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Protocol for use with NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645, E7103) V.2

New England Biolabs[1]

[1]New England Biolabs

New England Biolabs (NEB) NEBNext

Isabel Gautreau
New England Biolabs

ABSTRACT

The NEBNext Ultra II DNA Library Prep Kit for Illumina contains 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.
Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of an indexed 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 II DNA Library Prep Kit for Illumina

Protocol status: Working
We use this protocol and it's working

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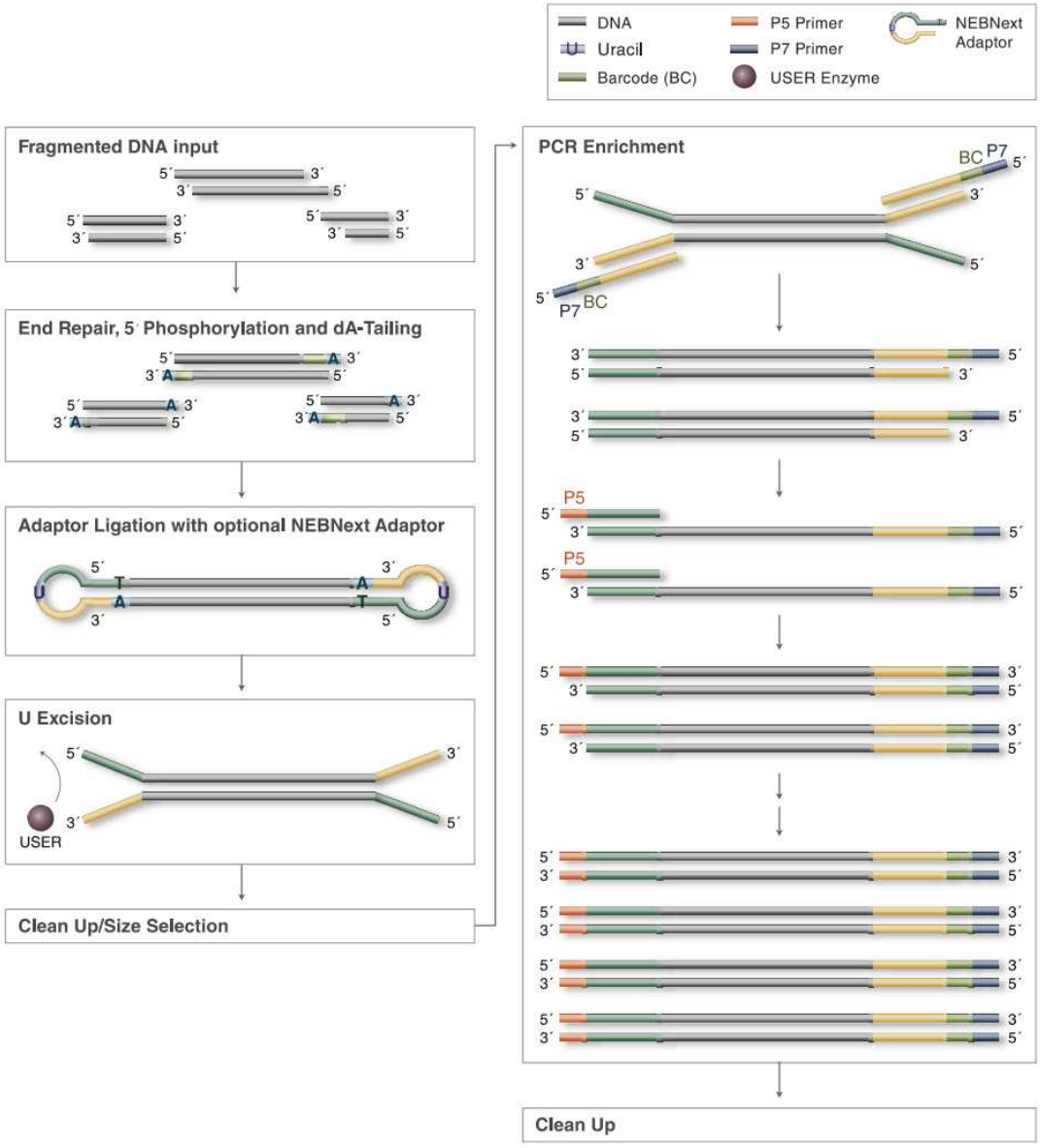

Adaptor trimming sequences:
The NEBNext libraries for Illumina resemble TruSeq libraries and can be trimmed similar to TruSeq:
Adaptor Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
Adaptor Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

GUIDELINES

Safe Stop Point: This is a point where you can safely stop the protocol.

Caution: This 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 amount of input DNA.

Color: A color listed before or after the reagent name indicates the cap color of the reagent to be added to the reaction.

MATERIALS

MATERIALS

This Library Kit Includes

The volumes provide are sufficient for preparation of up to 24 reactions (NEB #E7645S/ #E7103S) and 96 reactions (NEB #E7645L/ #E7103L). All reagents should be stored at -20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

Package 1: Store at -20°C

(green) NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #E7646**

(green) NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog #E7647**

(red) NEBNext Ultra II Ligation Master Mix **New England Biolabs Catalog #E7648**

(red) NEBNext Ligation Enhancer **New England Biolabs Catalog #E7374**

(blue) NEBNext Ultra II Q5 Master Mix **New England Biolabs Catalog #E7649**

Package 2: Store at room temperature. Do not freeze.

Supplied only with

NEBNext Ultra II DNA Library Prep with Sample Purification Beads - 24 rxns **New England Biolabs Catalog #E7103S**

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

Required Materials Not Included

NEBNext Oligo Kit options can be found at www.neb.com/oligos
Alternatively, customer supplied adaptor and primers can be used, please see information in link below:
https://www.neb.com/faqs/2019/03/08/can-i-use-this-nebnext-kit-with-adaptors-and-primers-from-other-vendors-than-neb

Please note: Separate instructions exist for UNIQUE DUAL INDEX UMI ADAPTORS. Please contact Technical Support at info@neb.com

- Magnetic rack (

NEBNext® Magnetic Separation Rack **New England Biolabs Catalog #S1515S**

) magnetic plate (

Alpaqua 96S Super Magnet Plate **Contributed by users Catalog #A001322**

) or equivalent.

- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- 0.1X TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- Thin wall 200 µl PCR tubes (for example

  TempAssure PCR 8-tube strip **USA Scientific Catalog #1402-4700**

)
- PCR machine
- Bioanalyzer®, TapeStation® (Agilent Technologies, Inc.) or similar fragment analyzer and consumables.

For NEB #E7645 only:

SPRIselect reagent kit **Beckman Coulter Catalog #B23317**

or

Ampure XP beads **Beckman Coulter Catalog #A63881**

Optional:

10 mM Tris-HCl, pH 7.5-8.0 with 10 mM NaCl (for adaptor dilution of DNA input < 100 ng) or

NEBNext Adaptor Dilution Buffer - 9.6 ml **New England Biolabs Catalog #B1430S**

PROTOCOL MATERIALS

NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog #E7647**

Materials, Step 1

NEBNext Ultra II Ligation Master Mix **New England Biolabs Catalog #E7648**

Materials, Step 5

NEBNext Ultra II Q5 Master Mix **New England Biolabs Catalog #E7649**

Materials, Step 27

Alpaqua 96S Super Magnet Plate **Catalog #A001322**

Materials

Ampure XP beads **Beckman Coulter Catalog #A63881** Materials

TempAssure PCR 8-tube strip **USA Scientific Catalog #1402-4700** Materials

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

In Materials and 2 steps

NEBNext Adaptor Dilution Buffer - 9.6 ml **New England Biolabs Catalog #B1430S**

Materials

NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #E7646**

Materials, Step 1

NEBNext Adaptor for Illumina **New England Biolabs Catalog #E7337 in Kits E7335, E7500, E771**

Step 5

NEBNext Ligation Enhancer **New England Biolabs Catalog #E7374**

Materials, Step 5

TE Buffer (1X) **New England Biolabs Catalog #E7808** Step 23

NEBNext Ultra II DNA Library Prep with Sample Purification Beads - 24 rxns **New England Biolabs Catalog #E7103S**

Materials

SPRIselect reagent kit **Beckman Coulter Catalog #B23317** Materials

NEBNext® Magnetic Separation Rack **New England Biolabs Catalog #S1515S**

Materials

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE START INSTRUCTIONS

Starting Material: 500 pg – 1 µg fragmented DNA. NEB recommends 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.

NEBNext End Prep

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

	A	B
	Component	Volume
	(green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
	(green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
	Fragmented DNA	50 µl
	Total Volume	60 µl

NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #E7646**

NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog #E7647**

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.

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

00:30:00 @ 20 °C
00:30:00 @ 65 °C
Hold at 4 °C

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

Adaptor Ligation

4 Determine whether adaptor dilution is necessary.

Caution: If DNA input is ≤ 100 ng, dilute the NEBNext Adaptor for Illumina in 10 mM Tris-HCl, pH 7.5-8.0 with 10 mM NaCl as indicated in the table.

	A	B	C
	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

Note

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.

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

	A	B
	Component	**Amount**
	End Prep Reaction Mixture (Step 3)	60 µl
	(red) NEBNext Adaptor for Illumina**	2.5 µl
	(red) NEBNext Ultra II Ligation Master Mix*	30 µl
	(red) NEBNext Ligation Enhancer	1 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 Oligo kits. NEB has several oligo options which are supplied separately from the library prep kit. Please see www.neb.com/oligos for additional information.

Note

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

NEBNext Adaptor for Illumina **New England Biolabs Catalog #E7337 in Kits E7335, E7500, E771**

NEBNext Ultra II Ligation Master Mix **New England Biolabs Catalog #E7648**

NEBNext Ligation Enhancer **New England Biolabs Catalog #E7374**

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.

7 Incubate at 20 °C for 00:15:00 in a thermal cycler with the heated lid off.

8 Add 3 µL of (red or blue) USER Enzyme to the ligation mixture from Step 6.

Note

Steps 8 and 9 are only required for use with non indexed NEBNext Adaptor. USER enzyme can be found in most NEBNext oligo kits. If you are using the indexed UMI adaptor, USER is not needed. Please see corresponding manual for use with UMI on the NEB #E7395 product page under the protocols, manuals, and usage tab.

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

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

Size Selection or Cleanup of Adaptor-ligated DNA

10 **Caution: If the starting material is > 50 ng, follow the protocol for size selection in Step Case: Input > 50 ng. For input ≤ 50 ng, size selection is not recommended to maintain library complexity. Follow the protocol for cleanup without size selection in Step Case: Input ≤ 50 ng.**

Size Selection of Adaptor-ligated DNA

STEP CASE

Input > 50 ng 33 steps

Size Selection of Adaptor-ligated DNA

11 **Caution: 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** 00:30:00 **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.**

Caution: 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:
Recommended Conditions for Bead Based Size Selection

	A	B	C	D	E	F	G	H
		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	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

12 Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

13 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 00:00:03 – 00:00:05 on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

14 Incubate samples on bench top for at least 00:05:00 at room temperature.

15 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.

16 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.

17 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 00:05:00 at room temperature.

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

18 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.

19 After 00:05:00 (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.

20 Add 200 µL of 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.

21 Repeat step 20 once for a total of two washes. Be sure to remove all visable 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.

22 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.

23 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.

TE Buffer (1X) **New England Biolabs Catalog #E7808**

24 Mix well on a vortex mixer or by pipetting up and down 10 times. Incubate for at least 00:02:00 a 2m 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.

25 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 Stop Point: Samples can be stored at -20 °C

PCR Enrichment of Adaptor-ligated DNA: PCR Amplification

26 Use **Option A** if you are using the following oligos:
Use Option A for any NEBNext Oligo Kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes. Primers are supplied at 10 µM each.

Use **Option B** if you are using the following oligos:
Use Option B for any kit where NEBNext Oligo Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 µM (5 µM each).

27 **PCR Amplification**

Add the following components to a sterile strip tube:

Option A: Forward and Reverse Primers NOT Already Combined

	A	B
	Component	**Volume**
	Adaptor Ligated DNA Fragments (Step 25 for Input > 50 ng or Step 22 for Input ≤ 50 ng)	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

* NEBNext Oligos must be purchased separately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" section (in abstract). 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.

Option B: Forward and Reverse Primer Already Combined

	A	B
	Comonent	**Volume**
	Adaptor Ligated DNA Fragments (Step 25 for Input > 50 ng or Step 22 for Input ≤ 50 ng)	15 µl

	A	B
	(blue) NEBNext Ultra II Q5 Master Mix	25 µl
	(blue) Index Primer Mix*	10 µl
	Total Volume	50 µl

*NEBNext Oligos must be purchased separately from the libray prep kit. For oligo purchasing options refer to "Required Materials Not Included" section (page 1).

NEBNext Ultra II Q5 Master Mix **New England Biolabs Catalog #E7649**

28 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.

29 Place the tube on a thermal cycler and perform PCR amplification using the following PCR cycling conditions.

	A	B	C	D
	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.

	A	B
	INPUT DNA IN THE END PREP REACTION	**# OF CYCLES REQUIRED FOR STANDARD LIBRARY PREP ~100 ng (30–100 nM)**

	A	B
	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

Table 4.1

* 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.

	A	B
	INPUT DNA IN THE END PREP REACTION	**# OF CYCLES REQUIRED FOR STANDARD 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

Table 4.2

* 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.

30 Proceed to Cleanup of PCR Amplification in the next Section.

Cleanup of PCR Reaction

31

Note

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.

32 Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

33 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 00:00:03 – 00:00:05 on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

34 Incubate samples on bench top for at least 00:05:00 at room temperature.

35 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.

36 After 00:05:00 (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.

37 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.

38 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.

39 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.

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

41 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.

42 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.

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

Note

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

Examples of libraries prepared with human DNA (NA19240):