B-Pure™

EasySeq™ PCR Plates

For BRCA1 and BRCA2 sequencing

- √ Based on the Gold Standard Sanger Sequencing
- √ Sample in -> Result out: No need for batching up samples
- √ Thorough coverage: 100% Gene resequencing
- √ Fast, Straightforward, Robust
- √ Minimal hands-on time
- √ Compatible with:
  - Standard DyeTerminator Sequencing workflows
  - BrightDye® Terminator Cycle Sequencing Kit
  - BigDye® Direct
- √ Primers tailed with M13 sequences: Universal Cycle Sequencing

Introduction

BRCA1 and *BRCA2* genes are tumor suppressor genes. The gene products are directly involved in the human DNA repair system and prevent uncontrolled cell growth.

Both *BRCA1* and *BRCA2* are comprehensive genes, comprising 23 and 27 exons, respectively. Mutations, found in both *BRCA* genes are not located in hotspots, but are distributed throughout the gene coding region. Functional mutations have been correlated with an increased cancer risk [1,2,3]. Ongoing *BRCA1* and *BRCA2* research has highlighted the need for a straightforward mutation detection workflow.

Principal

The EasySeq™ PCR plates were developed to facilitate an easy, robust, fast and cost effective sequencing workflow for detection of mutations in the BRCA genes. Other methods, like Next Generation Sequencing (NGS) or heteroduplex based pre-screening methods (for example dHPLC, HiRes Melting curve analysis, CSCE), introduce additional, non-robust and time consuming steps in the workflow. They also implicate the need for batching up samples. This can introduce unwanted delays in sample-to-result timelines.

Offering an off-the-shelf solution, this EasySeq™ approach provides an ultra-straightforward protocol. The workflow is based on on the industry-standard Sanger sequencing chemistry and Genetic Analyzers.

Kit content

The product is available as semi-skirted PCR plates, pre-spotted with dried-down primer pairs, to generate 80 PCR products covering both genes. Primers have been optimized for use with MCLAB's HoTaq PCR Mastermix, but are also fully compatible with other commercial available PCR mastermixes, like Amplitaq® GOLD360.

Primer Design

Making use of the Human Genome build NCBI36 (BRCA1: Acc. nr: cDNA: NM_007294.3, BRCA2: Acc. nr: NM_000059.3), M13 tailed PCR primers were designed and optimized for 100% coverage of the coding sequence of the *BRCA1* and *BRCA2* genes, including sections of approximately 50 bp up- and downstream of each exon. All amplicons can be amplified and sequenced using one universal set of PCR conditions. Cycle Sequencing is done with universal (-21M13 / M13REV) sequencing primers.

For more information about primer sequences and amplicon design, please contact NimaGen.

Non-Template control wells

To provide maximum flexibility and easy conversion of your workflow, the EasySeq™ plates contain 15 multiplex Non-Template control wells to check the specificity of the results (see table 1). This set of multiplexes contain all primer and can be verified by agarose gel electrophoresis or spectrophotometric DNA concentration measurement to prove that there is no amplification.

Quality

All primers are produced under highly controlled conditions and are of B-Pure™, PAGE purified quality. After LC/MS and functional quality tests, the primer pairs are spotted and dried down under controlled conditions in semi-skirted 96-well plates with barcode. We have shown average sequence quality of >QV40 in the Regions of Interest. This enables unidirectional sequencing for *BRCA1/2*[5] as an option.

References

1. Irminger-Finger, Siegel BD, Leung WC (1999) The functions of breast cancer susceptibility gene 1 (BRCA1) product and its associated proteins. BiolChem 380(2)117-128.

2. Wooster R, Bignell G, Lancaster J et al. [1995) Identification of the breast cancer susceptibility gene BRCA2. Nature 378:789-792.

3. Casey G (1997) The BRAC1 and BRAC2 breast cancer genes. CurrOpinOncol 9:88-93.

4. J. Theelen, M. Nelen, N. Arts, H. Ouchene, A. Felton, C. Davidson, W. Hettenna, R. Petraroli, M. Ligtenberg (2010). A robust and straightforward approach for screening of BRCA1 and BRCA2 genes by direct resequencing.ESHG poster P06.017

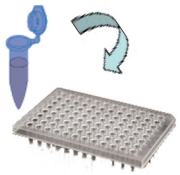
5. S. Ellard, B. Shields, C. Tysoe, R. Treacy, S. Yau, C. Mattocks, A. Wallace (2009]. Semi-automated Unidirectional Sequence Analysis for Mutation Detection in a Clinical Diagnostic Setting. GEN. TESTING AND MOL. BIOMARKERS Vo113 (3):381-386

	1	2	3	4	5	6	7	8	9	10	11	12
A	BRCA1 exon 1	BRCA1 exon 10	BRCA1 exon 11/8	BRCA1 exon 15	BRCA1 exon 23	BRCA2 exon 8	BRCA2 exon 11/3	BRCA2 exon 11/11	BRCA2 exon 13	BRCA2 exon 20	BRCA1 MP-1	BRCA1 MP-3
B	BRCA1 exon 2	BRCA1 exon 11/1	BRCA1 exon 11/9	BRCA1 exon 16	BRCA1 exon 24	BRCA2 exon 9	BRCA2 exon 11/4	BRCA2 exon 11/12	BRCA2 exon 14/1	BRCA2 exon 21	BRCA1 MP-2	BRCA2 MP-4
C	BRCA1 exon 3	BRCA1 exon 11/2	BRCA1 exon 11/10	BRCA1 exon 17	BRCA2 exon 1	BRCA2 exon 10/1	BRCA2 exon 11/5	BRCA2 exon 11/13	BRCA2 exon 14/2	BRCA2 exon 22	BRCA1 MP-3	BRCA2 MP-5
D	BRCA1 exon 5	BRCA1 exon 11/3	BRCA1 exon 11/11	BRCA1 exon 18	BRCA2 exon 2	BRCA2 exon 10/2	BRCA2 exon 11/6	BRCA2 exon 11/14	BRCA2 exon 15	BRCA2 exon 23&24	BRCA1 MP-4	BRCA2 MP-6
E	BRCA1 exon 6	BRCA1 exon 11/4	BRCA1 exon 11/12	BRCA1 exon 19	BRCA2 exon 3	BRCA2 exon 10/3	BRCA2 exon 11/7	BRCA2 exon 11/15	BRCA2 exon 16	BRCA2 exon 25	BRCA1 MP-5	BRCA2 MP-7
F	BRCA1 exon 7	BRCA1 exon 11/5	BRCA1 exon 12	BRCA1 exon 20	BRCA2 exon 4	BRCA2 exon 10/4	BRCA2 exon 11/8	BRCA2 exon 11/16	BRCA2 exon 17	BRCA2 exon 26	BRCA1 MP-6	BRCA2 MP-8
G	BRCA1 exon 8	BRCA1 exon 11/6	BRCA1 exon 13	BRCA1 exon 21	BRCA2 exon 5&6	BRCA2 exon 11/1	BRCA2 exon 11/9	BRCA2 exon 11/17	BRCA2 exon 18	BRCA2 exon 27/1	BRCA2 MP-1	BRCA2 MP-9
H	BRCA1 exon 9	BRCA1 exon 11/7	BRCA1 exon 14	BRCA1 exon 22	BRCA2 exon 7	BRCA2 exon 11/2	BRCA2 exon 11/10	BRCA2 exon 12	BRCA2 exon 19	BRCA2 exon 27/2	BRCA2 MP-2	EM PTY

Table 1: Positions of the Amplicon-Specific primers and No-Template Controls in the EasySeq™ plates

Steps:

1 Mix DNA with PCR Mastermix[1] and simply dispense In EasySeq Plate

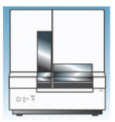


2 PCR amplification

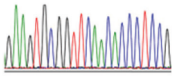
3 Cleanup[2] PCR Product and Cycle Sequence with -21M13 and M13REV primers

4 Purify Sequencing Reactions

5 Run and Analyse On 31xx, 35xx or 37xx series Genetic Analyzer



6 Data Analysis



[1] In the last two columns (multiplex NTC wells), add only PCR Mastermix with water
[2] When using BD Direct or C-Pure, the PCR cleanup can be eliminated

Name	Description	P/N
B-pure™ EasySeq™ PCR Plates 96-well	5 pcs / 5 analyses	350596
B-pure™ EasySeq™ PCR Plates 96-well	25 pcs / 25 analyses	352596

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EasySeq™ PCR Plates for BRCA1/2 Sequencing

User manual

Version: 4.0
Revision date: 17-08-2017

Product and Company Information

Product use: For Research Use Only
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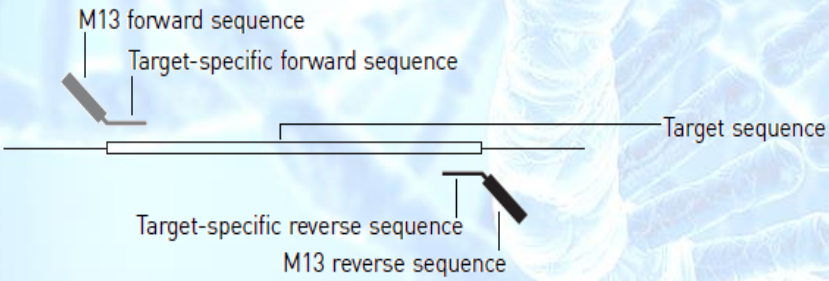
Store the kits at -18 to -22 °C. Use the EasySeq™ PCR plates for *BRCA1* and *BRCA2* for setting up PCR reactions, followed by cycle sequencing and capillary electrophoresis, to screen for mutations in the complete coding region including ±50 bp up- and downstream of each coding exon of the *BRCA1* and *BRCA2* genes. The product consists of semi skirted PCR plates, containing dried down primer pairs in optimized concentrations, covering the both BRCA genes. The columns 11 and 12 contain all amplicons, in a total of 15 PCR multiplexes, to be used as a No-Template Control (NTC), to prove all results are specific. All primer sets have been optimized in concentration and design to be compatible with the following chemistry:

- MCLAB's HotStart PCR Master Mix
- Amplitaq GOLD (360) (Applied Biosystems)

Al primers have been tailed with universal tails: Forward primers with -21M13 and reverse primers with M13Rev to enable cycle sequencing with universal primers:

M13 Forward primer (-21M13): 5' TGTAAAACGACGGCCAGT 3'
M13 Reverse primer (M13Rev): 5' CAGGAAACAGCTATGACC 3'



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Materials and equipment required but not included

Product	Source
MCLAB's HotStart PCR Master Mix, p/n HMM-100, 200, 300 *Or* Amplitaq GOLD (360) MasterMix	Nimagen ThermoFisher
Veriti® 96-Well Thermal Cycler (RECOMMENDED) *Or* 96-Well GeneAmp® PCR System 9700 *Or* 96-Well SimpliAmp® Thermal Cycler	ThermoFisher
Pipettors, multichannel (1-10 microliter and 20-200 microliter) and single 1-10, 1-20, 10-200, 100-1000 microliter)	Major Laboratory Suppliers (MLS)
General disposables (tubes, vials, filter tips)	MLS
Mini Plate Spinner MPS1000	Labnet
MicroAmp® 96-well optical reaction plates *Or* FrameStar® 96 Well ABI® Style Plates	ThermoFisher 4titude
Adhesive PCR seals	MLS
Molecular Biology Grade Water	MLS
M13(-21) Sequencing Primer, forward	NimaGen
M13REV Sequencing primer reverse	NimaGen
Ex'S-Pure PCR Cleanup kit	NimaGen
D-Pure DyeTerminator Cleanup kit	NimaGen
Magnetic ring magnet plate, 96 well, Alpaqua	NimaGen
BrilliantDye Terminator Cycle Sequencing kit	NimaGen
NimaPOP-7 polymer and NimaPOP Running Buffer	NimaGen
Genetic Analyzer (3100, 3130, 3500 or 3730 series)	ThermoFisher

Guidelines for Genomic DNA quality

The performance of the EasySeq™ PCR plates for *BRCA1* and *BRCA2* sequencing is **highly dependent** on the quality and quantity of the used genomic DNA. NimaGen strongly recommends to check quality and quantity of your DNA sample by A260/A280 ratio analysis. Follow the recommended quantity of DNA in the original PCR setup (25-50 ng/µl). Too much input DNA will result in possible inhibition and/or overloaded signals.

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Setting up the PCR reactions

1. For each sample (EasySeq plate), prepare two PCR Mixes in 2 mL vials:

A. PCR Mix

PCR Mastermix (2x)	880 µl
Human Genomic DNA (25 – 50 ng/µl)	90 µl
Water (molecular biology grade)	790 µl
TOTAL	***1760 µl***

B. No-Template-Control (NTC) Mix

PCR Mastermix (2x)	165 µl
Water (molecular biology grade)	165 µl
TOTAL	***330 µl***

2. Dispense 20 µl of the PCR (A) mix in all wells of columns 1 – 10 of the EasySeq PCR plate (yellow wells in picture 1)
3. Dispense 20 µl of the NTC (B) mix in all wells of columns 11 and 12 except well H12, of the EasySeq PCR plate (pink wells in picture 1)

	1	2	3	4	5	6	7	8	9	10	11	12
A	BRCA1 exon 1	BRCA1 exon 10	BRCA1 exon 11/8	BRCA1 exon 15	BRCA1 exon 23	BRCA2 exon 8	BRCA2 exon 11/3	BRCA2 exon 11/11	BRCA2 exon 13	BRCA2 exon 20	BRCA1 MP-1	BRCA1 MP-3
B	BRCA1 exon 2	BRCA1 exon 11/1	BRCA1 exon 11/9	BRCA1 exon 16	BRCA1 exon 24	BRCA2 exon 9	BRCA2 exon 11/4	BRCA2 exon 11/12	BRCA2 exon 14/1	BRCA2 exon 21	BRCA1 MP-2	BRCA2 MP-4
C	BRCA1 exon 3	BRCA1 exon 11/2	BRCA1 exon 11/10	BRCA1 exon 17	BRCA2 exon 1	BRCA2 exon 10/1	BRCA2 exon 11/5	BRCA2 exon 11/13	BRCA2 exon 14/2	BRCA2 exon 22	BRCA1 MP-3	BRCA2 MP-5
D	BRCA1 exon 5	BRCA1 exon 11/3	BRCA1 exon 11/11	BRCA1 exon 18	BRCA2 exon 2	BRCA2 exon 10/2	BRCA2 exon 11/6	BRCA2 exon 11/14	BRCA2 exon 15	BRCA2 exon 23&24	BRCA1 MP-4	BRCA2 MP-6
E	BRCA1 exon 6	BRCA1 exon 11/4	BRCA1 exon 11/12	BRCA1 exon 19	BRCA2 exon 3	BRCA2 exon 10/3	BRCA2 exon 11/7	BRCA2 exon 11/15	BRCA2 exon 16	BRCA2 exon 25	BRCA1 MP-5	BRCA2 MP-7
F	BRCA1 exon 7	BRCA1 exon 11/5	BRCA1 exon 12	BRCA1 exon 20	BRCA2 exon 4	BRCA2 exon 10/4	BRCA2 exon 11/8	BRCA2 exon 11/16	BRCA2 exon 17	BRCA2 exon 26	BRCA1 MP-6	BRCA2 MP-8
G	BRCA1 exon 8	BRCA1 exon 11/6	BRCA1 exon 13	BRCA1 exon 21	BRCA2 exon 5&6	BRCA2 exon 11/1	BRCA2 exon 11/9	BRCA2 exon 11/17	BRCA2 exon 18	BRCA2 exon 27/1	BRCA2 MP-1	BRCA2 MP-9
H	BRCA1 exon 9	BRCA1 exon 11/7	BRCA1 exon 14	BRCA1 exon 22	BRCA2 exon 7	BRCA2 exon 11/2	BRCA2 exon 11/10	BRCA2 exon 12	BRCA2 exon 19	BRCA2 exon 27/2	BRCA2 MP-2	EMPTY

Picture 1

4. Seal the plate with adhesive (PCR) film, then spin the plate briefly.
Note: make sure to tightly seal all wells, to prevent evaporation during PCR
5. Run the reactions in the thermal cycler:

PCR conditions

HOLD	95°C	10 min
Cycle (32 cycles)	95°C	15 sec
	60°C	30 sec
	72°C	60 sec
HOLD	72°C	7 min
HOLD	4°C	∞

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6. Check 5 µL of the wells in the NTC columns (A11-G12) on a 2% agarose gel to check for no amplification. Optionally check 5 µL of the sample PCR to check for amplification.

Recommendations for post-PCR processing

1. **Option 1: Cleanup of PCR by ExS-Pure:** The most easy and straightforward method is to use ExS-Pure for purification of the PCR products
    a. Open the plate and just add 2 µL of ExS-Pure to each well of column 1-10
    b. Close (seal) the plate and mix well
    c. Incubate in thermal cycler 15 minutes at 37°C, followed by a5 minutes at 80°.
    d. Add 25 µL water to each well to dilute PCR product and mix

    Note: The protocol described above is slightly modified from the original Ex'S-Pure protocol; incubation times have been extended to correct for larger volume, making the workflow more straightforward. The protocol works for cleaning up the full 20 µL reactions, as well as for 15 µL reactions, in case 5 µL was taken out for agarose gel analysis.

2. **Option 2: Cleanup of PCR by AmpliClean:** Alternatively, use the AmpliClean PCR cleanup kit from Nimagen according to this protocol:
    a. Gently shake the AmpliClean to resuspend the magnetic particles to obtain a homogeneous suspension.
    b. Add 36 µl of AmpliClean to the 20 µL PCR reactions
    c. Mix thoroughly by pipetting up and down and incubate for 3 - 5 minutes at Room Temperature
    d. Place the reaction plate onto a Alpaqua Magnetic Ring Plate for 2 minutes
    e. While sitting on the magnet, remove the cleared solution from the reaction plate and discard by pipetting from the center of the bottom of the wells. Make sure the removed solution is fully cleared and not to remove any magnetic particles
    f. While leaving the plate on the magnet, immediately dispense 150 µL of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature.
    g. Remove the ethanol and discard. Repeat step f followed by a complete removal of Ethanol with the last aspiration. Check if all wells are free of liquid after this step
    h. Dry for 5 minutes at RT. Take plate from the Magnet.
    i. While plate is **off the magnet**, add 40 µL water or elution buffer to each well of the reaction plate and homogenize the beads in the elution buffer.
    j. Place the reaction plate onto the Magnet Plate for 2 minutes to separate beads from the solution and transfer the max. 30 µL of the eluant, containing the purified PCR products to a new plate.

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3. Cycle Sequencing

Note: Optionally check the purified PCR products on agarose gel (apply 5 µL)
Note: For forward and reverse sequencing, perform steps a to c in duplo

a. Prepare Cycle Sequencing Mastermix:
   - 88 µL BrilliantDye™ Terminator v1.1 or v3.1 Sequencing Mix
   - 132 µL 5x BrilliantDye Sequencing Buffer
   - 88 µL -21M13 (Forward) **or** M13Rev (Reverse) primer (5 pMol/ µL)
   - 484 µL Molecular Biology grade water

   TOTAL 792 µL

b. Dispense 9 µL of this Mastermix in the 80 wells of column 1-10 of a new 96-well plate
c. By multichanner pipetting, Transfer 1 µL of the cleaned PCR products to the corresponding wells of the prepared sequencing plate
d. Seal the plate tightly and perform cycle sequencing:

initial denaturation	96°C	60 sec
28 cycles	96°C	10 sec
	50°C	5 sec
	60°C	1 min. 15 sec.
hold	4°C	∞

4. Sequence Cleanup: Nimagen recommends to use the D-Pure Cycle Sequencing cleanup kit (DP-005, DP-050, DP-500) according to the general protocol, or use a commercial available sephadex-based cleanup method.
5. Capillary Electrophoresis: Analyze the purified sequence samples on an Applied Biosystems / Hitachi Genetic Analyzer of the 3130, 3500 or 3730 series, preferably using a 50 cm array, NimaPOP-7 and with a *standard* or *fast* sequencing run module.
   Note: It might be needed to lower the injection voltage and/or time to prevent over exposure.

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