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We are still developing and optimizing this protocol

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NEBNext Ultra II Library Prep for Illumina Low Volume Version (Naturalis)

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ABSTRACT

Low volume version of NEBNext Ultra II DNA library protocol (Naturalis).

PROTOCOL MATERIALS

NEBNext dA-Tailing Reaction Buffer - 2.0 ml **New England Biolabs Catalog #B6059S**

In 2 steps

TE Buffer **Contributed by users** Step 1

NEBNext Ligation Master Mix **New England Biolabs Catalog #E7648AAVIAL**

Step 5

NEBNext® Ligation Enhancer **New England Biolabs Catalog #E7374AAVIAL**

Step 5

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

Step 5

NEB USER® Enzyme **New England Biolabs Catalog #M5505S/L** Step 5.4

NEBNext Ultra II Q5 Master Mix - 250 rxns **New England Biolabs Catalog #M0544L**

Step 7

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

In 2 steps

NEBNext Ultra II Library Prep for Illumina Low Volume Version...

1 Make sure that the DNA is eluted in TE Buffer **Contributed by users**. If this is not the case, samples can be diluted using 10nM Tris-HCl, pH 8.0 or 0.1X TE.

2 Check the DNA concentration of your samples using the Fragment Analyzer Genomic DNA 50Kb kit (DNF-467-0500).

3 Normalise all samples based on the Fragment Analyzer results. The DNA input (ng) should be close to equal for all samples before proceeding with the library prep.

3.1 Concentrate plates using the Eppendorf Concentrator (SpeedVac) if starting concentrations are low.

NEBNext End Prep

4 Thaw the NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #E7646** and NEBNext dA-Tailing Reaction Buffer - 2.0 ml **New England Biolabs Catalog #B6059S**, place the **Enzyme Mix** on ice.

Gently mix and spin down the components before use.

Prepare a **master mix** of the NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #E7646** and NEBNext dA-Tailing Reaction Buffer - 2.0 ml **New England Biolabs Catalog #B6059S** based on the volume option you choose to work with.

4.1

	A	B	C	D
		Volume option 1 (1:8)	Volume option 2 (1:12)	Volume option 3 (1:16)

	A	B	C	D
	NEBNext Ultra II End Prep Enzyme Mix	0,375	0,25	0,15
	NEBNext Ultra II End Prep Reaction Buffer	0,875	0,58	0,35
	Fragmented DNA (can be adjust with 0,1 xTE)	6,26	4,17	2,5
	Total volume	7,5	5	3

Table 1: Amount (µl) per volume option.

4.2

- Let the I.DOT Mini distribute the appropriate volume of the master mix:
- **Option 1**: 1.25 µL, **Option 2**: 0.83 µL, and **option 3**: 0.5 µL
- **I.DOT Liquid setting**: H_20.

4.3

- Manually add the appropriate amount of fragmented DNA (see ***Table 1***) using a P10 multichannel.
- Gently mix by slowly pipetting the entire volume up and down at least 10 times.

4.4 Place in a thermocycler, with the heated lid set to ≥ 75 °C, and run the following program: 1h

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

NEBNext Adaptor Ligation 30m

5 Thaw the NEBNext Ligation Master Mix **New England Biolabs Catalog #E7648AAVIAL**, NEBNext® Ligation Enhancer **New England Biolabs Catalog #E7374AAVIAL** & NEBNext Adaptor for Illumina **New England Biolabs Catalog #E7337 in Kits E7335, E7500, E771** and place on ice.

5.1 **Important note:** Before starting the Adapter Ligation, determine whether adaptor dilution* is necessary:

	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

	A	B	C
	less than 5 ng	25-Fold (1:25)	0.6 µM

Table 2: Input Adaptor Dilution.

- Prepare an Adaptor dilution in Tris/NaCl, pH 8.0 (10 mM Tris, 10mM NaCl) if necessary.

*The NEB adaptor sequence:
/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTC/ideoxyU/ACACTCTTTCCCTACACGACGCTCTTCCGATC*T

5.2

- Prepare a Master Mix of the Ligation Master Mix & Ligation Enhancer based on the volume option chosen (see ***Table 3***).
- Let the **I.DOT Mini** pipet the appropriate volume of your master mix to the End Prep Reaction Mixture.
- Option 1: 3.875 µL, option 2: 2.583 µL, and option 3: 1.55 µL.
  - **Liquid class**:
- Let the I.DOT Mini pipet the appropriate volume of your NEBNext Adaptor for Illumina.
  - **Liquid class**: H_20.

	A	B	C	D
		Volume option 1 (1:8)	Volume option 2 (1:12)	Volume option 3 (1:16)
	End Prep Reaction Mixture from step 1	7,5 µl	5 µl	3 µl
	NEBNext Ultra II Ligation Master Mix*	3,75 µl	2,5 µl	1,5 µl
	NEBNext Ligation Enhancer	0,125 µl	0,083 µl	0,05 µl
	NEBNext Adaptor for Illumina	0,31 µl	0,208 µl	0,0125 µl
	Total Volume	11,41 µl	7,79 µl	4,56 µl

Table 3: Amount (µl) per volume option.

- Mix by pipetting the entire volume up and down at least 10 times.
- Spin down briefly.

5.3 Incubate at 20 °C for 00:15:00 in a thermocycler with the **heated lid of** 15m

5.4

- Let the I.DOT pipet the appropriate volume (see ***Table 4***) of the

NEB USER® Enzyme **New England Biolabs Catalog #M5505S/L** to the **ligation mixture**:

	A	B	C	D
	USER Enzyme	0,5 µl	0,35 µl	0,2 µl

Table 4: Amount (µl) per volume option.

Liquid class: Glycerol 10%

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

5.6 Bring the volume of the total reaction to 15 µL before proceeding to the following step.

1,2X Reaction Clean-up

6 21m

- Let the MN beads get to room temperature (remove from fridge 15 min before use).
- Prepare fresh 80% EtOH (200 µL per sample, e.g. 41.7 mL EtOH 96% + 8.3 mL MilliQ).
- Mix the MN beads well by vortexing.
- Add 1,2x MN beads to the ligation reaction.
- Mix well by pipetting.
- Incubate at room temperature for 00:05:00 .
- Place the plate on the magnetic rack.
- When the solution is clear (00:05:00), carefully remove the supernatant (LEAVE THE PLATE ON THE MAGNET).
- Wash the beads 2x with 50 µL 80% ethanol (LEAVE THE PLATE ON THE MAGNET).
- Let the beads air dry for 00:01:00 (LEAVE THE PLATE ON THE MAGNET).
- Take the plate off the magnet and resuspend the beads with e.g. 12 µL 0,1x TE, by pipetting up and down.
- Wait 00:05:00 and put the plate back on the magnet.
- When the solution is clear (00:05:00), move 12 µL of the supernatant to a clean plate.

NOTE: The plate can be concentrated again in the Eppendorf Concentrator (SpeedVac) if volume **option 2** or volume **option 3** is chosen and low concentrations are expected.

NEBNext PCR Enrichment

30m

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- Distribute the NEBNext Ultra II Q5 Master Mix - 250 rxns **New England Biolabs Catalog #M0544L** with the chosen volume option to all wells of a new PCR plate.
- Add the **Adaptor Ligated DNA Fragments** and **IDT_10 primers** manually using a P10 multichannel.

	A	B	C	D
		Volume option 1 (1:8)	Volume option 2 (1:12)	Volume option 3 (1:16)
	Adaptor Ligated DNA Fragments	5,25 µl	3,15 µl	2,1 µl
	NEBNext Ultra II Q5 Master Mix 2X	6,25 µl	3.75 µl	2,5 µl
	Illumina IDT_10 i7 Primer*	0,5 µl	0,3 µl	0,2 µl
	Illumina IDT_10 i5 Primer*	0,5 µl	0,3 µl	0,2 µl
	Total volume	12,5 µl	7,5 µl	5 µl

Table 5: Amount (µl) per volume option.

7.1

- Mix by pipetting the entire volume up and down at least 10 times
- Place the plate in a thermocycler 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	∞	

Table 6: PCR program

7.2

The number of **PCR cycles** should be chosen based on input amount and sample type (see ***Table 7***). 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 TapeStation or Fragment Analyzer). Use the table below for applications requiring high library yields (~ 1 µg).

	A	B

	A	B
	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

Table 7: Number of cycles per input DNA.

Check PCR succes

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- Check the success of a few amplified samples on the **TapeStation using the D5000 High Sensitivity kit**:
- Dilute 1 µL of amplified sample in 9 µL of MilliQ (10x) and measure the sample.
  - Optional: add 0.2 µL of amplified sample and 1.8 µL of MilliQ directly to the TapeStation strip.
- If the amplification was not sufficient, add additional cycles.
- If overcycling has occurred, redo the amplification with the remaining sample from the previous step with fewer cycles.

- Bring the volume of the PCR reaction to at least 10 µL before proceeding to the next step.

Reaction Clean-up

16m

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- Let the MN beads get to room temperature (remove from fridge 15 min before use). 16m
- Prepare fresh 80% EtOH (200 µL per sample, e.g. 41.7 mL EtOH 96% + 8.3 mL MilliQ).
- Mix the MN beads well by vortexing.
- Add 0,9x MN beads* to the ligation reaction.
- Mix well by pipetting.
- Incubate at room temperature for 00:05:00 .
- Place the plate on the magnetic rack.
- When the solution is clear (00:05:00), carefully remove the supernatant (LEAVE THE PLATE ON THE MAGNET).
- Wash the beads 2x with 50 µL 80% ethanol (LEAVE THE PLATE ON THE MAGNET).

- Let the beads air dry for 00:01:00 (LEAVE THE PLATE ON THE MAGNET).
- Take the plate off the magnet and resuspend the beads with e.g. 12 µL 0,1x TE, by pipetting up and down.
- Place the plate back on the magnet.
- When the solution is clear (00:05:00), move 12 µL of the supernatant to a clean plate.

*Bead to sample ratio is dependent on the library size. Carefully choose the correct ratio.

Measuring libraries and equimolar pooling of samples

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- Measure the concentration of all samples on the Fragment Analyzer.
- Export a smear analysis and pool all samples equimolarly by either using the Qiagility pipetting robot or manually.

Measuring pools

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- Measure the pool(s) in triplicate on the TapeStation using the **High Sensitivity D5000** kit.
- Use the mean concentration per pool for calculations.
- If equimolar pooling of subpools is required, use the average molar concentration of the three measured subpools for pooling of these pools (calculate this based on the lowest pool concentration).