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We use this protocol and it's working

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Protocol for use with NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760, E7765) V.3

New England Biolabs[1], Isabel Gautreau[1]

[1]New England Biolabs

New England Biolabs (NEB)
Tech. support phone: **+1(800)632-7799** email: **info@neb.com**

Isabel Gautreau
New England Biolabs

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 integer ID:
81412

GUIDELINES

Section 1

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. For PolyA mRNA enrichment, high quality RNA with a RIN score > 7 is required.

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. Any residual activity of the DNase I may degrade the oligos necessary for the enrichment. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation.

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

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 **Contributed by users**
- 80% Ethanol (freshly prepared) **Contributed by users**
- Thermal cycler **Contributed by users**
- Agencourt RNAClean XP Beads **Beckman Coulter Catalog #A63987**
- DNase I (e.g., NEB #M0303) and DNase I Cleanup Reagants or Kit for Removal o DNA Prior to Depletion **New England Biolabs**
- Random Primers **New England Biolabs Catalog #E7422**

STEP MATERIALS

- NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**
- (0.1X) TE Buffer **New England Biolabs Catalog #E7763**
- Fresh 80% Ethanol **Contributed by users**
- NEBNext USER Enzyme **New England Biolabs Catalog #E7458**
- NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**
- 80% Ethanol (freshly prepared) **Contributed by users**
- NEBNext Sample Purification Beads **New England Biolabs Catalog #E6315**
- 80% Ethanol (freshly prepared) **Contributed by users**
- 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 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**

SAFETY WARNINGS

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

BEFORE START INSTRUCTIONS

Input Amount Requirement: 10 ng–1 µg DNA-free total RNA quantified by Qubit® Fluorometer and quality checked by Bioanalyzer. The protocol is optimized for approximately 200 bp RNA inserts. To generate libraries with longer RNA insert sizes, refer to Appendix (manual) for recommended fragmentation times and size selection conditions.

Keep all the buffers on ice, unless otherwise indicated.

Preparation of First Strand Reaction Buffer and Probe Hybr...

1 Prepare the First Strand Synthesis Reaction Buffer and Random Primer Mix in a nuclease-free microcentrifuge tube as follows:

	Component	Volume
	NEBNext First Strand Synthesis Reaction Buffer	8 µl
	NEBNext Random Primers	2 µl
	Nuclease-free Water	10 µl
	Total Volume	20 µl

You can prepare the first strand synthesis reaction buffer later in the protocol, but it is important that it is ready before the elution in step 38. **The beads should not be allowed to dry out.**

2 Mix thoroughly by pipetting up and down 10 times.

Note

Keep the mix on ice until mRNA is purified. It will be used in Step 38 and 41.

mRNA Isolation, Fragmentation and Priming Starting with T..

3 Dilute the total RNA with nuclease-free water to a final volume of 50 µL in a nuclease-free 0.2 ml PCR tube and keep on ice.

4 To wash the Oligo dT Beads, add the following to a 1.5 ml nuclease-free tube. If preparing multiple libraries, beads for up to 10 samples can be added to a single 1.5 ml tube for subsequent washes (use magnet NEB #S1506 for 1.5 ml tubes). The purpose of this step is to bring the beads from the storage buffer into the binding buffer. The 2X Binding Buffer does not have to be diluted for this step.

	Component	Volume Per One Library
	Oligo dT Beads d(T)25	20 µl
	RNA Binding Buffer	100 µl
	Total Volume	120 µl

5 Wash the beads by pipetting up and down 6 times.

6 Place the tube on the magnet and incubate at room temperature until the solution is clear (~ 00:02:00).

7 Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

8 Remove the tube from the magnetic rack.

9 Add 100 µL RNA Binding Buffer (2X) to the beads and wash by pipetting up and down six times. If preparing multiple libraries, add 100 µL RNA Binding Buffer (2X) per sample. The Binding Buffer does not have to be diluted.

10 Place the tubes on the magnet and incubate at room temperature until the solution is clear (~ 00:02:00).

11 Remove and discard the supernatant from the tube. Take care not to disturb the beads.

12 Add 50 µL RNA Binding Buffer (2X) to the beads and mix by pipetting up and down until beads are homogenous. If preparing multiple libraries, add 50 µL RNA Binding Buffer (2X) per sample. This first binding step removes most of the non target RNA.

13 Add 50 µl beads to each RNA sample from Step 3. Mix thoroughly by pipetting up and down 6 times.

14 Place the tube in a thermocycler and close the lid. Heat the sample at 65 °C **for** 00:05:00 **and cool to** 4 °C **with the heated lid set at ≥** 75 °C to denature the RNA and facilitate binding of the mRNA to the beads.

15 Remove the tube from the thermocycler when the temperature reaches 4 °C .

16 Mix thoroughly by pipetting up and down 6 times. Place the tube on the bench and incubate at

room temperature for 00:05:00 to allow the mRNA to bind to the beads.

17 Place the tube on the magnetic rack at room temperature until the solution is clear (~ 00:02:00).

18 Remove and discard all of the supernatant. Take care not to disturb the beads.

19 Remove the tube from the magnetic rack.

20 Wash the beads by adding 200 µL of Wash Buffer to the tube to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly.

21 Place the tube on the magnetic rack at room temperature until the solution is clear (~ 00:02:00).

22 Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

23 Remove the tube from the magnetic rack.

24 Repeat steps 20–23.
Repeat

25 Add 50 µl of Tris Buffer (provided in NEB #E7490 kit) to each tube. Gently pipette up and down 6 times to mix thoroughly.

26 Place the tube on the thermocycler. Close the lid and heat the samples at 80 °C **for** 00:02:00 **, then cool to** 25 °C **with the heated lid set at ≥** 90 °C to do the first elution of the mRNA from the beads.

27 Remove the tube from the thermocycler when the temperature reaches 25 °C.

28 Add 50 µl of RNA Binding Buffer (2X) to the sample to allow the mRNA to re-bind to the beads. Mix thoroughly by gently pipetting up and down six times.

29 Incubate the tube at **room temperature for** 00:05:00 .

30 Place the tube on the magnetic rack at room temperature until the solution is clear (~ 00:02:00).

31 Remove and discard the supernatant from the tube. Take care not to disturb the beads.

32 Remove the tube from the magnetic rack.

33 Wash the beads by adding 200 µL of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.

34 Spin down the tube briefly to collect the liquid from the wall and lid of the tube.

Note

It is important to spin down the tube to prevent carryover of the Wash Buffer in subsequent steps.

35 Place the tube on the magnet at room temperature until the solution is clear (~ 00:02:00).

36 Remove and discard all of the supernatant from the tube. Take care not to disturb the beads that contains the mRNA.

Note

It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps. Spin down the tube. Place the tube on the magnetic rack and with a 10 µl tip, remove all of the wash buffer. (Caution: Do not disturb beads that contain the mRNA). Avoid letting the beads dry out before adding elution buffer.

37 Remove the tube from the magnetic rack.

Note

[!] **The next step provides a fragmentation incubation time resulting in an RNA insert size of ~ 200 nt. For RNA insert sizes > 200 nt, refer to Appendix A in the manual for recommended fragmentation times in Step 39.**

38 To elute the mRNA from the beads and fragment, add 11.5 µL of the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) prepared in Step 2, pipette up and down six times to resuspend the beads.

39 Incubate the sample in a preheated thermocycler with the heated lid set at 105 °C as follows:
00:15:00 **at** 94 °C
Hold at 4 °C ***

*Immediately transfer the tube to ice for 00:01:00 as soon as it is cool enough to handle (~ 65 °C)

40 Quickly spin down the tube in a microcentrifuge to collect the liquid from the sides of the tube and place on the magnet right away until the solution is clear (~1–2 minutes).

41 Collect the fragmented mRNA by transferring 10 µL of the supernatant to a nuclease-free 0.2 ml PCR tube.

Note

If the supernatant volume recovered is less than 10 µl for any reason, bring the volume up to 10 µl by adding the First Strand Synthesis Reaction Buffer and Random Primer Mix prepared in Step 2 and continue with the protocol.

Note

Avoid transferring any of the magnetic beads.

42 Place the tube **on ice** and proceed directly to First Strand cDNA Synthesis.

First Strand cDNA Synthesis

43 Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 42:

	A	B
	First Strand Synthesis Reaction	**Volume**
	Fragmented and Primed RNA (Step 42)	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**

44 Mix thoroughly by pipetting up and down 10 times.

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

Note: If you are following recommendations in the Appendix (manual), 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

46 Proceed directly to Second Strand cDNA Synthesis.

Second Strand cDNA Synthesis

47 Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis product from Step 45).

	A	B
	Second Strand Synthesis Reaction	**Volume**
	First Strand Synthesis Product (Step 45)	20 µl
	NEBNext Second Strand Synthesis Reaction Buffer with dUTP	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**

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

49 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 Bea...

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

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

51 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.

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

53 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.

54 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.

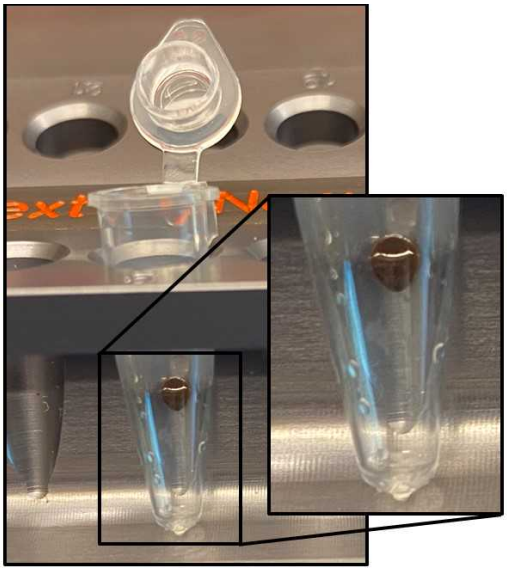
Fresh 80% Ethanol **Contributed by users**

55 Repeat Step 54 once for a total of 2 washing steps.

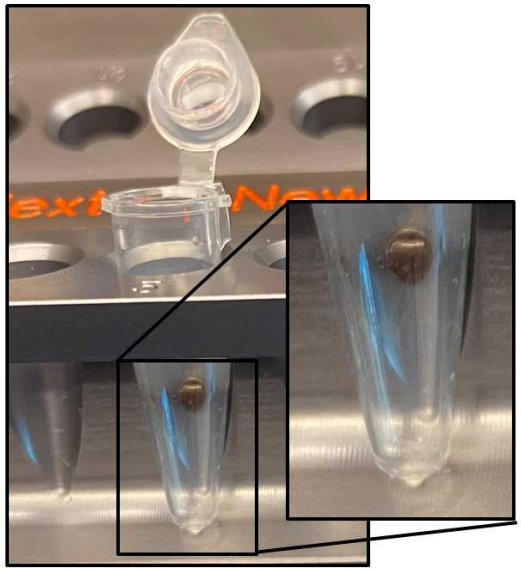
Repeat Step

56 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.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little glossy

57 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**

58 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

59 Assemble the end prep reaction on ice by adding the following components to the second strand synthesis product from Step 58.

	End Prep Reaction	Volume
	Second Strand Synthesis Product (Step 58)	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**

60 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.

61 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

62 Proceed immediately to Adaptor Ligation.

Adaptor Ligation

63 [!] 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–250 ng	5–fold dilution in Adaptor Dilution Buffer
	249 ng–100 ng	25–fold dilution in Adaptor Dilution Buffer
	99 ng–10 ng	100–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. Please see www.neb.com/oligos for additional information.

64 Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 61.

	A	B
	Ligation Reaction	Volume
	End Prepped DNA (Step 61)	60 µl
	Diluted Adaptor (Step 63)	2.5 µl
	NEBNext Ligation Enhancer	1 µl
	NEBNext Ultra II Ligation Master Mix	30 µl

	A	B
	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. Do not premix 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**

65 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.

66 Incubate **15 minutes at 20°C** in a thermocycler with the heated lid off.

00:15:00 Incubate

67 Add 3 µL (blue or red) USER™ Enzyme to the ligation mixture from Step 66, resulting in total volume of 96.5 µL

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

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

69 Proceed immediately to Purification of the Ligation Reaction.

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

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**

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

72 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.

73 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) **Contributed by users**

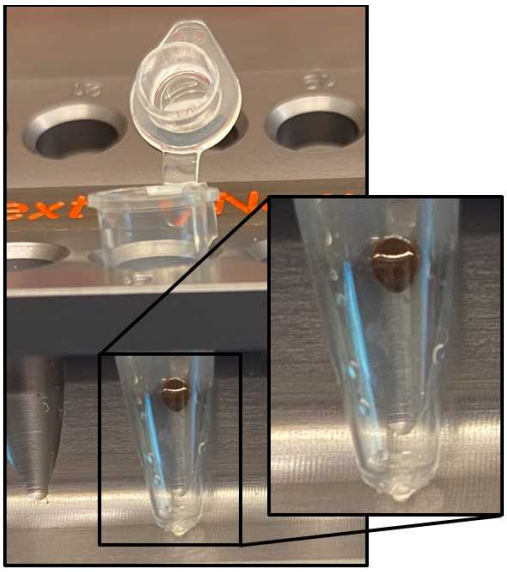
74 Repeat Step 73 once for a total of 2 washing steps.

Repeat Step

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

76 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.**



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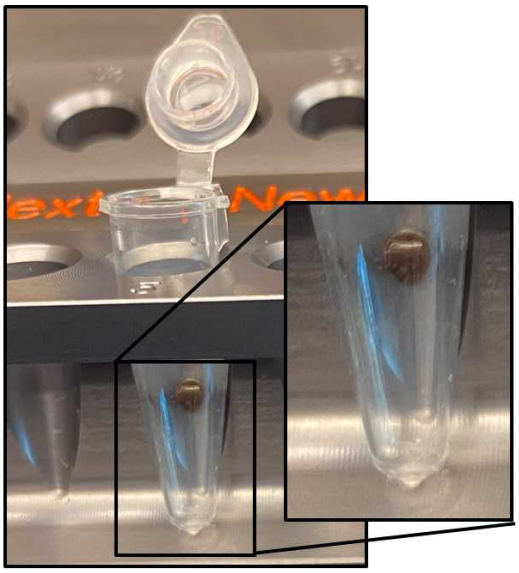


When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little glossy

77 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.

78 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

79

[!] 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. Primers are supplied at 10 µM each.

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. Primers are supplied at 10 µM combined (5 µM each).

80 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 78)	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 78)	15 µl
NEBNext Ultra II Q5 Master Mix	25 µl
Index (X)/Universal 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

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

82 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 82.A and Table 82.B):
Table 82.A:

A	B	C	D
Cycle Step	**Temp**	**Time**	**Cycles**

	A	B	C	D
	Initial Denaturation	98°C	30 seconds	1
	Denaturation	98°C	10 seconds	8–16*,**
	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 82.B: Recommended PCR cycles based on total RNA input amount:

	Total RNA Input	Recommended PCR Cycles
	1,000 ng	8–9
	100 ng	12–13
	10 ng	15–16

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.

Purification of the PCR Reaction using SPRIselect Beads or ...

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

84 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.

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

86 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.

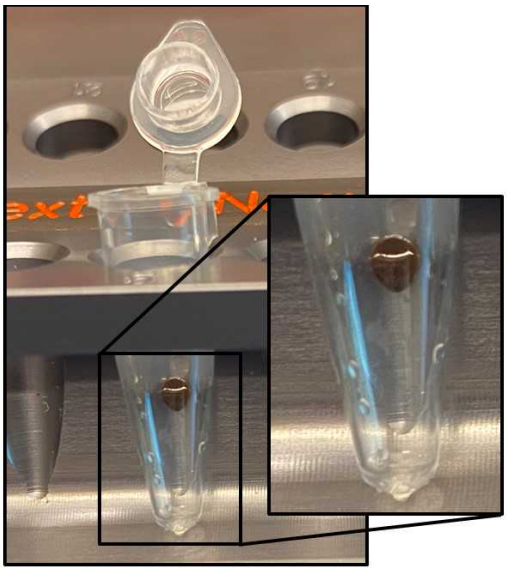
87 Add 200 µL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 00:00:30 , and then carefully remove and discard the supernatant.

80% Ethanol (freshly prepared) **Contributed by users**

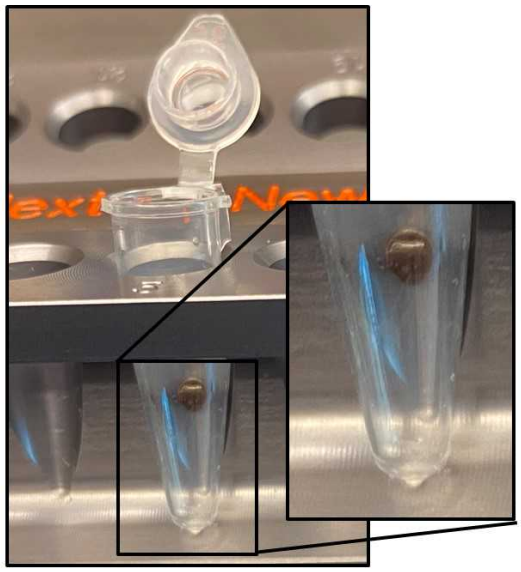
88 Repeat once for a total of 2 washing steps.

89 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.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little glossy

90 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.

91 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

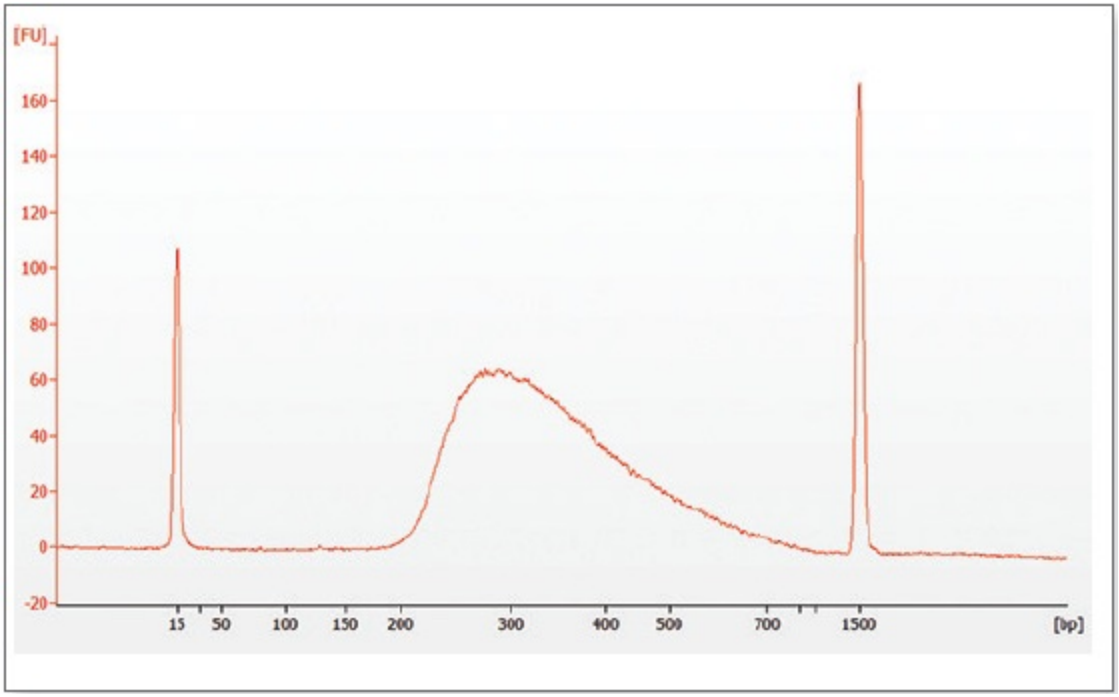
92 Run 1 µL library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip.

93 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 91) 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").

94 Figure 94: Example of RNA library size distribution on a Bioanalyzer.


[FU]
160
140
120
100
80
60
40
20
0
-20
15
50
100
150
200
300
400
500
700
1500
[bp]