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FOR REFERENCE PURPOSES

This manual is for Reference Purposes Only.
DO NOT use this protocol to run your assays.
Periodically, optimizations and revisions are made to the kit and protocol,
so it is important to always use the protocol included with the kit.

NEXTflex™ Pre - Capture Combo Kit

(NimbleGen SeqCap V3 Compatible)

Catalog #: 5140-53 (192 reactions)

V14.09

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GENERAL INFORMATION

Product Overview

The NEXTflex™ Pre - Capture Combo Kit contains the NEXTflex™ DNA Sequencing Kit, NEXTflex™ DNA Barcodes and NEXTflex™ DNA Barcode Blockers. The NEXTflex™ Pre - Capture Combo Kit simplifies workflow by using master mixed reagents and magnetic bead based cleanup, reducing pipetting and eliminating time consuming steps in library preparation.

There are five main steps involved in preparing genomic DNA for sequencing: DNA extraction, DNA fragmentation, DNA end repair, adapter ligation and PCR amplification. The barcoded adapters and barcode blockers provided are designed to be used with NimbleGen SeqCap V3 EZ Libraries for enrichment of whole exome or custom regions. The NEXTflex™ Pre - Capture Combo Kit allows for both pre-capture and post capture pooling / multiplexing. The kit contains the necessary material to take the user's purified and fragmented genomic DNA through preparation and amplification for target capture and enrichment.

Contents, Storage and Shelf Life

The NEXTflex™ Pre - Capture Combo Kit contains enough material to prepare 192 genomic DNA samples for NimbleGen SeqCap V3 capture and Illumina® compatible sequencing. The shelf life of all reagents is 12 months when safely stored at -20°C.

Kit Contents	Amount
CLEAR CAP	
NEXTflex™ End Repair Buffer Mix	(2) 672 µL
NEXTflex™ End Repair Enzyme Mix	(2) 288 µL
RED CAP	
NEXTflex™ Adenylation Mix	(2) 432 µL
PURPLE CAP	
NEXTflex™ Ligation Mix	(4) 1512 µL
NEXTflex™ DNA Barcode Adapter 1 - 12 (25 µM)	20 µL
RED CAP	
NEXTflex™ DNA Barcode Adapter 13 - 24 (25 µM)	20 µL
GREEN CAP	
NEXTflex™ PCR Master Mix	(2) 1152 µL
NEXTflex™ Primer Mix (12.5 µM)	384 µL
ORANGE CAP	
NEXTflex™ INV-HE Index 1 – 12 (250 µM)	32 µL
GREEN CAP	
NEXTflex™ INV-HE Index 13 – 24 (250 µM)	32 µL
WHITE CAP	
NEXTflex™ HE Universal Oligo 1 (500 µM)	384 µL

Kit Contents	Amount
BLUE CAP	
NEXTflex™ LM-PCR Oligo 1 (100 µM)	384 µL
NEXTflex™ LM-PCR Oligo 2 (100 µM)	384 µL
YELLOW CAP	
NEXTflex™ LM-PCR Master Mix (2X)	(8) 1200 µL
CLEAR CAP-BOTTLE	
Nuclease-free Water	(2) 10 mL
Resuspension Buffer	(2) 24 mL

Required Materials not Provided

- 1 µg of fragmented genomic DNA in up to 40 µL nuclease-free water.
- NimbleGen SeqCap V3 EZ Library
- All consumables required to carry out NimbleGen SeqCap V3 EZ Library
- NimbleGen SeqCap EZ Hybridization and Wash Kit (Roche, Cat. # 05634253001, 96 reactions)
- All consumables required to carry out NimbleGen SeqCap EZ Hybridization and Wash
- Ethanol 100% (room temperature)
- Ethanol 80% (room temperature)
- AIR™ DNA Fragmentation Kit (Bioo Scientific, Cat # 5135-01) / or / Covaris System (S2, E210)
- 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)
- Agencourt AMPure XP 60 mL (Beckman Coulter Genomics, Cat # A63881)
- Magnetic Stand -96 (Ambion, Cat # AM10027) / or / similar
- Heat block
- Thermocycler
- 2, 10, 20, 200 and 1000 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips
- Microcentrifuge
- 1.5 mL nuclease-free microcentrifuge tubes
- Low melt agarose such as Low Gelling Temperature Agarose with a melt point of 65ºC (Boston Bioproducts, Cat # P-730)
- 1X TAE buffer
- Clean razor or scalpel
- SYBR Gold (Invitrogen, Cat # S11494)
- UV transilluminator or gel documentation instrument
- Gel electrophoresis apparatus
- Electrophoresis power supply
- Vortex

Warnings and Precautions

Bioo Scientific 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 Bioo Scientific at nextgen@biooscientific.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 precipitate is in solution.
- Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
- Do not heat the DNA Barcode Adapters above room temperature.
- Try to 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.
- DNA fragmentation methods that physically break up DNA into pieces of less than 800 bp are compatible with this kit. These methods include the AIR™ DNA Fragmentation Kit (5135-01), based on the nebulization of DNA or acoustic technologies that fragment DNA in a controlled and accurate manner. We do not recommend any enzymatic methods of fragmentation as this may introduce sequence bias into the preparation.
- If starting with a DNA input amount greater than or less than 1 µg, adjust the DNA Adapter or DNA Barcoded Adapter volume to preserve the insert to adapter ratio.
- 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.

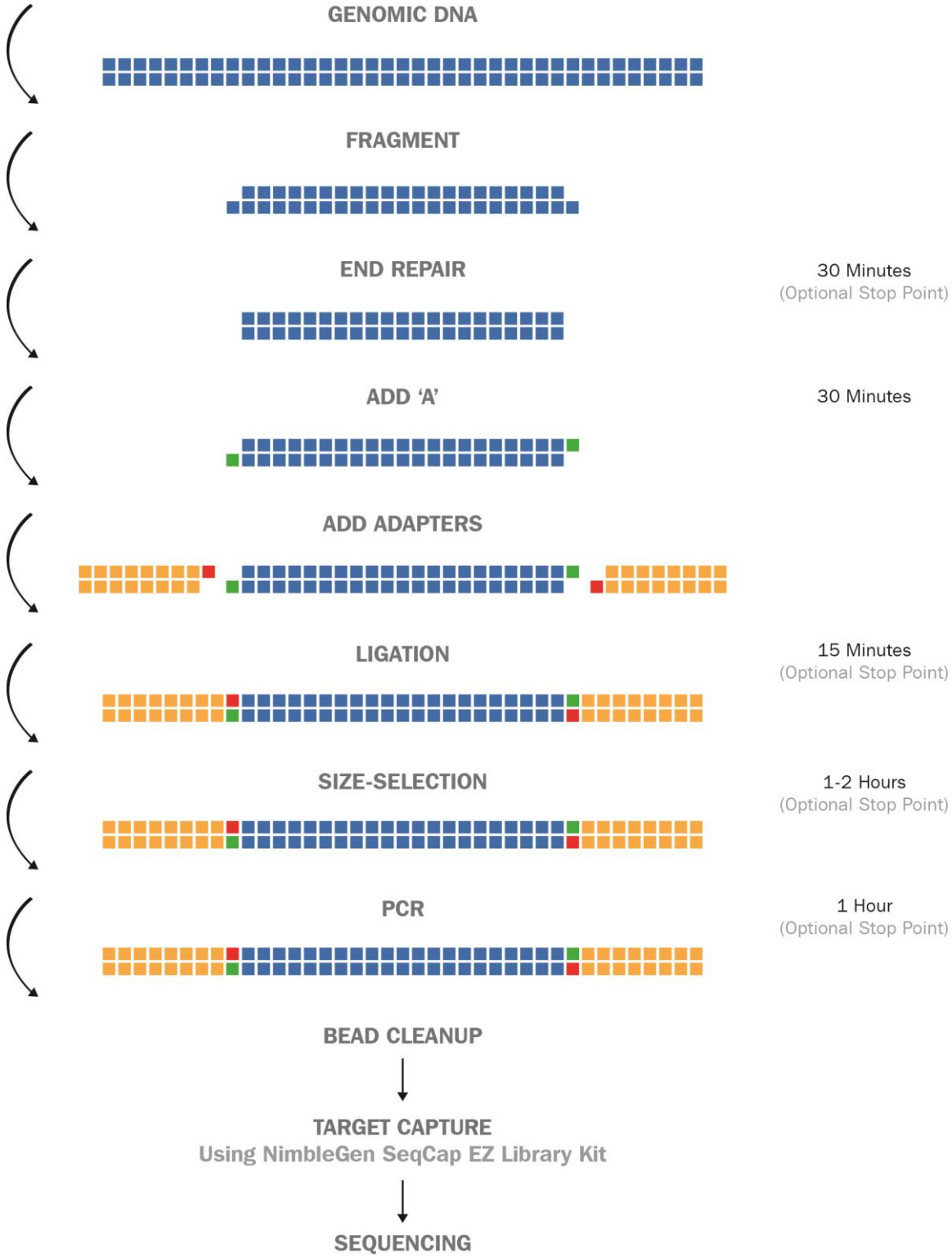
Revision History

Version	Date	Description of Change
V12.10	December 2010	Initial product launch.
V13.04	April 2013	Layout of Appendix A has been changed.
V14.09	September 2014	The index of NEXTflex™ INV-HE Index 6 has been corrected in the manual.

NEXTflex™ PRE - CAPTURE SAMPLE PREPARATION PROTOCOL

NEXTflex™ Pre - Capture Sample Preparation Flow Chart

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



Starting Material

The NEXTflex™ Pre - Capture Combo Kit has been optimized and validated using genomic DNA. Starting with 1 µg of high quality fragmented genomic DNA will allow you to perform at least 8 reactions per barcoded adapter (see page 3, Warnings and Precautions).

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 ™ Mix just prior to use.

2. DTT in buffers may precipitate after freezing. If precipitate is seen in any mix, vortex for 1 minute or until the precipitate is in solution. The performance of the mix is not affected once precipitate is in solution.

3. Allow Agencourt AMPure XP Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.

Sample Library Size Range

This protocol is designed for an optimal insert size range of 150 bp – 500 bp. The user should take into account the target type and the required sequencing read length before selecting a specific insert size for target capture experiments.

To select a specific insert size, the user can follow one of the options listed below:

Option 1: If your DNA fragments are already the appropriate size range, do not perform size selection at all. Follow the protocol in this manual starting with Step A without any modifications.

Option 2: Modify the Agencourt AMPure XP bead volume during bead cleanups in Step B or Step E to allow for a specific insert size.

Option 3: Perform an agarose gel size selection after Step E to allow for a specific insert size.

STEP A: End Repair

Materials

Bioo Scientific Supplied

CLEAR CAP
NEXTflex™ End Repair Buffer Mix
NEXTflex™ End Repair Enzyme Mix

WHITE CAP
Nuclease-free H_2O

User Supplied

Fragmented DNA in 40 µL (or less) nuclease-free water
96 well PCR Plate
Adhesive PCR Plate Seal
Agencourt AMPure XP Magnetic Beads
Microcentrifuge
Ice

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

_ µL	Nuclease-free H_2O
_ µL	Fragmented DNA (1 µg)
7 µL	NEXTflex™ End Repair Buffer Mix
3 µL	NEXTflex™ End Repair Enzyme Mix
50 µL	TOTAL

2. Set pipette to 50 µL, gently pipette the entire volume up and down 10 times.

3. Apply adhesive PCR plate seal and incubate on a thermocycler for 30 minutes at 22°C.

STEP B: Clean-Up

Materials

Bioo Scientific Supplied

CLEAR CAP
Resuspension Buffer

User Supplied

Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand

1. Add 90 µL of AMPure XP Beads to each sample and gently pipette the entire volume up and down 10 times.

2. Incubate 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. Set pipette to 137 µL, gently remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in wells.

5. With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.

6. Repeat step 5, for a total of 2 ethanol washes. Ensure all ethanol has been removed.

7. Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.

8. Resuspend dried beads with 17 µL Resuspension Buffer. Gently, pipette entire volume up and down 10 times mixing thoroughly. Ensure beads are no longer attached to the side of the well.

9. Incubate resuspended beads at room temperature for 2 minutes.

10. Place plate on magnetic stand at room temperature for 5 minutes or until the supernatant appears completely clear.

11. Gently transfer 16 µL of clear sample to new well.

12. If you wish to pause your experiment, the procedure may be safely stopped at this step and samples stored at -20°C. To restart, thaw frozen samples on ice before proceeding.

STEP C: 3' Adenylation

Materials

Bioo Scientific Supplied

RED CAP
NEXTflex™ Adenylation Mix

User Supplied

Thermocycler
16 µL of End Repaired DNA (from STEP B)

1. Combine the following in the PCR plate:

16 µL	End-Repaired DNA (from Step B)
4.5 µL	NEXTflex™ Adenylation Mix
20.5 µL	TOTAL

2. Set pipette to 20 µL, gently pipette the entire volume up and down 10 times.

3. Apply adhesive PCR plate seal and incubate on a thermocycler for 30 minutes at 37°C.

STEP D: Adapter Ligation

Materials

Bioo Scientific Supplied

PURPLE CAP
NEXTflex™ Ligation Mix
NEXTflex™ DNA Barcodes 1 – 12 (20 µM)

RED CAP
NEXTflex™ DNA Barcodes 13 – 24 (20 µM)

User Supplied

20.5 µL 3' Adenylated DNA (from STEP C)

1. For each sample, combine the following reagents (in this order) in the PCR plate:

20.5 µL	3' Adenylated DNA (from step C)
31.5 µL	NEXTflex™ Ligation Mix
2.5 µL	NEXTflex™ DNA Barcode Adapters
54.5 µL	TOTAL

2. Set pipette to 50 µL, gently pipette the entire volume up and down 10 times.

3. Apply adhesive PCR plate seal and incubate on a thermocycler for 15 minutes at 22°C.

STEP E: Clean-Up

Materials

Bioo Scientific Supplied

CLEAR CAP
Resuspension Buffer

User Supplied

Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand

1. Add 55 µL of AMPure XP Beads to each sample and gently pipette the entire volume up and down 10 times.
2. Incubate 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. Set pipette to 96 µL, gently remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in wells.
5. With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
6. Repeat step 5, for a total of 2 ethanol washes and ensure all ethanol has been removed.
7. Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
8. Resuspend dried beads with 57 µL Resuspension Buffer. Gently, pipette entire volume up and down 10 times mixing thoroughly and ensuring beads are no longer attached to the side of the well.
9. Incubate resuspended beads at room temperature for 2 minutes.
10. Place plate on magnetic stand for 5 minutes or until the sample appears completely clear.
11. Gently transfer 54.5 µL of clear sample to new well.
12. Repeat steps 1-7.
13. Resuspend dried beads with 22.5 µL Resuspension Buffer. Gently, pipette entire volume up and down 10 times mixing thoroughly. Ensure beads are no longer attached to the side of the well.
14. Incubate resuspended beads at room temperature for 2 minutes.
15. Place plate on magnetic stand for 5 minutes or until the sample appears completely clear.
16. Gently transfer 20 µL of clear sample to a new well.
17. If you wish to pause your experiment, the procedure may be safely stopped at this step and samples stored at -20°C. To restart, thaw frozen samples on ice before proceeding.

STEP F: PCR Amplification

Materials

Bioo Scientific Supplied

GREEN CAP
NEXTflex™ Primer Mix
NEXTflex™ PCR Master Mix

CLEAR CAP BOTTLE
Resuspension Water
Nuclease-free Water

User Supplied

Thermocycler
96 Well PCR Plate
***Ligation Product (from STEP E)**

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

20 µL	Ligation Product
16 µL	Nuclease-free H_2O
12 µL	NEXTflex™ PCR Master Mix
2 µL	NEXTflex™ Primer Mix
50 µL	TOTAL

2. Set pipette to 50 µL, gently pipette the entire volume up and down 10 times.

3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

2 min	98°C	
30 sec	98°C	
30 sec	65°C	Repeat 7 - 10 cycles*
60 sec	72°C	
4 min	72°C	

*PCR cycles will vary depending on the amount of starting material and quality of your sample. Further optimization may be necessary. Always use the least number of cycles possible.

4. Add 55 µL of AMPure XP Beads to each sample and gently pipette the entire volume up and down 10 times.

5. Incubate 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. Set pipette to 100 µL, gently remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in wells.

8. With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.

9. Repeat step 8, for a total of 2 ethanol washes and ensure all ethanol has been removed.

10. Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.

11. Resuspend dried beads with 33 µL **Nuclease-free Water**. Gently, pipette entire volume up and down 10 times mixing thoroughly. Ensure beads are no longer attached to the side of the well.

12. Incubate resuspended beads at room temperature for 2 minutes.

13. Place plate on magnetic stand for 5 minutes or until the sample appears clear.

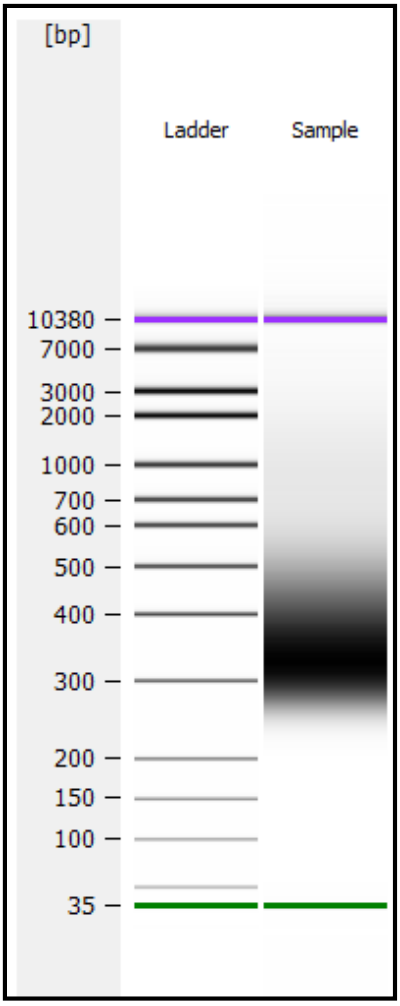
14. Gently transfer 30 µL of clear sample to a well of a new 96 well PCR Plate.

15. Verify the quality of the Amplified Sample Library by Agilent Bioanalyzer using a High Sensitivity DNA Chip and asses the quantity by Qubit® Fluorometer or Nanodrop.

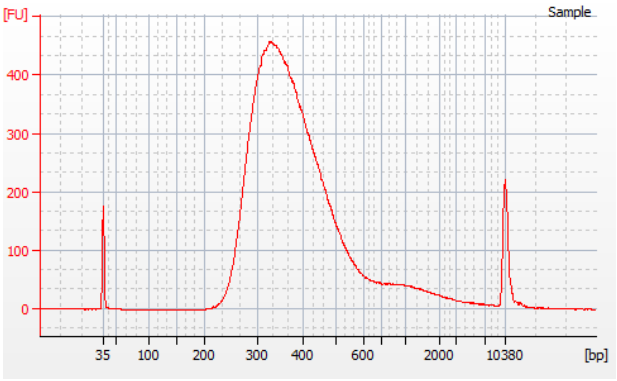
16. If you wish to pause your experiment, the procedure may be safely stopped at this step and samples stored at -20°C. To restart, thaw frozen samples on ice before proceeding to target capture using Roche Nimblegen SeqCap EZ Kits.

LIBRARY VALIDATION

Figure 2. Bioanalyzer validation of NEXTflex PCR product (7 cycles). Followed Option 1 to make library with an average insert size of 200 bp



High Sensitivity DNA Chip Gel Image



High Sensitivity DNA Electropherogram Gel

TARGET CAPTURE

NOTICE: Before starting Step G, prepare SeqCap EZ Library as described in Nimblegen SeqCap EZ Library User Guide V3 "Chapter 2. Storing the SeqCap EZ Library". (pg 13)

Note: *SeqCap EZ Library should be thawed and refrozen as little as possible. To ensure the highest quality of the SeqCap EZ Library, it is recommended that the user aliquot the entire volume into single-use 4.5 µL aliquots upon receipt.*

STEP G: Hybridizing the Sample and SeqCap EZ Libraries

NOTICE: This protocol is designed for post – capture pooling. If performing pre – capture pooling, proceed to Appendix B

Materials

Bioo Scientific Supplied

ORANGE CAP
NEXTflex™ INV-HE Index 1 – 12 (250 µM)

GREEN CAP
NEXTflex™ INV-HE Index 1 – 12 (250 µM)

WHITE CAP
NEXTflex™ Universal Oligo 1 (500 µM)

CLEAR CAP BOTTLE
Nuclease-free Water

User Supplied

NimbleGen SeqCap EZ Library
Heat Block
COT DNA
DNA Vacuum Concentrator
1.5 mL PCR tube
0.2 mL PCR tube
Centrifuge
Thermocycler
18-20 Gauge Needle
Laboratory tape
Amplified Sample Library in nuclease-free Water

1. Pre-heat heat block to 95°C.

2. For each multiplexed capture experiment, thaw one single-use 4.5 µL aliquot of SeqCap EZ

Library on ice.

3. Combine 5 µL of 1 mg/mL COT DNA and 1 µg amplified sample library in a new 1.5 mL tube.

4. For each target capture experiment, 2000 pmol of Hybridization Enhancing Oligo Pool must be prepared. The pool should contain 1000 pmol (2uL) of NEXTflex HE Universal Oligo 1, and 1000 pmol (4uL) of the NEXTflex INV-HE Index Oligo that corresponds to the NEXTflex™ DNA Adapter used during library construction.

Amount	Component
2 µL (1000 pmol)	NEXTflex™ HE Universal Oligo 1 (500 µM)
4 µL (250 pmol)	NEXTflex™ INV-HE Index Oligo (250 µM)
6 µL (2000 pmol)	**TOTAL**

5. Close the tube lid and pierce the tube's cap with an 18 – 20 gauge or finer needle. This is done to minimize contamination in the DNA vacuum concentrator.

6. Dry COT DNA / Multiplex DNA Library Pool / Multiplex Hybridization Enhancing Oligo Pool in a DNA vacuum concentrator on high heat (60°C).

7. To each dried down "Amplified Sample Library/COT DNA/NEXTflex™ INV-HE Index Oligos, add the following components :
   - 7.5 µL 2X Hybridization Buffer (NimbleGen)
   - 3 µL Hybridization Component A (NimbleGen)

The tube with the Amplified Sample Library / COT DNA / NEXTflex™ INV-HE Index Oligos should now contain the following components:

Amount	Component
5 µg	COT DNA
1 µg	Amplified Sample Library
2000 pmol*	Hybridization Enhancing Oligo Pool
7.5 µL	2X Hybridization Buffer (Vial 5)
3 µL	Hybridization Component A (Vial 6)
10.5 µL	**TOTAL**

*1000 pmol of NEXTflex™ HE Universal Oligo 1 and 1000 pmol 'mixture' of appropriate NEXTflex™ INV-HE Index Oligos.

8. Cover the hole in the tube cap with a sticker or small piece of laboratory tape.

9. Vortex the mix for 10 seconds and centrifuge at maximum speed for 10 seconds.

10. Place mix in a 95°C heat block for 10 minutes to denature the DNA.

11. Centrifuge mix at maximum speed for 10 seconds at room temperature.

12. Transfer mix to the 4.5 µL aliquot of SeqCap EZ Library in a 0.2 mL PCR tube (Entire volume can also be transferred to a 96-well PCR plate).

13. Vortex for 3 seconds and centrifuge at maximum speed for 10 seconds.

    The Hybridization Sample should now contain the following components:

Amount	Component
5 µg	COT DNA
1 ug	Amplified Sample Library
2000 pmol*	Hybridization Enhancing Oligo Pool
7.5 µL	2X Hybridization Buffer (Vial 5)
3 µL	Hybridization Component A (Vial 6)
4.5 µL	SeqCap EZ Library
15.0 µL	**TOTAL**

*1000 pmol of NEXTflex™ HE Universal Oligo 1 and 1000 pmol 'mixture' of appropriate NEXTflex™ INV-HE Index Oligos.

14. Incubate in a thermocycler at 47°C for 64 - 72 hours. The thermocycler's heated lid should be turned on and set to maintain 57°C, 10°C above the hybridization temperature.

STEP H: Washing and Recovering Captured DNA

Materials

User Supplied

NimbleGen SeqCap EZ Hybridization and Wash Kit (Roche, Cat. # 05634253001, 96 reactions)
Streptavidin Dynabeads
Dynamag-2 Device
1.5 mL Microcentrifuge Tube
Hybridization Samples from Step G

Follow the procedure in NimbleGen SeqCap EZ Library User Guide V3: "**Chapter 6**: Washing and Recovering Captured DNA." Follow all steps listed under Chapter 6.

STEP I: Amplifying Captured DNA Using LM-PCR

Materials

Bioo Scientific Supplied

BLUE CAP
NEXTflex™ PCR Oligo 1 (100 µM)
NEXTflex™ PCR Oligo 2 (100 µM)

YELLOW CAP
NEXTflex™ LM-PCR Master Mix (2X)

CLEAR CAP BOTTLE
Nuclease-free Water

User Supplied

Qiagen QIAquick PCR Purification Kit
Thermocycler
96 Well PCR Plate
Resuspension Buffer
Bead-bound Captured DNA from Step H

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

Amount	Component
20 µL	Bead-bound Captured DNA
26 µL	Nuclease-free Water
50 µL	NEXTflex™ LM - PCR Master Mix
2 µL	NEXTflex™ PCR Oligo 1
2 µL	NEXTflex™ PCR Oligo 2
100 µL	TOTAL

2. Set pipette to 90 µL, gently pipette the entire volume up and down 10 times.

3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

30 sec	98°C	
10 sec	98°C	
30 sec	65°C	Repeat 18 cycles
30 sec	72°C	
5 min	72°C	
Hold	4°C	

4. Follow the procedure in NimbleGen SeqCap EZ Library User Guide V3: "**Chapter 7**: Amplifying Captured DNA Using LM-PCR," starting with **"Step 3.** Cleaning up the Amplified Captured DNA**"**and ending with "**Step 4.** Determining the Concentration, Size Distribution, and Quality of the Amplified Captured DNA."

STEP J: Measuring Enrichment Using qPCR

Materials

User Supplied

qPCR NSC Forward and Reverse Oligos
SYBR Green Master Mix (2X)

To assess the quality of the captured DNA by qPCR, follow Nimblegen SeqCap EZ Library User Guide V3.0 "**Chapter 8.** Using qPCR for Amplified Sample Library Quality Control."

STEP K: Sequencing Amplified Captured Libraries

To perform multiplexed sequencing of amplified captured libraries, follow Nimblegen SeqCap EZ Library User Guide V3 "**Appendix C**: Multiplexed Sequencing."

APPENDIX A

Oligonucleotide Sequences

NEXTflex™	Sequence
Primer 1	5'AATGATACGGCGACCACCGAGATCTACAC
Primer 2	5'CAAGCAGAAGACGGCATACGAGAT
DNA Adapter	5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 5'GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**XXXXXX**[1]ATCTCGTATGCCGTCTTCTGCTTG

[1]**XXXXXX** denotes the index region of the adapter. The index sequence of each adapter is listed below.

Index 1	**CGATGT**
Index 2	**TGACCA**
Index 3	**ACAGTG**
Index 4	**GCCAAT**
Index 5	**CAGATC**
Index 6	**CTTGTA**
Index 7	**ATCACG**
Index 8	**TTAGGC**
Index 9	**ACTTGA**
Index 10	**GATCAG**
Index 11	**TAGCTT**
Index 12	**GGCTAC**

Index 13	**AGTCAA**
Index 14	**AGTTCC**
Index 15	**ATGTCA**
Index 16	**CCGTCC**
Index 17	**GTAGAG**
Index 18	**GTCCGC**
Index 19	**GTGAAA**
Index 20	**GTGGCC**
Index 21	**GTTTCG**
Index 22	**CGTACG**
Index 23	**GAGTGG**
Index 24	**GGTAGC**

NEXTflex™	Sequence
HE Universal Oligo 1	5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
INV-HE Index	5'CAAGCAGAAGACGGCATACGAGAT**XXXXXX**[2]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT/3INVdT/
PCR Oligo 1	5'AATGATACGGCGACCACCGAGATCTACAC
PCR Oligo 2	5'CAAGCAGAAGACGGCATACGAGAT

[2]**XXXXXX** denotes the inverse index region of the blocker strand. The inverse index sequences are listed below.

Index 1	**ACATCG**
Index 2	**TGGTCA**
Index 3	**CACTGT**
Index 4	**ATTGGC**
Index 5	**GATCTG**
Index 6	**TACAAG**
Index 7	**CGTGAT**
Index 8	**GCCTAA**
Index 9	**TCAAGT**
Index 10	**CTGATC**
Index 11	**AAGCTA**
Index 12	**GTAGCC**

Index 13	**TTGACT**
Index 14	**GGAACT**
Index 15	**TGACAT**
Index 16	**GGACGG**
Index 17	**CTCTAC**
Index 18	**GCGGAC**
Index 19	**TTTCAC**
Index 20	**GGCCAC**
Index 21	**CGAAAC**
Index 22	**CGTACG**
Index 23	**CCACTC**
Index 24	**GCTACC**

For an electronic list of the DNA barcodes contact: nextgen@biooscientific.com.

APPENDIX B

Hybridization of Multiplexed Samples (Pre – Capture)

NOTICE: This protocol is for pre – capture pooling of up to 24 uniquely barcoded samples, and hybridization of the pool with SeqCap EZ Libraries.

Materials

Bioo Scientific Supplied

ORANGE CAP
NEXTflex™ INV-HE Index 1 – 12 (250 µM)

GREEN CAP
NEXTflex™ INV-HE Index 1 – 12 (250 µM)

WHITE CAP
NEXTflex™ Universal Oligo 1 (500 µM)

User Supplied

NimbleGen SeqCap EZ Exome Library Kit (Roche, Cat. # 06465692001, 48 reactions) / or/
NimbleGen SeqCap EZ Choice Library Kit (Roche, Cat. # 06266312001, 48 reactions)
Thermocycler
1 mg/mL COT Human DNA (Roche Applied Science, Cat. # 05480647001)
DNA Vacuum Concentrator
Heat block for 95° incubation
Uniquely Barcoded Amplified Sample Libraries in Nuclease-free Water

1. Thaw on ice each of the uniquely barcoded amplified sample library to be used in the multiplex target capture experiment.

2. To prepare your Multiplex DNA Sample Library Pool, combine equal mass of each of the amplified sample so that the mass of the pooled library is 1.1 µg.

   Example: If the multiplex pool contains 4 samples barcoded with NEXTflex™ DNA Adapter 1, 2, 3 and 4 respectively, then setup pooling as detailed below:

Amount	Component
0.275 µg	Sample barcoded with NEXTflex™ DNA Adapter 1
0.275 µg	Sample barcoded with NEXTflex™ DNA Adapter 2
0.275 µg	Sample barcoded with NEXTflex™ DNA Adapter 3
0.275 µg	Sample barcoded with NEXTflex™ DNA Adapter 4
1.1 µg	**TOTAL**

3. Thaw the following on ice: NEXTflex™ HE Universal Oligo 1 (500 µM) and all the NEXTflex™ INV-HE Index oligos (250 µM) that match each of the barcoded samples in the Multiplex DNA Library Pool from Step 2.

   Note: For optimal target capture results, it is important to pool the correct NEXTflex™ INV-HE Index Oligos based on the NEXTflex™ DNA Adapters in the Multiplex DNA Library Pool.

4. For each target capture experiment, 2000 pmol of Hybridization Enhancing Oligo Pool must be prepared. The pool should contain 1000 pmol (2uL) of NEXTflex HE Universal Oligo 1, and the remaining 1000 pmol should comprise equal mass of the appropriate NEXTflex INV-HE Index Oligos.

   Example: If the Multiplex DNA Sample Library Pool contains 4 samples barcoded with NEXTflex™ DNA Adapter 1, 2, 3 and 4 respectively, then setup hybridization pooling as detailed below:

Amount	Component
2 µL (1000 pmol)	NEXTflex™ HE Universal Oligo 1 (500 µM)
1 µL (250 pmol)	NEXTflex™ INV-HE Index Oligo 1 (250 µM)
1 µL (250 pmol)	NEXTflex™ INV-HE Index Oligo 2 (250 µM)
1 µL (250 pmol)	NEXTflex™ INV-HE Index Oligo 3 (250 µM)
1 µL (250 pmol)	NEXTflex™ INV-HE Index Oligo 4 (250 µM)
6 µL (2000 pmol)	**TOTAL**

5. For each multiplexed capture experiment, thaw one single-use 4.5 µL aliquot of SeqCap EZ Library on ice.

   Note: SeqCap EZ Library should be thawed and refrozen as little as possible. To ensure the highest quality of the SeqCap EZ Library, it is recommended that the user aliquot the entire volume into single-use 4.5 µL aliquots upon receipt.

6. Combine 5 µL of 1 mg/mL COT DNA and 1 µg of Multiplex DNA Sample Library Pool in a new 1.5 mL tube.

7. Add 6 µL of Hybridization Enhancing Oligo Pool (2000 pmol) to the tube containing COT DNA and 'Multiplex DNA Library Pool'.

8. Close the tube lid and pierce the tube's cap with an 18 – 20 gauge or finer needle. This is done to minimize contamination in the DNA vacuum concentrator.

9. Dry COT DNA / Multiplex DNA Sample Library Pool / Multiplex Hybridization Enhancing Oligo Pool in a DNA vacuum concentrator on high heat (60°C)

10. To each dried down COT DNA / Multiplex DNA SampleLibrary Pool / Multiplex Hybridization Enhancing Oligo Pool, add the following components:
    - 7.5 µL of 2X Hybridization Buffer (Vial 5)
    - 3 µL of Hybridization Component A (Vial 6)

    The tube with COT DNA / Multiplex DNA Library Pool / Multiplex Hybridization Enhancing Oligo Pool now contains the following components:

Amount	Component
5 µg	COT DNA
1 µg	Multiplex DNA Sample Library Pool
2000 pmol*	Multiplex Hybridization Enhancing Pool
7.5 µL	2X Hybridization Buffer (Vial 5)
3 µL	Hybridization Component A (Vial 6)
10.5 µL	**TOTAL**

*1000 pmol of NEXTflex™ HE Universal Oligo 1 and 1000 pmol 'mixture' of appropriate NEXTflex™ INV-HE Index Oligos.

11. Seal the hole on the tube cap with small sticker or laboratory tape.

12. Vortex the tube 10 seconds and centrifuge at maximum speed for 10 seconds.

13. Place the tube in a heat block at 95°C for 10 minutes to denature the DNA.

14. Centrifuge the tube for 10 seconds at room temperature.

15. Transfer COT DNA / Multiplex DNA Sample Library Pool / Multiplex Hybridization Enhancing Oligo Pool / Hybridization Cocktail (2X Hybridization Buffer and Hybridization Component A) to the 4.5 µL aliquot of SeqCap EZ Library in a 0.2 mL PCR tube.

16. Vortex the tube for 3 seconds and centrifuge at maximum speed for 10 seconds.

    The hybridization sample now contains the following components:

Amount	Component
5 µg	COT DNA
1 µg	Multiplex DNA Sample Library Pool
2000 pmol*	Multiplex Hybridization Enhancing Pool
7.5 µL	2X Hybridization Buffer (Vial 5)
3 µL	Hybridization Component A (Vial 6)
4.5 µL	SeqCap EZ Library
15 µL	**TOTAL**

*1000 pmol of NEXTflex™ HE Universal Oligo 1 and 1000 pmol 'mixture' of appropriate NEXTflex™ INV-HE Index Oligos.

17. Incubate in a thermocycler at 47°C for 64 - 72 hours. The thermocycler's heated lid should be turned on and set to maintain 57°C, 10°C above the hybridization temperature.

18. After hybridization, proceed to **STEP H: Washing and Recovering Captured DNA** (pg 15 of this User Manual). Follow procedure through **Target Capture**.

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RELATED PRODUCTS

Illumina Compatible DNA NGS Kits and Adapters

Product	Catalog Number
NEXTflex™ Msp 1 (8 reactions)	511921
NEXTflex™ Msp 1 (48 reactions)	511922
NEXTflex™ Bisulfite-Seq kit (8 reactions)	5119-01
NEXTflex™ Bisulfite-Seq kit (48 reactions)	5119-02
NEXTflex™ Bisulfite-Seq Barcodes – 6	511911
NEXTflex™ Bisulfite-Seq Barcodes – 12	511912
NEXTflex™ DNA Sequencing Kit (8 reactions)	5140-01
NEXTflex™ DNA Sequencing Kit (48 reactions)	5140-02
NEXTflex™ DNA Barcodes – 6	514101
NEXTflex™ DNA Barcodes – 12	514102
NEXTflex™ DNA Barcodes – 24	514103
NEXTflex™ DNA Barcodes – 48	514104
NEXTflex-96™ DNA Barcodes	514106
NEXTflex™ ChIP-Seq Kit (8 reactions)	5143-01
NEXTflex™ ChIP-Seq Kit (48 reactions)	5143-02
NEXTflex™ ChIP-Seq Barcodes – 6	514120
NEXTflex™ ChIP-Seq Barcodes – 12	514121
NEXTflex™ ChIP-Seq Barcodes – 24	514122
NEXTflex™ ChIP-Seq Barcodes – 48	514123
NEXTflex-96™ ChIP-Seq Barcodes	514124
NEXTflex™ PCR-Free DNA Sequencing Kit (8 reactions)	5142-01
NEXTflex™ PCR-Free DNA Sequencing Kit (48 reactions)	5142-02
NEXTflex™ PCR-Free Barcodes – 6	514110
NEXTflex™ PCR-Free Barcodes – 12	514111
NEXTflex™ PCR-Free Barcodes – 24	514112
NEXTflex™ PCR-Free Barcodes – 48	514113
NEXTflex™ Methyl-Seq 1 kit (8 reactions)	5118-01
NEXTflex™ Methyl-Seq 1 kit (48 reactions)	5118-02

DNA Fragmentation

Product	Catalog Number
AIR™ DNA Fragmentation Kit (10 reactions)	5135-01
AIR™ DNA Fragmentation Kit (40 reactions)	5135-02

Illumina Compatible RNA NGS Kits and Adapters

Product	Catalog Number
NEXTflex™ RNA-Seq Kit (8 reactions)	5129-01
NEXTflex™ RNA-Seq Kit (48 reactions)	5129-02
NEXTflex™ RNA-Seq Barcodes – 6	512911

NEXTflex™ RNA-Seq Barcodes – 12	512912
NEXTflex™ RNA-Seq Barcodes – 24	512913
NEXTflex™ RNA-Seq Barcodes – 48	512914
NEXTflex-96™ RNA-Seq Barcodes	512916
NEXTflex™ Directional RNA-Seq Kit (dUTP-Based) (8 reactions)	5129-05
NEXTflex™ Directional RNA-Seq Kit (dUTP-Based) (48 reactions)	5129-06
NEXTflex™ Directional RNA-Seq Kit (Ligaton-Based) (8 reactions)	5134-01
NEXTflex™ Directional RNA-Seq Kit (Ligaton-Based) (24 reactions)	5134-02
NEXTflex™ Directional RNA-Seq Kit (Ligaton-Based) (48 reactions)	5134-03
NEXTflex™ Directional RNA-Seq Barcodes – Set A	513311
NEXTflex™ Directional RNA-Seq Barcodes – Set B	513312
NEXTflex™ Directional RNA-Seq Barcodes – Set C	513313
NEXTflex™ Directional RNA-Seq Barcodes – Set D	513314
NEXTflex™ Small RNA Sequencing Kit (24 reactions)	5132-01
NEXTflex™ Small RNA Sequencing Kit (48 reactions)	5132-02
NEXTflex™ Small RNA Barcodes – Set A	513301
NEXTflex™ Small RNA Barcodes – Set B	513302
NEXTflex™ Small RNA Barcodes – Set C	513303
NEXTflex™ Small RNA Barcodes – Set D	513304

Bioo Scientific also offers library prep kits and barcodes for the Ion Torrent, 5500 SOLiD and SOLiD 4 sequencing platforms. For more information about any of these kits visit our website at www.biooscientific.com.

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