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 6000, NextSeq 500, MiSeq, MiSeqDx, MiniSeq, iSeq 100

Amoy Diagnostics Co., Ltd.
39 Dingshan Road, Haicang District,
Xiamen 361027, P. R. China
Tel: +86 592 6806835
Fax: +86 592 6806839
E-mail: sales@amoydx.com
Website: http://www.amoydx.com

EC REP **Qarad EC-REP BV**
Pas 257,
2440 Geel, Belgium

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Background

BRCA1 and *BRCA2* 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 *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 College of Medical Genetics (ACMG) (Appendix II), with Class 4 and 5 being effective populations to 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* variants using DNA isolated from peripheral whole blood, fresh-frozen tissue or formalin-fixed paraffin-embedded (FFPE) tissue samples. The kit provides full and uniform coverage of both *BRCA1* and *BRCA2* genes, covering all coding exons, exon/intron junctions, some introns and UTR regions. The kit allows the detection of single nucleotide variants (SNVs) and insertions and deletions (Indels) from tissue-derived DNA, and the detection of SNVs, Indels, and large rearrangements (LRs) from blood-derived DNA.
The kit is intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

The test kit is based on the Halo-shape ANnealing and Defer-Ligation Enrichment (HANDLE) system technology which is an improved Molecular Inversion Probe (MIP) technology to capture the target gene region (Figure 1). During the library construction process, each individual DNA molecule is tagged with a unique molecular index (UMI) at both ends, which allows high sensitivity in variant detection by eliminating any library amplification and sequencing bias. The test kit uses DNA extracted from tissue or blood samples, and it offers a time saving protocol that can be completed within 5 hours, and requires just about 1 hour of hands-on time.

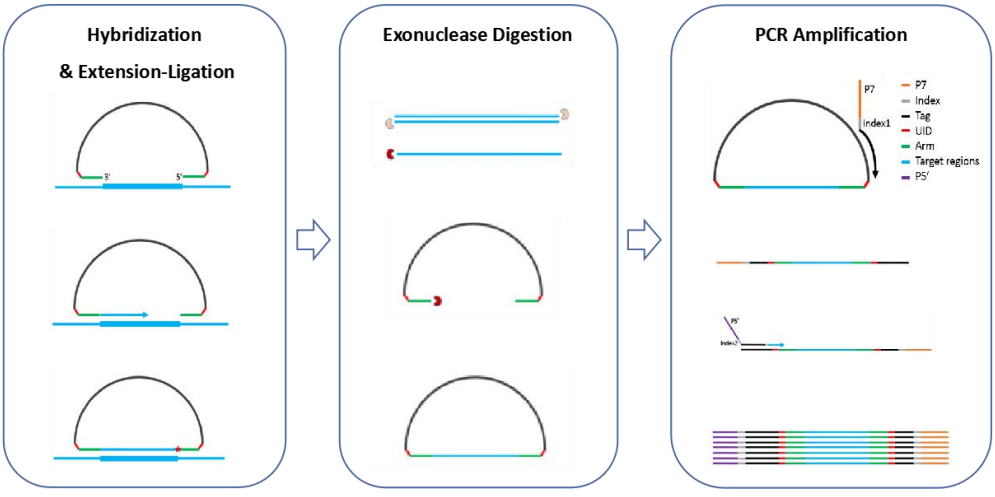

Figure 1 Principle of library construction (HANDLE system)

The probe contains an extension arm and a ligation arm which are complementary to the target gene region. Firstly, the probe anneals onto the DNA template of the target region. Secondly, the DNA is extended from the extension arm to the ligation arm with the help of DNA polymerase, then the nicks are repaired to generate the circular products with the help of DNA ligase. Next, the remaining linear probes, single-strand and double-strand DNA are digested with the help of enzyme exonuclease, and only the target circular DNA will be kept for PCR amplification. Finally, the universal PCR amplification is performed to enrich the target DNA, and the magnetic bead-based purification is performed to obtain the final library.

After quality control (QC), the qualified libraries could be sequenced on Illumina sequencing platform. The sequencing data can be analyzed by AmoyDx NGS data analysis system (ANDAS) to detect the genomic variants in the target region.

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	28 μL/tube ×1
2	HS-Hybridization Buffer	Tris-HCl, K^+, Mg^{2+}	28 μL/tube ×1
3	HS-Extension Ligation Master Mix	DNA polymerase, dNTPs, DNA Ligase, Ligation buffer	28 μL/tube ×1
4	HS-Exonuclease A	DNA Exonuclease	40 μL/tube ×1
5	HS-Exonuclease B	DNA Exonuclease	28 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 Table S1.

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°C.

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

Additional Reagents and Equipment Required but Not Supplied

1) PCR instrument: Applied Biosystems™ 2720 Thermal Cycler, MiniAmp™ Thermal Cycler or Bio-Rad T100™ Thermal Cycler is recommended.
2) DNA quantification kit: QuantiFluor dsDNA System (Promega, Cat. No. E2670) or Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851/Q32854) is recommended.
3) Fluorometer: Quantus™ Fluorometer (Promega, Cat. No. E6150) or Qubit® 2.0/3.0/4 Fluorometer (Thermo Fisher Scientific, Cat. No. Q32866/Q33216/Q33226) is recommended.
4) DNA extraction kit: AmoyDx® Blood DNA kit (Amoy Diagnostics, Cat No.: 8.02.24201X036G) is recommended for DNA extraction from whole blood sample; AmoyDx® FFPE DNA Kit (Amoy Diagnostics, Cat No.8.02.23501X036G) or MagPure FFPE DNA LQ Kit (Magentec, Cat No. D6323-01B) is recommended for DNA extraction from FFPE tissue sample; AmoyDx® Tissue DNA Kit (Amoy Diagnostics, Cat No.: 8.02.24301X036G) is recommended for DNA extraction from fresh-frozen tissue sample. It is recommended to use RNase A (Thermo Fisher Scientific, Cat. No. EN0531) to degrade RNA during the FFPE DNA extraction.
5) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter, Cat. No. A63880/A63881/A63882) is recommended.
6) Capillary electrophoresis analyzer and related kit: Agilent 2100 Bioanalyzer system and Agilent DNA 1000 Reagents (Agilent Technologies, Cat. No. 5067-1504) or Agilent High Sensitivity DNA Kit (Agilent Technologies, Cat. No. 5067-4626), or E-Gel™ Power Snap Electrophoresis System (Thermo Fisher Scientific, Cat. No. G8300) and E-Gel™ EX Agarose Gels, 2% (Thermo Fisher Scientific, Cat. No. G4010-02) are recommended.
7) Sequencing Instrument: Illumina NovaSeq 6000/NextSeq 500/MiSeq/MiSeqDx/MiniSeq/iSeq 100 is recommended.
8) Sequencing reagent: Illumina 300 cycles (Paired-End Reads, 2×150 cycles) is recommended.
9) Illumina PhiX Control V3 (Cat. No. FC-110-3002).
10) Magnetic stand: DynaMag™-2 Magnet (Thermo Fisher Scientific, Cat. No. 12321D) is recommended.
11) Mini centrifuge.
12) Vortex mixer.

13) Ice box for 0.2 mL and 1.5 mL tubes.
14) Nuclease-free 1.5 mL centrifuge tubes.
15) Nuclease-free 0.2 mL PCR tubes.
16) Nuclease-free filtered pipette tips.
17) Absolute ethanol (AR).
18) PCR-grade water (nuclease-free).
19) 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 reagents 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 whole blood, fresh-frozen tissue or FFPE tissue samples of ovarian cancer or breast cancer patients.
- The FFPE tissue sample should be fixed by 10% neutral buffered formalin for 6~24 hours (no more than 24 hours). The freshly cut sections of FFPE tissue should be used for DNA extraction immediately. The storage time for the FFPE tissue should be less than 12 months.
- It is recommended that the tumor cell content is no less than 20%.
- The peripheral whole 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.
- It is recommended to use a commercialized DNA extraction kit to perform the DNA extraction according to the sample type. And it is

recommended to use RNase A (Thermo Fisher Scientific, Cat. No. EN0531) to degrade RNA during the FFPE DNA extraction. After DNA extraction, measure the concentration of extracted DNA using Quantus™ or Qubit® Fluorometer. For fresh-frozen 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 peripheral whole blood sample, the DNA concentration should be more than 1.25 ng/μL, and total DNA should be more than 10 ng. For unqualified samples, re-collection or re-extraction are required.

- The qualified 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 HS-Positive Control (PC) in the process of library preparation, sequencing, data analysis and a No-template Control (NTC) in the process of library preparation. For NTC, it is just for quality control of the library construction process, and no need to run the sequencing or data analysis process.*
- *During the following DNA library preparation process, please use the corresponding adapter in the PCR instrument to avoid PCR product evaporation.*
- *It is recommended to use fluorescent dye method (Quantus™ or Qubit® Fluorometer) for all the DNA concentration measurement steps.*

1. Hybridization:

1.1. Take out the **HS-Probe** and **HS-Hybridization Buffer** and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer and centrifuge briefly, then keep the tube on ice.

1.2. Assemble the hybridization reaction on ice by adding the following components according to Table 2.

Table 2 Hybridization reaction

Reagent	Volume
PCR-grade water (nuclease-free)	8-χ μL
DNA	χ μL
HS-Probe	1 μL
HS-Hybridization Buffer	1 μL
Total	**10 μL**

Note:

- *For blood samples, "χ" stands for the volume of 10~50 ng DNA (50 ng is recommended).*
- *For fresh-frozen tissue or FFPE tissue samples, "χ" stands for the volume of 30~100 ng DNA (for 2% sensitivity with 300Mb/sample, 50 ng is recommended; for 5% sensitivity with 100Mb/sample, 100ng is recommended).*
- *For PC, the DNA concentration of HS-Positive Control is 5 ng/μL, take 8 μL to construct library (χ=8).*
- *For NTC, use PCR-grade water (nuclease-free) (not provided) of 8 μL (χ=0).*
- *It is recommended to prepare **freshly ready-to-use premix** of HS-Probe and HS-Hybridization Buffer for precise pipetting when perform three or more samples simultaneously.*

1.3. Mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler. Set the reaction volume as 10 μL and perform the following program: 98℃ for 5 min, 60℃ for 2 h, 4℃ hold.

***Note**:*

- *Keep the tubes at low temperature after hybridization is finished, as high temperature like room temperature may increase the non-specificity. It is recommended to place the ice box besides the thermocycler, and when it is finished, take out the reaction tube and put it in ice box immediately.*
- *The hybridization products should be stored at 2~8℃ for no more than 20 hours if not proceed to the next step.*

2. Extension-Ligation

2.1. Take out the **HS-Extension Ligation Master Mix** and thaw the reagent at room temperature. When the reagents are completely thawed, mix well on a vortex mixer and centrifuge briefly, then keep the tube on ice.

2.2. Take out the above hybridization product from the thermocycler and keep the tube on ice. Add 1 μL **HS-Extension Ligation Master Mix** into the PCR tube, mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 11 μL and perform the following program: 60°C for 10 min, 4°C hold.

***Note**:*

- *Keep the tubes at low temperature after extension-ligation is finished, as high temperature like room temperature may increase the non-specificity. It is recommended to place the ice box besides the thermocycler, and when it is finished, take out the reaction tube and put it in ice box immediately.*
- ***Perform the subsequent exonuclease digestion step immediately when the extension-ligation step is finished.***

3. Exonuclease Digestion

3.1. Take out the **HS-Exonuclease A** and **HS-Exonuclease B**, mix well on a vortex mixer and centrifuge briefly, then keep the tube on ice.

3.2. Assemble the exonuclease digestion reaction on ice by adding the following components according to Table 3.

Table 3 Exonuclease digestion reaction

Reagent	Volume
Extension-Ligation product (from step 2.2)	11 μL
HS-Exonuclease A	1.5 μL
HS-Exonuclease B	1 μL
Total	**13.5 μL**

3.3. Mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 13 μL and perform the following program: 37°C for 30 min, 95°C for 10 min, 4°C hold.

***Note**: The products of exonuclease digestion should be stored at 2~8℃ for no more than 20 hours if not proceed to the next step.*

4. PCR Amplification

4.1. 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, mix well on a vortex mixer and centrifuge briefly, then keep the tube on ice.

4.2. Assemble the PCR amplification reaction on ice by adding the following components according to Table 4.

Table 4 PCR amplification reaction

Reagent	Volume
Exonuclease digestion product (from step 3.3)	13.5 μL
HS-PCR Master Mix	25 μL
PCR-grade water (nuclease-free)	8.5 μL
HS-S5 Primer	1.5 μL
HS-N7 Primer	1.5 μL
Total	**50 μL**

***Note**:*

- *Each of the HS-S5 Primer or HS-N7 Primer has a different index sequence. Use different combination of HS-S5 Primer and HS-N7 Primer for each sample library. **Do not** use the same combination of index for two or more sample libraries in one sequencing run. The detailed information for the index sequence is shown in Appendix Table S1.*
- *Transfer the prepared tubes to the amplification room to perform PCR amplification and the following purification to avoid contamination.*

4.3. Mix the solution in each PCR tube thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 50 μL, and then perform the following program according to Table 5.

Table 5 PCR program

Temperature	Time	Cycles
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 different sample type. 25 cycles for whole blood sample DNA and 27 cycles for tissue DNA is recommended.*
- *The PCR products should be stored at 2~8℃ for no more than 20 hours if not proceed to the next step.*

5. Purification

5.1. Take out the AMPure XP beads and equilibrate them to room temperature for 30 min, and vortex the bottle of the beads to resuspend any magnetic particles that may have settled.

5.2. Add **34 μL beads** and **40 μL PCR products** into a clean 1.5 mL centrifuge tube, mix thoroughly by vortexing or pipetting, then incubate for 5 min at room temperature.

5.3. 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.

5.4. Keep the tubes on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to the tube. Incubate at room temperature for at least 30 seconds, and then carefully remove and discard the supernatant.

5.5. Repeat step 5.4 once for a total of 2 washing steps.

5.6. Briefly spin the tube, and put the tube back in the magnetic stand. Completely remove the residual ethanol, and air dry the beads for 2~3 min while the tube is on the magnetic stand with the lid open.

***Note**: **Do not** over-dry the beads. This may result in lower recovery of DNA target.*

5.7. Remove the tube from the magnet. Elute DNA target from the beads by adding 30 μL TE-low solution (pH 8.0) or 10 mM Tris solution (pH 8.0) (not provided), mix thoroughly by vortexing or pipetting, and incubate for 3 min at room temperature.

5.8. Put the tube in the magnetic stand for 3~5 min until the solution turns clear. Without disturbing the bead pellet, transfer the supernatant into a clean 1.5 mL centrifuge tube to obtain the final library.

***Note**: The purified library should be stored at -20±5 ℃ for no more than two weeks if not proceed directly to sequencing.*

6. Library Quality Control (QC)

6.1. Library concentration QC: Quantify the DNA library concentration with a recommended kit. For the Quantus™ or Qubit® Fluorometer, the library concentration should be more than 5 ng/μL.

6.2. Library fragment size QC: Assess the library quality on an Agilent Bioanalyzer DNA chip, the main peak size of the DNA fragment should be at 260~400 bp, as shown in Figure 2.

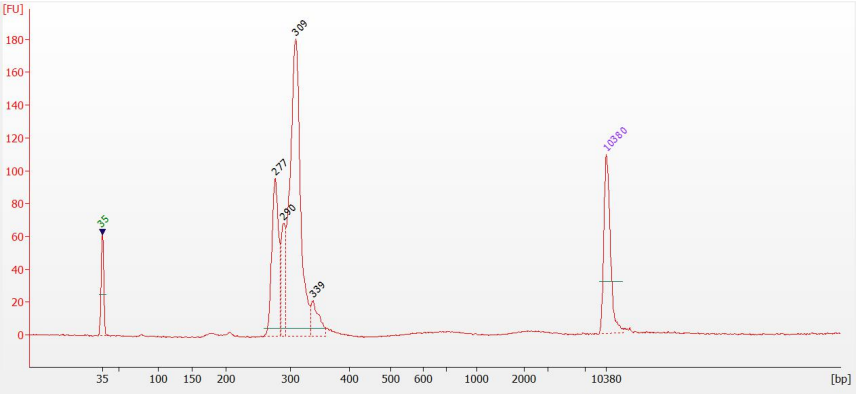

Figure 2. Example of library size distribution on a Bioanalyzer

Note:

- *If the library QC pass, then move to sequencing. If not, the library should be reconstructed.*
- *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 original DNA concentration should be retested, and it is recommended to input 100 ng DNA and perform overnight hybridization (60℃ for 12~18 h) to rebuild the library.*
- *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.*

7. Sequencing

Illumina 300 cycles (Paired-End Reads, 2×150 cycles) and the matched reagents and instruments are recommended for the sequencing. The recommended percentage of Illumina PhiX Control v3 is 1%. For blood sample, the sequencing data per sample should be no less than 60 Mb; for fresh-frozen tissue and FFPE sample, the sequencing data per sample should be no less than 300 Mb with sensitivity of 2%, or no less than 100 Mb with sensitivity of 5%. The suggested sample quantity per run is listed in Table 6.

Table 6 Recommended sequencing instruments and sample quantity per run

Illumina Sequencer			**Sample Quantity/Run**		
Sequencer	**Flow Cell**	**Read Length**	**For Germline Variants**	**For Somatic Variants**	**For Somatic Variants**
			0.06 Gb/sample	**0.1 Gb/sample (5% Sensitivity)**	**0.3 Gb/sample (2% Sensitivity)**
NextSeq 500	Mid	2×150bp	at least 96	at least 96	at least 96
MiSeq/ MiSeqDx	V3	2×150bp	at least 96	~75	~25
	V2	2×150bp	~80	~45	~15
	V2 Micro	2×150bp	~20	~12	~4
	V2 Nano	2×150bp	~5	~3	~1
MiniSeq	High	2×150bp	at least 96	~72	~24
	Mid	2×150bp	~40	~24	~8
iSeq 100	i1	2×150bp	~20	~12	~4

Perform the denaturation and dilution of the libraries according to the instrument's instructions. The final concentration of sequencing library is recommended in Table 7.

Table 7 Recommended final concentration of sequencing library

Sequencing Instrument	**Final Concentration**
iSeq 100	30~40 pM
MiSeq/MiSeqDx	6~8 pM
MiniSeq	0.6~0.9 pM
NextSeq 500	0.6~0.8 pM

Note: *The concentration converting formula:*

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

8. Data Analysis

When the sequencing is finished, adopt AmoyDx ANDAS Data Analyzer to analyze the sequencing data.

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.

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

Select the analysis module:

If the Q30 value is qualified, select the appropriate analysis module according to the sample type and sensitivity parameter, as shown in Table 8.

Table 8 Analysis modules for different samples

Sample Type	Data Throughput	Sensitivity	Analysis Module	Detected Variants
Blood	60 Mb/sample	NA	ADXHS-gBRCA-CNV	SNVs, Indels, LRs
Fresh-frozen tissue / FFPE	300 Mb/sample	2%	ADXHS-tBRCA	SNVs, Indels
	100 Mb/sample	5%	ADXHS-tBRCA-5p	SNVs, Indels

***Note**:*

- *The detection of large rearrangements (LRs) applies to blood sample only, and requires **at least five blood samples** to be extracted, library constructed, sequenced and analyzed simultaneously with reagents of the same lot, and the **HS-Positive Control** should be analyzed separately from the blood samples.*
- *If all the tested samples are blood samples, it is recommended to use the ADXHS-gBRCA-CNV module to analyze the positive control (PC), and the PC needs to be analyzed separately from blood samples.*
- *If the tested samples include tissue samples (fresh-frozen tissue or FFPE tissue), it is recommended to use the ADXHS-tBRCA or ADXHS-tBRCA-5p module to analyze the PC.*

Result Interpretation:

The mutations are detected if meeting the following requirements.

- For blood samples

  The minAmp (minimum depth) should be no less than 100×, the mutant allele frequency should be no less than 20%.
- For fresh-frozen tissue and FFPE samples

  The effectiveDepth (average effective depth) should be no less than 300×.

  The filtering thresholds are listed in Table 9.

Table 9 Filtering thresholds

Analysis Module	Depth	Non-Polymer Filter Threshold		Polymer Filter Threshold
		Non(C>T, G>A) Filter Threshold	Filter Threshold of C>T, G>A	
ADXHS-tBRCA (2% Sensitivity)	≥50×	1%	2%	2%
ADXHS-tBRCA-5p (5% Sensitivity)	≥100×	3%	3%	4%

***Note**:*

- *Q30: one base call in 1,000 is predicted to be incorrect meaning a base call accuracy of 99.9%.*
- *effectiveDepth: The average depth of the target region after UMI calibration, the effective depth calibration algorithm is different in ADXHS-tBRCA and ADXHS-tBRCA-5p analysis module.*
- *Depth: The effective depth of the detected variant.*
- *Polymer means the regions with 5 or more consecutive identical nucleotides.*
- *The PC should be detected as positive result for the corresponding variants as shown in Table S3. Otherwise, the BRCA testing is unqualified, it is necessary to check if there is any operational error and the experiment should be repeated.*
- *The clinical classification of BRCA1/2 gene variants are listed in Table S2.*

Performance

1) Limit of Detection (LoD)

The LoD of SNV/Indel was 5% allele frequency at polymer region, and 2% allele frequency at non-polymer region.

2) Accuracy

Studies of whole blood samples demonstrated 100% positive percent agreement (PPA), 100% negative percent agreement (NPA), and 100% overall percent agreement (OPA) for all variants (SNV, SNP, Indel and LR). The lower bounds of the 95% confidence intervals (CI) for PPA and NPA were 99.2% and 99.8%, respectively. Studies of clinical FFPE samples demonstrated 100% PPA, 100% NPA and 100% OPA for all variants (SNV, SNP and Indel). The lower bounds of the 95% CI for PPA and NPA were 97.9% and 98.6%, respectively.

3) Precision

Repeatability studies of whole blood samples demonstrated 100% PPA and 100% NPA for all variants (SNV, SNP, Indel and LR) assessed across lots of reagents, operators, and days. Repeatability studies of clinical FFPE samples demonstrated 100% PPA and 100% NPA for all variants (SNV, SNP and Indel) assessed across lots of reagents, operators, and days.

4) Inteference impact

Interference studies of whole blood samples demonstrated 100% PPA and 100% NPA for all variants (SNV, SNP, Indel and LR) by the presence of potential interfering substances (Bilirubin at a final concentration of 342 μM, Hemoglobin at 2 g/L, Triglyceride at 37 mM), when compared samples without additional interferents. Interference studies of clinical FFPE samples demonstrated 100% PPA and 100% NPA for all variants (SNV, SNP and Indel) by the presence of potential interfering substances (xylene at a final concentration of 35 mmol/L, ethanol at 21.7 mmol/L, and proteinase K at 0.08 mg/mL), when compared samples without additional interferents. These results demonstrate that the assay are minimally impacted or not impacted by the presence of any of the substances tested in this study.

Limitations

1) The kit is to be used only by personnel specially trained in the techniques of PCR and NGS.
2) The kit has been only validated for use with human peripheral whole blood samples, fresh-frozen tissue and FFPE tissue samples.
3) If a positive large rearrangement is detected, it should be verified by MLPA for double check.
4) Negative results can not completely exclude the existence of gene variants. Low tumor cell content, severe DNA degradation or the frequency under the limit of detection may also cause a false negative result.
5) A negative results of large rearrangement cannot completely exclude the existence of large rearrangement amplification and deletion of *BRCA1* and *BRCA2*, too few an amplified or deleted exon region may cause negative a false results.
6) The exon 2 sequence of the *BRCA1* gene contains complex secondary structure, high AT content and pseudogenes, may cause a false positive for large rearrangement detection.
7) False negative results may occur when the deletion occurs on two adjacent probes of two consecutive amplicons.
8) The kit can only be used for qualitative detection of *BRCA1/2* variants, it's not intended for measuring therapeutic efficacy.
9) Different parts of the tumor tissue or different sampling times may cause different mutation results due to tumor heterogeneity.
10) Reliable results are dependent on proper sample processing, transport, and storage.

References

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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

Table S1. Index Sequence Information for Primers

Primer Name	Primer Index Information	Illumina Nextera XT v2 Set D No.
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
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

Table S2. Clinical Classification of *BRCA1/2* Gene Variants

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

Note: *Class of Variant: according to the classification criteria from International Agency of Research on Cancer (IARC), Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) and American College of Medical Genetics and Genomics (ACMG).*

Table S3. Pathogenic or likely pathogenic variants in HS-Positive Control

No.	Gene	CDS Change
1	*BRCA2*	NM_000059.3:exon11:c.3599_3600delGT:p.C1200*
2	*BRCA2*	NM_000059.3:exon11:c.5351delA:p.N1784Tfs*7
3	*BRCA2*	NM_000059.3:exon19:c.8351G>A:p.R2784Q
4	*BRCA1*	NM_007294.3:intron20:c.5277+1G>A:p.?
5	*BRCA1*	NM_007294.3:exon20:c.5266_5267insC:p.Q1756Pfs*74

Note: *Variants in No. 4~5 are germline variants and used for the quality control when using ADXHS-gBRCA-CNV module for data analysis; Variants in No. 1~5 are used for quality control when using ADXHS-tBRCA or ADXHS-tBRCA-5p module for data analysis.*