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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™ BRCA1 & BRCA2 Amplicon Panel

(Ion Torrent)

Catalog #4040-01 (8 reactions)

 V15.11

This product is for research use only.

NEXTflex™ BRCA1 & BRCA2 Amplicon Panel - 4040-01

Product Overview

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel produces barcoded amplicon libraries compatible with Ion Torrent platforms. Libraries are constructed using high quality genomic DNA extracted from blood or cell samples. FFPE or cfDNA samples are currently not compatible with this kit. This panel contains a total of 131 primer pairs in two pools that allow for the amplification and sequencing of all *BRCA1* and *BRCA2* coding exons. Amplicon insert sizes (without primers) range from 101-243 bp. These target regions are amplified in PCR I, which is followed by end repair and and adapter ligation. PCR II then enriches for the product of interest necessary for downstream Ion sequencing (Fig. 1). NEXTflex™ BRCA Amplicon Ion Primer Mixes have been optimized to achieve high coverage uniformity and reduction of off-target reads.

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel covers 20.9 kilobases comprising 48 coding *BRCA1* and *BRCA2* exons. Libraries have 100% uniformity at ≥0.2x mean coverage and > 97.5% on-target reads. Up to 20 samples can be multiplexed with at least 100x coverage on a single Ion 318™ Chip Kit v2.

Contents, Storage and Shelf Life

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel contains enough material to prepare 8 sample libraries. The shelf life of all reagents is 12 months when stored properly. All of the components can be safely stored at -20°C.

Kit Contents	Amount
PINK CAP	
NEXTflex™ BRCA Amplicon Ion Primer Mix 1	32 µL
BLUE CAP	
NEXTflex™ BRCA Amplicon Ion Primer Mix 2	32 µL
CLEAR CAP	
NEXTflex™ Hot Start PCR I Master Mix	192 µL
RED CAP	
NEXTflex™ End Repair Buffer Mix	56 µL
NEXTflex™ End Repair Enzyme Mix	24 µL
PURPLE CAP	
NEXTflex™ Ligation Mix	252 µL
NEXTflex™ DNA P1 Adapter	16 µL
NEXTflex™ DNA Barcoded Adapters 1-8	4 µL each

GREEN CAP	
NEXTflex™ Primer Mix	16 µL
NEXTflex™ PCR II Master Mix	80 µL

WHITE CAP	
Nuclease-free Water	1.5 mL
Resuspension Buffer	1.5 mL

Required Materials not Provided

- 20-100 ng of high-quality genomic DNA in up to 34 µL nuclease-free water
- 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)
- Agencourt AMPure XP 5 mL (Beckman Coulter Genomics, Cat # A63880)
- Magnetic Stand -96 (Ambion, Cat # AM10027) / or / similar
- Thermocycler
- 2, 10, 20, and 200 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips
- Microcentrifuge
- 1.5 mL nuclease-free microcentrifuge tubes
- 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.
- Ensure pipettes are properly calibrated, as library preparation is highly sensitive to pipetting error.
- Do not heat the DNA Adapters above room temperature.
- Try to 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.
- NEXTflex™ BRCA Amplicon FFPE Primer Mixes are required for PCR I amplification.

NEXTflex™ BRCA1 & BRCA2 Amplicon Panel Preparation Flow Chart

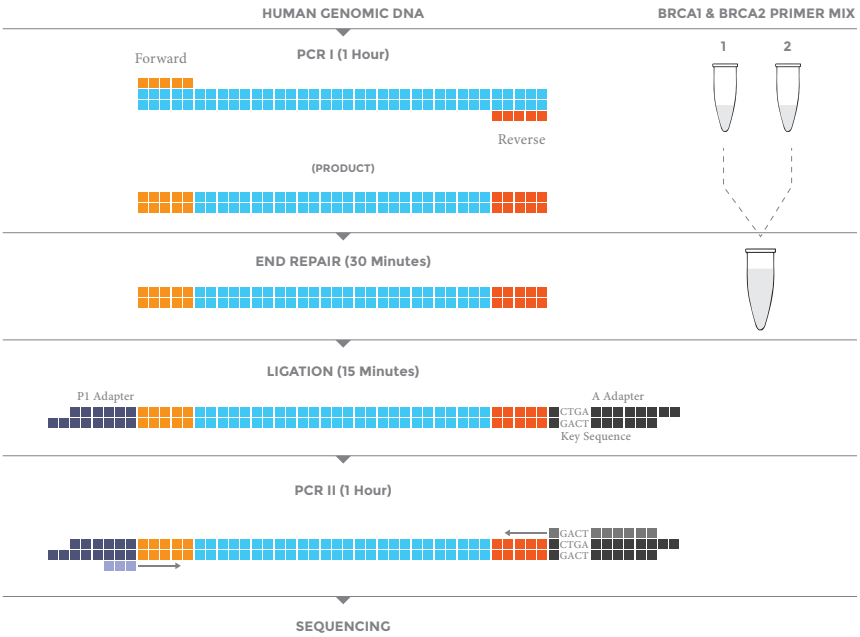

Figure 1: Sample flow chart with the approximate time necessary for each step.

Starting Material

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel has been optimized and validated using 20-100 ng of high-quality genomic DNA. This kit is not compatible with FFPE or cfDNA samples.

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 tube 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.

STEP A: PCR I – *BRCA1* and *BRCA2* Amplification

Materials

Bioo Scientific Supplied

PINK CAP - NEXTflex™ BRCA Amplicon Ion Primer Mix 1
BLUE CAP - NEXTflex™ BRCA Amplicon Ion Primer Mix 2
CLEAR CAP - NEXTflex™ Hot Start PCR I Master Mix
WHITE CAP - Nuclease-free Water

User Supplied

Thermocycler
96 Well PCR Plate
Adhesive PCR Plate Seal
10-50 ng of genomic DNA for each reaction in up to 34 µL of water

1. For each sample, prepare two separate reactions using BRCA Amplicon Ion Primer Mix 1 and 2 by combining the following reagents in adjacent wells in a PCR plate:

	Reaction 1
_ µL	Genomic DNA (10-50 ng in up to 34 µL)
_ µL	Nuclease-free water
4 µL	NEXTflex™ BRCA Amplicon Ion Primer Mix 1
12 µL	NEXTflex™ Hot Start PCR I Master Mix
50 µL	TOTAL

	Reaction 2
_ µL	Genomic DNA (10-50 ng in up to 34 µL)
_ µL	Nuclease-free water
4 µL	NEXTflex™ BRCA Amplicon Ion Primer Mix 2
12 µL	NEXTflex™ Hot Start PCR I Master Mix
50 µL	TOTAL

2. Mix thoroughly by pipette.
3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

2 min	98°C	
20 sec	98°C	Repeat for a total of 15 (10 ng input) or 12 cycles (50 ng input)
4 min	62°C	

4. Proceed to Step B: PCR I Cleanup.

STEP B: PCR I Cleanup

Materials

Bioo Scientific Supplied
WHITE CAP - Resuspension Buffer

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
96 Well PCR Plate
Magnetic Stand
PCR I Product (from Step A)

1. Add 75 µL of AMPure XP Beads to each sample. Mix thoroughly until homogenized.
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. Remove and discard the supernatant. Do not 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. Repeat this step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
6. Remove the plate from the magnetic stand and let dry at room temperature for 5 minutes or until bead pellet is visibly dry.
7. Resuspend dried beads with 22 µL of Resuspension Buffer. Mix thoroughly by pipette. Ensure beads are no longer attached to the side of the well.
8. Incubate resuspended beads at room temperature for 3 minutes.
9. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.
10. Transfer 20 µL of clear supernatant (purified PCR I product) to new well.
11. Proceed to Step C: End Repair.

STEP C: End Repair

Materials

Bioo Scientific Supplied

RED CAP - NEXTflex™ End Repair Buffer Mix, NEXTflex™ End Repair Enzyme Mix

User Supplied

96 well PCR Plate
Adhesive PCR Plate Seal
Thermocycler
Ice
Purified PCR I Reactions 1 and 2 (from Step B)

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

20 µL	Purified PCR I Reaction 1
20 µL	Purified PCR I Reaction 2
7 µL	NEXTflex™ End Repair Buffer Mix
3 µL	NEXTflex™ End Repair Enzyme Mix
50 µL	TOTAL

2. Mix thoroughly by pipette.
3. Apply adhesive PCR plate seal and incubate at 22°C for 30 minutes.
4. Proceed to Step D: Cleanup.

STEP D: Cleanup

Materials

Bioo Scientific Supplied
WHITE CAP - Resuspension Buffer

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
96 Well PCR Plate
Magnetic Stand
End Repaired DNA (from Step C)

1. Add 90 µL of AMPure XP Beads to each sample. Mix thoroughly until homogenized.
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. Remove and discard the supernatant. Do not 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. Repeat this step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
6. Remove the plate from the magnetic stand and let dry at room temperature for 5 minutes or until bead pellet is visibly dry.
7. Resuspend dried beads with 21 µL of Resuspension Buffer. Mix thoroughly by pipette. Ensure beads are no longer attached to the side of the well.
8. Incubate resuspended beads at room temperature for 3 minutes.
9. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.
10. Gently transfer 19 µL of clear sample to new well.
11. 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.
12. Proceed to Step E: Adapter Ligation.

STEP E: Adapter Ligation

Materials

Bioo Scientific Supplied

PURPLE CAP - NEXTflex™ Ligation Mix (thaw on ice right before use and store immediately after use at -20°C), NEXTflex™ DNA P1 Adapter, NEXTflex™ DNA Barcoded Adapter for Ion platforms (1-8)

User Supplied

96 well PCR Plate
Adhesive PCR Plate Seal
Thermocycler
Ice
Purified End Repaired DNA (from Step D)

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

19 µL	Purified End Repaired DNA
2 µL	NEXTflex™ DNA P1 Adapter
2 µL	NEXTflex™ DNA Barcoded Adapter
31.5 µL	NEXTflex™ Ligation Mix
54.5 µL	TOTAL

2. Mix thoroughly by pipette.
3. Apply adhesive PCR plate seal and incubate at 22°C for 15 minutes.
4. Proceed to Step F: Cleanup.

STEP F: Cleanup

Materials

Bioo Scientific Supplied
WHITE CAP - Resuspension Buffer

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
96 Well PCR Plate
Magnetic Stand
Adapter Ligated DNA (from Step E)

1. Add 43 µL of AMPure XP Beads to each sample. Mix thoroughly until homogenized.
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. Remove and discard the supernatant. Do not 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. Repeat this step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
6. Remove the plate from the magnetic stand and let dry at room temperature for 5 minutes or until bead pellet is visibly dry.
7. Resuspend dried beads with 40 µL of Resuspension Buffer. Mix thoroughly by pipette. Ensure beads are no longer attached to the side of the well.
8. Incubate resuspended beads at room temperature for 5 minutes.
9. Place the 96 well PCR plate on the magnetic stand at room temperature for 3 minutes, or until sample appears clear.
10. Gently transfer 38 µL of clear sample to new well.
11. 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 Step G: PCR II Amplification and Cleanup.

STEP G: PCR II Amplification and Cleanup

Materials

Bioo Scientific Supplied

GREEN CAP - NEXTflex™ Primer Mix, NEXTflex™ PCR II Master Mix
WHITE CAP - Resuspension Buffer

User Supplied

Thermocycler
96 Well PCR Plate
Adhesive PCR Plate Seal
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand
Purified Adapter Ligated DNA (from STEP F)

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

38 µL	Purified Adapter Ligated DNA (from Step F)
2 µL	NEXTflex™ Primer Mix
10 µL	NEXTflex™ PCR II Master Mix
50 µL	TOTAL

2. Mix thoroughly by pipette.
3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

20 min	65°C	Nick Translation
2 min	95°C	
30 sec	95°C	} Repeat for a total of 15 (10 ng input) or 12 cycles (50 ng input)
30 sec	58°C	
60 sec	72°C	
4 min	72°C	

4. Remove PCR plate from the thermocycler. Add 40 µL of AMPure XP Beads to each sample and mix thoroughly until homogenized.
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. Remove and discard the supernatant. Do not 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. Repeat this step for a total of 2 ethanol washes. Ensure all ethanol has been removed.
9. Remove the plate from the magnetic stand and let dry at room temperature for 5 minutes or until bead pellet is visibly dry.
10. Resuspend dried beads with 22 µL of Resuspension Buffer. Mix thoroughly by pipette. Ensure beads are no longer attached to the side of the well.
11. Incubate resuspended beads at room temperature for 5 minutes.
12. Place the 96 well PCR plate on the magnetic stand at room temperature for 3 minutes, or until sample appears clear.
13. Gently transfer 20 µL of clear sample to a new well. Proceed to library analysis and template preparation or seal plate with adhesive PCR plate seal and store at -20°C
14. To determine the Library Dilution factor required for Template Preparation, accurate quantification of the library is necessary. Qubit and Bioanalyzer are recommended to quantify and analyze quality of the library.
15. If significant high molecular weight products remain, use the following cleanup steps:
16. Bring sample volume to 30 µL with Nuclease-free Water.
17. Add 15 µL AMPure XP beads and mix well by pipetting.
18. Incubate at room temperature for 5 minutes.
19. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until the supernatant appears completely clear.
20. Gently transfer 45 µL of clear sample to a new well, being careful to not disturb the bead pellet. Do not discard the supernatant.
21. Remove the plate from the magnetic stand.
22. Add 45 µL of AMPure XP Beads to each sample and mix thoroughly until homogenized.
23. Repeat steps 5 - 14.

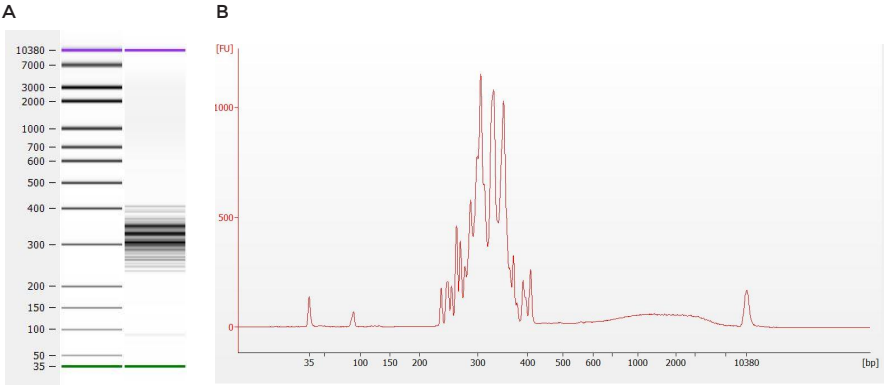

Fig. 2 NEXTflex™ BRCA1 & BRCA2 Amplicon Panel Libraries. *Libraries were visualized using Agilent Bioanalyzer High Sensitivity DNA Chip Output:*
A) NEXTflex™ BRCA1 & BRCA2 Amplicon Panel Library – 100 ng input (Bioanalyzer gel image).
B) NEXTflex™ BRCA1 & BRCA2 Amplicon Panel Library – 100 ng input (electropherogram).

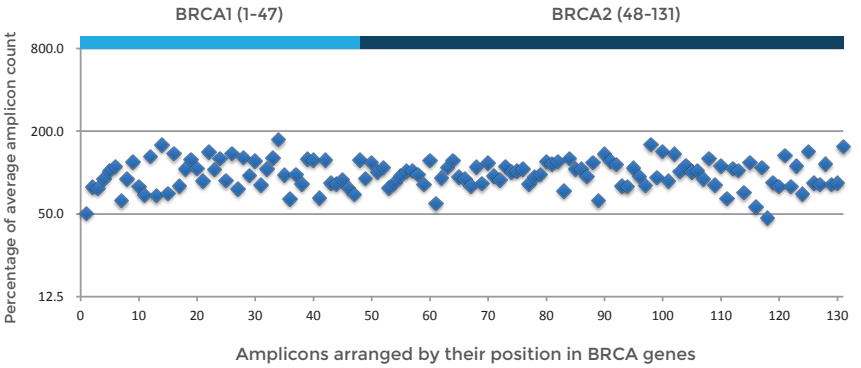

Figure 3. Performance of 131 amplicons from NEXTflex™ BRCA 1 & BRCA 2 Amplicon Panel for detection of germline mutations.

Oligonucleotide Sequences

NEXTflex™	Sequence 5' → 3'
P1 Adapter	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT ATCACCGACTGCCCATAGAGAGGAAAGCGGAGGCGTAGTGGTT
A1 Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACGAT ATCGTTACCTTAGCTGAGTCGGAGACACGC
A2 Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACGAT ATCGTTCTCCTTACTGAGTCGGAGACACGC
A3 Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCGAT ATCGAATCCTCTTCTGAGTCGGAGACACGC
A4 Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCGAT ATCGATCTTGGTACTGAGTCGGAGACACGC
A5 Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACGAT ATCGTTCCTTCTGCTGAGTCGGAGACACGC
A6 Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCGAT ATCGAACTTGCAGCTGAGTCGGAGACACGC
A7 Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCGAT ATCGAATCACGAACTGAGTCGGAGACACGC
A8 Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACGAT ATCGTTATCGGAACTGAGTCGGAGACACGC
Primer 1	CCTCTCTATGGGCAGTCGGTGAT
Primer 2	CCATCTCATCCCTGCGTGTCTCCGAC

Underlined sequences denote the index regions of adapters.

For a complete electronic list of the BED and FASTA files for this kit, please follow the instructions on the label on the inside of the kit box.

Ion PGM™ & Ion Proton™ Compatible DNA NGS Kits

Catalog #	Product
4001-01	NEXTflex™ DNA-Seq Kit for Ion PGM™ & Ion Proton (8 reactions)
4001-02	NEXTflex™ DNA-Seq Kit for Ion PGM™ & Ion Proton (48 reactions)
401001	NEXTflex™ DNA-Seq Kit Barcodes for Ion PGM™ & Ion Proton - 8
401002	NEXTflex™ DNA-Seq Kit Barcodes for Ion PGM™ & Ion Proton - 16
401003	NEXTflex™ DNA-Seq Kit Barcodes for Ion PGM™ & Ion Proton - 32
401004	NEXTflex™ DNA-Seq Kit Barcodes for Ion PGM™ & Ion Proton - 64

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THE NGS EXPERTS™

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