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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

(Illumina Compatible)

Catalog #4220-01 (8 reactions)

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NEXTflex™ BRCA1 & BRCA2 Amplicon Panel - 4220-01

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Product Overview

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel produces Illumina™ compatible, barcoded amplicon libraries in two PCR steps. Libraries are constructed using high quality genomic DNA extracted from blood or cell samples. FFPE or cfDNA samples are not compatible with this kit. This panel contains a total of 130 primer pairs in two pools that allow for the amplification and sequencing of all *BRCA1* and *BRCA2* coding exons. Amplicon regions of interest range between 101 – 229 bp. The regions of interest plus primer pad sites, which comprise the read portion of the libraries, range between 152 – 284 bp. These target regions are amplified and barcoded in two PCR steps (Fig. 1). NEXTflex™ BRCA Amplicon Primer Mixes have been optimized to achieve high coverage uniformity and reduction of off-target reads.

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel covers 20.4 kilobases comprising 48 coding *BRCA1* and *BRCA2* exons. Libraries have 100% uniformity at ≥0.2x mean coverage and > 99% on-target reads. Up to 384 samples can be multiplexed with at least 100x coverage on a single Illumina 2x300 MiSeq lane for detection of germline mutations. Up to 14 samples can be multiplexed at 2000x coverage for detection of somatic mutations. Standard Illumina sequencing primers may be used with this kit.

Contents, Storage and Shelf Life

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel contains enough material to prepare 8 samples for Illumina® compatible sequencing. The shelf life of all reagents is 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, which should be stored at 4°C.

Kit Contents	Amount
ORANGE CAP	
NEXTflex™ BRCA Amplicon Primer Mix 1	32 µL
RED CAP	
NEXTflex™ BRCA Amplicon Primer Mix 2	32 µL
YELLOW CAP	
NEXTflex™ PCR II Barcoded Primer Mix 1 – 8	4 µL each
GREEN CAP	
NEXTflex™ PCR Master Mix	288 µL
WHITE CAP	
Nuclease-free Water	1.5 mL
Resuspension Buffer	1 mL
BROWN CAP	
NEXTflex™ Cleanup Beads	(2) 900 µL

Required Materials not Provided

- 20-100 ng of high-quality genomic DNA (two 10-50 ng aliquots in up to 34 µL nuclease-free water each)
- 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
- Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
- Magnetic Stand -96 (Ambion, Cat # AM10027) or similar
- Thermocycler
- 2, 10, 20, and 200 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips
- Vortex
- 80% Ethanol, freshly prepared (room temperature)

Revision History

Version	Date	Description
v15.11	November 2015	Initial Product Launch
v16.02	February 2016	NEXTflex™ Cleanup Beads are now included with this kit.
v16.04	April 2016	PCR I Reaction and Cleanup volumes have been optimized. PCR I Reactions 1 & 2 are now pooled in PCR II Reaction.

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 PCR is highly sensitive to pipetting error.
- Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
- Genomic DNA sample quality may vary between preparations. It is the user's responsibility to utilize high quality genomic DNA. Genomic 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™ BRCA1 & BRCA2 Amplicon Panel Preparation Flow Chart

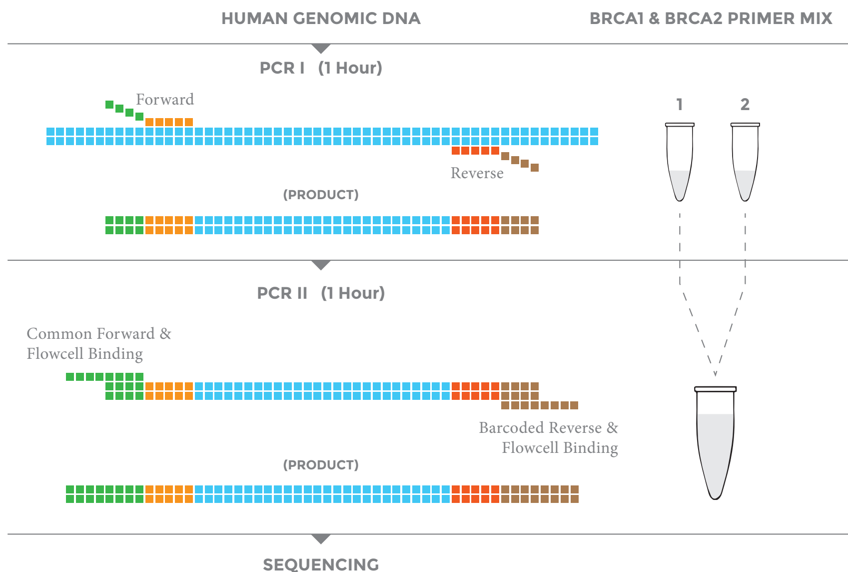

Figure 1: Sample flow chart with the approximate time necessary for each step.

Starting Material

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel has been optimized and validated using 20-100 ng of high-quality genomic DNA (Fig. 2). This kit is not compatible with FFPE or cfDNA samples.

Reagent Preparation

1. Thaw all tubes on ice.
2. Briefly mix and centrifuge each component just prior to use to ensure material has not lodged in the cap or side of tube.
3. Allow NEXTflex™ Cleanup 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

ORANGE CAP - NEXTflex™ BRCA Amplicon Primer Mix 1
RED CAP - NEXTflex™ BRCA Amplicon Primer Mix 2
GREEN CAP - NEXTflex™ PCR Master Mix
WHITE CAP - Nuclease-free Water

User Supplied

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

1. For each sample prepare two separate reactions in adjacent wells of a 96-well PCR Plate on ice as described below. Note: It is recommended to combine these reagents as a master mix if processing multiple samples.

	Reaction 1
_ µL	Genomic DNA (10-50 ng)
_ µL	Nuclease-free Water
4 µL	NEXTflex™ BRCA Amplicon Primer Mix 1
12 µL	NEXTflex™ PCR Master Mix
50 µL	TOTAL

	Reaction 2
_ µL	Genomic DNA (10-50 ng)
_ µL	Nuclease-free Water
4 µL	NEXTflex™ BRCA Amplicon Primer Mix 2
12 µL	NEXTflex™ PCR 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 9 cycles
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
BROWN CAP - NEXTflex™ Cleanup Beads (room temperature)

User Supplied

80% Ethanol, freshly prepared (room temperature)
96 Well PCR Plate
Magnetic Stand
PCR I Reaction 1 & 2 (from Step A)

1. Add 75 µL of NEXTflex™ Cleanup Beads to each reaction. 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 for each sample 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 pellets are visibly dry.
7. Resuspend dried beads with 20 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 samples appear clear.
10. Transfer 18 µL of clear supernatant from each sample to a new well.
11. Proceed to Step C: PCR II Amplification (Indexing) and Cleanup.

STEP C: PCR II Amplification (Indexing) and Cleanup

Materials

Bioo Scientific Supplied
YELLOW CAP - NEXTflex™ PCR II Barcoded Primer Mix (1-8)
GREEN CAP - NEXTflex™ PCR Master Mix
WHITE CAP - Resuspension Buffer
BROWN CAP - NEXTflex™ Cleanup Beads (room temperature)

User Supplied
Thermocycler
96 Well PCR Plate
Adhesive PCR Plate Seal
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand
Ice
Purified PCR I Reactions 1 & 2 (from step B)

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

18 µL	Purified PCR I Reaction 1
18 µL	Purified PCR I Reaction 2
2 µL	NEXTflex™ PCR II Barcoded Primer Mix
12 µL	NEXTflex™ PCR 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	
30 sec	98°C	Repeat for a total of 21 cycles
30 sec	60°C	
30 sec	72°C	
4 min	72°C	

4. Remove plate from thermocycler. Add 40 µL of NEXTflex™ Cleanup Beads to each sample. 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 3 minutes.

12. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.

13. Transfer 20 µL of clear supernatant to a new well.

14. To ensure optimal cluster generation, library concentration must be determined. To determine the library dilution factor required for template preparation, Qubit and/or Bioanalyzer analyses are necessary (Fig 2).

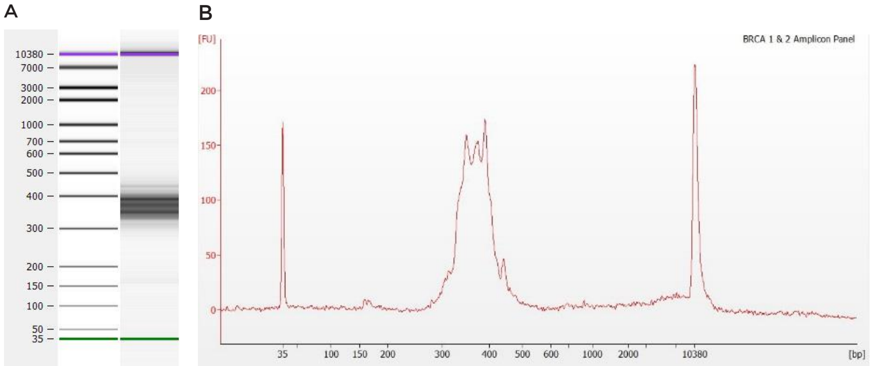

Figure 2. ***NEXTflex™ BRCA 1 & BRCA 2 Amplicon Panel Libraries.*** *Libraries were visualized using Agilent Bioanalyzer. High Sensitivity DNA Chip Ladder / Electropherogram:*
A) 100 ng input NEXTflex™ BRCA1 & BRCA2 Amplicon Panel Library (Bioanalyzer gel image)
B) 100 ng input NEXTflex™ BRCA1 & BRCA2 Amplicon Panel Library (electropherogram)

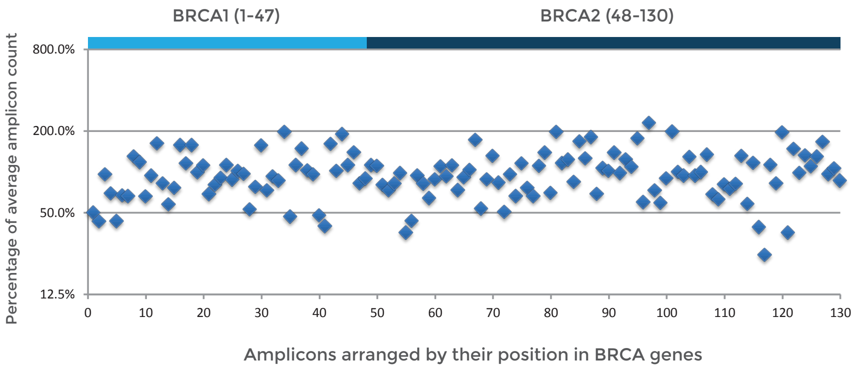

Figure 3. Performance of 130 amplicons from NEXTflex™ BRCA 1 & BRCA 2 Amplicon Panel for detection of germline mutations.

Oligonucleotide Sequences

NEXTflex™ PCR II Barcoded Primer Mix	
NEXTflex™	**Sequence 5' → 3'**
PCR II Forward Primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCT
PCR II Reverse Barcoded Primer	CAAGCAGAAGACGGCATACGAGAT**XXXXXXXXXXXX**[1]GTGACTGGAGTT CAGACGTGTGCTCTTCCGATCT

[1]**XXXXXXXXXXXX** denotes the index region of primer. The index sequences and the respective reverse complement sequences contained in each primer are listed below. The reverse complement is the sequence reported in the index read.

Reverse Primer Index Sequences and Reverse Complements

Barcoded Primer	Sequence 5' → 3'	Reverse Complement
1	GGCCGGCTAGAT	ATCTAGCCGGCC
2	AAGGAAGAGATA	TATCTCTTCCTT
3	GGACGGCATCTA	TAGATGCCGTCC
4	AAGGAAGGAGCG	CGCTCCTTCCTT
5	GGACGGCGCTCG	CGAGCGCCGTCC
6	CCGGACTCTCGA	TCGAGAGTCCGG
7	GGCCGGCCGAGC	GCTCGGCCGGCC
8	CCGGACTGAGCT	AGCTCAGTCCGG

Low Level Multiplexing

Every combination of sequential odd and even numbered barcodes is fully color balanced at all positions of the index. For example, barcodes 5 and 6 offer opposite colors at every position, but barcodes 6 and 7 do not. Larger pools can be made by combining multiple sets of color balanced pairs. For pools of odd numbers of samples, any barcode can be added to a balanced pool. For example, for a pool of 3 samples, pooling barcodes 5, 6, and any other barcode is acceptable.

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.

RELATED PRODUCTS

Illumina Compatible RNA NGS Kits and Adapters
NEXTflex™ Rapid Directional RNA-Seq Kit
NEXTflex™ RNA-Seq Barcodes
NEXTflex-96™ RNA-Seq Barcodes
NEXTflex™ Rapid Directional qRNA-Seq™ Kit
NEXTflex™ Small RNA Sequencing Kit v2
NEXTlfex™ Small RNA Barcode Primers
NEXTflex™ Poly(A) Beads

Illumina Compatible DNA NGS Kits and Adapters
NEXTflex™ 16S V4 Amplicon-Seq Kit
NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0
NEXTflex™ 16S V1-V3 Amplicon-Seq Kit
NEXTflex™ 18S ITS Amplicon-Seq Kit
NEXTflex™ Rapid DNA-Seq Kit
NEXTflex™ Cell Free DNA-Seq Kit
NEXTflex™ DNA Barcodes
NEXTflex-96™ DNA Barcodes
NEXTflex-HT™ Barcodes
NEXTflex™ Dual-Indexed DNA Barcodes
NEXTflex™ Bisulfite-Seq Kit
NEXTflex™ Bisulfite-Seq Barcodes
NEXTflex™ Methyl-Seq 1 Kit
NEXTflex™ Msp 1
NEXTflex™ ChIP-Seq Kit
NEXTflex™ ChIP-Seq Barcodes
NEXTflex-96™ ChIP-Seq Barcodes
NEXTflex™ Pre-Capture Combo Kit
NEXTflex™ Rapid Pre-Capture Combo Kit
NEXTflex™ DNA Barcode Blockers
NEXTflex™ PCR-Free DNA Sequencing Kit
NEXTflex™ PCR-Free Barcodes

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