



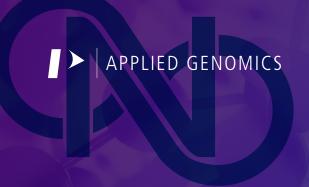
► APPLIED GENOMICS

USE MANUAL FOR:

*NOVA-5198-01 *NOVA-5198-02 *NOVA-5198-03

NEXTFLEX® Rapid Directional RNA-Seq Kit 2.0





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NEXTFLEX® Rapid Directional RNA-Seq Kit 2.0 NOVA-5198-01; -02; -03

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//// 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 at least 6 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
NEXTFLEX* RNA-Seq PCR Master Mix 2.0	GREEN CAP	$200/1200/(2)~1200~\mu L$	-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 CLEAR CAP BOTTLES			

^{*} reagents will be in CLEAR 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@perkinelmer.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@perkinelmer.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. The
 NEXTFLEX® RNA-Seq Barcodes (NOVA-51291X series) are not compatible with the NEXTFLEX®
 Rapid Directional RNA-Seq 2.0 Kit as they are at a starting concentration of 0.6 μM. 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@perkinelmer.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@perkinelmer.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



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. 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.

RNA should be free of DNA, divalent cations, salts such as guanidinium salts, organics such as phenol, reducing agents such as DTT, and chelators such as EDTA. We recommend examining total RNA integrity using a LabChip® GXII Touch HT instrument or equivalent. If total RNA has an RNA Quality Score (RQS)/RNA Integrity Number (RIN) that is greater than or equal to 7, then NEXTFLEX® Poly(A) Beads 2.0 or NEXTFLEX® RiboNaut™ rRNA Depletion Kit (Human, Mouse, Rat) should be used for poly(A) enrichment or rRNA depletion, respectively. If total RNA has an RQS/RIN that is less than 7, then NEXTFLEX® RiboNaut™ rRNA Depletion Kit (Human, Mouse, Rat) should be used for rRNA depletion. Alternatively, total RNA may be run on a 1 - 2% agarose gel and integrity examined by staining with ethidium bromide. High quality total RNA from mammalian cells 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@perkinelmer.com.

The NEXTFLEX® RNA-Seq 2.0 Unique Dual Index barcodes (6.25 $\mu M)$ 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.







MATERIALS

● BROWN CAP - NEXTFLEX® RNA-Seq Fragmentation Buffer 2.0 OWHITE CAP or CLEAR 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

Step A: RNA Fragmentation

- · 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.
- 缴 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 μΙ.	TOTAL

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

	RQS/R 10 -		RQS/RI 6.99 -		RQS/RIN: < 3
	15 min	94°C	10-12 min	94°C	Fragmentation no
_	HOLD	4°C	HOLD	4°C	recommended

4. Proceed to Step B: First Strand Synthesis.

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

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

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

10 min	25°C
15 min	50°C
10 min	70°C
HOLD	1°C

Proceed to Step C: Second Strand Synthesis.

40 MINS 101



Step C: Second Strand Synthesis

MATERIALS

● BLUE CAP - NEXTFLEX® RNA-Seq Directional Second Strand Synthesis Mix 2.0 ○ WHITE CAP or CLEAR CAP BOTTLE - Resuspension Buffer ○ WHITE CAP or CLEAR CAP BOTTLE - NEXTFLEX® Cleanup Beads XP (room temp)

STEP A

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.
- 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.
 - 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 μ L 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.

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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
- Ic



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

$32~\mu L$	Second strand synthesis DNA (from Step C)
15 μL	NEXTFLEX® RNA-Seq Adenylation Buffer Mix 2.0
$3\mu L$	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.

45 MINS



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
- OWHITE CAP or CLEAR CAP BOTTLE Resuspension Buffer
- OWHITE CAP or CLEAR CAP BOTTLE- NEXTFLEX® Cleanup Beads XP (room temp)

User Supplied

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

Total	RNA or Previously Enriched/Depleted F	RNA
Previously Poly (A) enriched RN Previously rRNA depleted RNA Total RNA, or FFPE Total RNA	A, Desired Adapter Concentrati	on Adapter Dilution Required
1 ng	0.3125 μΜ	1/20
10 ng	1.56 µM	1/4
50 ng	3.125 μΜ	1/2
100 ng	6.25 µM	None
Poly(A) enrich	ned total RNA using NEXTFLEX® Poly(A) Beads 2.0
Amount of total RNA used for poly(A) enrichment	Desired Adapter Concentration	Adapter Dilution Required
10 ng	0.104 μΜ	1/60
100 ng	0.3125 μΜ	1/20
1,000 ng	1.56 μΜ	1/4
5,000 ng	6.25 μΜ	None
rRNA depleted RNA	A using NEXTFLEX® RiboNaut™ rRNA D	epletion (H/M/R)
Amount of total RNA used for rRNA depletion	Desired Adapter Concentration	Adapter Dilution Required
5 ng	0.104 μΜ	1/60
100 ng	0.3125 μΜ	1/20
1,000 ng	3.125 μΜ	1/2
rRNA depleted degraded/F	FPE RNA using NEXTFLEX® RiboNaut™	rRNA Depletion (H/M/R)
Amount of FFPE/degraded total RNA used for rRNA depletion	Desired Adapter Concentration	Adapter Dilution Required
5 ng	0.104 μΜ	1/60
50 ng	0.104 μΜ	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.

If using NEXTFLEX® Poly(A) Beads 2.0 or NEXTFLEX® RiboNaut™ rRNA Depletion Kit (H/M/R) for enrichment/depletion and are going directly into NEXTFLEX Rapid Directional RNA-Seq Kit 2.0, use the full volume of recovered enriched/depleted RNA in Step A: Fragmentation (14 μ L). Then refer to these recommendations based on your total RNA input before poly(A) enrichment/rRNA depletion.

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STEP F

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)
$44.5~\mu L$	NEXTFLEX® RNA-Seq Ligation Buffer Mix 2.0*
2.5 μL	NEXTFLEX® adapter barcodes (one unique barcode per sample, ensure proper concentration)
$3.0~\mu\mathrm{L}$	NEXTFLEX® RNA-Seq Ligase Enzyme 2.0*
100 uL	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.

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

15 min	20°C
HOLD	4°C

- 3. Incubate on the thermal cycler with above settings with heated lid turned off or left open.
- 4. 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.
- 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.
- 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.
 - 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 μ L of Resuspension Buffer by pipetting volume up and down. Ensure that the beads are completely resuspended.
 - 11. Add 45 μ L 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 μ L 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 μ L of supernatant to a new PCR plate.
 - 21. The procedure may be stopped at this point and the samples stored at -20°C.
 - 22. Proceed to Step F: PCR Amplification.

40 MINS 52-6



Step F: PCR Amplification

MATERIALS

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

OWHITE CAP or CLEAR CAP BOTTLE - Resuspension Buffer

OWHITE CAP or CLEAR 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	
Poly(A) enriched total RNA using NEXTFLEX® Poly(A) Beads 2.0		
Amount of total RNA used for poly(A) enrichment	PCR Cycles	
10 ng	16–17	
100 ng	13–14	
1,000 ng	9–10	
5,000 ng	7-8	
rRNA depleted RNA using NEXTFLEX® RiboNaut™ rRNA Depletion (H/M/R)		
Amount of total RNA used for rRNA depletion	PCR Cycles	
5 ng	16-17	
100 ng	12–13	
1,000 ng	8-9	
rRNA depleted degraded/FFPE RNA using NEXTFLEX® RiboNaut™ rRNA Depletion (H/M/R)		
Amount of FFPE/degraded total RNA used for rRNA depletion	PCR Cycles	
5 ng	17-20	
50 ng	14-17	

If using NEXTFLEX® Poly(A) Beads 2.0 or NEXTFLEX® RiboNaut™ rRNA Depletion Kit (H/M/R) for enrichment/depletion and are going directly into NEXTFLEX® Rapid Directional RNA-Seq Kit 2.0, use the full volume of recovered enriched/depleted RNA in Step A: Fragmentation (14 μ L). Then refer to these recommendations based on your total RNA input before poly(A) enrichment/rRNA depletion.

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

$23~\mu L$	Adapter Ligated DNA (from Step E)
25 μL	NEXTFLEX® RNA-Seq PCR Master Mix 2.0
$2~\mu L$	NEXTFLEX® RNA-Seq Primer Mix 2.0 (50 $\mu M)$
50 μL	TOTAL

Rapid Directional RNA-Seq Kit 2.0 | *NOVA-5198-01; -02; -03

3 MINS

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

30 sec	98°C	_
15 sec	98°C)
30 sec	65°C	}
30 sec	72°C	J
2 min	72°C	

Repeat 7 - 20 cycles

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.

- 4. Add 45 μ L 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.
- 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 $50~\mu L$ of Resuspension Buffer by pipetting volume up and down. Ensure that the beads are completely resuspended.
 - 11. Add 45 μ L 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 μ L 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 (PerkinElmer).

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.

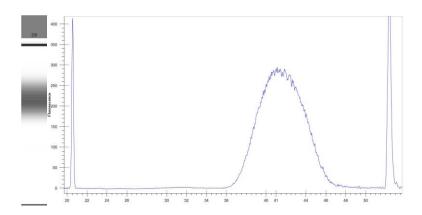


Figure 1: Example of Poly(A) enriched RNA-Seq library size distribution using 10 ng of Universal Human Reference RNA (Agilent* #740000). 3 µL of the library was run on the LabChip* GXII Touch™ HT instrument to verify size. Using a Qubit* 2.0 Fluorometer & Qubit* dsDNA HS Assay Kit, the concentration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

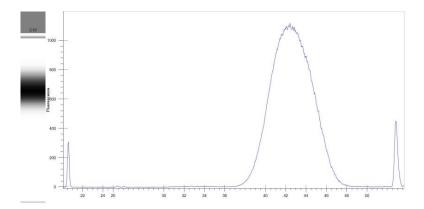


Figure 2: Example of rRNA depleted RNA-Seq library size distribution using 100 ng of of Universal Human Reference RNA (Agilent* #740000). 3 μ L of the library was run on the LabChip* GXII Touch* HT instrument to verify size. Using a Qubit* 2.0 Fluorometer & Qubit* dsDNA HS Assay Kit, the concentration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

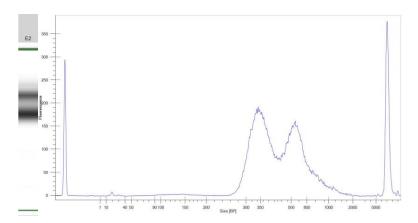


Figure 3: Example of rRNA depleted FFPE RNA-Seq library size distribution using 50 ng of FFPE total RNA (Biochain #R2234149). 3 µL of the library was run on the LabChip® GXII Touch™ HT instrument to verify size. Using a Qubit® 2.0 Fluorometer & Qubit® dsDNA HS Assay Kit, the concentration of the library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

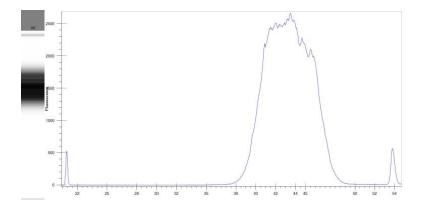


Figure 4: Example of unenriched/undepleted RNA-Seq library size distribution using 5 ng of Universal Human Reference RNA (Agilent* #740000). 3 μ L of the library was run on the LabChip* GXII Touch* HT instrument to verify size. Using a Qubit* 2.0 Fluorometer & Qubit* dsDNA HS Assay Kit, the concentration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



//// Oligonucleotide Sequences

NEXTFLEX®	Sequence
PCR Primer 1	S'AATGATACGGCGACCACCGAGATCTACAC
PCR Primer 2	S'CAAGCAGAAGACGGCATACGAGAT



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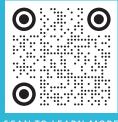
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