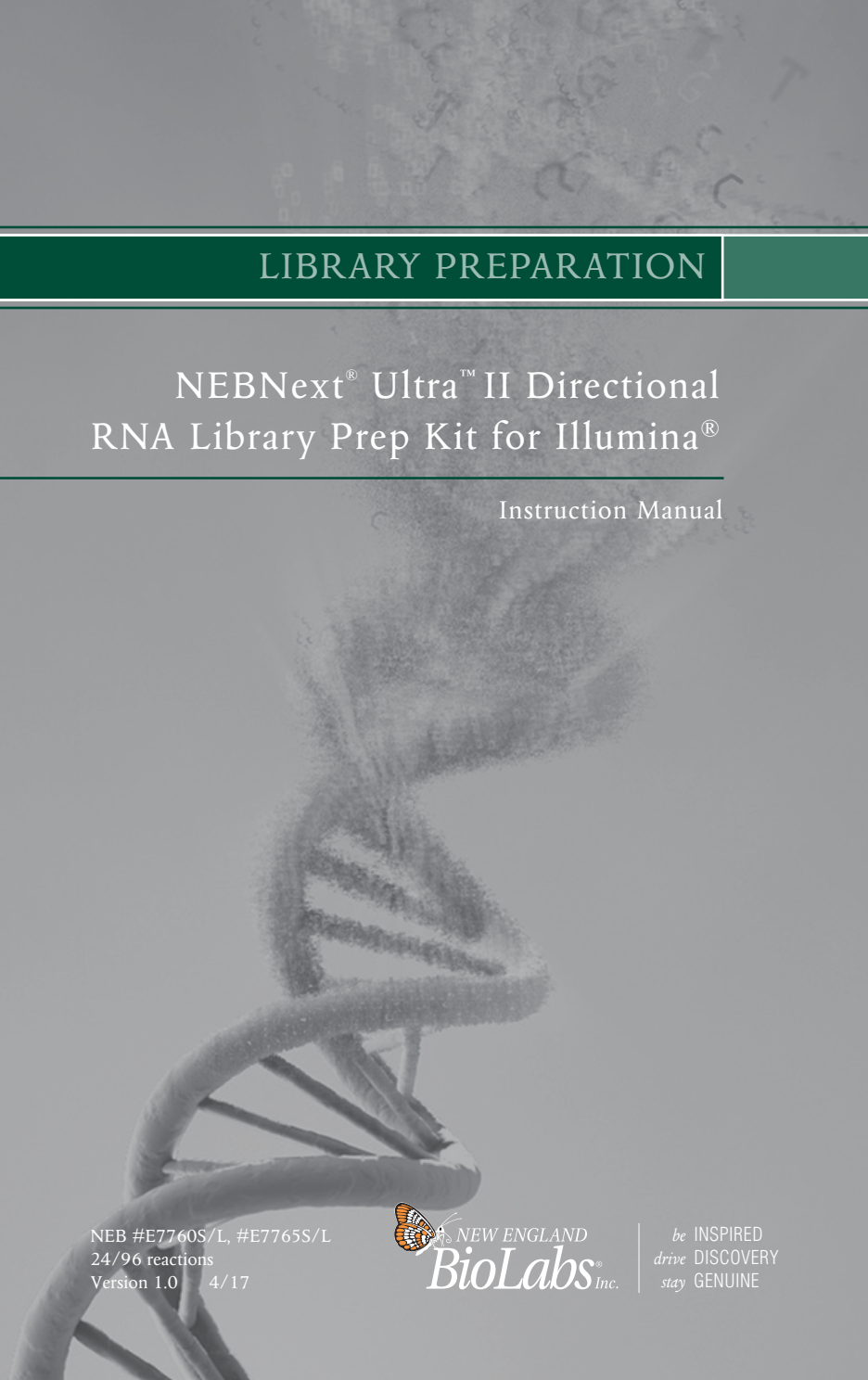
LIBRARY PREPARATION

NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®

Instruction Manual



NEB #E7760S/L, #E7765S/L
24/96 reactions
Version 1.0 4/17

NEW ENGLAND BioLabs® Inc.

be INSPIRED
drive DISCOVERY
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The Library Prep Kit Includes:

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7760S/#E7765S) and 96 reactions (NEB #E7760L/#E7765L).

Package 1: Store at –20°C.

- (lilac) NEBNext First Strand Synthesis Reaction Buffer
- (lilac) Random Primers
- (lilac) NEBNext First Strand Synthesis Enzyme Mix
- (brown) NEBNext Strand Specificity Reagent
- (orange) NEBNext Second Strand Synthesis Enzyme Mix
- (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)
- (green) NEBNext Ultra II End Prep Enzyme Mix
- (green) NEBNext Ultra II End Prep Reaction Buffer (10X)
- (red) NEBNext Ultra II Ligation Master Mix
- (red) NEBNext Ligation Enhancer
- (blue) NEBNext USER™ Enzyme
- (blue) NEBNext Ultra II Q5® Master Mix

NEBNext Adaptor Dilution Buffer

TE Buffer (0.1X)

Nuclease-free Water

Package 2: Store at room temperature. Do not freeze.

Supplied only with NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads, NEB #E7765.

NEBNext Sample Purification Beads

Required Materials Not Included:

NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina or customer supplied oligos

Magnetic Rack (Alpaqua®, cat. #A001322 or equivalent)

80% Ethanol (freshly prepared)

Thermal Cycler

For NEB #E7760 only:

SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)

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Protocol for use with Purified mRNA or rRNA Depleted RNA

NEBNext Ultra II Directional
RNA Library Prep Kit for Illumina
Instruction Manual

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

RNA Sample Recommendations

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 library preparation protocol in Chapter 4 (current chapter). See Table 4.1.1 for the recommended the fragmentation times.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which do not require fragmentation, follow the library preparation protocol in Chapter 5.

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 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 (Chapter 6) for recommended fragmentation times and size selection conditions.

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

4.1. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 4.1.1.

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

FRAGMENTATION AND PRIMING MIX	VOLUME
Purified mRNA or rRNA Depleted RNA	5 µl
(lilac) NEBNext First Strand Synthesis Reaction Buffer (5X)	4 µl
(lilac) Random Primers	1 µl
Total Volume	10 µl

4.1.2. Mix thoroughly by pipetting up and down several times.

4.1.3. Place the sample in a thermal cycler and incubate the sample at 94°C following the recommendations in Table 4.5.1 below for fragment sizes ~200 nt.

Table 4.1.1 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 (Chapter 6) only apply for intact RNA.

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

4.2 First Strand cDNA Synthesis Reaction

4.2.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 4.1.4:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 4.1.4)	10 µl
(brown) NEBNext Strand Specificity Reagent	8 µl
(lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

4.2.2. Mix thoroughly by pipetting up and down several times.

4.2.3. ⚠ 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 (Chapter 6), for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2.

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

4.2.4. Proceed directly to Second Strand cDNA Synthesis.

4.3. Second Strand cDNA Synthesis

4.3.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 4.2.4.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First Strand Synthesis Product (Step 4.2.4)	20 µl
● (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
● (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

4.3.2. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down several times.

4.3.3. Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set at ≤ 40°C.

4.4. Purification of Double-stranded cDNA Using SPRIselect Beads or NEBNext Sample Purification Beads

4.4.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

4.4.2. Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

4.4.3. Incubate for 5 minutes at room temperature.

4.4.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA.

4.4.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

4.4.6. Repeat Step 4.4.5 once for a total of 2 washing steps.

4.4.7. Air dry the beads for **5 minutes** while the tube is on the magnetic rack with lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

4.4.8. Remove the tube from the magnetic rack. Elute the DNA from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

4.4.9. Remove 50 µl of the supernatant and transfer to a clean nuclease free PCR tube.

Stop Here For the Day!
Be sure your tubes are well labelled (include group letter) and bring them to the wet lab staff for overnight storage

4.5. End Prep of cDNA Library

4.5.1. Assemble the end prep reaction **on ice** by adding the following components to second strand synthesis product from Step 4.4.9.

END PREP REACTION	VOLUME
Second strand cDNA Synthesis Product (Step 4.4.9)	50 µl
● (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
● (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	60 µl

4.5.2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

4.5.3. Incubate the sample in a thermal cycler with the heated lid set at ≥ 75°C as follows:

30 minutes at 20°C
30 minutes at 65°C
Hold at 4°C

4.5.4. Proceed immediately to Adaptor Ligation.

4.6. Adaptor Ligation

4.6.1. ⚠ **Dilute the ● (red) NEBNext Adaptor prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the diluted adaptors on ice.**

PURIFIED RNA	DILUTION REQUIRED
100 ng–11 ng	5-fold dilution in Adaptor Dilution Buffer
10 ng–1 ng	25-fold dilution in Adaptor Dilution Buffer

*The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.

4.6.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 4.5.4:

Read Carefully: Add diluted adapter DIRECTLY to each sample. You can make a master mix of Ligation Enhancer and Ligation Master Mix, but it should not include adapter

LIGATION REACTION	VOLUME PER ONE LIBRARY
End Prepped DNA (Step 4.5.4)	60 µl
Diluted Adaptor (Step 4.6.1)	2.5 µl
● (red) NEBNext Ligation Enhancer	1 µl
● (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

4.6.3. Set a 100 µl or 200 µl pipette to 80 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.**

4.6.4. Incubate **15 minutes at 20°C** in a thermal cycler.

4.6.5. Add 3 µl ● (blue) USER Enzyme to the ligation mixture from Step 4.6.4, resulting in total volume of 96.5 µl.

4.6.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to ≥ 45°C.

4.6.7. Proceed immediately to Purification of the Ligation Reaction.

4.7 Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads

Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A, Chapter 6.

4.7.1. Add 87 µl (0.9X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

4.7.2. Incubate for 10 minutes at room temperature.

4.7.3. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments **(Caution: do not discard the beads)**.

4.7.4. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

4.7.5. Repeat Step 4.7.4 once for a total of 2 washing steps.

4.7.6. Briefly spin the tube, and put the tube back in the magnetic rack.

4.7.7. Completely remove the residual ethanol, and air dry beads until the beads are dry for 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

4.7.8. Remove the tube from the magnet. Elute DNA target from the beads by adding 17 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times, and incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

4.7.9. Without disturbing the bead pellet, transfer 15 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

Note: If you need to stop at this point in the protocol samples can be stored at –20°C.

4.8. PCR Enrichment of Adaptor Ligated DNA

⚠ Note: Check and verify that the concentration of your oligos is 10 µM on the label.

⚠ **Follow Section 4.8.1A** if you are using the following oligos (10 µM):

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow Section 4.8.1B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

4.8.1. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

4.8.1A Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.7.9)	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
Universal PCR Primer /i5 Primer*, **	5 µl
Index (X) Primer /i7 Primer*, ***	5 µl
Total Volume	50 µl

4.8.1B Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.7.9)	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X)/Universal Primer Mix****	10 µl
Total Volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

*** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7710, #E7730 or #E7500) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

**** The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

4.8.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.

4.8.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 4.8.3A and Table 4.8.3B):

Table 4.8.3A:

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

* The number of PCR cycles should be adjusted based on RNA input.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (see page 76).

Table 4.8.3B: Recommended PCR cycles based on input amount:

PURIFIED mRNA or rRNA DEPLETED RNA (QUANTIFIED AFTER PURIFICATION)	RECOMMENDED PCR CYCLES
100 ng	6–7
50 ng	7–8
10 ng	10
1 ng	12–13

Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality to prevent PCR over-amplification.

4.9. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

4.9.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

4.9.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

4.9.3. Incubate for 5 minutes at room temperature.

4.9.4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and

discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

4.9.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

4.9.6. Repeat Step 4.9.5 once for a total of 2 washing steps.

4.9.7. Air dry the beads for 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

4.9.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

4.9.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

Be sure your tubes are well labelled (include group letter) and bring them to the wet lab staff

4.10.1. Run 1 µl of each prepared library sample on a DNA High Sensitivity chip.

4.10.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces; Bring up the sample volume (from Step 4.9.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 4.9).

Revision History:

Revision #	Description
1.0	N/A