Instructions For Use

Version: 1.0 *Ref: IFU_BRCA1/2* *Revision date: 2023-10-27*

EasySeq™

BRCA1 and *BRCA2*

+ *CHEK2* HS Sequencing Kit

For NGS Library Prep by Reverse Complement PCR

NimaGen.

Innovators in DNA Sequencing Technologies

Product and Company Information

EasySeq™ *BRCA1* and *BRCA2* + *CHEK2* HS Sequencing Kit

REF RC-BRCA096

Research Use Only

NimaGen B.V.
Hogelandseweg 88
6545 AB Nijmegen
The Netherlands
Tel: +31 (0)24 820 02 41
Email: info@nimagen.com

Symbols Used on Product Labels and in Instruction For Use

Symbol	Description
	Manufacturer
	Use-by date
LOT	Lot number
REF	Reference number
	Temperature limit for storage
	Contains sufficient for <*n*> tests
	Matrix code containing the reference number, lot number and use-by date

Product Description

The intended purpose of this assay is NGS library preparation for Illumina® sequencing of the human *BRCA1* and *BRCA2* gene coding exons including a minimum of 20 bases upstream and downstream of each exon. The assay also includes the CHEK2 1100delC (NM_001005735) hotspot.

The assay provides reagents for Multiplex Amplicon-based NGS library preparation and is for research use only, not for diagnostic purposes. The kit is tested and validated for an input DNA amount of 20 ng.

The kit is based on the patented Reverse Complement PCR technology, providing a safe, robust and simple workflow, combining multiplex amplification in a single reaction, decreasing the risk of PCR contamination and sample swapping.

Reverse Complement PCR Kit Contents

NimaGen Part# RC-BRCA096 (store at -20 °C)	Contents
BRCA1/2 Probe Panel A (REF: PM-BRCA-A)	1x Tube (24 µL)
BRCA1/2 Probe Panel B (REF: PM-BRCA-B)	1x Tube (24 µL)
2x PCR Master Mix (Hot Start HiFi) (REF: MMHS096)	2x Tube (1150 µL)
Probe Dilution Buffer enhanced (REF: PDB-Enh-2x)	1x Tube (432 µL)

Required Materials, Not Included

Description	Vendor
Index Primer Plate, dehydrated. Choose one of the 8 available EasySeq™ Unique Dual Index plates for Illumina. Available REF: IDX96-U01D, IDX96-U02D, IDX96-U03D, IDX96-U04D, IDX96-U05D, IDX96-U06D, IDX96-U07D IDX96-U08D. **Note:** The index sequences are available from the download section of the NimaGen website.	NimaGen
Adjustable Pipette Set (P10, P20, P100, P200, P1000)	Multiple Vendors
TapeStation, Bioanalyzer Instrument, incl. consumables.	Agilent
Ethanol Absolute, Molecular Biology Grade	Multiple Vendors
AmpliClean™ or AMPure XP Bead Solution	NimaGen / Beckman Coulter
General plasticware, DNAse free (1.5 mL tubes, pipette tips etc.)	Multiple Vendors
Mini Spinner for 1.5 mL tubes and 8-well PCR strips or PCR plates	Multiple Vendors
Magnetic stand for 1.5 mL Eppendorf tubes and/or 96-wells plates	Multiple Vendors / NimaGen
Water, PCR Grade	Multiple Vendors
Qubit Fluorometer incl. High Sensitivity consumables	Thermo Fisher Scientific
Thermocycler with heated lid, (0.2 mL standard PCR tubes), compatible with semi-skirted ABI style PCR plates and option for ramp rate programming. **Note:** Kit is validated for Applied Biosystems™ Veriti™, MiniAmp™ and SimpliAmp™ Thermal Cyclers.	Multiple Vendors
NaOH Solution (2 N)	Multiple Vendors
Tris-HCl (200 mM), pH 7	Multiple Vendors
Low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Multiple Vendors
Illumina® NGS Sequencing Instrument	Illumina®
Illumina® Reagent kit	Illumina®

General Precautions

Read the Material Safety Data Sheet (MSDS) and follow the handling instructions. Adhere to good laboratory practice and wear protective eyewear, gloves and lab coat when handling both the reagents supplied in this kit and other reagents required. Wash body parts with ample amount of water immediately if they come in contact with the reagents. Seek medical help if needed.

Use a Pre-PCR environment for setting up the RC-PCR. Sample pooling, purification and library quantification should be performed in a Post-PCR environment.

Protocol

1. Thermocycling Program

Temp	Duration	Ramp Rate (from previous step)	Cycles
98 °C	2 minutes	N/A	1 x
98 °C	10 seconds	Max	1 x
58 °C	10 minutes	0.1 °C/sec (or 2% of Max)	
72 °C	1 minute	Max	
95 °C	10 seconds	Max	2 x
80 °C	1 second	Max	
62 °C	90 minutes	0.1 °C/sec (or 2% of Max)	
72 °C	30 seconds	Max	
95 °C	10 seconds	Max	34 x
80 °C	1 second	Max	
62 °C	2 minutes	0.5 °C/sec (or 10% of Max)	
72 °C	30 seconds	Max	
4 °C	∞	Max	1 x

Heated lid at 105 °C.

Note: This protocol takes approximately 6-7 hours to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program as a dummy run, to check the predicted duration of 6-7 hours.

2. Reverse Complement PCR

In a single, closed tube reaction, the target specific RC-probes are working as a template to extend the UDI primers to synthesize functional, tailed and indexed PCR primers. This will be followed by two long hybridization/extension steps of 90 minutes and subsequently a further DNA amplification of the target regions, meanwhile synthesizing more primers.

2.1 Thaw on ice:
- RC-PCR Probe Panel A (Black cap)
- RC-PCR Probe Panel B (Red cap)
- Probe Dilution Buffer (Blue cap)
- HiFi Master Mix (White cap)

Note: The HiFi Master Mix contains iso-stabilizers and may not freeze completely, even when stored at -15 °C to -25 °C. It may contain precipitates when thawed at +2 °C to +8 °C. Always ensure that the Master Mix is fully thawed and thoroughly mixed before use.

2.2. Take two identical IDX PCR plates and break off the number of strips needed. Mark the plates with 'A' and 'B'.

Note: Register the indexes used (IDX set/strip-column number and well position for each sample). Download the index details for setting up the Illumina sample sheet.

Note: For each sample two PCR reactions are needed (Panel A and Panel B). Always use the same well position for the same sample in order to generate identical indexes for each sample in both panels.

Note: Before breaking off 8-well strips, cut the seal at the breaking line with a sharp knife.

2.3. Prepare in a fresh 1.5 mL Eppendorf tube the RC-PCR mix panel A by combining and mixing:
- 0.2 µL RC-PCR Probe Panel A per reaction (Black cap)
- 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 10 µL HiFi Master Mix per reaction (White cap)
- 4 µL PCR grade H_2O per reaction

2.4. Prepare in a fresh 1.5 mL Eppendorf tube the RC-PCR mix Panel B by combining and mixing:
- 0.2 µL RC-PCR Probe Panel B per reaction (Red cap)
- 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 10 µL HiFi Master Mix per reaction (White cap)
- 4 µL PCR grade H_2O per reaction

Example: 24 samples + 10% extra volume*
- 5.28 µL RC-PCR Probe Panel
- 47.52 µL Probe Dilution Buffer
- 264 µL HiFi Master Mix
- 105.6 µL PCR grade H_2O

*It is recommended to allow for a 10% excess when preparing the RC-PCR mix to correct for any pipetting loss. The kit contains extra reagent to facilitate this.

2.5. Remove the seal from the PCR plates or strip(s).

2.6. Dispense 16 µL of the RC-PCR mix Panel A (from step 2.3) to each well of the plate/strip(s) A.

2.7. Dispense 16 µL of the RC-PCR mix Panel B (from step 2.4) to each well of the plate/strip(s) B

2.8. Add to each well: 4 µL of DNA solution (optimal: 20 ng total DNA input).

2.9. Close the tube strips **carefully** with the caps provided, there should be an audible click. Mix by short vortexing, followed by a quick spin. Verify that the colour of the reaction mix is homogenously pink.

2.10. Place the samples in the thermal cycler(s) and start the RC-PCR program.

After the RC-PCR, samples have been amplified and tagged with sample-specific indexes and sequencing adapters. From this point, RC-PCR product purification is performed using a magnetic bead based purification to remove primers, dimers and salts.

3. Purification

Note: Before pooling, optionally check the unpurified PCR products on agarose.

3.1. Bring the magnetic bead solution (AmpliClean™ or AMPure XP) to room temperature.

3.2. Perform steps 3.3 to 3.7 for both Panel A and Panel B individually.

3.3. Pool 5 µL RC-PCR products from each reaction into a 1.5 mL Eppendorf tube.

3.4. Mix well and transfer 40 µL of this pool to a new 1.5 mL Eppendorf tube.

3.5. Add 60 µL Low TE buffer or molecular grade H_2O to the tube and mix well (total volume is now 100 µL).

3.6. Beads purification:

a. Vortex the beads thoroughly to resuspend.
b. Add 80 µL bead solution to the 100 µL pool (from step 3.4) and mix well immediately by pipetting up and down 5 times.
c. Incubate for 5 minutes.

On magnet:

d. Place the tube for 3 minutes on the magnet, or until the solution is fully cleared.

e. Remove and discard all liquid carefully, without disturbing the beads.
f. Add 200 µL (freshly prepared) 75% ethanol, without disturbing the beads.
g. Wait for 1 minute.

h. Repeat steps **e.**, **f.** and **g.** for a second ethanol wash step.
i. Carefully remove all liquid without leaving traces of ethanol. (Optionally: quick spin, then place the tube back on the magnet and remove the last traces of ethanol)
j. Dry with open cap for 2-3 minutes at room temperature. **Do not over-dry.**

3.7. Elution:

a. **On Magnet:** Add 80 µL Low TE buffer to the tube.
b. **Off Magnet:** Resuspend the beads by flicking, or by short vortexing.
c. **Off Magnet:** Incubate for 2 minutes.
d. **On Magnet:** Wait for 1-3 minutes, or until the solution is fully cleared.
e. **On Magnet:** Carefully bring 70 µL of the clear solution into a new 1.5 mL Eppendorf tube, making sure not to transfer any of the beads.

The libraries are now ready for a quantitative and qualitative check, followed by NGS.

4. Sequencing

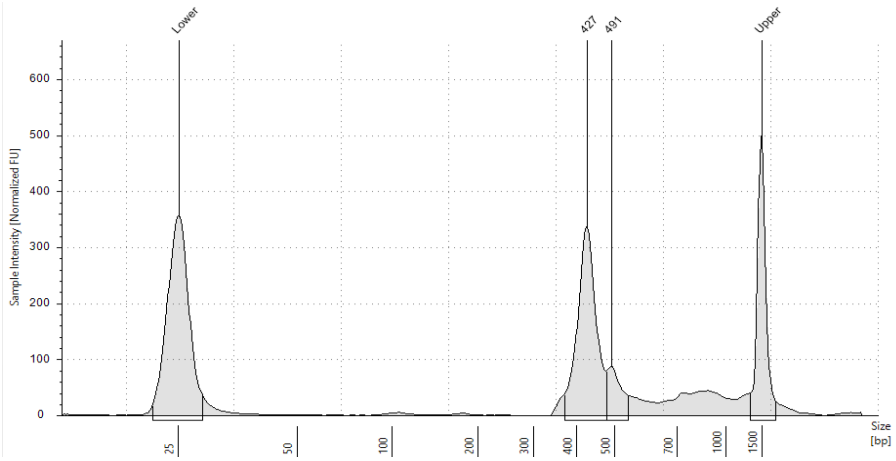
4.1. Determine the final concentration of the libraries by a double Qubit (HS) measurement:

- **a.** Bring the Qubit reagents to room temperature.
- **b.** Label the Qubit tubes on the lid according to the number of samples to be used plus 2 standards.
- **c.** Dilute the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer for each sample/ standard. It is recommended to allow for >10% excess when preparing the working solution to correct for any pipetting loss.
- **d.** For the standards: mix 190 µL of the working solution with 10 µL of the standard.
- **e.** For the samples: mix 180-199 µL of the working solution with 1-20 µL sample (total 200 µL).
- **f.** Vortex the tubes thoroughly and incubate the tubes for 2 minutes.
- **g.** Measure the standards and the samples using the 'dsDNA High Sensitivity' settings making sure to select the correct sample volume used in step **e.**.

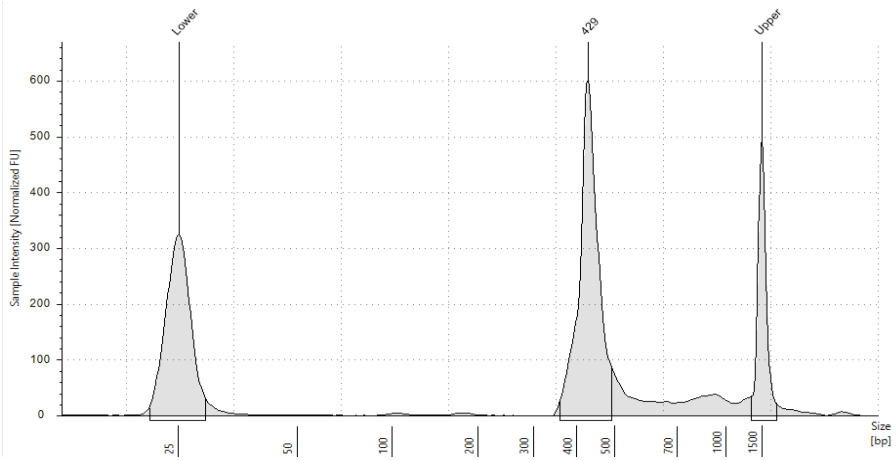
4.2. **Optional but recommended:** Perform a qualitative verification of the libraries on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute the pool. E.g. for TapeStation High Sensitivity kit, dilute to ~2 ng/µL.

Example of clean libraries on TapeStation:

Panel A



Panel B



4.3. Perform sequencing on an Illumina® platform, according to the manufacturer's manual. Use a minimal of 151-8-8-151 sequencing scheme.
Use the following guidelines for running the libraries:

- For calculation of the library concentrations, use an average fragment size of 445 bp for both libraries.
- Combine 10 µL of each of the equimolar Panel A and B dilutions before addition of the NaOH solution.
- The required reads per samples for minimal 50x coverage is 50 000 total reads per sample.
- Dilute the library pool to the required loading concentration for your Illumina instrument. We advise to start with a lower loading concentration for their initial sequence run and adjust in subsequent runs if needed. This avoids overclustering and potentially failure of the run.
- A spike-in of 5% PhiX is recommended for QC purposes.

Customer Support

For technical questions, assistance, or to suggest enhancements, please contact us at techsupport@nimagen.com.

Revision History

Section	Summary of changes	Version	Date
All	New document.	1.0	2023-10-27

Legal Notice

Disclaimer

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NimaGen B.V.
Hogelandseweg 88
6545 AB Nijmegen
The Netherlands
www.nimagen.com