RingCap®

BRCA1/2 Gene Mutations Detection Kit

High-Throughput Sequencing

Instruction for Use(Ion torrent)

Product Name

BRCA1/2 Gene Mutations Detection Kit (High-Throughput Sequencing)

Packing Specification

16 Tests/Kit, 32 Tests/Kit

Intended Use

The kit is intended for the detection of whole exon and exon-intron junctions in BRCA1/2 with FFPE pathological tissue and peripheral blood samples from patients with breast cancer or ovarian cancer.

BRCA1/2 is the abbreviation of Breast Cancer Susceptibility Gene 1/2; Both of them are tumor suppressor genes and play important roles in DNA damage repair, cell cycle regulation, gene transcription activation, and chromatin stability. BRCA1 gene(NM_007294) locates in human chromosome 17q21, is composed of 23 exons; BRCA2 gene(NM_000059) locates in human chromosome 13q12.3, is composed of 27 exons.

Among women suffer from breast cancer and ovarian cancer, about 2% and 10-15% respectively of the patients have been reported to carry the mutations of BRCA1/2, moreover, the mutation status is closely related to the familial breast cancer, namely, people with BRCA1/2 mutations may be more susceptible to breast cancer and/or ovarian cancer. It is studied that people who're mutation carriers may suffer from breast cancer at the probability of 40-80% and ovarian cancer at 16-60%, while the probability is 12% and 1% respectively among people with wild-type phenotype. Furthermore, BRCA2 mutation may also lead to the occurrence of breast cancer in male with the occurrence rate at about 6%.

Neither BRCA1 nor BRCA2 gene has mutations of high frequency, which means that hotspot sites sequencing is inadequate, but call for complete genome sequencing then. This kit facilitates library construction of whole exome of BRCA1/2 genes before quick detection of 2229 mutation sites (see Attached Table 1) with the assist of high throughput sequencer. Scientific and clinical trials have shown that the new drug, olaparib, could be a target therapy for cancer patients with BRCA1 or BRCA2 mutation. The detection of BRCA1/2 gene mutation status has gradually become an aid of individualized therapy in clinic.

Technological Principle

High-Throughput Sequencing, also known as Next Generation Sequencing (NGS), can be divided into semiconductor sequencing, DNA nanosphere sequencing and so on according to the different sequencing principles. NGS enables the sequencing of up to millions of target nucleic acids at once, providing abundant variation information in a short time and at relatively low cost. Highlighting the characteristics of high output and high resolution, NGS has drawn more and more attention in multiple signaling pathways and targets studies of cancer.

The construction of sample library relies on specific modified primers and RingCap® mediated amplification technology with the employment of PCR apparatus. Specific modified primers enable the precise PCR amplification of target sequences, RingCap® mediated amplification allows terminal modification of the products with specific sequences. With the combination of particular PCR program and Ring-Cap® enzyme, library construction of target sequences could be achieved on common PCR apparatus before they are ready for high-throughput sequencing.

Kit Contents

Table 1. Kit Contents

No.	Content Name	Main Content	Strip Color	16 Tests/Kit			32 Tests/Kit			Note
				Volume	Quantity	8-Tube Strip	Volume	Quantity	8-Tube Strip	
1	**BRCA enriching PCR strip 1**	Primer, dNTPs, Mg^{2+}, buffer	Blue	20 μL	16 tubes	2 strips	20 μL	32 tubes	4 strips	Each tube contains same reagent.

2	**BRCA enriching PCR strip 2**	Primer, dNTPs, Mg^{2+}, buffer	Pink	20 μL	16 tubes	2 strips	20 μL	32 tubes	4 strips	Each tube contains same reagent.
3	**Barcode 1-8 ligation reaction strip**	Barcode, dNTPs, Mg^{2+}, buffer	Purple	20 μL	8 tubes	1 strip	20 μL	8 tubes	1 strip	Each tube represents a barcode.
4	**Barcode 9-16 ligation reaction strip**	Barcode, dNTPs, Mg^{2+}, buffer	Green	20 μL	8 tubes	1 strip	20 μL	8 tubes	1 strip	Each tube represents a barcode.
5	**Barcode 17-24 ligation reaction strip**	Barcode, dNTPs, Mg^{2+}, buffer	White	——	——	——	20 μL	8 tubes	1 strip	Each tube represents a barcode.
6	**Barcode 25-32 ligation reaction strip**	Barcode, dNTPs, Mg^{2+}, buffer	Yellow	——	——	——	20 μL	8 tubes	1 strip	Each tube represents a barcode.
7	**RingCap-Taq (1#)**	Taq enzyme	——	20 μL	1 tube	——	20 μL	2 tubes	——	——
8	**BRCA Negative Control**	Purified water	——	1.0 mL	1 tube	——	1.0 mL	1 tube	——	——
9	**BRCA Positive Control**	Wild-type cell line DNA	——	20 μL	1 tube	——	40 μL	1 tube	——	——

Note: In the Barcode ligation reaction strip, different serial numbers respectively contain 32 different IonDx recognition sequences (see Appendix Table 1); The reaction solution has been pre-loaded in the eight strips, and the left oblique position of the cap of 8-tube strip is oriented in the forward direction, from left to right followed by Barcode 1, 2, 3, 4, 5, 6, 7, 8 (Figure 1).

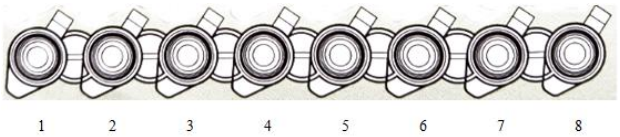

Figure 1. Barcode numbers of 8-tube strips

Note: The components of different batches of reagents cannot be mixed.

Equipment and Reagents Required

1. Fluorometer: Promega Quantus™ Fluorometer, Cat. No. E6150; Thermo Fisher Scientific Qubit® 4.0 Fluorescence Meter, Cat. No. Q32866;
2. Nucleic acid extraction kit: commercial nucleic acid extraction kits are recommended;
3. Quantification kit of nucleic acids: Promega, QuantiFluor® dsDNA System, Cat. No. E2670; Thermo Fisher Scientific Qubit® dsDNA HS Assay Kit, Cat. No. Q32851/Q32854;
4. Magnetic beads: Xiamen Spacegen Co., Ltd SGpure beads, Cat. No. SPG-PB001; Magnetic beads Kit from Beckman Coulter, Cat. No. A63880/A63881/A63882;
5. Sequencing Reagents: Selecting the corresponding sequencing reagent according to the gene sequencer;
6. TE buffer (pH 8.0);
7. Absolute ethanol (Analytical Grade);
8. Nuclease-free water;
9. Nuclease-free pipettes and tips;

Storage and Stability

1. Storage Condition. Store the kit away from light at - 20±5°C, valid for 9 months and is not influence for bottle openings, moreover, do not use the reagents after 5 repeated freeze-thaw cycles.
2. Transportation Condition. The kit should be transported in foam cases with ice bags, with transporting time less than one week and transporting temperature under 25°C.
3. Check labels for the production date and expiration date of the kit.

Applicable Instruments

1. PCR system/ thermal cycler: ABI9700, ABI 2720, ABI Veriti, ABI MiniAmp;
2. Sequencing instruments: Ion Torrent PGM; Ion Proton; Ion S5.

Specimen Material

The quality of the DNA is critical. Therefore, collecting samples according to the following recommended sample types and requirements, followed DNA extraction:

1. Recommended sample types: FFPE pathological tissue or slices, peripheral blood;
2. FFPE samples: ensure that at least 20% of the collected pathological tissue is tumor lesions; choose FFPE samples that have not been stored for more than 2 years; extract DNA with at least 8 slices of 5 μm section or at least 5 slices of 10 μm section;
3. Peripheral blood: Peripheral blood should be collected with a non-heparin anticoagulant with volumes no less than 2 mL;
4. Commercial kits are highly recommended to extract genomic DNA from the samples. Quantify sample DNA with a fluorescence meter, the concentration should be ≥ 2 ng/μL, total amount of DNA should be ≥ 20 ng. Once the DNA quantity or quality did not conform to the above requirements, re-extract DNA with a resample or a larger amount of samples. Proceed to library construction after DNA extraction or store at -20±5 °C for less than 12 months.

Experimental Procedure

Note: Parallel analysis of BRCA Positive Control (PC) and BRCA Negative Control (NTC) with tested sample is suggested.

I. Library Enrichment

1. Reagent preparation: Unfreeze the **BRCA enriching PCR strip 1 (blue)** and **BRCA enriching PCR strip 2 (pink)** at room temperature according to total number of samples, briefly centrifuge the tubes before use; Place the **RingCap-Taq (1#)** on ice after centrifugation;
2. Sample preparation: Dilute sample DNA to 2 ng/μL with TE buffer (pH 8.0), and prepare ≥ 10 μL of the diluted sample;
3. Enriching PCR reaction:
   a) Pipet 0.5 μL **RingCap-Taq (1#)** to 10 μL of the "DNA Sample", "BRCA-PC", "BRCA-NTC", vortex slightly followed by a brief centrifugation;
   b) Gently remove the cap of enriching PCR strip, for one sample, respectively pipet 5 μL of the template prepared above (10 μL) into BRCA enriching PCR tube 1 (blue) and BRCA enriching PCR tube 2 (pink), replace the cap carefully;
   c) Centrifuge the tubes / strips slightly to dislodge bubbles;
4. Load the PCR reaction tubes / strips into the PCR instrument, Set up and perform the amplification procedure according to Table 2.

Table 2. PCR amplification procedure

Phase	Temperature	Time	Cyclic number
Predenaturation	98 °C	2 minutes	1
Denaturation	98 °C	15 seconds	15
Annealing	65 °C	4 minutes	
Storage	4 °C	∞	1

Note: Proceed to "Purification of Enriching Products", or store the products at 2 - 8°C within 8 hours or at - 20±5°C within 24 hours. Storing for more than 24 hours is not suggested.

II. Purification of Enriching Products

Note: Bring magnetic beads to room temperature and vortex thoroughly to disperse magnetic beads before use; prepare fresh 70% ethanol with nuclease-free water.

1. Mix the two PCR enrichment products of one sample into a new 1.5 mL eppendorf tube, add 50 μL magnetic beads to each tube, pipet up and down to mix the bead suspension thoroughly with the product;
2. Incubate the mixture for 5 minutes at room temperature;
3. Place the tube on a magnet rack, then incubate for 2 minutes, carefully remove and discard the supernatant without disturbing magnetic beads;
4. Pipet 150 μL of freshly prepared 70% ethanol into each tube, rotate the tube side-to-side in the two positions of magnetic rack for 5 times to wash magnetic beads, place the tube on magnetic rack for 2 minutes, carefully remove and discard the supernatant without

disturbing magnetic beads;

5. Repeat step 4 for a second wash;
6. Remove all the ethanol from the tube, and keep the tube on magnetic rack for 5 minutes to air-dry magnetic beads (avoid over-dry);
7. Remove the tube from the magnetic rack, add 35 μL of TE buffer (pH 8.0) to each tube, and vortex thoroughly (alternatively, mix by pipetting at least half the total volume up and down), briefly centrifuge to collect the droplets, Incubate the mixture for 5 minutes at room temperature;
8. Place the tube on magnetic rack for 2 minutes until the solution is clear, carefully remove and store the supernatant (i.e. **purified product**) at - 20±5°C or proceed to "Library Construction".

III. Library Construction

Note: Using different barcodes for different samples is suggested.

1. Reagent preparation: Unfreeze the **barcode ligation reaction strips** at room temperature, briefly centrifuge the tubes before use; Place the **RingCap-Taq(1#)** on ice after centrifugation;
2. BRCA library construction :
   a) Add 0.25 μL **RingCap-Taq** (1#) to 5 μL of the purified products of DNA samples, BRCA- PC and BRCA-NTC, vortex slightly followed by a brief centrifugation;
   b) Gently remove the cap of **barcode ligation reaction strips**, sequentially add 5 μL of the template prepared above into respective tube, replace the cap carefully;
   c) Centrifuge the tubes / strips slightly to dislodge bubbles;
3. Load the PCR reaction tubes/ strips into the PCR instrument, and set up and perform the amplification procedure according to Table 3.

Table 3. PCR amplification procedure

Phase	Temperature	Time	Cyclic number
Predenaturation	98 °C	2 minutes	1
Denaturation	98 °C	15 seconds	20
Annealing	65 °C	4 minutes	
Storage	4 °C	∞	1

Note: Proceed to "Library Purification", or store the products at 2 - 8°C within 8 hours or at - 20±5°C within 24 hours. Storing for more than 24 hours is not suggested.

IV. Library Purification

Note: Bring the beads to room temperature and vortex thoroughly to disperse the beads before use; Prepare fresh 70% ethanol with nuclease-free water.

1. Transfer 25 μL of the PCR library product to a new 1.5mL Eppendorf tube, add 25 μL of magnetic beads to each tube, and pipet up and down to mix the bead suspension thoroughly with the product;
2. Incubate the mixture for 5 minutes at room temperature;
3. Place the tube on a magnet rack, then incubate for 2 minutes, carefully remove and discard the supernatant without disturbing magnetic beads;
4. Pipet 150 μL of freshly prepared 70% ethanol into each tube, rotate the tube side-to-side in the two positions of magnetic rack for 5 times to wash magnetic beads, place the tube on magnetic rack for 2 minutes, carefully remove and discard the supernatant without disturbing magnetic beads;
5. Repeat step 4 for a second wash;
6. Remove all the ethanol from the tube, keep the tube on magnetic rack for 5 minutes to air-dry magnetic beads (avoid over-dry);
7. Remove the tube from magnetic rack, add 35 μL of TE buffer (pH 8.0) to each tube, and vortex thoroughly (alternatively, mix by pipetting at least half the total volume up and down), briefly centrifuge to collect the droplets; Incubate the mixture for 5 minutes at room

temperature;

8. Place the tube on magnetic rack for 2 minutes until the solution is clear, carefully remove and store the supernatant (i.e. **library**) at -20±5°C or proceed to "Library Quantification and Dilution".

V. Library Quantification and Dilution

1. Quality control (QC) of sample library: A bioanalyzer is recommended for the quality control of library fragments; For BRCA-NTC library, no fragments shall be detected; For BRCA-PC library and all sample libraries, the main fragments should be around 250-350 bp; for library concentration lowers than 1 ng/μL (measured by fluorometer), either of which is decided unqualified;
2. Per the concentration measured, dilute sample library to 34 ng/mL with Nuclease-free water;
3. Mix equal amount of each sample library, then centrifuge slightly for further use;
4. Take the above-mixed library for library enrichment, sequencing (see instrument and reagent procedure).

VI. Library enrichment, sequencing

1. Perform the emulsion PCR reaction to the above mixed library on the Ion One Touch 2 instrument, following the matching sequencing reaction universal kit; the entire program takes about 5 hours, the product can be placed at room temperature for 8 hours after the operation, and can be run overnight;
2. The product obtained by the emulsion PCR reaction can be transferred to the Ion One Touch ES instrument for purification, the operation should be performed according to the instructions of the matching sequencing reaction general kit; The instrument automatically completes the purification operation, the running time takes about 40 minutes;
3. Complete the cleaning and initialization of the gene sequencer according to the operation instructions of the gene sequencer;
4. Log in to the gene sequencer server, click the "Plan" tab, select the "Templates" module, and find the corresponding template program (for the first use, you can create a template program and save it with the help of technical support), select "Templates" from the drop-down menu.
5. Name the experiment in the "Plan New Run" text box, enter the number of samples, information and the corresponding Barcode number, and click "Plan Run";
6. At the end of the operation of the Ion One Touch ES instrument, the purified product is automatically transferred to a 0.2 mL EP tube. Refer to the instruction manual of the gene sequencer to complete the chip calibration and loading, add sequencing polymerase, and incubate at room temperature for 5 mins;
7. Transfer the chip loaded with the library template to the chip holder and fix it, close the chip chamber, start to run the experimental program set in step 5 and start sequencing;
8. After sequencing, perform a water wash and turn off the instrument.

VII. Bioinformatics Analysis

Transfer the Excel files（amplicon.cov）obtained by Torrent Server variant Caller, followed mutation annotation analysis-based on the Ion Platform Automated Reporting and Analysis System (abbreviated as analysis system below) of Xiamen Spacegen Co., Ltd.

Positive Judgment Value

Mutated positive judging criteria:

1. Standard quality control: For DNA sample libraries, the main fragments should be at 250 ~ 350 bp, Uniformity ≥ 75%, mean Depth ≥ 500×
2. Mutated positive judging criteria
   a) Peripheral blood sample: in the result of germline variation analysis, if effective depth > 100× and mutation frequency > 25%, this mutations site is judged as positive mutation; Otherwise, it is judged as negative.
   b) FFPE: in the result of somatic variation analysis, if effective depth > 100× and mutation frequency > 5%, this mutations site is judged as positive mutation; Otherwise, it is judged as negative or below the detection limit.

Interpretation of Results

1. NTC libraries should be not any fragment; Otherwise, this test is invalidated;
2. For DNA positive control library, the target fragment should be in 250 ~ 350 bp as well as Uniformity should be more than 75%, moreover, Mean Depth more than 500×;

3. For DNA tested sample library, the target fragment should be in 250 ~ 350 bp as well as Uniformity should be more than 75%, moreover, Mean Depth more than 500×; Otherwise, this test is invalidated;
4. The grade of somatic variation based on the "Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer: A Joint Consensus Recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists" jointly formulated by AMP/ASCO/CAP in 2017 could divide into 4 types:
   a) Clear clinical significance: Diagnostic\prognostic marker of specific tumor or drugs recommended\approved in the professional guidelines;
   b) Potential clinical significance: Diagnostic\prognostic marker of specific tumor or drugs that has level A evidence of other tumor in the multiple small research;
   c) Unknown clinical significance: It is not found higher rates of variants in the general population and tumor databases, moreover, not has clear published evidence;
   d) Harmless or may be harmless clinical significance: It is found higher rates of variants in the general population and not has published evidence.
5. The grade of germline variation based on the "Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology" and other standard of genetic variation, variation types and conserved biological functional prediction, evidence of databases(e.g.ClinVar、COSMIC) and literature could divide into 5 levels
   a）Pathogenic: There is clear evidence that this variant is associated with an increased risk of cancer;
   b）Likely pathogenic: There is moderate evidence that this variant is associated with an increased risk of cancer;
   c）Uncertain significance: There is unclear evidence that this variant is associated with an increased risk of cancer;
   d）Likely benign: There is moderate evidence that this variant is unrelated to an increased risk of cancer;
   e）Benign: There is clear evidence that this variant is unrelated to an increased risk of cancer.

Limitation of the Kit

Testing results obtained from the kit should only be taken as a scientific reference. The instruction shall not interpret the results for mutation sites that were not included in the kit or DNA extracted from samples that were not collected according to designated requirements..

Physical Performance of Products

1. The kit should be of neat appearance, clear marking, and of no leakage. When unfrozen, the reagents shall be clear, without sediment.
2. The consistency rates of both positive reference samples are 100%.
3. The consistency rates of both negative reference samples are 100%
4. The kit allows the detection of 5% of specific gene mutations in 10 ng DNA sample.
5. The repeatability is 100% by detecting designated sample for 10 consecutive times.

Precautions and Warning

1. Please read the instruction carefully in prior to experiments.
2. Conduct experiments abided by laboratory regulations to reduce cross-contaminations of products or reagents; Divide experiment areas into different function zones if possible.
3. Clean experiment areas before experiment with 10% hypochlorous acid followed by water rinsing. Sterilize the environment and pipettes with 10% hypochlorous acid, 75% ethanol, or UV radiation.
4. Avoid using peripheral wells of PCR instrument; Vacate holes or columns between samples to avoid cross-contamination.
5. Testing results might be influenced by sample sources, sampling process, sample quality, carriage conditions, sample handling, etc; Also might it be limited by the quality of DNA, instrument types, operating environment, and the limitation of current molecular biotechnology, all of which may lead to false positive/ negative results. The users should thoroughly be informed of potential errors as well as the limitation of accuracy.
6. Avoid unnecessary freezing-thawing the reagents, the reagents were allowed to undergo no more than 5 freeze-thaw cycles.
7. The quality of DNA matters experimental results to a great extent, hence, purification of extracted DNA with magnet beads is highly suggested. Purified DNA should be stored as required (- 20±5°C) or proceed to further steps immediately;
8. Do not substitute any original reagents contained in the kit. Do not mix reagents of different lots.

9. The use of filter tips is highly recommended to avoid false-positive results caused by contamination of reagents.
10. Be cautious of contamination from external DNA; Use specific pipettes and tips for reagents preparation and template addition.
11. All reagents in use have potential hazard. Only people who have work permit for PCR laboratories are allowed to use this kit. For first-use of this kit, you may receive training by our technical supports. All used contents of the kit should be considered as clinical dessert and should be disposed properly.
12. All samples including positive control in the kit should be considered potential infectious substances. They should be handled carefully.

Notes

Symbol	Legend
	Indicates the need for the user to consult the instructions for use.
	Indicates the date when the medical device was manufactured.
	Indicates the manufacturer's batch code so that the batch or lot can be identified.
	Indicates the temperature limits to which the medical device can be safely exposed.
	Indicates the date after which the medical device is not to be used.
	This is the correct upright position of the distribution packages for transport or storage.
	Indicates a medical device that needs to be protected from moisture.
	Indicates a medical device that needs protection from light sources.
	Indicates the medical device manufacturer.
	Indicates the authorized representative in the European Community/European Union.
	The product meets the basic requirements of European in vitro diagnostic medical devices directive 98/79/EC.

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EC REP

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Appendix table 1:

Information of 32 IonDx recognition sequences based on Ion torrent tech

Strip Color	Barcode Number	Sequence
Purple	Barcode_001	CTAAGGTAAC
	Barcode_002	TTACAACCTC
	Barcode_003	CCTGCCATTCGC
	Barcode_004	TGGAGGACGGAC
	Barcode_005	TGAGCGGAAC
	Barcode_006	CCTTAGAGTTC
	Barcode_007	TCCTCGAATC
	Barcode_008	AACCTCATTC
Green	Barcode_009	CGGACAATGGC
	Barcode_010	TCCTGAATCTC
	Barcode_011	TAAGCCATTGTC
	Barcode_012	CTGAGTTCCGAC
	Barcode_013	CGGAAGAACCTC
	Barcode_014	TCTTACACAC
	Barcode_015	AAGGAATCGTC
	Barcode_016	TAGGTGGTTC

Strip Color	Barcode Number	Sequence
White	Barcode_017	TAAGGAGAAC
	Barcode_018	AAGAGGATTC
	Barcode_019	TACCAAGATC
	Barcode_020	CAGAAGGAAC
	Barcode_021	CTGCAAGTTC
	Barcode_022	TTCGTGATTC
	Barcode_023	TTCCGATAAC
	Barcode_024	CTGACCGAAC
Yellow	Barcode_025	TCTAACGGAC
	Barcode_026	TTGGAGTGTC
	Barcode_027	TCTAGAGGTC
	Barcode_028	TCTGGATGAC
	Barcode_029	TCTATTCGTC
	Barcode_030	AGGCAATTGC
	Barcode_031	TTAGTCGGAC
	Barcode_032	CAGATCCATC