INSTRUCTION MANUAL

NEW ENGLAND Biolabs®

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

NEB #E6420S/L

24/96 reactions

Version 6.0_01/24

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The NEBNext Single Cell/Low Input RNA Library Prep Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E6420S) and 96 reactions (NEB #6420L). All reagents should be stored at –20°C. Colored bullets indicate the cap color of the reagent to be added to a reaction.

- (white) Murine RNase Inhibitor
- (white) NEBNext Cell Lysis Buffer
- (lilac) NEBNext Single Cell RT Primer Mix
- (lilac) NEBNext Single Cell RT Buffer
- (lilac) NEBNext Template Switching Oligo
- (lilac) NEBNext Single Cell RT Enzyme Mix
- (orange or white) NEBNext Single Cell cDNA PCR Master Mix
- (orange) NEBNext Single Cell cDNA PCR Primer
- (yellow) NEBNext Ultra II FS Enzyme Mix
- (yellow) NEBNext Ultra II FS Reaction Buffer
- (red) NEBNext Ultra II Ligation Master Mix
- (red) NEBNext Ligation Enhancer
- (blue) NEBNext Ultra II Q5® Master Mix
- (white) NEBNext Bead Reconstitution Buffer
- (white) NEBNext Adaptor Dilution Buffer
- (white) TE Buffer
- (white) Nuclease-free Buffer

Required Materials Not Included

- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf® #022431021) or DNase RNase free PCR strip tubes (USA Scientific 1402-1708)
- NEBNext Oligos
    - NEBNext Oligo Kit options can be found at www.neb.com/oligos. Alternatively, customer supplied adaptor and primers can be used, please see information at: http://www.neb.com/faq-nonNEB-adaptors.
- Magnetic rack/stand (NEB #S1515S, 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

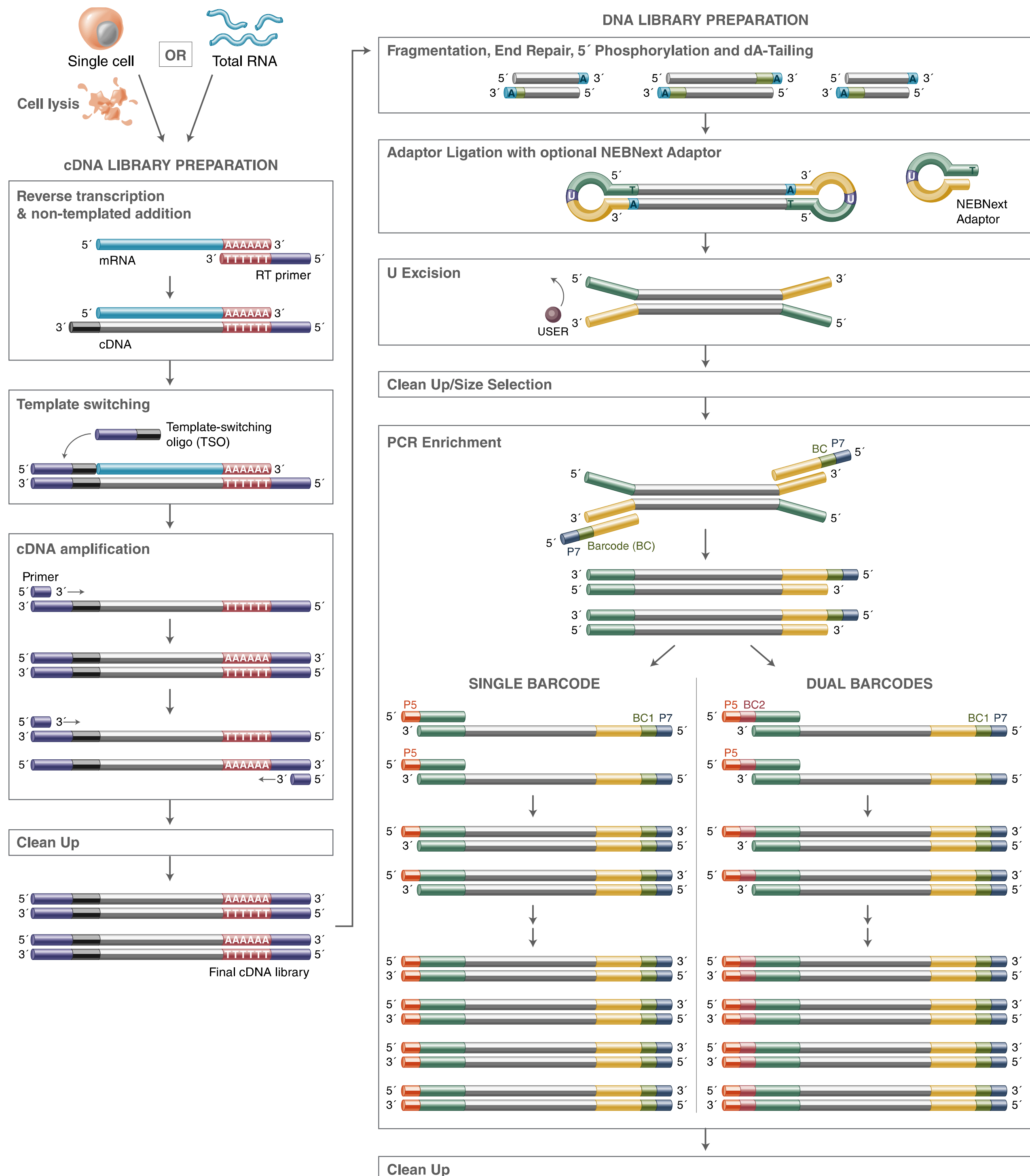
Overview

The NEBNext Single Cell/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 Customized Solutions Team at NEB. Please contact custom@neb.com for further information.

Workflow: Library Preparation for Illumina



Section 1
Protocol for Cells: cDNA Synthesis, Amplification and Library Generation

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.

Colored bullets indicate the cap color of the reagent to be added.

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 into cold cell lysis buffer provided in the kit. See Section 1.2 for cell lysis buffer dilution and recommended volumes before use. Cells should be washed and resuspended in PBS prior to isolation/sorting, because 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 cDNA yields will depend on the quality and quantity of the cell and the mRNA content of the sample. Typical cDNA yields range between 1–20 ng based on the PCR cycle recommendations provided in Section 1.5.

Typical Library 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 1.11.

Notes

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

1.1. Sample and Reagents Preparation

1.1.1. 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.

1.1.2. 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).

1.1.3. 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 bottle at 4°C or at room temperature for storage.

1.2. Cell Collection and Lysis

1.2.1. If the PBS carryover volume from cell isolation/sorting is < 1 µl, cells can be dispensed directly into cold 1X NEBNext Cell Lysis Buffer (without accounting for added volume). If PBS carryover volume from cell isolation/sorting is ≥ 1 µl, skip to Step 1.2.5.

On Ice prepare 1X NEBNext Cell Lysis Buffer in an RNase-free tube as follows:

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**

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

1.2.3. Dispense cells directly into cold 5 µl 1X Cell Lysis Buffer.

Safe Stopping Point: After dispensing, cells can be flash-frozen and stored at -80°C for future use or lysed as outlined in Step 1.2.4.

1.2.4. Incubate at room temperature for 5 minutes and then proceed immediately to Section 1.3

1.2.5. 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 1X Cell Lysis Buffer (on ice) according to the table below, accounting for the carryover cell volume.

Safe Stopping Point: Cells can be flash frozen and stored at -80°C for future use or lysed as outlined in Step 1.2.6.

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**

1.2.6. Incubate at room temperature for 5 minutes and then proceed immediately to Section 1.3.

1.3. Primer Annealing for First Strand Synthesis

Follow Step 1.3.1A for carryover volumes < 1 µl.

Follow Step 1.3.1B for carryover volumes ≥ 1 µl.

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

1.3.1A. Carryover Volume < 1 µl

COMPONENT	VOLUME (µl) PER REACTION
Lysed Cell(s) (Step 1.2.4)	5 µl
● (lilac) NEBNext Single Cell RT Primer Mix	1 µl
Nuclease-free Water	3 µl
Total Volume	**9 µl**

Continue to Step 1.3.2.

1.3.1B. Carryover Volume ≥ 1 µl

COMPONENT	VOLUME (µl) PER REACTION
Lysed Cell(s) (Step 1.2.6)	8 µl
● (lilac) NEBNext Single Cell RT Primer Mix	1 µl
Total Volume	**9 µl**

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

1.3.3. Incubate for 5 minutes at 70°C in a thermal cycler with the heated lid set to 105°C, then hold at 4ºC until next step. During the above annealing step, prepare the components for the following step.

1.4. Reverse Transcription (RT) and Template Switching

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

COMPONENT	VOLUME (µl) PER REACTION
• (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**

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

1.4.3. Combine 11 µl of the RT mix (above) with 9 µl of the annealed sample (Step 1.3.3.). Mix well by pipetting up and down at least 10 times and centrifuge briefly.

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

90 minutes at 42°C
10 minutes at 70°C
Hold at 4°C

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

1.5. cDNA Amplification by PCR

1.5.1. Prepare cDNA amplification mix as follows:

COMPONENT	VOLUME (µl) PER REACTION
• (orange or white) 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**

1.5.2. Add 80 µl cDNA amplification mix to 20 µl of the sample from Step 1.4.4. Mix by pipetting up and down at least 10 times.

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

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	45 seconds	1
Denaturation	98°C	10 seconds	11-21* (see table next page)
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

RNA CONTENT OF CELL OR CELL TYPE	RECOMMENDED NUMBER OF PCR CYCLES*
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

* 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 (Section 1.6) before proceeding to the library preparation (Sections 1.8–1.13). 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.

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

1.6. Cleanup of Amplified cDNA

1.6.1. Allow the NEBNext Bead Reconstitution Buffer and the SPRI® beads (if stored at 4°C) to warm to room temperature for at least 30 minutes before use. Vortex SPRI Beads to resuspend well and prepare fresh 80% ethanol.

1.6.2. 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.

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

1.6.4. 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.

1.6.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA **(Caution: do not discard the beads)**.

1.6.6. 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 cDNA.

1.6.7. Repeat Step 1.6.6 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.

1.6.8. 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 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.

1.6.9. Remove the tube/plate from the magnetic stand. Elute the cDNA from the beads by adding 50 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in nuclease free water).

1.6.10. 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.

1.6.11. Add 45 µl of (room temperature) NEBNext Bead Reconstitution Buffer to the eluted cDNA + bead mixture from Step 1.6.10. for a second sample clean up. Mix well by pipetting up and down at least 10 times **(Caution: Skipping this additional cleanup step may reduce overall cDNA purity)**.

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

1.6.13. 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.

1.6.14. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA **(Caution: do not discard the beads)**.

1.6.15. 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 cDNA.

1.6.16. Repeat Step 1.6.15 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.

1.6.17. 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 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.

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

1.6.19. 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.

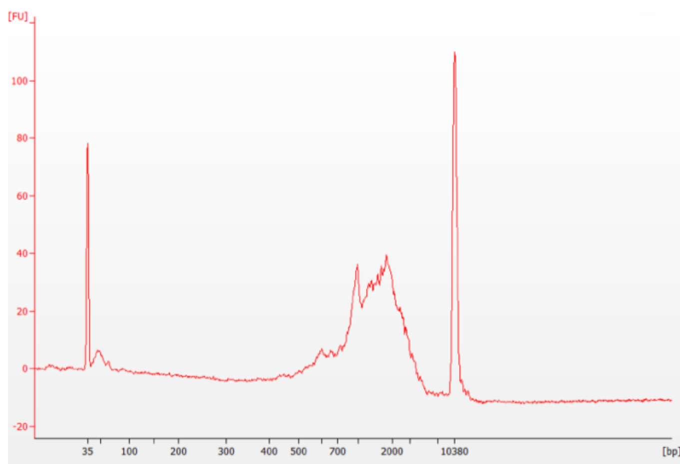
1.6.20. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube.

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

1.7. Assess Amplified cDNA Quality and Quantity on a Bioanalyzer

1.7.1. Run 1 µl of amplified cDNA from Step 1.6.20 on a DNA High Sensitivity Chip.

Figure 1.7.1: 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 1.8–1.13). If using cDNA outside the range of 1 ng–20 ng (as determined in Section 1.7), adjust the PCR cycles to amplify the adaptor ligated DNA. For details, see Step 1.11 in this protocol. If the cDNA yield is variable, the samples can be normalized to the same concentration prior to Step 1.8 in order to treat all of the samples with the same number of PCR cycles.

Recommendation Based on cDNA PCR Yield

cDNA PCR YIELD	RECOMMENDATION FOR STEP 1.8–1.13
100 pg–1 ng	Use all of the cDNA and adjust PCR cycles (see table in Step 1.11.)
1 ng–20 ng	Typical cDNA yield. Use 8 cycles for the library enrichment PCR. cDNA input into library prep can be normalized.
20 ng–100 ng	cDNA input into library prep can be normalized. Adjust PCR cycles per table in Step 1.11.
> 100 ng	Normalize cDNA so that at least 3 PCR cycles will be used in the library enrichment PCR (Step 1.11.)

1.8. Fragmentation/End Prep

1.8.1. 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.

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

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

1.8.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

COMPONENT	VOLUME (µl) PER REACTION
cDNA (Step 1.6.20)	26 µl
(yellow) NEBNext Ultra II FS Reaction Buffer	7 µl
(yellow) NEBNext Ultra II FS Enzyme Mix	2 µl
Total Volume	**35 µl**

1.8.4. Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.

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

25 minutes at 37°C
30 minutes at 65°C
Hold at 4°C

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.

1.9. Adaptor Ligation

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

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

1.9.3. Add the following components directly to the FS Reaction Mixture on ice:

COMPONENT	VOLUME (µl) PER REACTION
FS Reaction Mixture (Step 1.8.5.)	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. Please see www.neb.com/oligos for additional information.

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. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

1.9.4. 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. **(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).**

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

1.9.6. Add 3 µl of ● (red) USER® Enzyme to the ligation mixture from Step 1.9.5.

Note: Steps 1.9.6 and 1.9.7 are only required for use with NEBNext Adaptors. USER enzyme can be found in most NEBNext oligo kits. Indexed UMI adaptor does not require USER, but these oligos are not recommended for the NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina workflow.

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

Safe Stopping Point: Samples can be safely stored overnight at –20°C.

1.10. Cleanup of Adaptor-ligated DNA

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.

1.10.1. If stored at 4°C allow the SPRI beads to warm to room temperature for at least 30 minutes before use. Vortex SPRI beads to resuspend well and prepare fresh 80% ethanol.

1.10.2. 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.

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

1.10.4. 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.

1.10.5. 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).**

1.10.6. 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.

1.10.7. Repeat Step 1.10.6 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.

1.10.8. 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.

1.10.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in water).

1.10.10. 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.

1.10.11. 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.

1.10.12. Proceed to PCR Enrichment of Adaptor-ligated DNA in Section 1.11.

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

1.11. PCR Enrichment of Adaptor-ligated DNA

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. Primers are supplied at 10 µM each.

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. Primers are supplied at 10 µM combined (5 µM each).

1.11.1. Combine the following components in a sterile tube:

1.11.1A. Forward and Reverse Primers Supplied Separately

COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 1.9.11)	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.

Proceed to Step 1.11.2.

1.11.1B. Forward and Reverse Primers Already Combined

COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 1.9.11)	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.

1.11.2. 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.

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

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
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 input of 1 ng–20 ng, the typical Illumina library yield, using 8 PCR cycles, is 100 ng–1 µg.

INPUT IN THE FRAGMENTATION/ END PREP REACTION*	# 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. See Step 1.7.1.

1.12. Cleanup of PCR Reaction

1.12.1. If stored at 4°C allow the SPRI beads to warm to room temperature for at least 30 minutes before use. Vortex SPRI beads to resuspend well and prepare fresh 80% ethanol.

1.12.2. 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.

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

1.12.4. 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.

1.12.5. 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)**.

1.12.6. 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.

1.12.7. Repeat Step 1.12.6 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.

1.12.8. 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.

1.12.9. 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).

1.12.10. 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.

1.12.11. 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. Libraries can be stored at –20°C.

1.13. Assess Library Quality and Quantity on a Bioanalyzer

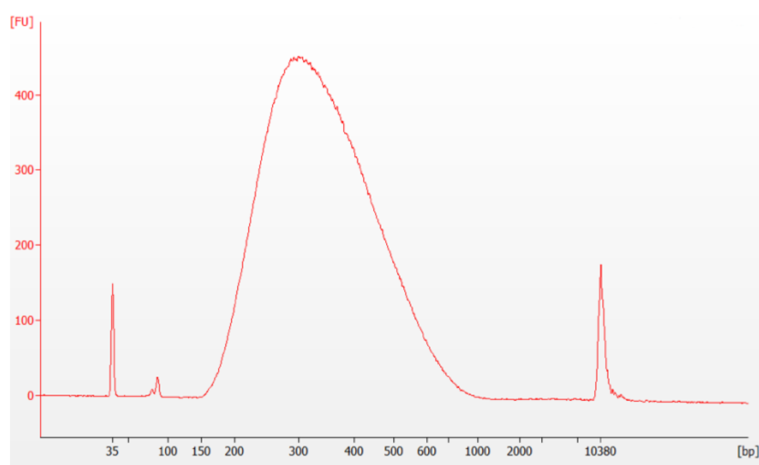
1.13.1. Dilute library (from Step 1.12.11) 5-fold in 0.1X TE Buffer (inputs ≤ 1 ng may not require dilution to run on a Bioanalyzer).

1.13.2. Run 1 µl on a DNA High Sensitivity Chip.

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

Note: If a peak ~80 bp (primers) or 128–140 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 1.12.11.) to 50 µl with 0.1X TE Buffer and repeat the cleanup of PCR Reaction as described in Section 1.12. You may see adaptor-dimer when starting with inputs ≤ 1 ng.

Figure 1.13.1: 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.

Please read the FAQ section on NEB.com for additional information about this product.

Section 2
Protocol for Low Input RNA: cDNA Synthesis, Amplification and Library Generation

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.

Colored bullets indicate the cap color of the reagent to be added.

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 performed. 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 1–20 ng (for the lower RNA inputs) based on the PCR cycle recommendations provided in Section 2.4.

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 2.10.

Notes

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

2.1. Sample and Reagents Preparation

2.1.1. Briefly centrifuge the tubes containing NEBNext Single Cell RT Enzyme Mix to collect solutions to the bottom of the tubes, then place on ice.

2.1.2. 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).

2.1.3. Mix each component thoroughly, centrifuge briefly to collect solutions to the bottom of the tube, and then place on ice. Leave the NEBNext Cell Lysis Buffer bottle at 4°C or at room temperature for storage.

2.1.4. Thaw total RNA on ice prior to starting the protocol.

2.2. Primer Annealing for First Strand Synthesis

2.2.1. To anneal cDNA Primer with total RNA samples, prepare the reaction as follows (on ice):

COMPONENT	< 5 ng RNA VOLUME (µl) PER RXN	≥ 5 ng RNA VOLUME (µl) PER RXN
Total RNA	Up to 8 µl	Up to 7 µl
• (lilac) NEBNext Single Cell RT Primer	1 µl	2 µl
Nuclease-free Water	Variable	Variable
Total Volume	**9 µl**	**9 µl**

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

2.2.3. Incubate for 5 minutes at 70°C in a thermal cycler with the heated lid set to 105°C, then hold at 4ºC until next step.

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

2.3. Reverse Transcription (RT) and Template Switching

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

COMPONENT	VOLUME (µl) PER REACTION
• (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**

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

2.3.3. Combine 11 µl of the RT mix (above) with 9 µl of the annealed sample (Step 2.2.3.). Mix well by pipetting up and down at least 10 times and centrifuge briefly.

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

90 minutes at 42°C
10 minutes at 70°C
Hold at 4°C

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

2.4. cDNA Amplification by PCR

2.4.1. Prepare cDNA amplification mix as follows:

COMPONENT	VOLUME (µl) PER REACTION
• (orange or white) 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**

2.4.2. Add 80 µl cDNA amplification mix to 20 µl of the sample from Step 2.3.4. Mix by pipetting up and down at least 10 times.

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

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	45 seconds	1
Denaturation	98°C	10 seconds	7–21* (see table below)
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

TOTAL RNA	RECOMMENDED NUMBER OF PCR CYCLES*
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.

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 (Step 2.5) before proceeding to the library preparation (Sections 2.7–2.12). 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.

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

2.5. Cleanup of Amplified cDNA

2.5.1. Allow the NEBNext Bead Reconstitution Buffer and the SPRI® beads (if stored at 4°C) to warm to room temperature for at least 30 minutes before use. Vortex SPRI Beads to resuspend well and prepare fresh 80% ethanol.

2.5.2. 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.

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

2.5.4. 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.

2.5.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA **(Caution: do not discard the beads)**.

2.5.6. 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 cDNA.

2.5.7. Repeat Step 2.5.6. 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.

2.5.8. 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 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.

2.5.9. Remove the tube/plate from the magnetic stand. Elute the cDNA from the beads by adding 50 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in nuclease free water).

2.5.10. 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.

2.5.11. Add 45 µl of (room temperature) NEBNext Bead Reconstitution Buffer to the eluted cDNA + bead mixture from Step 2.5.10 for a second sample clean up. Mix well by pipetting up and down at least 10 times **(Caution: Skipping this additional cleanup step may reduce overall cDNA purity)**.

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

2.5.13. 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.

2.5.14. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA **(Caution: do not discard the beads)**.

2.5.15. 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 cDNA.

2.5.16. Repeat Step 2.5.15 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.

2.5.17. 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 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.

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

2.5.19. 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.

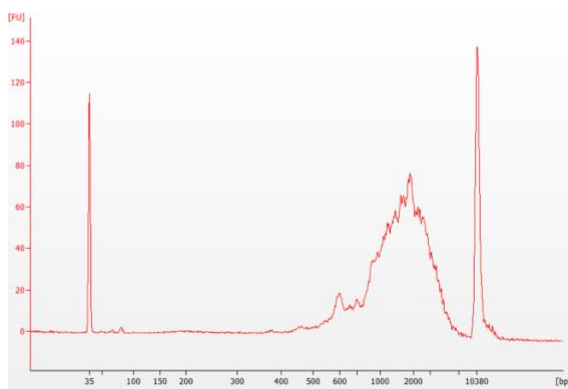
2.5.20. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube.

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

2.6. Assess Amplified cDNA Quality and Quantity on a Bioanalyzer

2.6.1. Run 1 µl of amplified cDNA from Step 2.5.20 on a DNA High Sensitivity Chip.

Figure 2.6.1: 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 2.7–2.12). If using cDNA outside the range of 1 ng–20 ng (as determined in Step 2.6), adjust the PCR cycles to amplify the adaptor ligated DNA. For details, see Step 2.10 in this protocol. If the cDNA yield is variable, the samples can be normalized to the same concentration prior to Step 2.7 in order to treat all of the samples with the same number of PCR cycles.

Recommendations for cDNA PCR Yield

cDNA PCR YIELD	RECOMMENDATION FOR STEP 2.7–2.12
100 pg–1 ng	Use all of the cDNA and adjust PCR cycles (see table in Step 2.10)
1 ng–20 ng	Typical cDNA yield. Use 8 cycles for the library enrichment PCR. cDNA input into library prep (Step 2.7–2.12) can be normalized.
20 ng–100 ng	cDNA input into library prep can be normalized. Adjust PCR cycles per table in Step 2.10.
> 100 ng	Normalize cDNA so that at least 4 PCR cycles will be used in the library enrichment PCR (Step 2.10.)

2.7. Fragmentation/End Prep

2.7.1. 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.

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

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

2.7.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

COMPONENT	VOLUME (µl) PER REACTION
cDNA (Step 2.5.20)	26 µl
○ (yellow) NEBNext Ultra II FS Reaction Buffer	7 µl
○ (yellow) NEBNext Ultra II FS Enzyme Mix	2 µl
Total Volume	**35 µl**

2.7.4. Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.

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

25 minutes at 37°C
30 minutes at 65°C
Hold at 4°C

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.

2.8. Adaptor Ligation

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

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

2.8.3. Add the following components directly to the FS Reaction Mixture on ice:

COMPONENT	VOLUME (µl) PER REACTION
FS Reaction Mixture (Step 2.7.5)	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. Please see www.neb.com/oligos for additional information.

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. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

2.8.4. 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. **(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).**

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

2.8.6. Add 3 µl of ● (red) USER® Enzyme to the ligation mixture from Step 2.8.5.

Note: Steps 2.8.6 and 2.8.7 are only required for use with NEBNext Adaptors. USER enzyme can be found in most the NEBNext oligo kits. Indexed UMI adaptor does not require USER, but these oligos are not recommended for the NEBNext Single Cell/Low RNA Library Prep Kit for Illumina workflow.

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

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

2.9. Cleanup of Adaptor-ligated DNA

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.

2.9.1. If stored at 4°C allow the SPRI beads to warm to room temperature for at least 30 minutes before use. Vortex SPRI beads to resuspend well and prepare fresh 80% ethanol.

2.9.2. 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.

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

2.9.4. 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.

2.9.5. 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).**

2.9.6. 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.

2.9.7. Repeat Step 2.9.6 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.

2.9.8. 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.

2.9.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in nuclease free water).

2.9.10. 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.

2.9.11. 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. Proceed to PCR Enrichment of Adaptor-ligated DNA in Section 2.10.

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

2.10. PCR Enrichment of Adaptor-ligated DNA

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. Primers are supplied at 10 µM each.

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. Primers are supplied at 10 µM combined (5 µM each).

2.10.1. Combine the following components in a sterile tube:

2.10.1A. Forward and Reverse Primers Supplied Separately

COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 2.9.11.)	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.

Proceed to Step 2.10.2.

2.10.1B. Forward and Reverse Primers Already Combined

COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 2.9.11.)	15 µl
● (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer/Universal Primer*	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.

2.10.2. 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.

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

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
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.

INPUT IN THE FRAGMENTATION/ END PREP REACTION*	# 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. See Step 2.6.1.

2.11. Cleanup of PCR Reaction

2.11.1. If stored at 4°C allow the SPRI beads to warm to room temperature for at least 30 minutes before use. Vortex SPRI beads to resuspend well and prepare fresh 80% ethanol.

2.11.2. 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.

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

2.11.4. 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.

2.11.5. 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)**.

2.11.6. 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.

2.11.7. Repeat Step 2.11.6 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.

2.11.8. 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.

2.11.9. 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 nuclease free water).

2.11.10. 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.

2.11.11. 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. Libraries can be stored at –20°C.

2.12. Assess Library Quality and Quantity on a Bioanalyzer

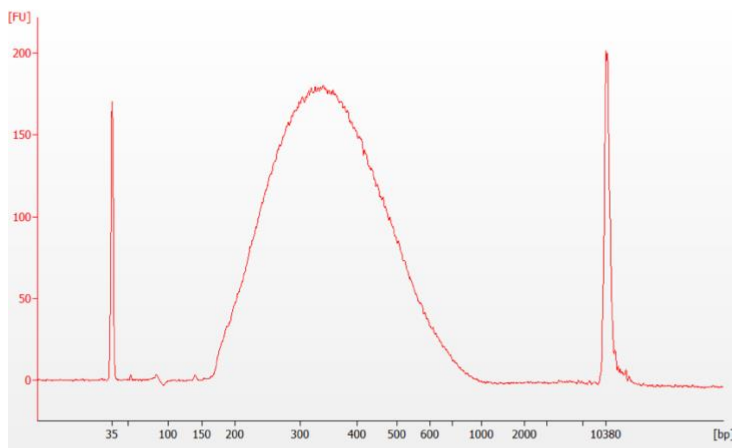
2.12.1. Dilute library (from Step 2.11.11) 5-fold in 0.1X TE Buffer (inputs ≤ 1 ng may not require dilution to run on a Bioanalyzer).

2.12.2. Run 1 µl on a DNA High Sensitivity Chip.

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

Note: If a peak ~80 bp (primers) or 128-140 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 2.11.11.) to 50 µl with 0.1X TE Buffer and repeat the cleanup of PCR Reaction as described in Section 2.11. You may see adaptor-dimer when starting with inputs ≤ 1 ng.

Figure 2.12.1: 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.

Please read the FAQ section on NEB.com for additional information about this product.

Oligo Sequences

PRODUCT	OLIGO SEQUENCE
NEBNext Template Switching Oligo	5´-GCT AAT CAT TGC AAG CAG TGG TAT CAA CGC AGA GTA CAT rGrGrG-3´
NEBNext Single Cell RT Primer Mix	5´-AAG CAG TGG TAT CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TV-3´
NEBNext Single Cell cDNA PCR Primer	5´-AAG CAG TGG TAT CAA CGC AGA GT-3´

Adaptor Trimming Sequences

The NEBNext libraries for Illumina resemble TruSeq libraries and can be trimmed similar to TrueSeq:
AdaptorRead1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
AdaptorRead2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Read Length and Depth

The typical read length employed for single cell sequencing is 2x75 bp but this can be changed depending on insert size distribution. The sequencing depth requirement is highly dependent on the biological questions that one is trying to answer. Typically, single cell sequencing requires about 500,000 reads per cell, however to detect low abundance transcripts or to identify novel features increasing read depth may be helpful.

Kit Components

NEB #E6420S Table of Components

NEB #	PRODUCT	VOLUME
E6429A	Murine RNase Inhibitor	0.024 ml
E6428A	NEBNext Cell Lysis Buffer	0.048 ml
E6422A	NEBNext Single Cell RT Primer Mix	0.048 ml
E6423A	NEBNext Single Cell RT Buffer	0.120 ml
E6424A	NEBNext Template Switching Oligo	0.024 ml
E6425A	NEBNext Single Cell RT Enzyme Mix	0.048 ml
E6426A	NEBNext Single Cell cDNA PCR Master Mix	1.2 ml
E6427A	NEBNext Single Cell cDNA PCR Primer	0.048 ml
E7806A	NEBNext Ultra II FS Enzyme Mix	0.048 ml
E7807A	NEBNext Ultra II FS Reaction Buffer	0.168 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.72 ml
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7649A	NEBNext Ultra II Q5 Master Mix	0.6 ml
E6430A	NEBNext Bead Reconstitution Buffer	1.08 ml
E6431A	NEBNext Adaptor Dilution Buffer	0.576 ml
E6432A	TE Buffer	1.2 ml
E6433A	Nuclease-free Water	1.44 ml

NEB #E6420L Table of Components

NEB #	PRODUCT	VOLUME
E6429AA	Murine RNase Inhibitor	0.096 ml
E6428AA	NEBNext Cell Lysis Buffer	0.192 ml
E6422AA	NEBNext Single Cell RT Primer Mix	0.192 ml
E6423AA	NEBNext Single Cell RT Buffer	0.48 ml
E6424AA	NEBNext Template Switching Oligo	0.096 ml
E6425AA	NEBNext Single Cell RT Enzyme Mix	0.192 ml
E6426AA	NEBNext Single Cell cDNA PCR Master Mix	4.8 ml
E6427AA	NEBNext Single Cell cDNA PCR Primer	0.192 ml
E7806AA	NEBNext Ultra II FS Enzyme Mix	0.192 ml
E7807AA	NEBNext Ultra II FS Reaction Buffer	0.672 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	0.96 ml
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7649AA	NEBNext Ultra II Q5 Master Mix	1.2 ml
E6430AA	NEBNext Bead Reconstitution Buffer	4.32 ml
E6431AA	NEBNext Adaptor Dilution Buffer	2.304 ml
E6432AA	TE Buffer	4.8 ml
E6433AA	Nuclease-free Water	5.76 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	
2.0	Added DNase RNase free PCR strip tubes to Required Materials Not Included. Updated Step 1.6.10, 2.1.1 and 2.5.10. Updated volume for component E6423A to read 0.120 ml. Updated Step 2.1.1 on Checklist. Update Chapter 2 starting material text.	10/18
3.0	Updated Step 1.6.14. and 2.5.14. on Checklist.	1/19
4.0	New Format applied	2/20
4.1	Update workflow drawing	3/20
5.0	Edits to protocols. Updated license information.	5/20
6.0	Edits to protocols. Updated license information.	01/24

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Methods for avoidance of adaptor dimer formation are covered by pending patent (New England Biolabs, Inc.).

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