AmoyDx
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CE IVD

AmoyDx® HANDLE HRR NGS Panel

Instructions for Use

REF 8.0680701X024I 24 tests/kit For Illumina NovaSeq 6000, NextSeq 500, MiSeq, MiSeqDx, MiniSeq, iSeq 100

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Background

The Homologous Recombination Repair (HRR) pathway plays an important role in double strand break, which is the major cause of cancer development. It has been demonstrated that loss of function of HRR genes (e.g. *BRCA1, BRCA2, PALB2*) and homologous recombination deficiency (HRD) will cause a higher risk of developing cancer, and patients with HRR gene mutations showed higher response to PARPi and platinum-containing therapies[1-5].

Intended Use

The AmoyDx® HANDLE HRR NGS Panel is intended for qualitative detection of single nucleotide variants (SNVs) and insertions and deletions (InDels) variants in protein coding regions and intron/exon boundaries of 27 HRR genes (*AR, ATM, ATR, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CDK12, CHEK1, CHEK2, ESR1, FANCA, FANCL, HDAC2, HOXB13, MRE11, NBN, PALB2, PPP2R2A, PTEN, RAD51B, RAD51C, RAD51D, RAD54L, STK11* and *TP53*), and SNVs/InDels variants in hotspot regions of 5 driver genes (*BRAF, ERBB2, KRAS, NRAS* and *PIK3CA*) using DNA isolated from peripheral whole blood samples, fresh-frozen tumor tissue or formalin-fixed paraffin embedded (FFPE) tumor tissue specimens. In addition, the kit also allows the detection of large rearrangements (LRs) of the *BRCA1* and *BRCA2* genes 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 Halo-shape ANnealing and Defer-Ligation Enrichment system (HANDLE system) technology to capture the target gene region (Figure 1). During the library construction process, each individual DNA molecule is tagged with an 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.

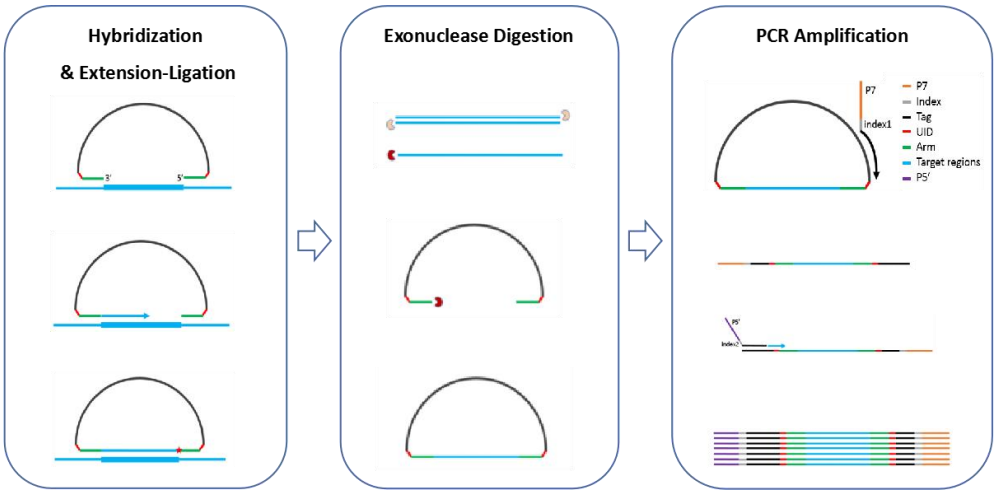

Figure 1. Principle of library construction (HANDLE system)

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	HRR-Probe	Oligonucleotides	28 μL/tube ×1
2	HRR-Hybridization Buffer	Tris-HCl, K^+, Mg^{2+}	28 μL/tube ×1
3	HRR-Extension Ligation Master Mix	DNA polymerase, dNTPs, DNA Ligase, Ligation buffer	28 μL/tube ×1
4	HRR-Exonuclease A	DNA Exonuclease	40 μL/tube ×1
5	HRR-Exonuclease B	DNA Exonuclease	28 μL/tube ×1
6	HRR-PCR Master Mix	Tris, Mg^{2+}, dNTPs, DNA polymerase	650 μL/tube ×1
7	HRR-N7 Primer *	Oligonucleotides	5 μL/tube ×12
8	HRR-S5 Primer *	Oligonucleotides	5 μL/tube ×8
9	HRR-Positive Control	DNA	60 μL/tube ×1

* For labeling and sequence information of the primers, refer to Appendix Table S2.

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 twelve months. The recommended maximum freeze-thaw cycle is five cycles.

Materials 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) or Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific) is recommended.
3) Fluorometer: Quantus™ Fluorometer (Promega) or Qubit® 2.0/3.0/4 Fluorometer (Thermo Fisher Scientific) is recommended.
4) DNA extraction kit: AmoyDx® Blood DNA kit (Amoy Diagnostics) is recommended for DNA extraction from whole blood sample; AmoyDx® FFPE DNA Kit (Amoy Diagnostics), GeneRead DNA FFPE Kit (Qiagen), or MagPure FFPE DNA LQ Kit (Magen Biotech) is recommended for DNA extraction from FFPE tissue sample; AmoyDx® Tissue DNA Kit (Amoy Diagnostics) is recommended for

DNA extraction from fresh-frozen tissue sample. It is recommended to use RNase A (Thermo Fisher Scientific) to degrade RNA during the FFPE DNA extraction.

5) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter) or CleanNGS magnetic beads (Vdobiotech) is recommended.
6) Capillary electrophoresis analyzer and related kit: Agilent 2100 Bioanalyzer system and Agilent DNA 1000 Reagents (Agilent Technologies) or Agilent High Sensitivity DNA Kit (Agilent Technologies); or Agilent 2200 TapeStation and D1000 ScreenTape/Reagents (Agilent Technologies) or High Sensitivity D1000 ScreenTape/Reagents (Agilent Technologies); or LabChip GX Touch and DNA High Sensitivity Reagent Kit (PerkinElmer); or E-Gel™ Power Snap Electrophoresis System (Thermo Fisher Scientific) and E-Gel™ EX Agarose Gels, 2% (Thermo Fisher Scientific) 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.
10) Magnetic stand: DynaMag™-2 Magnet (Thermo Fisher Scientific) 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) Nuclease-free water.
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 from another 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, breast cancer, pancreatic cancer, or prostate cancer patients.
- The FFPE tissue sample should be fixed in 10% neutral buffered formalin for 6~24 hours (no more than 24 hours). 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 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, or equivalent) 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 tissue sample, the DNA concentration should be no less than 3.8 ng/μL, and total DNA should be no less than 30 ng; for peripheral whole blood sample, the DNA concentration should be no less than 2.5 ng/μL, and total DNA should be no less than 20 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 HRR-Positive Control (PC) in the process of library preparation, sequencing, and data analysis.*
- *When using the kit for the first time, or when necessary, it is recommended to use a no template control (NTC) to verify the absence of contamination. The NTC could be used for the 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. Pre-denaturation

1.1. Take out the **DNA samples** and **TE-low solution** (not provided) and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer for 5 to 10 seconds and centrifuge briefly, then keep the tube on ice.

1.2. Assemble the pre-denaturation reaction on ice by adding the following components according to Table 2.

Table 2. Pre-denaturation reaction

Reagent	**Volume**
TE-low solution	8-χ μL
DNA	χ μL
Total	**8 μL**

Note:

- *For blood samples, "χ" stands for the volume of 20~100 ng DNA (100 ng is recommended).*
- *For fresh-frozen tissue and FFPE tissue samples, "χ" stands for the volume of 30~100 ng DNA (100 ng is recommended).*
- *For PC, take 8 μL to construct library (χ=8).*
- *For NTC, use the TE-low solution of 8 μL (χ=0).*

1.3. Mix the solution thoroughly by vortexing for 5 to 10 seconds or pipetting, and centrifuge briefly, then place the sample in a thermocycler. Set the reaction volume as 8 μL and perform the following program: 98 ℃ for 5 min, then put the tubes on ice immediately.

2. Hybridization

2.1. Take out the **HRR-Probe** and **HRR-Hybridization Buffer** and thaw the reagent at room temperature. When the reagents are completely thawed, mix well on a vortex mixer for 5 to 10 seconds and centrifuge briefly, then keep the tube on ice.

2.2. Take out the above pre-denaturation product from the thermocycler and keep the tube on ice. Assemble the hybridization reaction on ice by adding the following components according to Table 3.

Table 3. Hybridization reaction

Reagent	Volume
Pre-denaturation product (from step 1.3)	8 μL
HRR-Probe	1 μL
HRR-Hybridization Buffer	1 μL
Total	**10 μL**

***Note**: It is recommended to prepare **freshly ready-to-use premix** of HRR-Probe and HRR-Hybridization Buffer for precise pipetting when perform three or more samples simultaneously.*

2.3. Mix the solution thoroughly by vortexing for 5 to 10 seconds or pipetting, and centrifuge briefly, then place the sample in a thermocycler. Set the reaction volume as 10 μL and perform the following program: 95℃ for 5 min, 60℃ for 2 hours, 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.*

3. Extension-Ligation

3.1. Take out the **HRR-Extension Ligation Master Mix** and thaw the reagent on ice. When the reagents are completely thawed, mix well on a vortex mixer for 5 to 10 seconds and centrifuge briefly, then keep the tube on ice.

3.2. Take out the above hybridization product from the thermocycler and keep the tube on ice. Add 1 μL **HRR-Extension Ligation Master Mix** into the PCR tubes, mix the solution thoroughly by vortexing for 5 to 10 seconds 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**:*

- *It is recommended to put in the reaction tube when the thermocycler is heated to above 50℃ after starting the PCR program.*
- ***Perform the subsequent exonuclease digestion step immediately when the extension-ligation step is finished.***

4. Exonuclease Digestion

4.1. Take out the **HRR-Exonuclease A** and **HRR-Exonuclease B**, mix well on a vortex mixer for 5 to 10 seconds and centrifuge briefly, then keep the tube on ice.

4.2. Assemble the exonuclease digestion reaction on ice by adding the following components according to Table 4.

Table 4. Exonuclease digestion reaction

Reagent	Volume
Extension-Ligation product (from step 3.2)	11 μL
HRR-Exonuclease A	1.5 μL
HRR-Exonuclease B	1 μL
Total	**13.5 μL**

4.3. Mix the solution thoroughly by vortexing for 5 to 10 seconds or pipetting, and centrifuge briefly, then place the sample in a

thermocycler, set the reaction volume as 14 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.*

5. PCR Amplification

5.1. Take out the **HRR-N7 Primer, HRR-S5 Primer** and **HRR-PCR Master Mix** and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer for 5 to 10 seconds and centrifuge briefly, then keep the tube on ice.

5.2. Assemble the PCR amplification reaction on ice by adding the following components according to Table 5.

Table 5. PCR amplification reaction

Reagent	Volume
Exonuclease digestion product (from step 4.3)	13.5 μL
HRR-PCR Master Mix	25 μL
Nuclease-free water	8.5 μL
HRR-N7 Primer	1.5 μL
HRR-S5 Primer	1.5 μL
Total	**50 μL**

***Note**:*

- *Each of the HRR-N7 Primer or HRR-S5 Primer has a different index sequence. Use a different combination of HRR-S5 Primer and HRR-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 S2.*
- *Transfer the prepared tubes to the amplification room to perform PCR amplification and the following purification to avoid contamination.*

5.3. Mix the solution in each PCR tube thoroughly by vortexing for 5 to 10 seconds 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 6.

Table 6. PCR program

Temperature	Time	Cycles
98℃	30 s	1
98℃	10 s	23~25
61℃	30 s	
72℃	20 s	
72℃	5 min	1
4℃	∞	1

***Note**:*

- *Amplification cycle number differs according to different sample type. 23 cycles for whole blood sample DNA and 25 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.*

6. Purification

6.1. Take out the AMPure XP beads (or CleanNGS magnetic beads) and equilibrate them to room temperature for 30 min, and vortex the

bottle of the beads for 5 to 10 seconds to resuspend any magnetic particles that may have settled.

6.2. Add **34 µL beads** and **40 µL PCR products** into a clean 1.5 mL centrifuge tube, mix thoroughly by vortexing for 5 to 10 seconds or pipetting, then incubate for 5 min at room temperature.

6.3. 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.

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

6.5. Repeat step 6.4 once for a total of 2 washing steps.

6.6. Briefly spin the tube, and put the tube back in the magnetic rack. 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.*

6.7. Remove the tube from the magnet. Elute DNA target from the beads by adding 20 µL TE-low solution (pH 8.0) or 10 mM Tris solution (pH 8.0), mix thoroughly by vortexing for 5 to 10 seconds or pipetting, and incubate for 3 min at room temperature.

6.8. Put the tube in the magnetic rack 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**: It is recommended to store the purified library at -20℃±5℃ for no more than one week if not proceed directly to sequencing.*

7. DNA Library Quality Control (QC)

7.1. Library concentration QC: Quantify the DNA library concentration with a recommended kit. For the Quantus™ or Qubit® Fluorometer, the library concentration should be no less than 10 ng/µL.

7.2. Library fragment size QC: Assess the library quality with a recommended kit (Agilent DNA 1000 kit, Agilent High Sensitivity DNA Kit, Agilent D1000 ScreenTape/Reagents, Agilent High Sensitivity D1000 ScreenTape/Reagents, PerkinElmer DNA High Sensitivity Reagent Kit, or E-Gel™ EX Agarose Gels (2%)), the main peak size of the DNA library fragment should be at 260~400 bp, as shown in Figure 2.

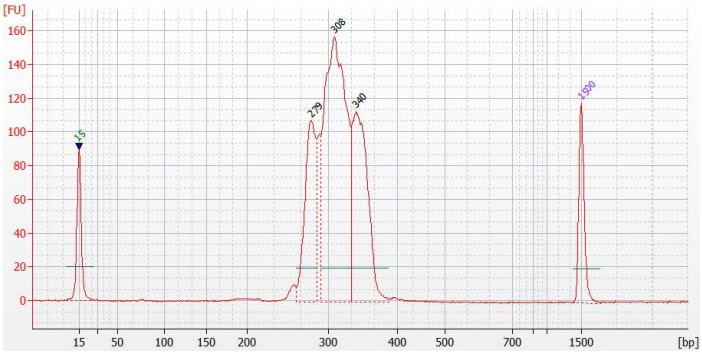

Figure 2. Example of library size distribution on Agilent 2100 Bioanalyzer

Note:

- *If the library QC pass, then move to the sequencing. If not, the library should be reconstructed.*

- *If the library concentration is less than 10 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, and it is recommended to increase the hybridization time (up to 16 hours at 60 ℃) 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.*

8. Sequencing:

Illumina 300 cycles (Paired-End Reads, 2×150 cycles) and the matched reagents and instrument are recommended for the sequencing. The recommended percentage of Illumina PhiX Control v3 is 1% (no more than 50%). For blood sample, the data output per sample should be no less than 350 Mb; for fresh-frozen tissue and FFPE sample, the data output per sample should be no less than 700 Mb with sensitivity of 5% mutant allele frequency. The suggested sample quantity per run is listed in Table 7.

Table 7. Recommended sequencing instruments and sample quantity per run

Illumina Sequencer			**Sample Quantity/Run**	
Sequencer	**Flow Cell**	**Read Length**	**For Germline Variants**	**For Somatic Variants**
			350 Mb/sample	**700 Mb/sample (5% Sensitivity)**
NextSeq 500	Mid	2×150 bp	up to 96[#]	~55
	High	2×150 bp	up to 96[#]	up to 96[#]
MiSeq/ MiSeqDx	v3	2×150 bp	~21	~10
	v2	2×150 bp	~12	~6
	v2 Micro	2×150 bp	~3	1
MiniSeq	High	2×150 bp	~21	~10
	Mid	2×150 bp	~6	~3
iSeq 100	i1	2×150 bp	~3	1

[#] *Maximum 96 indexes available.*

Perform the denaturation and dilution of the libraries according to the instrument's instructions. The final concentration of sequencing library is recommended in Table 8.

Table 8. 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:*

$$\text{Library Concentration [nM]} = \frac{\text{Library Concentration [ng/μL]} \times 10^6}{660 \times 319}$$

9. Data Analysis:

When the sequencing is finished, adopt AmoyDx ANDAS Data Analyzer to analyze the sequencing data and detect the variants of the

32 genes (Table S1).

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 (accuracy of 99.9%).*

Select the analysis module:

If the Q30 value is qualified, select the appropriate analysis module according to the sample type, as shown in Table 9.

Table 9. Analysis modules for different samples

Sample Type	Data Output	Sensitivity	Analysis Module	Variants Detected
Blood	350 Mb/sample	NA	ADXHS-gHRR-EN	SNVs, InDels, LRs
Fresh Tissue/FFPE	700 Mb/sample	5%	ADXHS-tHRR-EN	SNVs, InDels

Note*: The detection of large rearrangements (LRs) of the BRCA1 and BRCA2 genes applies to blood samples 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* ***HRR-Positive Control*** *should be analyzed separately from the blood samples.*

Check the Depth of the analyzing data:

- For blood samples, the Depth (mean depth of target region after UMI calibration) should be no less than 100×.
- For fresh tissue and FFPE samples, the Depth (mean depth of target region after UMI calibration) should be no less than 300×.

Result Interpretation

The mutations are detected if meeting the following requirements.

- For blood samples

  The Depth (depth of both allele at the mutation position) should be no less than 50×, the mutant allele frequency should be no less than 20%.

  If a positive large rearrangement is detected, it should be confirmed by MLPA.

- For fresh tissue and FFPE samples

  The Depth (depth of both allele at the mutation position) should be no less than 100×, the mutant allele frequency should be no less than 3%, the depth of mutant allele (AltDepth) should be no less than 5 copies.

Note*:*

- *The PC should be detected as positive result for the corresponding variants as shown in Table S3. Otherwise, the HRR testing is unqualified, it is necessary to check if there is any operational error and the experiment should be repeated.*

Performance

1) Limit of Detection (LoD)

The LoD of SNVs/InDels was 5% allele frequency for somatic variants.

2) Accuracy

The positive percent agreement (PPA) of SNVs/InDels (both germline and somatic) was 100%, and the concordance between the HRR assay and Multiples ligation-dependent probe amplification (MLPA) was 93.75% for determining the large rearrangements (LRs) of the *BRCA1* and *BRCA2* genes from blood-derived DNA.

3) Specificity

The negative percent agreement (NPA) of SNVs/InDels/LRs was 100%.

4) Precision

The overall analytical concordance of SNVs/InDels/LRs across 5 replicates was 100%.

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) Reliable results are dependent on proper sample processing, transport, and storage.
4) If a positive large rearrangement is detected, it should be verified by MLPA for double check.
5) A negative result 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.
6) A negative result of large rearrangement cannot completely exclude the existence of large rearrangement amplification and deletion of *BRCA1* and *BRCA2*, amplified or deleted exon regions that are too small may cause a false negative results.
7) The exon 2 sequence of the *BRCA1* gene contains complex secondary structure, high AT content and pseudogenes, which may cause a false positive for large rearrangement detection.
8) InDels ≤ 26 bp in length can be detected by this assay. For InDels with the length longer than 26 bp, the detection ability may decrease as the length increases.
9) Different parts of the tumor tissue or different sampling times may lead to different mutation results due to tumor heterogeneity.
10) The test results of this kit are for clinical reference only and should not be used as the sole basis for individualized treatment of patients. Clinicians should make comprehensive judgments on the test results based on factors such as the patient's condition, drug indications, treatment response and other laboratory test indicators.

References

1. Farmer H, Mccabe N, Lord CJ, Tutt A, Johnson DA, et al. (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 434: 917-921.
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3. Lord CJ, Ashworth A (2016) BRCAness revisited. Nat Rev Cancer 16: 110-120.
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5. McCabe N, Turner NC, Lord CJ, Kluzek K, Bialkowska A, et al. (2006) Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. Cancer Res 66: 8109-8115.

Symbols

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

Appendix

Table S1. Target regions

Gene	Target Regions*	Variants Detected
BRCA1 / *BRCA2*	All coding exons and exon-intron boundaries	SNVs, InDels, LRs
AR / *ATM* / *ATR* / *BARD1* / *BRIP1* / *CDH1* / *CDK12* / *CHEK1* / *CHEK2* / *ESR1* / *FANCA* / *FANCL* / *HDAC2* / *HOXB13* / *MRE11* / *NBN* / *PALB2* / *PPP2R2A* / *PTEN* / *RAD51B* / *RAD51C* / *RAD51D* / *RAD54L* / *STK11* / *TP53*	All coding exons and exon-intron boundaries	SNVs, InDels
BRAF	Hotspots in Exon 11/12/15/18	SNVs, InDels
ERBB2	Hotspots in Exon 1/3/8/9/11/16~21/23/25/27	SNVs, InDels
KRAS	Hotspots in Exon 2/3/4	SNVs, InDels
NRAS	Hotspots in Exon 2/3/4	SNVs, InDels
PIK3CA	Hotspots in Exon 2/5/6/8/10/14/21	SNVs, InDels

** Some regions in following exons are excluded from detection due to consistently low coverage: AR:NM_000044:exon1, CDK12:NM_016507:exon2, exon14, ESR1:NM_000125:exon1, PPP2R2A:NM_002717:exon1, RAD51B:NM_133509:exon5, RAD51D:NM_002878:exon3-5, STK11:NM_000455:exon3, TP53:NM_000546: exon3, exon4.*

Table S2. Index sequence information for primers

Primer Name	Primer Index Information	Illumina Nextera XT v2 Set B No.
HRR-N716	TAGCGAGT	N716
HRR-N718	GTAGCTCC	N718
HRR-N719	TACTACGC	N719
HRR-N720	AGGCTCCG	N720
HRR-N721	GCAGCGTA	N721
HRR-N722	CTGCGCAT	N722
HRR-N723	GAGCGCTA	N723
HRR-N724	CGCTCAGT	N724
HRR-N726	GTCTTAGG	N726
HRR-N727	ACTGATCG	N727
HRR-N728	TAGCTGCA	N728
HRR-N729	GACGTCGA	N729

Primer Name	Primer Index Information	Illumina Nextera XT v2 Set B No.
HRR-S502	CTCTCTAT	S502
HRR-S503	TATCCTCT	S503
HRR-S505	GTAAGGAG	S505
HRR-S506	ACTGCATA	S506
HRR-S507	AAGGAGTA	S507
HRR-S508	CTAAGCCT	S508
HRR-S510	CGTCTAAT	S510
HRR-S511	TCTCTCCG	S511

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

No	Gene	CDS Change
1	*BRCA2*	NM_000059.3:exon11:c.3599_3600del:p.(C1200*)
2	*BRCA2*	NM_000059.3:exon11:c.5351del:p.(N1784Tfs*7)
3	*BRCA2*	NM_000059.3:exon19:c.8351G>A:p.(R2784Q)
4	*CDH1*	NM_004360.3:intron8:c.1138-1G>A:p.?
5	*CHEK2*	NM_007194.3:exon6:c.740C>A:p.(A247D)
6	*CHEK2*	NM_007194.3:exon3:c.433C>T:p.(R145W)
7	*FANCA*	NM_000135.2:exon36:c.3532G>T:p.(E1178*)
8	*PALB2*	NM_024675.3:exon5:c.2422G>T:p.(G808*)
9	*PIK3CA*	NM_006218:Exon10:c.1633G>A:p.(E545K)
10	*TP53*	NM_000546.5:intron10:c.1101-2A>C:p.?
11	*TP53*	NM_000546.5:exon7:c.722C>T:p.(S241F)

Note: Variant No.4 (CDH1 Intron8:c.1138-1G>A:p.?) is for the quality control only when using ADXHS-tHRR; All other variants can be used for the quality control when using both ADXHS-tHRR and ADXHS-gHRR.

*Class of variant: according to the classification criteria from American College of Medical Genetics and Genomics (ACMG).