LIBRARY PREPARATION

NEBNext® Ultra™ DNA Library Prep Kit for Illumina®

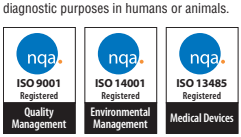
Instruction Manual

NEB #E7370S/L
24/96 reactions
Version 5.0 5/17

NEW ENGLAND BioLabs® Inc.

be INSPIRED
drive DISCOVERY
stay GENUINE

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.



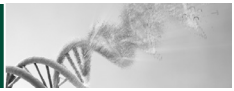


Table of Contents:

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)

5 M NaCl (required for DNA input <100 ng)

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 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.

Protocol:

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.

Colored bullets indicate the cap color of the reagent to be added

Starting Material: 5 ng–1 µg fragmented DNA.

1. NEBNext End Prep

1.1. 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

1.2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with the performance.

1.3. Place in a thermocycler, with the heated lid set to ≥ 75°C, and run the following program:

30 minutes @ 20°C

30 minutes @ 65°C

Hold at 4°C

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 with 10 mM NaCl to a final concentration of 1.5 µM, use immediately.

2.1. 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, #E7710, #E7730, #E7600, #E6609) Oligos for Illumina.

Note: Ligation Enhancer and Blunt/TA Ligase Master Mix can be mixed ahead of time and is stable for at least 8 hours at 4°C. We do not recommend adding adaptor to a premix in the adaptor ligation step. For best results add adaptor last and mix well immediately or premix adaptor and sample and then add the other ligation reagents.

2.2. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. Caution: The blunt/TA Ligase Master Mix is viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

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

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

Note: Steps 2.4 and 2.5 are only required for use with NEBNext Adaptors. USER Enzyme can be found in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600 and #E6609) Oligos for Illumina.

2.5. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.

Safe Stopping Point: Samples can be stored overnight at -20°C.

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.

3. Size Selection or Cleanup of Adaptor-ligated DNA

3A. Size Selection of Adaptor-ligated DNA

Note: The volumes of SPRIselect or AMPure XP reagent provided here are for use with the sample contained in the exact buffer at this step. These volumes may not work properly for a size selection at a different step in the workflow, or if this is a second size selection at this step. For size selection of samples contained in different buffer conditions the volumes may need to be experimentally determined.

The following size selection protocol is for libraries with 200 bp inserts only. For libraries with different size fragment inserts, refer to the Table below for the appropriate volumes of beads to be added. The size selection protocol is based on a starting volume of 100 µl. Size selection conditions were optimized with AMPure XP beads; however, SPRIselect beads can be used following the same conditions.

To select a different insert size than 200 bp, please use the volumes in this table:

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 + primer)	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

3A.1. Vortex SPRIselect beads to resuspend. AMPure XP beads can be used as well. If using AMPure XP beads, please allow the beads to warm to room temperature for at least 30 minutes before use.

3A.2. Add 13.5 µl of dH_2O to the ligation reaction for a 100 µl total volume.

3A.3. Add 55 µl (0.55X) of resuspended SPRIselect beads to the 100 µl ligation reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

3A.4. Incubate samples on bench top for at least 5 minutes at room temperature.

3A.5. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

3A.6. After 5 minutes (or when the solution is clear), 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.

3A.7. Add 25 µl (0.25X) resuspended SPRIselect beads to the supernatant and **mix at least 10 times**. Be careful to expel all of the liquid from the tip during the last mix. Then incubate samples on the bench top for at least 5 minutes at room temperature.

3A.8. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

3A.9. After 5 minutes (or when the solution is clear), 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)**.

3A.10. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

3A.11. Repeat Step 3A.10 once. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

3A.12. Air dry the beads for up to 5 minutes while the tube/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. 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.

3A.13. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 17 µl of 10 mM Tris-HCl or 0.1X TE.

3A.14. Mix well on a vortex mixer or by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

3A.15. Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 15 µl to a new PCR tube for amplification.

Safe Stopping Point: It is safe to store the library at -20°C.

3B. Cleanup of Adaptor-ligated DNA without Size Selection

Note: the volumes of SPRIselect or AMPure XP reagent provided here are for use with the sample contained in the exact buffer at this step. These volumes may not work properly for a cleanup at a different step in the workflow, or if this is a second cleanup at this step. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

3B.1. Vortex SPRIselect beads to resuspend (AMPure XP beads can be used as well). If using AMPure XP beads, allow the beads to warm to room temperature for at least 30 minutes before use.

3B.2. Add 86.5 µl (1X) resuspended SPRIselect beads to the Adaptor Ligation reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

3B.3. Incubate samples on bench top for at least 5 minutes at room temperature.

3B.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

3B.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads).**

3B.6. Add 200 µl of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

3B.7. Repeat Step 3B.6 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

3B.8. Air dry the beads for up to 5 minutes while the tube/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.

3B.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 10 mM Tris-HCl or 0.1X TE.

3B.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

3B.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 15 µl to a new PCR tube.

Samples can be stored at –20°C.

4. PCR Enrichment of Adaptor-ligated DNA

⚠ **Note: Check and verify that the concentration of your oligos is 10 µM.**

⚠ **Follow Section 4.1A if you are using the following oligos (10 µM primer):**

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

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

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

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

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

Follow Section 4.1B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609)

4.1. Add the following components to a sterile strip tube:

4.1A. **Forward and Reverse Primer not already combined**

Adaptor Ligated DNA Fragments (Step 3A.15 or 3B.11)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
• (blue) Index Primer/i7 Primer*,**	5 µl
• (blue) Universal PCR Primer/i5 Primer*,***	5 µl
Total volume	50 µl

4.1B. **Forward and Reverse Primer already combined**

Adaptor Ligated DNA Fragments (Step 3A.15 or 3B.11)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
• (blue) Index/Universal Primer****	10 µl
Total volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #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, #E7500, #E7710 or #E7730) 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.

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

4.2. Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

4.3. Place the tube on a thermocycler and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	4–12*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles recommended in Table 4.1 are to be seen as a starting point to determine the number of PCR cycles best for your samples. The number of PCR cycles should be chosen based on input amount and sample type. Thus, samples prepared with a different method prior to library prep may require re-optimization of the number of PCR cycles. The number of cycles should be high enough to provide sufficient library fragments for a successful sequencing run, but low enough to avoid PCR artifacts and over-cycling (high molecular weight fragments on Bioanalyzer).

Table 4.1.

INPUT DNA IN THE END PREP REACTION	# OF CYCLES
1 µg	4
50 ng	7-8
5 ng	12

NEBNext adaptors contain a unique truncated design. Libraries constructed with NEBNext adaptors require a minimum of 3 amplification cycles to add the complete adaptor sequences for downstream processes.

4.4. Proceed to Cleanup of PCR Amplification in Section 5.

5. Cleanup of PCR Reaction

Note: the volumes of SPRIselect or AMPure XP reagent provided here are for use with the sample contained in the exact buffer at this step. These volumes may not work properly for a cleanup at a different step in the workflow. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

5.1. Vortex SPRIselect beads to resuspend. AMPure XP beads can be used as well. If using AMPure XP beads, allow the beads to warm to room temperature for at least 30 minutes before use.

5.2. Add 45 µl (0.9X) resuspended SPRIselect beads to the PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

5.3. Incubate samples on bench top for at least 5 minutes at room temperature.

5.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

5.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

5.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

5.7. Repeat Step 5.6 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

5.8. Air dry the beads for up to 5 minutes while the tube/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.

5.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 0.1X TE.

5.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

5.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube for and store at –20°C.

5.12. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA chip. The sample may need to be diluted before loading.

Samples can be stored at –20°C.

Checklist:

1. NEBNext End Prep

[_] 1.1. Add End Prep Reagents to sample (55.5 µl):

- [_] 3 µl End Prep Enzyme Mix
- [_] 6.5 µl End Prep Reaction Buffer

[_] 1.2. Pipette mix 10 times with pipette set to 50 µl, quick spin

[_] 1.3. Thermal cycle (Heated lid ≥ 75°C; 30 min 20°C, 30 min 65°C, Hold at 4°C)

2. Adaptor Ligation

[_] 2.0. Dilute adaptor if necessary

[_] 2.1. Add Ligation reagents to sample:

- [_] 15 µl Blunt/TA Ligase Master Mix
- [_] 1 µl Ligation Enhancer
- [_] 2.5 µl diluted adaptor

[_] 2.2. Pipette mix 10 times with pipette set to 80 µl, quick spin

[_] 2.3. Incubate 15 min at 20°C (heated lid off)

[_] 2.4. Add 3 µl USER

[_] 2.5. Pipette mix 10 times with pipette set to 80 µl, quick spin; incubate 15 min 37°C (heated lid ≥ 47°C)

3. Cleanup or Size Selection

3A. Size Selection of Adaptor-ligated DNA

[_] 3A.1. Vortex beads

[_] 3A.2. Add 13.5 µl of water to sample.

[_] 3A.3. Add __ µl of beads to sample and mix by pipetting 10 times.

[_] 3A.4. Incubate 5 min

[_] 3A.5. Place tubes on magnet

[_] 3A.6. Wait 5 min then transfer the supernatant to a new tube (keep the supernatant)

[_] 3A.7. Add ___ µl of beads to the supernatant and mix by pipetting 10 times. Incubate 5 min.

[_] 3A.8. Place tubes on magnet

[_] 3A.9. Wait 5 min then remove the supernatant (keep the beads)

[_] 3A.10. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 3A.11. Repeat Step 3A.10. once

[_] 3A.12. Air dry beads, do not overdry

[_] 3A.13. Off magnet add 17 µl 10 mM Tris-HCl or 0.1 x TE

[_] 3A.14. Mix by pipetting 10 times. Incubate 2 min.

[_] 3A.15. Place tubes on magnet. Wait 5 min and transfer 15 µl to a new tube

Skip to 4

3B. Cleanup of Adaptor-ligated DNA without Size Selection

[_] 3B.1. Vortex beads

[_] 3B.2. Add 86.5 µl of beads to sample and mix by pipetting 10 times.

[_] 3B.3. Incubate for 5 min

[_] 3B.4. Place tubes on magnet

[_] 3B.5. Wait 5 min and remove supernatant (keep the beads)

[_] 3B.6. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 3B.7. Repeat Step 3B.6. once

[_] 3B.8. Air dry beads, do not overdry

[_] 3B.9. Off magnet add 17 µl 10 mM Tris-HCl or 0.1 x TE

[_] 3B.10. Mix by pipetting 10 times. Incubate 2 min.

[_] 3B.11. Place tubes on magnet. Wait 5 min and transfer 15 µl to a new tube

4. PCR Enrichment of Adaptor-ligated DNA

[_] 4.1. Add PCR Reagents to sample

4.1A. [_] A (25 µl Q5 Master Mix, 5 µl index primer/ i7 primer; 5 µl Universal primer i5 primer)

OR

4.1B. [_] B (25 µl Q5 Master Mix, 10 µl Index and universal primer)

[_] 4.2. Pipette mix 10 times with pipette set to 40 µl, quick spin

[_] 4.3. Thermal cycle (Heated lid ≥ 103°C; 98°C 30 sec, 3-15 cycles of 98°C for 10 sec and 65°C for 75 sec, 65°C for 5 min, Hold at 4°C)

5. Cleanup of PCR Amplification

[_] 5.1. Vortex beads

[_] 5.2. Add 45 µl of beads to sample and mix by pipetting 10 times

[_] 5.3. Incubate for 5 min

[_] 5.4. Place tubes on magnet

[_] 5.5. Wait 5 min and remove supernatant (keep the beads)

[_] 5.6. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 5.7. Repeat Step 5.6 once

[_] 5.8. Air dry beads, do not overdry

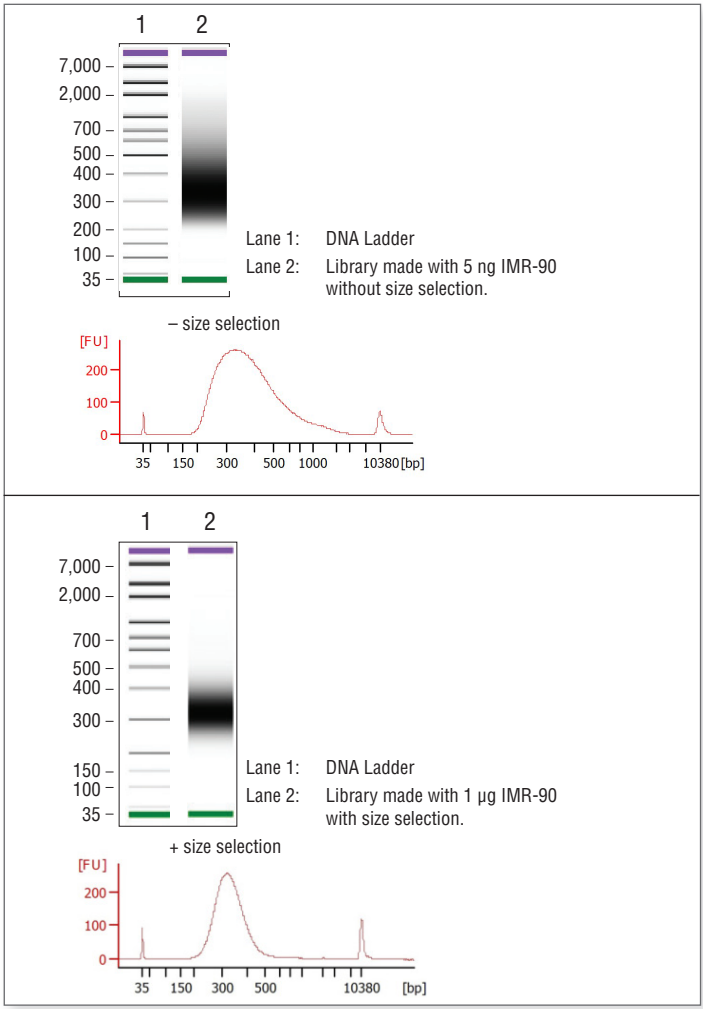
[_] 5.9. Off magnet add 33 µl 10 mM Tris-HCl or 0.1 x TE

[_] 5.10. Mix by pipetting 10 times. Incubate 2 min.

[_] 5.11. Place tubes on magnet. Wait 5 min and transfer 30 µl to a new tube

[_] 5.12. Check size distribution on Bioanalyzer

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	$> 1 \times 10^7$	$> 2.5 \times 10^6$
Uncut vector	$> 1 \times 10^8$	

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.

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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

Revision History:

REVISION #	DESCRIPTION	DATE
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).	
4.0	Protocol updated to include NEB #E7710 and NEB #E7730.	6/16
5.0	Section C in the PCR setup step was removed because all of the 25 µM primers are now expired. Protocol steps were assigned new numbering system.	5/17

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

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