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Ultra II DNA Library Prep Kit with Inputs >100 ng V.1

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melissa.m.brandner Brandner[1]

[1]university of Tromsø

Celine Richard
The Arctic University of Norway

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We use this protocol and it's working

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Abstract

Ultra II DNA Library Prep Kit with Inputs >100 ng

Pre-tasks

1

Note

1. You need to start by defrosting the chemistry you will use, cleaning the lab and hood and putting the UV on for 30 minutes.
Make sure that once defrosted the chemicals are stored in the fridge or on a cool block from the freezer.
2. If you have less than 100 ng starting DNA, you will also need to prep adapter to 0.6 µM. You do this by:
Diluting one of the 15 µM adapters in the freezer 25 fold, add 1 µl of adapter to 24 µl of 10 mM Tris-Hcl / NaCl.(this may be found in the fridge or made fresh from the chemical store room if it is 1 year or older).

Starting Material: 100 pg–100 ng purified, genomic DNA.
If the input DNA is less than 50 µl, add TE (provided) to a final volume of 50 µl.

Sonication of DNA for fragmentation

2 Replace the water in the minichiller and sonicator.

3 Turn mini chiller on to chill H_2O for 30 minutes

4 Turn on the sonicator and set to the following program: 30 secs on, + 90 secs off, 3x cycles

5 Prep your sample: Add 200 ng of DNA in 100 µl total volume H_2O in the sonicator tubes

6 Vortex the samples well and chill in a freezer block for 10 minutes before placing in the sonicator

7 Follow the instructions on the screen to start.

Safety information

Wear ear protection and change the sign on the door

8 Each run of 12 samples should take 6 minutes

Note

Take 1 µl of each sample to run on a gel if it is the first run and check the fragmentation!!

Speedvac of fragmented samples for concentration to 50 µl

9 Set the machine on at least 30 minutes before use, turn on the cool safe (both top and bottom switch at 1)

10 Set machine to 2000 RPM, 30 mins 30°C

11 When at 100°C open tubes and place symmetrically, place the upside down triangle

12 Make sure it gets up to 2000 RPM + vacuum pump

13 Turn on

14 Quantify with Qubit after the run

NEB Next End Prep

15 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

16 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.

17 Place in a thermal cycler, with the heated lid set to ≥ 75°C, and run the following program:
30 minutes at 20°C
30 minutes at 65°C
Hold at 4°C

Note

SAFE STOP
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.

18 Adaptor ligation:
Determine whether adaptor dilution is necessary

Note

If DNA input is < 100 ng, dilute the **(red)** NEBNext Adaptor for Illumina in 10 mM Tris-HCl, pH 7.5-8.0 with 10 mM NaCl as indicated in Table below

This is what you already prepared!

	A	B	C
	INPUT	ADAPTOR DILUTION	WORKING ADAPTOR CONCENTRATION
	100 ng - 500 ng	No dilution	15 µM
	5 ng - 99 ng	10-fold (1:10)	1.5 µM
	less than 5 ng	25-fold (1:25)	0.6 µM

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

	A	B
	COMPONENT	VOLUME
	End Prep Reactiion Mixture (step 15)	60 µl
	(red) NEBNext Adaptor for Ilumina	2.5 µl
	(red) NEBNext Ultra II Lifation 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

20 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.

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

Note

SAFE STOP
Samples can be stored overnight at -20°C

Size Selection or Cleanup of Adaptor-ligated DNA

22

Note

If the starting material is **> 50 ng**, follow the protocol for size selection in **Section A.**
For input **≤ 50 ng**, size selection is not recommended to maintain library complexity.
Follow the protocol for cleanup without size selection in **Section B.**

A: Size Selection if input > 50 ng

23 Vortex magnetic beads thoroughly until they are homogenous

24 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.

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

26 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.

27 After 5 minutes (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.

28 Add 20 µl (0.2X) resuspended **SPRIselect** or **NEBNext Sample Purification Bead**s 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

29 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.

30 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).

31 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.

32 Repeat Step 31 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.

33 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 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.

34 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**.

35 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.

36 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).

B: Cleanup of Adaptor-ligated DNA if input ≤ 50ng

37

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 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.

38 Vortex **SPRIselect** or **NEBNext Sample Purification Beads** to resuspend

39 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.

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

41 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.

42 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 beads).

43 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.

44 Repeat Step 43 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.

45 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.

46 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.**

47 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.

48 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.

Note

SAFE STOP
Samples can be stored overnight at -20°C

PCR Enrichment of Adaptor-ligated DNA

49 Add the following components to a sterile strip tube
Forward and Reverse primers not already combined (you will find these in the purple plates, they say i7 and i5 on the bags they are in).

	A	B
	Adaptor Ligated DNA Fragments	15 µl
	(blue) NEBNext Ultra II Q5 Master Mix	25 µl
	(blue) Index Primer / i7 Primers	5 µl
	(blue) Universal PCR Primer / i5 Primers	5 µl
	Total Volume	50 µl

50 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.

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

	Cycle Step	Temperature	Time	Cycles
	Initial Denaturation	98°C	30 secondes	1
	Denaturation	98°C	10 secondes	4
	Annealing / Extension	65°C	75 secondes	
	Final extension	65°C	5 minutes	1
	Hold	4°C	hold	

Proceed to cleanup of PCR reaction

Cleanup of PCR Reaction

52

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.

53 Vortex **SPRIselect** or **NEBNext Sample Purification Beads** to resuspend.

54 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

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

56 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.

57 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).

58 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.

59 Repeat Step 58 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.

60 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. 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.

61 Remove the tube/plate from the magnetic stand.
Elute the DNA target from the beads by adding 33 µl of **0.1X TE** (dilute 1X TE Buffer 1:10 in water).

62 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.

63 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.

Assess Library Quality on Bioanalyzer

64 Dilute library 5-fold in **0.1X TE Buffer** (inputs ≤ 1 ng may not require dilution to run on a Bioanalyzer).

65 Run 1 µl on a DNA High Sensitivity Chip.

66 Check that the library size shows a narrow distribution with an expected peak size based on fragmentation time

Note

If a peak ~80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume to 50 µl with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section 1.5. You may see adaptor-dimer when starting with inputs ≤ 1 ng