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WORKS FOR ME 1

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

COMMENTS 0

DOI
dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/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.e6nvw5k49vmk/v1

EXTERNAL LINK

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

PROTOCOL CITATION

New England Biolabs 2022. NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina Protocol for Low Input RNA E6420. **protocols.io** https://dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/v1

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Nov 03, 2020		Liz Brydon	Protocols.io
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44162

GUIDELINES

Overview

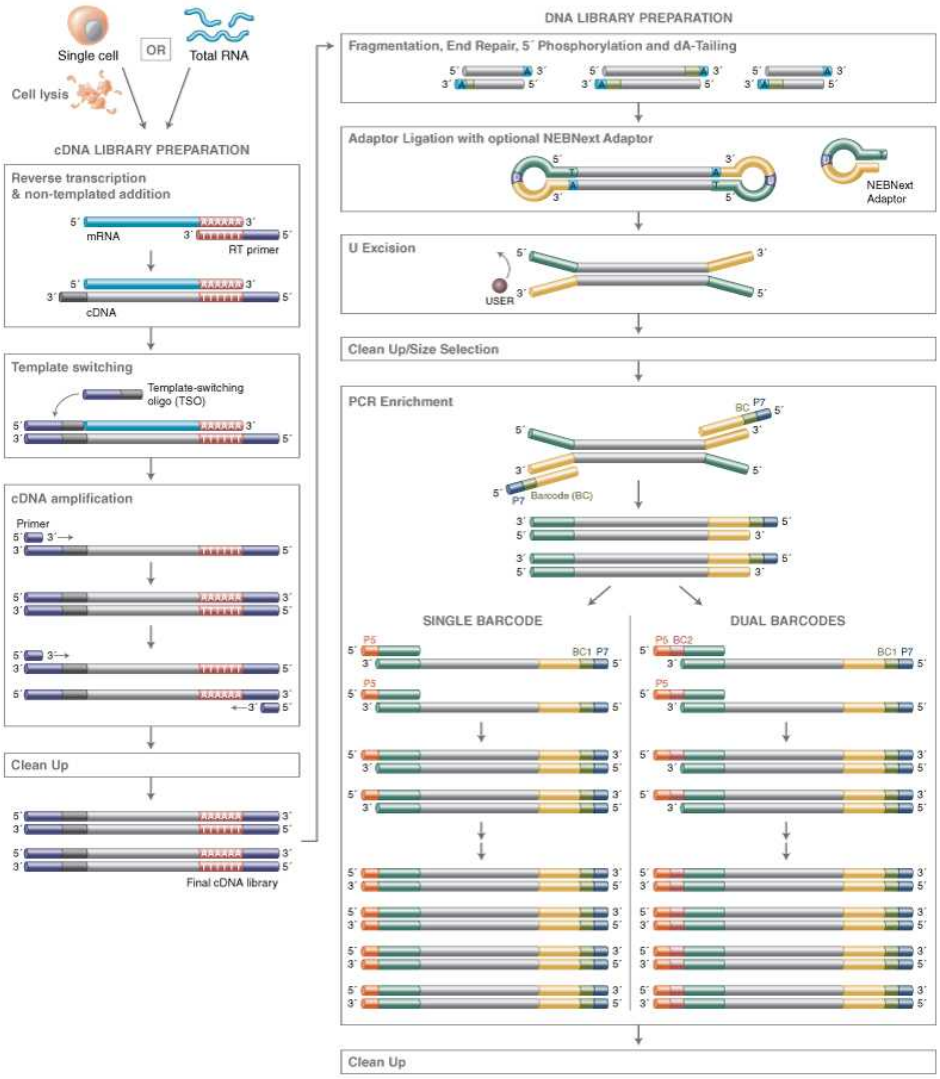
The NEBNext Low Input RNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of total RNA inputs or RNA from cultured and primary cells into high quality libraries for next-generation sequencing on the Illumina platform. The fast, user- friendly workflow 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 Protocol for Low Input RNA E6420 https://dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/v1



Oligo Sequences

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

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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 to be used for total RNA.

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol). If an excess amount of genomic DNA is present in RNA samples, an optional DNase I treatment could be peformed. Inactivate/remove DNase I after treatment.
Assess quality of the input RNA by running input RNA on an Agilent Bioanalyzer to determine the RNA Integrity Number (RIN).

Starting Material

2 pg–200 ng poly(A) tail-containing total RNA (DNA free), RIN score ≥ 8.0.

Typical Yield of cDNA from a Reaction

Actual yields will depend on the quality and quantity of the input RNA, the mRNA content of the sample, and the method used to purify the RNA. Typical cDNA yields range between 5–15 ng (for the lower RNA inputs) 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".

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina Protocol for Low Input RNA E6420 https://dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/v1

Notes
Keep all buffers and enzymes on ice, unless otherwise indicated.

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 [M] 10 X NEBNext Cell Lysis Buffer at Room temperature.

4 Thaw total RNA On ice prior to starting the protocol.

5m

Primer Annealing for First Strand Synthesis

5 To anneal cDNA Primer with total RNA samples, prepare the reaction as follows (On ice):

A	B	C
COMPONENT	< 5 ng RNA VOLUME	≥ 5 ng RNA VOLUME (µl) PER
Total RNA	Up to 8 µl	Up to 7 µl
(lilac) NEBNext Single Cell RT Primer M	1 µl	2 µl
Nuclease-free Water	Variable	Variable

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A	B	C
Total Volume	9 µl	9 µl

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

7 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. 5m

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

Reverse Transcription (RT) and Template Switching

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

Note

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

A	B
COMPONENT	VOLUME (µl) PER REA
(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

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

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina Protocol for Low Input RNA E6420 https://dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/v1

10 Combine 11 µL RT mix (above) with 9 µL annealed sample (Step 7). Mix well by pipetting up and down at least 10 times, and centrifuge briefly.

11 1h 40m

Incubate the reaction mix 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

12 Prepare cDNA amplification mix as follows:

A	B
COMPONENT	VOLUME (µl) PER REAC
(orange) NEBNext Single Cell cDNA PCR Master Mix	50 µl
(orange) NEBNext Single Cell cDNA PCR Primer	2 µl
(white) NEBNext Cell Lysis Buffer (10X)	0.5 µl
Nuclease-free Water	27.5 µl
Total Volume	80 µl

13 Add 80 µL cDNA amplification mix to 20 µL sample from Step 11. Mix by pipetting up and down at least 10 times.

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina Protocol for Low Input RNA E6420 https://dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/v1

14 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	7-21* (See 'Recommendec
Annealing	62°C	15 seconds	
Extension	72°C	3 minutes	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

Recommended Number of PCR Cycles

A	B
TOTAL RNA	RECOMMENDED NUMBER OF PCR
2 pg	20-21
10 pg	17-18
100 pg	14-15
1 ng	10-11
10 ng	8-9
100 ng/200 ng	7-8

*Note: The amount of RNA in your sample should be used to determine the appropriate number of PCR cycles.

Note

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 cDNA after the cleanup (next section) before proceeding to the library preparation (Sections "Fragmentation/End Prep" - "Assess Library Quality and Quantity on a Bioanalyzer"). The higher RNA input (> 100 ng) may yield > 15 ng cDNA. The total RNA used for the above recommendations is Universal Human Reference (UHR) RNA.

Note

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

Cleanup of Amplified cDNA

15 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.

16 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.

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

18 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.

19 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.

Note

Caution: do not discard the beads

20 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.

21 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.

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22 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.

23 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).

24 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.

25 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.

Note

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

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

27 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 Protocol for Low Input RNA E6420 https://dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/v1

28 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.

Note

Caution: do not discard the beads

29 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.

30 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.

31 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.

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

33 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.

34 Place the tube/plate on the magnetic stand. After 00:05:00 (or when the solution is clear), transfer

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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

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

Expected result

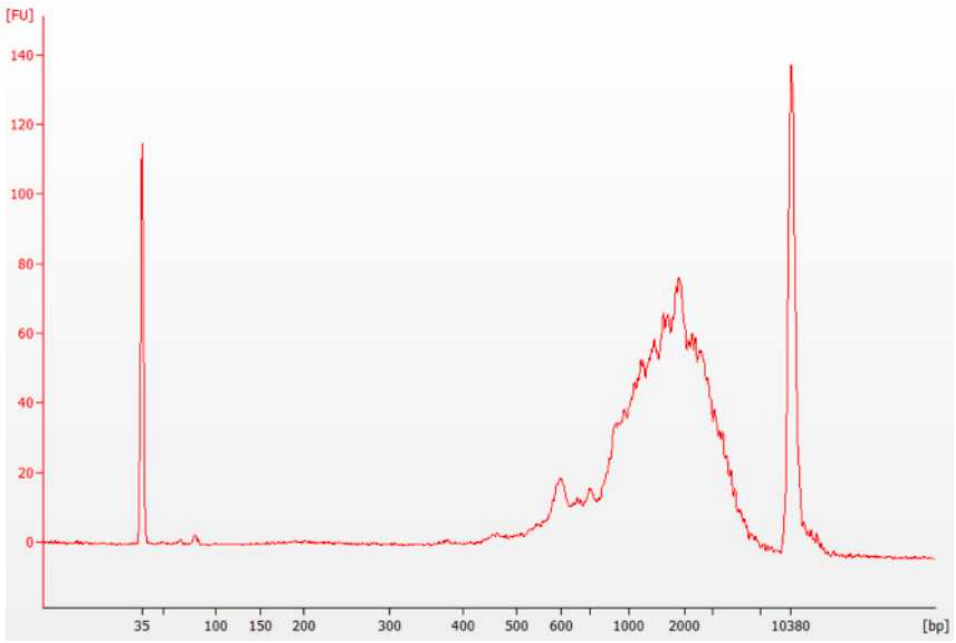

Figure 35. Examples of cDNA size distribution on a Bioanalyzer.

2 pg Total RNA (UHR) was used to synthesize cDNA and amplified using 21 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.

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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 FOR SECTIONS "Fragmentation,
100 pg–1 ng	Use all of the cDNA and adjust PCR cycles (see table
1 ng–20 ng	Typical cDNA yield. Use 8 cycles for the library enrichı
20 ng–100 ng	cDNA input into library prep can be normalized. Adjus
> 100 ng	Normalize cDNA so that at least 4 PCR cycles will be ı

Fragmentation/End Prep

36

Note

Please note, there are some videos describing how to handle the reagents on the NEB.com website:
Preparing Ultra II FS Reagents: https://www.neb.com/tools-and-resources/video-library/quick-tips---preparing-the-nebnext-ultra-ii-fs-dna-reaction-buffer-and-enzyme-mix Making a Ultra II FS master mix https://www.neb.com/tools-and-resources/video-library/quick-tips---preparing-nebnext-ultra-ii-fs-dna-reaction-buffer-and-enzyme-mix-master-mix

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.

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

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Note

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

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

A	B
COMPONENT	VOLUME (µl) PER REACTION
cDNA (Step 34)	26 µl
(yellow) NEBNext Ultra II FS Reaction Buffer	7 µl
(yellow) NEBNext Ultra II FS Enzyme Mix	2 µl
Total Volume	35 µl

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

55m

40 In a thermal cycler, with the heated lid set to 75 °C

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.

Adaptor Ligation

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

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

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

A	B
COMPONENT	VOLUME (µl) PER REACTION
FS Reaction Mixture (Step 40)	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.

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.

44 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.

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

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

Note

Note: Steps 46 and 47 are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext oligo kits.

47 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** 4 °C **or** -20 °C**.**

Cleanup of Adaptor-ligated DNA

48

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.

49 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.

50 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

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stop the centrifugation before the beads start to settle out.

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

52 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.

53 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

54 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.

55 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.

56 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 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.

57 Remove the tube/plate from the magnetic stand. Elute the DNA from the beads by adding 17 µL 0.1X TE

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(dilute [M] 1 X TE Buffer 1:10 in water).

58 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.

59 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.

60 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.

61 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.

PCR Enrichment of Adaptor-ligated DNA

62

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:

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A	B
COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 60)	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 REACTION
Adaptor Ligated DNA Fragments (Step 60)	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
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

63 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.

64 Place the tube on a thermal cycler 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

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A	B	C	D
Denaturation	98°C	10 seconds	8*
Annealing/ Extension	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 yield of 100 pg we recommend testing 12 PCR cycles. 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 PRE	# CYCLES REQUIRED
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.

Cleanup of PCR Reaction

65 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.

66 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. 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.

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

Citation: New England Biolabs NEBNext Single Cell/ Low Input RNA Library Prep Kit for Illumina Protocol for Low Input RNA E6420 https://dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/v1

68 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.

69 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.

70 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.

71 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.

72 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 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.

73 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).

74 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.

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75 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

76 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).

77 Run 1 µL on a DNA High Sensitivity Chip.

78 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 75) 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.

Expected result

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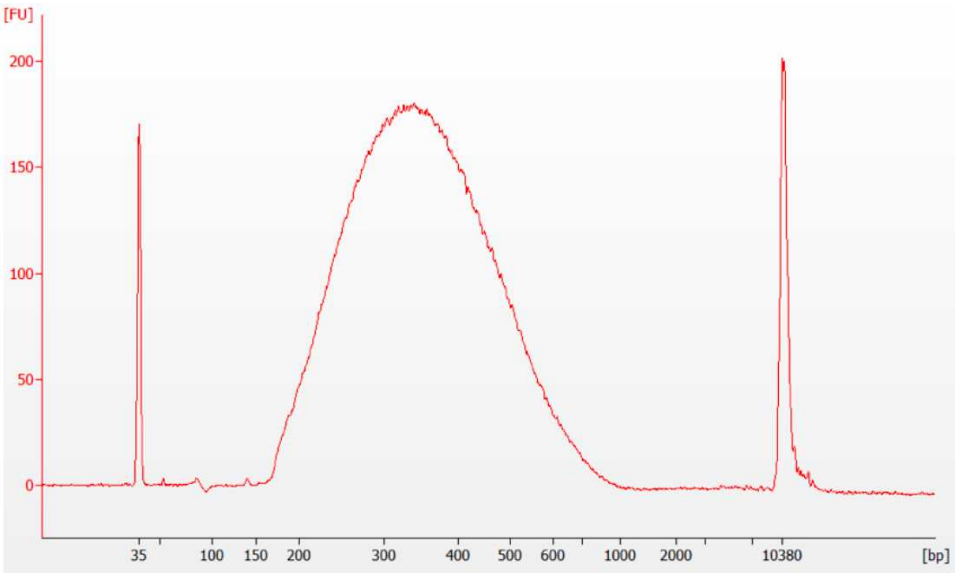

Figure 78. Example of final library size distribution on a Bioanalyzer.

cDNA from 2 pg total RNA (UHR) 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 Protocol for Low Input RNA E6420 https://dx.doi.org/10.17504/protocols.io.e6nvw5k49vmk/v1