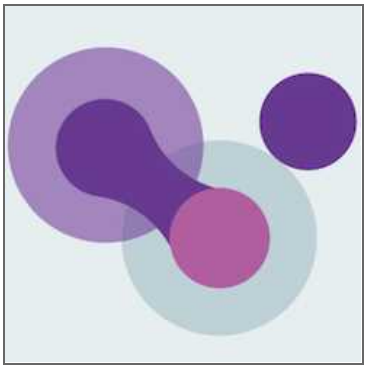

OCT 17, 2023

OPEN ACCESS

External link:
https://international.neb.com/products/e7805-nebnext-ultra-ii-fs-dna-library-prep-kit-for-illumina#Protocols,%20Manuals%20&%20Usage

Protocol Citation: Mihir Kekre, Ben Pascoe 2023. C-SOP-301: DNA Library Preparation using the NEBNext Ultra II FS DNA Kit (≥100 ng DNA input). **protocols.io** https://protocols.io/view/c-sop-301-dna-library-preparation-using-the-nebnex-c3hyyj7w

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

C-SOP-301: DNA Library Preparation using the NEBNext Ultra II FS DNA Kit (≥100 ng DNA input)

Forked from C-SOP-301: DNA Library Preparation using the NEBNext Ultra II FS DNA Kit (≥100 ng DNA input)

Ben
Mihir Kekre[1], Pascoe[1]

[1]The Centre for Genomic Pathogen Surveillance, Oxford, United Kingdom

CGPS

Ben Pascoe
The Centre for Genomic Pathogen Surveillance, Oxford, United...

DISCLAIMER

This protocol has been adapted from the NEBNext®Ultra™II FS DNA Library Prep Kit for Illumina developed by New England BioLabs (NEB).

Created: Oct 16, 2023

Last Modified: Oct 17, 2023

PROTOCOL integer ID: 89368

Keywords: DNA library, library prep, library preparation, NEB, enzymatic fragmentation, illumina DNA library preparation

ABSTRACT

The NEBNext®Ultra™ II FS DNA Library Prep Kit meets the dual challenges of constructing high quality libraries from ever-decreasing input material, and scalability of library construction compatible with Illumina sequencing platforms. The fragmentation protocol can tolerate any input amount from 100 picograms–500 nanograms of DNA and varying amounts (%) of GC content.

Some of the operational advantages that this kit offers are:

- Perform fragmentation, end repair and dA-tailing with a single enzyme mix
- Experience reliable fragmentation with a single protocol, regardless of DNA input amount or GC content
- Prepare high quality libraries from a wide range of input amounts: 100 pg–500 ng
- Generate high yields with increased reaction efficiencies and minimised sample loss
- Use with DNA in standard buffers (TE, Tris-HCl) and water
- Vary incubation time to generate a wide range of insert sizes

This protocol has been adapted from the NEBNext®Ultra™II FS DNA Library Prep Kit for Illumina developed by New England BioLabs (NEB).

GUIDELINES

E6177: Product Specifications

MATERIALS

1. Double-stranded genomic DNA (quantified using Qubit and purity-checked with Nanodrop)

2. NEBNext®Ultra™II FS DNA Library Prep for Illumina w/ Sample Purification Beads (New England BioLabs, Cat no. E6177L)

Required Materials that are not included in (2):
3. 80% Ethanol (freshly prepared solution)

4. Nuclease-free Water

5. 96-well PCR-plate, low-profile, full skirted (ThermoFisher Scientific, Cat no. AB0800)

6. NEBNext® Multiplex Oligos (New England BioLabs, Cat no. E6609S, www.neb.com/oligos)

7. Magnetic rack/stand (ThermoFisher Scientific DynaMag™-96 Side Skirted Magnet, Cat no. 12027 **or** Alpaqua Catalyst™ 96 Slotted Ring Magnet Plate, Cat no. A000550)

8. PCR Thermal Cycler

9. Vortex

10. Microcentrifuge

11. 2.0 mL microcentrifuge tubes

12. 15 mL Falcon tubes

13. Single- and multi-channel pipettes (P2, P10, P200, P1000) with compatible, sterile filtered pipette tips

SAFETY WARNINGS

Safety Data Sheets (NEBNext®Ultra™II FS):

1. Ligation Enhancer
2. Ultra II Ligation Master Mix
3. NEBNext® Ultra II Q5 Master Mix
4. NEBNext® Ultra II FS Enzyme Mix
5. NEBNext® Ultra II FS Reaction Buffer
6. TE Buffer

Before Starting

1

Prior to initiating the protocol, ensure that all active workbenches are cleaned with 80% ethanol, all relevant personal protective clothing is worn and the work area is prepared according to local GLP guidelines for molecular methods.

2 **Starting Material:** 200 ng purified, genomic DNA.

> **Note**
>
> i. It is recommended that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA), however, 10 mM Tris pH 7.5–8, low EDTA TE or nuclease-free H_2O are also acceptable.
>
> ii. If the input DNA (of 200 ng) is less than 26 µl, add 1X TE (provided) to a final volume of 26 µl.

3 Create an organised bench space by clearing away all clutter in order to maximize work efficiency and to avoid unnecessary movements that will minimise exposure of sterile materials to airborne and liquid contaminants.

Prepare a large bucket of ice to store reagents and samples temporarily during use.

Fragmentation/End Prep

13s

4 **Fragmentation occurs during a 37°C incubation step.**
Ensure that the Ultra II FS Reaction Buffer is completely thawed before placing it on ice.

Note

If a precipitate is seen in the buffer, pipette up and down several times to break it up.

5 Vortex the Ultra II FS Enzyme Mix 00:00:08 prior to use and place on ice. 8s

Note

It is important to vortex the enzyme mix prior to use for optimal performance.

6 In a 15 mL Falcon tube, prepare a fresh mastermix of the following components (for each DNA sample + 1 sample overage) as indicated below in Table 1:

	A	B
	Component	Volume per library
	(yellow) NEBNext Ultra II FS Reaction Buffer	7 µl
	(yellow) NEBNext Ultra II FS Enzyme Mix	2 µl
	Total Volume	9 µl

Table 1. Fragmentation Mastermix

7 Gently mix the reaction by pipetting up/down 15 times and then briefly spin in a microcentrifuge.

Avoid frothing the solution.

8 Using a P10, pipette:

- 9 µL of mastermix into each required well of the 96-plate PCR microplate.
- 26 µL of DNA from each sample (normalised to 200ng from step 2)

Note

i. Using a permanent marker, demarcate these wells as 'FRAG' (short for fragmentation). Make sure that the marker ink does not get close to any of the sample wells.

ii. The total volume in each sample well should be the same (35 µL)

Gently mix the reaction by pipetting up/down 15 times and then briefly spin in a microcentrifuge to collect liquid from the sides of the well..

Avoid frothing of the solution.

9 Place the sealed PCR microplate in the thermocycler. With a heated lid set to 75 °C, run the following program: 40m

1. 00:10:00 @ 37 °C
2. 00:30:00 @ 65 °C
3. **Infinite Hold** @ 4 °C

Using these conditions, we will obtain a mean fragment size of approx. 500 bp for each library sample.

Note

Use Table 2 to determine the incubation time required to generate any custom-desired fragment size.
Incubation time may need to be optimised for individual samples.

See Fig. 1 for a typical fragmentation pattern.

FRAGMENTATION SIZE	INCUBATION @ 37°C	OPTIMIZATION
100 bp-250 bp	30 min	30-40 min
150 bp-350 bp	20 min	20-30 min
200 bp-450 bp	15 min	15-20 min
300 bp-700 bp	10 min	5-15 min
500 bp-1 kb	5 min	5-10 min

Table 2.

Example of size distribution on a Bioanalyzer®. Human DNA (NA19240) was fragmented for 5-40 min.

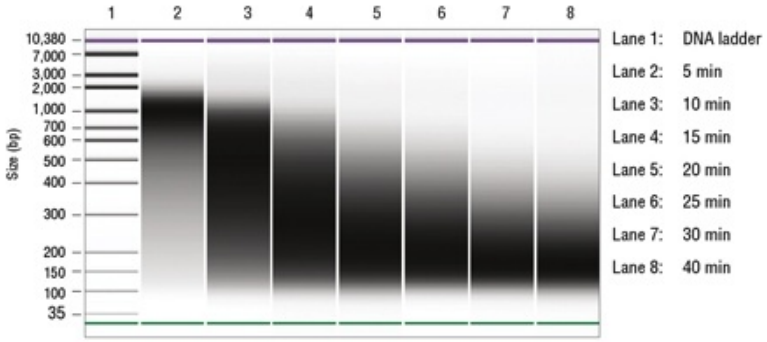

Figure 1.

Adaptor Ligation

30m

10 Remove the microplate from the thermocycler and immediately place on ice or a cold block.

> **Note**
>
> At this stage, it is recommended that a volume check is performed on a few random wells to ensure that the reaction volume for each sample is still 35 µL (+/- 1 µL).

11 In a 15 mL Falcon tube, prepare a fresh mastermix of the following components (for each Frag sample + 1 sample overage) as indicated below in Table 3:

	A	B
	Component	Volume per library
	(red) NEBNext Ultra II Ligation Master Mix*	30 µl
	(red) NEBNext Ligation Enhancer	1 µl
	(red) NEBNext Adaptor for Illumina**	2.5 µl
	Total Volume	33.5 µl

Table 3.
* The Ultra II Ligation Master Mix is highly viscous. Mix it by pipetting up and down several times prior to adding to the reaction
** The NEBNext adaptor is provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335,#E7500,#E7710,#E7730,#E7600,#E7535and#E6609) Oligos for Illumina.

12 Gently mix the reaction by pipetting up/down 15 times and then briefly spin in a microcentrifuge.

Avoid frothing of the solution.

13 Using a P100 or P200, pipette:

- 33.5 µL of mastermix into each Frag mix-occupied well of the microplate.

Note

i. Using a permanent marker, strikethrough FRAG and **replace with 'LIG'**. Make sure that the marker ink does not get close to any of the sample wells.

ii. The total volume in each sample well should now be the same (68.5 µL)

Gently mix the reaction by pipetting up/down 10 times and then briefly spin in a microcentrifuge to collect liquid from the sides of the well.

Avoid frothing of the solution.

14 Place the sealed PCR microplate in the thermocycler. With a heated lid **OFF**, run the following program: 15m

00:15:00 @ 20 °C

Remove the plate from the thermocycler immediately upon program completion.

15 To each well, now add 3 µL of (red) USER®Enzyme. 15m

Mix by gentle repeated pipetting.

Place the sealed PCR microplate in the thermocycler.
With a heated lid set at 47 °C, run the following program:

00:15:00 @ 37 °C

Note

i. Use a fresh tip for subsequent samples if you deposit the enzyme by touching the liquid surface.

ii. Step 15 is only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335,#E7500,#E7710,#E7730,#E7600,#E7535and#E6609) Oligos for Illumina.

16 Remove the microplate from the thermocycler, place on ice and proceed to the next section (Size Selection of Adaptor-ligated DNA)

Note

The protocol can be **paused** here provided the samples are stored at -20 °C Overnight.

Size Selection of Adaptor-ligated DNA

27m 30s

17 In order to select in the right length of library fragments, the volumes of SPRIselect or NEBNext Sample Purification Beads provided are to be used in exact volume ratios to sample in each well. **A shift in the volume ratios (of sample : beads) may lead to selection of a different fragment size range.**

Note

At this stage, it is recommended that a volume check is performed on the wells to ensure that the reaction volume for each sample is 71.5 µL.

18 The size selection protocol is based on a total starting volume of 100 µl.

Bring the volume of each well up to 100 µl by adding 28.5 µL **0.1X TE** (1X TE Buffer diluted 1:10 with water).

19 Vortex SPRIselect or NEBNext Sample Purification Beads for 00:00:30 to resuspend. 30s

Note

Size selection conditions were optimized with SPRIselect or NEBNext Sample Purification Beads; however, AMPure XP beads can be used following the same conditions.

If using AMPure XP beads, please allow the beads to warm to Room temperature for at least 00:30:00 before use.

20 Table 4 shows the recommended conditions (and volumes) for bead-based size selection:

	Approximate Insert Size Distribution	150-250 bp	200-350 bp	275-475 bp	350-600 bp
Library Size	Approx. Final Library Size Distribution (Insert + Adaptor + Primers)	270-370 bp	320-470 bp	400-600 bp	470-800 bp
Volume to be added (µl)	1st Bead Selection	40	30	25	20
	2nd Bead Selection	20	15	10	10

Table 4.

For this protocol, we will aim for insert sizes between 350-600 bp.

21 Add 20 µL (0.2X) resuspended beads to the 100 µl samples from step 18.

Mix well by pipetting up and down very gently at least 10 times.

Note

i. The SPRI beads tends to be viscous and slow-aspirating. Take extra care to to expel all of the liquid out of the tip during the last mix.

ii. Pipette gently to avoid droplet sprays on the inner sides of the well.

22 Incubate the mix on the bench for 00:07:00 at Room temperature. 7m

23 Place the plate on the magnetic stand/rack to separate the beads from the supernatant.

Note

If necessary, quickly spin (3 seconds) the samples to collect the liquid from the sides of the plate wells before placing it on the magnetic stand.

24 After 7 minutes (or when the solution is clear), carefully **transfer the supernatant** (~ 120 µL) containing your DNA to a new tube.

Note

Do NOT discard the supernatant. Discard the beads that will now contain the unwanted fragment clusters.

25 Add 10 µL (~0.1X) resuspended SPRIselect or Sample Purification Beads to the supernatant.

Note

If the SPRIselect have separated out at this stage, vortex for 30 seconds once again.

Mix well by pipetting up and down very gently at least 10 times. Be careful to expel all of the liquid from the tip during the last mix.

26 Incubate samples on the bench for 00:07:00 at Room temperature. 7m

27 Place the plate on the magnetic stand to separate the beads from the supernatant.

Note

If necessary, quickly spin (3 seconds) the samples to collect the liquid from the sides of the plate wells before placing on the magnetic stand.

28 After 00:07:00 (or when the solution is clear), carefully **remove and discard the supernatant** that contains unwanted fragments. 7m

Note

Do NOT discard the beads.

Be careful not to disturb the beads that contain the desired DNA when draining the supernatant.

29 Add 200 µL of freshly prepared 80% ethanol to the plate wells while they are still on the magnetic stand.

30 Incubate at Room temperature for 00:00:30, and then carefully remove and discard the liquid. 30s

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

31 Repeat the ethanol wash steps 29 and 30 **two additional times**.

32 After the third wash, be sure to remove all visible traces/droplets of ethanol. 1m

If necessary, briefly spin the tube/plate, place back on the magnet for 00:01:00 and remove traces of ethanol at the bottom of each well with a p10 pipette tip.

33 Air dry the beads for up to 5 minutes while the plate is on the magnetic stand exposed to the air.

> **Note**
>
> **CAUTION: Do not over-dry the beads as this will 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.

34 Remove the plate from the magnetic stand.

Elute the DNA target from the beads by adding 17 µL **0.1X TE** directly onto the clumped beads.

35 Mix well by gently pipetting up and down 10 times. 5m

Incubate for 00:05:00 at Room temperature .

36 Place the plate on the magnetic stand. 7m

After 00:07:00 or when the solution is clear, transfer 15 µL of each eluate to a fresh well on the plate.

37 Place the microplate on ice and proceed to the next section (PCR Enrichment of Adaptor-ligated DNA).

Note

The protocol can be **paused** here provided the samples are stored at -20 °C Overnight .

PCR Enrichment of Adaptor-ligated DNA 1m 55s

38 On a fresh 96-PCR microplate, pipette 25 µL of the (blue) **NEBNext Ultra II Q5 Master Mix** into each corresponding sample well from the previous reaction.

Note

i. Using a permanent marker, demarcate the fresh wells as 'LIB PCR'. Make sure that the marker ink does not get close to any of the sample wells.

39 To the Q5 Master Mix in each well, add 10 µL of (blue) Index/Universal Primer combination mix.

Ensure that a fresh tip is used for every well.

Note

The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB Cat no. E6609).

Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

40 To each well, now add 15 µL of the corresponding size-selected eluates generated in step 36. The PCR reactions in each well will now contain the following:

Adaptor Ligated DNA Fragments	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
(blue) Index/Universal Primer****	10 µl
Total Volume	**50 µl**

Table 5.

The total volume in each sample well should be the same (50 µL)

41 Using a P100/200 pipette set to 40ul, gently pipette the entire PCR mix up and down at least 10 times to mix thoroughly.

Perform a quick spin to collect all liquid from the sides of the tube.

42 Place the sealed PCR microplate in the thermocycler. With a heated lid set to 98 °C, run the following program:

	A	B	C	D
	CYCLE STEP	TEMPERATURE	TIME	CYCLES
	Initial Denaturation	98°C	30 seconds	1
	Denaturation	98°C	10 seconds	3-7
	Annealing/Extension	65°C	75 seconds	
	Final Extension	65°C	5 minutes	1
	Hold	4°C	∞	

Table 6.
* The number of PCR cycles recommended for denaturation/annealing-extension need to be custom determined and should be chosen based on input amount and sample type. Thus, samples prepared with a different method prior to library prep may require re-optimisation 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 artefacts and over-cycling (high molecular weight fragments on Bioanalyzer or Tapestation).

For standard library prep samples, Table 7 could be used as a starting point to determine the number of PCR cycles.

	A	B
	INPUT DNA IN THE FS REACTION	# OF CYCLES REQUIRED FOR STANDARD LIBRARY PREP: YIELD ~100 ng (5–35 nM)*
	500 ng	3**
	200 ng	3-4
	100 ng	4-5

Table 7.
* Cycle number was determined for size selected libraries.
** 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.

Note

For the aims and outputs of this protocol, our recommendation would be to apply **5 CYCLES**.

Cleanup of Amplified Libraries

20m 30s

43 Remove the PCR amplicons from the thermocycler and immediately place on ice.

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.

44 Vortex SPRIselect or NEBNext Sample Purification Beads for 00:00:30 to resuspend. 30s

45 Add 45 µL (0.9X) resuspended beads to the PCR reaction.

Mix well by gently pipetting up and down at least 10 times.

Note

i. The SPRI beads tends to be viscous and slow-aspirating. Take extra care to to expel all of the liquid out of the tip during the last mix.

ii. Pipette gently to avoid droplet sprays on the inner sides of the well.

46 Incubate the samples on the bench for 00:07:00 at Room temperature. 7m

47 Place the plate on the magnetic stand to separate the beads from the supernatant.

Note

If necessary, quickly spin (3 seconds) the samples to collect the liquid from the sides of the plate wells before placing on the magnetic stand.

48 After 00:07:00 (or when the solution is clear), carefully **remove and discard the supernatant**. 7m

Note

Do NOT discard the beads.

Be careful not to disturb the beads that contain the desired DNA when draining the supernatant.

49 Add 200 µL of freshly prepared 80% ethanol to the plate wells while they are still on the magnetic stand.

50 Incubate at Room temperature for 00:00:30, and then carefully remove and discard the liquid. 30s

Note

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

51 Repeat the ethanol wash steps 49 and 50 **two additional times**.

52 After the third wash, be sure to remove all visible traces/droplets of ethanol. 1m

If necessary, briefly spin the tube/plate, place back on the magnet for 00:01:00 and remove traces of ethanol at the bottom of each well with a p10 pipette tip.

53 Air dry the beads for up to 5 minutes while the plate is on the magnetic stand exposed to the air.

> Note
>
> **CAUTION: Do not over-dry the beads as this will 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.

54

Remove the plate from the magnetic stand.

Elute the DNA target from the beads by swiftly adding 33 µL **0.1X TE** directly onto the clumped beads.

55 Mix well by gently pipetting up and down 10 times. 7m

Incubate for 00:07:00 at Room temperature.

56 Place the plate on the magnetic stand. 7m

After 00:07:00 (or when the solution is clear), transfer 30 µL of each eluate to a fresh well.

57 WGS Library preparation is now complete.
Store the prepared libraries at -20 °C until they are ready for QC.

Assessing Library Quality

58 Library QC can be performed using a combination of fragment analysis and real-time PCR to estimate qualitative measurements of 1. median fragment size, 2. distributions of fragments sizes alongside quantitative estimates like mean library concentration.

Recommended assays:
Fragment analysis -
Agilent High Sensitivity (HS) Assay on the Bioanalyzer 2100
or
Agilent D1000 ScreenTape Assay on the Tapestation 4150/4200

qPCR (using SYBR fluorescent dyes) -
NEBNext Library Quant Kit for Illumina (NEB)
or
KAPA Library Quantification Kit Illumina® Platforms (Roche)

> **Note**
>
> For a guide to library QC for Illumina sequencing, refer to:
> "**C-SOP-401: Quality Control (QC) of DNA Libraries for Whole Genome Sequencing**" on protocols.io

Additional Information & Troubleshooting

59 Troubleshooting Guide for NEBNext®Ultra™ II FS DNA Library Prep Kit

Technical Note: SPRI select: DNA Ratios Affect the Size Range of Library Fragments (10x Genomics)