Bioo Scientific
a PerkinElmer company

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

(For Illumina® Platforms)

Catalog #NOVA-5149-02 (Kit contains 48 reactions)

 V18.09

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

NEXTFLEX® Rapid XP DNA-Seq Kit (1 ng – 1 µg) - NOVA-5149-02

Product Overview

The NEXTFLEX® Rapid XP DNA-Seq Kit is designed for ~2.5 hour DNA library construction from 1 ng – 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. An optional bead-based size selection protocol eliminates the need for agarose gel size selection. In addition, the availability of up to 384 unique 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 PCR amplification. The NEXTFLEX® Rapid XP DNA-Seq Kit contains the necessary material to take the user's purified DNA through preparation and amplification for loading onto flow cells for sequencing.

Contents, Storage, and Shelf Life

The NEXTFLEX® Rapid XP DNA-Seq Kit contains enough material to prepare 48 DNA 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	Amount
CLEAR CAP	
NEXTFLEX® Fragmentation Buffer	240 µL
NEXTFLEX® Fragmentation Enzyme Mix	528 µL
PURPLE CAP	
NEXTFLEX® Ligase Buffer Mix XP	(4) 534 µL
NEXTFLEX® Ligase Enzyme XP	144 µL
GREEN CAP	
NEXTFLEX® PCR Master Mix XP	1200 µL
NEXTFLEX® Primer Mix XP	96 µL
CLEAR CAP BOTTLE	
Nuclease-free Water	6 mL
Resuspension Buffer	6 mL
NEXTFLEX® Cleanup Beads XP	7 mL

Required Materials Not Provided

- 1 ng - 1 µg of DNA in up to 34 µL nuclease-free water.
- If multiplexing: NEXTFLEX® DNA Barcodes – 6 / 12 / 24 / 48 (Cat # 514101, 514102, 514103, 514104) or NEXTFLEX-96™ DNA Barcodes (Cat # 514106) or NEXTFLEX® ChIP-Seq Barcodes – 6 / 12 / 24 / 48 (Cat # 514120, 514121, 514122, 514123) or NEXTFLEX-96™ ChIP-Seq Barcodes (Cat # 514124) or NEXTFLEX-HT™ Barcodes (Cat # 514170, 514174, 514175, 514176, 514177) or NEXFLEX® Unique Dual Index Barcodes (Cat # 514150, 514151)
- 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
- Vortex

Warnings and Precautions

We strongly recommends 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 bioo.ngs@perkinelmer.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 DNA-Seq Adapter above room temperature.
- 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.
- Maintain a laboratory temperature of 20º–25ºC (68º–77ºF).
- 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.
- 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.
- 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.

NEXTFLEX® RAPID XP DNA Sample Preparation Flow Chart

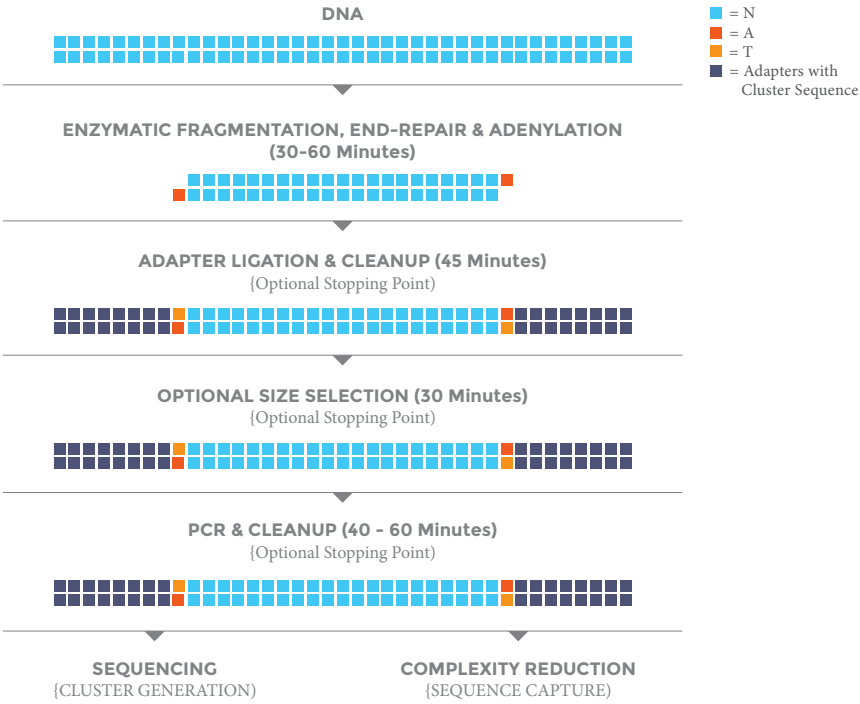

Figure 1: Sample flow chart with approximate times necessary for each step.

Starting Material

The NEXTFLEX® Rapid XP DNA-Seq Kit has been optimized and validated using high quality genomic DNA inputs ranging from 1 ng - 1 µg. This kit will allow you to perform at least 48 reactions (see page 4, Warnings and Precautions).

There are two Rapid XP DNA-Seq 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

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 except the NEXTFLEX® Fragmentation Enzyme Mix just prior to use. Nuclease-free Water and Resuspension Buffer should be stored at room temperature. NEXTFLEX® Cleanup Beads XP 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. Allow NEXTFLEX® Cleanup Beads XP to come to room temperature and vortex the beads until homogenous.

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: FRAGMENTATION, END-REPAIR & ADENYLATION

Materials

Bioo Scientific Supplied

CLEAR CAP - NEXTFLEX® Fragmentation Buffer, NEXTFLEX® Fragmentation Enzyme Mix
CLEAR CAP BOTTLE - Nuclease-free Water

User Supplied

DNA in 34 µL (or less) nuclease-free water
Thermal Cycler
96 well PCR Plate
Adhesive PCR Plate Seal
Microcentrifuge
Ice

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.

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

_ µL	Nuclease-free Water
_ µL	DNA (1 ng - 1 µg)
5 µL	NEXTFLEX® Fragmentation Buffer
39 µL	TOTAL

Ensure thorough mixing by pipetting up and down. Proceed with adding the enzyme.

39 µL	DNA + NEXTFLEX® Fragmentation Buffer mixture
11 µL	NEXTFLEX® Fragmentation Enzyme Mix (DO NOT VORTEX)
50 µL	TOTAL

Note: Do NOT vortex the final NEXTFLEX® Fragmentation reaction. Mix by pipette only. It is important to mix the reaction on ice.

2. 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
end	4 °C

Note: The initial 4 °C step is to pre-chill the instrument temperature. Place samples into thermal cycler after the temperature reaches 4 °C and follow the program. A full one-minute incubation at 4 °C is not necessary.

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	Target Fragment Peak Size			
	200 - 300	300 - 400	400 - 500	500 - 600
	Fragmentation Time (min) at 35 °C			
1 ng	24	15	8	4
10 ng	18	8	5	3
100 ng	15	8	5	3
500 ng	15	8	5	3
1000 ng	14	7	5	3

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

3. The procedure may be safely stopped at this step with samples stored at -20 °C if needed. To restart the protocol, thaw frozen samples on ice before proceeding to **Step B1**.

STEP B1: Adapter Ligation

Materials

Bioo Scientific Supplied

PURPLE CAP - NEXTFLEX® Ligase Buffer Mix XP, NEXTFLEX® Ligase Enzyme XP
CLEAR CAP BOTTLE - Nuclease-free Water, Resuspension Buffer, NEXTFLEX® Cleanup Beads XP

User Supplied

50 µL of Fragmented, 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) or
NEXTFLEX® Dual Index Barcodes – 96 (Cat # 514160, 514161) or
NEXTFLEX-HT™ Barcodes – 6 / 96 (Cat # 514170, 514174, 514175, 514176, 514177) or
NEXTFLEX® DNA Barcodes – 6 / 12 / 24 / 48 (Cat # 514101, 514102, 514103, 514104) or
NEXTFLEX-96™ DNA Barcodes (Cat # 514106) or
NEXTFLEX® ChIP-Seq Barcodes – 6 / 12 / 24 / 48 (Cat # 514120, 514121, 514122, 514123) or
NEXTFLEX-96™ ChIP-Seq Barcodes (Cat # 514124)

1. Thaw NEXTFLEX® Ligase Buffer Mix XP 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 barcoded adapter concentration dilutions for various input amounts for all listed barcoded adapter except ChIP-Seq barcoded adapters::

Input DNA	Desired Adapter	Adapter Dilution Concentration
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

* ChIP-Seq barcoded adapter may be used for only 1ng inputs. Please inquire for details.

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

The following reaction must be mixed thoroughly. The NEXTFLEX® Ligase Buffer Mix XP 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	Fragmented, End Repaired & Adenylated DNA (from Step A1)
44.5 µL	NEXTFLEX® Ligase Buffer Mix XP*
2.5 µL	NEXTFLEX® Barcoded Adapter
3 µL	NEXTFLEX® Ligase Enzyme Mix XP*
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 of NEXTFLEX® Cleanup Beads XP to each sample. Mix thoroughly until homogenized. The NEXTFLEX® Cleanup Beads XP 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 the supernatant appears completely clear.
7. Remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in well.
8. 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.
9. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
10. Remove the 96 well plate from the magnetic stand and let dry at room temperature for 3 minutes.
11. Resuspend dried beads with 28 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 sample 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 the protocol, thaw frozen samples on ice before proceeding to **Step C1**. If input DNA amount was 500 ng or greater, PCR Amplification may not be necessary, depending on the 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 C1: PCR Amplification

Materials

Bioo Scientific Supplied

GREEN CAP - NEXTFLEX® PCR Master Mix XP, NEXTFLEX® Primer Mix XP
CLEAR CAP BOTTLE - Nuclease-free Water, Resuspension Buffer, NEXTFLEX® Cleanup Beads XP

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

*The following table lists recommended PCR cycles:

	Number of PCR cycles to produce	
Input DNA	**100 ng libraries**	**1 µg libraries**
1 ng	10 - 12	13 - 15
10 ng	6 - 8	9 - 11
100 ng	2 - 3	6 - 7
250 ng	1 - 2	4 - 5
500 ng	0	3 - 4
1000 ng	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 XP*
2 µL	NEXTFLEX® Primer Mix XP*
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	Repeat as suggested in above table
15 sec	98°C	
30 sec	65°C	
30 sec	72°C	
2 min	72°C	

3. Add 45 µL of NEXTFLEX® Cleanup Beads XP 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 plate from 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 your 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®)].
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.
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: FRAGMENTATION, END-REPAIR & ADENYLATION

Materials

Bioo Scientific Supplied

CLEAR CAP - NEXTFLEX® Fragmentation Buffer, NEXTFLEX® Fragmentation Enzyme Mix
CLEAR CAP BOTTLE - Nuclease-free Water

User Supplied

DNA in 34 µL (or less) nuclease-free water
Thermal Cycler
96 well PCR Plate
Adhesive PCR Plate Seal
Microcentrifuge
Ice

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.

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

_ µL	Nuclease-free Water
_ µL	DNA (1 ng - 1 µg)
5 µL	NEXTFLEX® Fragmentation Buffer
39 µL	TOTAL

Ensure thorough mixing by pipetting up and down. Proceed with adding the enzyme.

39 µL	DNA + NEXTFLEX® Fragmentation Buffer mixture
11 µL	NEXTFLEX® Fragmentation Enzyme Mix (DO NOT VORTEX)
50 µL	TOTAL

Note: Do NOT vortex the final NEXTFLEX® Fragmentation reaction. Mix by pipette only. It is important to mix the reaction on ice.

2. 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
end	4 °C

Note: The initial 4 °C step is to pre-chill the instrument temperature. Place samples into thermal cycler after the temperature reaches 4 °C and follow the program. A full one-minute incubation at 4 °C is not necessary.

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	Target Fragment Peak Size			
	200 - 300	300 - 400	400 - 500	500 - 600
	Fragmentation Time (min) at 35 °C			
1 ng	24	15	8	4
10 ng	18	8	5	3
100 ng	15	8	5	3
500 ng	15	8	5	3
1000 ng	14	7	5	3

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

3. The procedure may be safely stopped at this step with samples stored at -20 °C if needed. To restart the protocol, thaw frozen samples on ice before proceeding to **Step B2**.

STEP B2: Adapter Ligation

Materials

Bioo Scientific Supplied

PURPLE CAP - NEXTFLEX® Ligase Buffer Mix XP, NEXTFLEX® Ligase Enzyme XP
CLEAR CAP BOTTLE - Nuclease-free Water, Resuspension Buffer, NEXTFLEX® Cleanup Beads XP

User Supplied

50 µL of Fragmented, 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) or
NEXTFLEX® Dual Index Barcodes – 96 (Cat # 514160, 514161) or
NEXTFLEX-HT™ Barcodes – 6 / 96 (Cat # 514170, 514174, 514175, 514176, 514177) or
NEXTFLEX® DNA Barcodes – 6 / 12 / 24 / 48 (Cat # 514101, 514102, 514103, 514104) or
NEXTFLEX-96™ DNA Barcodes (Cat # 514106) or
NEXTFLEX® ChIP-Seq Barcodes – 6 / 12 / 24 / 48 (Cat # 514120, 514121, 514122, 514123) or
NEXTFLEX-96™ ChIP-Seq Barcodes (Cat # 514124)

1. Thaw NEXTFLEX® Ligase Buffer Mix XP 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 barcoded adapter concentration dilutions for various input amounts for all listed barcoded adapter except ChIP-Seq barcoded adapters::

Input DNA	Desired Adapter	Adapter Dilution Concentration
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

* ChIP-Seq barcoded adapters may be used for only 1ng inputs. Please inquire for details.

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

The following reaction must be mixed thoroughly. The NEXTFLEX® Ligase Buffer Mix XP 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	Fragmented, End Repaired & Adenylated DNA (from Step A2)
44.5 µL	NEXTFLEX® Ligase Buffer Mix XP*
2.5 µL	NEXTFLEX® Barcoded Adapter
3 µL	NEXTFLEX® Ligase Enzyme Mix XP*
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 on 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 of NEXTFLEX® Cleanup Beads XP to each sample. Mix thoroughly until homogenized. The NEXTFLEX® Cleanup Beads 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 the supernatant appears completely clear.
7. Remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in well.
8. 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.
9. Repeat previous step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
10. Remove the 96 well plate from the magnetic stand and let dry at room temperature for 3 minutes.
11. Resuspend dried beads with 55 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 sample 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 the protocol, thaw frozen samples on ice before proceeding to **Step C2**.

STEP C2: Bead Size Selection

Materials

Bioo Scientific Supplied

CLEAR CAP BOTTLE - Resuspension Buffe, NEXTFLEX® Cleanup Beads XP

User Supplied

100 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 XP 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 XP 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 XP 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 XP 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

Bioo Scientific Supplied

GREEN CAP - NEXTFLEX® PCR Master Mix XP, NEXTFLEX® Primer Mix XP
CLEAR CAP BOTTLE - Nuclease-free Water, Resuspension Buffer, NEXTFLEX® Cleanup Beads XP

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

*The following table lists recommended PCR cycles:

	Number of PCR cycles to produce	
Input DNA	**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 XP*
2 µL	NEXTFLEX® Primer Mix XP*
50 µL	TOTAL

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

2. Apply adhesive PCR plate seal and place in thermocycler 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 XP 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 plate from 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 your 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®)].
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.
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.

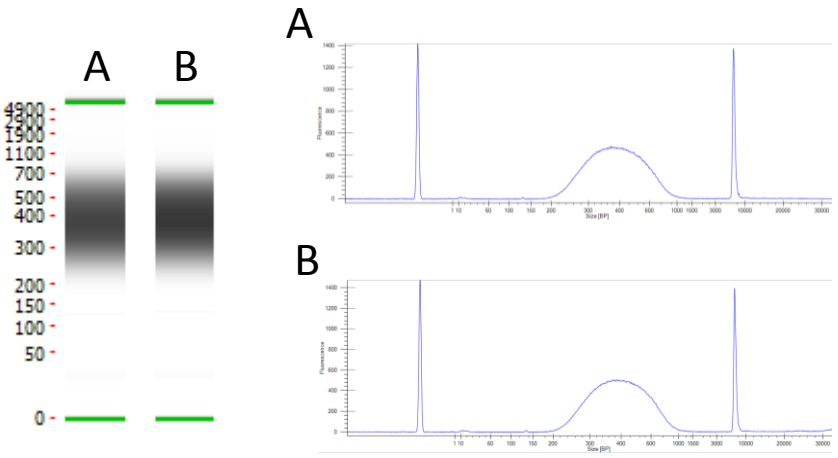

Figure 2: Library Validation
5ng of libraries were loaded onto the LabChip® GXII Touch™ HT instrument (PerkinElmer®)
A) 1 µg input of Human Jurkat, 2-cycle PCR product with 7 minutes fragmentation
B) 1 ng input of Human Jurkat, 12-cycle PCR product with 15 minutes fragmentation

Oligonucleotide Sequences

NEXTFLEX™	Sequence
NEXTFLEX™ DNA-Seq Adapter 1	5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 5'GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTTG
Primer 1	5'AATGATACGGCGACCACCGAGATCTACAC
Primer 2	5'CAAGCAGAAGACGGCATACGAGAT

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