NEBNext® Ultra™ DNA Library Prep Kit for Illumina®

NEB #E7370L

96 reactions

Version 9.1_1/23

Table of Contents

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 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
- (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)
- NEBNext Multiplex Oligos for Illumina® (NEB.com/oligos)
- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
- PCR machine
- SPRIselect® Reagent (Beckman Coulter®, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- DNase RNase free PCR strip tubes (USA Scientific® 1402-1708)

Applications

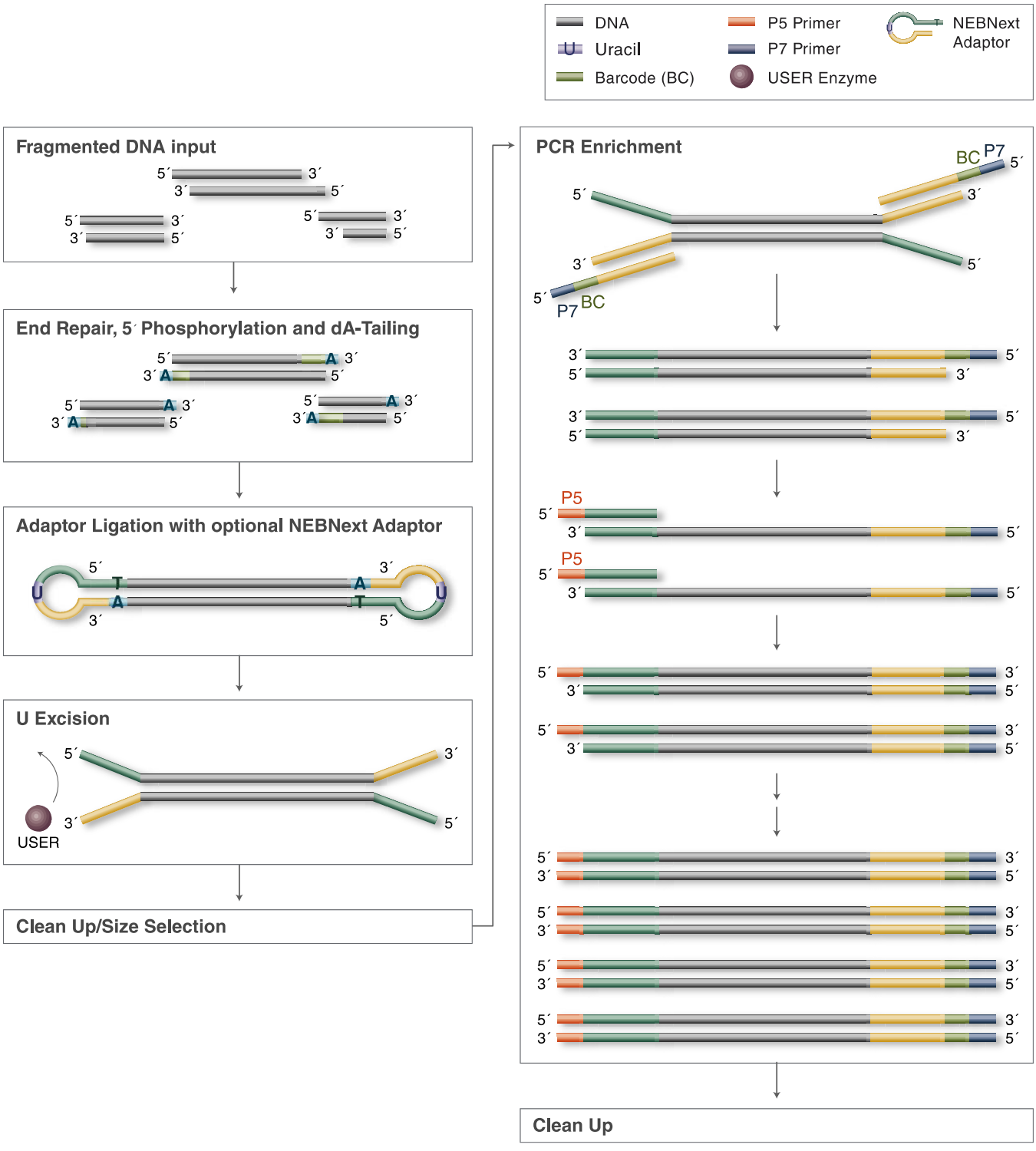
The NEBNext Ultra DNA Library Prep Kit for Illumina contains enzymes and buffers that are ideal to convert a small amount of DNA input into indexed libraries for next-generation sequencing on the Illumina platform (Illumina, Inc). The workflow of NEBNext Ultra DNA Library Prep Kit for Illumina is very 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.

Figure 1. Workflow demonstrating the use of NEBNext Ultra DNA Library Prep Kit for Illumina



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.

●

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. Add the following components to a sterile nuclease-free tube:

COMPONENT	VOLUME
● (green) End Prep Enzyme Mix	3.0 µl
● (green) End Repair Reaction Buffer	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 performance.

1.3. Place in a thermal cycler, 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

If necessary, samples can be stored at -20°C; however, a slight loss in yield (~20%) may be observed.
We recommend continuing with adaptor ligation before stopping.

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:

COMPONENT	VOLUME
● (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**

* Mix the Blunt/TA Ligase Master Mix by pipetting up and down several times prior to adding to the reaction.
** The NEBNext adaptor is provided in NEBNext oligos kit. NEB has several oligo kit options, which are supplied separately from the library prep kit.

Note: The Ligation Enhancer and Blunt/TA Ligase Master Mix can be mixed ahead of time and is stable for at least 8 hours @ 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 2.3.

Note: Steps 2.4. and 2.5. are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Oligo kits.

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

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

Size selection is optional. If the starting material is > 50 ng, follow the protocol for size selection in Section 3A. For input less than 50 ng, size selection is not recommended. Follow the protocol for cleanup without size selection in Section 3B.

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 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 DISTRIBUTION	150 bp	200 bp	250 bp	300-400 bp	400-500 bp	500-700 bp
LIBRARY PARAMETERS	Approx. Final Library Size Distribution (insert + adaptor + primers)	270 bp	320 bp	400 bp	400-500 bp	500-600 bp	600-800 bp
BEAD VOLUME TO BE ADDED (µl)	1st Bead Addition	65	55	45	40	35	30
	2nd Bead Addition	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 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

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 plate format. These kits have the forward and reverse (i7 and i5) primers combined.

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

4.1A. Forward and Reverse Primers supplied separately

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 3A.15. or 3B.11)	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**

Proceed to Step 4.2.

4.1B. Forward and Reverse Primers already combined

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 3A.15. or 3B.11)	15 µl
• (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
• (blue) Index/Universal Primer*	10 µl
Total Volume	**50 µl**

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

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 thermal cycler 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	# 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.

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. 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.

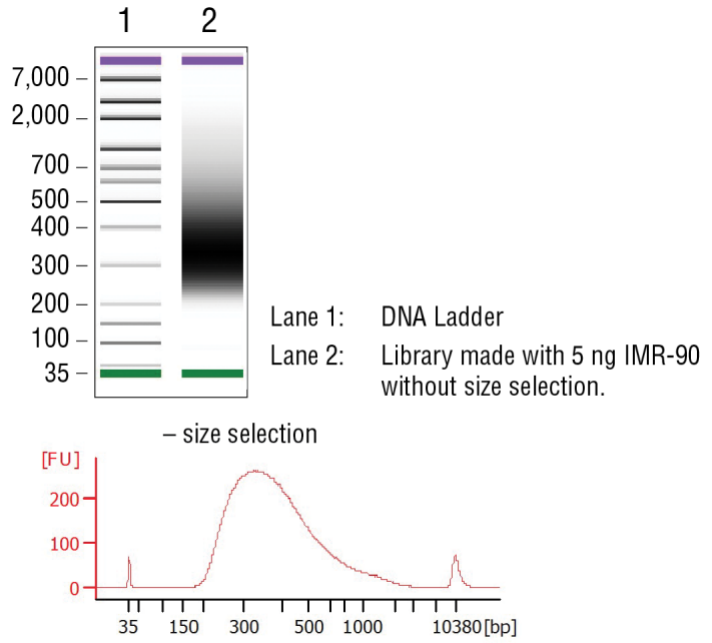
5.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

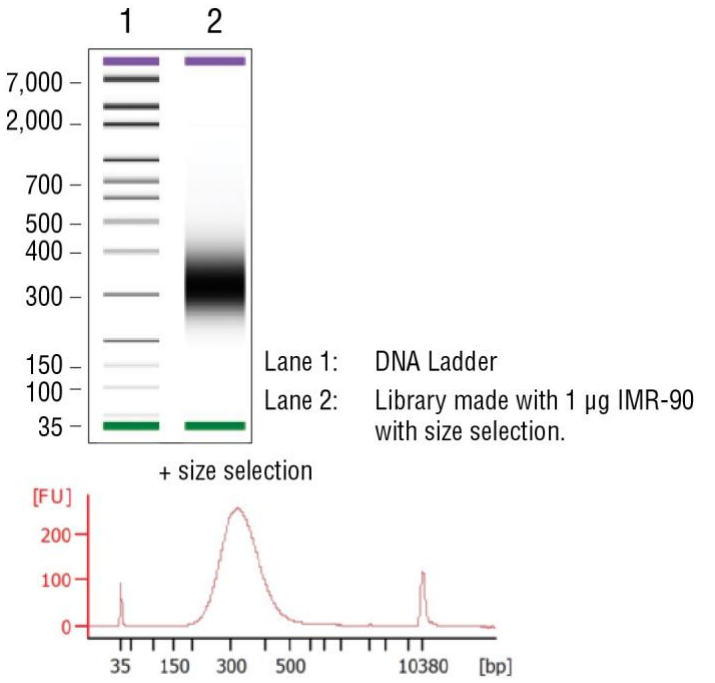
5.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube and store at –20°C.

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.

Figure 5.1: Examples of libraries prepared with human IMR-90 gDNA.





Kit Components

NEB #E7370L Table of Components

NEB #	PRODUCT	VOLUME
E7371AA	NEBNext End Prep Enzyme Mix	0.288 ml
E7372AA	NEBNext End Repair Reaction Buffer	0.624 ml
E7373AA	Blunt T/A Ligase Master Mix	0.720 ml
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E6625AA	NEBNext Q5 Hot Start HiFi PCR Master Mix	1.2 ml

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

[_] 2.1. Dilute adaptor if necessary

[_] 2.2. Add Ligation reagents to sample:

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

[_] 2.3. Pipette mix 10 times with pipette set to 80 µl, quick spin

[_] 2.4 Incubate 15 min at 20°C (heated lid off)

[_] 2.5 Add 3 µl USER

[_] 2.6 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

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
5.1	Functional activity Assay for M0543 part (E6625) has been adjusted from 4-plex to 3-plex. Remove the Endonuclease Activity Assay from the Blunt/TA Master Mix.	9/17
6.0	Added a caution note to Section 3 Size Selection or Cleanup of Adaptor-ligated DNA. Added NEBNext Q5 Hot Start HiFi PCR Master Mix to Step 4.1A and 4.1B and delete NEBNext Ultra II Q5 Master Mix. Move Figure 1 to page 12.	2/18
7.0	Delete quality control information. Insert kit components table.	3/18
8.0	Update manual format.	7/19
8.1	Updated product license information	5/20
9.0	Remove small size, update required materials not included and update protocol	8/22
9.1	Table formatting and legal footer updated.	1/23

www.neb.com

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