Ultra II DNA Library Prep Kit for Illumina E7645/E7103

New England Biolabs

Abstract

The NEBNext Ultra II DNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of input amounts of DNA into high quality libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow also has minimal hands-on time.

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Before start

Starting Material: 500 pg–1 µg fragmented DNA. We recommend that DNA be sheared in 1X TE. If the DNA volume post shearing is less than 50 µl, add 1X TE to a final volume of 50 µl. Alternatively, samples can be diluted with 10 mM Tris-HCl, pH 8.0 or 0.1X TE.

Materials

- NEBNext Ultra II End Prep Reaction Buffer E7647 by New England Biolabs
- NEBNext Ultra II Ligation Master Mix E7648 by New England Biolabs
- NEBNext Ligation Enhancer E7374 by New England Biolabs
- NEBNext Ultra II Q5 Master Mix E7649 by New England Biolabs
- NEBNext Ultra II End Prep Enzyme Mix E7646 by New England Biolabs
- 80% Ethanol (freshly prepared) by Contributed by users
- Nuclease-free Water by Contributed by

users

0.1X TE (1mM Tris-HCl, pH 8.0, 0.1 mM EDTA) by Contributed by users

DNA LoBind Tubes #022431021 by Eppendorf

NEBNext Singleplex or Multiplex Oligos for Illumina by New England Biolabs

Magnetic rack/stand by Contributed by users

Only for E7645: SPRIselect Reagent Kit or AMPure XP Beads by Beckman Coulter

Protocol

NEBNext End Prep

Step 1.

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

Component	Volume
NEBNext Ultra II End Prep Enzyme Mix	3 µl
NEBNext Ultra II End Prep Reaction Buffer	7 µl
Fragmented DNA	50 µl
Total volume	60 µl

REAGENTS

NEBNext End Prep Enzyme Mix E7646 by New England Biolabs

NEBNext Ultra II End Prep Reaction Buffer E7647 by New

England Biolabs

NEBNext End Prep

Step 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.

NOTES

It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

NEBNext End Prep

Step 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

NOTES

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.

Adaptor Ligation

Step 4.

Determine whether adaptor dilution is necessary.

If DNA input is ≤ 100 ng, dilute the NEBNext Adaptor for Illumina in Tris/NaCl, pH 8.0 as indicated in Table 2.1.

Table 2.1: Adaptor Dilution

Input	Adaptor Dilution (Volume of Adaptor: Total Volume)	Working Adaptor Concentration
1 µg–101 ng	No Dilution	15 µM
100 ng–5 ng	10-Fold (1:10)	1.5 µM
less than 5 ng	25-Fold (1:25)	0.6 µM

NOTES

The appropriate adaptor dilution for your sample input and type may need to be optimized experimentally. The dilutions provided here are a general starting point. Excess adaptor should be removed prior to PCR enrichment.

Adaptor Ligation

Step 5.

Add the following components directly to the End Prep Reaction Mixture:

Component	Amount
End Prep Reaction Mixture (Step 3)	60 µl
NEBNext Ultra II Ligation Master Mix *	30 µl
NEBNext Ligation Enhancer	1 µl
NEBNext Adaptor for Illumina **	2.5 µl
Total Volume	93.5 µl

* Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.

** The NEBNext adaptor is provided in NEBNext Singleplex or Multiplex Oligos for Illumina.

READAGENTS

NEBNext Adaptor for Illumina
View by New England Biolabs

NEBNext Ligation Enhancer E7374 by New England Biolabs

NEBNext Ultra II Ligation Master Mix E7648 by New England Biolabs

NOTES

The Ligation Master Mix and Ligation Enhancer 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.

Adaptor Ligation

Step 6.

Set a 100 µl or 200 µl pipette to 80 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.

Caution: The NEBNext Ultra II Ligation Master Mix is very 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.

Adaptor Ligation

Step 7.

Incubate at 20°C for 15 minutes in a thermocycler with the heated lid off.

Adaptor Ligation

Step 8.

Add 3 µl of USER™ Enzyme to the ligation mixture from Step 7.

READAGENTS

USER™ Enzyme by New England Biolabs

NOTES

Steps 8 and 9 are only required for use with NEBNext Adaptors. USER™ enzyme can be found in the NEBNext Singleplex or Multiplex Oligos for Illumina.

Adaptor Ligation

Step 9.

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

NOTES

Samples can be stored overnight at –20°C.

Attention: Choose Size Selection or Cleanup

Step 10.

Please select whether your starting material is:

- GREATER than 50 ng to follow the protocol for size selection
- LESS than or EQUAL to 50 ng to follow the protocol for cleanup without size selection.

For input LESS THAN or EQUAL to 50 ng, size selection is NOT recommended to maintain library complexity.

Size Selection of Adaptor-ligated DNA

Step 11 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

The following section is for cleanup of the ligation reaction. The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. 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. These bead 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.

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 96.5 µl.

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

Table 3.1: Recommended conditions for bead based size selection.

LIBRARY PARAMETERS	APPROXIMATE INSERT SIZE	150 bp	200 bp	250 bp	300-400 bp	400-500 bp	500-700 bp
	Approx. Final Library Size (insert + adaptor + primers)	270 bp	320 bp	370 bp	480 bp	600 bp	750-800 bp
BEAD VOLUME TO BE ADDED (µl)	1st Bead Addition	50	40	30	25	20	15
	2nd Bead Addition	25	20	15	10	10	10

Size Selection of Adaptor-ligated DNA

Step 12 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

Size Selection of Adaptor-ligated DNA

Step 13 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Add 40 µl (0.4X) of resuspended beads to the 96.5 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.

Size Selection of Adaptor-ligated DNA

Step 14 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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

Size Selection of Adaptor-ligated DNA

Step 15 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Size Selection of Adaptor-ligated DNA

Step 16 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

After 5 minutes (or when the solution is clear), carefully transfer the supernatant containing your DNA to a new tube. Discard the beads that contain the unwanted large fragments.

Caution: Do not discard the supernatant.

Size Selection of Adaptor-ligated DNA

Step 17 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Add 20 µl (0.2X) resuspended SPRIselect or NEBNext Sample Purification 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.

Size Selection of Adaptor-ligated DNA

Step 18 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Size Selection of Adaptor-ligated DNA

Step 19 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Size Selection of Adaptor-ligated DNA

Step 20 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Size Selection of Adaptor-ligated DNA

Step 21 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Repeat Step 20 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.

GOTO

Repeat Step -> go to step #20

Size Selection of Adaptor-ligated DNA

Step 22 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Size Selection of Adaptor-ligated DNA

Step 23 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
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.

Size Selection of Adaptor-ligated DNA

Step 24 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
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.

Size Selection of Adaptor-ligated DNA

Step 25 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
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. Proceed to PCR Amplification in Step 26.

NOTES

Samples can be stored at –20°C.

PCR Amplification

Step 26 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
Use **Option A** for any kit where NEBNext index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

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

PCR Amplification

Step 27 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
Add the following components to a sterile strip tube:

Option A: Forward and Reverse Primer NOT Already Combined

Component	Volume per Reaction
Adaptor Ligated DNA Fragments	15 µl
NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer/i7 Primer*,**	5 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	50 µl

Option B: Forward and Reverse Primer Already Combined

Comonent	Volume per Reaction
Adaptor Ligated DNA Fragments	15 µl
NEBNext Ultra II Q5 aster Mix	25 µl
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/index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

PCR Amplification

Step 28 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
Set 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.

PCR Amplification

Step 29 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
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	3-15 *
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* 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). 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 standard library prep samples. Use Table 4.2 for applications requiring high library yields (1 µg) such as target enrichment.

Table 4.1.

INPUT DNA IN THE END PREP REACTION	# OF CYCLES REQUIRED FOR STANDART LIBRARY PREP 100 ng (30–100 nM)
1 µg *	3**
500 ng *	3**

100 ng *	3
50 ng	3–4
10 ng	6–7
5 ng	7–8
1 ng	9–10
0.5 ng	10–11

* These input ranges will work best when size selection is done
** 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.

Table 4.2.

INPUT DNA IN THE END PREP REACTION	# OF CYCLES REQUIRED FOR STANDART LIBRARY PREP 100 ng (30–100 nM)
1 µg *	3–4*,**
500 ng *	4–5**
100 ng *	6–7
50 ng	7–8
10 ng	9–10
5 ng	10–11
1 ng	12–13
0.5 ng	14–15

* These input ranges will work best when size selection is done
** 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.

PCR Amplification

Step 30 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
Proceed to Cleanup of PCR Amplification in next Section: **Cleanup of PCR Reaction**.

Cleanup of PCR Reaction

Step 31 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).
The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. 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. 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.

Cleanup of PCR Reaction

Step 32 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

Cleanup of PCR Reaction

Step 33 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Add 45 µl (0.9X) resuspended 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.

Cleanup of PCR Reaction

Step 34 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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

Cleanup of PCR Reaction

Step 35 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Cleanup of PCR Reaction

Step 36 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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

Caution: Do not discard the beads.

Cleanup of PCR Reaction

Step 37 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Add 200 µl or 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.

Cleanup of PCR Reaction

Step 38 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Repeat Step 37 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.

GOTO

Repeat -> go to step #37

Cleanup of PCR Reaction

Step 39 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Cleanup of PCR Reaction

Step 40 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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

Cleanup of PCR Reaction

Step 41 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Cleanup of PCR Reaction

Step 42 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

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.

Cleanup of PCR Reaction

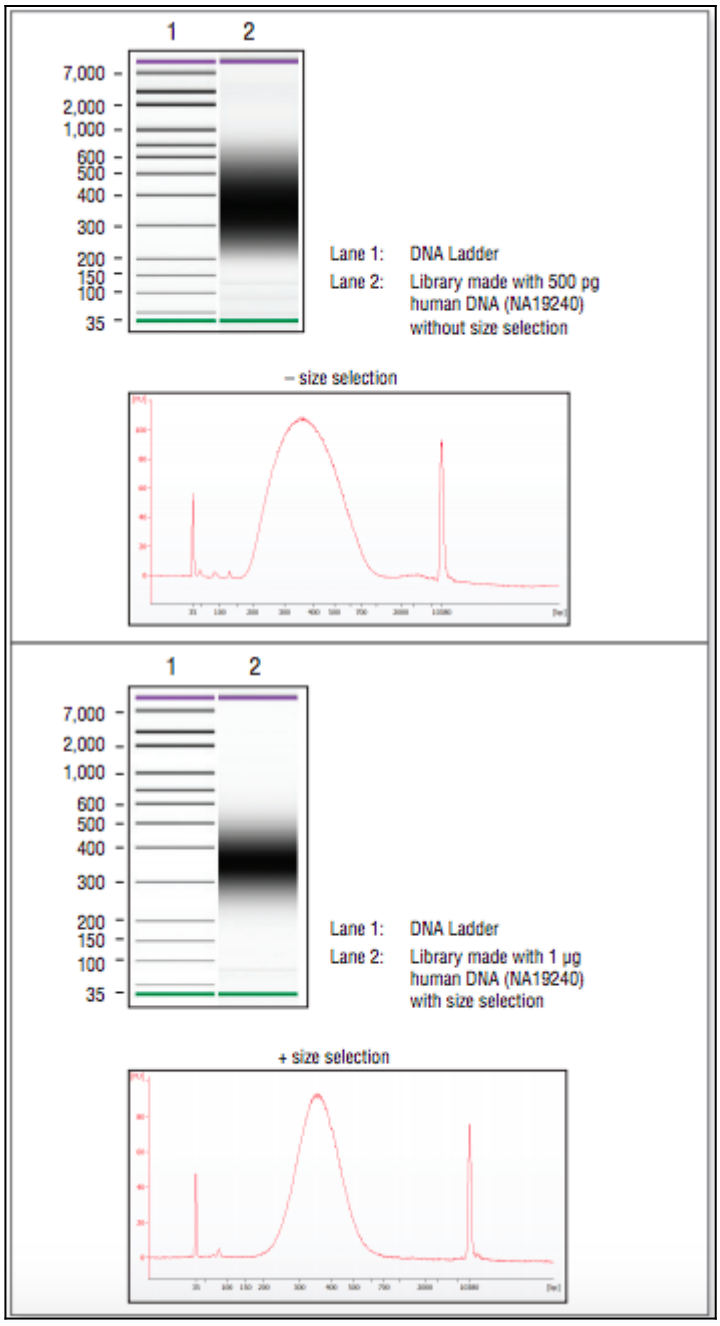
Step 43 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Check the size distribution on an Aglient Bioanalyzer High Sensitivity DNA chip. The sample may need to be diluted before loading. See Figure 5.1 in next step for examples.

Cleanup of PCR Reaction

Step 44 - Input > 50 ng (Size Selection of Adaptor-ligated DNA).

Figure 5.1: Examples of libraries prepared with human DNA (NA19240).



Cleanup of Adaptor-ligated DNA without Size Selection

Step 11 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
The following section is for cleanup of the ligation reaction. If your input DNA is > 50 ng, follow the size selection protocol by selecting > 50 ng in previous step.

The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. 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. These bead 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.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 12 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 13 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Add 87 µl (0.9X) resuspended 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.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 14 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Incubate samples on bench top for at least 5 minutes at room temperature.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 15 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 16 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 17 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 18 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Repeat Step 17 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.

GOTO

Repeat -> go to step #17

Cleanup of Adaptor-ligated DNA without Size Selection

Step 19 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 20 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 21 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of Adaptor-ligated DNA without Size Selection

Step 22 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

NOTES

Samples can be stored at –20°C.

PCR Amplification

Step 23 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Use **Option A** for any kit where NEBNext index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

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

PCR Amplification

Step 24 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Add the following components to a steribe strip tube:

Option A: Forward and Reverse Primer NOT Already Combined

Component	Volume per Reaction
Adaptor Ligated DNA Fragments	15 µl
NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer/i7 Primer *,**	5 µl
Universal PCR Primer/i5 Primer *,**	5 µl
Total Volume	50 µl

Option B: Forward and Reverse Primer Already Combined

Component	Volume per Reaction
Adaptor Ligated DNA Fragments	15 µl
NEBNext Ultra II Q5 aster Mix	25 µl
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/index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

PCR Amplification

Step 25 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Set 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.

PCR Amplification

Step 26 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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	3-15 *
Annealing/Extension	65°C	75 seconds	3-15 *
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* 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). 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 standard library prep samples. Use Table 4.2 for applications requiring high library yields (1 µg) such as target enrichment.

Table 4.1.

INPUT DNA IN THE END PREP REACTION	# OF CYCLES REQUIRED FOR STANDARD LIBRARY PREP 100 ng (30–100 nM)
1 µg*	3**
500 ng*	3**
100 ng*	3
50 ng	3–4
10 ng	6–7
5 ng	7–8
1 ng	9–10
0.5 ng	10–11

* These input ranges will work best when size selection is done
** 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.

Table 4.2.

INPUT DNA IN THE END PREP REACTION	# OF CYCLES REQUIRED FOR TARGET ENRICHMENT LIBRARY PREP (1 µg)
1 µg*	3–4*, **
500 ng*	4–5*
100 ng*	6–7*
50 ng	7–8
10 ng	9–10
5 ng	10–11
1 ng	12–13
0.5 ng	14–15

* These input ranges will work best when size selection is done
** 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.

PCR Amplification

Step 27 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Proceed to Cleanup of PCR Amplification in next Section: **Cleanup of PCR Reaction**.

Cleanup of PCR Reaction

Step 28 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. 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. 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.

Cleanup of PCR Reaction

Step 29 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

Cleanup of PCR Reaction

Step 30 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Add 45 µl (0.9X) resuspended 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.

Cleanup of PCR Reaction

Step 31 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Incubate samples on bench top for at least 5 minutes at room temperature.

Cleanup of PCR Reaction

Step 32 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of PCR Reaction

Step 33 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

Caution: Do not discard the beads.

Cleanup of PCR Reaction

Step 34 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Add 200 µl or 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 tot disturb the beads that contain DNA targets.

Cleanup of PCR Reaction

Step 35 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Repeat Step 49 once for a total of two washes. e 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.

Cleanup of PCR Reaction

Step 36 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of PCR Reaction

Step 37 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 0.1X TE.

Cleanup of PCR Reaction

Step 38 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of PCR Reaction

Step 39 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
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.

Cleanup of PCR Reaction

Step 40 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Check the size distribution on an Aglient Bioanalyzer High Sensitivity DNA chip. The sample may need to be diluted before loading. See Figure 5.1 in next step for examples.

Cleanup of PCR Reaction

Step 41 - Input ≤ 50 ng (Cleanup of Adaptor-ligated DNA without Size Selection).
Figure 5.1: Examples of libraries prepared with human DNA (NA19240).