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WORKS FOR ME 1

NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells

COMMENTS 0

DOI
dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

New England Biolabs[1]

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ABSTRACT

The NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina® uses a template switching method to generate full length cDNAs directly from single cells or 2 pg – 200 ng RNA, followed by conversion to sequence-ready libraries using the Ultra™ II FS workflow. This unique workflow enables generation of the highest yields from a broad range of inputs, and superior transcript detection, while providing reliably consistent performance.

ATTACHMENTS

NEBNext Single Cell_Low Input RNA Library Prep Kit for Illumina.pdf

DOI

dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

EXTERNAL LINK

https://www.neb.com/protocols/2018/04/25/protocol-for-cells-cdna-synthesis-amplification-and-library-generation-e6420

PROTOCOL CITATION

New England Biolabs 2022. NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells. **protocols.io** https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

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Nov 01, 2020  Liz Brydon Protocols.io

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GUIDELINES

Overview

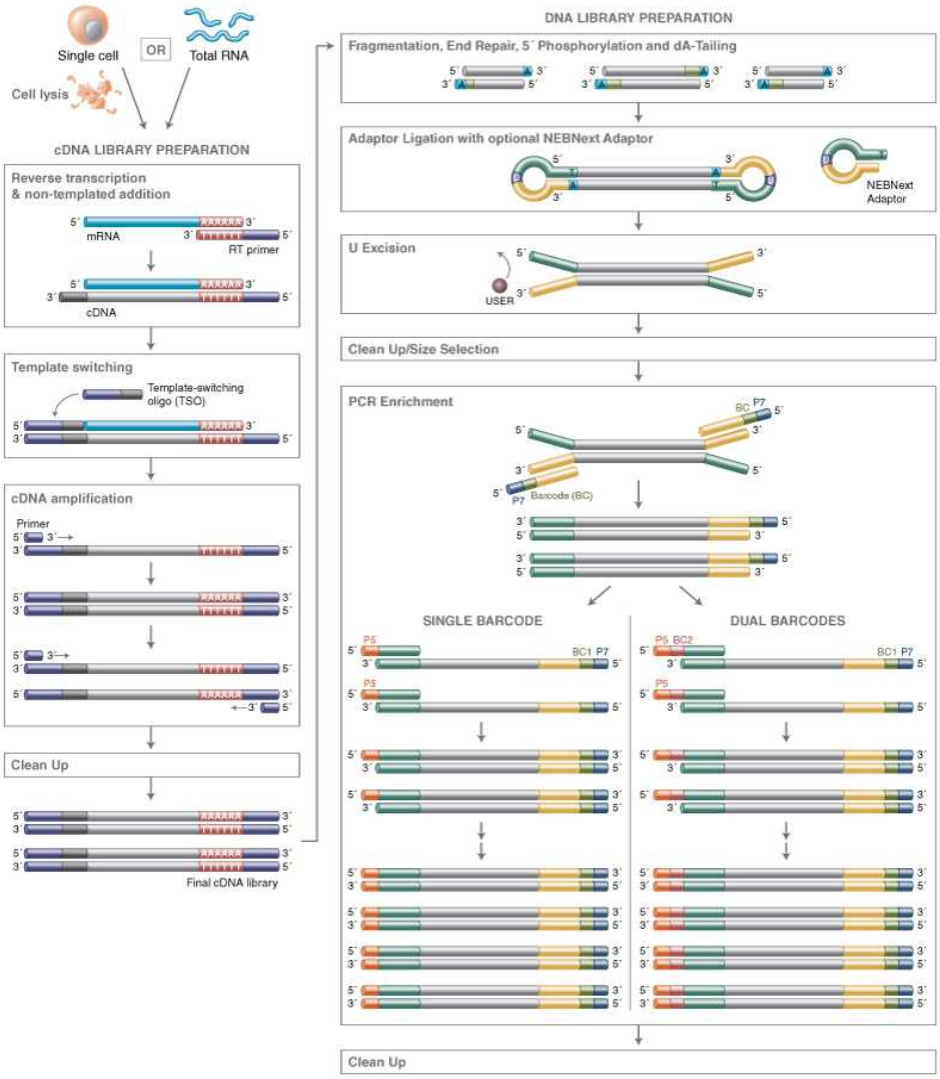
The NEBNext Single Cell Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of total RNA inputs or RNA from cultured and primary cells into high quality libraries for next-generation sequencing on the Illumina platform. The fast, user- friendly workflow also 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 of indexed libraries made from single cells and commercially available RNA and sequenced on an 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.

Workflow: Library Preparation for Illumina

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1



Oligo Sequences

PRODUCT	OLIGO
NEBNext Template S	5´-GCT
NEBNext Single Cell	5´-AAG
NEBNext Single Cell	5´-AAG

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells
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MATERIALS TEXT

Kit Required:

NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina - 24 rxns **New England Biolabs Catalog #E6420S**

NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs Catalog #E6420L**

Please note that adaptors and primers are not included in the kit and are available separately.

Required Materials Not Included:

- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf® #022431021)
- NEBNext Oligos
- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Thermal cycler
- Vortex Mixer
- Microcentrifuge
- SPRIselect® Reagent (Beckman Coulter®, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
- DNase RNase free PCR strip tubes (USA Scientific 1402-1708)

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

BEFORE STARTING

Please review the important information under the "Guidelines" & "Warnings" tabs before beginning.

Sample Recommendations

This protocol is intended for isolated cultured or primary cells, but is not compatible with fixed cells. Cells should be intact and sorted in cell lysis buffer provided in the kit. See Section "Cell Collection and Lysis" for cell lysis buffer dilution and recommended volumes before use. Cells should be washed and resuspended in PBS prior to isolation/sorting. Carryover of media may affect the cDNA synthesis efficiency.

Starting Material

Isolated single, tens or hundred cells.

Typical Yield of cDNA from a Reaction

Actual yields will depend on the quality and quantity of the cell and the mRNA content of the sample. Typical cDNA yields range between 5–15 ng based on the PCR cycle recommendations provided in Section "cDNA Amplification by PCR".

Typical Yield of Illumina Library from a Reaction

Actual yields will depend on the quality and quantity of the input cDNA. Typical library yields range between 100 ng–1 µg based on the PCR cycle recommendations provided in Section "PCR Enrichment of Adaptor-ligated DNA".

Notes

Keep all buffers and enzymes on ice, unless otherwise indicated

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells
https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

ATTACHMENTS

NEBNext Single Cell_Low Input RNA Library Prep Kit for Illumina.pdf

Sample and Reagents Preparation

1 Briefly centrifuge the tubes containing NEBNext Single Cell RT Enzyme Mix and Murine RNase Inhibitor to collect solutions to the bottom of the tubes, then place On ice.

2 Thaw all other frozen components at Room temperature (if the 10X NEBNext Cell Lysis Buffer appears cloudy after thawing, incubate briefly at 37 °C to clear up the solution).

3 Mix each component thoroughly, centrifuge briefly to collect solutions to the bottom of the tube, and then place On ice. Leave the 10X NEBNext Cell Lysis Buffer at Room temperature.

10m

Cell Collection and Lysis

4 If the carryover volume from cell isolation/sorting is < 1 µl, cells can be dispensed directly into 1X NEBNext Cell Lysis Buffer (without accounting for added volume). If carryover volume from cell isolation/sorting is ≥ 1 µl, skip to Step 8.

Prepare 1X NEBNext Cell Lysis Buffer in an RNase-free tube as follows:

A	B
COMPONENT	VOLUME (µl) PER REACTION
(white) NEBNext Cell Lysis Buffer (10X)	0.5 µl
(white) Murine RNase Inhibitor	0.25 µl
Nuclease-free Water	4.25 µl
Total Volume	5 µl

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

5 Mix solution thoroughly by pipetting, avoiding bubbles. Centrifuge briefly to collect solution to the bottom of the tube.

6 Dispense cells directly into 5 µL 1X Cell Lysis Buffer. After dispensing, cells can be flash-frozen and stored at -80 °C for future use, or lysed as outlined in the next step.

5m

7 Incubate at Room temperature for 00:05:00 and then proceed immediately to the next section ("Primer Annealing for First Strand Synthesis").

8 If the carryover volume from cell isolation/sorting is ≥ 1 µl or the cells have already been collected in a solution with a volume ≥ 1 µl, prepare a Cell Lysis Buffer according to the table below, accounting for the carryover cell volume. Cells can be flash frozen and stored at -80 °C for future use or lysed as outlined in the next step.

A	B
COMPONENT	VOLUME (µl) PER REACTION
Carryover Cell Volume	1-5 µl
(white) NEBNext Cell Lysis Buffer (10X)	0.8 µl
(white) Murine RNase Inhibitor	0.4 µl
Nuclease-free Water	Variable (based on carryover cell volume)
Total Volume	8 µl

5m

9 Incubate at Room temperature for 00:05:00 and then proceed immediately to the next section ("Primer Annealing for First Strand Synthesis").

5m

Primer Annealing for First Strand Synthesis

10 For carryover volumes **<** 1 µl or **≥** 1 µl, follow the corresponding protocol:
Step 10 includes a Step case.
< 1 µl
≥ 1 µl

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells
https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

step case

< 1 µl

To anneal cDNA Primer with RNA templates in the sample, prepare the reaction as follows (on ice):

COMPONENT	VOLUM
Lysed Cell (Step 7)	5 µl
(lilac) NEBNext Single Cell RT Primer Mix	1 µl
Nuclease-free Water	3 µl
Total Volume	9 µl

11

Mix well by pipetting up and down gently at least 10 times, then centrifuge briefly to collect solution to the bottom of the tube.

5m

Incubate for 00:05:00 at 70 °C in a thermal cycler with the heated lid set to 105 °C, then hold at 4 °C until next step.

During the above annealing step, prepare the components for the following step.

Reverse Transcription (RT) and Template Switching

13

Vortex the NEBNext Single Cell RT Buffer, then prepare the RT mix in a separate tube as follows (adding NEBNext Single Cell RT Enzyme Mix last) On ice.

Note

Note: It is important to vortex the buffer prior to use for optimal performance.

A	B
COMPONENT	VOLUME (µl) PER
(lilac) NEBNext Single Cell RT Buffer	5 µl
(lilac) NEBNext Template Switching Oligo	1 µl
(lilac) NEBNext Single Cell RT Enzyme Mix	2 µl
Nuclease-free Water	3 µl
Total Volume	11 µl

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

14 Mix thoroughly by pipetting up and down several times, then centrifuge briefly to collect solutions to the bottom of tubes.

15 Combine 11 µL RT mix (above) with 9 µL annealed sample (Step 12). Mix well by pipetting up and down at least 10 times, and centrifuge briefly.

1h 40m

16 Incubate the reaction in a thermal cycler with the following steps and the heated lid set to 105 °C:

01:30:00 at 42 °C
00:10:00 at 70 °C
Hold at 4 °C

Note

Safe Stopping Point: Samples can be safely stored Overnight **at** 4 °C **or** -20 °C.

cDNA Amplification by PCR

17 Prepare cDNA amplification mix as follows:

COMPONENT	VOLUM
(orange) NEBNext Single Cell cDNA PCR Master Mix	50 µl
(orange) NEBNext Single Cell cDNA PCR Primer	2 µl
Nuclease-free Water	28 µl
Total Volume	80 µl

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

18 Add 80 µL cDNA amplification mix to 20 µL sample from Step 16. Mix by pipetting up and down at least 10 times.

19 Incubate the reaction in a thermal cycler with the following PCR cycling conditions and the heated lid set to 105 °C:

A	B	C	D
CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	45 seconds	1
Denaturation	98°C	10 seconds	11-21* (see 'Recomme
Annealing	62°C	15 seconds	
Extension	72°C	3 minutes	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

Note

Recommended Number of PCR Cycles

A	B
RNA CONTENT OF CELL OR CELL TYI	RECOMMENDED NUMBER OF PC
Hek293 Single Cell	18
HeLa Single Cell	17
Jurkat Single Cell	20
Mouse M1 Cells	20
10 cells	14–17
100 cells	11–14
2 pg	20–21
10 pg	17–18
100 pg	14–15

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*Note: The amount of RNA in your sample should be used to determine the appropriate number of PCR cycles.
If you are starting with single cells not listed above, a PCR cycle titration can be done to determine the appropriate number of PCR cycles for your sample.
For the various inputs listed above, the recommended PCR cycles will typically result in cDNA yields between 1–20 ng (in most cases 5-15 ng). We recommend quantifying the cDNA after the cleanup (Cleanup of Amplified cDNA Section) before proceeding to the library preparation (from Fragmentation/End Prep Section starting step 41 through until Assess Library Quality and Quantity on a Bioanalyzer section starting step 80). The total RNA used for the above recommendations is Universal Human Reference (UHR) RNA. When using other sources of starting material or a different cell type, some optimization may be necessary due to variations in mRNA amounts.

Note

Safe Stopping Point: Samples can be safely stored Overnight **at** 4 °C **or** -20 °C.

43m

Cleanup of Amplified cDNA

30m

20 Allow the NEBNext Bead Reconstitution Buffer and the SPRI® beads (if stored at 4 °C) to warm to Room temperature for at least 00:30:00 before use. Vortex SPRI Beads to resuspend well and prepare fresh [M] 80 % ethanol.

8s

21 Add 60 µL (0.6X of sample volume) 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. Alternatively, samples can be mixed by vortexing for 3–5 seconds on high. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

5m

22 Incubate samples on the bench top for at least 00:05:00 at Room temperature.

23 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.

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells
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24 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 cDNA. 5m

Note

Caution: do not discard the beads

25 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 cDNA. 30s

26 Repeat previous step 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.

27 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of cDNA. 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 cDNA from the beads by adding 50 µL 0.1X TE (dilute [M] 1 X TE Buffer 1:10 in water).

29 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. 2m

30 Add 45 µL NEBNext Bead Reconstitution Buffer (Room temperature) to the eluted cDNA + bead mixture from the previous step for a second sample clean up. Mix well by pipetting up and down at least 10 times.

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

Note

Caution: Skipping this additional cleanup step may reduce overall cDNA purity.

31 Incubate samples on the bench top for at least 00:05:00 at Room temperature. 5m

32 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.

33 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 cDNA. 5m

Note

Caution: do not discard the beads.

34 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 cDNA. 30s

35 Repeat previous step 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.

36 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of cDNA. 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.

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37 Remove the tube/plate from the magnetic stand. Elute the cDNA from the beads by adding 33 µL 1X TE (provided in kit).

2m

38 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.

5m

39 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.

Note

Safe Stopping Point: Samples can be safely stored overnight at 4 °C or -20 °C.

Assess Amplified cDNA Quality and Quantity on a Bioanalyzer

40 Run 1 µL amplified cDNA from the previous step on a DNA High Sensitivity Chip.

Expected result

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

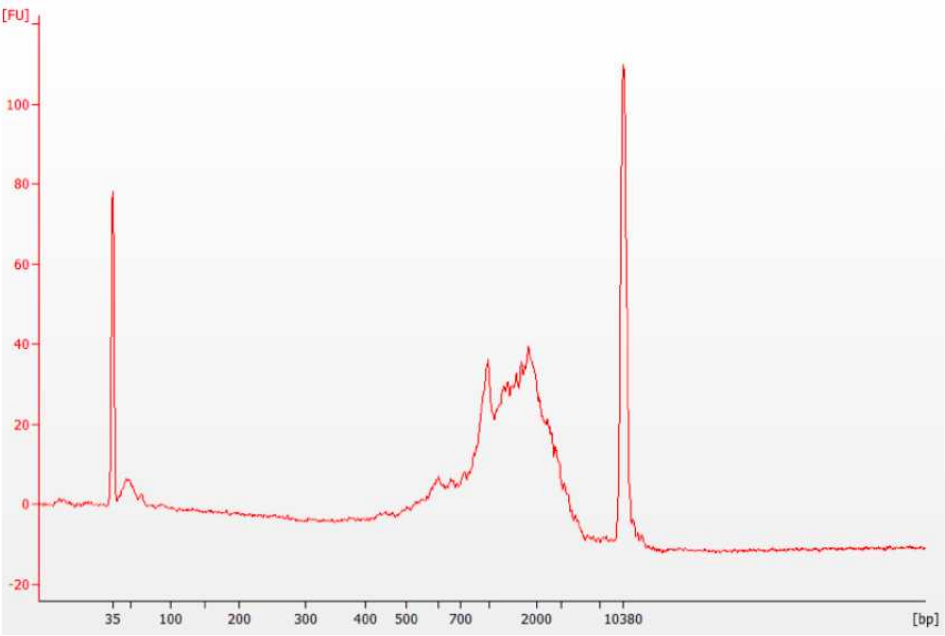

Figure 40: Examples of cDNA size distribution on a Bioanalyzer.

HeLa single cell was used to synthesize cDNA and amplified using 17 PCR cycles.

Quantitation (recommended) and Normalization (optional): While 1 ng–20 ng cDNA yield is typical, 100 pg–20 ng purified cDNA can be used in the library construction protocol (Sections "Fragmentation/End Prep" – "Assess Library Quality and Quantity on a Bioanalyzer"). If using cDNA outside the range of 1 ng–20 ng (as determined in Section "Assess Amplified cDNA Quality and Quantity on a Bioanalyzer"), adjust the PCR cycles to amplify the adaptor ligated DNA. For details, see Section "PCR Enrichment of Adaptor-ligated DNA" in this protocol.

Note

If the cDNA yield is variable, the samples can be normalized to the same concentration prior to the next step in order to treat all of the samples with the same number of PCR cycles.

Note

A	B
cDNA PCR YIELD	RECOMMENDATION FOF
100 pg–1 ng	Use all of the cDNA and a

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A	B
1 ng–20 ng	Typical cDNA yield. Use 8
20 ng–100 ng	cDNA input into library pr
> 100 ng	Normalize cDNA so that a

13s

Fragmentation/End Prep

41 Ensure that the NEBNext Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place On ice until use.

42 Vortex the NEBNext Ultra II FS Enzyme Mix 5–8 seconds prior to use and place On ice.

Note

Note: It is important to vortex the enzyme mix prior to use for optimal performance.
Please see these videos Quick Tips - Preparing the NEBNext Ultra II FS DNA Reaction Buffer and Enzyme Mix | NEB
Quick Tips - Preparing NEBNext Ultra II FS DNA Reaction Buffer and Enzyme Mix Master Mix | NEB

43 Add the following components to a 0.2 ml thin wall PCR tube On ice:

A	B
COMPONENT	VOLUME (µl) PER F
cDNA (Step 39)	26 µl
(yellow) NEBNext Ultra II FS Reaction Buffer	7 µl
(yellow) NEBNext Ultra II FS Enzyme Mix	2 µl
Total Volume	35 µl

5s

44 Vortex the reaction for 00:00:05 and briefly spin in a microcentrifuge.

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55m

45 In a thermal cycler, with the heated lid set to 75 °C, run the following program:

00:25:00 at 37 °C

00:30:00 at 65 °C

Hold at 4 °C

Note

Safe Stopping Point: 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.

30m

Adaptor Ligation

46 Dilute (red) NEBNext Adaptor for Illumina by 25-fold (0.6 micromolar (µM)) in the NEBNext Adaptor Dilution Buffer (provided).

47 Mix the NEBNext Ultra II Ligation Master Mix by pipetting up and down several times.

48 Add the following components directly to the FS Reaction Mixture On ice:

A	B
COMPONENT	VOLUME (µl) PER
FS Reaction Mixture (Step 45)	35 µl
(red) NEBNext Ultra II Ligation Master Mix	30 µl
(red) NEBNext Ligation Enhancer	1 µl
(red) NEBNext Adaptor for Illumina* (diluted 1:25)	2.5 µl
Total Volume	68.5 µl

*The NEBNext adaptor is provided in the NEBNext Oligo kit. NEB has several Oligo kit options, which are supplied separately from the library prep kit.

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

Note

Note: The ligation master mix and ligation enhancer can be mixed ahead of time and the mixture is stable for at least 8 hours @ 4 °C. We do not recommend adding adaptor to a premix in the Adaptor Ligation Step.

49 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

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.

50 15m

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

51 Add 3 µL (red) USER® Enzyme to the ligation mixture from the previous step.

Note

Note: Steps 51 and 52 are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext oligo kits.

52 15m

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

Note

Safe Stopping Point: Samples can be safely stored Overnight at -20 °C.

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53m

Cleanup of Adaptor-ligated DNA

53

Note

Note: The following 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.

30m

54 If stored at 4 °C allow the SPRI beads to warm to Room temperature for at least 00:30:00 before use. Vortex SPRI beads to resuspend well and prepare fresh [M] 80 % ethanol.

55 Add 57 µL (0.8X of sample volume) 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. Alternatively, samples can be mixed by vortexing for 3–5 seconds on high. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

5m

56 Incubate samples on the bench top for at least 00:05:00 at Room temperature.

57 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.

5m

58 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 DNA targets.

Note

Caution: do not discard the beads.

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59 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. 30s

60 Repeat previous step 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.

61 Air dry the beads for up to 00:05:00 while the tube/plate is on the magnetic stand with the lid open. 5m

Note

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.

62 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µL 0.1X TE (dilute [M] 1 X TE Buffer 1:10 in water).

63 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. 2m

64 Place the tube/plate on the magnetic stand. After 00:05:00 (or when the solution is clear), transfer 15 µL to a new PCR tube. 5m

65 Proceed to PCR Enrichment of Adaptor-ligated DNA in the next section.

Note

Safe Stopping Point: Samples can be safely stored overnight at 4 °C or -20 °C.

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66

Note

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.

Use Option B for any 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.

Option A (Forward and Reverse Primers Supplied Separately)
Combine the following components in a sterile tube and then proceed to the next step:

A	B
COMPONENT	VOLUME (µl) PER R
Adaptor Ligated DNA Fragments (Step 64)	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. 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 Primers Already Combined)
Combine the following components in a sterile tube and then proceed to the next step:

A	B
COMPONENT	VOLUME (µl) PER
Adaptor Ligated DNA Fragments (Step 64)	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells
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A	B
Index Primer Mix *	10 µl
Total Volume	50 µl

*NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations

67 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.

68 Place the tube on a thermal cycler and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLE
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	8*
Annealing	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* If your cDNA input is outside the input range of 1 ng–20 ng, adjust the PCR cycle numbers accordingly. We recommend a minimum of 3 PCR cycles for all of the original molecules to make it into the final library. For cDNA input of 1 ng–20 ng, the typical Illumina library yield, using 8 PCR cycles, is 100 ng–1 µg.

A	B
INPUT IN THE FRAGMENTATION/END PREP REA	# CYCLES REQ
100 pg–1 ng	9–12
1 ng–20 ng	6–9
20 ng–100 ng	3–6

* It is possible to normalize the cDNA input into the Fragmentation/End Prep Reaction so that all libraries start out with a similar amount of cDNA

53m

Cleanup of PCR Reaction

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1

69 If stored at 4 °C allow the SPRI beads to warm to Room temperature for at least 00:30:00 before use. Vortex SPRI beads to resuspend well and prepare fresh [M] 80 % ethanol. 30m

70 
Add 45 µL (0.9X of sample volume) 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. Alternatively, samples can be mixed by vortexing for 3–5 seconds on high. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

71 
Incubate samples on the bench top for at least 00:05:00 at Room temperature. 5m

72 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.

73 
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 DNA targets. 5m

Note

Caution: do not discard the beads.

74 
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. 30s

75 
Repeat previous step 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.

76 Air dry the beads for up to 00:05:00 while the tube/plate is on the magnetic stand with the lid open. 5m

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the

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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.

77 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µL 0.1X TE (dilute [M] 1 X TE Buffer 1:10 in water).

78 2m

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.

79 5m

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. Libraries can be stored at -20 °C.

Assess Library Quality and Quantity on a Bioanalyzer

80 Dilute library (from previous step) 5-fold in [M] 0.1 X TE Buffer (inputs ≤ 1 ng may not require dilution to run on a Bioanalyzer).

81 Run 1 µL on a DNA High Sensitivity Chip.

82 Check that the electropherogram shows a narrow distribution with a peak size of 300–350 bp.

Note

Note: If a peak ~80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 79) to 50 µL with [M] 0.1 X TE Buffer and repeat the cleanup of PCR Reaction as described in Section "Cleanup of PCR Reaction". You may see adaptor-dimer when starting with inputs ≤ 1 ng.

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Expected result

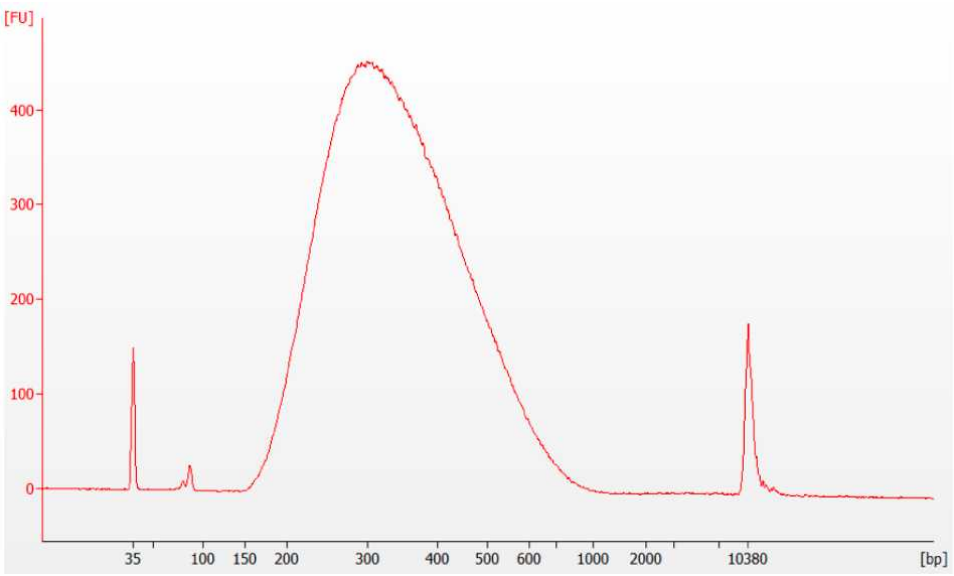

Figure 82. Example of final library size distribution on a Bioanalyzer.

HeLa single cell cDNA was used in library preparation. Shown here is a 1:5 dilution of final library.

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina E6420 Protocol for Cells https://dx.doi.org/10.17504/protocols.io.81wgb762ovpk/v1