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NEXTFLEX® Small RNA-Seq Kit v3

(Illumina® Compatible)

Catalog #NOVA-5132-05 (8 reactions)

GEL-FREE & LOW INPUT OPTIONS

 V19.01

This product is for research use only.
Not for use in diagnostic procedures.

NEXTFLEX® Small RNA-Seq Kit v3 - 5132-05

Product Overview

The NEXTFLEX® Small RNA-Seq Kit v3 can be used to prepare small RNA libraries from total RNA or purified small RNA. The NEXTFLEX® Small RNA-Seq Kit v3 is designed to greatly reduce formation of adapter-dimer product in small RNA-seq library preparation, allowing completely gel-free library preparation from typical input amounts, or allowing libraries to be created from low input amounts with a PAGE-based size selection of the final library. This kit utilizes patent-pending adapters with randomized ends to greatly reduce sequence bias in small RNA sequencing library construction (1), allowing more accurate identification and quantification of microRNAs, piRNAs, and other small RNAs.

This manual includes protocols for size selection using a gel-free (Step H1) or PAGE-based (Step H2) method. The gel-free protocol described in this manual is recommended for library preparations where a sufficient amount of ~150 bp product and no ~130 bp adapter-dimer product is seen after 18 cycles or fewer of PCR, which is typically achieved when using 200 ng - 2 µg of total RNA starting material. For lower input amounts or samples with low small RNA content, libraries may be successfully created by using up to 25 cycles of PCR; however, size selection by PAGE will likely be necessary due to the presence of adapter-dimer product in the post-PCR sample. PCR introduces very little bias into small RNA sequencing libraries (1,2), so reliable data can be generated with up to 25 cycles of PCR.

Kit Overview

This kit can be used to generate 8 small RNA libraries. The kit contains the necessary reagents to process the user's purified RNA sample through library preparation.

Contents, Storage and Shelf Life

The NEXTFLEX® Small RNA-Seq Kit v3 contains enough material to prepare 8 RNA samples for Illumina-compatible next-generation sequencing. The shelf life of all reagents is 6 months when stored properly. All components can safely be stored at -20°C, except: NEXTFLEX® Adapter Depletion Solution, Resuspension Buffer, Elution Buffer, and Nuclease-free Water, which can be stored at room temperature, and NEXTFLEX® Cleanup Beads, which should be stored at 4°C.

Kit Contents	Amount
RED CAP	
NEXTFLEX® 3' 4N Adenylated Adapter	8 µL
NEXTFLEX® 3' Ligation Buffer	56 µL
NEXTFLEX® 3' Ligation Enzyme Mix	12 µL
NEXTFLEX® Adapter Depletion Solution	480 µL
PINK CAP	
NEXTFLEX® Adapter Inactivation Buffer	16 µL
NEXTFLEX® Adapter Inactivation Enzyme	4 µL

LIGHT PURPLE CAP	
NEXTFLEX® 5' 4N Adapter	12 µL
NEXTFLEX® 5' Ligation Buffer	60 µL
NEXTFLEX® 5' Ligation Enzyme Mix	16 µL

BLUE CAP	
M-MuLV Reverse Transcriptase	16 µL
NEXTFLEX® RT Buffer	104 µL

GREEN CAP	
NEXTFLEX® Universal Primer (12.5 µM)	8 µL
NEXTFLEX® Barcode Primer 1-8* (12.5 µM)	2 µL each (16 µL total)
NEXTFLEX® Small RNA PCR Master Mix	40 µL

ORANGE CAP	
6X Loading Dye	50 µL
Ready to Load Low MW Ladder	100 µL

YELLOW CAP	
Resuspension Buffer	1 mL

WHITE CAP	
Nuclease-free Water	1.5 mL

CLEAR CAP	
microRNA Control	5 µL

BROWN CAP	
NEXTFLEX® Cleanup Beads	(2) 1 mL

CLEAR CAP BOTTLE	
NEXTFLEX® Elution Buffer	5 mL

*Barcodes 1, 2, 4, 5, 9, 10, 12, 19

Required Materials Not Provided

- 1 ng - 2 µg total RNA or purified small RNA from 1-10 µg total RNA in up to 10.5 µL Nuclease-free Water
- Isopropanol
- 80% Ethanol
- 2, 10, 20, 200 and 1000 µL pipettes
- RNase-free pipette tips
- Microcentrifuge
- 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
- Thin-wall nuclease-free PCR tubes
- Thermocycler
- Heat block
- Vortex
- Magnetic Stand -96 (Ambion, Cat # AM10027) or similar

Optional Materials Not Provided

- 6% TBE PAGE gels (1.0 mm) (Life Technologies Cat # EC6265BOX)
- 1X TBE buffer
- Clean razor or scalpel
- Nucleic acid stain such as SYBR Gold (Invitrogen)
- UV transilluminator or gel documentation instrument
- Gel Electrophoresis apparatus
- Electrophoresis power supply
- 0.45µm, 2 mL Spin-X Centrifuge tube (Sigma Cat # CLS8162)
- Nuclease-free 1.7 mL microcentrifuge tubes
- Sterile disposable pestles (Fisher Cat # K749521-1500 or similar)
- Magnetic stand for microcentrifuge tubes (Life Technologies DynaMag™-2 or similar)

Revision History

Version	Date	Description
V15.11	November 2015	Initial Product Launch.
V16.06	June 2016	• Reagents for 3' adapter ligation, adapter inactivation, 5' adapter ligation, and reverse transcription have been master-mixed to increase ease of use. • The minimum recommended input has been reduced to 1 ng of total RNA. • The Data Analysis section has been updated. • A link to an alternative protocol for Preparing Libraries without Size Selection has been added. • Recommended temperature for 3' ligation is now 25°C. • It is now recommended to perform 3' and 5' adapter ligations with the thermocycler heated lid turned off or left open. • Reference to the 65 bp peak sometimes seen in final libraries has been added to the legend of Figure 4. • 5132-06 only: A note has been added that barcodes 11 and 24 are not compatible with sequencing on the Illumina NextSeq 500.

V18.07	July 2018	• Additional reagent handling recommendations have been added.
V19.01	January 2019	• Additionional recommendations added for use of tRNA/YRNA blockers as an alternative protocol for cfRNA.

Warnings and Precautions

We strongly recommends that you read the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local distributor or contact us at bioo.ngs@perkinelmer.com.

- Do not use the kit past the expiration date.
- Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
- RNA sample quality may vary between preparations. It is the user's responsibility to optimize the initial RNA input amount to obtain desired PCR bands for purification and sequencing. Refer to the Starting Material section for additional information.
- Vortex and centrifuge each component prior to use. To ensure material has not lodged in the cap or side of the tube, centrifuge in a microcentrifuge at >12,000 x g for 5 seconds.
- Do not remove NEXTFLEX® 3' Ligation Enzyme Mix orNEXTFLEX® 5' Ligation Enzyme Mix from -20°C until immediately before use and return to -20°C immediately after use.
- Some total RNA extraction and purification methods may not efficiently isolate small RNAs. Users should verify that their extraction and purification method also isolates small RNAs (see Figure 3 in Appendix B).
- Do not freeze NEXTFLEX® Cleanup Beads. NEXTFLEX® Cleanup Beads should be stored at 4°C and brought to room temperature before use.

Data Analysis

(For more detailed information on data analysis see BiooScientific.com/Resources)
The 3' and 5' adapters included in this kit both contain 4 random bases that will appear immediately 5' and 3' to the insert in sequencing data. The presence of these random bases should be considered when choosing an alignment strategy. When using "end-to-end" alignment, we recommend processing data in the following manner:

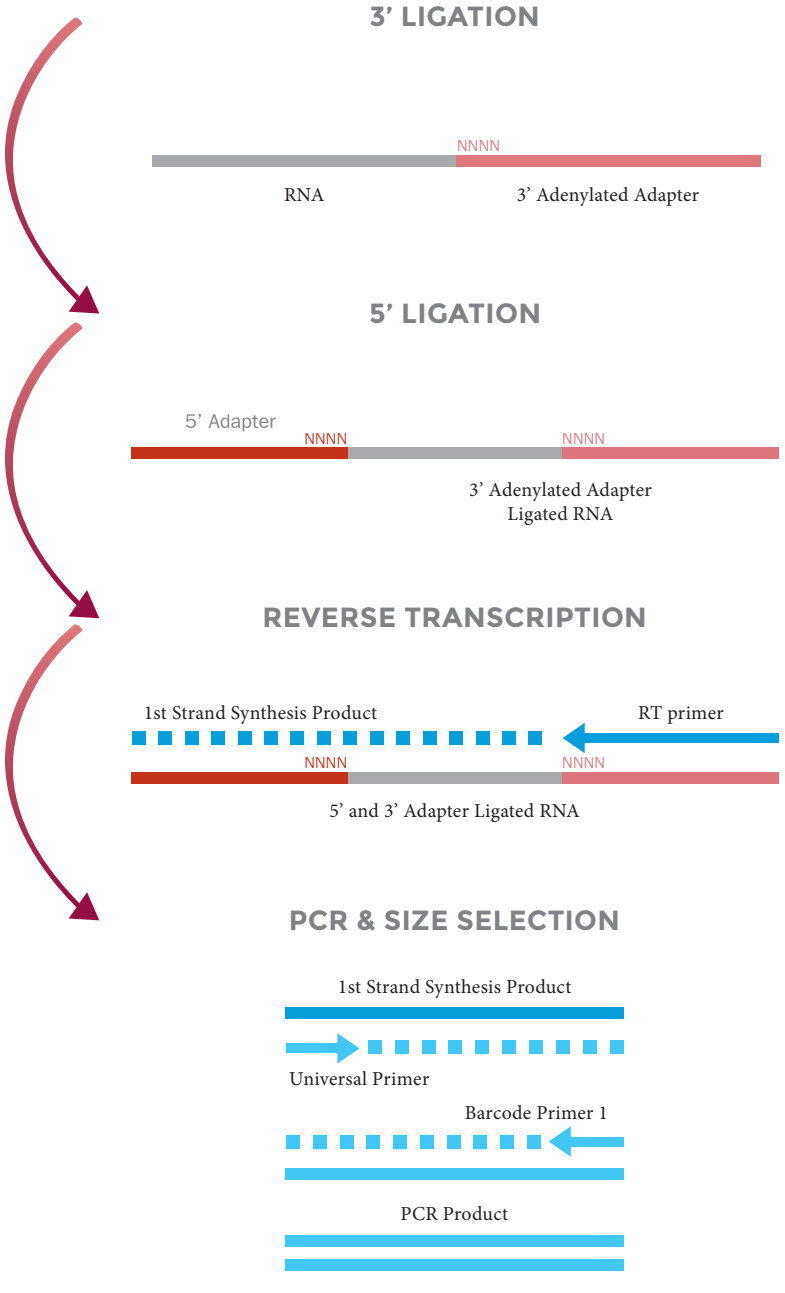
1. Clip the 3' adapter sequence (TGGAATTCTCGGGTGCCAAGG).
2. Trim the first and last 4 bases from the adapter-clipped reads.
3. Perform alignments as normal.

Alternatively, alignment may be performed in "local" mode.

Note: We do not recommend enabling the adapter trimming option or an adapter sequencing into Illumina Experiment Manager, as it may lead to unwanted masking of reads.

NEXTFLEX® Small RNA Sample Preparation Flow Chart

Figure 1: Sample flow chart.



Starting Material

The NEXTFLEX® Small RNA-Seq Kit v3 has been optimized and validated using total RNA (1 ng - 2 µg), purified small RNA (from 1 - 10 µg total RNA), and a synthetic miRNA pool (≥100 pg). Best results are obtained with high quality starting material. The use of degraded RNA may result in poor yields or lack of sequencing output data. We recommends running total RNA on a 1 - 2% agarose gel or examining its integrity using an Agilent Bioanalyzer. High quality total RNA preparations should have a 28S band that is twice as intense as the 18S band of ribosomal RNA. At low concentrations, small RNA is difficult to detect on a gel; however, it can be detected using an Agilent Bioanalyzer Small RNA assay (see Figure 3 in Appendix B). For low input library preparation, We recommends diluting the NEXTFLEX® 3' 4N Adenylated adapter and the NEXTFLEX® 5' 4N adapter with nuclease-free water. See Table 1 below for more details.

The NEXTFLEX® Small RNA-Seq™ Kit v3 is compatible with cell-free RNA, such as RNA isolated from plasma. Users who wish to deplete the abundant tRNA fragments and Y RNA fragments found in many types of cell-free RNA should use the NEXTFLEX® tRNA/YRNA Blocker (not included in kit). Please contact us at Bioo.NGS@PerkinElmer.com if interested. Instructions for use of the blocker can be found at BiooScientific.com/AltProtocols .

If the user is performing the procedure for the first time, we recommend using the microRNA Control included in the kit. This positive control sample consists of 21 RNA nucleotides and does not match any known sequence in miRBase. When running a positive control reaction, the user should add 1 µL of the microRNA Control in STEP A instead of their small RNA sample and expect to observe a strong 147 bp PCR product following 15 cycles of PCR. The microRNA control may degrade with multiple freeze thaw cycles or exposure to nucleases. If you plan on using the control multiple times, we recommend aliquoting into several tubes and storing at -20°C. For a total RNA positive control, human brain total RNA (Ambion catalog number AM7962 or similar) is recommended.

Table 1. Guidelines for different input amounts. Some optimization may be required.

Input Amount	Adapter Dilution*	PCR Cycles	Gel-Free Size Selection**
2 µg - 200 ng	None	12-18	+
200 ng - 50 ng	1/2-1/4	16-22	+/-
50 ng - 1 ng	1/4	22-25	-

*Only enough diluted adapter for the number of samples being processed should be prepared at the time of use, and any excess should be discarded.
**Refer to the following section, Determining Which Size Selection Method to Use, and Appendix B for more details on size selection

Determining Which Size Selection Method to Use

Typically, gel-free library preparation can be achieved with 200 ng - 2 µg of total RNA starting material and 18 or fewer cycles of PCR. PAGE-based size selection will be necessary when using less than 200 ng of total RNA starting material and up to 25 cycles of PCR. However, the small RNA fraction of total RNA can vary greatly depending on the cell/tissue type and the extraction method used, so it is the user's responsibility to determine optimal input amounts and PCR cycle numbers. Following PCR, products may be analyzed by TBE-PAGE gel, Agilent

Bioanalyzer HS DNA Assay, or similar (see Figure 6 & 7 in Appendix B). For analysis by PAGE gel, we recommend mixing 5 µL of PCR product with 1 µL of 6x Loading Dye and running on a 6% TBE-PAGE gel alongside 5 µL of Ready to Load Low Molecular Weight Ladder, and staining with SYBR Gold or ethidium bromide. For analysis by Bioanalyzer, we recommend running 1 µL of PCR product diluted 1/4 with nuclease-free water. The Bioanalyzer software may not correctly identify the peak sizes, so it is recommended to also run a library created with miRNA control to help identify the ~150 bp peak. Presence of a strong ~150 bp band indicates a successful library preparation, and absence of a band ~130 bp indicates that gel-free size selection may be used. See Table 2 & Appendix B for more information.

Table 2

~150 bp band	~130 bp band	Recommended Action
Strong	Absent or very weak	Gel-free size selection
Strong	Weak	PAGE size selection or repeat experiment with fewer PCR cycles
Strong	Strong	PAGE size selection
Absent/Weak	Absent	Additional PCR cycles
Absent/Weak	Strong	Repeat experiment with adapter dilution (1/2 - 1/4) and with additional PCR cycles

Preparing Libraries without Size Selection

Libraries can be prepared without size selection using the alternative protocol available at BiooScientific.com/AltProtocols .

Reagent Preparation

1. Vortex and centrifuge each component prior to use, to ensure material has not lodged in the cap or the side of the tube.
2. Allow NEXTFLEX® Cleanup Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.
3. Due to the viscosity of certain materials, attempting to prepare more than the stated number of reactions may result in a shortage of materials. All NEXTFLEX® enzyme components must be centrifuged at 600xg for 5 seconds before opening the tube(s). Adapters/barcoded primers supplied in a 96-well plate must be centrifuged at 280xg for 1 minute before removing the plate seal. Adapters/barcoded primers supplied in individual tubes must be centrifuged at 600xg for 5 seconds before opening the tube(s).

IMPORTANT - PLEASE READ

The NEXTFLEX® Small RNA Sequencing Kit v3 protocol requires 1.5-2 days for completion. Approximate times to complete each step and safe Stopping Points are noted in the manual; however, careful planning and time management are important for efficient and successful small RNA library preparation. If performing the protocol for the first time, we highly recommend preparing a library with the included microRNA control.

STEP A: NEXTFLEX® 3' 4N Adenylated Adapter Ligation

Approximate time to complete: 2.5 hours

Materials

Bioo Scientific Supplied

RED CAP - NEXTFLEX® 3' 4N Adenylated Adapter, NEXTFLEX® 3' Ligation Buffer, NEXTFLEX® 3' Ligation Enzyme Mix
WHITE CAP - Nuclease-free Water

User Supplied

RNA (1 ng - 2 µg total RNA or small RNA isolated from total RNA) in up to 10.5 µL Nuclease-free Water
96-well PCR plate
Adhesive PCR Plate Seal
Thermocycler
Ice

1. For each sample, combine the following reagents on ice in a nuclease-free 96-well PCR plate:

_ µL	RNA
_ µL	Nuclease-free Water
10.5 µL	TOTAL

2. Heat at 70°C for 2 minutes then immediately place on ice.
3. Incubate on ice for 2 - 5 minutes.
4. Note: Be sure to mix the following reaction until visibly homogenous by pipetting or brief vortexing. For each sample, combine the following reagents on ice in a nuclease-free 96-well PCR plate:

10.5 µL	Denatured RNA (from Step 1)
1 µL	NEXTFLEX® 3' 4N Adenylated Adapter* (Up to 1/4 dilution may be used. See Table 1 in Starting Material)
7 µL	NEXTFLEX® 3' Ligation Buffer
1.5 µL	NEXTFLEX® 3' Ligation Enzyme Mix
20 µL	TOTAL

5. Mix thoroughly by pipetting.
6. Incubate at 25°C for 2 hours in a thermocycler with heated lid turned off or left open. Incubation overnight at 20°C may increase yield in some cases. For ligations to 2' O-methylated small RNAs, such as those found in plants, incubate at 16°C overnight.
7. Proceed immediately to Step B: Excess 3' Adapter Removal.

STEP B: Excess 3' Adapter Removal

Approximate time to complete: 1 - 2 hours

Materials

Bioo Scientific Supplied
RED CAP - NEXTFLEX® Adapter Depletion Solution
YELLOW CAP - Resuspension Buffer
WHITE CAP - Nuclease-free Water
BROWN CAP - NEXTFLEX® Cleanup Beads

User Supplied
Isopropanol
80% Ethanol, freshly prepared
Magnetic Stand
***20 µL of** NEXTFLEX® **3' 4N Adenylated Adapter Ligated RNA (from Step A)**

1. To each sample, add 25 µL of NEXTFLEX® Adapter Depletion Solution and mix well by pipette.
2. Add 40 µL of NEXTFLEX® Cleanup Beads and mix well by pipette.
3. Immediately add 60 µL of isopropanol and mix well by pipette.
4. Incubate for 5 minutes.
5. Magnetize sample for 5 minutes or until solution appears clear.
6. Remove and discard supernatant.
7. Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant. Repeat this step for a total of 2 ethanol washes. IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.
8. Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.
9. Remove plate from magnetic stand and resuspend bead pellet in 22 µL of Resuspension Buffer by pipetting volume up and down. Ensure that beads are completely resuspended.
10. Incubate for 2 minutes.
11. Magnetize sample for 3 minutes or until solution appears clear.
12. Transfer 20 µL of supernatant to a new well.
13. Add 25 µL of NEXTFLEX® Adapter Depletion Solution and mix well by pipette.
14. Add 40 µL of NEXTFLEX® Cleanup Beads and mix well by pipette.
15. Immediately add 60 µL of isopropanol and mix well by pipette.
16. Incubate for 5 minutes.
17. Magnetize sample for 5 minutes or until solution appears clear.

18. Remove and discard supernatant.
19. Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant. Repeat this step for a total of 2 ethanol washes. IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.
20. Incubate sample for 3 minutes. After one minute, remove any residual liquid that may have collected at the bottom of the well.
21. Remove plate from magnetic stand and resuspend bead pellet in 13 µL of Nuclease-free Water by pipetting. Ensure that beads are completely resuspended.
22. Incubate for 2 minutes.
23. Magnetize sample for 3 minutes or until solution appears clear.
24. Transfer 11.5 µL of supernatant to a new well.
25. Proceed immediately to Step C: Excess Adapter Inactivation.

    STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored overnight at -20°C. To restart, thaw frozen samples on ice before proceeding to Step C: Excess Adapter Inactivation.

STEP C: Excess Adapter Inactivation

Approximate time to complete: 45 minutes

Materials

Bioo Scientific Supplied

PINK CAP - NEXTFLEX® Adapter Inactivation Buffer, NEXTFLEX® Adapter Inactivation Enzyme

User Supplied

96 well PCR plate
Adhesive PCR Plate Seal
Thermocycler
Ice
***11.5 µL of Purified** NEXTFLEX® **3' 4N Adenylated Adapter Ligated RNA (from Step B)**

1. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR plate:

11.5 µL	Purified NEXTFLEX® 3' 4N Adenylated Adapter Ligated RNA (from Step B)
2.0 µL	NEXTFLEX® Adapter Inactivation Buffer
0.5 µL	NEXTFLEX® Adapter Inactivation Enzyme
14 µL	TOTAL

2. Mix thoroughly by pipetting.
3. Incubate as follows:

15 min	12°C
20 min	50°C
hold	4°C

4. Proceed immediately to Step D: NEXTFLEX® 5' 4N Adapter Ligation.

STEP D: NEXTFLEX® 5' 4N Adapter Ligation

Approximate time to complete: 1.5 hours

Materials

Bioo Scientific Supplied

LIGHT PURPLE CAP - NEXTFLEX® 5' 4N Adapter, NEXTFLEX® 5' Ligation Buffer, NEXTFLEX® Ligation Enzyme Mix

User Supplied

96 well PCR plate
Adhesive PCR Plate Seal
Thermocycler
Ice
***14 µL of Purified NEXTFLEX® 3' 4N Adenylated Adapter Ligated RNA (from Step C)**

1. Heat 1.5 uL of NEXTFLEX® 5' 4N adapter per reaction at 70°C for 2 minutes, then immediately place on ice.
2. Note: Be sure to mix the following reaction until visibly homogenous by pipetting or brief vortexing. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR plate:

14 µL	Purified NEXTFLEX® 3' 4N Adenylated Adapter Ligated RNA (from Step C)
1.5 µL	NEXTFLEX® 5' 4N Adapter (Up to 1/4 dilution may be used. See Table 1 in Starting Material)
7.5 µL	NEXTFLEX® 5' Ligation Buffer
2 µL	NEXTFLEX® 5' Ligation Enzyme Mix
25 µL	TOTAL

3. Mix thoroughly by pipetting.
4. Incubate at 20°C for 1 hour in a thermocycler with heated lid turned off or left open.
5. Proceed immediately to Step E: Reverse Transcription - First Strand Synthesis.

   STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored overnight at -20°C. To restart, thaw frozen samples on ice before proceeding to Step E: Reverse Transcription - First Strand Synthesis.

STEP E: Reverse Transcription-First Strand Synthesis

Approximate time to complete: 1 hour

Materials

Bioo Scientific Supplied

BLUE CAP - NEXTFLEX® RT Buffer, M-MuLV Reverse Transcriptase

User Supplied

96 well PCR plate
Adhesive PCR Plate Seal
Thermocycler
Ice
***25 µL of 5' and 3' NEXTFLEX® Adapter Ligated RNA (from Step D)**

1. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR plate:

25 µL	5' and 3' NEXTFLEX® Adapter Ligated RNA (from Step D)
13 µL	NEXTFLEX® RT Buffer
2 µL	M-MuLV Reverse Transcriptase
40 µL	TOTAL

2. Mix thoroughly by pipetting.
3. Incubate as follows:

30 min	42°C
10 min	90°C

4. Proceed immediately to to Step F: Bead Cleanup.

   STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored overnight at 4°C or up to one week at -20°C. To restart, thaw frozen samples on ice before proceeding to Step F: Bead Cleanup.

STEP F: Bead Cleanup

Approximate time to complete: 30 - 45 minutes

Materials

Bioo Scientific Supplied

RED CAP - NEXTFLEX® Adapter Depletion Solution
BROWN CAP - NEXTFLEX® Cleanup Beads
WHITE CAP - Nuclease-Free Water

User Supplied

Isopropanol
80% Ethanol, freshly prepared
Magnetic Stand
***40 µL of First Strand Synthesis product (from Step E)**

1. To each sample, add 20 µL of NEXTFLEX® Cleanup Beads and mix well by pipette.
2. Add 22 µL isopropanol and mix well by pipette.
3. Incubate for 5 minutes.
4. Magnetize sample for 5 minutes or until solution is clear.
5. Transfer 70 µL of supernatant to a new well. DO NOT DISCARD SUPERNATANT, this solution contains the cDNA product. Take care to not transfer beads along with clear supernatant.
6. Remove plate from magnetic stand.
7. Add 10 µL NEXTFLEX® Adapter Depletion Solution and mix well by pipette.
8. Add 20 µL of NEXTFLEX® Cleanup Beads and mix well by pipette.
9. Add 68 µL of isopropanol and mix well by pipette.
10. Incubate for 5 minutes.
11. Magnetize sample for 5 minutes, or until solution appears clear.
12. Remove and discard supernatant.
13. Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant. Repeat this step for a total of 2 ethanol washes. IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.
14. Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.
15. Remove plate from magnetic stand and resuspend bead pellet in 20 µL Nuclease-free Water by pipetting volume up and down. Ensure that beads are completely resuspended.
16. Incubate for 2 minutes.
17. Magnetize sample for 3 minutes or until solution appears clear.
18. Transfer 18 µL of supernatant to a new well.
19. Proceed immediately to Step G: PCR Amplification.

    STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored overnight at -20°C. To restart, thaw frozen samples on ice before proceeding to Step G: PCR Amplification.

STEP G: PCR Amplification

Approximate time to complete: 40 - 60 minutes

Materials

Bioo Scientific Supplied

GREEN CAP - NEXTFLEX® Barcode Primers, NEXTFLEX® Universal Primer, NEXTFLEX® Small RNA PCR Master Mix

User Supplied

96 well PCR plate
Adhesive PCR Plate Seal
Thermocycler
Ice
***18 µL Purified First Strand Synthesis Product (from Step F)**

1. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR plate:

18 µL	Purified First Strand Synthesis Product (From Step F)
1 µL	NEXTFLEX® Universal Primer
1 µL	NEXTFLEX® Barcoded Primer (a different barcoded primer should be used for each sample that will be multiplexed for sequencing)
5 µL	NEXTFLEX® Small RNA PCR Master Mix
25 µL	TOTAL

2. Cycle as follows (make sure thermocycler is above 80°C before placing samples on block):

2 min	95°C	
20 sec	95°C	Repeat 12 - 25 cycles (see Table 1 in Starting Material on pg. 7 for recommendations)
30 sec	60°C	
15 sec	72°C	
2 min	72°C	

3. Proceed immediately to Step H1: Gel-Free Size Selection Cleanup or Step H2: Gel Electrophoresis & Nucleic Acid Elution and Purification.

STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored up to one week at -20°C. To restart, thaw frozen samples on ice before proceeding to Step H1: Gel-Free Size Selection Cleanup or Step H2: Gel Electrophoresis & Nucleic Acid Elution and Purification.

STEP H1: Gel-Free Size Selection & Cleanup

Note: This option is recommended if ≤18 cycles of PCR were performed in Step G (see Table 2).

Approximate time to complete: 45 minutes

Materials

Bioo Scientific Supplied

WHITE CAP - Nuclease-Free Water
YELLOW CAP - Resuspension Buffer
BROWN CAP - NEXTFLEX® Cleanup Beads

User Supplied

80% Ethanol, freshly prepared
Magnetic Stand
***25 µL of PCR Product (from Step G)**

1. Ensure the volume of all samples is 25 µL. If less, add Nuclease-free Water to bring the entire volume up to 25 uL.
2. Add 32.5 µL of NEXTFLEX® Cleanup Beads and mix well by pipetting.
3. Incubate for 5 minutes.
4. Magnetize sample for 5 minutes or until solution is clear.
5. Transfer 52.5 µL of supernatant to a new well. DO NOT DISCARD SUPERNATANT, this solution contains the amplified product. Take care to not transfer beads along with clear supernatant.
6. Remove plate from magnetic stand.
7. Add 30 µL of NEXTFLEX® Cleanup Beads to each sample and mix well by pipette.
8. Incubate for 5 minutes.
9. Magnetize sample for 5 minutes, or until solution appears clear.
10. Remove and discard supernatant.
11. Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant. Repeat this step for a total of 2 ethanol washes. IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.
12. Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.
13. Remove plate from magnetic stand and resuspend bead pellet in 13.5 µL of Resuspension Buffer by pipetting volume up and down. Ensure that beads are completely resuspended.
14. Incubate for 2 minutes.
15. Magnetize sample for 3 minutes or until solution appears clear.
16. Transfer 12 µL of supernatant to a new well or clean microcentrifuge tube. This is your sequencing library.
17. Check the size distribution of the final library by Bioanalyzer High Sensitivity DNA Assay (Agilent) and the concentration by Qubit dsDNA HS Assay (Life Technologies). See Figure 4A in Appendix A for sample Bioanalyzer trace. **(If significant high molecular weight products remain, bring total sample volume to 25 µL with nuclease free water and repeat Step H1. See Figure 5 in Appendix A)**

STEP H2: PAGE Size Selection & Cleanup

Note: This option is recommended if >18 cycles of PCR were performed in Step G (see Table 2).
Note: This step can be replaced with selection by Pippin Prep or Blue Pippin (See Resources tab at http://www.biooscientific.com/SmallRNA)

Approximate time to complete: 3 hours - overnight

Materials

Bioo Scientific Supplied

YELLOW CAP - Resuspension Buffer
ORANGE CAP - 6X Gel Loading Dye, Ready to Load Low MW Ladder
CLEAR CAP BOTTLE - NEXTFLEX® Elution Buffer
BROWN CAP - NEXTFLEX® Cleanup Beads

User Supplied

Isopropanol
80% Ethanol
Nuclease-free 1.7 mL microcentrifuge tubes
Spin-X Centrifuge tube (Sigma)
Sterile disposable pestles (Fisher Cat # K749521-1500 or similar)
Magnetic stand for micrcentrifuge tubes (Life Technologies DynaMag™-2 or similar)
6% TBE-PAGE Gel
1X TBE Buffer
Nucleic acid stain such as SYBR Gold (Invitrogen)
UV transilluminator or other visualization tool
Clean razor or scalpel
***25 µL of PCR Product (from Step G)**

1. Add 5 µL of 6X Gel Loading Dye to each PCR product and mix well.
2. Load PCR products onto a 6% TBE-PAGE gel. We recommend leaving 1-2 lanes between samples prepared with the same barcode primer to avoid cross contamination. Samples prepared with different barcodes and that will be sequenced together may be run in adjacent lanes.
3. In an adjacent lane, load 10 µL of Ready to Load Low MW Ladder.
4. Run the gel with 1X TBE buffer at 200 V until the lower dye band is near the bottom of the gel (0.5-1 cm). The gel should run for approximately 30 minutes. Run times may vary depending on individual equipment.
5. Carefully remove the gel from the glass plates and stain with a nucleic acid stain such as SYBR Gold (Invitrogen) per manufacturer instructions.
6. Visualize gel bands on a UV transilluminator or other gel documentation instrument.
7. Using a clean razor, cut out the ~150 bp band and place into clean 1.7 mL tube. Do not cut out the ~130 bp band; this is adapter dimer product (see Figure 2). The ladder band at 200 bp is twice as intense as the other bands and can be used for orientation.
8. Briefly centrifuge the microcentrifuge tube containing the gel slice to collect the gel slice at the bottom of the tube.

9. Crush the gel slice thoroughly with a disposable pestle. Leave the pestle in the tube.
10. Add 300 µL of NEXTFLEX® Elution Buffer to each tube and then remove the pestle, ensuring that as much gel as possible has been washed from the pestle.
11. Let gel pieces soak at least 2 hours or overnight at room temperature with agitation. DO NOT incubate longer than overnight.
12. Pulse spin tubes to collect all eluate from wall and lid.
13. Carefully transfer the eluate (including crushed gel) to the top of a Spin-X Centrifuge tube (Sigma). Cutting the end off of a P1000 tip can help for transfers of larger gel pieces. Centrifuge the Spin-X tube at 16,000 x g for 2 minutes. Dispose of the spin filter.
14. Add to each tube and mix well:

50 µL	NEXTFLEX® Cleanup Beads
350 µL	Isopropanol

15. Incubate at room temperature for 10 minutes. Agitation during this incubation may increase efficiency of recovery.
16. Pulse spin tubes to collect solution from walls and lid of tube and to pellet beads.
17. Magnetize sample for 2 minutes or until solution appears clear.
18. Carefully remove and discard the supernatant.
19. Add 950 µL 80% ethanol, incubate for 30 seconds, then remove all of the supernatant. Repeat this step for a total of two ethanol washes. IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.
20. Dry sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the tube.
21. Remove plate from magnetic stand and resuspend bead pellet in 13 µL of Resuspension Buffer by pipetting volume up and down. Ensure that beads are completely resuspended and rehydrated.
22. Incubate for 2 minutes.
23. Magnetize for 3 minutes or until supernatant appears clear.
24. Transfer 12 µL of supernatant to a clean 1.7 mL tube. This is your sequencing library.
25. Check the size distribution of the final library by Bioanalyzer High Sensitivity DNA Assay (Agilent) and the concentration by Qubit dsDNA HS Assay (Life Technologies). See Figure 4B in Appendix A for sample Bioanalyzer trace.

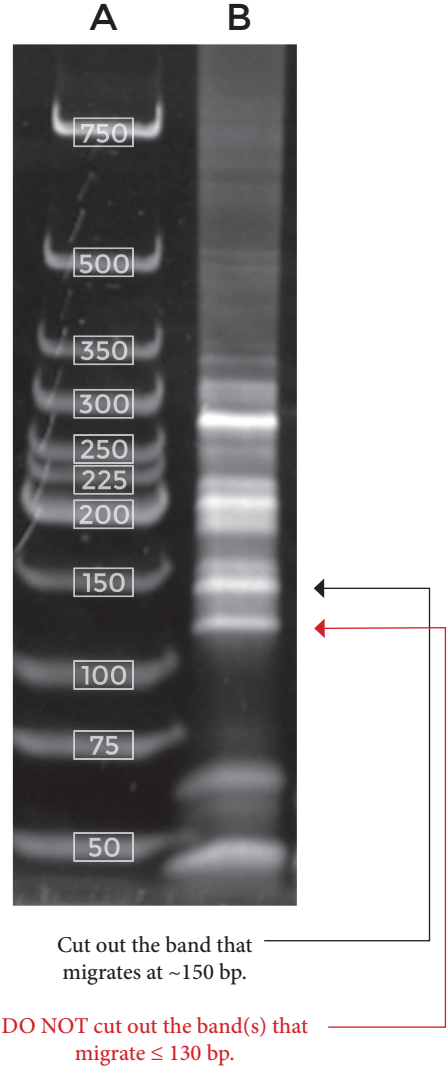
Figure 2

6% TBE-PAGE gel

A. Ready to Load Low MW Ladder

B. PCR product from library constructed from human brain total RNA

NOTE: 225 bp & 250 bp ladder bands may run as a single band (25 bp not shown).



Oligonucleotide Sequences

NEXTFLEX®	Sequence
NEXTFLEX® 3'4N Adenylated Adapter	5' rApp /NNNNTGGAATTCTCGGGTGCCAAGG/ 3ddC/
NEXTFLEX® 5'4N Adapter	5' GUUCAGAGUUCUACAGUCCGACGAUCNNNN
NEXTFLEX® RT Primer	5' GCCTTGGCACCCGAGAATTCCA
NEXTFLEX® Barcode Primers	5' CAAGCAGAAGACGGCATACGAGAT<u>XXXXXX</u>[1]GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
NEXTFLEX® Universal Primer	5' AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTC-CGA
microRNA Control	5'Phos/CUCAGGAUGGCGGAGCGGUCU/3'

[1]The XXXXXX denotes the index region of adapter, which is the reverse complement of the index sequence listed below.

NEXTFLEX® Small RNA Barcodes	Index Sequence
PCR Primer 1	ATCACG
PCR Primer 2	CGATGT
PCR Primer 4	TGACCA
PCR Primer 5	ACAGTG
PCR Primer 9	GATCAG
PCR Primer 10	TAGCTT
PCR Primer 12	CTTGTA
PCR Primer 19	GTGAAA

See BiooScientific.com/Resources for a spreadsheet containing all barcode sequences.

Low Level Multiplexing

The set of barcoded primers included with this kit is designed to allow low level multiplexing. Please see the table below for recommended low-level multiplexing options.

Number of Samples	NEXTFLEX® Small RNA Barcodes
2	5, 19
3	1, 9, 12, or 2, 4, 10, or 5, 19, and any other barcode
4 or more	1, 9, 12, and any other barcodes, or 2, 4, 10, and any other barcodes, or 5, 19, and any other barcodes

Figure 3: Small RNA Traces from Agilent Bioanalyzer

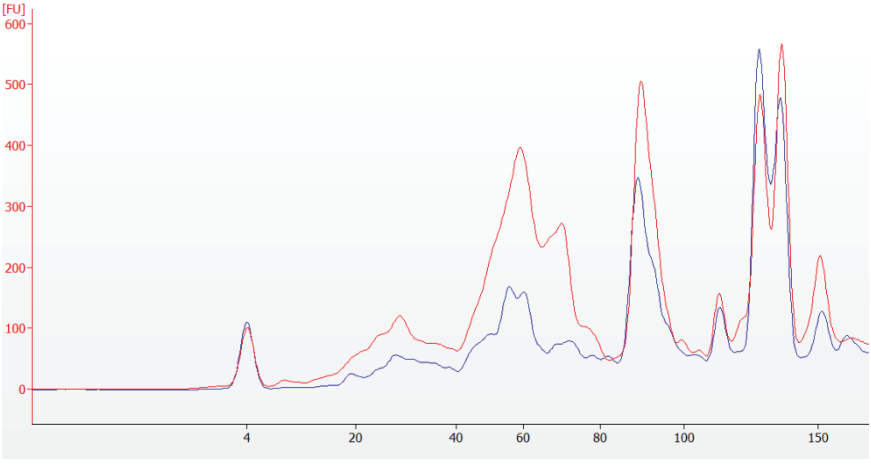


Figure 3. Bioanalyzer Small RNA assay results from 100 ng of human brain total RNA (red line) and MCF-7 total RNA (blue line). MicroRNAs are shown in the region from ~10 to 40 nts. Both of these RNA samples are suitable for library preparation with the NEXTFLEX® *Small RNA-Seq Kit, but greater input amount or more PCR cycles will be required for library preparation from the MCF-7 RNA sample versus the human brain RNA sample.*

Figure 4: Final Library Traces

Figure 4A

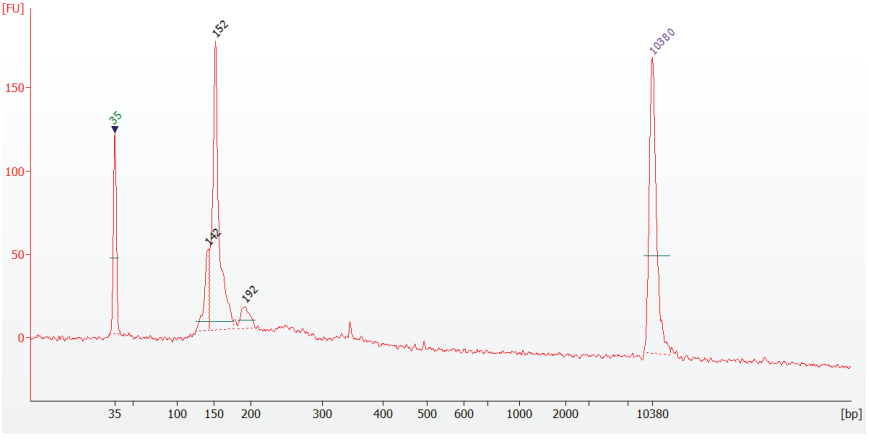
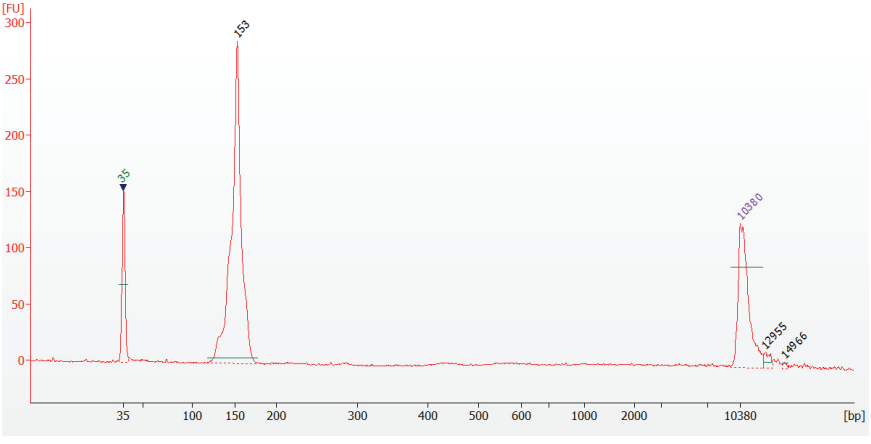


Figure 4B



Figures 4A & 4B. Sample Bioanalyzer HS DNA traces from libraries created from MCF-7 total RNA using gel-free (4A) or gel electrophorisis (4B) protocols. Occasionally, a peak of ~65 bp will be seen. This peak represents excess PCR primer and will not negatively affect sequencing or quantification by Qubit dsDNA HS Assay.

Figure 5: Library with High Molecular Weight Contamination

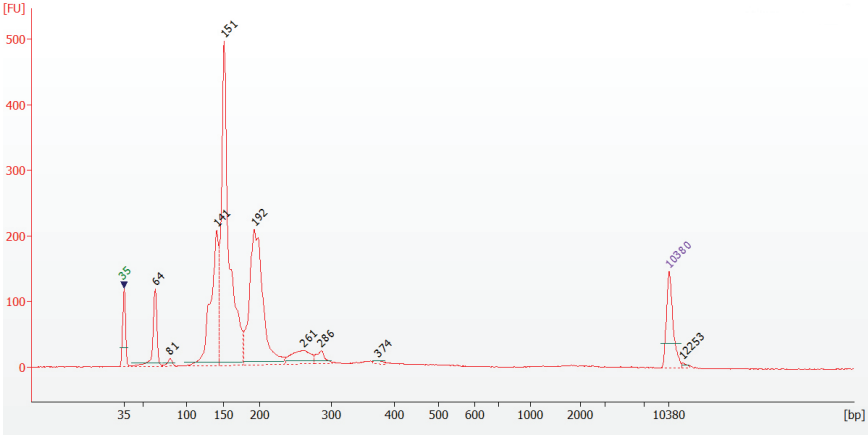


Figure 5. Sample Bioanalyzer HS DNA trace of a library that would benefit from repeating Step H1: Gel-Free Size Selection Cleanup. Elimination of the products ≥180 bp will result in a greater proportion of reads representing small RNAs. Note that repeating step H1 will result in some overall loss.

Figure 6: Choosing a Size Selection Method by PAGE

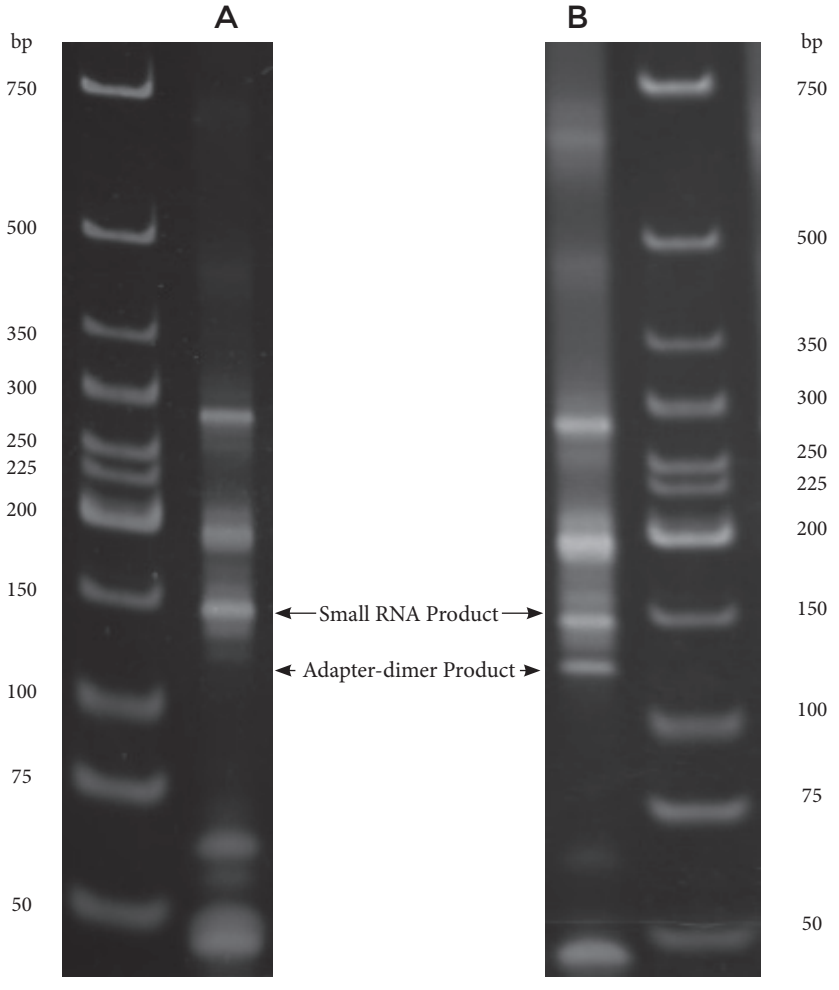


Figure 6. Sample PAGE images of samples that could be size selected with option H1: Gel-Free Size Selection Cleanup (A) or H2: PAGE Size Selection and Cleanup (B).

NOTE: 225 bp & 250 bp ladder bands may run as a single band (25 bp not shown).

Figure 7: Choosing a Size Selection Method by Agilent Bioanalyzer

Figure 7A

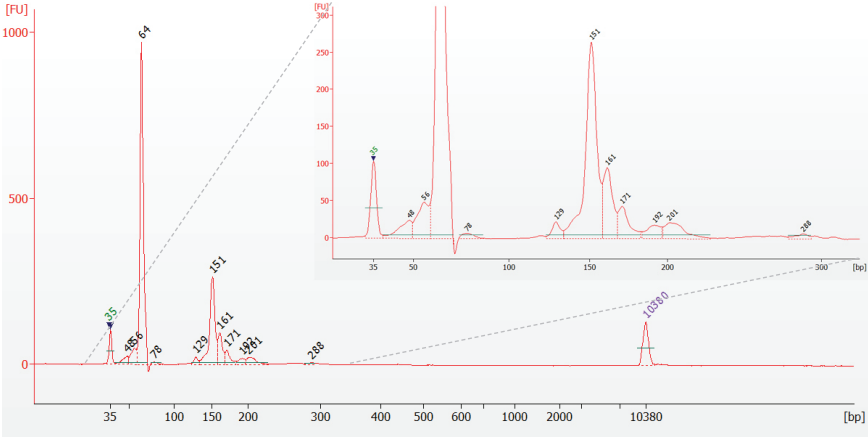


Figure 7B

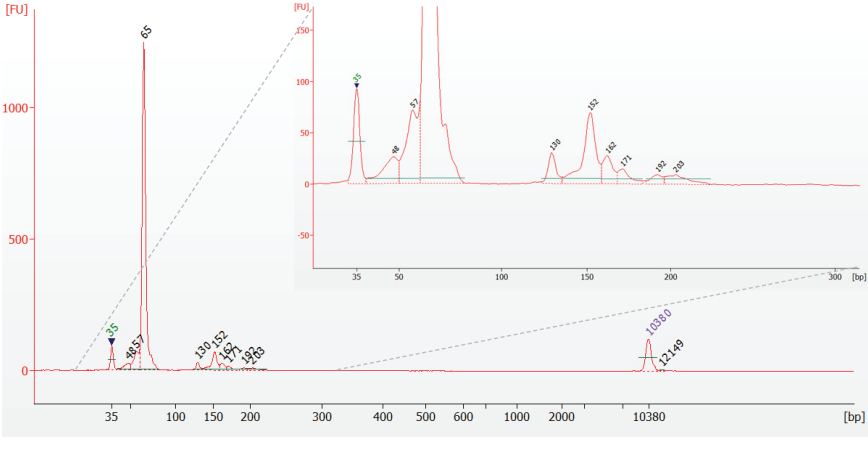


Figure 7. Sample Bioanalyzer HS DNA images of samples that could be size selected with option H1: Gel-Free Size Selection Cleanup (A) or H2: PAGE Size Selection and Cleanup (B).

References

1. Jayaprakash, A.D., et al., Identification and remediation of biases in the activity of RNA ligases in small-RNA deep sequencing. Nucleic Acids Res, 2011. 39(21): p. e141.

2. Hafner, M., et al., RNA-ligase-dependent biases in miRNA representation in deep-sequenced small RNA cDNA libraries. RNA, 2011. 17(9): p. 1697-712.

RELATED PRODUCTS

Illumina Compatible RNA NGS Kits and Adapters

- NEXTflex™ Rapid Directional RNA-Seq Kit
- NEXTflex™ RNA-Seq Barcodes
- NEXTflex-96™ RNA-Seq Barcodes
- NEXTflex™ Rapid Directional qRNA-Seq™ Kit
- NEXTlfex™ Small RNA Barcode Primers
- NEXTflex™ Poly(A) Beads

Illumina Compatible DNA NGS Kits and Adapters

- NEXTflex™ 16S V4 Amplicon-Seq Kit
- NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0
- NEXTflex™ 16S V1-V3 Amplicon-Seq Kit
- NEXTflex™ 18S ITS Amplicon-Seq Kit
- NEXTflex™ Rapid DNA-Seq Kit
- NEXTflex™ Cell Free DNA-Seq Kit
- NEXTflex™ DNA Barcodes
- NEXTflex-96™ DNA Barcodes
- NEXTflex-HT™ Barcodes
- NEXTflex™ Dual-Indexed DNA Barcodes
- NEXTflex™ Bisulfite-Seq Kit
- NEXTflex™ Bisulfite-Seq Barcodes
- NEXTflex™ Methyl-Seq 1 Kit
- NEXTflex™ Msp 1
- NEXTflex™ ChIP-Seq Kit
- NEXTflex™ ChIP-Seq Barcodes
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- NEXTflex™ Rapid Pre-Capture Combo Kit
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NOTES

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