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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 for FFPE

(Illumina Compatible)

Catalog #4222-01 (8 reactions)

 V16.04

This product is for research use only.

NEXTflex™ BRCA1 & BRCA2 Amplicon Panel for FFPE Illumina-Compatible - 4222-01

Product Overview

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel for FFPE produces barcoded amplicon libraries compatible with Illumina platforms. Libraries are constructed using DNA isolated from FFPE samples. This panel contains a total of 261 primer pairs in four pools that allow for the amplification and sequencing of all coding exons of the *BRCA1* and *BRCA2* loci. Amplicon regions of interest range in size from 63-128 bp. The regions of interest plus primer pad sites, which comprise the read portion of the libraries, range from 121-195 bp. These target regions are amplified in PCR I, which is followed by end repair and adapter ligation. PCR II then enriches for the product of interest, as well as introduces unique barcodes and sequences necessary for downstream sequencing (Fig. 1). NEXTflex™ Cleanup Beads are included, and have been validated with amplicon library preparation. NEXTflex™ BRCA Amplicon FFPE Primer Mixes are optimized to achieve high coverage uniformity and reduce off-target reads using DNA isolated from FFPE samples.

Contents, Storage and Shelf Life

The NEXTflex™ BRCA1 & BRCA2 Amplicon Panel for FFPE contains enough material to prepare 8 sample libraries. 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
BLUE CAP	
NEXTflex™ BRCA Amplicon FFPE Primer Mix 1 - 4	32 µL each
CLEAR CAP	
NEXTflex™ Hot Start PCR I Master Mix	384 µL
RED CAP	
NEXTflex™ End Repair Buffer Mix	56 µL
NEXTflex™ End Repair Enzyme Mix	8 µL
LIGHT PURPLE CAP	
NEXTflex™ Ligation Mix	252 µL
NEXTflex™ Amplicon DNA Adapter	16 µL
YELLOW CAP	
NEXTflex™ PCR II Barcoded Primer Mix 1-8	4 µL each

GREEN CAP	
NEXTflex™ PCR II Master Mix	80 µL

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

CLEAR CAP BOTTLE	
NEXTflex™ Cleanup Beads	5 mL

Required Materials not Provided

- 40 - 200 ng of DNA isolated from FFPE samples (four 10 – 50 ng aliquots in up to 34 µL nuclease-free water each)
- 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
- Thermocycler
- 2, 10, 20, 200 and 1000 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 preparations are highly sensitive to pipetting error.
- Do not heat NEXTflex™ Adapters above room temperature.
- Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
- DNA sample quality may vary between preparations. 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 FFPE Preparation Flow Chart

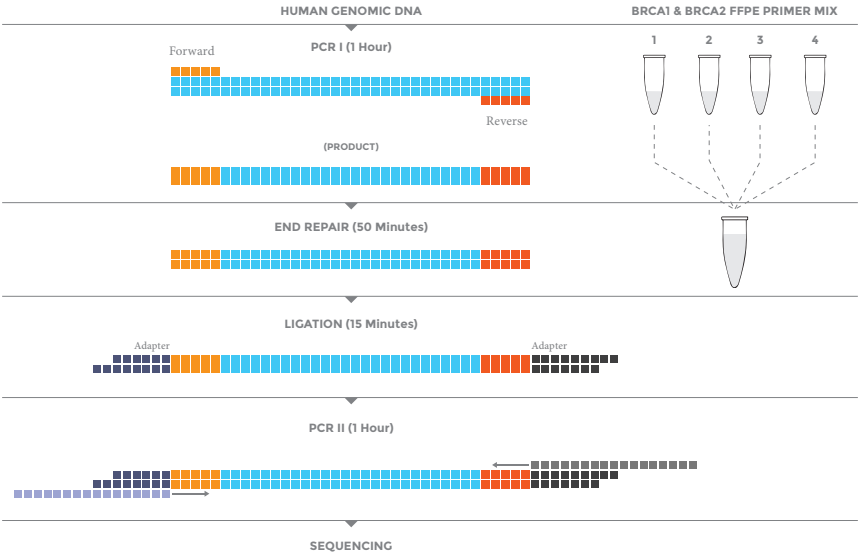

Figure 1: Sample flow chart with approximate times necessary for each step.

Starting Material

The NEXTflex™ BRCA1 and BRCA2 Amplicon Panel for FFPE has been optimized and validated using 10 - 50 ng of DNA isolated from FFPE samples for each PCR I primer pool.

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. Before every use, allow NEXTflex™ Cleanup Beads to come to room temperature and vortex until liquid appears homogenous.

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

Materials

Bioo Scientific Supplied

CLEAR CAP - NEXTflex™ Hot Start PCR I Master Mix
BLUE CAP - NEXTflex™ BRCA Amplicon FFPE Primer Mix 1 - 4
WHITE CAP - Nuclease-Free Water

User Supplied

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

1. For each sample, prepare four separate reactions using BRCA Amplicon FFPE Primer Mix 1, 2, 3, and 4 by combining the following reagents in adjacent wells in a PCR plate. **Note: It is recommended to combine these reagents as a master mix if processing multiple samples.**

	Reactions 1 - 4
_ µL	FFPE DNA (10-50 ng in up to 34 µL)
_ µL	Nuclease-free Water
12 µL	NEXTflex™ Hot Start PCR I Master Mix
4 µL	NEXTflex™ BRCA Amplicon FFPE Primer Mix 1,2,3, or 4
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 20 cycles
4 min	62°C	

4. Proceed immediately to Step B: PCR I Cleanup.

STEP B: PCR I Cleanup

Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer
CLEAR CAP BOTTLE - NEXTflex™ Cleanup Beads (room temperature)

User Supplied

80% Ethanol, freshly prepared (room temperature)
Magnetic Stand
50 µL PCR I Reactions 1 - 4 (from Step A)

1. Add 45 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 appears completely clear.
4. Do not discard the supernatant. Carefully transfer supernatant to a new well, being careful not to disturb the bead pellet.
5. Add 45 µL of NEXTflex™ Cleanup Beads to supernatant. Mix thoroughly until homogenized.
6. Incubate 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 the supernatant. Do not disturb beads. Some liquid may remain in wells.
9. 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.
10. Repeat previous step, for a total of 2 ethanol washes. Ensure all ethanol has been removed.
11. Remove the plate from the magnetic stand and let dry at room temperature for 5 minutes or until bead pellet is visibly dry.
12. Resuspend dried beads with 12 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
13. Incubate resuspended beads at room temperature for 3 minutes.
14. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.
15. Transfer 10 µL of clear supernatant (purified PCR I product) to new well.
16. Proceed immediately 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
WHITE CAP - Nuclease-Free Water

User Supplied

Adhesive PCR Plate Seal
Thermocycler
Ice
10 µL Purified PCR I Reactions (from Step B)

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

10 µL	Purified PCR I Reaction 1
10 µL	Purified PCR I Reaction 2
10 µL	Purified PCR I Reaction 3
10 µL	Purified PCR I Reaction 4
2 µL	Nuclease-free Water
7 µL	NEXTflex™ End Repair Buffer Mix
1 µL	NEXTflex™ End Repair Enzyme Mix
50 µL	TOTAL

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

STEP D: Cleanup

Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer
CLEAR CAP BOTTLE - NEXTflex™ Cleanup Beads (room temperature)

User Supplied

80% Ethanol, freshly prepared (room temperature)
96 Well PCR Plate
Magnetic Stand
50 µL End Repaired DNA (from Step C)

1. Add 90 µL of NEXTflex™ Cleanup 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.
6. Repeat previous step, 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 5 minutes, or until bead pellet is visibly dry.
8. Resuspend dried beads with 23 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
9. Incubate resuspended beads at room temperature for 3 minutes.
10. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.
11. Transfer 21 µL of clear supernatant to new well.
12. Proceed immediately to Step E: Adapter Ligation.

    STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored at -20°C. To restart, thaw frozen samples on ice before proceeding to Step E: Adapter Ligation.

STEP E: Adapter Ligation

Materials

Bioo Scientific Supplied

LIGHT PURPLE CAP - NEXTflex™ Ligation Mix (remove right before use and store at -20°C immediately after use), NEXTflex™ Amplicon DNA Adapter

User Supplied

Thermocycler
Adhesive PCR Plate Seal
Ice
21 µL 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:

21 µL	Purified End-Repaired DNA (from Step D)
2 µL	NEXTflex™ Amplicon DNA Adapter
31.5	NEXTflex™ Ligation Mix
54.5 µL	TOTAL

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

STEP F: Cleanup

Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer
CLEAR CAP BOTTLE - NEXTflex™ Cleanup Beads (room temperature)

User Supplied

80% Ethanol, freshly prepared (room temperature)
Magnetic Stand
54.5 µL Adapter Ligated DNA (from Step E)

1. Add 43 µL of NEXTflex™ Cleanup 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.
6. Repeat previous step, 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 5 minutes or until bead pellet is visibly dry.
8. Resuspend dried beads with 40 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
9. Incubate resuspended beads at room temperature for 3 minutes.
10. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.
11. Gently transfer 38 µL of clear sample to new well.
12. Proceed immediately to Step G: PCR II Amplification.

    STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored at -20°C. To restart, thaw frozen samples on ice before proceeding to Step G: PCR II Amplification.

STEP G: PCR II Amplification

Materials

Bioo Scientific Supplied

YELLOW CAP - NEXTflex™ PCR II Barcoded Primer Mix (1-8)
GREEN CAP - NEXTflex™ PCR II Master Mix
WHITE CAP - Resuspension Buffer
CLEAR CAP BOTTLE - NEXTflex™ Cleanup Beads (room temperature)

User Supplied

Thermocycler
Adhesive PCR Plate Seal
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand
Ice
38 µL 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™ PCR II Barcoded 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	
2 min	98°C	
30 sec	98°C	} Repeat for a total of 9 cycles
30 sec	65°C	
60 sec	72°C	
4 min	72°C	

4. Remove PCR plate from the thermocycler. Add 40 µL of NEXTflex™ Cleanup 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.

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

10. Remove the plate from the magnetic stand and let dry at room temperature for 5 minutes or until bead pellet is visibly dry.

11. Resuspend dried beads with 22 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.

12. Incubate resuspended beads at room temperature for 3 minutes.

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

14. Gently transfer 20 µL of clear sample to a new well and proceed to library analysis, or seal plate with adhesive PCR plate seal and store at -20°C. Qubit (Life Technologies) and Bioanalyzer (Agilent) are recommended to quantify and analyze quality of the library.

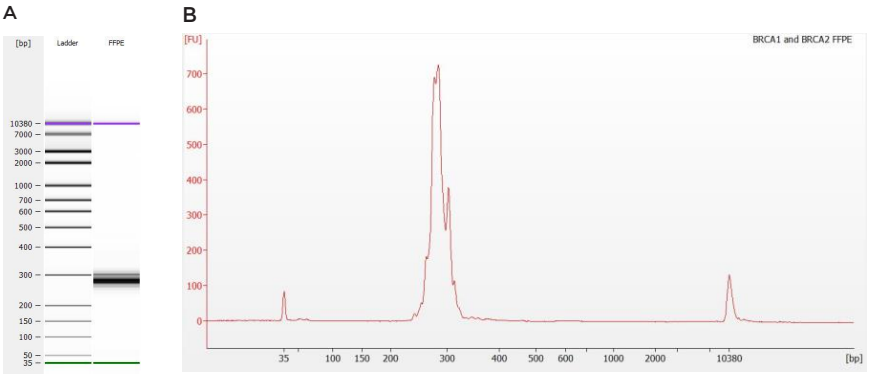

Figure 2. High Sensitivity DNA Chip Output:
A) NEXTflex™ BRCA1 and BRCA2 Amplicon Panel for FFPE library - 40 ng input (Bioanalyzer gel image)
B) NEXTflex™ BRCA1 and BRCA2 Amplicon Panel for FFPE library - 40 ng input (electropherogram)

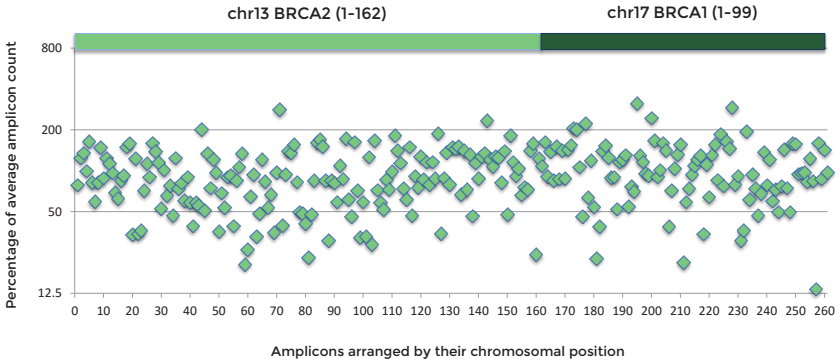

Figure 3. Performance of 261 amplicons from NEXTflex™ BRCA1 & BRCA2 Amplicon Panel for detection of mutations in FFPE samples

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 the 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.

A BED file of the covered regions is available for download on our webpage.

To receive a complete electronic list of the BED and FASTA files for this kit, please follow the instructions on the label inside 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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THE NGS EXPERTS™

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