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Apr 12, 2022

NEBNext® Ultra™ DNA Library Prep Protocol for Illumina® (E7370) V.3

New England Biolabs[1]

[1]New England Biolabs

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dx.doi.org/10.17504/protocols.io.j8epv5edv1bz/v3

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The NEBNext®Ultra™ DNA Library Prep Kit for Illumina®contains reagents for preparation of libraries for next-generation sequencing on the Illumina platform from 5 ng – 1 µg input DNA, in a streamlined workflow. Please note that adaptors and primers are not included in the kit and are available separately.

Each kit component must pass rigorous quality control standards, and each set of reagents is functionally validated together by construction and sequencing of a 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 custom@neb.com for further information.

DOI

dx.doi.org/10.17504/protocols.io.j8epv5edv1bz/v3

https://www.neb.com/protocols/2014/05/22/protocol-for-use-with-nebnext-ultra-dna-library-prep-kit-for-illumina-e7370

New England Biolabs 2022. NEBNext® Ultra™ DNA Library Prep Protocol for Illumina® (E7370). **protocols.io**
https://dx.doi.org/10.17504/protocols.io.j8epv5edv1bz/v3
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Illumina, DNA library , Size selection

protocol ,

Apr 15, 2020

Apr 12, 2022

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Ultra DNA Library Preparation Workflow for Illumina

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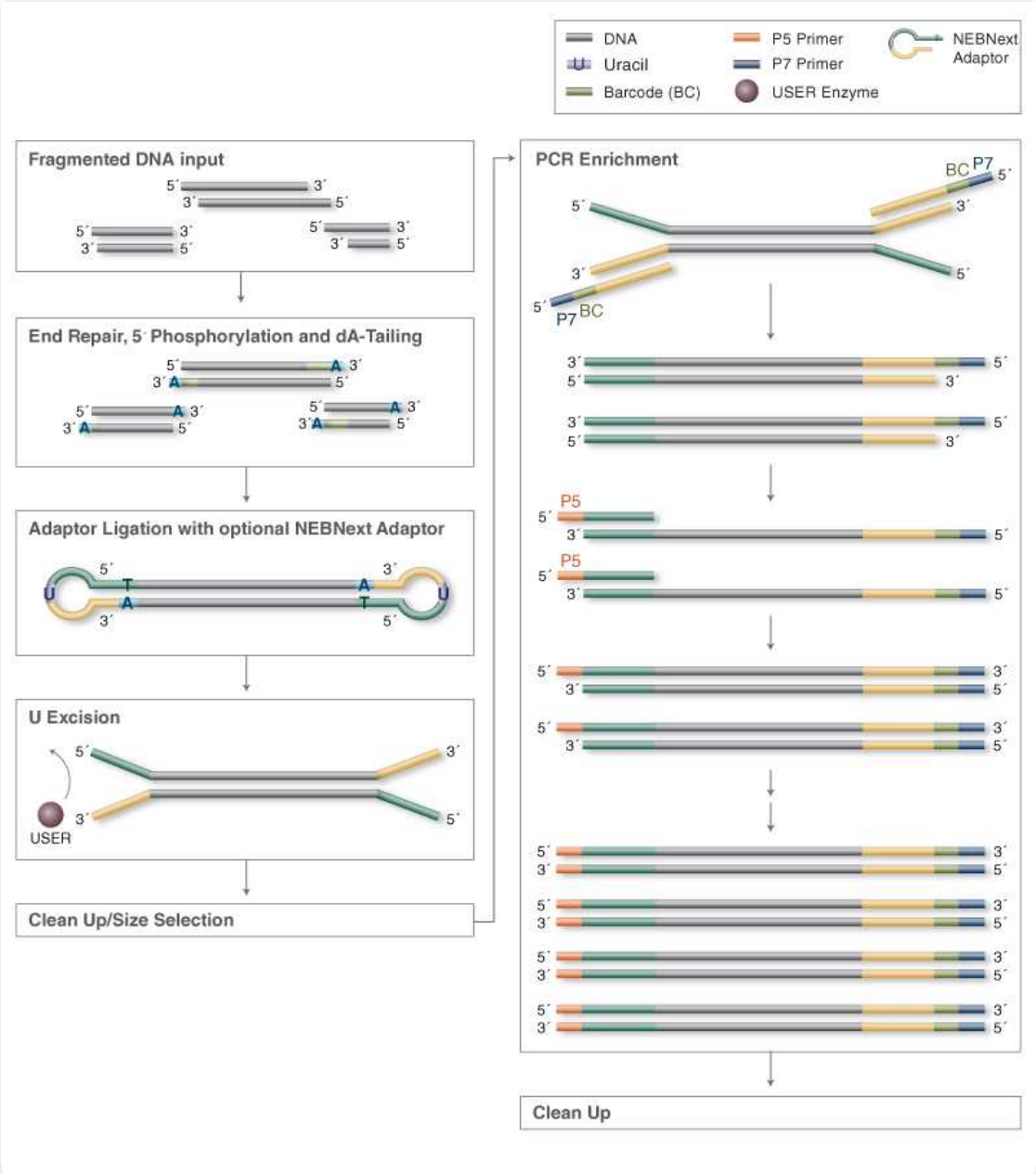

NEBNext Ultra™ DNA provides high library yields even with low inputs and difficult samples

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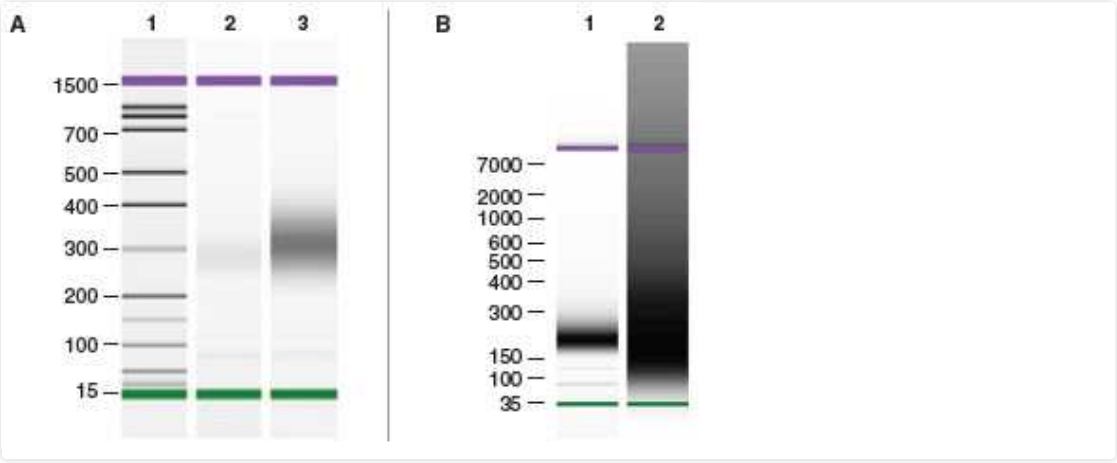
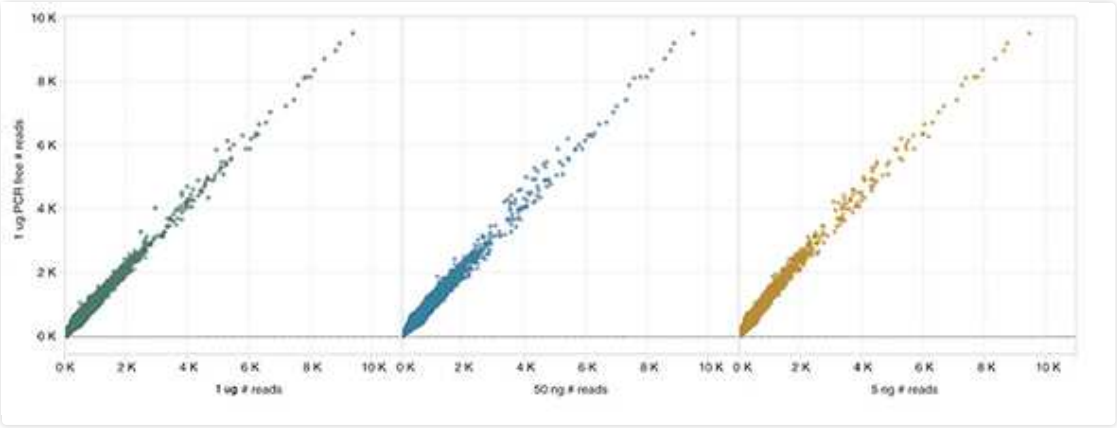


All libraries were prepared with 5 ng of input DNA and run on an Agilent Bioanalyzer®.
A.E. coligenomic DNA libraries. 1: Ladder; 2: Library prepared using NEBNext DNA Library Prep Master Mix Set (E6040); 3: Library prepared using NEBNext Ultra DNA Library Prep Kit (E7370). With 5 ng inputs, library yields are significantly higher using the NEBNext Ultra™ DNA Kit.
B. FFPE sample. Human lung tumor genomic DNA from a 66-year old male, extracted from a 10-year old FFPE block (Biochain, Inc.). 1: Library prepared using NEBNext Ultra DNA Library Prep Kit (E7370). 2: Input DNA (highly degraded FFPE genomic DNA).

Read depth correlation shows consistent high coverage for 5 ng - 1 µg input amounts



Libraries were prepared with various amounts of human IMR90 gDNA (5, 50, and 1000 ng) and PCR amplified. A PCR-free library was also prepared with 1000 ng human IMR90 gDNA. Sequence coverage of 10 kb windows of the human genome was analyzed on Galaxy, and Pearson correlation coefficient (R2 values) were calculated using Microsoft® Excel®. All libraries are highly correlated with each other, independent of DNA input as well as PCR amplification. High coverage was achieved for all DNA input amounts.

NEBNext Ultra DNA Kits display strong performance with input amounts as low as 5 ng

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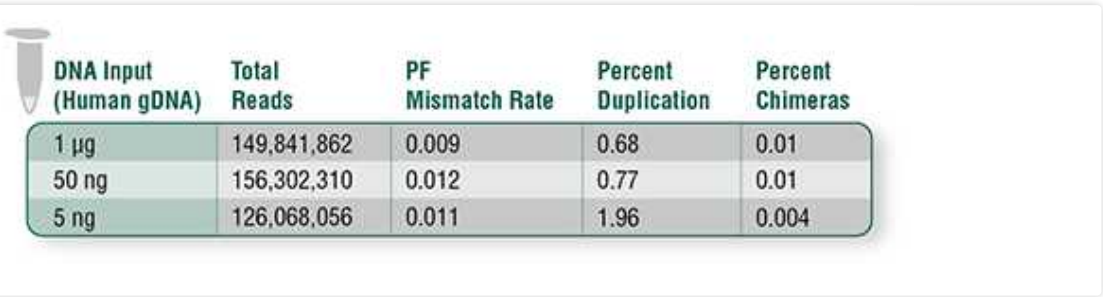

DNA Input (Human gDNA)	Total Reads	PF Mismatch Rate	Percent Duplication	Percent Chimeras
1 µg	149,841,862	0.009	0.68	0.01
50 ng	156,302,310	0.012	0.77	0.01
5 ng	126,068,056	0.011	1.96	0.004

Input DNA: IMR90 genomic DNA (human fibroblast).

PF Mismatch Rate: The rate of bases mismatching the reference for all bases aligned to the reference sequence.

Percent Duplication: The percentage of mapped sequence that is marked as duplicate.

Percent Chimeras: The percentage of reads that map outside of a maximum insert size or that have the two ends mapping to different chromosomes.

This data illustrates the strong performance of the NEBNext Ultra DNA Library Prep Kit for Illumina, even with 5 ng of human genomic DNA input. Mismatch rate, % duplication and % chimeras are all low, and similar, regardless of input amount.

Lot Control
The lots provided 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

MATERIALS

NEBNext Ultra DNA Library Prep Kit for Illumina - 24 rxns **New England Biolabs Catalog #E7370S**

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

NEBNext End Prep

1

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

A	B	C
Reagent	**Cap Color**	**Volume**
Fragmented DNA (5 ng–1 µg)		55.5 µl
End Prep Enzyme Mix	Green	3.0 µl
End Repair Reaction Buffer (10x)	Green	6.5 µl
Total Volume		65 µl

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2 

Set a 100 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 the liquid from the side of the tube.

Note: it is important to mix well. The presence of a small amount of bubbles will not interfere with the performance. See the guidelines section for a picture of acceptable amounts of bubbles.

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

A	B
Time	**Temperature**
30 minutes	20°C
30 minutes	65°C
Hold	4°C

Adaptor Ligation

4 

Add the following components directly to the End Prep reaction mixture:

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

A	B	C
Reagent	**Cap Color**	**Volume**
Blunt/TA Ligase Master Mix	Red	15 µl
Ligation Enhancer	Red	1 µl
NEBNext Adaptor For Illumina*	Red	2.5 µl
Total volume		83.5 µl

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

The NEBNext adaptor is provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB

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#E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina.

Ligation enhancer and Blunt TA Ligase Master Mix can be mixed ahead of time are 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 immediately mix well, or premix adaptor and sample and then add the other ligation reagents.

5

Set a 100 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 the liquid from the side of the tube.

Note: 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 the performance.

6

Incubate at **20 °C** for **00:15:00** in a thermal cycler.

7

Add **3 µL USER enzyme** to the ligation mixture.

Steps 7 and 8 are only required for use with NEBNext Adaptors. USER Enzyme can be found in the NEBNext Singleplex or Multiplex Oligos for Illumina.

8

Mix well and incubate at **37 °C** for **00:15:00** with heated lid set to ≥ **47 °C** .

9

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

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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.

10 Size selection is optional. If the starting material is > 50 ng, follow the protocol for size selection. For input less than 50 ng, size selection is not recommended. Follow the protocol for cleanup without size selection.
Step 10 includes a Step case.
Size Selection
Cleanup

Size Selection

step case

Size Selection

Follow this protocol if DNA starting material is > 50 ng

11

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:

A	B	C	D	E	F	G	H
LIBRARY PARAMETERS	**APPROXIMATEINSERT SIZE**	**150 bp**	**200 bp**	**250 bp**	**300-400 bp**	**400-500 bp**	**500-700 bp**
	Total Library Size (insert + adaptor)	270 bp	320 bp	400 bp	400-500 bp	500-600 bp	600-800 bp
VOLUME TO BE ADDED (µl)	1st Bead Selection	65	55	45	40	35	30
	2nd Bead Selection	25	25	25	20	15	15

12 Vortex SPRIselect beads to resuspend. AMPure XP beads can be used as well. If using AMPure XP

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beads, please allow the beads to warm to **Room temperature** for at least **00:30:00** before use.

13 

Add **13.5 µL dH2O** to the ligation reaction for a 100 µl total volume.

14 

Add **55 µL (0.55X) resuspended SPRIselect beads** to the **100 µL ligation reaction** (**for 200bp selection** -- for other size selections, refer to the table above for the amount of 1st bead selection to add).

15 

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.

16 

Incubate samples on bench top for at least **00:05:00** at **Room temperature** .

17 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.

18 After **00:05:00** (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.

19 

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Add **25 µL (0.25 x) resuspended SPRIselect beads** to the supernatant (**for 200bp selection** -- for other size selections, refer to the table above for the amount of 2nd bead selection to add).

20

Mix **at least** 10 times.

Be careful to expel all of the liquid from the tip during the last mix.

21

Incubate samples on the bench top for at least **00:05:00** at **Room temperature** .

22

Place the tube/plate on an appropriate magnetic stand for **00:05:00** (or until solution is clear) 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.

23 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant that contains unwanted DNA. **(Caution: do not discard beads).**

Be careful not to disturb the beads that contain the desired DNA targets.

24

Add **200 µL 80% freshly prepared ethanol** to the tube/plate while in the magnetic stand. Incubate at **Room temperature** for **00:00:30** , and then carefully remove and discard the supernatant.

Be careful not to disturb the beads that contain DNA targets.

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25

Repeat the previous step: Add **200 µL 80% freshly prepared ethanol** to the tube/plate while in the magnetic stand. Incubate at **Room temperature** for **00:00:30** , and then carefully remove and discard the supernatant.

Be careful not to disturb the beads that contain DNA targets.

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.

26 Air the dry beads for **up to** **00:05:00** while the tube is on the magnetic stand with the lid open.

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

27

Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into **17 µL** of **10 Milimolar (mM) Tris-HCl** or **0.1 X TE** .

28

Mix well on a vortex mixer or by pipetting up and down 10 times. Incubate for at least **00:02:00** 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.

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29

Place the tube/plate on a magnetic stand. After **00:05:00** (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 .

PCR Enrichment of Adaptor-ligated DNA

30

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

Mix the following components in sterile strip tubes:

- For index kits with primers separate:

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

A	B	C
Reagent	**Cap Color**	**Volume**
Adaptor Ligated Fragments	n/a	15 µl
NEBNext Q5 Hot Start HiFi PCR Master Mix	Blue	25 µl
Index Primer/ i7 primer*,**	Blue	5 µl
Universal PCR primer/ i5 Primer*,**	Blue	5 µl
Total volume	n/a	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 a single index kits) per sample.

- For Index kits where the primers are already combined:

Use this Option 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.

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A	B	C
Reagent	**Cap Color**	**Volume**
Adaptor Ligated Fragments	n/a	15 µl
NEBNext Q5 Hot Start HiFi PCR Master Mix	Blue	25 µl
Index/ Universal Primer*	Blue	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.

31 

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.

32 

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

A	B	C	D
Cycle Step	**Temperature**	**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	∞	

* Please note the number of PCR cycles recommended in the following table are to be seen as s starting point to determine the number of PCR cycles best for your samples. The number of cycles should be chosen based on the input amount and sample type. Thus, samples prepared with a different method prior to library prep may require further 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).

A	B
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

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adaptors require a minimum of 3 amplification cycles to add the complete adaptor sequences for downstream processes.

Cleanup of PCR Amplification

33

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.

34 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 **00:30:00** before use.

35 

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.

36 

Incubate for at least **00:05:00** at **Room temperature** .

37 Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant (**00:05:00** or until the solution is clear).

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.

38 After the solution is clear (about **00:05:00**), carefully remove and discard the supernatant. Be

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careful not to disturb the beads that contain DNA targets (**Caution do not discard beads**).

39

Add **200 µL 80% freshly prepared ethanol** to the tube/plate while in the magnetic stand. Incubate at **Room temperature** for **00:00:30** , and then carefully remove and discard the supernatant.

Be careful not to disturb the beads that contain DNA targets.

40

Repeat the previous step once for a total of two washes: Add **200 µL 80% freshly prepared ethanol** to the tube/ plate while in the magnetic stand. Incubate at **Room temperature** for **00:00:30** , and then carefully remove and discard the supernatant.

Be careful not to disturb the beads that contain DNA targets.

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.

41 Air dry the beads for **up to** **00:05:00** 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.

42

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

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43

Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least **00:02:00** 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.

44

Place the tube/plate on the magnetic stand. After **00:05:00** (or when the solution is clear), transfer **30 µL** to a new PCR tube and store at **-20 °C** .

45

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

46

Safe Stop: Samples can be stored at **-20 °C** .

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