CE IVD

AmoyDx® *BRCA1* and *BRCA2* Gene Mutation Detection Kit (Reversible Terminator Sequencing)

Instruction for Use

REF 8.06.27203X024I	24 tests	For Illumina NovaSeq, HiSeq, NextSeq Series, MiSeq, MiSeq Dx, MiniSeq, iSeq 100	

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Background

BRCA1 gene and *BRCA2* gene are tumor suppressor genes, which are involved in DNA damage repair and transcriptional regulation by homologous recombination. Type I poly ADP-ribose polymerase (PARP-1) is a modified enzyme that plays an important role in DNA damage repair and apoptosis. Tumor cells with mutations in the *BRCA1* gene and the *BRCA2* gene often rely on PARP-1 to repair DNA damage. Therefore, PARP inhibitors can inhibit the repair of DNA damage in tumor cells by inhibiting the function of PARP-1. Clinical studies have shown that multiple tumors with *BRCA1* or *BRCA2* mutations can benefit from the treatment of PARP inhibitors [1-3]. Currently, the US FDA has approved PARP (poly ADP-ribose polymerase) inhibitors for the treatment of ovarian cancer and breast cancer patients with *BRCA1* gene or *BRCA2* gene mutation. The *BRCA1* gene and the *BRCA2* gene are located on human chromosomes 17 and 13, respectively, each containing 22 and 26 exon regions with coding functions. According to current clinical studies and databases, mutation types include insertions, deletions, and point mutation. Mutations affecting gene function are up to more than 3,000, distributing in various functional areas of genes, and there is no hotspot mutation region. These mutations can be classified into five categories according to the classification criteria of the International Agency for Research on Cancer (IARC) and the American Society for Genetic Genetics (ACMG) (Appendix II), with Class 4 and 5 being effective populations to targeting PARP inhibitors.

Intended Use

The AmoyDx® *BRCA1* and *BRCA2* Gene Mutation Detection Kit (Reversible Terminator Sequencing), is intended for qualitative detection of *BRCA1* and *BRCA2* gene mutations (including all coding exons, exon-intron boundaries) in extracted DNA from human blood samples, fresh tissue or neutral formalin-fixed paraffin-embedded (FFPE) tissue samples. The kit is intended to be used to assess *BRCA1* and *BRCA2* gene status in ovarian cancer and breast cancer patients.

The kit is for *in vitro* diagnostic use, and intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

The test kit is based on Halo-shape ANnealing and Defer-Ligation Enrichment system (HANDLE system) technology which is improved Molecular Inversion Probe (MIP) technology to capture the target gene region, and then the prepared DNA libraries are sequenced on Illumina sequencing platform to detect the *BRCA1/2* gene mutations.

The probe contains an extension arm and a ligation arm which are complementary to the target gene region. First, the extension arm and ligation arm are anchored to the target gene region, and the DNA is extended from the extension arm to the ligation arm by the function of DNA polymerase. Next, the nicks are connected with the ligase to generate the circular products. The remaining linear probes, single-strand and double-strand nucleic acid are digested with the exonuclease. Finally, the universal PCR amplification is performed to enrich the target libraries.

Kit Contents

This kit contains the following components in Table 1.

Table 1 Kit Contents

Serial No.	Components	Main Ingredient	Quantity
1	HS-Probe	Oligonucleotides	24 μL/tube ×1
2	HS-Hybridization Buffer	Tris-HCl, K^+, Mg^{2+}	24 μL/tube ×1
3	HS-Extension Ligation Master Mix	DNA polymerase, dNTPs, DNA Ligase, Ligation buffer	24 μL/tube ×1
4	HS-Exonuclease A	DNA Exonuclease	36 μL/tube ×1
5	HS-Exonuclease B	DNA Exonuclease	24 μL/tube ×1
6	HS-PCR Master Mix	Tris, Mg^{2+}, dNTPs, DNA polymerase	600 μL/tube ×1
7	HS-S5 Primer *	Oligonucleotides	5 μL/tube ×8
8	HS-N7 Primer *	Oligonucleotides	5 μL/tube ×12
9	HS-Positive Control	DNA	60 μL/tube ×1

* For labeling and sequence information of the primers, refer to Appendix I.

Storage and Stability

The kit requires shipment on frozen ice packs and the shipping time should be less than one week. All contents of the kit should be stored immediately upon receipt at -20±5℃.

The shelf-life of the kit is eight months. The recommend maximum freeze-thaw cycle is five cycles.

Additional Reagents and Equipment Required but Not Supplied

1) DNA quantification kit, we recommend to use QuantiFluor dsDNA System (Promega, Ct. No. E2670) or Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851 or 32854).
2) DNA Extraction Kit. We recommend: for blood sample, use AmoyDx® Blood DNA kit (Amoy Diagnostics, Cat No.: 8.02.24201X036G); for FFPE tissue sample, use AmoyDx® FFPE DNA Kit (Amoy Diagnostics, Cat No.8.02.23501X036G) or GeneRead DNA FFPE Kit (Qiagen, Cat. No. 180134); for fresh tissue, use AmoyDx® Tissue DNA Kit (Amoy Diagnostics, Cat No.: 8.02.24301X036G).
3) DNA purification kit, we recommend to use Agencourt AMPure XP Kit from Beckman Coulter Genomics, Cat. No. A63880/A63881/A63882.
4) Capillary electrophoresis analyzer kit, we recommend to use 2100 Bioanalyzer system and Agilent 2100 High Sensitivity DNA Kit from Agilent Technologies (Cat. No. 5067-4626) or Agilent DNA Kit (Cat. No. 5067-1504).
5) PCR Instrument.
6) Illumina PhiX Control V3, Cat. No. FC-110-3002.
7) Sequencing reagent: Illumina 300 cycles (Paired-End Reads, 2×150 cycles) is recommended.
8) Sequencing Instrument: Illumina platform: NovaSeq/HiSeq/NextSeq Series/MiSeq/MiSeq Dx/MiniSeq/iSeq 100.
9) Photofluorometer, we recommend to use Quantus™ Fluorometer (Promega, Cat. No. E6150), Qubit 2.0 (Thermo Fisher Scientific, Cat. No. Q32866) or Qubit 3.0 (Thermo Fisher Scientific, Cat. No. Q33216).
10) Magnetic stand, we recommend to use DynaMag™-2 Magnet from Thermo Fisher Scientific, Cat. No. 12321D.
11) Mini centrifuge.
12) Vortex mixer.
13) Nuclease-free centrifuge tubes.
14) Nuclease-free PCR tubes.
15) Nuclease-free filtered pipette tips
16) Absolute ethanol (AR).
17) Nuclease-free water, TE-low solution (10 mM Tris, 0.1 mM EDTA, pH 8.0) or 10 mM Tris solution (pH 8.0).

Precautions and Handling Requirements

For *in vitro* diagnostic use.

Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use, and strictly follow the instruction during operation.
- DO NOT use the kit or any kit component after their expiry date.
- DO NOT use any other reagents from different lots in the tests.
- DO NOT use any other reagent in the other test kits.

Safety Information

- Handle all specimens and components of the kit as potentially infectious material using safe laboratory procedures.
- Only trained professionals can use this kit. Please wear suitable lab coat and disposable gloves while handling the reagents.
- Avoid skin, eyes and mucous membranes contact with the chemicals. In case of contact, flush with water immediately.
- DO NOT pipet by mouth.

Decontamination and Disposal

- The kit contains positive control; strictly distinguish the positive control from other reagents to avoid contamination which may cause

false positive results.

- PCR amplification is extremely sensitive to cross-contamination. The flow of tubes, racks, pipets and other materials used should be from pre-amplification to post-amplification, and never backwards.
- Gloves should be worn and changed frequently when handling samples and reagents to prevent contamination.
- Use separate, dedicated pipettes and filtered pipette tips when handling samples and reagents to prevent exogenous nucleic acid contamination to the reagents.
- All disposable materials are for one time use. DO NOT reuse.
- The unused reagents, used kit, and waste must be disposed properly.

Cleaning

- After the experiment, wipe down the work area, spray down the pipettes and equipment with 75% ethanol or 10% hypochlorous acid solution.

Specimen Preparation

- Sample DNA should be extracted from peripheral blood, fresh tissue or FFPE samples.
- The FFPE tissue sample should be fixed in 10% neutral buffered formalin for 6-24 hours. It's important to make sure that there are at least 20% tumor cells existing in the tissue sample. It's recommended to use the central section of paraffin blocks. The freshly cut sections of FFPE tissue should be used for DNA extraction immediately. The storage time of FFPE tissue samples should be less than 12 months.
- The peripheral blood should be more than 2 mL. The EDTA anticoagulant is recommended during the blood collection, avoid using heparin anticoagulant. The blood sample should be extracted immediately or transported at 2~8℃, if not, store the blood sample at -20±5℃ for no more than 3 years.
- We recommend to use a commercialized extraction kit to perform DNA extraction. After the extraction, measure the concentration of extracted DNA. For fresh tissue and FFPE sample, the DNA concentration should be more than 3.75 ng/μL, and total DNA should be more than 30 ng; for blood sample, the DNA concentration should be more than 1.25 ng/μL, and total DNA should be more than 10 ng.
- The extracted DNA should be used for DNA library preparation immediately, if not, it should be stored at -20±5℃ for no more than 12 months, avoid repeated freezing and thawing.

Assay Procedure

Note:

- *It is recommended to include a Positive Control (PC) and a No-template Control (NTC) in the process of library preparation, sequencing and data analysis.*
- *During the following DNA library preparation process, please use the corresponding adaptor in the PCR instrument to avoid the PCR products evaporation.*

1. Hybridization:

1) Take out the **HS-Probe** and **HS-Hybridization Buffer** and thaw the reagents at room temperature. When the reagents are completely thawed, vortex each tube for 5~10 seconds and centrifuge briefly, then put them on the ice.
2) Prepare the hybridization reaction solution in a 0.2 mL PCR tube according to the ratio in Table 2.

Table 2 Hybridization reaction solution

Reagent	Volume
Purified water	8-χ μL
DNA	χ μL
HS-Probe	1 μL
HS-Hybridization Buffer	1 μL

Note:

- *For blood samples, "χ" stands for the volume of 10~50 ng DNA (50 ng is recommended).*
- *For fresh tissue and FFPE samples, "χ" stands for the volume of 30~50 ng DNA (50 ng is recommended).*

- *For PC, the DNA concentration of HS-Positive Control is 5 ng/μL, take 8 μL to construct library.*
- *For NTC, use the sterile and nuclease-free purified water.*
- *If the sample is degraded severely, try to increase the amount of DNA or perform the hybridization step overnight.*
- *If there are more samples, HS-Probe and HS-Hybridization Buffer can be premixed.*

3) Mix the solution thoroughly by vortexing or pipetting up and down more than 10 times, and centrifuge briefly, then put the tubes in the PCR instrument. Set the reaction volume as 10 μL and perform the following program: 95℃ for 5 min, 60℃ for 2 h, then incubate at 4℃.

***Note**: keep the tubes at low temperature after hybridization is finished, as high temperature like room temperature may increase the non-specificity.*

2. Extension-Ligation

1) Take out the **HS-Extension Ligation** Master Mix and thaw the reagent at room temperature. When the reagents are completely thawed, vortex each tube for 5~10 seconds and centrifuge briefly, then put them on the ice.
2) Take out the above PCR tubes from the PCR instrument and put them on the ice. Add 1 μL **HS-Extension Ligation** Master Mix into the PCR tubes, vortex or pipette to mix well, centrifuge briefly, then put the tubes in the PCR instrument. Set the reaction volume as 11 μL and perform the following program: 60℃ for 10 min, then incubate at 4℃.

***Note**: keep the tubes at low temperature after extension-ligation is finished, as high temperature like room temperature may increase the non-specificity.*

3. Exonuclease Digestion

1) Take out the **HS-Exonuclease A** and **HS-Exonuclease B**, vortex each tube for 5~10 seconds and centrifuge briefly, then put them on the ice.
2) Take out the above PCR tubes from the PCR instrument and put them on the ice. Add the following reagents listed in Table 3:

Table 3 The amount of Exonuclease

Reagent	Volume
HS-Exonuclease A	1.5 μL
HS-Exonuclease B	1 μL

3) Put the tubes in the PCR instrument. Set the reaction volume as 13 μL and run the following program: 37℃ for 30 min, 95℃ for 10 min, then incubate at 4℃.

4. PCR Amplification

1) Take out the **HS-S5 Primer, HS-N7 Primer** and **HS-PCR Master Mix** and thaw the reagents at room temperature. When the reagents are completely thawed, vortex each tube for 5~10 seconds and centrifuge briefly, then put them on the ice.
2) Take out the above PCR tubes from the PCR instrument and put them on the ice . Add the following reagents listed in Table 4:

Table 4 Preparation of PCR Reaction Solution

Reagent	Volume
HS-PCR Master Mix	25 μL
Purified Water	8.5 μL
HS-S5 Primer	1.5 μL
HS-N7 Primer	1.5 μL

***Note**:*

- *Each of the HS-S5 Primer or HS-N7 Primer has a different index sequence. Use a combination of HS-S5 Primer and HS-N7 Primer for every one sample library, the same combination cannot be used for two or more sample libraries in one sequencing run. The detailed information for the index sequence is shown in Appendix I.*
- *Transfer the prepared tubes to the amplification room to perform PCR amplification and the following purification to avoid contamination.*

3) Mix the reagents in each PCR tube thoroughly by vortexing or pipetting up and down more than 10 times, centrifuge briefly, then put the tubes in the PCR instrument. Set the reaction volume as 50 μL, then perform the following program in Table 5 to obtain PCR

products.

Table 5 PCR Program

Temperature	Time	Cycle Number
98℃	30 s	1
98℃	10 s	25~27
61℃	30 s	
72℃	20 s	
72℃	5 min	1
4℃	∞	1

***Note**: Amplification cycle number differs according to sample type. 25 cycles for whole blood sample DNA and 27 for tissue DNA.*

5. Purification

1) Take the AMPure XP beads at room temperature for 30 min, and shake the bottle of the beads to resuspend any magnetic particles that may have settled.
2) Add 34 μL beads and 40 μL PCR products into 1.5 mL centrifuge tubes, Mix thoroughly by vortexing or pipetting up and down more than 10 times, then incubate the mixture at room temperature for 5 min.
3) Place the centrifuge tubes onto the magnetic stand for 3~5 min until the solution turns clear. Gently remove and discard the supernatant while the centrifuge tube is on the magnetic stand. Do not touch the beads with pipette tip.
4) Keep the tubes on the magnetic stand, add 200 μL fresh 80% ethanol into the centrifuge tube, and keep it at room temperature for at least 30 seconds. Remove and discard the supernatant while the tube is on the magnetic stand.
5) Repeat the above step (4).
6) Allow the Beads to dry at room temperature with lid open for 2~3 min until the beads show matt surface. Over drying of the beads may decrease elution efficiency.
7) Remove the centrifuge tubes away from the magnetic stand, add 30 μL TE-low solution or 10 mM Tris solution, mix thoroughly by vortexing or pipetting up and down more than 10 times. Then incubate the mixture at room temperature for 3 min.
8) Place the tube onto the magnetic stand for 3~5 min until the solution turns clear. Transfer the supernatant into a new 1.5 mL centrifuge tube to obtain the DNA library. Do not touch the beads with pipette tip.

6. DNA Library Quality Control

1) DNA Library Quality Control: quantify the DNA library with a recommended kit. For the Qubit® Kit or Quantus Kit, the concentration should be more than 5 ng/μL, for the qPCR library Quantification kit, the concentration should be more than 25.25 nM.
The concentration converting formula:

$$\text{Library Concentration}\ [\text{nM}] = \frac{\text{Library Concentration}\ [\text{ng/uL}] \times 10^6}{660 \times 300}$$

2) DNA fragment Quality Control: the Agilent 2100 Bioanalyzer is recommended. The main peak of the library should be at 260~400 bp, shown in Figure 1.

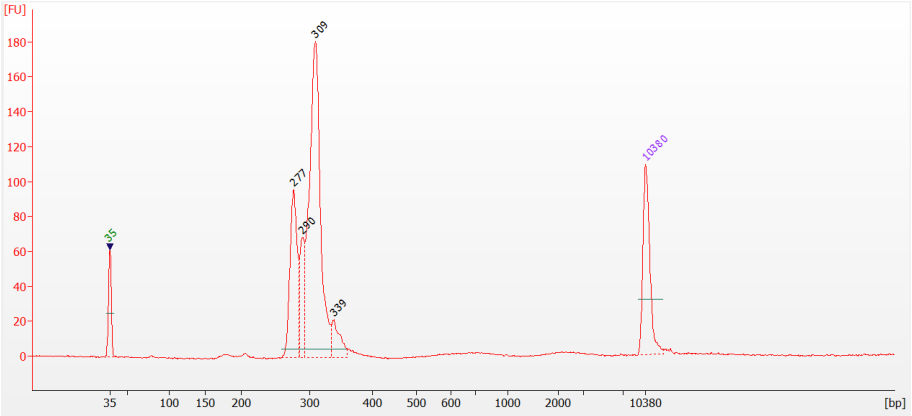

Figure 1 Diagram of the quality control of the library on Agilent 2100 Bioanalyzer High Sensitivity Chip

7. Sequencing:

Illumina 300 cycles (Paired-End Reads, 2×150 cycles) and the matched reagents and instrument are recommended for the sequencing. The percentage of Phix is 1%. It is recommended that the sequencing data for blood sample is not less than 60Mb, for fresh tissue and FFPE tissue sample is not less than 300 Mb.

Perform the denaturation and dilution of the libraries according to the instrument's instructions. The final concentration of sequencing library is recommended in Table 6.

Table 6 Recommended Final Concentration of Sequencing Library

Sequencing Instrument	Final Concentration
iSeq 100	30~40 pM
MiSeq/MiSeqDx	5~7 pM
MiniSeq	0.6~0.8 pM
NextSeq 500	0.6~0.8 pM

8. Data Analysis:

When the sequencing is finished, adopt AmoyDx ANDAS Data Analyzer to analyze the sequencing data and assess the mutation status of *BRCA1* and *BRCA2* genes.

Check Q30 value for the sequencing data:

If Q30 value of the sequencing data is ≥75%, the run data is qualified. If not, the sequencing data is unqualified.

Note: For base calls with a quality score of Q30, one base call in 1,000 is predicted to be incorrect.

Result Interpretation

The mutations are detected if meeting the following requirements.

- For Blood samples

  The raw depth of sequencing is not less than 100×, the mutant allele frequency is not less than 20%.

- For fresh tissue and FFPE samples

  The effective depth of sequencing is not less than 300×, the mutant allele frequency is not less than 1%.

Note:

- *For FFPE tissue sample, the deamination of cytosine bases to deoxyuracil results in numerous C>T and G>A conversion in sequencing data. Therefore, the cut-off frequencies of C>T and G>A are 2%.*
- *If the library concentration is less than 5 ng/μL, the original DNA may be of poor quality or the DNA concentration may be inaccurate or there may be operational errors during the experiment. The DNA concentration should be retested and the DNA libraries should be re-constructed.*
- *If the NTC library detects the target length fragment (260-400 bp), there may be contamination during the experiment and the experiment should be repeated.*
- *Test results for the Positive control should be positive for the corresponding mutation type, if not, repeat the test.*

Limitations

1) The kit is to be used only by personnel specially trained in the techniques of PCR and NGS.
2) The results can be used to assist clinical diagnosis, combining with other clinical and laboratory findings.
3) The kit has been only validated for use with human blood samples, fresh tissue and FFPE samples.
4) The kit can only be used for qualitative detection of *BRCA1/2* mutations, it's not intended for measuring therapeutic efficacy.
5) Reliable results are dependent on proper sample processing, transport, and storage.
6) The kit is based on PCR amplification, sample DNA degradation may affect the kit performance.

Reference

1) Kaufman B, Shapira-Frommer R, Schmutzler RK, et al. Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. J Clin Oncol 33:244-50, 2015
2) Mateo J, Carreira S, Sandhu S, et al. DNA-Repair Defects and Olaparib in Metastatic Prostate Cancer. N Engl J Med 373:1697-708, 2015
3) Oza AM, Cibula D, Benzaquen AO, et al. Olaparib combined with chemotherapy for recurrent platinum-sensitive ovarian cancer: a randomised phase 2 trial. Lancet Oncol 16:87-97, 2015

Symbols

EC REP	Authorized Representative in the European Community	IVD	In Vitro Diagnostic Medical Device
	Manufacturer	REF	Catalogue Number
LOT	Batch Code		Use By
Σ	Contains Sufficient for <n> Tests		Temperature Limitation
	Consult Instructions For Use		Keep Dry
	This Way Up		Fragile, Handle With Care

Appendix I

Index Sequence Information for Primers

Name	Primer Index Information	Illumina Nextera XT v2 Set D No.
HS-S513	TCGACTAG	S513
HS-S515	TTCTAGCT	S515
HS-S516	CCTAGAGT	S516
HS-S517	GCGTAAGA	S517
HS-S518	CTATTAAG	S518
HS-S520	AAGGCTAT	S520
HS-S521	GAGCCTTA	S521
HS-S522	TTATGCGA	S522
HS-N716	TAGCGAGT	N716
HS-N718	GTAGCTCC	N718
HS-N719	TACTACGC	N719
HS-N720	AGGCTCCG	N720
HS-N721	GCAGCGTA	N721
HS-N722	CTGCGCAT	N722
HS-N723	GAGCGCTA	N723
HS-N724	CGCTCAGT	N724
HS-N726	GTCTTAGG	N726
HS-N727	ACTGATCG	N727
HS-N728	TAGCTGCA	N728
HS-N729	GACGTCGA	N729

Appendix II

Clinical Classification of *BRCA1/2* Gene Mutations

*Class of Variant	Example of Classification Basis	
5- Pathogenic	a b c	Premature termination of protein expression Non-coding functional region affecting protein function Amino acid alteration affecting protein function
4- Likely pathogenic	d	There is evidence that this variant is a dominant high-risk pathogenic variant and no substantial conflicting evidence.
3- VUS (variant with uncertain significance)	e	Missense mutations and mutations occurring in the intron region The clinical significance of these mutations has not been confirmed
2- Likely benign	f	There is evidence against this variant being a dominant high-risk pathogenic variant and no substantial conflicting evidence
1- Benign	g	Harmless variation in the coding region of the protein, these Mutation does not change the amino acid sequence or does not change significantly Protein function

*Class of Variant: according to the classification criteria from International Agency of Research on Cancer (IARC) and American College of Medical Genetics and Genomics (ACMG).

Appendix III

Class 4 and Class 5 mutation information in HS-Positive Control

No.*	Gene	Class 4 and 5 mutation information
1	*BRCA2*	exon11:c.3599_3600delGT:p.C1200*
2	*BRCA2*	exon11:c.5351delA:p.N1784Tfs*7
3	*BRCA2*	exon19:c.8351G>A:p.R2784Q
4	*BRCA1*	intron20:c.5277+1G>A:p.?
5	*BRCA1*	exon20:c.5266_5267insC:p.Q1756Pfs*74

No.*: Mutations in No. 1-5 are quality control sites for somatic mutation testing, mutations in No.4 and 5 are quality control sites on germline mutation testing.