NEXTFLEX® Cell Free DNA-Seq Kit 2.0

(For Illumina® & Element® platforms)

KIT CONTAINS : 8 or 48 reactions

USER MANUAL FOR :
#NOVA-5150-01
#NOVA-5150-02

NEXTFLEX® Cell Free DNA-Seq Kit 2.0

This product is for research use only.

Not for use in diagnostic procedures.

GENERAL INFORMATION

Product Overview

Cell free DNA has become a powerful marker in clinical research applications due to the unique origin of DNA molecules present in plasma or serum. Detection of fetal DNA from maternal plasma has proven to be a viable, non-invasive option to identify a variety of fetal traits including sex determination, sex chromosome-linked disorders and aneuploidy events. Circulating tumor DNA extracted from plasma of symptomatic patients can be used as a non-invasive resource for research towards diagnosis, prognosis, treatment decisions, and follow-up monitoring of cancer patients. If performing quantitative sequencing applications such detecting low-frequency variants or rare somatic mutations, consider the use of our NEXTFLEX UDI-UMI Barcodes.

The NEXTFLEX® Cell Free DNA-Seq Kit 2.0 is designed for 3-hour DNA library construction of cell free fetal or circulating tumor DNA. The kit can be used to prepare single, paired end and multiplexed DNA libraries for sequencing using Illumina® and Element® platforms. NEXTFLEX® 1-step End-Repair and Adenylation protocol simplifies workflow and shortens hands-on library construction time. In addition, the availability of up to 384 unique adapter barcodes facilitates high-throughput applications.

There are three main steps involved in preparing cell free DNA for sequencing: DNA End Repair & Adenylation, Adapter Ligation and PCR Amplification. The optional Gel-Free Nucleosome Enrichment step, performed before End Repair & Adenylation, is designed to enrich for mono-nucleosomes, di-, and tri-nucleosomes. The NEXTFLEX® Cell Free DNA-Seq Kit 2.0 contains the necessary material, except barcodes, to take the user's purified cell free DNA through library preparation and amplification for loading onto Illumina and Element® flow cells for sequencing. The NEXTFLEX® Cell Free DNA-Seq Kit 2.0 is intended for research use only.

Kit Overview

The NEXTFLEX® Rapid DNA-Seq Kit 2.0 contains enough material to prepare 8, or 48 Cell Free DNA samples for Illumina® and Element® compatible sequencing.

Contents, Storage and Shelf Life

The shelf life of all reagents is at least 6 months when stored properly. The Resuspension Buffer and Nuclease-free Water can be stored at room temperature. The NEXTFLEX® cfDNA Enrichment Beads and Cleanup Beads 2.0 should be stored at 4°C, and all other components should be stored at -20°C.

Kit Contents	Amount
BROWN CAP	
NEXTFLEX® cfDNA Enrichment Beads	1.5 / 8* mL
CLEAR CAP	

NEXTFLEX® End-Repair & Adenylation Buffer Mix 2.0	120 / 720 µL
NEXTFLEX® End-Repair & Adenylation Enzyme Mix 2.0	24 / 144 µL

PURPLE CAP	
NEXTFLEX® Ligase Buffer Mix 2.0	356 / (4) 534 µL
NEXTFLEX® Ligase Enzyme 2.0	24/ 144 µL

GREEN CAP	
NEXTFLEX® PCR Master Mix 2.0	200 / 1200 µL
NEXTFLEX® Primer Mix 2.0 (50 µM)	16 / 96 µL

WHITE CAP	
Nuclease-free Water	(2) 1.5 / 10* mL
Resuspension Buffer	1.5 / 6* mL
NEXTFLEX® Cleanup Beads 2.0	1.5 /* 7 mL

*Reagents well be in WHITE CAP BOTTLES

Required Materials Not Provided

- 10 ng of cell free DNA in up to 32 µL nuclease-free water. Cell free DNA can be in up to 50 µL nuclease-free water if performing optional size selection step.
- NEXFLEX® Unique Dual Index Barcodes (Cat #NOVA-514150, NOVA-514151, NOVA-514152, NOVA-514153, NOVA-534100, NOVA-534101, NOVA-534102, NOVA-534103, NOVA-534104).
- NEXFLEX® UDI-UMI Barcodes (NOVA-734100, NOVA-734101, NOVA-734102, NOVA-734103
- Ethanol 100% (room temperature) Ethanol 80% (room temperature)
- 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
- 96 well Library Storage and Pooling Plate (Fisher Scientific, Cat # AB-0765) or similar Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
- Magnetic Stand -96 (Ambion, Cat # AM10027) / or / similar Thermal cycler
- 2, 10, 20, 200 and 1000 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips

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.
- 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.
- Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
- Do not heat the DNA Adapter above room temperature.
- To enable multiplexing, please use the appropriate combination of DNA Barcodes during the Adapter Ligation step.
- Maintain a laboratory temperature of 20°–25°C (68°–77°F).
- Cell free DNA sample quality may vary between preparations. It is highly recommended fluorescent dyes be used as a means for cell free DNA sample quantification, as the NanoDrop cannot accurately detect nucleic acids at concentrations found in pure cell free DNA sample preps (0.05 ng/µL to 1 ng/µL). The user should be aware that contaminating RNA, nucleotides, and single-stranded DNA may affect the amount of usable DNA in a sample preparation.
- It is highly recommended that NEXTFLEX® Primer Mix be used during PCR amplification. Inadvertent use of an incorrect primer sequence can potentially result in elimination of the index.

Revision History

Version	Date	Description
V14.07	July 2014	Initial Product Launch
V14.09	September 2014	Added Optional Gel-Free Size Selection
V19.02	February 2019	Updated product name and optional Gel-Free Size Selection to Nucleosome Enrichment protocol. Updated core chemistries for an improved library prep experience.
V22.08	August 2022	Manual Update
V23.11	November 2023	Rebrand to Revvity
V25.07	July 2025	Manual Update

NEXTFLEX® CELL FREE DNA-SEQ 2.0 SAMPLE PREPARATION PROTOCOL

NEXTFLEX® Cell Free DNA-Seq 2.0 Flow Chart

Figure 1: Sample flow chart with approximate times necessary for each step.

Starting Material

The NEXTFLEX® Cell Free DNA-Seq Kit 2.0 has been optimized and validated using 10 ng of cell free DNA.

Reagent Preparation

1. Briefly spin down each component to ensure material has not lodged in the cap or side of tube. Keep on ice and vortex each NEXTFLEX® component just prior to use. The Resuspension Buffer and Nuclease-free Water can be stored at room temperature.
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. Allow NEXTFLEX® Enrichment and NEXTFLEX® Cleanup Beads to come to room temperature and vortex the beads until homogenous.

Gel-Free Nucleosome Enrichment (Optional)

This optional size selection step is designed to isolate mono-nucleosomes or mono-, di-, and tri-nucleosomes. Users who are not interested in enriching for mono-, di-, and tri-nucleosomes may begin the protocol at Step A: End-Repair and Adenylation. A minimum 10ng of cfDNA input is required for enrichment.

Materials

Revvity Supplied

WHITE CAP or WHITE CAP BOTTLE Resuspension Buffer

BROWN CAP or WHITE CAP BOTTLE NEXTFLEX® cfDNA Enrichment Beads

User Supplied

Cell free DNA in 50 µL or less Nuclease-free Water 96 well PCR Plate

Adhesive PCR Plate Seal

80% Ethanol, freshly prepared (room temperature) Magnetic Stand

Desired Enrichment	Mono-nucleosomes	Mono-, di-, and tri-nucleosomes
Bead Volume #1	45	30
Bead Volume #2	60	75

If sample volume is < 50 µL, bring to 50 µL with Nuclease-free Water

1. Add Bead Volume #1 to 50 µL cell-free DNA samples as indicated in the corresponding column for your desired enrichment. Mix thoroughly until homogenized by pipetting up and down.
4. Incubate sample at room temperature for 5 minutes.
5. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes or until the supernatant appears completely clear.
6. Do not discard the supernatant in this step. Transfer the clear supernatant to a new well. Be careful not to disrupt the magnetic bead pellet or transfer any magnetic beads with the sample.
7. Add Bead Volume #2 to samples as indicated in the corresponding column for your desired enrichment. Mix thoroughly until homogenized by pipetting up and down.
8. Incubate sample at room temperature for 5 minutes.
9. Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes or until the supernatant appears completely clear.
10. Remove and discard the clear supernatant, taking care not to disturb the beads. Some liquid may remain in the wells.
11. With the 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 the ethanol by pipette.
12. Repeat the previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.

13. Remove the 96 well PCR plate from the magnetic stand and let dry at room temperature for 3 minutes.
14. Resuspend dried beads with 34 µL Resuspension Buffer. Mix thoroughly until homogenized by pipetting up and down.
15. Incubate sample at room temperature for 2 minutes.
16. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until sample is clear.
17. Do not discard the sample (supernatant) in this step. Transfer 32 µL of clear sample to a new well.
18. Proceed to Step A: End-Repair and Adenylation.

STEP A: End-Repair & Adenylation

Materials

Revvity Supplied

CLEAR CAP NEXTFLEX® End-Repair & Adenylation Buffer Mix 2.0,
NEXTFLEX® End- Repair & Adenylation Enzyme Mix 2.0

WHITE CAP or WHITE CAP BOTTLE Nuclease-free Water

User Supplied

Cell free DNA in 32 µL (or less) nuclease-free water Thermal cycler

96 well PCR Plate Adhesive PCR Plate Seal Microcentrifuge

Ice

1. Thaw NEXTFLEX® End-Repair & Adenylation Buffer Mix on ice, and vortex for 5-10 seconds.
2. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR Plate:

_ µL	Nuclease-free Water
_ µL	Cell free DNA
15 µL	NEXTFLEX® End-Repair & Adenylation Buffer Mix 2.0*
3 µL	NEXTFLEX® End-Repair & Adenylation Enzyme Mix 2.0*
50 µL	TOTAL

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

3. Apply adhesive PCR plate seal and incubate in a thermal cycler using the following program:

30 min	20 °C
30 min	65 °C
end	4 °C

4. Proceed to Step B: Adapter Ligation.

STEP B: Adapter Ligation

Materials

Revvity Supplied

PURPLE CAP NEXTFLEX® Ligase Buffer Mix 2.0, NEXTFLEX Ligase Enzyme 2.0

WHITE CAP or WHITE CAP BOTTLE Nuclease-free Water, Resuspension Buffer, NEXTFLEX® Cleanup Beads 2.0

User Supplied

50 µL of End-Repaired and Adenylated DNA (from Step A)

Thermal cycler

Adhesive PCR Plate Seal

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

NEXTFLEX® Unique Dual Index Barcodes – (Cat # NOVA-514150, NOVA-514151, NOVA-514152, NOVA-514153, NOVA-534100, NOVA-534101, NOVA-534102, NOVA-534103, NOVA-534104)

NEXTFLEX® UDI-UMI Barcodes (NOVA-734100, NOVA-734101, NOVA-734102, NOVA-734103)

1. Thaw NEXTFLEX® Ligase Buffer Mix 2.0 to room temperature, and vortex for 5-10 seconds. Do not spin down tube, as this may cause components of the mix to separate and affect performance.
2. Perform adapter dilutions with Nuclease-free Water. Dilute the adapter concentration to 0.156 µM (a 1/40 dilution if using standard UDI barcodes. UDI-UMI barcodes do not need to be diluted) and add 2.5 µL of adapter to each sample.

   The following reaction must be mixed thoroughly. The NEXTFLEX® Ligase Enzyme 2.0 is very viscous. Thorough mixing of the reaction below is critical to obtaining optimal results.

   Suggestion: To mix, pipette up and down 15 times; visually inspect tubes to ensure proper homogenization. Combine the following in the PCR plate and mix thoroughly by pipette:

50 µL	End Repaired & Adenylated DNA (from Step A1)
44.5 µL	NEXTFLEX® Ligase Buffer Mix 2.0*
2.5 µL	NEXTFLEX® Barcode or UDI-UMI
3.0 µL	NEXTFLEX® 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.*

3. Apply adhesive PCR plate seal and incubate in a thermal cycler with heated lid turned off or open for 15 minutes at 20°C, followed by a 4°C hold.

4. Add 65 µL of Nuclease-free water and 35 µL NEXTFLEX® Cleanup Beads 2.0 to each sample. Mix thoroughly until homogenized. The NEXTFLEX® Cleanup Beads 2.0 and Nuclease-free water can be premixed and added in a single step.
5. Incubate sample at room temperature for 5 minutes.
6. Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes or until supernatant appears completely clear.
7. Remove and discard clear supernatant, taking care not to disturb beads. Some liquid may remain in wells.
8. 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.
9. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
10. Remove the 96 well PCR plate from the magnetic stand and let dry at room temperature for 3 minutes.
11. Resuspend dried beads with 25 µL of Resuspension Buffer. Mix thoroughly until homogenized.
12. Incubate sample at room temperature for 2 minutes.
13. Place the 96 well PCR Plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
14. Do not discard the supernatant in this step. Transfer 23 µL of clear sample to a new well. Remove the 96 well PCR plate from the magnetic stand.
15. The procedure may be safely stopped at this step with samples stored at -20°C if needed. To restart, thaw the frozen samples on ice before proceeding with Step C.

STEP C: PCR Amplification

Materials

Revvity Supplied

GREEN CAP NEXTFLEX® PCR Master Mix 2.0, NEXTFLEX® Primer Mix 2.0

WHITE CAP or WHITE CAP BOTTLE Nuclease-free Water, Resuspension Buffer, NEXTFLEX Cleanup Beads 2.0

User Supplied

23 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

Note: The NEXTFLEX® Primer Mix that is included in the NEXTFLEX® NGS Barcodes are NOT compatible with this kit and should NOT be used in place of the Primer Mix 2.0.

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

23 µL	Adapter Ligated DNA (from Step B)
25 µL	NEXTFLEX® PCR Master Mix 2.0*
2 µL	NEXTFLEX® Primer Mix 2.0*
50 µL	TOTAL

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

30 sec	98°C	
15 sec	98°C	
30 sec	65°C	Repeat for a total of 12 cycles
30 sec	72°C	
2 min	72°C	

2. Apply adhesive PCR plate seal and place in thermal cycler for the following PCR cycles:
3. Add 45 µL of NEXTFLEX® Cleanup Beads 2.0 to each sample. Mix thoroughly until homogenized.
4. Incubate at room temperature for 5 minutes.
5. Place the 96 well PCR Plate on the magnetic stand at room temperature for 5

minutes or until the supernatant appears completely clear.

6. Remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in wells.
7. 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.
8. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
9. Remove the 96 well PCR plate from the magnetic stand and let dry at room temperature for 3 minutes.
10. Resuspend dried beads with 33 µL of Resuspension Buffer. Mix thoroughly until homogenized.
11. Incubate resuspended beads at room temperature for 2 minutes.
12. Place the 96 well PCR Plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
13. Do not discard the supernatant in this step. Transfer 30 µL of clear sample to a new well. Remove the 96 well PCR plate from the magnetic stand.
14. 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) or equivalent.
15. qPCR is recommended to quantify DNA library templates for optimal cluster density. This can be performed using any qPCR quantification kit for Illumina® and Element® platforms and the NEXTFLEX® Primer Mix 2.0 as needed.
16. The library is now ready for cluster generation per the standard Illumina® and Element® protocol. Proceed to cluster generation or seal with adhesive PCR Plate Seal and store at -20°C.

LIBRARY VALIDATION

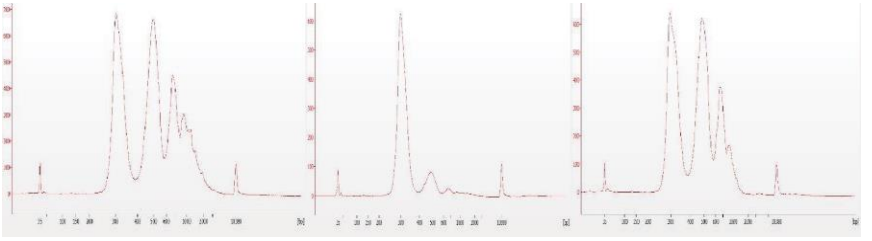

Figure 2: Nucleosome Enriched Library

10ng input of cfDNA was used for the enrichment (or no enrichment) and prepared using the rapid cell-free DNA-seq kit standard protocol:

A) No enrichment

B) Mono-nucleosome enrichment

C) Mono-, di-, tri-nucleosome enrichment

APPENDIX A

Oligonucleotide Sequences

NEXTFLEX®	Sequence (5′ → 3′)
PCR Primer 1	AATGATACGGCGACCACCGAGATCTACAC
PCR Primer 2	CAAGCAGAAGACGGCATACGAGAT
NEXTFLEX® UDI Barcode	AATGATACGGCGACCACCGAGATCTACACXXXXXXXX[1]AC ACTCTTTCCCTACACGACGCTCTTCCGATCT GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXX[2]ATCTCGTATGCCGTCTTCTGCTTG
NEXTFLEX® UDI-UMI Barcodes	AATGATACGGCGACCACCGAGATCTACACXXXXXXXX[1]ACACTCTTTCCCTACA CGACGCTCTTCCGATCT GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXX[2]NNNNNNNN[3]AT CTCGTATGCCGTCTTCTGCTTG

XXXXXXXX[1] denotes the P5 index region of adapter. The index sequences contained in each adapter are listed below.

XXXXXXXX[2] denotes the P7 index region of the adapter. The index sequences contained in each adapter are listed below.

NNNNNNNN[3] denotes the UMI region of the Barcode.

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.

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