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Library Prep for CUT&RUN with NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (E7645) V.1

Forked from NEBNext® Ultra™ DNA Library Prep Protocol for Illumina® With Size Selection (E7370)

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

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Abstract

Choose your protocol:

For Henikoff lab protocol, please see their Nature Protocol paper.

Why?
The difference between NEB stem loop adaptors and illumina adaptors are their lengths. When ligated to both ends of DNA, they add 64bp and 124bp respectively. This makes huge difference when you do beads based cleaning up.

What is this protocol?
This protocol is based on library preparation manual of “NEBNext® Ultra™ II DNA Library Prep Kit for Illumina”, NEB E7645, and is specifically modified to make libraries from small DNA fragments (25-70bp). We have successfully applied this protocol to make libraries for **CUT&RUN** experiments and showed that it greatly increased the preservation of small fragments (Liu, Cell 2018. Fig. S2B [**pdf**])

Attachments

PDF

2016SCOPE_AnnualMtg_...

4.7MB

Guidelines

Step1: End prep. 2 hours. Do not recommend to stop.
Step2: Adaptor ligation and USER treatment. 1 hour. **Safe stop point** after this step.
Step3: Cleanup of adaptor-ligated DNA. 30 min. **Safe stop point** after this step.
Step4: PCR amplification. 40 min. **Safe stop point** after this step.
Step5: Cleanup of PCR reaction. 30 min. **Safe stop point** after this step.
Step6: QC of libraries.

Materials

MATERIALS

NEBNext Ultra II DNA Library Prep Kit for Illumina - 24 rxns **New England Biolabs Catalog #**E7645S

Agencourt Ampure XP **Beckman Coulter Catalog #**A63880

Troubleshooting

Before start

This is version 1.0. Please check the online version of this document occasionally for update.

- Quantify DNA with fluoresence based method such as Qubit.
- Use less than 30 ng DNA to avoid saturation of enzyme activities.
- Sometimes there is very large DNA fragment contamination from CUT&RUN. Please use bioanalyzer to quantify the relative amount of short fraction and only use this fraction as input if there are significant amount of large fraction. For example, Qubit determines the concentration as 5 ng/ul, and bioanalyzer shows that 80% is large DNA, then you should use the DNA as 1 ng/ul.

NEBNext End Prep

1 **Mix components**

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

	Reagent	Volume
	CUT&RUN DNA (6ng)	25 µl
	(green) NEBNext Ultra II End Prep Enzyme Mix	1.5 µl
	(green) NEBNext Ultra II End Prep Reaction Buffer	3.5 µl

Note

Starting Material <30 ng fragmented DNA.

Note

Set a 100 or 200 µl pipette to 25 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.

2 Place in a thermocycler, with the heated lid set to ≥ 60°C, and run the following program:

	Time	Temperature
	30 minutes	20°C
	60 minutes	50°C
	Hold	4°C

CRITICAL: Decreasing temperature to 50°C can significantly help saving very small fragments (25-70 bp) as seen in our sequencing data.

Adaptor Ligation

3 Dilute the Adaptor based on the amount you need to use. I dilute the original 15 µM adaptor to 3 µM when I work with 6 ng input DNA.

Note

If your input is 6 ng, its molarity is 0.2 pmol. I typically use ~4 pmol (3 µM x 1.25 µL) of Adaptor (20x).
Lower amount of Adaptor can reduce the amount of final PCR dimers. If you have severe dimer contamination, you can use as low as 0.6 pmol Adaptor for 0.2 pmol of input DNA (3x).

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

	Reagent	Volume
	(red) NEBNext Ultra II Ligation Master Mix	15 µl
	(red) NEBNext Ligation Enhancer	0.5 µl
	(red) NEBNext Adaptor For Illumina	1.25 µl

Note

The NEBNext adaptor is provided in NEBNext Singleplex (**NEB #E7350**) or Multiplex (**NEB #E7335**, **#E7500**, **#E7710**, **#E7730**, **#E6609**, #E7600) Oligos for Illumina. **There is only one universal Adaptor, and the barcodes are embedded in the PCR primers.**
The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 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.

Note

Set a 100 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 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.

5 Incubate at 20°C for 15 minutes in a thermal cycler.

6 Add 1.5 µl of USER™ enzyme to the ligation mixture.
Mix well and incubate at 37°C for 15 minutes with heated lid set to ≥ 47°C.

Note

USER addition and USER incubation are only required for use with NEBNext adaptors. USER is provided in the NEBNext Index Primer kits. It is also available as a separate product NEB# M5505 which can be used in NEBNext library prep workflows.

Cleanup of Adaptor-ligated DNA

7 Vortex AMPure XP beads to resuspend. Please allow the beads to warm to room temperature for at least 30 minutes before use.

Note

We haven't tested SPRIselect beads. Only substitute if you are sure they work exactly the same.

8 Add 80 µl (~1.75x) AMPure XP beads to ligation reaction. Mix well by pipetting up and down at least 10 times.
Incubate samples on bench top for at least 5 minutes at room temperature.

Note

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.
CRITICAL: Use of 80 µl AMPure XP beads is essential to purify short DNA.

9 Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.

Note

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.

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

Note

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

11 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.

Note

Be careful not to disturb the beads that contain DNA targets. Beads can be left with 80% ethanol for minutes but we do not recommend to leave them too long.

12 Repeat the previous step once. 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.

13 Air the dry beads for **up to** 5 minutes while the tube is on the magnetic stand with the lid open. **Caution: Do not overdry the beads.**

Note

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.

14 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 15 µl of 10 mM Tris-HCl or 0.1X TE. Mix well on a vortex mixer or by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature.

Note

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.

15 Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 13 µl to a new PCR tube for (amplification).

Note

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

PCR Amplification

16 Mix the following components in sterile strip tubes (**Total volume 30 µl**):

	Reagent	Volume
	Adaptor Ligated Fragments	13 µl
	(blue) NEBNext Ultra II Q5 Master Mix	15 µl
	(blue) Index Primer/i7 Primer	1 µl
	(blue) Universal PCR Primer/i5 Primer	1 µl

Note

The primers are provided in NEBNext Singleplex (**NEB #E7350**) or Multiplex (**NEB #E7335**, **#E7500**, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina.

17 PCR using the following cycling conditions:

	Cycle Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	98°C	30 Seconds	1
	Denaturation	98°C	10 Seconds	12*
	Annealing/ Extension	65°C	10 Seconds	
	Final Extension	65°C	5 Minutes	1
	Hold	4°C	∞	

Note

When starting with 6 ng DNA, I've obtained 10-40 ng of DNA with 12 cycles of amplification with different samples.
If you use less input, consider increasing the cycle number.

Cleanup of PCR Amplification

18 Vortex AMPure XP beads to resuspend. Please allow the beads to warm to room temperature for at least 30 minutes before use.

Note

We haven't tested SPRIselect beads. Only substitute if you are sure they work exactly the same.

19 **First round size selection: remove PCR products that are >350 bp.**
Add 24 µl (0.8X) resuspended AMPure XP beads to the PCR reaction. Mix well by pipetting up and down at least 10 times.
Incubate for at least 5 minutes at room temperature.

Note

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.

20 Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant (5 min or when the solution is clear).

Note

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.

21 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.

22 **Second round of size selection: remove PCR products shorter than 150 bp.**
Add 12 µl (final 1.2x) resuspended AMPure XP beads to the supernatant and mix *at least 10 times.* Then incubate samples on the bench top for at least 5 minutes at room temperature.

Note

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

23 Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.

Note

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.

24 After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution do not discard beads**).

25 Wash #1: 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.

Note

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

26 Wash #2: Repeat the previous 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.

27 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.

28 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 15 µl of 0.1X TE. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature.

Note

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.

29 Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 13 µl to a new PCR tube for and store at -20ºC.

QC of libraries

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

Note

The size should be 160-350bp.
Since the size of library is so near to the PCR dimer (128 bp), sometimes there are residual dimers. The ratio of dimers is mostly 1-20% in my experiments (please see attached screenshots of Tapestation analysis of two libraries). You can easily get rid of them by doing another round of 1.2X AMPure XP beads clean up.
If you get too many dimer contamination, I recommend doing gel based size selection using a 2-3% agarose gel. Pippin Prep is a good option to do gel based size selection. Also consider reduce the Adaptor to as low as 3X when you make libraries for similar experiments in future.

Expected result

1.2X AMPure XP purification can completely remove all dimers in this sample↑.

This library needs gel based purification to remove dimers.

Sequencing and data processing tips:

31 Be sure to do pair end sequencing. This will give you the information of fragment size. Fragments that are <120 bp (library size <250 bp) contain most of the protein binding information while fragments >140 bp (library size >270 bp) contain surrounding nucleosome positioning information.

32 Be aware that there will be several bases of adapter sequences in some of the reads, known as read through adaptors. For example, I sequence 42 bp each read. For all fragments that are shorter than 42bp, there will be adapter sequences at 3′ of the reads. You need to trim them. My collaborator Qian Zhu has developed an easy to use pipeline for CUT&RUN data processing **here**. Please contact me or Qian with any questions. Some details can be found in the method section in our 2018 **paper**.