NEXTFLEX® Rapid Directional RNA-Seq Kit 2.0

KIT CONTAINS: 8, 48, or 96 RXNS

USER MANUAL FOR:
#NOVA-5198-01
#NOVA-5198-02
#NOVA-5198-03

Compatible with Illumina® platforms

NEXTFLEX® Rapid Directional RNA-Seq Kit 2.0

This product is for research use only.

Not for use in diagnostic procedures.

GENERAL INFORMATION

Product Overview

The NEXTFLEX® Rapid Directional RNA-Seq kit 2.0 is designed to prepare directional, strand specific RNA libraries for sequencing using Illumina® sequencers. The NEXTFLEX® Rapid Directional RNA-Seq kit 2.0 streamlines the sample prep procedure required for single, paired-end, and multiplexed sequencing of RNA. The NEXTFLEX® Rapid Directional RNA-Seq kit 2.0 simplifies workflow by using master mixed reagents and magnetic bead-based cleanup, reducing pipetting and eliminating time consuming steps in library preparation. In addition, the availability of up to 384 unique barcode adapters upon request and gel-free size selection allows for high-throughput, multiplexed sequencing. The procedure is ideal for insert sizes of >150 bp.

Directionality is retained by adding dUTP during the second strand synthesis step as only the cDNA strand is sequenced. This kit contains the necessary reagents to process the user's purified RNA sample through preparation and amplification for sequencing.

Kit Contents, Storage & Shelf Life

There are six steps involved in preparing RNAs for sequencing: RNA extraction and enrichment, fragmentation, first strand and second strand synthesis, adenylation, adapter ligation, and PCR amplification.

The kit contains enough material to prepare 8, 48, or 96 RNA samples for Illumina® compatible sequencing. The shelf life of all reagents is 12 months when stored properly. The Nuclease-free Water and Resuspension Buffer can be stored at room temperature. The NEXTFLEX® Cleanup Beads XP should be stored at 4°C, and all other components should be stored at -20°C.

Kit Contents	Cap Color	Amount (8/48/96 rxn)	Storage Temp.
NEXTFLEX® RNA-Seq Fragmentation Buffer Mix 2.0	BROWN CAP	48/288/576 µL	-20°C
NEXTFLEX® RNA-Seq Directional First Strand Synthesis Buffer Mix 2.0	RED CAP	32/192/384 µL	-20°C
NEXTFLEX® RNA-Seq Rapid Reverse Transcriptase 2.0	RED CAP	8/48/96 µL	-20°C
NEXTFLEX® RNA-Seq Directional Second Strand Synthesis Mix 2.0	BLUE CAP	200/1200/(2) 1200 µL	-20°C
NEXTFLEX® RNA-Seq Adenylation Buffer Mix 2.0	CLEAR CAP	120/720/1440 µL	-20°C
NEXTFLEX® RNA-Seq Adenylation Enzyme 2.0	CLEAR CAP	24/144/288 µL	-20°C
NEXTFLEX® RNA-Seq Ligase Buffer Mix 2.0	LIGHT PURPLE CAP	356/(2) 1068/(4) 1068 µL	-20°C
NEXTFLEX® RNA-Seq Ligase Enzyme 2.0	LIGHT PURPLE CAP	24/144/288 µL	-20°C
NEXTFLE®X RNA-Seq PCR Master Mix 2.0	GREEN CAP	200/1200/(2) 1200 µL	-20°C
NEXTFLEX® RNA-Seq Primer Mix 2.0 (50 µM)	GREEN CAP	16/96/192 µL	-20°C
Nuclease-free Water	WHITE CAP	1.5/10*/20* mL	Room temp
Resuspension Buffer	WHITE CAP	1.5/9*/18* mL	Room temp
NEXTFLEX® Cleanup Beads XP	WHITE CAP	(2) 1.5/18*/35* mL	4°C

** reagents will be in WHITE CAP BOTTLES*

Required Materials Not Provided

- Options for Poly(A) enrichment
    - NEXTFLEX® Poly(A) Beads 2.0 (Cat #NOVA-512991, 512992, 512993)
- Options for rRNA Depletion
    - NEXTFLEX® RiboNaut™ rRNA Depletion Kits (Human, Mouse, Rat) (Cat #NOVA-512961, 512962, 512963)
- NEXTFLEX RNA-Seq 2.0 Unique Dual Index Barcodes (6.25 µM) (Cat #NOVA-51292X series)
- Total RNA, Poly(A) Enriched RNA, rRNA Depleted RNA
- Most NEXTFLEX® adapter barcodes of choice diluted to 6.25 µM starting concentration (optional)
- 80% Ethanol (stored at room temperature)
- 10, 20, 200 and 1000 µL pipettes
- RNase-free barrier pipette tips
- Nuclease-free 1.5 mL microcentrifuge tubes
- Thin wall nuclease-free 0.5 mL microcentrifuge tubes
- 96 well PCR Plate Non-skirted (Phenix Research™, # MPS-499) or similar
- Adhesive PCR Plate Seal (BioRad®, # MSB1001)
- Magnetic Stand (Thermo Fisher® Scientific, # AM10027) or similar for post PCR cleanup
- Microcentrifuge
- Thermal cycler
- Vortex

Optional Materials Not Provided

- For additional barcodes for increased multiplexing options, please contact NGS@revvity.com.

Warnings & 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 NGS@revvity.com

- Do not use the kit past the expiration date.
- The NEXTFLEX® RNA-Seq Directional First Strand Synthesis Buffer Mix 2.0 may appear yellow in color.
- The NEXTFLEX® RNA-Seq 2.0 UDI barcodes (NOVA-51292X series) are intended to be used with the NEXTFLEX® Rapid Directional RNA-Seq 2.0 Kit and are at a 6.25 µM starting concentration. Most NEXTFLEX® adapter barcodes are typically provided at 25 µM and would require different dilution recommendations if using together with the NEXTFLEX® Rapid Directional RNA-Seq 2.0 Kit. Inquire for more details at NGS@revvity.com.

- Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
- Ensure that all pipette tips, microcentrifuge tubes, and other consumables are RNase-free.
- DTT in buffers may precipitate after freezing. If precipitate is seen, vortex buffer for 1-2 minutes or until the precipitate is in solution. The performance of the buffer is not affected once precipitate is in solution.
- Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
- Vortex and micro-centrifuge each component prior to use, to ensure material has not lodged in the cap or the side of the tube.
- Do not remove enzymes from -20°C until immediately before use; return to -20°C immediately after use.
- Do not freeze NEXTFLEX® Cleanup Beads XP.
- Thermal cycling should be performed with a heated lid except where specified.
- Do not heat the NEXTFLEX® Adapters above room temperature.
- For multiplexing options, please use the appropriate NEXTFLEX® Adapters during STEP E: Adapter Ligation. Inquire at NGS@revvity.com.
- Vortex beads until they are a uniform suspension.
- Allow beads to come to room temperature for 30 minutes prior to use.
- Keep beads in liquid suspension during storage and handling. Never freeze the beads.
- Ensure beads pellet on magnet before removing clear supernatant. Completely remove all washing buffer before eluting RNA.
- Vortex and micro-centrifuge each component prior to use, to ensure material has not lodged in the cap or the side of the tube.

Revision History

Version	Date	Description
V19.06	June 2019	Early Market Release
V19.10	October 2019	Product Launch
V20.03	March 2020	Added optional stop points
V20.08	August 2020	Updated barcodes
V23.10	October 2023	Rebrand to Revvity

SAMPLE PREP SETUP

Starting Materials

The NEXTFLEX® Rapid Directional RNA-seq Kit 2.0 has been optimized and validated using poly(A) enriched, rRNA depleted RNA, and total RNA. 10 ng - 5 µg of total RNA is required if NEXTFLEX® Poly(A) Beads 2.0 are used to enrich for mRNA. The NEXTFLEX® RiboNaut™ rRNA Depletion Kit (Human / Mouse / Rat) has been optimized and validated using 5 ng - 1 µg of total RNA for RNA-seq applications. 5 ng - 1 µg of total RNA is required if the NEXTFLEX® RiboNaut™ rRNA Depletion Kit (Human / Mouse / Rat) is used to deplete rRNA from human, mouse, or rat total RNA. The NEXTFLEX® RiboNaut rRNA Depletion Kit (H/M/R) has been optimized and validated using 5 ng – 1 µg of total RNA from human, mouse and rat for RNA-seq applications.

We recommend examining total RNA integrity using a LabChip® GXII Touch HT instrument or equivalent. High quality total RNA preparations should have an RNA Quality Score (RQS)/RNA Integrity Number (RIN) greater than or equal to 7. Alternatively, total RNA may be run on a 1 - 2% agarose gel and integrity examined by staining with ethidium bromide. High quality RNA should have a 28S band that is twice as intense as the 18S band of ribosomal RNA. Lower amounts of starting material may result in higher duplication rates, reduced library complexity, and other changes in sequencing data quality. To request additional information, email NGS@revvity.com.

The NEXTFLEX® RNA-Seq 2.0 Unique Dual Index barcodes (6.25 µM) are recommended to be used with this kit. Other NEXTFLEX® adapters are typically at 25 µM and should be diluted down to stock concentration for RNA- seq at 6.25 µM. Please inquire for recommendations of which NEXTFLEX® adapter barcodes to use.

SAMPLE PREP WORKFLOW

FRAGMENTATION USING POLY (A) ENRICHED, rRNA DEPLETED, or TOTAL RNA

FIRST STRAND SYNTHESIS

SECOND STRAND SYNTHESIS

BEAD CLEANUP (Optional Stopping Point)

ADD 'A'

ADD ADAPTERS

LIGATION

BEAD CLEANUP (Optional Stopping Point)

BEAD CLEANUP

SAMPLE PREP PROTOCOL

Step A: RNA Fragmentation

MATERIALS

BROWN CAP - NEXTFLEX® RNA-Seq Fragmentation Buffer 2.0

WHITE CAP or WHITE CAP BOTTLE - Nuclease-free Water

User Supplied

- mRNA or rRNA-depleted RNA (Refer to Required Materials Not Provided, pg. 5)
- Nuclease-free microcentrifuge tube or plate
- Thermal cycler
- Ice

! NOTE: Fragmentation times are dependent on RQS/RIN. Always check total RNA RQS/ RIN using a LabChip® GXII Touch HT instrument or equivalent before starting library preparation and/or upstream poly(A) enrichment/rRNA depletion.

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

14 µL	RNA (in Nuclease-free Water or Elution Buffer)
6 µL	NEXTFLEX RNA-Seq Fragmentation Buffer Mix 2.0
20 µL	TOTAL

2. Mix thoroughly by pipetting.
3. Program a thermal cycler as follows:

RQS/RIN: 10 - 7		RQS/RIN: 6.99 - 3		RQS/RIN: < 3
15 min	94°C	10-12 min	94°C	Fragmentation not recommended
HOLD	4°C	HOLD	4°C	

4. Proceed to Step B: First Strand Synthesis.

Step B: First Strand Synthesis

MATERIALS

RED CAP - NEXTFLEX RNA-Seq Directional First Strand Synthesis Buffer Mix 2.0

RED CAP - NEXTFLEX RNA-Seq Rapid Reverse Transcriptase 2.0

User Supplied

- Fragmented RNA (from Step A)
- Thermal cycler
- Ice

! NOTE: 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). Take care to only pipette the necessary volume, as excess material on the exterior of the pipette tip may cause shortage.

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

20 µL	Fragmented RNA (from Step A)
4 µL	NEXTFLEX® RNA-Seq Directional First Strand Synthesis Buffer Mix 2.0
1 µL	NEXTFLEX® RNA-Seq Rapid Reverse Transcriptase 2.0
25 µL	TOTAL

2. Mix thoroughly by pipetting.
3. Program a thermal cycler as follows:

10 min	25°C
15 min	50°C
10 min	70°C
HOLD	4°C

4. Proceed to Step C: Second Strand Synthesis.

Step C: Second Strand Synthesis

MATERIALS

BLUE CAP - NEXTFLEX® RNA-Seq Directional Second Strand Synthesis Mix 2.0

WHITE CAP or WHITE CAP BOTTLE - Resuspension Buffer

WHITE CAP or WHITE CAP BOTTLE - NEXTFLEX® Cleanup Beads XP (room temp)

User Supplied

- First Strand Synthesis product (from Step B)
- Thermal cycler
- Ice
- Adhesive PCR Plate Seal
- 80% Ethanol, freshly prepared (room temperature)
- Magnetic Stand

1. For each reaction combine the following in a nuclease-free microcentrifuge tube or plate:

25 µL	First Strand Synthesis product (from Step B)
25 µL	NEXTFLEX® RNA-Seq Directional Second Strand Synthesis Mix 2.0 (contains dUTP)
50 µL	TOTAL

2. Mix thoroughly by pipetting.
3. Program a thermal cycler as follows:

60 min	16°C
HOLD	4°C

4. Incubate on the thermal cycler with above settings with heated lid turned off or left open.
5. Add 90 µL of well mixed NEXTFLEX® Cleanup Beads XP to each sample and mix thoroughly by pipetting.
6. Incubate for 5 minutes.
7. Place the plate on the magnetic stand for 5 minutes or until solution is clear.
8. Remove and discard supernatant taking care not to disturb the beads.
9. With plate on stand, add 200 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.

10. Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.
11. Remove plate from magnetic stand and resuspend bead pellet in 34 µL of Resuspension Buffer by pipetting volume up and down. Ensure that the beads are completely resuspended.
12. Incubate sample for 2 minutes.
13. Place the plate on the magnetic stand for 3 minutes or until the supernatant appears clear.
14. Transfer 32 µL of supernatant to a new PCR plate.
15. The procedure may be stopped at this point and the samples stored at -20°C.
16. Proceed to Step D: Adenylation.

Step D: Adenylation

MATERIALS

CLEAR CAP - NEXTFLEX® RNA-Seq Adenylation Buffer Mix 2.0

CLEAR CAP - NEXTFLEX® RNA-Seq Adenylation Enzyme 2.0

User Supplied

- Purified Second Strand Synthesis DNA (from Step C)
- Thermal cycler
- Ice

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

32 µL	Second strand synthesis DNA (from Step C)
15 µL	NEXTFLEX® RNA-Seq Adenylation Buffer Mix 2.0
3 µL	NEXTFLEX® RNA-Seq Adenylation Enzyme 2.0
50 µL	TOTAL

2. Mix thoroughly by pipetting.
3. Program a thermal cycler as follows:

30 min	65°C
HOLD	4°C

4. Proceed to Step E: Adapter Ligation.

Step E: Adapter Ligation

MATERIALS

LIGHT PURPLE CAP - NEXTFLEX® RNA-Seq Ligation Buffer Mix 2.0

LIGHT PURPLE CAP - NEXTFLEX® RNA-Seq Ligase Enzyme 2.0

WHITE CAP or WHITE CAP BOTTLE - Resuspension Buffer

WHITE CAP or WHITE CAP BOTTLE- NEXTFLEX® Cleanup Beads XP (room temp)

User Supplied

- 50 µL Adenylated DNA (from Step D)
- Thermal cycler
- Adhesive PCR Plate Seal
- 80% Ethanol, freshly prepared (room temperature)
- Magnetic Stand

Total RNA or Previously Enriched/Depleted RNA		
Previously Poly (A) enriched RNA, Previously rRNA depleted RNA, Total RNA, or FFPE Total RNA	Desired Adapter Concentration	Adapter Dilution Required
1 ng	0.3125 µM	1/20
10 ng	1.56 µM	1/4
50 ng	3.125 µM	1/2
100 ng	6.25 µM	None

Total RNA		
Total RNA enriched using NEXTFLEX Poly(A) Beads 2.0	Desired Adapter Concentration	Adapter Dilution Required
10 ng	0.104 µM	1/60
100 ng	0.3125 µM	1/20
1,000 ng	1.56 µM	1/4
5,000 ng	6.25 µM	None

Total RNA		
Total RNA depleted using NEXTFLEX RiboNaut rRNA Depletion Kit (H/M/R)	Desired Adapter Concentration	Adapter Dilution Required
5 ng	0.104 µM	1/60
100 ng	0.3125 µM	1/20
1,000 ng	3.125 µM	1/2

FFPE Total RNA		
FFPE Total RNA depleted using NEXTFLEX RiboNaut rRNA Depletion Kit (H/M/R)	Desired Adapter Concentration	Adapter Dilution Required
5 ng	0.104 µM	1/60
50 ng	0.104 µM	1/60

! IMPORTANT: The NEXTFLEX® Ligase Buffer Mix 2.0 is very viscous. Be sure to mix the following reaction until visibly homogenous by pipetting or brief vortexing.

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

50 µL	3′ Adenylated DNA (from Step D)
44.5 µL	NEXTFLEX RNA-Seq Ligation Buffer Mix 2.0*
2.5 µL	NEXTFLEX adapter barcodes (one unique barcode per sample, ensure proper concentration)
3.0 µL	NEXTFLEX RNA-Seq Ligase Enzyme 2.0*
100 µL	TOTAL

**These components can be premixed and added in a single step. Adapter should not be premixed in order to prevent excess adapter dimer formation.*

2. Mix thoroughly by pipetting.
3. Program a thermal cycler as follows:

15 min	20°C
HOLD	4°C

4. Incubate on the thermal cycler with above settings with heated lid turned off or left open.
5. Add 65 µL of Nuclease-free Water and 35 µL of well mixed NEXTFLEX® Cleanup Beads XP to each well containing sample. Mix thoroughly by pipetting.
6. Incubate for 5 minutes.
7. Place the plate on the magnetic stand for 5 minutes or until solution is clear.
8. Remove and discard supernatant, taking care not to disturb the beads.
9. Add 200 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.

10. Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.
11. Remove plate from magnetic stand and resuspend bead pellet in 50 µL of Resuspension Buffer by pipetting volume up and down. Ensure that the beads are completely resuspended.
12. Add 45 µL of well mixed NEXTFLEX® Cleanup Beads XP to each well containing sample. Mix thoroughly by pipetting.
13. Incubate for 5 minutes.
14. Place the plate on the magnetic stand for 5 minutes or until solution is clear.

15. Remove and discard supernatant.
16. Add 200 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.

17. Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.
18. Remove plate from magnetic stand and resuspend bead pellet in 25 µL of Resuspension Buffer by pipetting volume up and down. Ensure that the beads are completely resuspended.
19. Incubate sample for 2 minutes.
20. Place the plate on the magnetic stand for 3 minutes or until the supernatant appears clear.
21. Transfer 23 µL of supernatant to a new PCR plate.
22. The procedure may be stopped at this point and the samples stored at -20°C.
23. Proceed to Step F: PCR Amplification.

STEP F: PCR Amplification

MATERIALS

GREEN CAP - NEXTFLEX® RNA-Seq Primer Mix 2.0

GREEN CAP - NEXTFLEX® RNA-Seq PCR Master Mix 2.0

WHITE CAP or WHITE CAP BOTTLE - Resuspension Buffer

WHITE CAP or WHITE CAP BOTTLE - NEXTFLEX® Cleanup Beads XP (room temp)

User Supplied

- 23 µL Purified Adapter Ligated DNA (from Step E)
- Thermal cycler
- 96 Well PCR Plate
- 80% Ethanol, freshly prepared (room temperature)
- Magnetic Stand

Total RNA or Previously Enriched/Depleted RNA	
Previously Poly (A) enriched RNA, Previously rRNA depleted RNA, Total RNA, or FFPE Total RNA	PCR Cycles
1 ng	13–14
10 ng	10–11
50 ng	8–9
100 ng	7–8

Total RNA	
Total RNA enriched using NEXTFLEX® Poly(A) Beads 2.0	PCR Cycles
10 ng	16–17
100 ng	13–14
1,000 ng	9–10
5,000 ng	7-8

Total RNA	
Total RNA depleted using NEXTFLEX® RiboNaut rRNA Depletion Kit (H/M/R)	PCR Cycles
5 ng	16-17
100 ng	12–13
1,000 ng	8-9

FFPE Total RNA	
FFPE Total RNA depleted using NEXTFLEX® RiboNaut rRNA Depletion Kit (H/M/R)	PCR Cycles
5 ng	17-20
50 ng	14-17

❄

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

23 µL	Adapter Ligated DNA (from Step E)
25 µL	NEXTFLEX® RNA-Seq PCR Master Mix 2.0
2 µL	NEXTFLEX® RNA-Seq Primer Mix 2.0 (50 µM)
50 µL	TOTAL

2. Mix thoroughly by pipetting.
3. Program a thermal cycler as follows:

30 sec	98°C	*Repeat 7 - 20 cycles*
15 sec	98°C	*PCR cycles will vary depending on the amount of starting material and quality of your sample. Further optimization may be necessary. Always use the least number of cycles possible.*
30 sec	65°C	
30 sec	72°C	
2 min	72°C	

4. Add 45 µL of well mixed NEXTFLEX® Cleanup Beads XP to each well containing sample. Mix thoroughly by pipetting.
5. Incubate for 5 minutes.
6. Place the plate on the magnetic stand for 5 minutes or until solution is clear.
7. Remove and discard supernatant, taking care not to disturb the beads.
8. Add 200 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.

9. Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.
10. Remove plate from magnetic stand and resuspend bead pellet in 50 µL of Resuspension Buffer by pipetting volume up and down. Ensure that the beads are completely resuspended.
11. Add 45 µL of well mixed NEXTFLEX® Cleanup Beads XP to each well containing sample. Mix thoroughly by pipetting.
12. Incubate for 5 minutes.
13. Place the plate on the magnetic stand for 5 minutes or until solution is clear.
14. Remove and discard supernatant.
15. Add 200 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.

16. Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.

17. Remove plate from magnetic stand and resuspend bead pellet in 25 µL of Resuspension Buffer by pipetting volume up and down. Ensure that the beads are completely resuspended.
18. Incubate sample for 2 minutes.
19. Place the plate on the magnetic stand for 3 minutes or until the supernatant appears clear.
20. Transfer 23 µL of supernatant to a new PCR plate.
21. Examine library by electrophoresis to ensure proper library sizing and to verify exclusion of contaminating small and large fragments (recommended: LabChip® GXII Touch™ HT instrument (Revvity).

    The library is now ready for cluster generation per the standard Illumina protocol. Proceed to cluster generation or store at -20°C.

    qPCR quantification is recommended to quantify DNA library templates for optimal cluster density. This can be performed using any qPCR quantification kit for Illumina® platforms and NEXTFLEX® Primer Mix 2.0 as needed.

LIBRARY VALIDATION

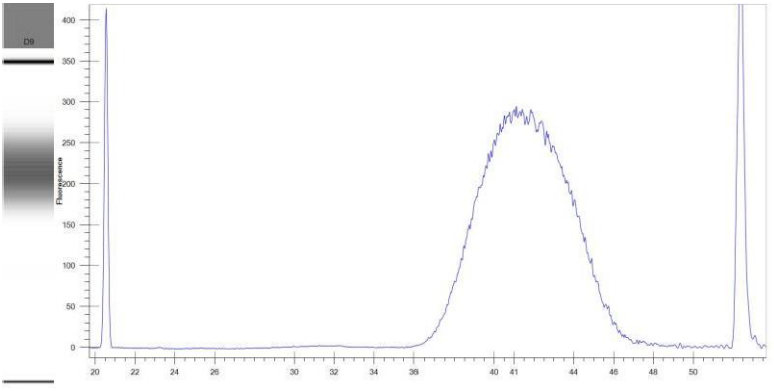

Figure 1: Example of Poly(A) enriched RNA-Seq library size distribution using 10 ng of Universal Human Reference RNA (Agilent® #740000). 3 µL of the library was run on the LabChip® GXII Touch™ HT instrument to verify size. Using a Qubit® 2.0 Fluorometer & Qubit® dsDNA HS Assay Kit, the concentration of the library was determined to be > 4 nM.

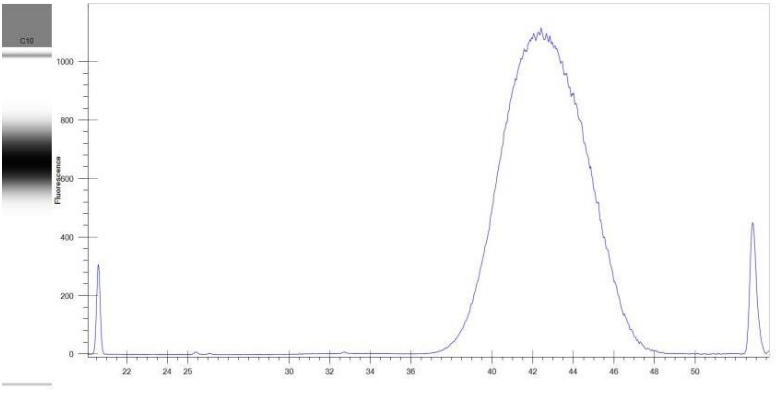

Figure 2: Example of rRNA depleted RNA-Seq library size distribution using 100 ng of of Universal Human Reference RNA (Agilent® #740000). 3 µL of the library was run on the LabChip® GXII Touch™ HT instrument to verify size. Using a Qubit® 2.0 Fluorometer & Qubit® dsDNA HS Assay Kit, the concentration of the library was determined to be > 4 nM.

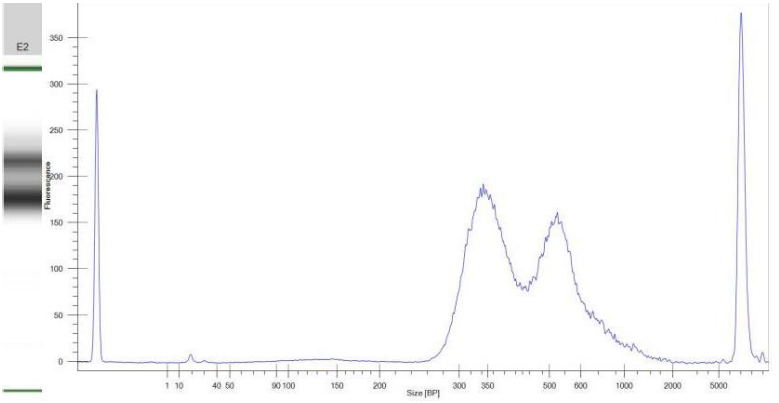

Figure 3: Example of rRNA depleted FFPE RNA-Seq library size distribution using 50 ng of FFPE total RNA (Biochain #R2234149). 3 µL of the library was run on the LabChip® GXII Touch™ HT instrument to verify size. Using a Qubit® 2.0 Fluorometer & Qubit® dsDNA HS Assay Kit, the concentration of the library was determined to be > 4 nM.

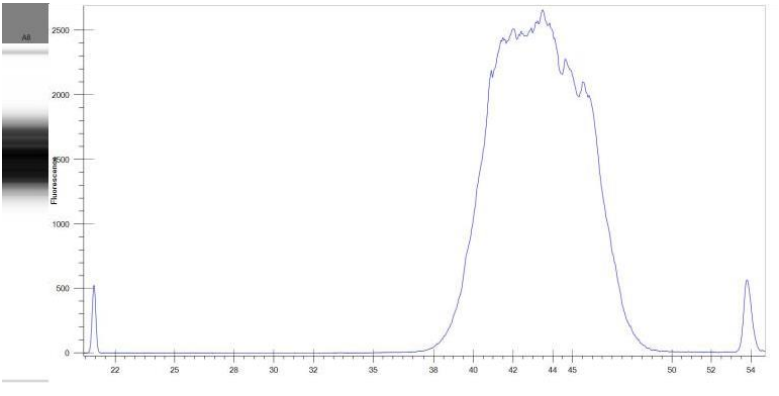

Figure 4: Example of unenriched/undepleted RNA-Seq library size distribution using 5 ng of Universal Human Reference RNA (Agilent® #740000). 3 µL of the library was run on the LabChip® GXII Touch™ HT instrument to verify size. Using a Qubit® 2.0 Fluorometer & Qubit® dsDNA HS Assay Kit, the concentration of the library was determined to be > 4 nM.

APPENDIX A

Oligonucleotide Sequences

NEXTFLEX	Sequence
PCR Primer 1	5'AATGATACGGCGACCACCGAGATCTACAC
PCR Primer 2	5'CAAGCAGAAGACGGCATACGAGAT

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