INSTRUCTION MANUAL

NEBNext® Low-bias Small RNA Library Prep Kit

NEB #E3420S/L

24/96 reactions

Version 2.0_5/26

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The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E3420S) or 96 reactions (NEB #E3420L). The NEBNext Sample Purification Beads should be stored at room temperature and all other reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

Package 1: Store at -20°C.

- (green) NEBNext LB Ligation Reaction Buffer
- (green) NEBNext LB 3′ Adaptor
- (green) NEBNext LB 3′ Ligase
- (lilac) NEBNext LB Adaptor Removal Enzyme Mix
- (red) 10 mM ATP
- (red) NEBNext LB 5′ Adaptor
- (red) NEBNext LB 5′ Ligase
- (red) NEBNext LB Ligation Enhancer
- (yellow) NEBNext LB RT Reaction Buffer
- (yellow) NEBNext LB RT Enzyme Mix
- (yellow) 100 mM DTT
- (blue) NEBNext Ultra™ II Q5® Master Mix
- (white) NEBNext LB Adaptor Dilution Buffer
- (white) 0.1X TE

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

NEBNext Sample Purification Beads

Required Materials Not Included

- NEBNext LV Unique Dual Index Primer Pairs (Select from Sets 1-5; see www.neb.com/oligos for available options)
- Nuclease-free Water
- 0.2 ml PCR tube, such as TempAssure 0.2 ml PCR Flex-Free 8-Tube Strips (USA Scientific®, #1402-4708)
- Isopropanol, such as Isopropyl Alcohol 99% (Pharmco, #231HPLC99)
- 80% Ethanol (freshly prepared)
- Magnetic rack (NEB #S1515S) or magnetic plate (Alpaqua®, #A001322) or equivalent
- Thermal Cycler
- Microcentrifuge
- Vortex Mixer
- Metal cooling block (e.g., Diversified Biotech®, #CHAM-1000)
- Agilent® TapeStation®, Bioanalyzer®, or other fragment analyzer and associated reagents and consumables

Overview

The NEBNext Low-bias Small RNA Library Prep Kit provides the necessary enzymes and buffers to make high-quality small RNA Illumina® libraries using 1,000–0.5 ng of total RNA or 5–0.05 ng of enriched small RNA. The streamlined workflow can be completed in ~3.5 hours and does not require gel purification (Figure 1). Libraries are amplified using a unique dual index (UDI) scheme, providing compatibility with Illumina sequencers that use patterned flow cells — platforms where index hopping may occur. Multiplexing is supported with up to 480 UDI primer pairs, available separately. NEB recommends the NEBNext LV Unique Dual Index Primer Pairs, Sets 1-5 (NEB #E3400, E3402, E3404, E3406, E3408) each include 96 primer pairs, while Sets 2A and 2B (NEB #E3390, E3392) each include 24 primer pairs. Sets 2A and 2B are subsets of Set 2 and, therefore, care should be taken not to duplicate primer pairs in the same sequencing run. Please refer to www.neb.com/oligos for additional information.

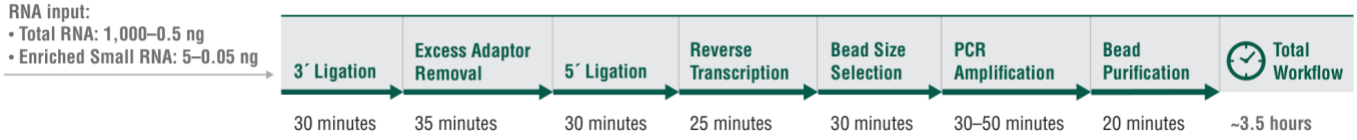
The NEBNext Low-bias Small RNA Library Prep Kit minimizes biased representation of small RNA species, more accurately reflecting the number and proportion of unique small RNAs present. It does this via a randomized splint ligation-based approach. The kit captures small RNAs (< 120 nt) with a 5′ phosphate and 3′ hydroxyl group, including microRNAs (miRNAs), tRNAs and tRNA-derived fragments, small nucleolar RNAs (snoRNAs), and other small RNAs. Additionally, this kit robustly captures 2'-O-methylated (2'-O-Me) small RNAs, such as piwi-interacting RNAs (piRNAs) and plant miRNAs, without any protocol modifications.

The NEBNext Low-bias Small RNA Library Prep Kit allows for both intact total RNA inputs and enriched small RNA inputs. For a sample with most of its input mass compatible with the ligation chemistry (5′ phosphate and 3′ hydroxyl group) and within the size range for capture with this kit (< 120 nt), please follow the enriched small RNA recommendations. Examples of input material that should be treated as enriched small RNA include isolated endogenous small RNAs, ribosome-protected fragments that have been properly end repaired (Ribo-seq), total RNA that has been fragmented within size range and end repaired, and appropriately sized synthetic RNAs (e.g. guide RNAs).

Each kit component must pass rigorous quality standards and, for each new lot, the entire set of reagents is functionally validated together by construction and sequencing of an indexed library on the Illumina sequencing platform.

Where larger volumes, customized or bulk packaging are required, we encourage consultation with the Customized Solutions team at NEB. Please complete the NEB Custom Contact Form at www.neb.com/CustomContactForm to learn more.

Figure 1. NEBNext Low-bias Small RNA Library Prep Kit Workflow



Library construction with the NEBNext Low-bias Small RNA Library Prep Kit is robust and streamlined and can be completed in less than 4 hours. Initially, a 3′ adaptor is ligated to the RNA followed by removal of excess adaptor molecules. Next, two reactions happen simultaneously: the 5′ adaptor is ligated and the previously ligated 3′ adaptor is enzymatically modified to create a primer for reverse transcription. cDNA is then generated, and a bead-based size selection ensures removal of non-ligated adaptor molecules and larger cDNA molecules. Libraries are amplified using the NEBNext LV Unique Dual Index Primer Pairs and are bead purified prior to sequencing on an Illumina platform. Two different bead purification approaches can be used, as described in Step 8. Users choose between a bead-based size selection that leads to a more focused miRNA enrichment or a bead-based cleanup that results in a more generalized small RNA library prep.

Protocol

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 or more paths leading to the same end point, but is dependent on a user variable, like the type of input small RNA.

●

Colored bullets indicate the cap color of the reagent to be added to a reaction

Starting Material: 1,000–0.5 ng of total RNA or 5–0.05 ng of enriched small RNA.

Note: Small RNA fragments must have 5′ monophosphate and 3′ hydroxyl ends. Follow enriched small RNA recommendations if most of the input mass is within the size range (< 120 nt) and has compatible ends. Examples include isolated small RNAs, end-repaired ribosome protected fragments (Ribo-seq), fragmented and end-repaired total RNAs, and synthetic RNAs (e.g. guide RNAs).

This protocol was developed using human brain total RNA (RIN ~7). Further optimization of adaptor dilutions and PCR cycles may be required for other sample types and applications. Samples with lower RIN values can be used to generate small RNA libraries, however any RNA fragments less than ~120 nt with 5′ monophosphate and 3′ hydroxyl ends will also be captured in those samples.

Protocol Tips

- Keep RNA on ice.
- Pipette RNA carefully to avoid potential degradation of RNA.
- We recommend setting up reactions in PCR strip tubes, making use of multichannel pipettes more convenient when processing multiple samples.
- To ensure even temperature distribution between samples, especially while working with RNA on ice, we recommend setting up reactions on a metal cooling block (e.g., Diversified Biotech, #CHAM-1000).
- The ● (green) NEBNext LB Ligation Reaction Buffer is very viscous. Thawing and handling at room temperature can facilitate dispensing. Mix the ● (green) NEBNext LB Ligation Reaction Buffer by vortex or pipette thoroughly prior to use.
- Adaptor dilutions may be necessary based on input. The adaptor dilutions provided are recommendations and a good starting point but can be further optimized based on specific samples and inputs.
- Vortex times listed throughout the protocol have been tested and recommended to ensure proper mixing of all reactions. All vortex steps should be done at ~3,000 rpm or on high setting.
- Master mixes can be prepared for most reaction steps in the protocol. Best practice is to add components to a master mix in the order listed in the table. **Do not add adaptors to any master mixes**. When preparing master mixes for adaptor ligation steps, first add the master mix to the sample, then add the adaptor.
- We recommend pre-wetting tips when adding the isopropanol in Step 6.9.
- Ensure there are no beads carried over after bead purification steps. If beads are present in sample following a bead purification step, we recommend holding the sample against a magnet and transferring the solution to a new tube.
- The NEBNext LV Unique Dual Index Primer Pairs (NEB #E3390, #E3392, #E3400, #E3402, #E3404, #E3406, #E3408) must be purchased separately to the NEBNext Low-bias Small RNA Library Prep Kit. There are 480 unique dual indexes. Refer to www.neb.com/oligos for additional information.

1. RNA Denaturation

1.1. Dilute the input RNA sample in nuclease-free water to a final volume of 8 µl in a nuclease-free 0.2 ml PCR tube and keep on ice.

1.2. Mix gently by pipette. Centrifuge briefly to collect all liquid from sides of the tube.

1.3. Incubate sample in a pre-heated thermal cycler with heated lid set to 80°C as follows:
2 minutes at 70°C
Hold at 4°C

1.4. Immediately transfer tubes to ice. Allow samples to cool for at least 2 minutes.

2. NEBNext LB 3′ Adaptor Ligation

2.1. Determine if adaptor dilution is necessary.

If total RNA input is < 100 ng or enriched small RNA input is < 5 ng, dilute the • (green) NEBNext LB 3′ Adaptor in the provided ∘ (white) NEBNext LB Adaptor Dilution Buffer according to Table 2.1 or Table 2.2 respectively. For example, to make a 10-fold dilution, combine 1 µl of the • (green) NEBNext LB 3′ Adaptor and 9 µl of the ∘ (white) NEBNext LB Adaptor Dilution Buffer, scale as needed. Place the diluted adaptor on ice. These adaptor dilutions are guidelines and a good starting point but may need further optimization based on specific samples and inputs.

Note: Each small RNA library prep reaction requires 1 µl of • (green) NEBNext LB 3′ Adaptor (undiluted or diluted).

Table 2.1: Total RNA

TOTAL RNA INPUT (ng)	ADAPTOR DILUTION (VOLUME OF ADAPTOR:TOTAL VOLUME)
1,000–100 ng	No dilution
99–10 ng	10-fold (1:10)
9–0.5 ng	30-fold (1:30)

Table 2.2: Enriched small RNA

ENRICHED SMALL RNA INPUT (ng)	ADAPTOR DILUTION (VOLUME OF ADAPTOR:TOTAL VOLUME)
5 ng	No dilution
0.5 ng	10-fold (1:10)
0.05 ng	30-fold (1:30)

Note: Adaptor dilutions for enriched small RNA samples are based on testing equivalence to human brain total RNA where the small RNA fraction (< 200 nt) is assumed to comprise ~ 5% of total RNA. Small RNAs enriched through different methods or from different samples may require further optimizations.

2.2. On ice, add the following components directly to the Denatured RNA:

COMPONENT	VOLUME
Denatured RNA (Step 1.4.)	8 µl
• (green) NEBNext LB 3′ Adaptor	1 µl
• (green) NEBNext LB Ligation Reaction Buffer*	10 µl
• (green) NEBNext LB 3′ Ligase	1 µl
Total Volume	20 µl

* The NEBNext LB Ligation Reaction Buffer is very viscous. Thawing and handling at room temperature facilitates dispensing. Mix the NEBNext LB Ligation Buffer by vortex or pipette prior to use.

- The • (green) NEBNext LB Ligation Reaction Buffer and • (green) NEBNext LB 3′ Ligase can be mixed ahead of time as a master mix.
- Do not premix the • (green) NEBNext LB 3′ Adaptor with either the • (green) NEBNext LB Ligation Reaction Buffer or • (green) NEBNext LB 3′ Ligase.
- If making a master mix, first add the master mix to the sample then add the adaptor.

2.3. Vortex to mix the sample for 5–10 seconds. Alternatively, set a 20 µl pipette to 14 µl and pipette up and down at least 10 times to mix thoroughly. Centrifuge briefly to collect all liquid from the sides of the tube. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency.

2.4. Incubate the sample in a thermal cycler with the heated lid off as follows:
30 minutes at 25°C
Hold at 4°C

3. Excess NEBNext LB 3′ Adaptor Removal

3.1. Determine if NEBNext Adaptor Removal Enzyme Mix dilution is necessary.

If total RNA input is < 100 ng or enriched small RNA input is < 1 ng, dilute the ● (lilac) NEBNext Adaptor Removal Enzyme Mix in nuclease free water according to Table 3.1 or Table 3.2 respectively. To make a 4-fold dilution, combine 1 µl of the ● (lilac) NEBNext Adaptor Removal Enzyme Mix and 3 µl of nuclease free water, scale as needed. Place the diluted Adaptor Removal Enzyme Mix on ice. Once used, do not store the diluted enzyme.Discard after use.

Note: Each small RNA library prep reaction requires 1 µl of ● (lilac) NEBNext Adaptor Removal Enzyme Mix *(undiluted or diluted).*

Table 3.1: Total RNA

TOTAL RNA INPUT (ng)	ADAPTOR REMOVAL ENZYME MIX DILUTION (VOLUME OF ENZYME : TOTAL VOLUME)
1,000–100 ng	No dilution
99–0.5 ng	4-fold (1:4)

Table 3.2: Enriched Small RNA

ENRICHED SMALL RNA INPUT (ng)	ADAPTOR REMOVAL ENZYME MIX DILUTION (VOLUME OF ENZYME : TOTAL VOLUME)
5–1 ng	No dilution
1–0.05 ng	4-fold (1:4)

3.2 On ice, add the following components directly to the LB 3′ Adaptor Ligated RNA.

COMPONENT	VOLUME
LB 3′ Adaptor Ligated RNA (Step 2.4.)	20 µl
● (lilac) NEBNext Adaptor Removal Enzyme Mix	1 µl
Total Volume	21 µl

3.3. Vortex to mix the sample 5–10 seconds. Alternatively, set a 20 µl pipette to 14 µl and pipette up and down at least 10 times to mix thoroughly. Centrifuge briefly to collect all liquid from the sides of the tube.

3.4. Incubate the sample in a preheated thermal cycler with the heated lid set to 85°C as follows:
15 minutes at 30°C
5 minutes at 75°C
Hold at 4°C

4. NEBNext LB 5′ Adaptor Ligation

4.1. Determine if adaptor dilution is necessary.

If total RNA input is < 100 ng or enriched small RNA input is < 5 ng, dilute the ● (red) NEBNext LB 5′ Adaptor in the provided ○ (white) NEBNext LB Adaptor Dilution Buffer according to Table 4.1 or Table 4.2, respectively. For example, to make a 10-fold dilution, combine 1 µl of the ● (red) NEBNext LB 5′ Adaptor and 9 µl of the ○ (white) NEBNext LB Adaptor Dilution Buffer, scale as needed. Place the diluted adaptor on ice. These adaptor dilutions are guidelines and a good starting point but may need further optimization based on specific samples and inputs.

Note: Each small RNA library prep reaction requires ***2 µl*** *of* ● *(red) NEBNext LB 5′ Adaptor (undiluted or diluted).*

Table 4.1: Total RNA

TOTAL RNA INPUT (ng)	ADAPTOR DILUTION (VOLUME OF ADAPTOR: TOTAL VOLUME)
1,000–100 ng	No dilution
99–10 ng	10-fold (1:10)
9–0.5 ng	30-fold (1:30)

Table 4.2: Enriched small RNA

ENRICHED SMALL RNA INPUT (ng)	ADAPTOR DILUTION (VOLUME OF ADAPTOR: TOTAL VOLUME)
5 ng	No dilution
0.5 ng	10-fold (1:10)
0.05 ng	30-fold (1:30)

Note: Adaptor dilutions for enriched small RNA samples are based on testing equivalence to human brain total RNA where the small RNA fraction (< 200 nt) is assumed to comprise ~ 5% of total RNA. Small RNAs enriched through different methods or from different samples may require further optimizations based on application.

4.2. On ice, add the following components directly to the Excess Adaptor Removed RNA:

COMPONENT	VOLUME
Excess Adaptor Removed RNA (Step 3.3.)	21 µl
• (red) NEBNext LB 5′ Adaptor	2 µl
• (red) 10 mM ATP	3 µl
• (red) NEBNext LB Ligation Enhancer	3 µl
• (red) NEBNext LB 5′ Ligase	2 µl
Total Volume	31 µl

- The • (red) 10 mM ATP, • (red) NEBNext LB Ligation Enhancer, and • (red) NEBNext LB 5′ Ligase can be mixed ahead of time as a master mix.
- Do not premix the • (red) NEBNext LB 5′ Adaptor with the • (red) 10 mM ATP, • (red) NEBNext LB Ligation Enhancer, or • (red) NEBNext LB 5′ Ligase.
- If making a master mix, first add the master mix to the sample then add the adaptor.

4.3. Vortex to mix the sample for 5–10 seconds. Alternatively, set a 100 µl or 200 µl pipette to 22 µl and pipette up and down at least 10 times to mix thoroughly. Centrifuge briefly to collect all liquid from the sides of the tube.

4.4. Incubate sample in a preheated thermal cycler with the heated lid set to 47°C as follows:
30 minutes at 37°C
Hold at 4°C

5. Reverse Transcription

5.1. On ice, add the following components directly to the 5′ and 3′ Adaptor Ligated RNA:

COMPONENT	VOLUME
5′ and 3′ Adaptor Ligated RNA (Step 4.4.)	31 µl
◦ (yellow) NEBNext LB RT Reaction Buffer	15 µl
◦ (yellow) 100 mM DTT	5 µl
◦ (yellow) NEBNext LB RT Enzyme Mix	2 µl
Total Volume	53 µl

- The ◦ (yellow) NEBNext LB RT Reaction Buffer, ◦ (yellow) 100 mM DTT, and ◦ (yellow) NEBNext LB RT Enzyme Mix can be mixed ahead of time as a master mix.

5.2. Vortex to mix the sample 5–10 seconds. Alternatively, set a 100 µl or 200 µl pipette to 40 µl and pipette up and down at least 10 times to mix thoroughly. Centrifuge briefly to collect all liquid from the sides of the tube.

5.3. Incubate sample in a preheated thermal cycler with the heated lid set to 80°C as follows:
15 minutes at 42°C
10 minutes at 70°C
Hold at 4°C

Safe Stopping Point: Samples can be stored overnight at -20°C.

6. Bead Size Selection of Reverse Transcription Reaction

The ratios recommended for NEBNext Sample Purification Beads 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 reverse transcription recommendations will not apply to samples post PCR). Please adhere to these guidelines and not those recommended by other sources or for other kits.

6.1. Vortex NEBNext Sample Purification Beads to resuspend.

6.2. Add 53 µl (1X) of resuspended NEBNext Sample Purification Beads to each sample. Vortex to mix the sample 5–10 seconds or pipette up and down at least 10 times. If pipetting, be careful to expel all the liquid from the tip during the last mix.

6.3. Incubate samples on bench top for 5 minutes at room temperature.

6.4. Perform a quick spin to collect all liquid from the sides of the tube.

6.5. Place the tubes against an appropriate magnetic rack to separate the beads from the supernatant.

6.6. After 5 minutes (or when the solution is clear), carefully transfer the supernatant containing the cDNA into a new PCR tube (**Caution: do not discard the supernatant**). Discard the beads that contain the unwanted large fragments.

6.7. Add 53 µl (1X) of resuspended NEBNext Sample Purification Beads to the transferred supernatant. Vortex to mix the sample 5–10 seconds or pipette up and down at least 10 times. If pipetting, be careful to expel all the liquid from the tip during the last mix.

6.8. Perform a quick spin to collect all liquid from the sides of the tube.

6.9. Add 40 µl of isopropanol and vortex to mix the sample 5–10 seconds or pipette up and down at least 10 times. If pipetting, be careful to expel all the liquid from the tip during the last mix.

6.10. Perform a quick spin to collect all liquid from the sides of the tube.

6.11. Incubate samples on bench top for 10 minutes at room temperature.

6.12. Place the tubes against an appropriate magnetic rack to separate the beads from the supernatant.

6.13. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA **(Caution: do not discard the beads)**.

6.14. Add 200 µl of freshly prepared 80% ethanol to the tubes while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA.

6.15. Repeat the ethanol wash for a total of two washes. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

6.16. Air dry the beads for up to 2 minutes while the tubes are 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.

6.17. Remove the tubes from the magnetic rack. Elute the cDNA from the beads by adding 22 µl of 0.1X TE.

6.18. Vortex to mix the sample 5–10 seconds or pipette up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, centrifuge briefly to collect the liquid from the sides of the tube before placing back on the magnetic rack.

6.19. Place the tube on the magnetic rack. After 3 minutes (or whenever the solution is clear), transfer 20 µl of the supernatant to a new PCR tube.

Safe Stopping Point: Samples can be stored overnight at -20°C.

7. PCR Amplification

7.1. On ice, add the following components directly to the size-selected cDNA:

COMPONENT	VOLUME
cDNA (step 6.19.)	20 µl
NEBNext LV UDI Primer Pair**	5 µl
• (blue) NEBNext Ultra II Q5 PCR Master Mix	25 µl
Total Volume	50 µl

** NEBNext LV Unique Dual Index Primers (NEB #E3390, #E3392, #E3400, #E3402, #E3404, #E3406, #E3408) must be purchased separately to the library prep kit. Refer to www.neb.com/oligos for additional information.

7.2. Vortex to mix the sample for 5–10 seconds. Alternatively, set a 100 µl or 200 µl pipette to 40 µl and pipette up and down at least 10 times to mix thoroughly. Centrifuge briefly to collect all liquid from the sides of the tube.

7.3. Place the sample in a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	9-22* For specific guidance, based on input type, see Table 7.1 or 7.2
Annealing	62°C	30 seconds	
Extension	72°C	30 seconds	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be chosen based on input amount and sample type. Cycle number should be high enough to provide sufficient library yield, but low enough to minimize PCR artifacts and over-cycling.

Refer to the tables below for suggested cycling conditions:

- Table 7.1 – Total RNA (Human brain total RNA)
- Table 7.2 – Enriched small RNA (Human brain small RNA fraction)

Note: These suggested amplification conditions can vary based on RNA input amount, tissue, and species. PCR cycle number may need to be re-optimized for your sample of interest.

Table 7.1: Total RNA PCR Cycle Recommendations

TOTAL RNA INPUT (ng)	PCR CYCLE RECOMMENDATION
1,000 ng	9 – 11
500 ng	10 – 12
100 ng	11 – 13
10 ng	15 – 17
1 ng	19 – 21
0.5 ng	20 – 22

Table 7.2: Enriched Small RNA PCR Cycles Recommendations

ENRICHED SMALL RNA INPUT (ng)	PCR CYCLE RECOMMENDATION
5 ng	11 – 13
0.5 ng	15 – 17
0.05 ng	19 – 21

Note: PCR cycle recommendations for enriched small RNA samples are based on testing equivalence to human brain total RNA where the small RNA fraction (< 200 nt) is assumed to comprise ~ 5% of total RNA. Small RNAs enriched through different methods or from different samples may require further optimizations based on application.

Safe Stopping Point: Samples can be stored overnight at -20°C.

8. Bead Purification of PCR Amplification Reaction

There are two potential post-PCR sample-bead purification strategies. Choose the option that aligns closest with the types of small RNA that you are interested in.

- **Section 8A. Bead Size Selection of PCR Amplification Reaction:** for capture of libraries with the shortest small RNA species (< 50 nt), including miRNA (size 18–22 nt), piRNA (size 24–33 nt), and tRNA-derived fragments (10–50 nt).
- **Section 8B. Bead Cleanup of PCR Amplification Reaction Without Size Selection:** for capture of libraries with a diverse range of small RNA species (< 120 nt).

8A. Bead Size Selection of PCR Amplification Reaction

The ratios recommended for NEBNext Sample Purification Beads 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 reverse transcription recommendations will not apply to samples post PCR). Please adhere to these guidelines and not those recommended by other sources or for other kits.

Note: This bead size selection has been optimized for recovery of a final library with primarily miRNAs (size 18–22 nt), piRNA (size 24–33 nt), and tRNA-derived fragments (10–50 nt) as inserts from human brain total RNA.

8A.1. Vortex the NEBNext Sample Purification Beads to resuspend.

8A.2. Add 60 µl (1.2X) of resuspended NEBNext Sample Purification Beads to each sample. Vortex to mix the sample 5–10 seconds or pipette up and down at least 10 times. If pipetting, be careful to expel all the liquid from the tip during the last mix.

8A.3. Incubate the samples on the bench top for 5 minutes at room temperature.

8A.4. Perform a quick spin to collect all liquid from the sides of the tube.

8A.5. Place the tubes against an appropriate magnetic rack to separate the beads from the supernatant.

8A.6. After 5 minutes (or when the solution is clear), carefully transfer supernatant containing the final library into a new tube (**Caution: do not discard the supernatant**). Discard the beads that contain the unwanted large fragments.

8A.7. Add 40 µl (0.8X) of resuspended NEBNext Sample Purification Beads to the transferred supernatant. Vortex to mix the sample 5–10 seconds or pipette up and down at least 10 times. If pipetting, be careful to expel all the liquid from the tip during the last mix.

8A.8. Incubate samples on bench top for 5 minutes at room temperature.

8A.9. Perform a quick spin to collect all liquid from the sides of the tube.

8A.10. Place the tubes against an appropriate magnetic rack to separate the beads from the supernatant.

8A.11. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant **(Caution: do not discard the beads)**.

8A.12. Add 200 µl of freshly prepared 80% ethanol to the tubes while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads.

8A.13. Repeat the ethanol wash for a total of two washes. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

8A.14. Air dry the beads for up to 2 minutes while the tubes are 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.

8A.15. Remove the tubes from the magnetic rack. Elute the DNA library from the beads by adding 17 µl of 0.1X TE.

8A.16. Vortex to mix the sample 5–10 seconds or pipette up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, centrifuge briefly to collect the liquid from the sides of the tube before placing back on the magnetic rack.

8A.17. Place the tube on the magnetic rack. After 3 minutes (or whenever the solution is clear), transfer 15 µl of the supernatant to a new PCR tube. This is your final library.

Safe Stopping Point: Final libraries can be stored at -20°C.

8B. Bead Cleanup of PCR Amplification Reaction Without Size Selection

The ratios recommended for NEBNext Sample Purification Beads 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 reverse transcription recommendations will not apply to samples post PCR). Please adhere to these guidelines and not those recommended by other sources or for other kits.

Note: This bead cleanup has been optimized for the recovery of a final library with all small RNA (< 120 nt) from human brain total RNA.

8B.1. Vortex the NEBNext Sample Purification Beads to resuspend.

8B.2. Add 100 µl (2X) of resuspended NEBNext Sample Purification Beads to each sample. Vortex to mix the sample 5–10 seconds or pipette up and down at least 10 times. If pipetting, be careful to expel all the liquid from the tip during the last mix.

8B.3. Incubate the samples on the bench top for 5 minutes at room temperature.

8B.4. Perform a quick spin to collect all liquid from the sides of the tube.

8B.5. Place the tubes against an appropriate magnetic rack to separate the beads from the supernatant.

8B.6. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant **(Caution: do not discard the beads)**.

8B.7. Add 200 µl of freshly prepared 80% ethanol to the tubes while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads.

8B.8. Repeat the ethanol wash for a total of two washes. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

8B.9. Air dry the beads for up to 2 minutes while the tubes are 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.

8B.10. Remove the tubes from the magnetic rack. Elute the DNA library from the beads by adding 17 µl of 0.1X TE.

8B.11. Vortex to mix the sample for 5–10 seconds or pipette up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, centrifuge briefly to collect the liquid from the sides of the tube before placing back on the magnetic rack.

8B.12. Place the tube on the magnetic rack. After 3 minutes (or whenever the solution is clear), transfer 15 µl of the supernatant to a new PCR tube. This is your final library.

Safe Stopping Point: Final libraries can be stored at -20°C.

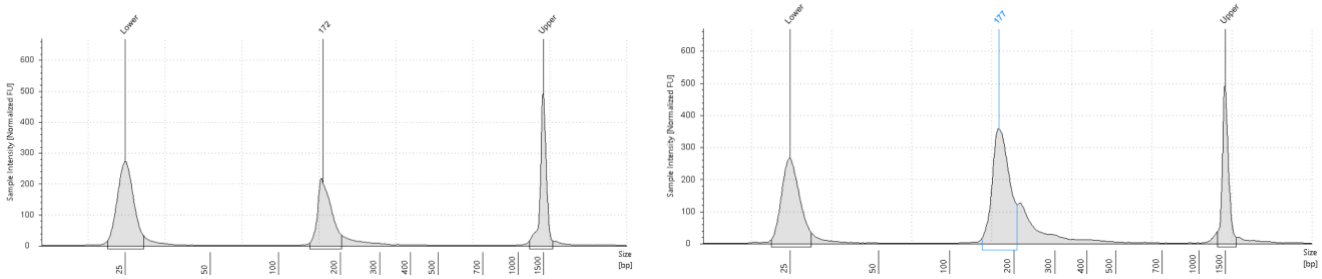
9. Library Quantification

Determine the size distribution and concentration of the libraries, for example, use an Agilent TapeStation or Bioanalyzer. A typical low-bias small RNA library would have the following TapeStation traces on the High Sensitivity D1000 ScreenTape®.

Note: Final libraries may need to be diluted to be within the quantitative range of the analysis system.

Figure 2. Final Library Profiles for Size Selection and Cleanup

A **B**



Representative TapeStation traces of libraries generated using the NEBNext Low-bias Small RNA Library Prep Kit with 500 ng input of human brain total RNA run on a HS D1000 tape. (A) Library generated using the miRNA focused bead-based size selection (Section 8A). (B) Library generated using the more generic small RNA, bead-based cleanup approach (Section 8B). Note the difference in the profiles with A demonstrating a more defined miRNA peak, whereas B displays a profile that encompasses miRNA peak and longer small RNAs.

Sequencing and Data Processing

This kit generates libraries compatible with Illumina instruments. A single 56-base read is typically sufficient to sequence small RNA libraries, however longer read lengths and paired-end reads can also be used.

The NEBNext Low-bias Small RNA libraries can be trimmed like standard TruSeq® libraries (not TruSeq Small RNA libraries):

Read 1: AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

Read 2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Note: Other vendors may use different adaptor sequences for small RNA libraries.

Kit Components

NEB #E3420S Table of Components

NEB #	PRODUCT	VOLUME
E3417AVIAL	NEBNext LB Ligation Reaction Buffer	0.24 ml
E3418AVIAL	NEBNext LB 3′ Adaptor	0.024 ml
E3419AVIAL	NEBNext LB Adaptor Dilution Buffer	0.72 ml
E3421AVIAL	NEBNext LB 3′ Ligase	0.024 ml
E3422AVIAL	NEBNext LB Adaptor Removal Enzyme Mix	0.024 ml
E3423AVIAL	NEBNext LB Ligation Enhancer	0.072 ml
E3424AVIAL	NEBNext LB 5′ Adaptor	0.048 ml
E3425AVIAL	10mM ATP	0.072 ml
E3426AVIAL	NEBNext LB 5′ Ligase	0.048 ml
E3427AVIAL	NEBNext LB RT Reaction Buffer	0.36 ml
E3428AVIAL	NEBNext LB RT Enzyme Mix	0.048 ml
E7139AAVIAL	100mM DTT	0.5 ml
E3429AVIAL	NEBNext Sample Purification Beads	4.95 ml
E7657AVIAL	0.1X TE	1.3 ml
E7649AVIAL	NEBNext Ultra II Q5 Master Mix	0.6 ml

NEB #E3420L Table of Components

NEB #	PRODUCT	VOLUME
E3417AAVIAL	NEBNext LB Ligation Reaction Buffer	0.96 ml
E3418AAVIAL	NEBNext LB 3′ Adaptor	0.096 ml
E3419AAVIAL	NEBNext LB Adaptor Dilution Buffer	2.88 ml
E3421AAVIAL	NEBNext LB 3′ Ligase	0.096 ml
E3422AAVIAL	NEBNext LB Adaptor Removal Enzyme Mix	0.096 ml
E3423AAVIAL	NEBNext LB Ligation Enhancer	0.288 ml
E3424AAVIAL	NEBNext LB 5′ Adaptor	0.192 ml
E3425AAVIAL	10mM ATP	0.288 ml
E3426AAVIAL	NEBNext LB 5′ Ligase	0.192 ml
E3427AAVIAL	NEBNext LB RT Reaction Buffer	1.44 ml
E3428AAVIAL	NEBNext LB RT Enzyme Mix	0.192 ml
E7139AAVIAL	100mM DTT	0.5 ml
E3429AAVIAL	NEBNext Sample Purification Beads	19.8 ml
E7657AAVIAL	0.1X TE	5.2 ml
E7649AAVIAL	NEBNext Ultra II Q5 Master Mix	1.2 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	6/25
2.0	Workflow updates; clarified definition of “enriched small RNA”	6/26

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