NEBNext® Ultra™ II RNA First Strand Synthesis Module

NEB #E7771S/L

24/96 reactions

Version 4.0_7/22

Table of Contents

The NEBNext Ultra II RNA First Strand Synthesis Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7771S) and 96 reactions (NEB #E7771L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

- (lilac) NEBNext First Strand Synthesis Reaction Buffer
- (lilac) Random Primers
- (lilac) NEBNext First Strand Synthesis Enzyme Mix
- (brown) NEBNext Strand Specificity Reagent

Required Materials Not Included

- NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) or NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400 or NEB #E7405) or NEBNext Globin & rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E7750 or NEB #E7755) or NEBNext rRNA Depletion Kit (Bacteria) (NEB #E7850 or NEB #E7860) or NEBNext RNA Depletion Core Reagent Set (NEB #E7865 or NEB #E7870) or NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310 or NEB #E6350)
- NEBNext Ultra II Directional RNA Second Strand Synthesis Module (NEB #E7550) or NEBNext Ultra II Non-Directional RNA Second Strand Symthesis Module (NEB #E6111)
- PCR machine
- Tempassure PCR flex-free 8-tube strips (USA Scientific #1402-4708)

Overview

The NEBNext Ultra II First Strand Synthesis Module contains the enzymes and buffers required to convert a broad range of input amounts of RNA into cDNA using random priming. The fast, user-friendly workflow has minimal hands-on time and is compatible with upstream poly(A) mRNA enrichment and rRNA depletion methods; it is also compatible with downstream 2nd strand cDNA synthesis for both directional and non-directional RNA-seq workflows.

Each module component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together with NEB #E6111 or NEB #E7550 and NEB #E7546, NEB #E7595 and NEB #M0544 to construct an indexed transcriptome library that is 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.

Section 1
Protocol for Directional RNA-seq Workflows

Symbols

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.

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.

●

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

RNA Sample Requirements

RNA Integrity:

RNA Integrity Number (RIN) is computed using ribosomal RNA (rRNA) amount in the sample. If rRNA is removed by any method, the RIN value should not be used to evaluate the integrity of the RNA sample. In this case, we recommend that the fragmentation time is empirically determined if the RNA sample is suspected to be low quality. **The following recommendation apply to the total RNA samples only.**

Assess the quality of the input RNA by running the RNA sample on an Agilent® Bioanalyzer® RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all.

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the First Strand Synthesis Protocol in Section 1 (current chapter). See Table 1.1.1 for the recommended fragmentation times to obtain insert sizes ~200 nt.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which do not require fragmentation, follow the library preparation protocol in Section 5 of the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760) manual.

RNA Purity:

The RNA sample should be free of DNA, salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

Input Amount Requirements

1 ng – 100 ng total RNA, purified mRNA or rRNA depleted RNA that is **quantified after the purification**. RNA should be DNA free in up to 5 µl of Nuclease-free Water, quantified by Qubit® Fluorometer and quality checked by Bioanalyzer.

The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to Appendix A (Section 3) for recommended fragmentation times.

This protocol has been optimized using Universal Human Reference Total RNA.

Note: This protocol is for total RNA, purified mRNA or rRNA depleted RNA only. For use with the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) or the NEBNext RNA Depletion Kits and the NEBNext Ultra II Directional RNA Library Prep Workflow, please follow the protocol in the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina® (NEB #E7760) manual.

1.1. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 1.1.1.

1.1.1. Assemble the fragmentation and priming reaction on ice in a nuclease-free tube by adding the following components on ice:

COMPONENT	VOLUME
Purified mRNA or rRNA Depleted RNA	5 µl
● (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
● (lilac) Random Primers	1 µl
Total Volume	10 µl

1.1.2. Mix thoroughly by pipetting up and down at least 10 times.

1.1.3. Place the sample in a preheated thermal cycler with the heated lid set to 105°C and incubate the sample at 94°C following the recommendations in Table 1.1.1 below for creating insert sizes ~200 nt.

Table 1.1.1 Suggested fragmentation times based on RIN value of RNA input.

RNA TYPE	RIN	FRAG. TIME
Intact RNA	> 7	15 min. at 94°C
Partially Degraded RNA	2–6	7–8 min. at 94°C

Note: Refer to Appendix A for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix A (Section 3) only apply for intact RNA.

1.1.4. Immediately transfer the tube to ice and proceed to First Strand cDNA Synthesis.

1.2. First Strand cDNA Synthesis Reaction

1.2.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 1.1.4:

COMPONENT	VOLUME
Fragmented and primed RNA (Step 1.1.4)	10 µl
● (brown) NEBNext Strand Specificity Reagent	8 µl
● (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

1.2.2. Mix thoroughly by pipetting up and down at least 10 times.

1.2.3. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows:

Note: If you are following recommendations in Appendix A (Section 3), for longer RNA fragments (creating inserts > 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

1.2.4. Proceed directly to Second Strand cDNA Synthesis, using the NEBNext Ultra II Directional RNA Second Strand Synthesis Module (NEB #E7550)

Section 2
Protocol for Non-Directional RNA-seq Workflows

Symbols

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.

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.

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

RNA Sample Requirements

RNA Integrity:

RNA Integrity Number (RIN) is computed using ribosomal RNA (rRNA) amount in the sample. If rRNA is removed by any method, the RIN value should not be used to evaluate the integrity of the RNA sample. In this case, we recommend that the fragmentation time is empirically determined if the RNA sample is suspected to be low quality. The following recommendation apply to the total RNA samples only.

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all.

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the First Strand Synthesis Protocol in Section 2 (current chapter). See Table 2.1.1 for the recommended fragmentation times to obtain insert sizes ~200 nt.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which do not require fragmentation, follow the library preparation protocol in the NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770) manual.

RNA Purity:

The RNA sample should be free of DNA, salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

Input Amount Requirement

1 ng–100 ng total RNA purified mRNA or rRNA depleted RNA that is **quantified after the purification**. RNA should be DNA free in up to 5 µl of Nuclease-free Water, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to Appendix A (Section 3) for recommended fragmentation times and size selection conditions.

This protocol has been optimized using Universal Human Reference Total RNA.

Note: This protocol is for total RNA, purified mRNA or rRNA depleted RNA only. For use with the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) or the NEBNext RNA Depletion Kits and the NEBNext Ultra II RNA Library Prep Workflow, please follow the protocol in the NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770) manual.

2.1. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 2.1.1.

2.1.1. Assemble the fragmentation and priming reaction **on ice** in a nuclease-free tube by adding the following components:

COMPONENT	VOLUME
Purified mRNA or rRNA Depleted RNA	5 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) Random Primers	1 µl
Total Volume	10 µl

2.1.2. Mix thoroughly by pipetting up and down 10 times.

2.1.3. Place the sample in a preheated thermal cycler with the heated lid set to 105°C and incubate the sample at 94°C following the recommendations in Table 2.1.1 below for creating insert sizes ~200 nt.

Table 2.1.1 Suggested fragmentation times based on RIN value of RNA input.

RNA TYPE	RIN	FRAG. TIME
Intact RNA	> 7	15 min. at 94°C
Partially Degraded RNA	2–6	7–8 min. at 94°C

Note: Refer to Appendix A (Section 3) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix A (Section 3) only apply for intact RNA.

2.1.4. Immediately transfer the tube to ice and proceed to First Strand cDNA Synthesis.

2.2. First Strand cDNA Synthesis Reaction

2.2.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 2.1.4:

COMPONENT	VOLUME
Fragmented and primed RNA (Step 2.1.4)	10 µl
Nuclease-free Water	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

1.2.2. Mix thoroughly by pipetting up and down at least 10 times.

1.2.3. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows:

Note: If you are following recommendations in Appendix A (Section 3), for longer RNA fragments (creating inserts > 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

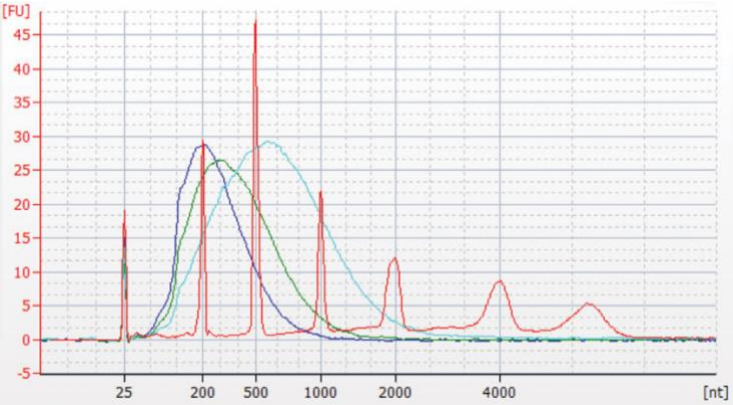
1.2.4. Proceed directly to Second Strand cDNA Synthesis, using the NEBNext Ultra II Non-directional RNA Second Strand Synthesis Module (NEB #E6111)

Section 3
Appendix A

3.1. Fragmentation

Note: These recommendations have been optimized using Universal Human Reference Total RNA. Other types of RNA may require different fragmentation times.

Modified fragmentation times for longer RNA inserts.



Red Ladder
Blue 150-300 bp, mRNA fragmented for 15 minutes at 94°C
Green 200-500 bp mRNA fragmented for 10 minutes at 94°C
Cyan 400-1,000 bp mRNA fragmented for 5 minutes at 94°C

Modified fragmentation times for longer RNA inserts. Bioanalyzer traces of RNA as shown in an RNA Pico Chip. mRNA isolated from Universal Human Reference RNA and fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) at 94°C for 5, 10 or 15 minutes, and purified using 2.2X volume of Agencourt® RNAClean® XP Beads. ***For libraries with RNA insert sizes larger than 300 bp, fragment RNA between 5–10 minutes.***

Kit Components

Each set of reagents is functionally validated together with NEB #E6111 or NEB #E7550 and NEB #E7546, NEB #E7595, and NEB #M0544, and compared to the previous lot through construction of libraries using the minimum and maximum amount of Universal Human Reference Total RNA. The previous and current lots are sequenced together on the same Illumina flow cell and compared across various sequence metrics including individual transcript abundances, 5´→3´ transcript coverage, and fraction of reads mapping to the reference

NEB #E7771S Table of Components

NEB #	PRODUCT	VOLUME
E7421A	NEBNext First Strand Synthesis Reaction Buffer	0.192 ml
E7422A	Random Primers	0.048 ml
E7761A	NEBNext First Strand Synthesis Enzyme Mix	0.048 ml
E7766A	NEBNext Strand Specificity Reagent	0.192 ml

NEB #E7771L Table of Components

NEB #	PRODUCT	VOLUME
E7421AA	NEBNext First Strand Synthesis Reaction Buffer	0.768 ml
E7422AA	Random Primers	0.192 ml
E7761AA	NEBNext First Strand Synthesis Enzyme Mix	0.192 ml
E7766AA	NEBNext Strand Specificity Reagent	0.768 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	
2.0	Add to materials required but not supplied. Update protocol.	4/18
3.0	New Format Applied.	4/20
4.0	Update protocol	7/22

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