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Protocol for use with NEBNext ULTRAEXPRESS® RNA Library Prep Kit (NEB#E3330) and NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and non indexed adaptor

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Protocol status: Working
We use this protocol and it's working

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Abstract

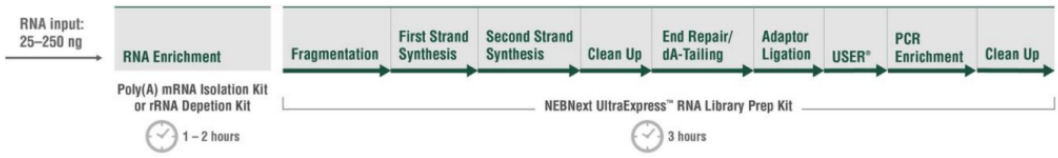
The NEBNext UltraExpress RNA Library Prep Kit contains the enzymes and buffers required to to rapidly convert 25–250 ng of total RNA into high-quality libraries for next-generation sequencing on the Illumina platform. The fast, simple workflow features minimal hands-on time and allows use of a single adaptor dilution and PCR cycling condition across the entire input range. In addition to the standard protocol, an appendix is included that details customized adaptor and cycling recommendations for varying RNA input amounts, if further optimization for library yields is required. If a stopping point is required between mRNA enrichment/ribosomal RNA depletion and library preparation, please refer to the FAQs tab for considerations and recommendations **the FAQ tab is on NEB.com.**

Guidelines

The FAQ tab is on NEB.com for additional information about this product.

For larger volume requirements, customized and bulk packaging is available by purchasing through the Customized Solutions Team at NEB. Please contact **custom@neb.com** for further information.

Figure:1 NEBNext UltraExpress RNA Workflow



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.

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

Materials

The Library Prep Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E3330S) and 96 reactions (NEB #E3330L)

Package 1: Store at -20 °C .

- (lilac) NEBNext UltraExpress RNA Fragmentation Mix (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (lilac) NEBNext UltraExpress First Strand Enzyme Mix (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (brown) NEBNext UltraExpress Strand Specificity Reagent (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (orange) NEBNext UltraExpress Second Strand Master Mix (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (green) NEBNext UltraExpress End Prep Enzyme Mix (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (green) NEBNext UltraExpress End Prep Reaction Buffer (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (red) NEBNext UltraExpress Ligation Master Mix (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (blue) NEBNext UltraExpress USER® Enzyme (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (blue) NEBNext MSTC High Yield Master Mix (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (white) NEBNext Adaptor Dilution Buffer (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (white) 0.1X TE (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (white) Nuclease-free Water (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)
- (white) NEBNext Bead Reconstitution Buffer (Protocol for use with NEBNext UltraExpress RNA Library Prep Kit (Neb #E3330), NEBNext rRNA Depletion Kits (NEB #E7400, #E7405) and NEBNext non-indexed adaptor)

Required Materials Not Included

- NEBNext Multiplex Oligos for Illumina®
- NEBNext Multiplex Oligos options can be found at www.neb.com/oligos. Alternatively, customer supplied adaptor and primers can be used; please see information in link below: https://www.neb.com/faqs/2019/03/08/can-i-use-this-nebnext-kit-with-adaptors-and-primers-from-other-vendors-than-neb
- SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Magnetic Rack (NEB S1515S, Alpaqua® cat. #A001322, or equivalent)
- 80% Ethanol (freshly prepared)
- Thermal cycler

- DNase-, RNase-free PCR strip tubes, for example TempAssure® PCR flex-free 8-tube strips (USA Scientific® #1402-4708)
- Bioanalyzer® or TapeStation® (Agilent® Technologies, Inc.) and associated reagents and consumables

For use with NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400) and other NEBNext RNA depletion kits that do not include beads (NEB #E7750, E7850, E7865):

- Agencourt® RNAClean® XP Beads (Beckman Coulter, Inc. #A63987)

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Before start

Considerations on Selecting Poly(A) mRNA Enrichment or rRNA Depletion

The library preparation protocol should be chosen based on the goals of the project and the quality of the RNA sample. Total cellular RNA is mainly composed of ribosomal RNA (rRNA) and often is not of interest. rRNA can be removed from total cellular RNA with either of two common methods. The first method uses oligo d(T) beads, which bind to the poly(A) tail of eukaryotic mRNA. Alternatively, rRNA can be depleted using rRNA-specific probes. NEB offers the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) and the NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) with or without RNA Sample Purification Beads (NEB #E7400/ #E7405) for the enrichment of non-ribosomal RNA.

In the oligo d(T) approach, only mRNA with poly(A) tails will be enriched; other cellular RNA without a poly(A) tail, such as non-coding RNA or mRNA lacking poly(A) will not bind to the beads. In addition, mRNA from some organisms (e.g., prokaryotes) or degraded RNA (e.g., FFPE RNA) do not have poly(A) tails and will not be captured by oligo d(T) beads. On the other hand, the probe-based rRNA depletion kit will remove the targeted rRNA, but it will preserve other biologically relevant cellular RNA such as non-coding RNA or mRNA.

The quality of an RNA sample should also be considered when deciding on a library preparation protocol. The NEBNext Poly(A) mRNA Magnetic Isolation Module should only be used with high-quality RNA samples (RIN > 7), since degradation results in a loss of poly(A) tails from mRNA molecules. For partially degraded or heavily degraded samples (e.g., RIN ≤ 7, FFPE RNA), the NEBNext rRNA Depletion Kit should be used.

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). For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in **sections in the kit manual on NEB.com**. For highly degraded samples (e.g., FFPE) which do not require fragmentation, follow the library preparation protocol recommendations in appendix B (in the library prep kit manual on NEB.com)

RNA Purity

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. Prior to depletion the RNA must be in nuclease free water. Some products, e.g., TURBO DNA-free™ Kit TURBO™ DNase Treatment and Removal Reagents do not produce RNA in nuclease free water and are not compatible with NEBNext rRNA depletion.

Input Amount

25–250 ng DNA-free total RNA quantified by Qubit Fluorometer or spectrophotometer and quality checked by Bioanalyzer. The protocol is optimized for approximately 200 bp RNA inserts.

Prior to Starting Enrichment

Remove the following components to thaw on ice:

• NEBNext UltraExpress RNA Fragmentation Mix
• NEBNext UltraExpress Second Strand Master Mix (Note: do not vortex reagent, mix only by inversion)

Preparation of 1X Fragmentation Mix for RNA elution

1 Thaw the Fragmentation Master Mix (2X) and prepare 1X composition as follows:

	A	B
	COMPONENT	VOLUME
	• (lilac) NEBNext UltraExpress RNA Fragmentation Mix	4 µl
	Nuclease-free Water	4 µl

Note

Keep the mix On ice until RNA is purified. It will be used in Step 26.

Probe Hybridization to RNA

2 Dilute 25 ng – 250 ng of total RNA with Nuclease-free Water to a final volume of 11 µL in a PCR tube. Keep the RNA On ice.

3 Assemble the following RNA/Probe hybridization reaction On ice:

	A	B
	RNA/PROBE HYBRIDIZATION REACTION	VOLUME
	Total RNA in Nuclease-free Water (25–250 ng)	11 µl
	• (white) NEBNext v2 rRNA Depletion Solution	2 µl
	• (white) NEBNext Probe Hybridization Buffer	2 µl
	Total Volume	15 µl

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

Note

It is crucial to mix well at this step.

5 Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

6 Place tube in a pre-heated thermocycler and run the following program with the heated lid set to 105 °C. This will take approximately 15–20 minutes to complete.

	A	B
	TEMPERATURE	TIME
	95°C	2 minutes
	Ramp Down to 22°C	0.1°C/sec
	Hold at 22°C	5 minutes

7 Briefly spin down the tube in a microcentrifuge and place On ice. Proceed immediately to the RNase H Digestion.

RNase H Digestion

30m

8 Assemble the following RNase H digestion reaction On ice:

	A	B
	RNASE H DIGESTION REACTION	VOLUME
	Hybridized RNA (Step 7)	15 µl
	• (white) RNase H Reaction Buffer	2 µl
	• (white) NEBNext Thermostable RNase H	2 µl
	Nuclease-free Water	1 µl
	Total Volume	20 µl

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

10 Briefly spin down the tube in a microcentrifuge.

11 Place in a pre-heated thermal cycler, with the heated lid set to 55 °C, and run the following program:

30m

00:30:00 at 50 °C.

Hold at 4 °C .

12 Briefly spin down the tube in a microcentrifuge and place On ice . Proceed immediately to DNase I Digestion.

DNase I Digestion

30m

13 Assemble the following DNase I digestion reaction On ice :

	A	B
	DNASE I DIGESTION REACTION	VOLUME
	RNase H treated RNA (Step 12)	20 µl
	• (white) DNase I Reaction Buffer	5 µl
	• (white) NEBNext DNase I (RNase-free)	2.5 µl
	Nuclease-free Water	22.5 µl
	Total Volume	50 µl

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

15 Briefly spin down the tube in a microcentrifuge.

16 Place in a thermal cycler, with the heated lid set at ≥ 45 °C , and run the following program:

30m

00:30:00 at 37 °C

Hold at 4 °C

17 Briefly spin down the tube in a microcentrifuge and place On ice . Proceed immediately to RNA Purification.

RNA Purification using Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads

38m

18 Vortex the Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads to resuspend.

19 Add 90 µL (1.8X) beads to the RNA sample from Step 17 and mix thoroughly by pipetting up and down at least 10 times.

20 Incubate for 00:15:00 On ice to bind RNA to the beads. 15m

21 Place the tube on a magnetic rack to separate the beads from the supernatant.

22 After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA.

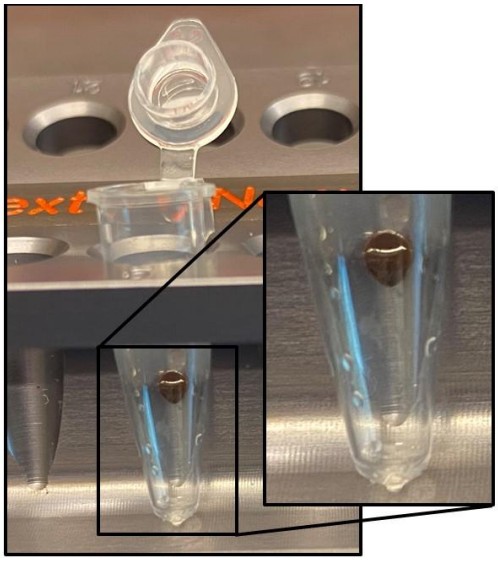
23 Add 200 µL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at Room temperature for 00:00:30 and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA. 30s

24 Repeat the previous step once for a total of 2 washes.

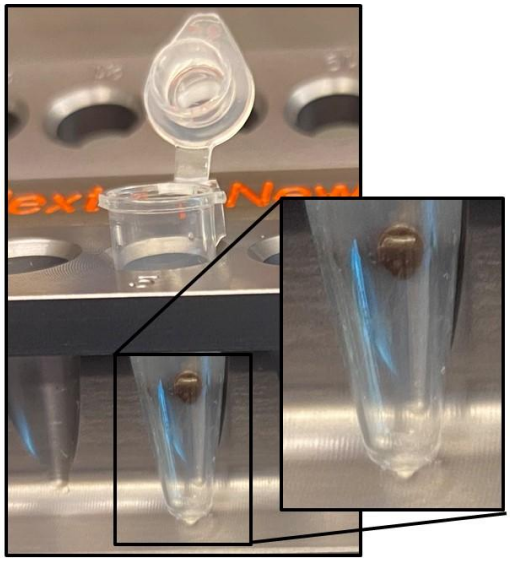
25 Completely remove residual ethanol and air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

Note

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.



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 matte

26 Remove the tube from the magnetic rack. Resuspend the RNA from the beads by adding 6.5 µL of Fragmentation Master Mix (1X). Mix thoroughly by pipetting up and down at least 10 times and briefly spin the tube. Incubate samples for 00:02:00 on bench top following elution and proceed to on-bead fragmentation.

2m

Note

The next step provides a fragmentation incubation time resulting in an RNA insert size of ~ 200 nt. For RNA insert sizes > 200 nt, see Appendix D (in the library prep kit manual on NEB.com)

27 Place in a thermal cycler, with the heated lid set at 105 °C, and run the following program: 15m

00:15:00 at 94 °C

Hold at 4 °C

28 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).

29 Collect the fragmented RNA by transferring 5 µL of the supernatant to a nuclease-free 0.2 ml PCR tube.

Note

1. **If the supernatant volume recovered is less than 5 µL for any reason, bring the volume up to 5 µL by adding additional 1X Fragmentation Master Mix and continue with the protocol.**
2. **Avoid transferring any of the magnetic beads.**

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

First Strand cDNA Synthesis 25m

31 Assemble the first strand synthesis reaction On ice by adding the following components to the fragmented and primed RNA from Step 30:

	A	B
	FIRST STRAND SYNTHESIS REACTION	VOLUME
	Fragmented and Primed RNA (Step 30)	5 µl
	• (brown) NEBNext UltraExpress Strand Specificity Reagent	4 µl
	• (lilac) NEBNext UltraExpress First Strand Enzyme Mix	1 µl
	Total Volume	10 µl

32 If processing multiple samples, prepare a master mix.

33 Mix thoroughly by pipetting up and down 10 times.

34 Place in a thermocycler, with the heated lid set at ≥ 80 °C, and run the following program: 25m

00:10:00 at 25 °C

00:10:00 at 42 °C

00:05:00 at 70 °C

Hold at 4 °C

Proceed directly to Second Strand cDNA Synthesis.

Second Strand cDNA Synthesis 43m

35 Thaw the second strand master mix On ice and mix by inverting tube 10 times, perform a quick spin to collect all liquid from the sides of the tube and place back On ice. Add the following components into the first strand synthesis reaction product from Step 34.

	A	B
	SECOND STRAND SYNTHESIS REACTION	VOLUME
	First-Strand Synthesis Product (Step 34)	10 µl
	• (orange) NEBNext UltraExpress Second Strand Master Mix	30 µl
	Total Volume	40 µl

36 Keeping the tube On ice, mix thoroughly by pipetting the reaction up and down at least 10 times.

37 Place in a thermal cycler, with the heated lid set at ≤ 40 °C (or off), and run the following program: 30m

00:30:00 at 16 °C

Hold at 4 °C

Purification of double-stranded cDNA using SPRIselect Beads or AMPure Beads.

38

Note

If using AMPure Beads, remove from 4 °C **and keep at** Room temperature **for 30 minutes prior to use.**

Vortex SPRIselect Beads or AMPure Beads to resuspend.

39 Add 72 µL (1.8X) of resuspended beads to the second strand synthesis reaction (~ 40 µL). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

40 Incubate samples on bench top for at least 00:05:00 at Room temperature . 5m

41 Briefly spin the tube in a microcentrifuge to collect any sample from 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.

Note

Caution: do not discard beads.

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

43 Repeat the previous step once for a total of 2 washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

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

Note

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.

45 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 22 µL 0.1X TE Buffer (provided) to the beads.

46 Mix well on a vortex mixer or by pipetting up and down 10 times. Incubate for at least 00:02:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 2m

47 Place the tube/plate on a magnetic stand. After 00:05:00 (or when the solution is clear), transfer 20 µL to a new PCR tube. 5m

Note

Safe Stop: If you need to stop at this point in the protocol, samples can be stored at -20 °C.

End Prep of cDNA Library 15m

48 Thaw the end prep master mix On ice. Add the following components to second strand synthesis product from Step 47.

	A	B
	END PREP REACTION	VOLUME
	Second Strand cDNA Synthesis Product (Step 47)	20 µl
	• (green) NEBNext UltraExpress End Prep Reaction Buffer	2.5 µl
	• (green) NEBNext UltraExpress End Prep Enzyme Mix	1.5 µl

49 If processing multiple samples, prepare a master mix.

50 Set a 20 µl or 100 µl pipette to 20 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.

51 Place in a thermal cycler, with the heated lid set at ≥ 75 °C, and run the following program: 15m

00:05:00 at 20 °C
00:10:00 at 65 °C
Hold at 4 °C

Proceed immediately to Adaptor Ligation.

Adaptor Ligation 20m

52 Dilute the • (red) NEBNext Adaptor* prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the diluted adaptor On ice.

	A	B
	TOTAL RNA INPUT	DILUTION REQUIRED
	25–250 ng	50-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.

Note

If customized adaptor titration per input is preferred use recommendations in Table 3.1.1. Appendix A (in the library prep kit manual on NEB.com)

53 Assemble the ligation reaction On ice by adding the following components, in the order given, to the end prep reaction product from Step 51.

	A	B
	LIGATION REACTION	VOLUME
	End Prepped DNA (Step 51)	24 µl
	Diluted Adaptor (Step 52)	2 µl
	• (red) NEBNext UltraExpress Ligation Master Mix	12 µl
	Total Volume	38 µl

Note

Do not premix the Ligation Master Mix and adaptor prior to use in the Adaptor Ligation Step.

54 Set a 100 µl pipette to 35 µl and then pipette the entire volume up and down at moderate speed at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note

Caution: NEBNext UltraExpress 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.

55 Place in a thermal cycler, with the heated lid set at ≤ 40 °C (or off), and run the following program: 15m

00:15:00 at 20 °C

Hold at 4 °C

56 Add 2 µL (• blue) NEBNext UltraExpress USER Enzyme to the ligation mixture from Step 55, resulting in total volume of 40 µL. Mix well by gently pipetting up and down 10 times at 35 µL volume.

57 Place in a thermal cycler, with the heated lid set at ≥ 45 °C, and run the following program: 5m

37 °C for 00:05:00
Hold at 4 °C

Proceed immediately to PCR Enrichment of Adaptor Ligated DNA.

PCR Enrichment of Adaptor Ligated DNA

58 **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 (i5and i7) primers combined. Primers are supplied at 10 µM combined (5 µM each).

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

59.1 **Option A:**

Forward and Reverse Primers Separate

	A	B
	COMPONENT	VOLUME PER ONE LIBRARY
	Adaptor Ligated DNA (Step 57)	40 µl
	• (blue) NEBNext MSTC High Yield Master Mix	50 µl
	Index (X) Primer/i7 Primer*, **	5 µl
	Universal PCR Primer/i5 Primer*,**	5 µl
	Total Volume	100 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

Note

Skip the next step if you are choosing this step.

59.2 **Option B:**

Forward and Reverse Primers Combined

	A	B
	COMPONENT	VOLUME PER ONE LIBRARY
	Adaptor Ligated DNA (Step 57)	40 µl
	• (blue) NEBNext MSTC High Yield Master Mix	50 µl
	Index Primer Mix*	10 µl
	Total Volume	100 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.

Note

Skip 59.2 if choosing 59.1

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

61 Place the tube on a thermal cycler with the heated lid set to 105 °C and perform PCR amplification using the following PCR cycling conditions (refer to Table 61):

Table 61:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	11**
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

** PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality. If using customized cycling conditions per input is preferred, use recommendations in Table 3.2.2 in Appendix A (in the library prep kit manual on NEB.com).

Note

Take out NEBNext Bead Reconstitution Buffer and bring up to Room temperature prior to Phased Bead Cleanup. Allow the buffer (and beads if using AMPure XP) to warm to Room temperature for at least 30 minutes before use.

Phased Bead Cleanup of PCR Reaction

5m

62

Note

Note: The SPRIselect/AMPure Bead ratio recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g. post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure Beads, remove from 4 °C and keep at Room temperature for 30 minutes prior to use.

Phased Cleanups for NEBNext UltraExpress Kits | NEB

63 Vortex SPRIselect Beads or AMPure Beads to resuspend.

64 Add 70 µL (0.7X) of resuspended beads to the PCR reaction (~ 100 µL). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

65 Incubate samples on bench top for at least 00:05:00 at Room temperature. 5m

66 Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

67 After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. 5m

Note

Caution: do not discard the beads

68 Remove the tube/plate from the magnetic stand (**Note: do not need ethanol wash at this step).** Add 50 µL 0.1 X TE and resuspend the beads by pipetting. Add another 40 µL (0.8X) NEBNext Bead Reconstitution Buffer to the 50 µL resuspended beads. Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortex for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

69 Incubate samples on bench top for at least 00:05:00 at Room temperature. 5m

70 Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

71 After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. 5m

Note

Caution: do not discard the beads

72 Add 200 µL of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. 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 DNA targets. 30s

73 Repeat the previous step once for a total of 2 washes.
Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

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

Note

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.

75 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 23 µL 0.1X TE (provided) to the beads.

76 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 2m

77 Place the tube/plate on the magnetic stand. After 00:05:00 (or when the solution is clear), transfer 20 µL to a new PCR tube or plate and store at -20 °C. 5m

Assess Library Quality on an Agilent Bioanalyzer DNA Chip

78 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.

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

Note

If a peak at ~ 80 bp (primers) or 150 bp (adaptor-dimer) is visible in the traces, bring up the sample volume (from Step 77) to 50 µL with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step using 0.9X (45 µl) standard bead clean-up.

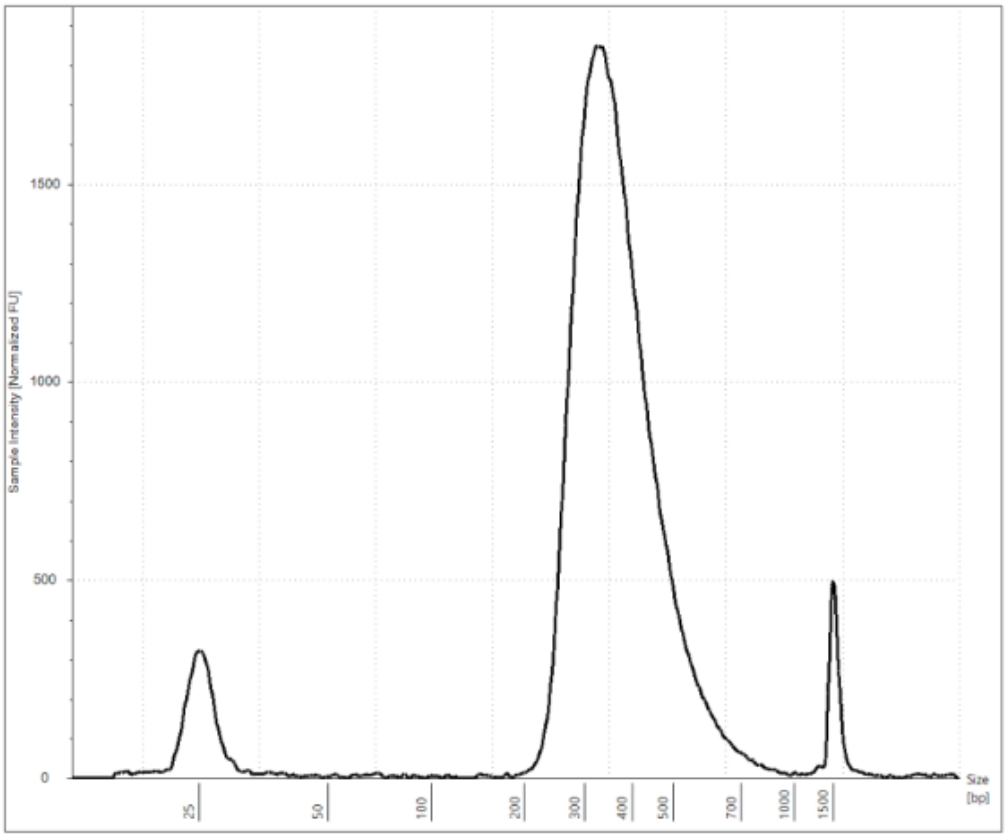


Figure 79. Example of library made using 250 ng of UHRR on a TapeStation.