resuspend.

2. Add 13.5 µl dH_2O to the ligation reaction for a 100 µl total volume.

3. Add 55 µl of resuspended AMPure XP Beads to the 100 µl ligation reaction. Mix well by pipetting up and down at least 10 times.

4. Incubate for 5 minutes at room temperature.

5. Quickly spin the tube and place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube **(Caution: do not discard the supernatant)**. Discard the beads that contain the unwanted large fragments.

6. Add 25 µl resuspended AMPure XP Beads to the supernatant, mix well and incubate for 5 minutes at room temperature.

7. Quickly spin the tube and place it on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets **(Caution: do not discard beads).**

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

9. Repeat Step 8 once.

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

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

11. Remove the tube/plate from the magnet. Elute the DNA target from the beads into 17 µl of 10 mM Tris-HCl or 0.1 X TE. Mix well on a vortex mixer or by pipetting up and down. Incubate for 2 minutes at room temperature.

12. Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 15 µl to a new PCR tube for amplification.

13. Proceed to PCR Amplification in Section 1.4.

Table 1.1: Recommended conditions for bead based size selection.

	APPROXIMATE INSERT SIZE	150 bp	200 bp	250 bp	300-400 bp	400-500 bp	500-700 bp
LIBRARY PARAMETERS	Total Library Size (insert + adaptor)	270 bp	320 bp	400 bp	400-500 bp	500-600 bp	600-800 bp
VOLUME TO BE ADDED (µl)	1st Bead Selection	65	55	45	40	35	30
	2nd Bead Selection	25	25	25	20	15	15

1.3B Cleanup of Adaptor-ligated DNA without Size Selection

1. Vortex AMPure XP Beads to resuspend
2. Add 86.5 µl resuspended AMPure XP Beads to the ligation reaction. Mix well by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube and place it 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)**.
5. Add 200 µl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open.

   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 17 µl of 10 mM Tris-HCl or 0.1X TE.
9. Mix well by pipetting up and down, or on a vortex mixer. Incubate for 2 minutes at room temperature.
10. Quickly spin the tube and place it on the magnetic stand.
11. After the solution is clear (about 5 minutes), transfer 15 µl to a new PCR tube for amplification.

1.4 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 1.4A 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 1.4B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

Follow Section 1.4C 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 or 0081407

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

1.4A PCR Amplification

1. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	15 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
(blue) Index Primer/i7 Primer*,**	5 µl
(blue) Universal PCR Primer/i5 Primer*,***	5 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	4–12*
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

**We suggest 4 PCR cycles for 1 µg DNA input 7-8 cycles for 50 ng, and 12 for 5 ng DNA input. Further optimization of PCR cycle number may be required.*

3. Proceed to Cleanup of PCR Amplification in Section 1.5.

1.4B PCR Amplification for use with NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609)

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	4–12*
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

** We suggest 4 PCR cycles for 1 µg DNA input 7-8 cycles for 50 ng, and 12 for 5 ng DNA input. Further optimization of PCR cycle number may be required.*

3. Proceed to Cleanup of PCR Amplification in Section 1.5

1.4C PCR Amplification

1. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	15 µl
(blue) Index Primer*,**	2 µl
(blue) Universal PCR Primer*	2 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
Sterile H_2O	6 µl
Total volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #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	4–12*
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

**We suggest 4 PCR cycles for 1 µg DNA input 7-8 cycles for 50 ng, and 12 for 5 ng DNA input. Further optimization of PCR cycle number may be required.*

3. Proceed to Cleanup of PCR Amplification in Section 1.5.

1.5 Cleanup of PCR Amplification

1. Vortex AMPure XP Beads to resuspend.
2. Add 45 µl of resuspended AMPure XP Beads to the PCR reactions (~ 50 µl). Mix well by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube and place it 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)**.
5. Add 200 µl of 80% ethanol to the 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 PCR plate is on the magnetic stand with the lid open.

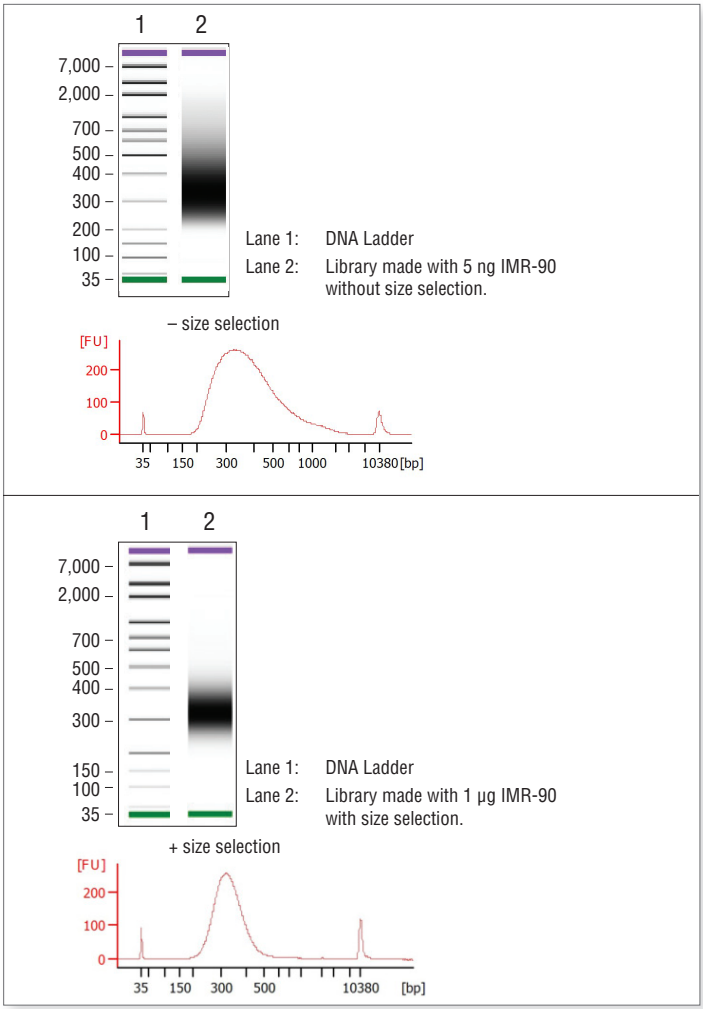
   Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8. Remove the tube/plate from the magnet. Elute DNA target from beads into 33 µl 0.1X TE. Mix well by pipetting up and down at least 10 times. Quickly spin the tube and incubate at room temperature for 2 minutes.

9. Place the sample on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer 28 µl supernatant to a new PCR tube. Libraries can be stored at –20°C.

10. Dilute 2-3 µl of the library 5 fold with 10 mM Tris-HCl or 0.1X TE and check the size distribution on an Agilent Bioanalyzer® (high sensitivity chip).

Figure 1.1: Examples of libraries prepared with human IMR-90 gDNA.



NEBNext End Prep Enzyme Mix

#E7371A: 0.072 ml
#E7371AA: 0.288 ml

Store at –20°C

Description: NEBNext End Prep Enzyme Mix is optimized to convert 5 ng–1 µg of fragmented DNA to repaired DNA having 5´-phosphorylated, dA-tailed ends.

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, Phosphorylation and dA-Tailing): 1 µl of this enzyme mix repairs and phosphorylates the ends of > 95% of 0.5ug of DNA fragments containing both 3´ and 5´ overhangs with 20 minutes at 25°C, in 1X End Repair Reaction buffer, as determined by capillary electrophoresis.

Lot Controlled

NEBNext End Repair Reaction Buffer

#E7372A: 0.156 ml **Concentration: 10X**
#E7372AA: 0.624 ml

Store at –20°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

Blunt/TA Ligase Master Mix

#E7373A: 0.360 ml
#E7373AA: 0.720 ml (2 vials provided)

Store at –20°C

Description: Blunt/TA Ligase Master Mix is a ready-to-use solution of T4 DNA Ligase, proprietary ligation enhancer, and optimized reaction buffer.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing Blunt/TA Ligase Master Mix 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 Blunt/TA Ligase Master Mix 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 Blunt/TA Ligase Master Mix 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 Blunt/TA Ligase Master Mix 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.

Transformation Assay: LITMUS™ 28 vector is cut with EcoRV (blunt), treated with calf intestinal phosphatase and gel purified. Blunt inserts from a HaeIII digest of ϕX174 DNA are ligated into the vector at a 3:1 insert:vector ratio using the Blunt/TA Ligase Master Mix Protocol. Ligation products are transformed as described.

Each lot exceeds the following standards:

	Efficiency (transformants/µg)	
	Recircularization	Insertion
Blunt ends	> 1×10^7	> 2.5×10^6
Uncut vector	> 1×10^8	

Lot Controlled

NEBNext Ligation Enhancer

#E7374A: 0.024 ml
#E7374AA: 0.096 ml

Store at –20°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µl NEBNext Ligation Enhancer and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1 µl NEBNext 5´ SR Adaptor 3 and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a 50 µl reaction containing 1 µl NEBNext Ligation Enhancer with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing 1 µl NEBNext Ligation Enhancer with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

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

Lot Controlled

NEBNext Q5 Hot Start HiFi PCR Master Mix

E6625A: 0.6 ml **Concentration: 2X**
E6625AA: 1.2 ml (2 vials provided)

Store at –20°C

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

Quality Control Assays

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

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

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

Lot Controlled

This product is covered by one or more Patents.

This product is licensed from Bio-Rad Laboratories, Inc. under U.S. Pat. Nos. 6,627,424; 7,541,170; 7,670,808; 7,666,645 and corresponding patents in other countries for use only in: (a) standard (non-real time) PCR in the research field only, but not real-time PCR or digital PCR; (b) any *in vitro* diagnostics application, except for applications using real time or digital PCR; and (c) any non-PCR applications in DNA sequencing, isothermal amplification and the production of synthetic DNA.

Revision History:

Revision #	Description
2.0	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 final AMPure Bead elutions to 0.1X TE. Changed ratio of AMPure Beads to 0.9X in final clean up after PCR reaction. Added 2 minute incubation after eluting DNA from AMPure beads.
3.0	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).

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

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

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

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

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

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

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

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

NEW ENGLAND BioLabs® Inc.

Version 3.0 8/15