NEXTFLEX® Small RNA-Seq Kit v4 with UDIs

KIT CONTAINS : 8, 48 or 96 UDIs

#NOVA-5132-31
#NOVA-5132-32
#NOVA-5132-41
#NOVA-5132-42
#NOVA-5132-43
#NOVA-5132-44

AUTOMATED ON :
SCICLONE® G3 NGSX WORKSTATION
ZEPHYR® G3 NGS WORKSTATION

Compatible with Illumina® and Element® platforms

NEXTFLEX® Small RNA-Seq Kit v4 with UDIs

This product is for research use only.

Not for use in diagnostic procedures.

GENERAL INFORMATION

Product Overview

The NEXTFLEX® Small RNA-Seq Kit v4 with UDIs can be used to prepare small RNA libraries from total RNA or purified small RNA and is designed to greatly reduce formation of adapter-dimer product in small RNA-Seq library preparation, allowing for complete gel-free library preparation from typical input amounts. This kit is designed to greatly reduce sequence bias in small RNA sequencing library construction, allowing more accurate identification and quantification of microRNAs, piRNAs, and other small RNAs.

Sequencing libraries generated by this kit contain Unique Dual Indices (UDIs), which are designed to specifically address the index-hopping phenomenon associated with Illumina platforms utilizing a patterned flow cell. These UDIs prevent mis-assigned reads from appearing in final datasets, allowing for the highest assurance of data integrity. These indexes work on Illumina® instruments such as the MiSeq®, HiSeq® 2000/2500, MiniSeq®, NextSeq®, and NovaSeq® as well as Element® platforms.

Kit Overview

This kit can be used to generate 8, 48, or 96 small RNA libraries. The kit contains the necessary reagents to process the user's purified RNA sample through library preparation.

Kit Contents, Storage & Shelf Life

The NEXTFLEX® Small RNA-Seq Kit v4 with UDIs contain enough material to prepare 8, 48, or 96 RNA samples for Illumina and Element® compatible next-generation sequencing. The shelf life of all reagents is at least 6 months when stored properly.

The Nuclease-free Water and Resuspension Buffer can be stored at room temperature. The NEXTFLEX® Cleanup Beads should be stored at 4°C, and all other components should be stored at -20°C.

Kit Contents	Cap Color	Amount (8, 48, 96†)	Storage Temp.
NEXTFLEX® 3′ Adenylated Adapter v4	RED CAP	8/48/96 µL	-20°C
NEXTFLEX® 3′ Ligation Buffer v4	RED CAP	100/600/1,200 µL	-20°C
NEXTFLEX® 3′ Ligation Enzyme Mix v4	RED CAP	12/72/144 µL	-20°C
NEXTFLEX® Adapter Depletion Solution	RED CAP	320/(2)960/4,000** µL	Room Temp.
NEXTFLEX® tRNA/YRNA Blockers v4	RED CAP	8/48/96 µL	-20°C
NEXTFLEX® Adapter Inactivation Mix v4	PINK CAP	32/192/384 µL	-20°C

NEXTFLEX® 5′ Adapter v4	LIGHT PURPLE CAP	8/48/96 µL	-20°C
NEXTFLEX® 5′ Ligation Buffer v4	LIGHT PURPLE CAP	24/144/288 µL	-20°C
NEXTFLEX® 5′ Ligation Enzyme Mix v4	LIGHT PURPLE CAP	16/96/192 µL	-20°C
NEXTFLEX® RT Enzyme Mix v4	BLUE CAP	16/96/192 µL	-20°C
NEXTFLEX® RT Buffer v4	BLUE CAP	56/336/672 µL	-20°C
NEXTFLEX® RT Primer v4	BLUE CAP	8/48/96 µL	-20°C
NEXTFLEX® UDI Barcoded Primer Mix v4* (6.25 µM)	GREEN CAP or PLATE	4 µL each	-20°C
NEXTFLEX® Small RNA PCR Master Mix v4	GREEN CAP	48/288/576 µL	-20°C
Resuspension Buffer	YELLOW CAP	1/1/(2)1 mL	Room Temp.
Nuclease-free Water	WHITE CAP	1/(2)1.5/10** mL	Room Temp.
microRNA Control	CLEAR CAP	10/(2)10/(2)10 µL	-20°C
NEXTFLEX® Cleanup Beads	BROWN CAP	1/5/10** mL	4°C

**in the 8 rxn kit the UDI barcoded primers are provided in tubes*

*** reagents will be in WHITE CAP BOTTLES*

† the 96 reaction kit contains the necessary overage to perform the assay on automation instrumentation.

Required Materials Not Provided

- 1 ng - 2 µg total RNA or purified small RNA from 1-10 µg total RNA in up to 5 µL Nuclease-free Water
- Isopropanol
- 80% Ethanol (Freshly made)
- 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
- Thermal Cycler
- Vortex
- Magnetic Stand -96 (Ambion, Cat # AM10027) or similar

Revision History

Version	Date	Description
V22.04	April 2022	Early Technology Release
V22.06	June 2022	Product Launch
V23.01	January 2023	Removal of dye from 3' Ligation Buffer
V23.04	April 2023	Manual Update
V23.10	October 2023	Rebrand to Revvity

Warnings and Precautions

- We strongly recommend 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 https://www.revvity.com/contact-us/technical-support and choose the "Next Gen Sequencing" category.
- 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 or NEXTFLEX® 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 methods also isolate small RNAs.
- Do not freeze NEXTFLEX® Cleanup Beads. NEXTFLEX® Cleanup Beads should be stored at 4°C and brought to room temperature before use.

SAMPLE PREP WORKFLOW

NEXTFLEX® Small RNA Sample Preparation Flow Chart

NEXTFLEX Small RNA Sample Preparation Flow Chart

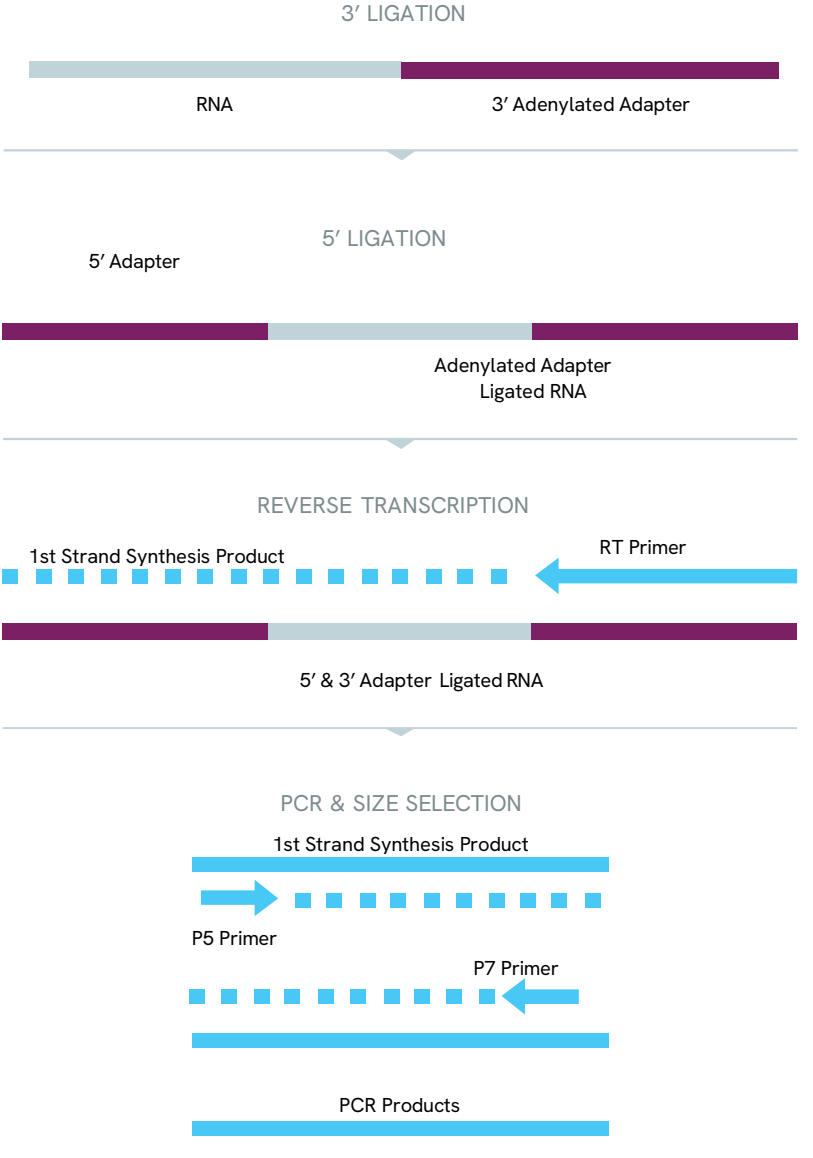


Figure 1. Sample flow chart.

SAMPLE PREP WORKFLOW

Starting Material

The NEXTFLEX® Small RNA-Seq Kit v4 with UDIs has been optimized and validated using total RNA (1 ng - 2 µg), RNA isolated from plasma & serum, 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 recommend 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 a LabChip® assay or similar.

For very poor-quality samples, diluting the NEXTFLEX® 3' Adenylated adapter and the NEXTFLEX® 5' Adapter 1/4 with Nuclease-free Water can help reduce adapter dimer formation. See Table 1 below for more details. The NEXTFLEX® Small RNA-Seq™ Kit v4 with UDIs is compatible with cell-free RNA, such as RNA isolated from plasma. Users who wish to deplete the abundant tRNA fragments and YRNA fragments found in many types of cell-free RNA should use the NEXTFLEX® tRNA/YRNA Blockers (included in kit). When working with cell-free RNA, it is recommended that users input 4 µl of extracted RNA, or 5 µl if not using tRNA/YRNA Blockers. Optionally, if samples prepared from blood or plasma are being used the NEXTFLEX® blood miRNA blockers can be used instead of or with tRNA/YRNA blockers.

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 sequences 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 ~165 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.

Input Amount	PCR Cycles
1 µg	12-14
100 ng	15-17
10 ng	18-20
1 ng	22-24*
Plasma/Serum	22-24*
miRNA Control	15-17

**When diluting adapters by 1/4, use 24 PCR cycles.*

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

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 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). Barcoded primers supplied in plates must be centrifuged at 600xg for 5 seconds before using.

! IMPORTANT – PLEASE READ

The NEXTFLEX® Small RNA Sequencing Kit v4 protocol requires ~6-7 hours 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.

SAMPLE PREP PROTOCOL

STEP A: NEXTFLEX® 3′ Adenylated Adapter Ligation

MATERIALS

RED CAP – NEXTFLEX® 3′ Adenylated Adapter v4

RED CAP – NEXTFLEX® 3′ Ligation Buffer v4

RED CAP – NEXTFLEX® 3′ Ligation Enzyme Mix v4

RED CAP – NEXTFLEX® tRNA/YRNA Blockers v4

WHITE CAP – Nuclease-free Water

User Supplied

- RNA (1 ng – 2 µg total RNA or small RNA isolated from total RNA) in up to 5 µL Nuclease- Free Water
- 96-well PCR Plate
- Adhesive PCR Plate Seal
- Thermal Cycler
- Ice
- NEXTFLEX® blood miRNA Blockers (P/N: NOVA-513103)

1. NOTE: Be sure to mix the following reaction until homogenous by brief vortexing. For each sample, combine the following reagents on ice in a nuclease-free 96-well PCR plate. It is recommended to combine these reagents as a master mix if processing multiple samples:

_ µL	RNA
_ µL	Nuclease-free Water
1 µL	NEXTFLEX® tRNA/YRNA Blockers v4 (optional)*
1 µL	NEXTFLEX® 3′ Adenylated Adapter v4
12.5 µL	NEXTFLEX® 3′ Ligation Buffer v4
1.5 µL	NEXTFLEX® 3′ Ligation Enzyme Mix v4
20 µL	TOTAL

*if processing samples from blood or plasma, the NEXTFLEX® blood miRNA blockers may be used instead of or with tRNA/YRNA blockers.

2. Mix viscous reaction by vortexing for at least 3 seconds until homogenized.
3. Incubate at 25°C for 1 hour in a thermal cycler. Hold at 4°C for at least 5 minutes. For ligations to 2′O-methylated small RNAs, such as those found in plants, incubate at 16°C overnight.
4. Proceed immediately to Step B: Adapter Inactivation.

STEP B: Excess 3′ Adapter Removal

MATERIALS

PINK CAP - NEXTFLEX Adapter Inactivation Mix v4

User Supplied

- 96 well PCR plate
- Adhesive PCR Plate Seal
- Thermal cycler
- Ice
- 20 µL of NEXTFLEX 3' Adapter Ligated RNA (from Step A)

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

20 µL	NEXTFLEX 3' Adapter Ligated RNA (from Step A)
4 µL	NEXTFLEX Adapter Inactivation Mix v4
24 µL	TOTAL

2. Mix viscous reaction by vortexing for at least 3 seconds until homogenized.
3. Incubate as follows:

2 min	70°C
5 min	4°C
HOLD	4°C

4. Proceed immediately to Step C: NEXTFLEX 5' Adapter Ligation.

STEP C: NEXTFLEX 5' Adapter Ligation

MATERIALS

LIGHT PURPLE CAP - NEXTFLEX 5' Adapter v4

LIGHT PURPLE CAP - NEXTFLEX 5' Ligation Buffer v4

LIGHT PURPLE CAP - NEXTFLEX Ligation Enzyme Mix v4

User Supplied

- 96 well PCR Plate
- Adhesive PCR Plate Seal
- Thermal Cycler
- Ice
- 24 µL of NEXTFLEX 3' Adapter Ligated RNA (from Step B)

1. NOTE: Be sure to mix the following reaction until visibly homogenous by brief vortexing. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR plate. It is recommended to combine these reagents as a master mix if processing multiple samples:

24 µL	NEXTFLEX 3' Adapter Ligated RNA (from Step B)
1 µL	NEXTFLEX 5' Adapter v4
3 µL	NEXTFLEX 5′ Ligation Buffer v4
2 µL	NEXTFLEX 5′ Ligation Enzyme Mix v4
30 µL	TOTAL

2. Mix reaction by vortexing for at least 3 seconds until homogenized.
3. Incubate at 20°C for 60 minutes in a thermal cycler.

60 min	20°C
5 min	4°C
HOLD	4°C

4. Proceed immediately to Step D: Reverse Transcription - First Strand Synthesis.

STEP D: Reverse Transcription-First Strand Synthesis

MATERIALS

BLUE CAP - NEXTFLEX® RT Buffer v4

BLUE CAP - NEXTFLEX® RT Enzyme Mix v4

BLUE CAP - NEXTFLEX® RT Primer v4

User Supplied

- 96 well PCR plate
- Adhesive PCR Plate Seal
- Thermal cycler
- Ice
- 30 µL of 5′ and 3′ NEXTFLEX® Adapter Ligated RNA (from Step C)

1. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR plate. It is recommended to combine these reagents as a master mix if processing multiple samples:

30 µL	5' and 3' NEXTFLEX® Adapter Ligated RNA (from Step C)
1 µL	NEXTFLEX® RT Primer v4
7 µL	NEXTFLEX® RT Buffer v4
2 µL	NEXTFLEX® RT Enzyme Mix v4
40 µL	TOTAL

2. Mix reaction by vortexing for at least 3 seconds until homogenized.
3. Incubate as follows:

60 min	50°C
5 min	90°C
5 min	4°C
HOLD	4°C

4. Proceed immediately to Step E: Bead Cleanup.

STOPPING POINT : It is encouraged to proceed through Step E: Bead Cleanup. However, samples may be stored at 4°C overnight, with minimal impact on data quality.

STEP E: Bead Cleanup

MATERIALS

RED CAP or WHITE CAP BOTTLE - NEXTFLEX® Adapter Depletion Solution

BROWN CAP or WHITE CAP BOTTLE - NEXTFLEX® Cleanup Beads

WHITE CAP or WHITE CAP BOTTLE - Nuclease-free Water

User Supplied

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

1. Add 40 µL NEXTFLEX® Adapter Depletion Solution and mix well by pipette.
2. Add 40 µL of NEXTFLEX® Cleanup Beads and mix well by pipette.
3. Add 90 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 200 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove 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 18 µL Nuclease-free Water 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 16 µL of supernatant to a new well.
13. Proceed immediately to Step F: 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 F: PCR Amplification.

STEP F: PCR Amplification

MATERIALS

GREEN CAP or PLATE - NEXTFLEX® UDI Barcoded Primer Mix v4

GREEN CAP - NEXTFLEX® Small RNA PCR Master Mix v4

User Supplied

- 96 well PCR plate
- Adhesive PCR Plate Seal
- Thermal cycler
- Ice
- 16 µL Purified First Strand Synthesis Product (from Step E)

1. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR plate. It is recommended to combine these reagents as a master mix if processing multiple samples:

16 µL	Purified First Strand Synthesis Product (From Step E)
4 µL	NEXTFLEX® UDI Barcoded Primer Mix v4 (a different barcoded primer should be used for each sample that will be multiplexed for sequencing)
6 µL	NEXTFLEX® Small RNA PCR Master Mix v4
26 µL	TOTAL

2. Cycle as follows:

30 sec	98°C	
10 sec	98°C	*(See Table 1 on page 9 in Starting Materials for recommendations)*
20 sec	65°C	
15 sec	72°C	
2 min	72°C	

3. Proceed immediately to Step G: Size Selection & Cleanup.

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 G: Size Selection Cleanup

STEP G: Size Selection & Cleanup

MATERIALS

WHITE CAP or CLEAR CAP BOTTLE - Nuclease-Free Water

YELLOW CAP - Resuspension Buffer

BROWN CAP or CLEAR CAP BOTTLE - NEXTFLEX® Cleanup Beads

User Supplied

- 80% Ethanol, freshly prepared
- Magnetic Stand
- 26 µL of PCR Product (from Step F)

1. Ensure the volume of all samples is 26 µL. If less, add Nuclease-free Water to bring the entire volume up to 26 µL.
2. Add 34 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 56 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 13 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 200 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove 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 17 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 15 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 LabChip® or equivalent and the concentration by Qubit dsDNA HS Assay (Life Technologies).

LIBRARY VALIDATION

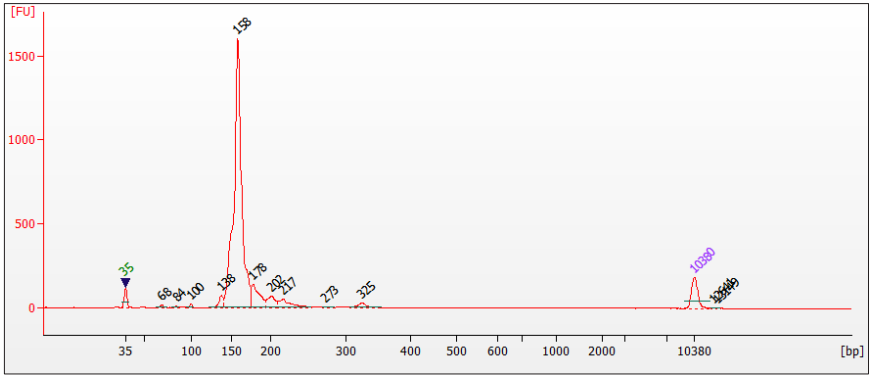


Figure 2. Sample Bioanalyzer HS DNA traces from libraries created from 1 ng human brain total RNA.

APPENDIX A

Oligonucleotide Sequences

NEXTFLEX® 3'Adenylated Adapter
5′ rApp /TGGAATTCTCGGGTGCCAAGG/ 3SpC3/
NEXTFLEX® 5'Adapter
5' UCUUUCCCUACACGACGCUCUUCCGAUCU
NEXTFLEX® RT Primer
5′ CCTTGGCACCCGAGAATTCCA
UDI primer 1 – P7 (included in NEXTFLEX® UDI Barcoded Primer Mix)
5' CAAGCAGAAGACGGCATACGAGATXXXXXXXX[1]GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
UDI primer 2 – P5 (included in NEXTFLEX® UDI Barcoded Primer Mix)
5' AATGATACGGCGACCACCGAGATCTACACXXXXXXXX[2]ACACTCTTTCCCTACACGACGCTCTTCCGATCT

XXXXXXXX[1] denotes the P7 index region of the primer. The index sequences that are added to each barcoded library via PCR are listed below. Note: UDI primer 1 contains the reverse complement of the sequence listed under P7 Index. The final library, however, will contain the P7 Index listed below, and the sequencer will read the index as listed below.

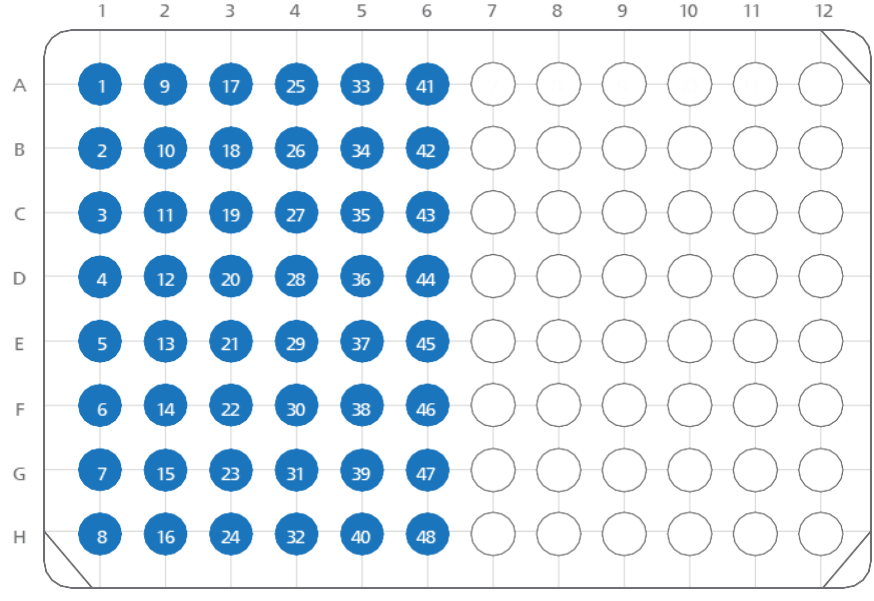
XXXXXXXX[2] denotes the P5 index region of the primer. The index sequences that are added to each barcoded library via PCR are listed below.

The complete index sequences can be found under the Technical Resources tab at https://resources.revvity.com/pdfs/NEXTFLEX_UDI_primers_small_RNA_seq_kit_v4.xlsx

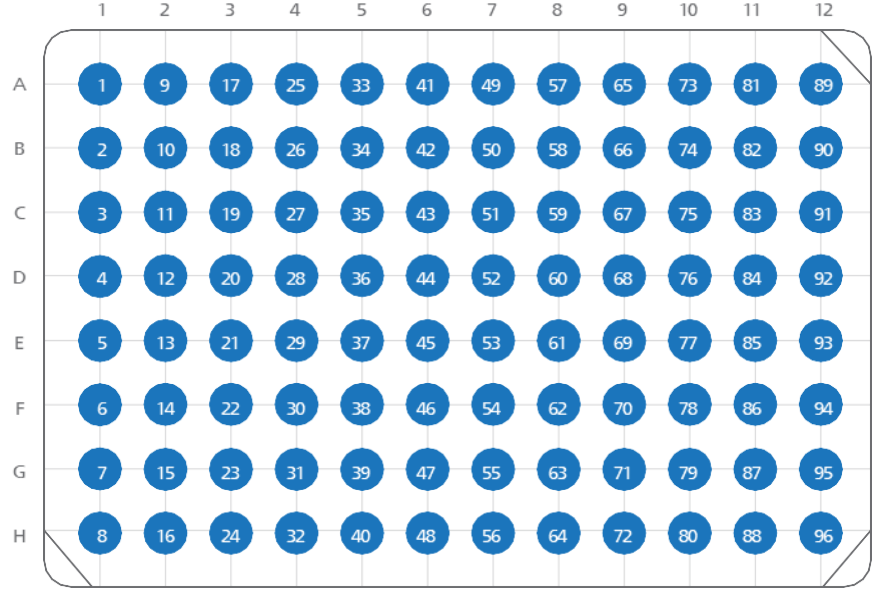
When entering index sequences for the Illumina® MiniSeq®, NextSeq®, HiSeq® 3000, or HiSeq® 4000 platforms, enter the P5 Index Reverse Complement. For all other Illumina® platforms, enter the P5 Index in the first column. For additional information, please contact https://www.revvity.com/contact-us/technical-support.

APPENDIX B

NOVA-5132-32 (UDI 1-48)



NOVA-5132-41 (UDI 1-96)



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