NEXTFLEX® Rapid DNA-Seq Kit 2.0 (1 ng – 1 µg)

(Compatible with Illumina® platforms)

KIT CONTAINS : 8, 48, or 96 reactions

USER MANUAL FOR :
#NOVA-5188-01
#NOVA-5188-02
#NOVA-5188-03

NEXTFLEX® Rapid DNA-Seq Kit 2.0 (1 ng – 1 µg)

This product is for research use only.

Not for use in diagnostic procedures.

GENERAL INFORMATION

Product Overview

The NEXTFLEX® Rapid DNA-Seq Kit 2.0 is designed for an approximately 3-hour DNA library construction with 1 ng – 1 µg of fragmented DNA. This kit is not recommended for FFPE samples. The kit can be used to prepare multiplexed libraries for single or paired-end sequencing using Illumina® platforms. In addition, the availability of up to 1536 unique adapter barcodes facilitates high-throughput applications.

There are five main steps involved in preparing DNA for sequencing: DNA extraction, DNA fragmentation, DNA end repair / adenylation, adapter ligation, and PCR amplification. The NEXTFLEX® Rapid 2.0 DNA Seq Kit contains the necessary material to take the user's purified and fragmented DNA through preparation and amplification for loading onto flow cells for sequencing.

Kit Overview

The NEXTFLEX® Rapid DNA-Seq Kit 2.0 contains enough material to prepare 8, 48, or 96 DNA samples for Illumina® compatible sequencing.

Kit Contents, Storage, and Shelf Life

The shelf life of all reagents is at least 12 months when stored properly. All components should be stored at -20°C, except the Nuclease-free Water and Resuspension Buffer, which can be safely stored at room temperature, and NEXTFLEX® Cleanup Beads 2.0, which should be stored at 4°C.

Kit Contents	Amount
CLEAR CAP	8 / 48 / 96 RXN's
NEXTFLEX® End-Repair & Adenylation Buffer Mix 2.0	120 / 720 / 1440 µL
NEXTFLEX® End-Repair & Adenylation Enzyme Mix 2.0	24 / 144 / 288 µL
PURPLE CAP	
NEXTFLEX® Ligase Buffer Mix 2.0	356 / (4) 534 / µL
NEXTFLEX® Ligase Enzyme 2.0	24 / 144 / (4) 1068 µL
GREEN CAP	
NEXTFLEX® PCR Master Mix 2.0	200 / 1200 / (2) 1200 µL
NEXTFLEX® Primer Mix 2.0 (50 µM)	16 / 96 / 192 µL
WHITE CAP	
Nuclease-free Water	(2) 1.5 / 10* / 20* mL
Resuspension Buffer	1.5 / 6* / 12* mL
NEXTFLEX® Cleanup Beads 2.0	1.5 / 7* / 14* mL

*Reagents will be in WHITE CAP BOTTLES

Required Materials Not Provided

- 1 ng - 1 µg of fragmented genomic DNA in up to 32 µL nuclease-free water.
- Ethanol 80% (room temperature)
- Covaris System (S2, E210) or other method for DNA fragmentation
- 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
- 96 well Library Storage and Pooling Plate (Thermo Fisher Scientific, Cat # AB-0765) or similar
- Adhesive PCR Plate Seal (Bio-Rad, 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, you may contact your local distributor, or contact us at ngs@revvity.com

- 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 NEXTFLEX® DNA Barcodes above room temperature.
- To enable multiplexing, please use the appropriate combination of NEXTFLEX Barcodes during the Adapter Ligation step.
- It is highly recommended that NEXTFLEX® Primer Mix 2.0 be used during PCR amplification. Inadvertent use of an incorrect primer sequence can potentially result in elimination of the index.
- 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.
- 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 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. Other quantification methods using fluorescent dyes may also be used. The user should be aware that contaminating RNA, nucleotides and single-stranded DNA may affect the amount of usable DNA in a sample preparation.
- DNA fragmentation methods that physically break up DNA into pieces of less than 800 bp are compatible with this kit.

Revision History

Version	Date	Description
V18.11	November 2018	Updated bead chemistry for Cleanup Beads 2.0. Protocol changes for Size Selection using the new Cleanup Beads 2.0.
V19.12	December 2019	Added FFPE sample language and additional comment at end about eluting in nuclease-free water for target-capture.
V23.10	October 2023	Rebrand to Revvity.

NEXTFLEX® Rapid 2.0 DNA Sample Preparation Flow Chart

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

Starting Material

The NEXTFLEX® Rapid DNA Seq Kit 2.0 has been optimized and validated using high quality fragmented genomic DNA ranging from 1 ng - 1 µg.

There are two Rapid DNA-Seq Kit 2.0 protocol options to choose from:

Option 1 is intended for users who do not wish to size select their libraries. Clean up steps throughout are designed to eliminate only unwanted low molecular weight material.

Option 2 is designed for users who wish to size select their libraries. The user can choose from five size selection ranges, found in Step C2: Bead Size Selection. Size Selection may not be optimal for inputs less than 10 ng. Please consider the amount of starting material that will be excluded by size selection when choosing input amount and desired size range.

Reagent Preparation

- 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. Nuclease-free water and Resuspension Buffer can be stored at room temperature. NEXTFLEX® Cleanup Beads 2.0 should be stored at 4°C but equilibrated to room temperature prior to use.
- DTT in buffers may precipitate after freezing. If precipitate is seen in any mix, vortex for 1-2 minutes or until the precipitate is in solution. The performance of the mix is not affected once the precipitate is in solution.
- Allow NEXTFLEX® Cleanup Beads 2.0 to come to room temperature and vortex the beads until homogenous.

OPTION 1: LIBRARY PREPARATION WITHOUT SIZE SELECTION

Option 1 is designed for users who do not wish to size-select their libraries. Clean-up steps throughout are designed to eliminate only unwanted low-molecular weight material. If you wish to size select your libraries, please follow Option 2.

STEP A1: 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

Fragmented 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	Fragmented DNA (1 ng - 1 µg)
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 B1: Adapter Ligation.

STEP B1: 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 A1)

Thermal cycler

Adhesive PCR Plate Seal

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

NEXTFLEX® Unique Dual Index Barcodes – 96 (Cat # 514150, 514151, 514152, 514153, or 534100)

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. The following table lists recommended adapter concentration dilutions for various input amounts for all listed barcodes:

Input DNA	Desired Adapter Concentration	Adapter Dilution Required
1 ng	0.3 µM	1 / 80
10 ng	0.6 µM	1 / 40
100 ng	6.25 µM	1 / 4
250 ng	25 µM	None
500 ng	25 µM	None
1 µg	25 µM	None

Each sample will require 2.5 µL of adapter to be added. Perform adapter dilutions if necessary with Nuclease-free Water, depending on input amount and starting adapter concentration.

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® Barcoded Adapter
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 C1. If input DNA amount was 500 ng or greater, PCR amplification may not be necessary depending on the sequencing application. Users starting with greater than 500 ng of input DNA are advised to quantify their unamplified libraries by qPCR at this point to determine if PCR amplification is necessary. This can be performed using any qPCR quantitation kit compatible with Illumina® platforms.

STEP C1: 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, NEXTFLE®X Cleanup Beads 2.0

User Supplied

23 µL of Adapter Ligated DNA (from Step B1)

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.

*The following table lists recommended PCR cycles:

	Number of PCR cycles to produce	
Input DNA (ng)	100 ng libraries	1 µg libraries
1	10 - 12	13 - 15
10	6 - 8	9 - 11
100	2 - 3	6 - 7
250	1 - 2	4 - 5
500	0	3 - 4
1000	0	2 - 3

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

23 µL	Adapter Ligated DNA (from Step B1)
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.

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

30 sec	98°C	
15 sec	98°C	Repeat as suggested in above table
30 sec	65°C	
30 sec	72°C	
2 min	72°C	

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

OPTION 2: LIBRARY PREPARATION WITH SIZE SELECTION

Option 2 is designed for users who wish to size-select their libraries. The user can choose from five selection ranges, found in Step C2: Bead Size Selection. If you do not wish to size select your libraries, please follow Option 1.

STEP A2: 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

Fragmented 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	Fragmented DNA (1 ng - 1 µg)
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 B2: Adapter Ligation.

STEP B2: 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 A2)

Thermal cycler

Adhesive PCR Plate Seal

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

NEXTFLEX® Unique Dual Index Barcodes – 96 (Cat # 514150, 514151, 514152, 514153, or 534100)

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. The following table lists recommended adapter concentration dilutions for various input amounts for all listed barcodes:

Input DNA	Desired Adapter Concentration	Adapter Dilution Required
1 ng	0.3 µM	1 / 80
10 ng	0.6 µM	1 / 40
100 ng	6.25 µM	1 / 4
250 ng	25 µM	None
500 ng	25 µM	None
1 µg	25 µM	None

Each sample will require 2.5 µL of adapter to be added. Perform adapter dilutions if necessary with Nuclease-free Water, depending on input amount and starting adapter concentration.

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 A2)
44.5 µL	NEXTFLEX® Ligase Buffer Mix 2.0*
2.5 µL	NEXTFLEX® Barcoded Adapter
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 52 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 50 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 C2.

STEP C2: Bead Size Selection

Materials

Revvity Supplied

WHITE CAP or WHITE CAP BOTTLE Resuspension Buffer, NEXTFLEX® Cleanup Beads 2.0

User Supplied

50 µL of Adapter Ligated DNA (from STEP B2)

80% Ethanol, freshly prepared (room temperature)

96 well PCR Plate

Magnetic Stand

Size Selection may not be optimal for inputs less than 10 ng. The size ranges listed in tables below reflect the total library size, including the insert and NEXTFLEX® Barcode Adapters. NEXTFLEX® Barcode Adapters add ~120bp to the insert length.

The following chart is a general recommendation for certain sizes. Yield and specificity of size selection is affected by size distribution of starting material. It is important to select for an insert size that is compatible with the size range of the starting material. The user should use this chart as a guideline with the expectation that optimization may be required for their specific application.

Note: During optimization, the user should keep in mind that adding more NEXTFLEX® Cleanup Beads 2.0 at the 1st cleanup step "Bead Volume #1" would decrease the library size for the upper size selection and as a result, the lower size selection as well. Adding more NEXFLEX® Cleanup Beads 2.0 at the 2nd cleanup step "Bead Volume #2" would decrease the library size of the lower size selection only.

The following table lists the appropriate volume of NEXTFLEX® Cleanup Beads 2.0 required to size select for library peak sizes (approximated) below:

Approximate Insert Peak Size (bp)	150 - 250	250 - 350	300 - 500	400 - 600	500 - 700
Approximate Library Peak Size (bp)	270 - 370	370 - 470	420 - 620	520 - 720	620 - 820
Bead Volume #1	35	32	30	27	24
Bead Volume #2	12	9	8	8	8

Ensure all reagents are at room temperature. Vortex the NEXTFLEX® Cleanup Beads 2.0 thoroughly prior to use. Use a fresh dilution of 80% ethanol during wash steps.

1. Add Bead Volume #1 to sample as indicated in the column corresponding to your desired size range in the chart above. Mix thoroughly until homogenized.
2. Incubate sample at room temperature for 5 minutes.
3. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes or until the supernatant appears completely clear.
4. 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.

5. Add Bead Volume #2 to sample as indicated in the column corresponding to your desired size range in the chart above. Mix thoroughly until homogenized.
6. Incubate sample at room temperature for 5 minutes.
7. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes or until the supernatant appears completely clear.
8. Remove and discard clear supernatant, taking care not to disturb beads. Some liquid may remain in wells.
9. 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.
10. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
11. Remove the 96 well PCR plate from the magnetic stand and let dry at room temperature for 3 minutes.
12. Resuspend dried beads with 25 µL of Resuspension Buffer. Mix thoroughly until homogenized.
13. Incubate sample at room temperature for 2 minutes.
14. Place the 96 well PCR plate on the magnetic stand at room temperature for 2 minutes or until the supernatant appears completely clear.
15. Transfer 23 µL of clear sample to a new well.
16. 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 D2. If input DNA amount was 500 ng or greater, PCR amplification may not be necessary depending on the sequencing application. Users starting with greater than 500 ng of input DNA are advised to quantify their unamplified libraries by qPCR at this point to determine if PCR amplification is necessary. This can be performed using any qPCR quantification kit compatible with Illumina® platforms.

STEP D2: 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 C2)

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.

*The following table lists recommended PCR cycles:

	Number of PCR cycles to produce	
Input DNA (ng)	100 ng libraries	1 µg libraries
10 ng	9 - 10	11 - 13
100 ng	4 - 5	8 - 9
250 ng	4 - 5	6 - 7
500 ng	0 - 4	4 - 5
1000 ng	0 - 4	4 - 5

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

23 µL	Adapter Ligated DNA (from Step C2)
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.

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

30 sec	98°C	
15 sec	98°C	
30 sec	65°C	Repeat as suggested in above table
30 sec	72°C	
2 min	72°C	

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. Note: If taking libraries into hybrid-capture, labs may choose to elute in nuclease-free water.
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).
15. qPCR is recommended to quantify DNA library templates for optimal cluster density. This can be performed using any qPCR quantification kit for Illumina® platforms and the NEXTFLEX® Primer Mix 2.0 as needed.
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.

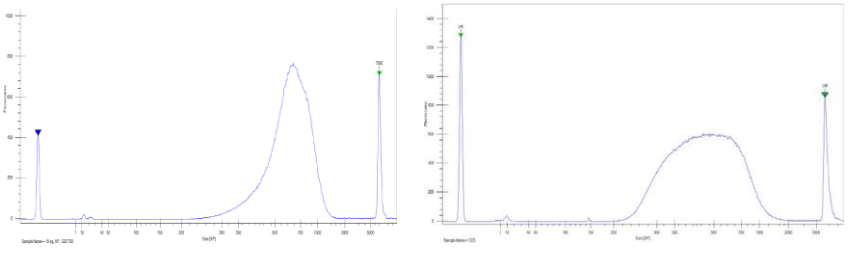

Figure 2: Library Validation

5ng of libraries were loaded onto the LabChip® GXII Touch™ HT instrument (Revvity)

A) 10 ng input of bacterial DNA (combined shear of 250 and 800bp), 13-cycle PCR product size selected for libraries of 520-720bp.

B) 500 ng input of bacterial DNA (combined shear of 250 - 800bp), 4-cycle PCR product with no size selection.

APPENDIX A

Oligonucleotide Sequences

NEXTFLEX	Sequence (5′ → 3′)
PCR Primer 1	AATGATACGGCGACCACCGAGATCTACAC
PCR Primer 2	CAAGCAGAAGACGGCATACGAGAT

For low level multiplexing recommendations, see Appendix A in the NEXTFLEX® barcode manual.

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revvity

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