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Protocol for use with NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (E6310) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760, E7765) V.2

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Abstract

The NEBNext rRNA Depletion Kit (Human/Mouse/Rat) depletes both cytoplasmic (5S rRNA, 5.8S rRNA, 18S rRNA and 28S rRNA) and mitochodrial ribosomal RNA (12S rRNA and 16S rRNA) from human, mouse and rat total RNA preparations. This product is suitable for both intact and degraded RNA (e.g. FFPE RNA). The resulting rRNA-depleted RNA is suitable for RNA-Seq, random-primed cDNA synthesis, or other downstream RNA analysis applications.

Attachments

PDF

E7760 E7765 final ve...

5.3MB

Guidelines

Section 2

RNA Sample Requirements

RNA Integrity:

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 2 (current Section). See Table 33 for the recommended fragmentation times, based on RIN.
For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which do not require fragmentation, follow the library preparation protocol in Section 3.

RNA Sample Requirements:

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) or organics (e.g., phenol and 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 (not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation.

Materials

MATERIALS

- NEBNext RNase H **New England Biolabs** **Catalog #**E6318
- RNase H Reaction Buffer **New England Biolabs** **Catalog #**E6312
- NEBNext rRNA Depletion Solution **New England Biolabs** **Catalog #**E6313
- NEBNext Probe Hybridization Buffer **New England Biolabs** **Catalog #**E6314
- DNase I (RNase-free) **New England Biolabs** **Catalog #**E6316
- DNase I Reaction Buffer **New England Biolabs** **Catalog #**E6315
- Nuclease-free Water **New England Biolabs** **Catalog #**E6317
- NEBNext RNA Sample Purification Beads **New England Biolabs** **Catalog #**E6315
- Magnetic Rack
- 80% Ethanol (freshly prepared)
- Thermal cycler
- Agencourt RNAClean XP Beads **Beckman Coulter** **Catalog #**A63987
- DNase I (e.g., NEB #M0303) and DNase I Cleanup Reagants or Kit for Removal of DNA Prior to Depletion **New England Biolabs**
- Random Primers **New England Biolabs** **Catalog #**E7422

STEP MATERIALS

- NEBNext rRNA Depletion Solution **New England Biolabs** **Catalog #**E6313
- NEBNext Probe Hybridization Buffer **New England Biolabs** **Catalog #**E6314
- NEBNext RNase H **New England Biolabs** **Catalog #**E6318
- RNase H Reaction Buffer **New England Biolabs** **Catalog #**E6312
- Nuclease-free Water **New England Biolabs** **Catalog #**E6317
- DNase I Reaction Buffer **New England Biolabs** **Catalog #**E6315
- DNase I (RNase-free) **New England Biolabs** **Catalog #**E6316
- Nuclease-free Water **New England Biolabs** **Catalog #**E6317
- NEBNext Sample Purification Beads **New England Biolabs** **Catalog #**E6315
- 80% Ethanol (freshly prepared)
- Nuclease-free Water **New England Biolabs** **Catalog #**E6317
- First Strand Synthesis Reaction Buffer **New England Biolabs** **Catalog #**E7421
- Random Primers **New England Biolabs** **Catalog #**E7422

NEBNext Strand Specificity Reagent **New England Biolabs Catalog #**E7766

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

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

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

Nuclease-free Water **New England Biolabs Catalog #**E7764

NEBNext Sample Purification Beads **New England Biolabs Catalog #**E6315

Fresh 80% Ethanol

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

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

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

NEBNext Ligation Enhancer **New England Biolabs Catalog #**E7374

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

NEBNext USER Enzyme **New England Biolabs Catalog #**E7458

NEBNext Sample Purification Beads **New England Biolabs Catalog #**E7767

80% Ethanol (freshly prepared)

NEBNext Sample Purification Beads **New England Biolabs Catalog #**E6315

80% Ethanol (freshly prepared)

Protocol materials

- NEBNext rRNA Depletion Solution **New England Biolabs** **Catalog** #E6313
- Random Primers **New England Biolabs** **Catalog** #E7422
- NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix **New England Biolabs** **Catalog** #E7426
- NEBNext Probe Hybridization Buffer **New England Biolabs** **Catalog** #E6314
- NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs** **Catalog** #E7646
- RNase H Reaction Buffer **New England Biolabs** **Catalog** #E6312
- Thermal cycler
- NEBNext rRNA Depletion Solution **New England Biolabs** **Catalog** #E6313
- NEBNext Ligation Enhancer **New England Biolabs** **Catalog** #E7374
- 80% Ethanol (freshly prepared)
- DNase I (RNase-free) **New England Biolabs** **Catalog** #E6316
- Agencourt RNAClean XP Beads **Beckman Coulter** **Catalog** #A63987
- NEBNext Sample Purification Beads **New England Biolabs** **Catalog** #E6315
- NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs** **Catalog** #E7647
- Nuclease-free Water **New England Biolabs** **Catalog** #E6317
- 80% Ethanol (freshly prepared)
- First Strand Synthesis Reaction Buffer **New England Biolabs** **Catalog** #E7421
- (0.1X) TE Buffer **New England Biolabs** **Catalog** #E7763
- NEBNext Sample Purification Beads **New England Biolabs** **Catalog** #E7767
- 80% Ethanol (freshly prepared)
- 80% Ethanol (freshly prepared)
- NEBNext RNase H **New England Biolabs** **Catalog** #E6318
- NEBNext Strand Specificity Reagent **New England Biolabs** **Catalog** #E7766
- Nuclease-free Water **New England Biolabs** **Catalog** #E7764
- NEBNext RNA Sample Purification Beads **New England Biolabs** **Catalog** #E6315
- Magnetic Rack
- Nuclease-free Water **New England Biolabs** **Catalog** #E6317
- NEBNext First Strand Synthesis Enzyme Mix **New England Biolabs** **Catalog** #E7761
- NEBNext Sample Purification Beads **New England Biolabs** **Catalog** #E6315
- Random Primers **New England Biolabs** **Catalog** #E7422

RNase H Reaction Buffer **New England Biolabs Catalog #**E6312

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

Fresh 80% Ethanol

NEBNext USER Enzyme **New England Biolabs Catalog #**E7458

Nuclease-free Water **New England Biolabs Catalog #**E6317

DNase I (e.g., NEB #M0303) and DNase I Cleanup Reagants or Kit for Removal of DNA Prior to Depletion **New England Biolabs**

DNase I Reaction Buffer **New England Biolabs Catalog #**E6315

DNase I (RNase-free) **New England Biolabs Catalog #**E6316

NEBNext Sample Purification Beads **New England Biolabs Catalog #**E6315

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

DNase I Reaction Buffer **New England Biolabs Catalog #**E6315

NEBNext Probe Hybridization Buffer **New England Biolabs Catalog #**E6314

NEBNext RNase H **New England Biolabs Catalog #**E6318

Nuclease-free Water **New England Biolabs Catalog #**E6317

NEBNext RNase H **New England Biolabs Catalog #**E6318

RNase H Reaction Buffer **New England Biolabs Catalog #**E6312

Nuclease-free Water **New England Biolabs Catalog #**E6317

DNase I (RNase-free) **New England Biolabs Catalog #**E6316

Nuclease-free Water **New England Biolabs Catalog #**E6317

DNase I Reaction Buffer **New England Biolabs Catalog #**E6315

NEBNext Sample Purification Beads **New England Biolabs Catalog #**E6315

Nuclease-free Water **New England Biolabs Catalog #**E6317

First Strand Synthesis Reaction Buffer **New England Biolabs Catalog #**E7421

Random Primers **New England Biolabs Catalog #**E7422

NEBNext rRNA Depletion Solution **New England Biolabs Catalog #**E6313

NEBNext Probe Hybridization Buffer **New England Biolabs Catalog #**E6314

80% Ethanol (freshly prepared)

NEBNext Strand Specificity Reagent **New England Biolabs Catalog #**E7766

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

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

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

Safety warnings

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

Before start

Input Amount Requirements
5 ng–1 µg total RNA (DNA free) in a 12 µl total volume, 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 6) for recommended fragmentation times and size selection conditions.

Keep all of the buffers on ice, unless otherwise indicated.

Probe Hybridization to RNA

1 Dilute the total RNA with Nuclease-free Water to a final volume of 12 µL in a PCR tube. Keep the RNA on ice.

2 Prepare a RNA/Probe master mix as follows:

	RNA Probe Master Mix	Volume
	NEBNext rRNA Depletion Solution	1 µl
	Probe Hybridization Buffer	2 µl
	Total Volume	**3 µl**

NEBNext rRNA Depletion Solution **New England Biolabs Catalog #**E6313

NEBNext Probe Hybridization Buffer **New England Biolabs Catalog #**E6314

3 Add 3 µL of the above mix to 12 µL total RNA (from Step 1), resulting in a total volume of 15 µL .

4 Mix by pipetting up and down at least 10 times.

5 Spin down briefly in a microcentrifuge.

6 Place samples in a thermocycler with a heated lid set to approximately 0 µL , and run the following program, which will take approximately 15–20 minutes to complete:

	Temp	Time
	95°C	2 min
	95-22°C	0.1°C/sec
	Hold at 22°C	5 minutes

7 Spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to RNase H Digestion Step.

RNase H Digestion

8 Assemble the RNAse H master mix **on ice** as follows.

	RNase H Master Mix	Volume
	NEBNext RNase H	2 µl
	RNase H Reaction Buffer	2 µl
	Nuclease-free Water	1 µl
	Total Volume	**5 µl**

NEBNext RNase H **New England Biolabs Catalog #**E6318

RNase H Reaction Buffer **New England Biolabs Catalog #**E6312

Nuclease-free Water **New England Biolabs Catalog #**E6317

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

10 Briefly spin down the samples in a microcentrifuge.

11 Add 5 µL of the RNase H master mix to the RNA sample from Step 7, resulting in a total volume of 20 µL .

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

13 Incubate the sample in a thermocycler for 00:30:00 **at** 37 °C with the lid set to 40 °C (or on).

14 Briefly spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to DNase I Digestion to prevent non-specific degradation of RNA.

DNase I Digestion

15 Assemble the DNase I master mix **on ice** in a nuclease-free tube.

	DNase I Master Mix	Volume
	DNase I Reaction Buffer	5 µl
	DNase I (RNase-free)	2.5 µl

	Nuclease-free Water	22.5µl
	Total Volume	**30 µl**

DNase I Reaction Buffer **New England Biolabs Catalog #**E6315

DNase I (RNase-free) **New England Biolabs Catalog #**E6316

Nuclease-free Water **New England Biolabs Catalog #**E6317

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

17 Briefly spin down the sample in a microcentrifuge.

18 Add 30 µL of DNase I master mix to 20 µL RNA sample from Step 14, resulting in a total volume of 50 µL.

19 Mix thoroughly by pipetting up and down 10 times.

20 Incubate the sample in a thermocycler for 00:30:00 **at** 37 °C with the heated lid set to 40 °C (or on).

21 Briefly spin down the sample in a microcentrifuge, and place on ice. Proceed immediately to RNA Purification.

RNA Purification after rRNA Depletion Using Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads

22 Vortex Agencourt RNAClean XP Beads or **RNA** Sample Purification Beads to resuspend.

23 Add 110 µL (2.2X) beads to the RNA sample from Step 21 and mix thoroughly by pipetting up and down at least 10 times.

NEBNext Sample Purification Beads **New England Biolabs Catalog #**E6315

24 Incubate the sample for 00:15:00 **on ice** to bind RNA to the beads.

25 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 RNA.

26 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. Be careful not to disturb the beads that contain the RNA.
80% Ethanol (freshly prepared)

27 Repeat Step 26 once for a total of 2 washing steps.
go to step #26 Repeat Step

28 Completely remove residual ethanol, and 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 RNA 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.

29 Remove the tube from the magnet. Elute the RNA from the beads by adding 7 µL Nuclease-free Water. Mix well by pipetting up and down at least 10 times and briefly spin the tube.
Nuclease-free Water **New England Biolabs Catalog #**E6317

30 Incubate for 00:02:00 at room temperature. Place the tube in the magnet until the solution is clear (~ 00:02:00).

31 Remove 5 µL of the supernatant containing RNA and transfer to a nuclease-free tube.

32 Place the sample on ice and proceed to RNA Fragmentation and Priming.

RNA Fragmentation and Priming

33 **RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 33.**

Table 33. Assemble the following fragmentation and priming reaction **on ice**:

	Fragmentation and Priming Reaction	Volume
	Ribosomal RNA Depleted Sample (Step 32)	5 µl
	NEBNext First Strand Synthesis Reaction Buffer	4 µl
	Random Primers	1 µl
	Total Volume	**10 µl**

First Strand Synthesis Reaction Buffer **New England Biolabs Catalog #**E7421

Random Primers **New England Biolabs Catalog #**E7422

34 Mix thoroughly by pipetting up and down 10 times.

35 Place the sample on a thermocycler and incubate the sample at 94 °C following the recommendations in **Table 35** below for libraries with inserts ~200 nt.

Table 35: Suggested fragmentation times based on RIN value of RNA input.

	RNA Type	RIN	Frag. Time
	Intact RNA	> 7	15 min. @ 94°C
	Partially Degraded DNA	2–6	7–8 min. @ 94°C

Note

Refer to Appendix A (Chapter 6) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix A only apply for intact RNA.

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

First Strand cDNA Synthesis

37 Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 36:

	First Strand Synthesis Reaction	Volume

Fragmented and Primed RNA (Step 36)	10 µl
NEBNext Strand Specificity Reagent	8 µl
NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

NEBNext Strand Specificity Reagent **New England Biolabs Catalog** #E7766

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

38 Mix thoroughly by pipetting up and down 10 times.

39 [!] 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

40 Proceed directly to Second Strand cDNA Synthesis.

Second Strand cDNA Synthesis

41 Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis product from Step 40.

	Second Strand Synthesis Reaction	**Volume**
	First Strand Synthesis Product (Step 40)	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 **New England Biolabs Catalog #**E7426

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

Nuclease-free Water **New England Biolabs Catalog #**E7764

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

43 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

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

45 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.

NEBNext Sample Purification Beads **New England Biolabs Catalog #**E6315

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

47 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.

48 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

49 Repeat Step 48 once for a total of 2 washing steps.

go to step #48 Repeat Step

50 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.

51 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 **New England Biolabs Catalog #**E7763

52 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

53 Assemble the end prep reaction on ice by adding the following components to the second strand synthesis product from Step 52.

	End Prep Reaction	**Volume**
	Second Strand Synthesis Product (Step 52)	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 10 µL of master mix to 50 µL of cDNA for the End Prep reaction.

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

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

54 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.

55 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

56 Proceed immediately to Adaptor Ligation.

Adaptor Ligation

57 **[!] Dilute the NEBNext Adaptor* prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the adaptor on ice.**

	Total RNA Input	Dilution Required
	1,000 ng–101 ng	5–fold dilution in Adaptor Dilution Buffer
	100 ng–10 ng	25–fold dilution in Adaptor Dilution Buffer
	5 ng	200–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.

58 Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 56.

	Ligation Reaction	Volume
	End Prepped DNA (Step 56)	60 µl
	Diluted Adaptor (Step 57)	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 **New England Biolabs Catalog #**E7374

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

59 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.

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

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

NEBNext USER Enzyme **New England Biolabs Catalog #**E7458

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

63 Proceed immediately to Purification of the Ligation Reaction.

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

64 **[!] 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 **New England Biolabs Catalog #**E7767

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

66 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.

67 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)

68 Repeat Step 67 once for a total of 2 washing steps.
go to step #67 Repeat Step

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

70 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.

71 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.

72 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

73 [!] 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**.

74 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 72)	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 72)	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

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

76 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 76.A and Table 76.B):

Table 76.A:

	Cycle Step	Temp	Time	Cycles
	Initial Denaturation	98°C	30 seconds	1
	Denaturation	98°C	10 seconds	7–16*,**
	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 in manual).

Table 76.B: Recommended PCR cycles based on total RNA input amount:

	Total RNA Input	Recommended PCR Cycles
	1,000 ng	7–8
	100 ng	11–12
	10 ng	14–15
	5 ng	15–16

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

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

78 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 **New England Biolabs Catalog #**E6315

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

80 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.

81 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)

82 Repeat Step 81 once for a total of 2 washing steps.
go to step #81 Repeat Step

83 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.

84 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.

85 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

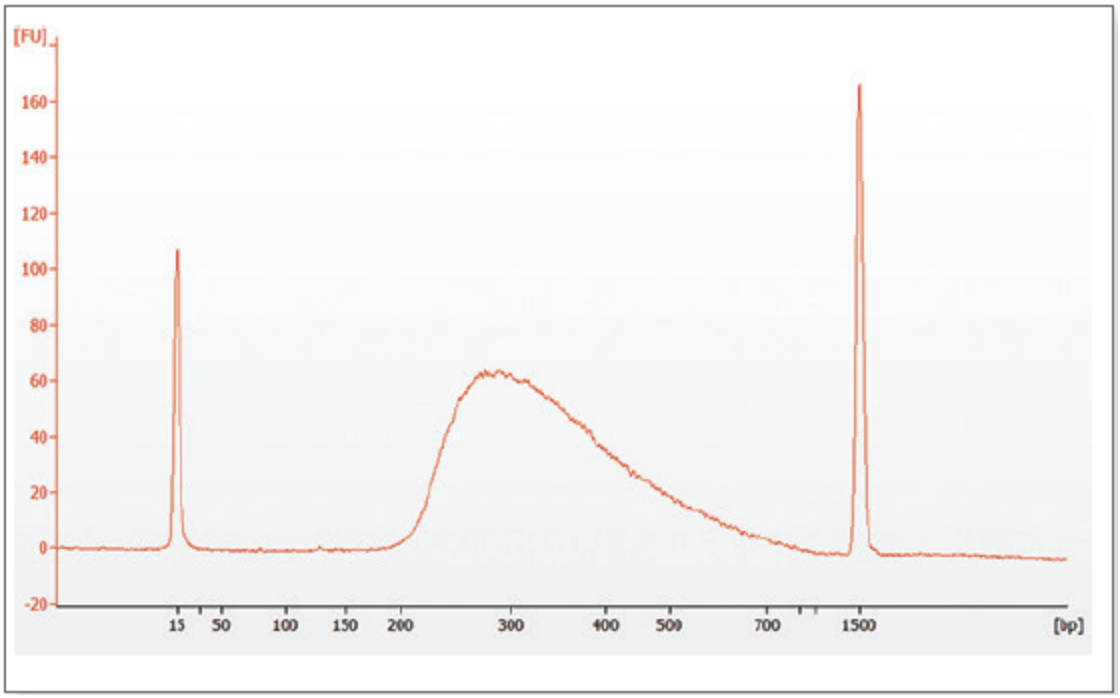
86 Run 1 µL library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip.

87 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 85) 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").

88 Figure 88: Example of RNA library size distribution on a Bioanalyzer.


[FU]
160
140
120
100
80
60
40
20
0
-20
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50
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200
300
400
500
700
1500
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