Protocol for use with rRNA Depleted FFPE RNA and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760, E7765)

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ABSTRACT

The NEBNext Ultra II Directional RNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of input amounts of RNA into high quality directional (strand-specific) libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow has minimal hands-on time and is compatible with poly(A) mRNA enrichment and rRNA depletion methods.

PROTOCOL STATUS

Working

GUIDELINES

Section 5

RNA Sample Requirements

This Section can be used for libraries without any enrichment or depletion of total RNA with RIN scores 1–2.

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. 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 Section 4.

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

RNA Sample Requirements:

The RNA sample should be free of salts (e.g. Mg^{2+}, or guanidinium salts, divalent cation chelating agents (e.g. EDTA or EGTA) or organics (e.g. phenol or ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (DNase is not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation.

MATERIALS

NAME	CATALOG #	VENDOR
NEBNext RNase H	E6318	New England Biolabs
RNase H Reaction Buffer	E6312	New England Biolabs
NEBNext rRNA Depletion Solution	E6313	New England Biolabs
NEBNext Probe Hybridization Buffer	E6314	New England Biolabs

NAME	CATALOG #	VENDOR
DNase I (RNase-free)	E6316	New England Biolabs
DNase I Reaction Buffer	E6315	New England Biolabs
Nuclease-free Water	E6317	New England Biolabs
NEBNext RNA Sample Purification Beads	E6315	New England Biolabs
Magnetic Rack	View	
80% Ethanol (freshly prepared)	View	
Thermal cycler	View	
Agencourt RNAClean XP Beads	A63987	Beckman Coulter
DNase I (e.g., NEB #M0303) and DNase I Cleanup Reagants or Kit for Removal of DNA Prior to Depletion	View	New England Biolabs
Random Primers	E7422	New England Biolabs

STEPS MATERIALS

NAME	CATALOG #	VENDOR
NEBNext Strand Specificity Reagent	E7766	New England Biolabs
NEBNext First Strand Synthesis Enzyme Mix	E7761	New England Biolabs
NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	E7426	New England Biolabs
NEBNext Second Strand Synthesis Enzyme Mix	E7425	New England Biolabs
Nuclease-free Water	E7764	New England Biolabs
NEBNext Sample Purification Beads	E7767	New England Biolabs
Fresh 80% Ethanol		
(0.1X) TE Buffer	E7763	New England Biolabs
NEBNext Ultra II End Prep Reaction Buffer	E7647	New England Biolabs
NEBNext Ultra II End Prep Enzyme Mix	E7646	New England Biolabs
NEBNext Ligation Enhancer	E7374	New England Biolabs
NEBNext Ultra II Ligation Master Mix	E7648	New England Biolabs
NEBNext USER Enzyme	E7458	New England Biolabs
NEBNext Sample Purification Beads	E7767	New England Biolabs
80% Ethanol (freshly prepared)		
NEBNext Sample Purification Beads	E6315	New England Biolabs
80% Ethanol (freshly prepared)		

SAFETY WARNINGS

Please refer to the SDS (Safety Data Sheet) for safety warnings and hazard information.

BEFORE STARTING

Starting Material:

1 ng – 100 ng alternative rRNA depleted FFPE RNA that is quantified after rRNA depletion. RNA should be DNA free in up to 5 µl of Nuclease-free Water, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

Priming of Highly Degraded RNA (FFPE) Which has a RIN ≤ 2 and Does not Require Fragmentation

1 Assemble the Priming Reaction **on ice** by adding the following components:

Priming Reaction	Volume
rRNA Depleted FFPE RNA (1 ng–100 ng)	5 µl
Random Primers	1 µl
Total Volume	6 µl

2 Mix thoroughly by pipetting up and down 10 times.

3 Briefly spin down the samples in a microcentrifuge.

4 Incubate the sample in a preheated thermocycler as follows:

00:05:00 **at** 65 °C **, with heated lid set at** 105 °C

Hold at 4 °C

5 Transfer the tube directly to ice and proceed to First Strand cDNA Synthesis.

First Strand cDNA Synthesis

6 Assemble the first strand synthesis reaction **on ice** by adding the following components:

First Strand Synthesis Reaction	**Volume**
Primed RNA (Step 5)	6 µl
NEBNext First Strand Synthesis Reaction Buffer	4 µl
NEBNext Strand Specificity Reagent	8 µl
NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

NEBNext Strand Specificity Reagent
by New England Biolabs
Catalog #: E7766

NEBNext First Strand Synthesis Enzyme Mix
by New England Biolabs
Catalog #: E7761

7 Mix thoroughly by pipetting up and down 10 times.

8 [!] Incubate the sample in a preheated thermocycler with the heated lid set at ≥ 80 °C as follows:

Note: If you are following recommendations in Appendix A (Chapter 6), for libraries with longer inserts (> 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 00:10:00 at 25 °C
Step 2: 00:15:00 at 42 °C
Step 3: 00:15:00 at 70 °C
Step 4: Hold at 4 °C

9 Proceed directly to Second Strand cDNA Synthesis.

Second Strand cDNA Synthesis

10 Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis product from Step 9).

Second Strand Synthesis Reaction	**Volume**
First Strand Synthesis Product (Step 9)	20 µl
NEBNext Second Strand Synthesis Reaction Buffer with dUTP (10X)	8 µl
NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix
by New England Biolabs
Catalog #: E7426

NEBNext Second Strand Synthesis Enzyme Mix
by New England Biolabs
Catalog #: E7425

Nuclease-free Water
by New England Biolabs
Catalog #: E7764

11 Keeping the tube on ice, mix thoroughly by pipetting up and down at least 10 times.

12 Incubate in a thermocycler for 01:00:00 **at** 16 °C with the heated lid set at ≤ 40 °C (or off).

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

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

NEBNext Sample Purification Beads
by New England Biolabs
Catalog #: E7767

14 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.

15 Incubate for 00:05:00 at room temperature.

16 Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnet 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.

Caution: Do not discard beads.

17 Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 00:00:30 , and then carefully remove and discard the supernatant.

Fresh 80% Ethanol

18 Repeat Step 17 once for a total of 2 washing steps.
go to step #17 Repeat Step

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

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

20 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 at least 10 times. Quickly spin the tube and incubate for 00:02:00 at room temperature. Place the tube on the magnetic rack until the solution is clear.

(0.1X) TE Buffer
by New England Biolabs
Catalog #: E7763

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

NOTE

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

End Prep of cDNA Library

22 Assemble the end prep reaction on ice by adding the following components to the second strand synthesis product from Step 21.

End Prep Reaction	Volume
Second Strand Synthesis Product (Step 21)	50 µl
NEBNext Ultra II End Prep Reaction Buffer	7 µl
NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	**60 µl**

If a master mix is made, add 0 µl of master mix to 50 µl of cDNA for the End Prep reaction.

NEBNext Ultra II End Prep Reaction Buffer
by New England Biolabs
Catalog #: E7647

NEBNext Ultra II End Prep Enzyme Mix
by New England Biolabs
Catalog #: E7646

23 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.

24 Incubate the sample in a thermocycler with the heated lid set at ≥ 75 °C as follows:

00:30:00 at 20 °C
00:30:00 at 65 °C
Hold at 4 °C

25 Proceed immediately to Adaptor Ligation.

Adaptor Ligation

26 **[!] Dilute the (red) NEBNext Adaptor* prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the adaptor 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 NEBNext adaptor is provided in NEBNext oligos kit. NEB has several oligo kit options, which are supplied separately from the library prep kit.

Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 24.

27

Ligation Reaction	Volume Per One Library
End Prepped DNA (Step 24)	60 µl
Diluted Adaptor (Step 26)	2.5 µl
NEBNext Ligation Enhancer	1 µl
NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	**93.5 µl**

NOTE

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.

NEBNext Ligation Enhancer
by New England Biolabs
Catalog #: E7374

NEBNext Ultra II Ligation Master Mix
by New England Biolabs
Catalog #: E7648

28 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.

29 Incubate 00:15:00 **at** 20 °C in a thermocycler.

30 Add 3 µl (blue) USER™ Enzyme to the ligation mixture from Step 60, resulting in total volume of 96.5 µl

NEBNext USER Enzyme
by New England Biolabs
Catalog #: E7458

31 Mix well and incubate at 37 °C **for** 00:15:00 with the heated lid set to ≥ 45 °C

32 Proceed immediately to Purification of the Ligation Reaction.

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

33 **[!] Note: If you are selecting for libraries with larger insert size (> 200 nt) follow the size selection recommendations in Appendix A, Chapter 6.**

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.

NEBNext Sample Purification Beads
by New England Biolabs
Catalog #: E7767

34 Incubate for 00:10:00 at room temperature.

35 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 (~ 00:05:00), discard the supernatant that contains unwanted fragments.

Caution: Do not discard beads.

36 Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 00:00:30 , and then carefully remove and discard the supernatant.

80% Ethanol (freshly prepared)

37 Repeat Step 36 once for a total of 2 washing steps.
go to step #36 Repeat step

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

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

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

40 Remove the tube from the magnetic rack. 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. Quickly spin the tube and incubate for 00:02:00 at room temperature. Put the tube in the magnet until the solution is clear.

41 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.

PCR Enrichment of Adaptor Ligated DNA

42 [!] Check and verify that the concentration of your oligos is 10 µM on the label.

[!] Use **Option A** for any NEBNext oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in **separate** tubes.

Use **Option B** for any NEBNext oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers **combined**.

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

Option A: Forward and Reverse Primers Separate:

Component	Volume Per One Library
Adaptor Ligated DNA (Step 41)	15 µl
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**

Option B: Forward and Reverse Primers Combined:

Component	Volume Per One Library
Adaptor ligated DNA (Step 41)	15 µl
NEBNext Ultra II Q5 Master Mix	25 µl
Index (X)/i7 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.
** Use only one i7 primer/ index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample

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

45 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 45.A and Table 45.B):

Table 45.A:

Cycle Step	Temp	Time	Cycles
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	7–13*,**
Annealing/Extension	65°C	75 seconds	
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 Figure 7.2 on page 79 in manual).

Table 45.B: Recommended PCR cycles based on total RNA input amount:

Total RNA Input	Recommended PCR Cycles
100 ng	7–8
50 ng	8–9
10 ng	10–11
1 ng	13–14

NOTE

PCR cycles are recommended based on internally tested FFPE RNA. It may require optimization based on the

sample quality to prevent PCR over-amplification.

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

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

47 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.

NEBNext Sample Purification Beads
by New England Biolabs
Catalog #: E6315

48 Incubate for 00:05:00 at room temperature.

49 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 (~ 00:05:00), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Caution: Do not discard beads.

50 Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 00:00:30 , and then carefully remove and discard the supernatant.

80% Ethanol (freshly prepared)

51 Repeat Step 50 once for a total of 2 washing steps.
go to step #50 **Repeat step**

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

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

53 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 ten times. Quickly spin the tube in a microcentrifuge and incubate for 00:02:00 at room temperature. Place the tube in the magnetic rack until the solution is clear.

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

Assess Library Quality on an Agilent Bioanalyzer DNA Chip

55 Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip.

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

NOTE

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 54) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section "Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads").

57 Figure 56: Example of RNA library size distribution on a Bioanalyzer.