

LIBRARY PREPARATION

NEBNext[®] DNA Library Prep Master Mix Set for Illumina[®]

Instruction Manual

NEB #E6040S/L
12/60 reactions
Version 8.0 9/18



be INSPIRED
drive DISCOVERY
stay GENUINE

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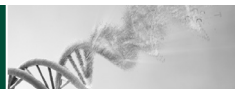


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The Library Kit Includes:

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⁻) **RI**
- (yellow) NEBNext dA-Tailing Reaction Buffer (10X)
- (red) Quick T4 DNA Ligase **RI**
- (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 (NEB #E7350, #E7335, #E7500, #E7710, #E7730, #E6609, #E7535 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.

Protocol:

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 ₂ O | variable |
| <hr/> | |
| 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 up to 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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 10 times 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' \rightarrow 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 up to 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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.

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

- 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 |
| <hr/> | |
| Total volume | 50 μl |

* The NEBNext adaptor is provided in NEBNext oligos kit. NEB has several oligo kit options which are supplied separately from the library prep kit.

- Incubate in a thermal cycler for 15 minutes at 20°C.
- Add 3 μl of ● (red) USER® Enzyme Mix by pipetting up and down at least 10 times, 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 or Multiplex 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

- Vortex AMPure XP Beads to resuspend.
- 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.
- Incubate for 5 minutes at room temperature.
- 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.
- 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.

- Repeat Step 5 once.
- Air dry beads for up to 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

- Remove the tube/plate from the magnet. Elute the DNA target by adding 105 μ l 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.

- Mix well on a vortex mixer or by pipetting up and down 10 times and incubate for 2 minutes at room temperature.
- Put the tube/PCR plate in the magnetic stand until the solution is clear. Transfer 100 μ l of supernatant (or desired volume) to a new tube/well, and proceed to bead based size selection.

| 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.

1.7 Size Select Adaptor Ligated DNA Using AMPure XP Beads



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 μ l

- Add 80 μ l (0.8X) resuspended AMPure XP Beads to 100 μ l DNA solution. Mix well on a vortex mixer or by pipetting up and down at least 10 times.
- Incubate for 5 minutes at room temperature.
- 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 up to 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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 at least 10 times 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



Use Option A for any NEBNext oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext oligos kit where index primers are supplied in a 96-well format. These kits have the forward and reverse (i7 and i5) primers combined.

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 |

1.8B PCR Enrichment of Adaptor Ligated DNA

1. Mix the following components in sterile strip tubes:

| | |
|---|------------|
| Adaptor Ligated DNA Fragments | 15 μ l |
| ● (blue) i7/i5 Primer Mix* | 10 μ l |
| ● (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix | 25 μ l |
| <hr/> | |
| Total volume | 50 μ l |

* NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

** Use only one i7 primer/index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

2. PCR cycling conditions:

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

*If library construction was performed with 5 μ g of starting material, use 2–4 cycles of amplification. If starting material was 1 μ g, 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

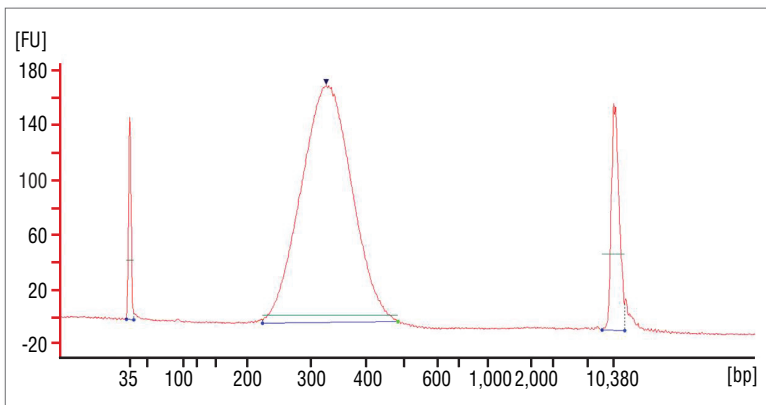
1.9 Cleanup Using AMPure XP Beads

1. Vortex AMPure XP Beads to resuspend.
2. Add 45 μ l (0.9X) of resuspended AMPure XP Beads to the PCR reactions (~50 μ l). 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 μ l 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 up to 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 30 μ l of 0.1X TE.
9. Mix well on a vortex mixer or by pipetting up and down at least 10 times 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 μ l of the supernatant to a clean LoBind[®] (Eppendorf AG) tube. Libraries can be stored at -20°C .
11. Dilute 2–3 μ l 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.



Kit Components

NEB #E6040S Table of Components

| NEB # | PRODUCT | VOLUME |
|--------|---|----------|
| E6041A | NEBNext End Repair Enzyme Mix | 0.06 ml |
| E6042A | NEBNext End Repair Reaction Buffer (10X) | 0.120 ml |
| E6044A | Klenow Fragment (3'→5' exo ⁻) | 0.036 ml |
| E6045A | NEBNext dA-Tailing Reaction Buffer (10X) | 0.06 ml |
| E6047A | Quick T4 DNA Ligase | 0.06 ml |
| E6048A | NEBNext Quick Ligation Reaction Buffer (5X) | 0.12 ml |
| E6630A | NEBNext Q5 Hot Start HiFi PCR Master Mix | 0.3 ml |

NEB #E6040L Table of Components

| NEB # | PRODUCT | VOLUME |
|---------|---|-----------------------|
| E6041AA | NEBNext End Repair Enzyme Mix | 0.3 ml |
| E6042AA | NEBNext End Repair Reaction Buffer (10X) | 0.6 ml |
| E6044AA | Klenow Fragment (3'→5' exo ⁻) | 0.180 ml |
| E6045AA | NEBNext dA-Tailing Reaction Buffer (10X) | 0.3 ml |
| E6047AA | Quick T4 DNA Ligase | 0.3 ml |
| E6048AA | NEBNext Quick Ligation Reaction Buffer (5X) | 0.6 ml |
| E6630AA | NEBNext Q5 Hot Start HiFi PCR Master Mix | (x 2 vials) or 3.5 ml |

Revision History:

| REVISION # | DESCRIPTION | DATE |
|------------|---|------|
| 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). | |
| 7.0 | Protocol updated to include NEB #E7710 and NEB #E7730. Section C in the PCR setup step was removed because all of the 25 μ M primers are now expired. | 6/16 |
| 8.0 | Create "Kit Component – Table of Components" for small and large size kits. Delete individual component information pages. Update the protocol. | 9/18 |



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

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.jp@neb.com
www.nebj.jp

SINGAPORE

New England Biolabs Pte. Ltd.
Telephone: +65 638 59623
Fax: +65 638 59617
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