NEXTFLEX® Rapid XP V2 DNA-Seq kit

(For Illumina® Platforms)

KIT CONTAINS : 8, 48 or 96 RXNS

USER MANUAL FOR :
#NOVA-5149-21
#NOVA-5149-22
#NOVA-5149-23

NEXTFLEX® Rapid XP V2 DNA-Seq kit

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

GENERAL INFORMATION

Product Overview

The NEXTFLEX® Rapid XP v2 DNA-Seq kit is designed for ~4 hours library construction and pooling from 100 pg – 1 µg of DNA. The kit can be used to prepare single, paired-end, and multiplexed DNA libraries for sequencing using Illumina® platforms. The NEXTFLEX® 1-step Fragmentation, End-Repair, and Adenylation simplifies workflow and shortens hands-on library construction time. In addition, the availability of up to 1,536 different Unique Dual Index adapter barcodes facilitates high-throughput applications.

There are three main steps involved in preparing DNA for sequencing: DNA fragmentation/end repair/adenylation, adapter ligation, and post-adapter ligation PCR amplification. The NEXTFLEX Rapid XP v2 DNA-Seq kit contains the necessary material to take the user's extracted nucleic acid sample through preparation and amplification for loading onto flow cells for sequencing.

Kit Contents, Storage & Shelf Life

The NEXTFLEX® Rapid XP v2 DNA-Seq kit contains enough material to prepare 8, 48 or 96 samples for Illumina® sequencing. The shelf-life of all reagents is at least 6 months when stored properly. The Nuclease-free Water, NEXTFLEX Conditioning solution V2 and Elution Buffer can be stored at room temperature. The NEXTFLEX Cleanup Beads XP and NEXTFLEX Normalization Beads V2 should be stored at 4°C, and all other components should be stored at -20°C.

Kit Contents	8 rxn	48 rxn	96 rxn
NEXTFLEX® Fragmentation Buffer V2	24 µL	144 µL	288 µL
NEXTFLEX® Fragmentation Enzyme V2	20 µL	120 µL	240 µL
NEXTFLEX® Ligation Master Mix V2	80 µL	480 µL	960 µL
NEXTFLEX® PCR Master Mix V2	100 µL	600 µL	1.2 mL
NEXTFLEX® Primer Mix V2	20 µL	120 µL	240 µL
NEXTFLEX® Conditioning solution V2	8 µL	48 µL	96 µL
Nuclease-free Water	1.5 mL	(2) 1.5 mL	6 mL
Elution Buffer	184 µL	1.2 mL	3 mL
NEXTFLEX® Cleanup Beads XP	1 mL	3 mL	5 mL
NEXTFLEX® Normalization Beads V2	872 µL	6 mL	11 mL

Required Materials Not Provided

- 100 pg - 1 µg of DNA in up to 17.5 µL nuclease-free water
- If multiplexing: NEXFLEX® Unique Dual Index Barcodes (Cat # 514150, 514151, 514152, 514153) or 1,536 NEXFLEX® Unique Dual Index Barcodes (Cat # 534100)
- Ethanol 100% (room temperature)
- 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
- Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
- Magnetic Stand -96 (Thermo Fisher Scientific, Cat # AM10027) or similar
- Thermal Cycler
- 2, 10, 20, 200 and 1000 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips
- Vortex

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, contact us at NGS@revvity.com

- Do not use the kit past the expiration date.
- Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
- Do not remove enzymes from -20°C until immediately before use; return to -20°C immediately after use.
- This kit does not contain Barcoded Adapter. To enable multiplexing, please use the appropriate concentration of the NEXTFLEX® barcoded adapters during the Adapter Ligation step.
- Do not freeze NEXTFLEX® Cleanup Beads XP or NEXTFLEX® Normalization Beads V2.
- Vortex beads until they are a uniform suspension.
- Thermal cycling should be performed with a heated lid (103°C-105°C) except where specified.
- Maintain a laboratory temperature of 20°–25°C (68°–77°F).
- DNA sample quality may vary between preparations. It is the user's responsibility to utilize high quality DNA. DNA that is heavily nicked or damaged may impact results or cause library preparation failure. Absorbance measurements at 260 nm are commonly used to quantify DNA and 260 nm / 280 nm ratios of 1.8 - 2.0 usually indicate relatively pure DNA. The user should be aware that contaminating RNA, nucleotides, and single-stranded DNA may affect the amount of usable DNA in a sample preparation.
- Presence of EDTA in starting input DNA can alter final library size. For optimal results, input DNA should be resuspended in ultrapure water or low TE buffer.

Revision History

Version	Date	Description
V22.06	June 2022	Product Launch
V22.08	August 2022	Manual Update
V22.11	November 2022	Added PCR-Free Recommendations Updated Fragmentation Volumes Updated Ligation times
V23.02	February 2023	Manual Update
V23.08	August 2023	Addition of Conditioning Solution
V23.10	October 2023	Rebranded to Revvity

SAMPLE PREP WORKFLOW

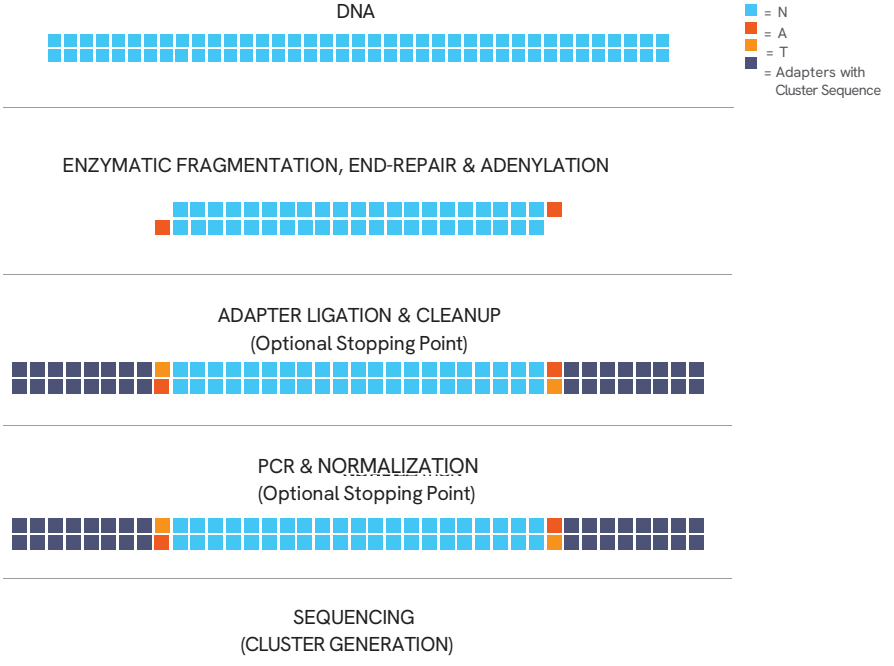


Figure 1. Sample flow chart showing the different steps of the protocol.

LIBRARY PREP PROTOCOL

Starting Material

The NEXTFLEX® Rapid XP V2 DNA-Seq kit has been optimized and validated using high quality genomic DNA inputs ranging from 100 pg - 1 µg.

If using the kit in PCR-Free workflow, the starting input is ≥100ng of high-quality genomic DNA. Follow step A and step B until Post Ligation Clean-up. For PCR-Free final clean-up, refer to Appendix A (PCR-Free Workflow Post-Ligation Clean-up).

Reagent Preparation

1. Briefly spin down each component to ensure material is not lodged in the cap or side of tube. Keep on ice and vortex each NEXTFLEX® component except NEXTFLEX® Fragmentation Enzyme Mix, NEXTFLEX® Ligation Master Mix V2, and NEXTFLEX® PCR Master Mix V2 prior to use. Nuclease-free Water and Elution Buffer should be stored at room temperature. NEXTFLEX® Cleanup Beads XP and NEXTFLEX® Normalization Beads V2 should be stored at 4°C but equilibrated to room temperature prior to use.
2. 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 the precipitate is in solution.
3. The 80% ethanol for bead washing steps should be freshly made prior to use.
4. Allow NEXTFLEX® Cleanup Beads XP to come to room temperature and vortex the beads until homogenous.
5. NEXTFLEX® Normalization Beads V2 preparation.

! NOTE: NEXTFLEX® Normalization Beads V2 should be made for immediate use. Once 100% EtOH has been added to NEXTFLEX® Normalization Beads V2, they should be used within 2 weeks.

SAMPLE PREP PROTOCOL

STEP A: Fragmentation, End-repair & Adenylation

MATERIALS

- NEXTFLEX® Fragmentation Buffer V2
- NEXTFLEX® Fragmentation Enzyme V2
- NEXTFLEX® Conditioning Solution V2 (See Appendix A)

User Supplied

- DNA in 19.5 µL (or less) nuclease-free water
- Thermal Cycler
- 96 well PCR Plate
- Adhesive PCR Plate Seal
- Microcentrifuge

! NOTE: The presence of EDTA in the DNA sample can result in larger sized fragments in the final library. We strongly recommend the removal of EDTA from the sample prior to fragmentation. However, if removal is not possible, this effect can be partially mitigated by increasing the fragmentation time or following the recommendations in Appendix A.

1. Set the thermocycler to 4°C paused while performing the following steps:
2. For each sample, combine the following reagents in a nuclease-free 96 well PCR plate:

_ µL	Nuclease-free water
_ µL	DNA (100 pg - 1 µg)
3 µL	NEXTFLEX® Fragmentation Buffer V2
22.5 µL	TOTAL

3. Transfer plate to thermal cycler at 4°C (paused).
4. Leave plate for 30 seconds in thermal cycler at 4°C.
5. Add 2.5 µL of NEXTFLEX Fragmentation Enzyme V2, pipette 10 times with pipette set to 17.5 µL.

! NOTE: Do NOT vortex the final NEXTFLEX® Fragmentation reaction. Mix by pipette only. Apply adhesive PCR plate seal and incubate on a thermal cycler using the following program:

1 min	4°C
See fragmentation table	35°C
30 min	65°C
hold	4°C

The following table lists the recommended incubation times as a guideline for fragmentation. The mode fragment size can be adjusted by changing the duration of incubation at this 35 °C step. These times are recommendations only, and incubation time may need to be optimized for different sample inputs and types to obtain desired mode fragment size.

Input DNA (ng)	Fragmentation Time (min) at 35°C
0.1 - 99	10
100 - 1,000	5

NOTE: The final library size will be approximately 120 bp larger than the fragment size.

6. Proceed immediately to Step B.

STEP B: Adapter Ligation

MATERIALS

- NEXTFLEX® Ligation Master Mix V2
- NEXTFLEX® Cleanup Beads XP
- NEXTFLEX® Unique Dual Index Barcodes Plate
- Nuclease-free Water

User Supplied

- 25 µL of Fragmented, End Repaired, and Adenylated DNA (from STEP A)
- Thermal Cycler
- Adhesive PCR Plate Seal
- 80% Ethanol, freshly prepared (room temperature)
- Magnetic Stand

Adapter Ligation

1. Invert the Ligation Master Mix 5 times to homogenize (DO NOT VORTEX) and place on ice.
2. Each sample will require 2.5 µL of barcoded adapter to be added. Combine the following in the PCR plate and mix thoroughly by pipette:

   *For starting input ≤10 ng, dilute NEXTFLEX® Barcoded Adapter 1:10 with ultrapure water.

25 µL	Fragmented, End Repaired & Adenylated DNA (from Step A)
10 µL	NEXTFLEX® Ligation Master Mix V2
2.5 µL	NEXTFLEX® Barcoded Adapter*
37.5 µL	TOTAL

   ! Adapter should not be premixed to prevent excess adapter dimer formation.

3. Apply adhesive PCR plate seal and run the following program on a thermal cycler with heated lid turned off or open:

30-60 min	20°C	*60 minutes produces optimal ligation efficiency
hold	4°C	

Post-Ligation Clean-up

! NOTE: If performing PCR-Free workflow, proceed to Appendix A (PCR-Free Workflow Post- Ligation Clean-up).

1. Remove plate from thermal cycler.
2. Add 30 µL of NEXTFLEX® Cleanup Beads XP to the 37.5 µL of Adapter Ligated DNA.
3. Incubate at room temperature for 5 minutes.
4. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
5. Remove and discard clear supernatant, taking care not to disturb beads. Some liquid may remain in well.
6. With the plate on the stand, add 200 µL of 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully remove ethanol by pipette.
7. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
8. With the plate on the magnetic stand, let beads air dry at room temperature for 3 minutes. Do not over dry beads or yield may be negatively impacted.
9. Resuspend dried beads with 12 µL of Nuclease-Free Water. Mix thoroughly until homogenized.

   If with the bead pellet is difficult to resuspend at this volume, increase resuspension volume to 20 µl of Nuclease-Free Water, transfer 10 µL in step 12 and increase PCR by 1 cycle. To accommodate for the lost material, use the maximum recommend PCR cycles for the starting input.
10. Incubate sample at room temperature for 2 minutes.
11. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
12. Do not discard the sample in this step. Transfer 10 µL of clear sample to a new well. Remove the 96 well PCR plate from the magnetic stand.
13. Proceed immediately to Step C.

STEP C: Post-Ligation PCR

MATERIALS

- NEXTFLEX® PCR Master Mix V2
- NEXTFLEX® Primer Mix V2

User Supplied

- 10 µL of Adapter Ligated DNA (from STEP B)
- Thermal Cycler
- Adhesive PCR Plate Seal
- 96 Well PCR Plate
- 80% Ethanol, freshly prepared (room temperature)
- Magnetic Stand

! *Thaw NEXTFLEX® PCR Master Mix V2 on ice. Once thawed invert several times or swirl to vigorously mix (DO NOT VORTEX).

1. For each sample, combine the following reagents on ice in the PCR plate. Mix thoroughly.

10 µL	Adapter Ligated DNA (from Step B)
12.5 µL	NEXTFLEX® PCR Master Mix V2*
2.5 µL	NEXTFLEX® Primer Mix V2*
25 µL	TOTAL

*These components can be premixed and added in a single step.

2. Apply adhesive PCR plate seal and place in thermal cycler for the following PCR cycles:

Time	Temperature	Cycles
45 sec	98°C	1
15 sec	98°C	
30 sec	60°C	See Table
30 sec	72°C	
1 min	72°C	1
HOLD	12°C	1

Genomic DNA input (ng)	PCR Cycles for bead-based normalization
0.1	13-15
0.5	12-14
0.75	11-13
1	9-11
5	7-9
10	6-8
50	4-6
100	3-5
500	2-4
1,000	2

*Expected yield is ~100-500ng, depending on the number of cycles and quality of input genomic DNA

3. Post-PCR, proceed to either Step D Bead-Based Library Normalization OR Step E Clean Up Beads.

STEP D: Bead-Based Library Normalization

MATERIALS

- NEXTFLEX® Normalization Beads V2
- Elution Buffer

! Reagent Preparation: 100% Ethanol must be added to the NEXTFLEX® Normalization Beads V2 prior to use. Only prepare enough beads for the number of library preparation reactions.

User Supplied

- 25 µL of Amplified Library (STEP C)
- 100% Ethanol (room temperature)
- 80% Ethanol, freshly prepared (room temperature)
- Magnetic Stand

1. Add 184 µL of NEXTFLEX® Normalization Beads V2 to each well containing supernatant. Mix thoroughly until homogenized.

   ! Ensure 100% Ethanol has been added to the NEXTFLEX® Normalization Beads V2. (See Reagent Preparation)
2. Incubate at room temperature for 8 minutes.
3. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes until the supernatant appears completely clear.
4. Remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in wells.
   Supernatant containing non-normalized library can be transferred to a clean tube and frozen. The tube may be thawed and purified for additional recovery. Contact NGS@revvity.com for assistance.
5. With plate on stand, add 200 µL of 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully remove ethanol by pipette.
6. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.

   *To aid in ethanol removal, use a clean set of p20 multichannel pipette tips, set the p20 volume to 15 µL and carefully remove any residual ethanol from the bottom of the tube.
7. With plate on magnetic stand, air dry beads at room temperature for 3 minutes.
8. Resuspend dried beads with 23 µL of Elution Buffer.
9. Mix thoroughly until homogenized.
10. Incubate resuspended beads at room temperature for 2 minutes.
11. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
12. Do not discard the supernatant in this step. Transfer 20 µL of clear sample to a new well.
13. Remove the 96 well PCR plate from the magnetic stand and discard.

14. Pool 5 µL of each eluted sample into single Pooled Library tube (1.5 mL tube). Library quantification of pooled material can be performed using fluorometric methods [recommended: Qubit] to determine concentration.

    ! NOTE: qPCR is recommended to quantify DNA library template for optimal cluster density. This can be performed using any qPCR quantification kit for Illumina platforms and the NEXTFLEX® Primer Mix V2 as needed.

15. Examine your single pooled library sample by electrophoresis to ensure proper library sizing [recommended: LabChip® GX Touch instrument (Revvity)].

    ! NOTE: Final pooled library size should be close to 425bp. If user does not have electrophoresis capabilities, this size may be used.

16. The library is now ready for cluster generation per the standard Illumina® protocol. Proceed to cluster generation or seal with adhesive PCR plate seal and store at -20 °C.

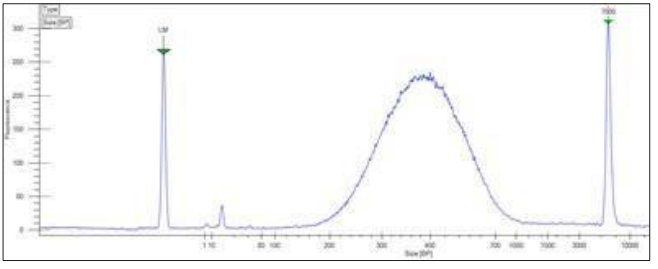
STEP E: Clean Up Beads

1. Add 20 µL of NEXTFLEX Cleanup Beads XP to the 25 µL of PCR-amplified libraries.
2. Incubate at room temperature for 5 minutes.
3. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
4. Remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in well.
5. With the plate on the stand, add 200 µL of 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully remove ethanol by pipette.
6. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
7. With the plate on the magnetic stand, let beads air dry at room temperature for 3 minutes. Do not over dry beads or yield may be negatively impacted.
8. Resuspend dried beads with 22 µL of Nuclease-Free Water. Mix thoroughly until homogenized.
9. Incubate sample at room temperature for 2 minutes.
10. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
11. Do not discard the sample in this step. Transfer 20 µL of clear sample to a new well. Remove the 96 well PCR plate from the magnetic stand.
    Quantification of each library can be performed using fluorometric methods [recommended: Qubit] to determine concentration.

    ! NOTE: qPCR is recommended to quantify DNA library template for optimal cluster density. This can be performed using any qPCR quantification kit for Illumina platforms and the NEXTFLEX Primer Mix as needed.
12. Examine your libraries by electrophoresis to ensure proper library sizing [recommended: LabChip® GX Touch instrument (Revvity)].

    ! NOTE: Library size should be close to 425bp. If user does not have electrophoresis capabilities, this size may be used.
13. The library is now ready for cluster generation per the standard Illumina® protocol. Proceed to cluster generation or seal with adhesive PCR plate seal and store at - 20 °C.

Library Validation



Libraries were analyzed using the LabChip® GX Touch™ instrument (Revvity).

APPENDIX A

PCR-Free Workflow Post-Ligation Clean-up

1. Remove plate from thermal cycler.
2. For starting inputs of 100ng-500ng, add 30 µL of NEXTFLEX® Cleanup Beads XP to the 37.5 µL of Adapter Ligated DNA proceed to step 3. For inputs ≥500ng proceed to step 2A.
    A. Add 17 µL of NEXTFLEX® Cleanup Beads XP to the 37.5 µL of Adapter Ligated DNA.
    B. Incubate at room temperature for 5 minutes.
    C. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
    D. Transfer supernatant to a new clean tube.
    E. Add 13 µL of NEXTFLEX® Cleanup Beads XP to the 54.5 µL of transferred supernatant.
3. Incubate at room temperature for 5 minutes.
4. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
5. With the plate on the stand, carefully remove and discard supernatant.
6. With the plate on the stand, add 200 µL of freshly made 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully remove ethanol by pipette.
7. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
8. With the plate on the magnetic stand, let beads air dry at room temperature for 3 minutes. Do not over dry beads or yield may be negatively impacted.
9. Resuspend dried beads with 22 µL of Nuclease-Free Water. Mix thoroughly until homogenized.
10. Incubate sample at room temperature for 2 minutes.
11. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
12. Do not discard the sample in this step. Transfer 20 µL of clear sample to a new well.
13. Proceed to library QC steps.

*PCR-Free libraries do not migrate accurately on the LabChip®/Bioanalyzer. For accurate sizing, a portion of the samples can be run through the PCR module (Step C) using 2 cycles. Alternatively, use 500bp as the default size.

*PCR-Free libraries require qPCR for accurate quantification.

Conditioning Solution for samples containing EDTA

If removal of EDTA is not possible, 1 µL of Conditioning Solution can be added to the fragmentation reaction of DNA samples containing up to 1 mM EDTA. Adding 1 µL of Conditioning Solution V2 will result in libraries within 200 bp of the desired fragment size. To achieve a more precise library size, further optimization of fragmentation time be required.

An example of adding Conditioning Solution into Step A:

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

_ µL	Nuclease-free water
_ µL	DNA (100 pg - 1 µg)
1 µL	NEXTFLEX® Conditioning Solution V2
3 µL	NEXTFLEX® Fragmentation Buffer V2
22.5 µL	TOTAL

Then proceed as stated in Step A.

APPENDIX B

Oligonucleotide Sequences

NEXTFLEX	Sequence (5'-3')
PCR Primer 1	AATGATACGGCGACCACCGAGATCTACAC
PCR Primer 2	CAAGCAGAAGACGGCATACGAGAT

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