NEBNext® Single Cell/Low Input cDNA Synthesis & Amplification Module

NEB #E6421S/L

24/96 reactions

Version 6.0_5/20

Table of Contents

The NEBNext Single Cell/Low Input cDNA Synthesis and Amplification Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E6421S) and 96 reactions (NEB #6421L). 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) NEBNext Single Cell cDNA PCR Master Mix
- ● (orange) NEBNext Single Cell cDNA PCR Primer
- ○ (white) NEBNext Bead Reconstitution Buffer
- ○ (white) TE Buffer
- ○ (white) Nuclease-free Water

Required Materials Not Included

- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf® #022431021)
- 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)

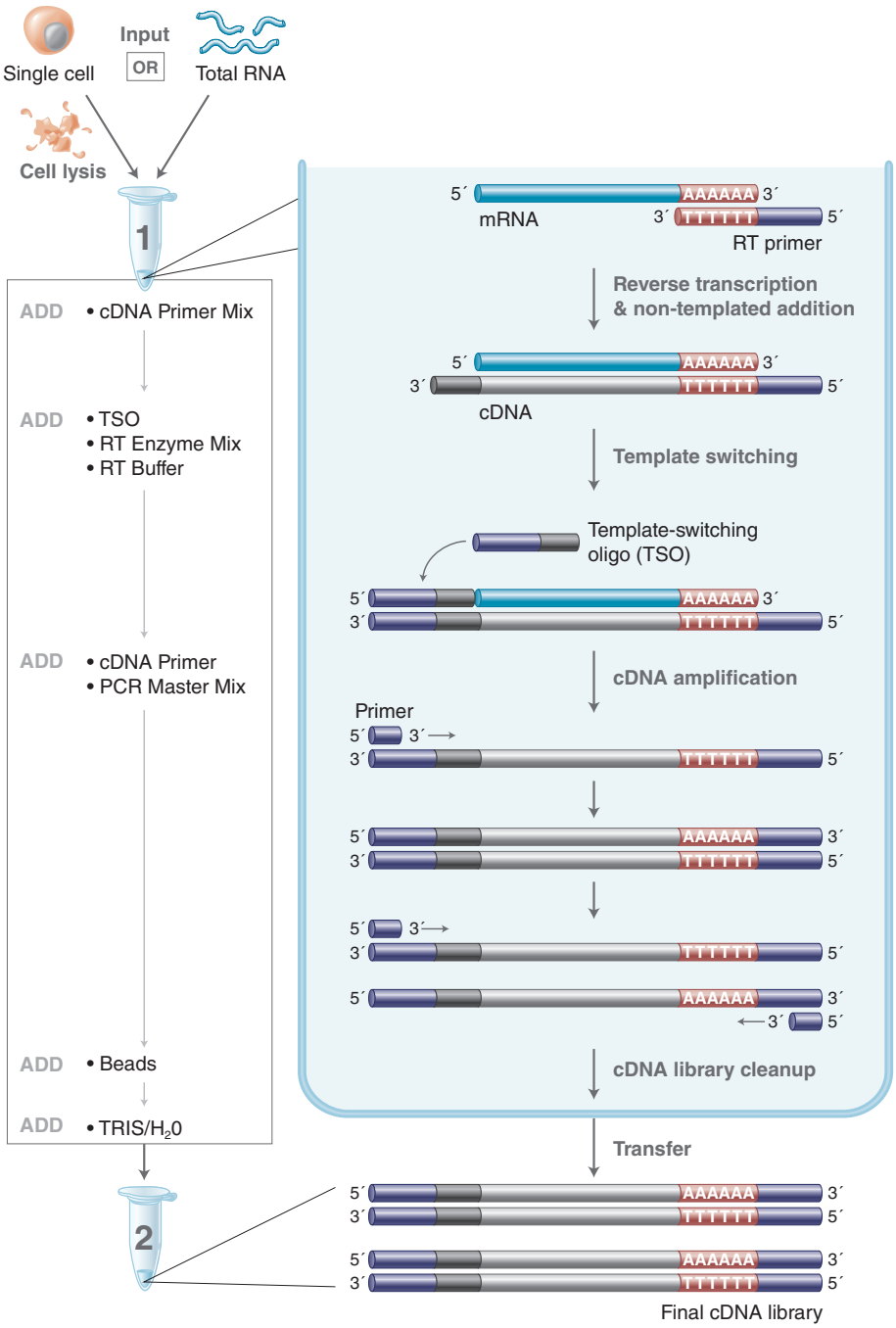
Overview

The NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module 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 amplified cDNA. 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



Section 1
Protocol for Cells: cDNA Synthesis and Amplification

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 in 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. 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 1.5.

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 at room temperature.

1.2. Cell Collection and Lysis

1.2.1. 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 1.2.5.

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 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 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 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 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 (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 (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) 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 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 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). Incubate at room temperature for 2 minutes to rehydrate the beads. (Note: if the downstream library construction protocol recommends that DNA be in solution free of EDTA, elute the cDNA in 10 mM Tris (pH 8.0) instead of TE).

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.

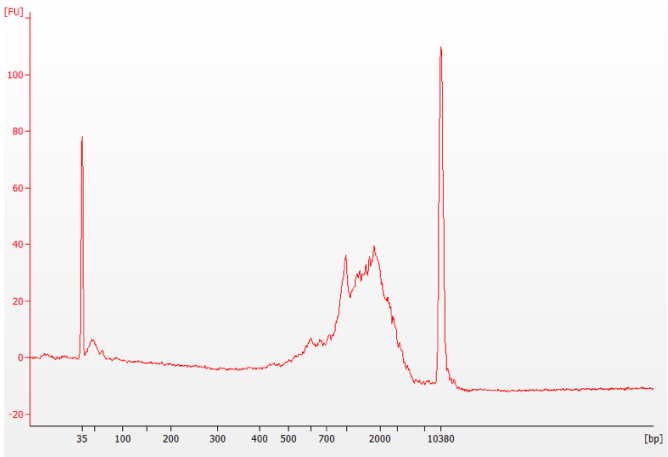
Safe Stopping Point: Samples can be safely stored overnight at 4°C or –20°C.

Note: For the subsequent library preparation protocols, we recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA), however, 10 mM Tris pH 7.5–8, low EDTA TE or H_2O are also acceptable.

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.

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

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 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 2.4.

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 and Murine RNase Inhibitor 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 10X NEBNext Cell Lysis Buffer at room temperature.

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 Mix	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) 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 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

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 5–15 ng. 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.

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 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.

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). Incubate at room temperature for 2 minutes to rehydrate the beads. (Note: if the downstream library construction protocol recommends that DNA be in solution free of EDTA, elute the cDNA in 10 mM Tris (pH 8.0) instead of TE).

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.

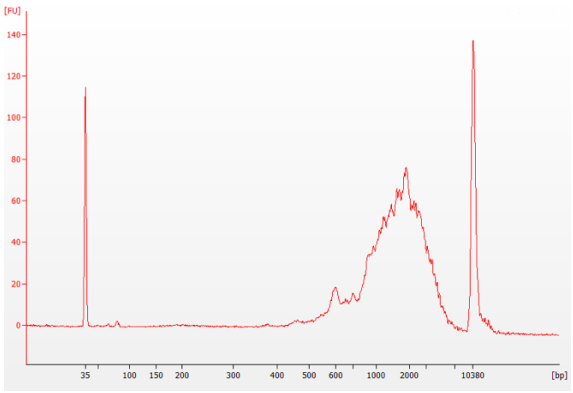
Safe Stopping Point: Samples can be safely stored overnight at 4°C or –20°C.

Note: For subsequent library preparation protocols, we recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA), however, 10 mM Tris pH 7.5–8, low EDTA TE or H_2O are also acceptable.

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.

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 TV-3´
NEBNext Single Cell cDNA PCR Primer	5´-AAG CAG TGG TAT CAA CGC AGA GT-3´

Kit Components

NEB #E6421S 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
E6430A	NEBNext Bead Reconstitution Buffer	1.08 ml
E6432A	TE Buffer	1.2 ml
E6433A	Nuclease-free Water	1.44 ml

NEB #E6421L 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
E6430AA	NEBNext Bead Reconstitution Buffer	4.32 ml
E6432AA	TE Buffer	4.8 ml
E6433AA	Nuclease-free Water	5.76 ml

CheckList

Section 1: Protocol for Cells

1.1 Sample Reagents Preparation

[_] 1.1.1. Spin NEBNext Single cell RT enzyme mix and Murine RNase inhibitor.

[_] 1.1.2. Thaw, mix and spin other reagents.

[_] 1.1.3. Place all reagents on ice until use, leave NEBNext Cell lysis buffer at room temperature.

[_] 1.1.4. Prepare cells for isolation or remove cells from storage.

1.2. Cell Collection and Lysis:

[_] **1.2.1A. If carryover volume from cell isolation is < 1 µl:**

[_] 1.2.1A.1. Prepare 1X Cell Lysis Buffer: (0.5 µl NEBNext 10X Cell Lysis Buffer, 0.25 µl Murine RNase Inhibitor, 4.25 µl Nuclease free water).

[_] 1.2.1A.2. Mix by pipetting 10 times.

[_] 1.2.1A.3. Dispense cells into 5 µl of 1X Cell Lysis Buffer from Step 1.2.1A.2. (Cells dispensed in the lysis buffer can be flash frozen and stored at -80°C for future use).

[_] 1.2.1A.4. Incubate for 5 minutes at room temperature.

[_] **1.2.1B. If carryover volume from cell isolation is ≥ 1 µl:**

[_] 1.2.1B.1. Dilute Cell Lysis Buffer: (0.8 µl NEBNext 10X Cell Lysis Buffer, 0.4 µl Murine RNase Inhibitor, variable amount of Nuclease free water, to 8 µl accounting for the carryover volume from cell isolation).

[_] 1.2.1B.2. Mix by pipetting 10 times.

[_] 1.2.1B.3. Dispense cells into diluted Cell Lysis Buffer from Step 1.2.1B.2. (Cells dispensed in the lysis buffer can be flash frozen and stored at -80°C for future use).

[_] 1.2.1B.4. Incubate for 5 minutes at room temperature.

1.3. Primer Annealing for First Strand Synthesis

[_] **1.3.1A. If carryover volume from cell isolation is < 1 µl:**

[_] 1.3.1A.1. To lysed cells (5 µl from Step 1.2.1A.4), add 1 µl Single Cell RT Primer Mix, 3 µl Nuclease-free water, to 9 µl. Mix by pipetting 10 times.

[_] **1.3.1B. If carryover volume from cell isolation is ≥ 1 µl:**

[_] 1.3.1A.1. To lysed cells (8 µl from Step 1.2.1B.4), add 1 µl Single Cell RT Primer Mix to 9 µl. Mix by pipetting 10 times.

[_] 1.3.2. Incubate for 5 minutes in thermal cycler at 70°C with heated lid at 105°C, then hold at 4°C.

[_] 1.3.3. During the annealing step, prepare the components for the following step (below).

1.4. Reverse Transcription and Template Switching

[_] 1.4.1. Prepare RT Reaction Mix (5 µl NEBNext Single Cell RT Buffer, 1 µl NEBNext Template Switching Oligo, 3 µl Nuclease-free water, 2 µl NEBNext Single Cell RT Enzyme Mix, to 11 µl)

[_] 1.4.2. Mix by pipetting 10 times.

[_] 1.4.3. Combine reaction mix (above) with the annealed sample (9 µl from 1.3.2.) by pipetting 10 times.

[_] 1.4.4. Place tubes in thermal cycler with heated lid at 105°C, incubate for 90 minutes at 42°C, 10 minutes at 70°C, hold at 4°C.

1.5. cDNA Amplification by PCR

[_] 1.5.1. To the first strand cDNA (20 µl from 1.4.4), add 50 µl NEBNext Single Cell cDNA PCR Master Mix, 2 µl NEBNext Single Cell cDNA PCR Primer, 28 µl Nuclease-free water

[_] 1.5.2. Mix by pipetting 10 times.

[_] 1.5.3. In thermal cycler with heated lid at 105°C, cycle with the following condition: Initial Duration 98°C for 45 seconds, 1 cycle; Denaturation 98°C for 10 seconds, Annealing 62°C for 15 seconds, Extension 72°C for 3 minutes, 11–21 cycles; Final extension 72°C for 5 minutes; hold 4°C.

1.6. Cleanup of Amplified cDNA

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

[_] 1.6.2. Add 60 µl (0.6X of sample volume) of beads to amplified cDNA mixture and mix by pipetting 10 times.

[_] 1.6.3. Incubate for 5 minutes at room temperature.

[_] 1.6.4. Place tubes on magnetic stand.

[_] 1.6.5 Wait 5 minutes and remove supernatant (keep the beads).

[_] 1.6.6. On magnetic stand, add 200 µl 80% ethanol, wait 30 seconds and remove.

[_] 1.6.7. Repeat Step 1.6.6. once.

[_] 1.6.8. Air dry the beads for 5 minutes, do not overdry.

[_] 1.6.9. Remove from magnet. Add 50 µl of 0.1X TE.

[_] 1.6.10. Mix by pipetting 10 times and incubate for 2 minutes.

[_] 1.6.11. Add 45 µl NEBNext Bead Reconstitution Buffer (warmed to room temperature) to cDNA and bead mixture and mix by pipetting 10 times (skipping may reduce cDNA purity).

[_] 1.6.12. Incubate for 5 minutes at room temperature.

[_] 1.6.13. Place tubes on magnetic stand.

[_] 1.6.14. Wait 5 minutes and remove supernatant (keep the beads).

[_] 1.6.15. On magnetic stand, add 200 µl 80% ethanol, wait 30 seconds and remove.

[_] 1.6.16. Repeat Step 1.6.15 once.

[_] 1.6.17. Air dry the beads for 5 minutes, do not overdry.

[_] 1.6.18. Remove tube from magnet and add 33 µl of 1X TE and incubate for 2 minutes.

[_] 1.6.19. Mix by pipetting 10 times and briefly spin.

[_] 1.6.20. Place tubes on magnetic stand. Wait 5 minutes and transfer 30 µl to fresh tube by pipetting, avoiding beads.

1.7. Run cDNA on Agilent Bioanalyzer for quantity and quality assessment.

CheckList

Section 2: Protocol for Low Input RNA

2.1. Sample Reagents Preparation

[_] 2.1.1. Spin NEBNext Single Cell RT Enzyme Mix and Murine RNase Inhibitor.

[_] 2.1.2. Thaw, mix and spin other reagents.

[_] 2.1.3. Place all reagents on ice until use, leave NEBNext Cell Lysis Buffer at room temperature.

[_] 2.1.4. Thaw total RNA on ice.

2.2. Primer Annealing for First Strand Synthesis:

[_] 2.2.1. Prepare annealing mix on ice then mix by pipetting 10 times.

[_] **2.2.1A. If RNA input is < 5 ng:**

[_] 2.2.1A.1. To 8 µl total RNA add, 1 µl Single Cell RT Primer Mix, Nuclease-free water (variable), to 9 µl.

[_] **2.2.1B. If RNA input is ≥ 5 ng:**

[_] 2.2.1B.1. To 7 µl total RNA add, 2 µl Single Cell RT Primer Mix, Nuclease-free water (variable), to 9 µl.

[_] 2.2.2. Mix by pipetting 10 times.

[_] 2.2.3. Incubate for 5 minutes in thermal cycler at 70°C with heated lid at 105°C, then hold at 4°C.

2.3. Reverse Transcription and Template Switching

[_] 2.3.1. Prepare RT reaction mix (5 µl NEBNext Single Cell RT Buffer, 1 µl NEBNext Template Switching Oligo, 3 µl Nuclease-free water, 2 µl NEBNext Single Cell RT Enzyme Mix, to 11 µl).

[_] 2.3.2. Mix by pipetting.

[_] 2.3.3. Combine reaction mix (above) with the annealed sample (9 µl from Step 2.2.3) by pipetting 10 times.

[_] 2.3.4. Place tubes in thermal cycler with heated lid at 105°C, incubate for 90 minutes at 42°C, 10 minutes at 70°C, hold at 4°C.

2.4. cDNA Amplification by PCR

[_] 2.4.1. To the first strand cDNA (20 µl from 2.3.4), add 0.5 µl NEBNext Cell Lysis Buffer (10X), 50 µl NEBNext Single Cell cDNA PCR Master Mix, 2 µl NEBNext Single Cell cDNA PCR Primer, 27.5 µl Nuclease-free water

[_] 2.4.2. Mix by pipetting 10 times.

[_] 2.4.3. In thermal cycler with heated lid at 105°C, cycle with the following condition: Initial Duration 98°C for 45 seconds, 1 cycle; Denaturation 98°C for 10 seconds, Annealing 62°C for 15 seconds, Extension 72°C for 3 minutes, 7–21 cycles; Final extension 72°C for 5 minutes; hold 4°C.

2.5. Cleanup of Amplified cDNA

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

[_] 2.5.2. Add 60 µl (0.6X of sample volume) of beads to cDNA mixture and mix 10 times by pipetting.

[_] 2.5.3. Incubate for 5 minutes at room temperature.

[_] 2.5.4. Place tubes on magnetic stand.

[_] 2.5.5. Wait for 5 minutes and remove supernatant (keep the beads).

[_] 2.5.6. On magnetic stand, add 200 µl 80% ethanol, wait 30 seconds and remove.

[_] 2.5.7. Repeat 2.5.6 once.

[_] 2.5.8. Air dry the beads for 5 minutes, do not overdry.

[_] 2.5.9. Remove from magnet. Add 50 µl of 0.1X TE.

[_] 2.5.10. Mix by pipetting 10 times and incubate for 2 minutes.

[_] 2.5.11. Add 45 µl NEBNext Bead Reconstitution Buffer (warmed to room temperature) to cDNA and bead mixture (skipping may reduce cDNA purity).

[_] 2.5.12. Incubate for 5 minutes at room temperature.

[_] 2.5.13. Place tubes on magnetic stand.

[_] 2.5.14. Wait 5 minutes and remove supernatant (keep the beads).

[_] 2.5.15. On magnetic stand, add 200 µl 80% ethanol, wait 30 seconds and remove.

[_] 2.5.16. Repeat Step 1.6.15. once.

[_] 2.5.17. Air dry the beads for 5 minutes, do not overdry.

[_] 2.5.18. Remove tube from magnet and add 33 µl of 1X TE and incubate for 2 minutes.

[_] 2.5.19. Mix by pipetting 10 times and briefly spin.

[_] 2.5.20. Place tubes on magnetic stand. Wait 5 minutes and transfer 30 µl to fresh tube by pipetting, avoiding the beads.

2.6. Run cDNA on Agilent Bioanalyzer for quantity and quality assessment.

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	4/18
2.0	Title of product is NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module	6/18
3.0	Add DNase RNase free PCR strip tubes to "Required Materials Not Included." Adjust text on Step 1.6.10, 2.1.1, 2.5.10. Update text for Starting Material of Section 2.	12/18
4.0	Update Step 1.6.14 and Step 2.5.14 on Checklist.	1/19
5.0	New format applied.	2/20
5.1	Update workflow drawing.	3/20
6.0	Update protocols.	5/20