

AmoyDx[®] HRD Complete Panel

Instructions for Use

For Research Use Only. Not for use in diagnostic procedures.

REF 8.06.0114 20 tests/kit For Illumina NovaSeq 6000, NextSeq 500, NextSeq 550, NextSeq 550Dx



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Background

The Homologous Recombination Repair (HRR) pathway plays an important role in the repair of double-strand breaks. Loss-of-function alterations in the genes involved in the HRR pathway could lead to the Homologous Recombination Deficiency (HRD) phenotype, which is characterized by the inability of a cell to effectively repair DNA double-strand breaks. It has been well-studied that the HRD phenotype could sensitize certain types of tumors to poly ADP ribose polymerase inhibitors (PARPi) and platinum-containing therapies, which target the destruction of cancer cells through synthetic lethality [1-7].

Intended Use

The AmoyDx® HRD Complete Panel is a next-generation sequencing (NGS) based assay intended to provide tumor profiling of the following purposes:

1. Determination of homologous recombination deficiency (HRD) status via qualitative detection and classification of single nucleotide variants (SNVs) and insertions and deletions (InDels) in protein coding regions and intron/exon boundaries of the *BRCA1* and *BRCA2* genes and the determination of the Genomic Scare Score (GSS) which is an algorithmic measurement of genomic instability status.
2. Qualitative detection and classification of SNVs, InDels, and homozygous deletions (HDs) in protein coding regions and intron/exon boundaries of the following 20 homologous recombination repair (HRR) genes: *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CDK12*, *CHEK1*, *CHEK2*, *FANCA*, *FANCL*, *HDAC2*, *PALB2*, *PPP2R2A*, *PTEN*, *RAD51B*, *RAD51C*, *RAD51D*, *RAD54L*, *TP53*.

The assay uses DNA isolated from formalin-fixed paraffin embedded (FFPE) tumour tissue specimens. The target regions are listed in Appendix Table S1.

This assay is for laboratory professional use only.

Principles of the Procedure

The test kit is based on Halo-shape ANnealing and Defer-Ligation Enrichment (HANDLE) system technology, capturing the targeted regions (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 variants detection by eliminating any library amplification and sequencing bias. The test kit uses DNA extracted from FFPE tissue, and it offers a time-saving protocol that can be completed within 5 hours, and requires just about 1 hour of hands-on time.

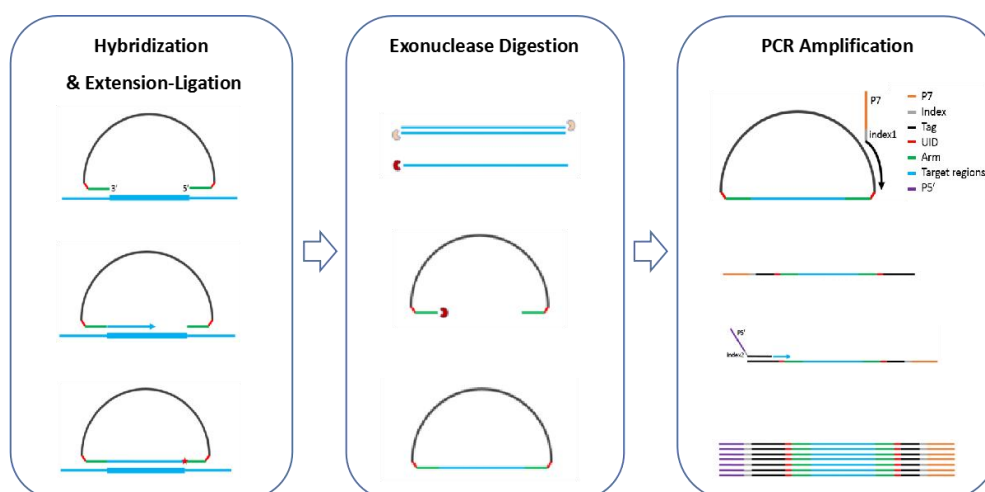


Figure 1. Principle of Library Construction (HANDLE System)

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. The DNA is then 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	HCOM-Probe	Oligonucleotides	23 μ L/tube \times 1
2	HCOM-Hybridization Buffer	Tris-HCl, K ⁺ , Mg ²⁺	23 μ L/tube \times 1
3	HCOM-Extension Ligation Master Mix	DNA polymerase, dNTPs, DNA Ligase, Ligation buffer	35 μ L/tube \times 1
4	HCOM-Exonuclease A	DNA Exonuclease	35 μ L/tube \times 1
5	HCOM-Exonuclease B	DNA Exonuclease	23 μ L/tube \times 1
6	HCOM-PCR Master Mix	dNTPs, DNA polymerase, PCR buffer	550 μ L/tube \times 1
7	HCOM-N7 Primer*	Oligonucleotides	5 μ L/tube \times 12
8	HCOM-S5 Primer*	Oligonucleotides	5 μ L/tube \times 8
9	HCOM-Positive Control**	DNA	36 μ L/tube \times 1
10	HCOM-Negative Control***	DNA	36 μ L/tube \times 1

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

** The positive variants in the HCOM-Positive Control are listed in Appendix Table S3.

*** The HCOM-Negative Control shows a negative result in the detection range of this kit.

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 at -20°C \pm 5°C immediately upon receipt.

The shelf-life of the kit is twelve months. The maximal number of freeze-thaw cycles is five.

Materials Required But Not Supplied

- 1) DNA quantification kit: QuantiFluor dsDNA System (Promega) or Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific) is recommended.
- 2) Fluorometer: Quantus™ Fluorometer (Promega) or Qubit Fluorometer (Thermo Fisher Scientific) is recommended.
- 3) DNA extraction kit: AmoyDx® Magnetic FFPE DNA Extraction Kit (Amoy Diagnostic), QIAamp DSP DNA FFPE Tissue Kit

(QIAGEN) or MagPure FFPE DNA LQ Kit (Magen Biotechnology) is recommended. It is recommended to use RNase A to degrade RNA during the DNA extraction.

- 4) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter Genomics) or CleanNGS magnetic beads (Vdo biotech) is recommended.
- 5) Capillary electrophoresis analyzer and related kit: Agilent 2100 Bioanalyzer System and Agilent DNA 1000 Kit (Agilent Technologies) are recommended.
- 6) Thermocycler: Applied Biosystems Veriti™ Dx 96-well Thermal Cycler is recommended.
- 7) Illumina PhiX Control Kit v3, or TG PhiX Control Kit v3.
- 8) Sequencing reagent: Illumina 300 cycles (Paired-End Reads, 2×150 cycles) is recommended.
- 9) Sequencer: Illumina NovaSeq 6000, NextSeq 500/550, NextSeq 550Dx.
- 10) Magnetic stand: DynaMag™-2 Magnet is recommended, or equivalent.
- 11) Mini centrifuge.
- 12) Vortex mixer.
- 13) Ice box for 0.2 mL and 1.5 mL tubes.
- 14) 1.5 mL nuclease-free centrifuge tubes (Axygen is recommended, or equivalent).
- 15) 0.2 mL nuclease-free PCR tubes, and colorless 0.5 mL PCR tubes (Axygen is recommended, or equivalent).
- 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).

Precautions and Handling Requirements

Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use; Strictly follow the instruction during operation.
- DO NOT use the kit or any kit component after their expiry date.
- DO NOT combine reagents from different kits or lots.
- 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 contact of skin, eyes and mucous membranes with the chemicals. In case of contact, flush with water immediately.

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, pipettes 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 single use. DO NOT reuse.
- Unused reagents, used kit, and waste must be disposed properly.
- Waste disposal shall follow local regulation.

Cleaning

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

Specimen Preparation

- Sample DNA should be extracted from FFPE tissue specimens of ovarian cancer, breast cancer or prostate cancer.
- The FFPE tissue sample should be fixed in 10% neutral buffered formalin for no more than 24 hours (recommend within 6 hours). It is recommended to use the central section of paraffin blocks. The freshly cut sections of FFPE tissue should be used for DNA extraction at the earliest convenience.
- Prior to testing, each FFPE tissue specimen should be subjected to independent pathology review to confirm to the presence and percentage of tumor cells. For HRR gene mutation (HRRm, including SNVs and InDels) detection, the tumor cell content should be no less than 20%; For HRD (BRCAm plus GSS) detection, the tumor cell content should be no less than 30%; For HD detection, the tumor cell content should be no less than 30% (at least 30% for HD detection at gene level, and at least 40% for HD detection at exon level). For samples with a tumor cell content lower than the requirement, it is recommended to perform microdissection or macrodissection to enrich the tumor cells, or re-collect samples if necessary. Microdissection/macrodissection is not applicable to core needle biopsy (CNB) samples.
- It is recommended to use adequate FFPE tissue (at least 1.2 mm³ of tumour) for DNA extraction, for example, using 3 unstained 4 μm FFPE slides with at least 1cm² of tumor. These recommendations are not applicable to CNB samples. 15 or more slides are recommended for use for the extraction of CNB samples.
- It is recommended to use a commercialized extraction kit to perform DNA extraction. It is recommended to use RNase A to digest RNA during the FFPE DNA extraction. After the extraction, measure the concentration of extracted DNA using Quantus™ or Qubit® Fluorometer.
- Different biomarkers (HRRm, HD, and HRD) have different sample requirements for DNA amount and tumor content, as shown in the table below. For each specific biomarker, if the sample requirements are not met, the results of that specific biomarker should be deemed unqualified and should not be used for report generation. For unqualified samples, re-collection or re-extraction are required.

Tumor Type	Biomarkers	DNA Amount	20% ≤tumor content< 30%	30% ≤tumor content< 40%	Tumor Content ≥ 40%
Ovarian cancer and breast cancer	HRRm	Total DNA ≥ 30 ng (Conc.≥ 3.75 ng/ul)	Qualified	Qualified	Qualified
	HRD (BRCAm plus GSS)	Total DNA≥ 50 ng (Conc.≥ 6.25 ng/ul)	Below threshold*	Qualified	Qualified
Prostate cancer	HRRm	Total DNA ≥ 30 ng (Conc.≥ 3.75 ng/ul)	Qualified	Qualified	Qualified
	HD	Total DNA ≥ 100 ng (Conc.≥ 12.5 ng/ul)	Below threshold*	Qualified for gene level HD	Qualified for exon level HD

HRRm: SNVs/InDels in 20 HRR genes (including BRCA1 and BRCA2 genes).

BRCAm: SNVs/InDels in BRCA1 and BRCA2 genes.

** Below threshold: For the samples with tumor content lower than the threshold, positive samples can be reported, while negative samples have risk of false negative result.*

- It is recommended to reduce the elution volume within the recommended range of extraction kit IFU during extraction to increase the DNA concentration.
- The extracted DNA should be used for DNA library preparation immediately, if not, it should be stored at -20°C±5°C for no more than 12 months, avoid repeated freezing and thawing.

Assay Procedure

Note:

- It is recommended to include a HCOM-Positive Control (PC) and a HCOM-Negative Control (NC) 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, using TE-low solution instead of DNA) to verify the absence of contamination. The NTC can 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 assemble each reaction mixture on ice to avoid non-specificity, and use the corresponding adaptor in the thermocycler to avoid the PCR products evaporation.
- It is recommended to use fluorescent dye method (Quantus™ or Qubit® Fluorometer) for all DNA concentration measurement steps.

1. Pre-denaturation

- Take out the **DNA samples**, **HCOM-Positive Control**, **HCOM-Negative Control** 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 and centrifuge briefly, then keep the tube on ice.
- Assemble the pre-denaturation reaction on ice in a clean nuclease-free 0.2 mL PCR tube by adding the following components according to Table 2.

Table 2. Pre-denaturation Reaction

Reagent	Volume
TE-low solution	8- χ μ L
DNA	χ μ L
Total	8 μL

Note:

- For FFPE samples, the addition volume χ represents the volume of 30–100 ng DNA sample (recommended 100 ng), please note that for HRRm (SNV/InDel) detection, the minimum DNA input is 30 ng; for HRD (BRCA plus GSS) detection, the minimum DNA input is 50 ng; for HD detection, the DNA input should be 100 ng.
- For HCOM-Positive Control (PC) or HCOM-Negative Control (NC), the addition volume χ is 8 μ L.
- If no template control (NTC) is required (Low TE solution), the added volume χ is 0 μ L.

1.3 Mix the solution thoroughly by vortexing, and centrifuge briefly, then place the sample in a thermocycler. Set the reaction volume as 8 μ L and perform the following program: 98°C for 5 min, then put the tubes on ice immediately.

2. Hybridization

- 2.1 Take out the **HCOM-Probe** and **HCOM-Hybridization Buffer**, 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 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 μ L
HCOM-Probe	1 μ L
HCOM-Hybridization Buffer	1 μ L
Total	10 μL

Note:

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

2.3 Mix the solution thoroughly by vortexing, and centrifuge briefly, then place the sample in a thermocycler. Set the reaction volume as 10 μ L and perform the following program: 95°C for 5 min, 60°C for 2 hours, 4°C hold.

Note:

- Important!** The hybridization duration at 60°C must be exactly 2 hours, otherwise the risk of QC failure may be increased.
- Keep the tubes at low temperature (2–8°C) after hybridization is finished, as high temperature (eg. room temperature) may increase the non-specificity. It is recommended to place the ice box besides the thermocycler in advance, when hybridization step is finished, take out the reaction tube and put it in the ice box immediately.
- The hybridization products should be stored at 2–8°C for no more than 20 hours if not proceed to the next step.

3. Extension-Ligation

- 3.1 Take out the **HCOM-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.
- 3.2 Take out the above hybridization product from the thermocycler and keep the tube on ice. Add 1.5 μ L **HCOM-Extension Ligation Master Mix** into the PCR tubes, mix the solution thoroughly by vortexing, and centrifuge briefly, then place the sample in a

thermocycler. Set the reaction volume as 12 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°C 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 **HCOM-Exonuclease A** and **HCOM-Exonuclease B**, mix well on a vortex mixer 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)	11.5 μL
HCOM-Exonuclease A	1.5 μL
HCOM-Exonuclease B	1 μL
Total	14 μL

4.3 Mix the solution thoroughly by vortexing, 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°C for no more than 20 hours if not proceed to the next step.

5. PCR Amplification

5.1 Take out the **HCOM-N7 Primer**, **HCOM-S5 Primer** and **HCOM-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.

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)	14 μL
HCOM-PCR Master Mix	25 μL
Nuclease-free water	8 μL
HCOM-N7 Primer	1.5 μL
HCOM-S5 Primer	1.5 μL
Total	50 μL

Note:

- There are 12 tubes of HCOM-N7 Primer and 8 tubes of HCOM-S5 Primer, and each of the HCOM-N7 Primer or HCOM-S5 Primer has a different index sequence, supporting a maximum of 96 indexes. For each sequencing run, it is necessary to ensure that each sample has a unique index (unique combination of N7 and S5) to distinguish between different samples. **Do not** use the same index (same combination of N7 and S5) for two or more samples in one sequencing run. The detailed information for the index sequence is shown in Appendix Table S2.
- It is recommended to 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, and centrifuge briefly, then place the sample in a thermocycler. Set the reaction volume as 50 μ L, and then perform the following program according to Table 6.

Table 6. PCR Program

Temperature	Time	Cycles
98°C	1 min	1
98°C	20 s	21
61°C	30 s	
72°C	20 s	
72°C	5 min	1
4°C	∞	1

Note:

- The PCR products should be stored at 2~8°C for no more than 20 hours if not proceed to the next step.

6. Purification

- 6.1 Take out the AMPure XP beads (or Clean NGS magnetic beads), and equilibrate them to room temperature for 30 min, and vortex the bottle of the beads before use to resuspend any magnetic particles that may have settled.
- 6.2 Add **37 μ L AMPure XP beads** and **40 μ L PCR products** into a clean 1.5 mL centrifuge tube, mix thoroughly by vortexing 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 μ L 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.
- 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 μ L TE-low solution, mix thoroughly by vortexing, 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 library at -20°C \pm 5°C if not proceed to the next step.*

7. DNA Library Quality Control (QC)

- DNA library concentration QC: Quantify the DNA library concentration using Quantus™ or Qubit® Fluorometer, the DNA library concentration should be no less than 20 ng/ μ L.
- DNA fragment QC (optional): Quantify the DNA library fragment size using Agilent 2100 Bioanalyzer System and Agilent DNA 1000 Kit (or other equivalent capillary electrophoresis analyzer and related kit which can identify DNA with targeting fragment size),

the main peak of the targeted DNA fragment size should be at 230~370 bp, without obvious non-specific peaks of small and big fragments, as shown in Figure 2.

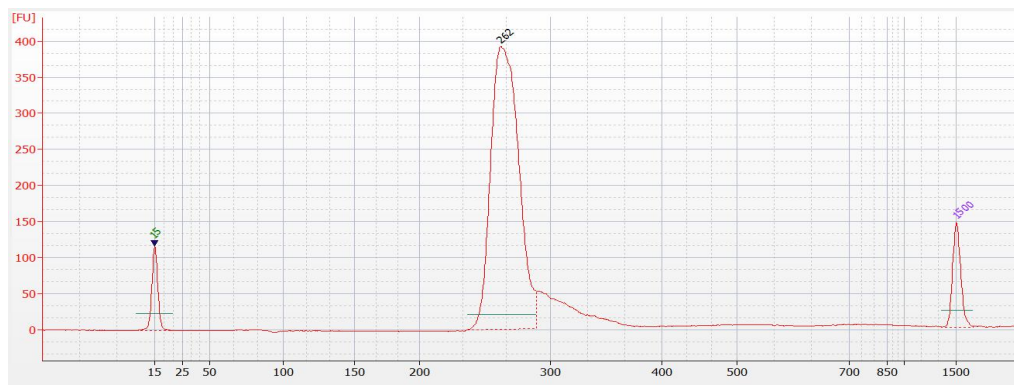


Figure 2. Example of Library Size Distribution on Agilent 2100 Bioanalyzer

Note:

- The library distribution shown in the Figure 2 above was assessed using Agilent 2100 Bioanalyzer system and Agilent DNA 1000 Reagents. The peak at 15 bp stands for the lower marker; and the peak at 1500 bp stands for the upper marker.
- The no template control (NTC) library should have a DNA concentration below 5 ng/μL, and no targeted DNA fragment size (230~370 bp). If not, there may be contamination during the experiment process, the test is unqualified and the test should be repeated.
- If the library concentration is less than 20 ng/μL, the library is unqualified. The DNA concentration should be re-tested and the DNA libraries should be re-constructed.
- It is recommended to apply the 3-tubes strategy when the single-tube-prepared library fails QC. For further information please refer to Point 11 in the Limitations Section.

8. 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%. The data output per sample (or PC/NC) should be no less than 4 Gb. The suggested sample quantity per run is listed in Table 7.

Note: When the number of samples loaded for one sequencing run is insufficient, it is recommended to adding additional library of PC, NC or samples as a supplement, or increasing the percentage of PhiX. In theory, the percentage of PhiX should be no more than 50%, according to the sequencer supplier's technical support.

Table 7. Recommended Sequencer and Sample Quantity per Run

Sequencer	Flow Cell	Read Length	Sample Quantity/Run
NextSeq 500/550/550Dx	Mid Output	2×150 bp	10
	High Output	2×150 bp	30
NovaSeq 6000	SP	2×150 bp	62
	S1	2×150 bp	up to 96 [#]

[#] 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

Illumina Sequencer	Final Concentration
NextSeq 500/550/550Dx	0.7~1.2 pM
NovaSeq 6000	1~1.4 nM

Note: The concentration converting formula:

$$\text{Library Concentration [nM]} = \frac{\text{Library Concentration [ng/}\mu\text{L]} \times 10^6}{660 \times 275}$$

9. Bioinformatics Analysis:

When the sequencing is finished, adopt AmoyDx ANDAS Data Analyzer to analyze the sequencing data to detect HRRm, HD and HRD. Select the module ADXHS-Complete for bioinformatics analysis.

Check Q30 value for the sequencing data:

If Q30 value of the sequencing run is $\geq 75\%$, the sequencing run is qualified. If not, the sequencing run is unqualified, re-sequencing or re-construct library is required.

If Q30 value of each library is $\geq 75\%$, the library sequencing data is qualified. If not, the library sequencing data is unqualified, re-sequencing or re-construct the library is required.

Data Quality Control (QC):

The qualified criteria for data QC is shown in Table 9.

Table 9. Acceptance Criteria for Data QC

Function	Qualified Criteria
Library Q30	CleanQ30 $\geq 75\%$
BRCAm (SNVs/InDels in <i>BRCA1/BRCA2</i> gene)	Coverage(180×)_BRCA $\geq 95\%$
HRRm (SNVs/InDels in 20 HRR genes)	Coverage(180×)_CDS $\geq 95\%$
HD (homozygous deletion in 20 HRR genes)	CDSDepthNoise ≤ 0.40 and CDSBAFNoise ≤ 0.05
GSS (genomic scar score in the whole genome)	DepthNoise ≤ 0.35 and BAFNoise ≤ 0.05

Note:

Q30: one base call in 1,000 is predicted to be incorrect meaning a base call accuracy of 99.9%.

CleanQ30: one base call in 1,000 is predicted to be incorrect meaning a base call accuracy of 99.9% in clean data.

*Coverage(180×)_BRCA: The proportion of the sequencing data mapped to the *BRCA1/2* gene region with a coverage depth of greater than 180×.*

Coverage(180×)_CDS: The proportion of the sequencing data mapped to the 20 genes CDS region with a coverage depth of greater than 180×.

CDSDepthNoise: The background depth noise of the CDS regions.

CDSBAFNoise: The background BAF noise of the CDS regions.

DepthNoise: The background depth noise of the whole genome regions.

BAFNoise: The background BAF noise of the whole genome regions.

Result Interpretation:

- **For HRRm (SNVs/InDels in 20 HRR genes, including *BRCA1/BRCA2*),** the mutations are detected if meeting the following requirements:

For non-polymer/STR variants, the mutant allele frequency (Freq) should be no less than 3.0%, the depth of mutant allele (AltDepth) should be no less than 8.

For polymer/STR variants, the mutant allele frequency (Freq) should be no less than 3.8%, the depth of mutant allele (AltDepth) should be no less than 8.

- **For Homozygous Deletion (HD)** status of 20 HRR genes, the HD is defined as positive if meeting the following requirements: Tumor content model interpretation (Fitness) ≥ 0.35 , the number of CNV events (Cnvs) ≥ 6 , and the probability (Prob) ≥ 0.70 .

- **For HRR Gene status**

A positive HRR Gene status result is defined by either the presence of a pathogenic/likely pathogenic variant in SNVs/InDels (HRRm positive) or a positive HD.

A negative HRR Gene status is defined by negative results in both SNVs/InDels and HD (HRRm negative and HD negative).

Otherwise it is unable to evaluate the HRR Gene status.

HRRm (SNVs/InDels) status	HD Status	Sample HRR Gene Status
HRRm Positive	HD Positive	HRR Gene Positive
HRRm Positive	HD Negative (or failed)	HRR Gene Positive
HRRm Negative (or failed)	HD Positive	HRR Gene Positive
HRRm Negative	HD Negative	HRR Gene Negative
HRRm Negative	HD failed	Unable to evaluate
HRRm failed	HD Negative (or failed)	Unable to evaluate

- **For Genomic Scar Score (GSS) status**

The range of GSS is from 0 to 100, and the cut-off of GSS is 50.0.

GSS ≥ 50.0 is defined as GSS positive; GSS < 50.0 is defined as GSS negative.

- **For HRD status**

A positive HRD status result is defined by either the presence of a pathogenic/likely pathogenic variant in *BRCA1* and *BRCA2* genes or a positive GSS (GSS ≥ 50.0).

A negative HRD status is defined by negative results in both *BRCA1/2* SNVs/InDels and GSS.

<i>BRCA</i> Status	GSS Status	Sample HRD Status
<i>BRCA1/2</i> Positive	GSS Positive	HRD Status Positive
<i>BRCA1/2</i> Positive	GSS Negative	HRD Status Positive
<i>BRCA1/2</i> Negative	GSS Positive	HRD Status Positive
<i>BRCA1/2</i> Negative	GSS Negative	HRD Status Negative

Note:

- Polymer means the regions with 5 or more consecutive identical nucleotides.
- STR (short tandem repeat) represents the regions with 5 or more consecutive repeat units comprising of 2-6 bases.
- The PC should be detected as positive result for the corresponding variants as shown in Table S3, and the NC should be detected as negative (no pathogenic or likely pathogenic variants in HRR genes, HD negative and GSS negative) in the detection range of this kit. Otherwise, the testing is unqualified, and it is necessary to check if there is any operational error and the experiment should be repeated.
- According to the classification standards of the International Agency of Research on Cancer (IARC) and the American College of Medical Genetics (ACMG), the variants of HRR genes can be divided into 5 classes: pathogenic variant (5), likely pathogenic variant (4), variant of uncertain significance (VUS) (3), likely benign variant (2), benign variant (1). Only pathogenic variant (5) or likely pathogenic variant (4) is defined as HRRm (including BRCA1/2 gene) positive.
- HRRm (or BRCAm), HD and HRD (GSS) are independent biomarkers, and the quality control parameters and requirements between them are different. When interpreting the results, they can be independently interpreted according to their corresponding parameters.

Performances

Limit of Detection (LoD)

For SNVs/InDels, the LoD was 5% mutant allele frequency at 30 ng DNA input.

For GSS detection, the LoD was 30% tumor content at 50 ng DNA input.

For HD detection, the LoD was 40% tumor content at 100 ng DNA input for exon level HD, and 30% tumor content at 100 ng DNA input for gene level HD.

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 FFPE tissue samples.
- 3) Reliable results are dependent on proper sample processing, transport, and storage.
- 4) A negative result does not rule out the presence of a mutation below the limits of detection of the assay.
- 5) Improper sample collection, transportation and processing, as well as improper experimental operation and experimental environment may lead to false negative or false positive results.
- 6) Different parts of the tumor tissue or different sampling times may cause different mutation results due to tumor heterogeneity.
- 7) This test identifies germline and somatic variants in the tumor but does not distinguish between the two.
- 8) InDels ≤ 38 bp in length can be correctly detected by this assay. InDels larger than 38 bp has not been fully validated, the detection ability may decrease as the length of the InDels increases.
- 9) When InDels occur on the primers of two consecutive probes, it may cause a false negative result.
- 10) A small portion of regions (corresponding amplicons) are difficult to amplify due to the low amplification depth, which cannot meet the detection requirements, which may lead to false negative results. False negative results may occur in tandemly repeated regions,

low-complexity regions of the human genome, or low quality sequence.

- 11) The performance of the assay was validated based on the one-tube library construction strategy. Under special conditions (eg. when the concentration is not sufficient for the single-tube strategy or when the single-tube-prepared library fails QC), the 3-tube strategy is recommended to improve the success rate of testing. The same total amount of input DNA as for the single-tube strategy (optimal 100 ng) is recommended for 3-tube strategy. The same indexes should be added for all 3 tubes of a sample when applying the 3-tube strategy. When taking 100 ng input DNA per sample for 3-tube preparation, aliquot the DNA into 3 tubes with 33.3 ng per tube (DNA concentration at 4.17 ng/μL with 8 μL per tube), then proceed to the library preparation process separately until the post-PCR purification step. For purification, take 40 μL of the PCR products from each of the 3 tubes from the same original DNA sample and pool them into one tube for purification by adding 37 μL AMPure XP beads (or CleanNGS magnetic beads) per 40 μL of PCR products for purification (eg. If aliquot 3 tubes per sample, there will be a total of 120 μL of PCR products, and 111 μL AMPure XP beads or CleanNGS magnetic beads should be added for purification). After purification, elute the DNA by adding 20 μL TE-low solution to obtain the final library. After the quality control of the libraries, sequencing can be performed according to the recommended data amount in the instructions.

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Symbols



Manufacturer



Batch Code



Contains Sufficient for <n> Tests



Consult Instructions For Use



This Way Up



Catalogue Number



Use By



Temperature Limitation



Keep Dry



Fragile, Handle With Care

Appendix

Table S1. Target Regions

NO.	Gene	Targeted Regions	Alteration Type	Tumor Type
1	<i>ATM, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CDK12, CHEK1, CHEK2, FANCA, FANCL, HDAC2, PALB2, PPP2R2A, PTEN, RAD51B, RAD51C, RAD51D, RAD54L, TP53</i>	All coding exons and exon-intron boundaries	HRR gene mutation (HRRm): SNVs, InDels	Breast cancer, ovarian cancer and prostate cancer
2	<i>ATM, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CDK12, CHEK1, CHEK2, FANCA, FANCL, HDAC2, PALB2, PPP2R2A, PTEN, RAD51B, RAD51C, RAD51D, RAD54L, TP53</i>	SNPs across the 20 genes	Homozygous Deletion (HD)	Prostate cancer
3	Whole genome	Genome-wide SNPs	Genomic Scar Score (GSS)	Breast cancer and ovarian cancer

Note:

- Some segments of the following gene exons may not be included in the detection range due to coverage reasons: *CDK12*: NM_016507: exon2, exon14; *CHEK2*: NM_007194: exon3, exon4; *FANCL*: NM_018062: exon14; *RAD51C*: NM_058216: exon1; *RAD51D*: exon3, exon4, exon5; *TP53*: NM_000546: exon2, exon3, exon4, exon11.
- For HRRm detection, the targeted regions of the 20 HRR genes are shown below.

No.	Gene	Transcript	Targeted Regions
1	<i>ATM</i>	NM_000051	Exon 2~63
2	<i>BARD1</i>	NM_000465	Exon 1~11
3	<i>BRCA1</i>	NM_007294	Exon 2, 3, 5~24; UTR3, UTR5
4	<i>BRCA2</i>	NM_000059	Exon 2~27; UTR5
5	<i>BRIP1</i>	NM_032043	Exon 2~20
6	<i>CDH1</i>	NM_004360	Exon 1~16
7	<i>CDK12</i>	NM_016507	Exon 1~14
8	<i>CHEK1</i>	NM_001274	Exon 2~13
9	<i>CHEK2</i>	NM_007194	Exon 2~15
10	<i>FANCA</i>	NM_000135	Exon 1~43
11	<i>FANCL</i>	NM_018062	Exon 1~14
12	<i>HDAC2</i>	NM_001527	Exon 2~14
13	<i>PALB2</i>	NM_024675	Exon 1~13
14	<i>PPP2R2A</i>	NM_002717	Exon 1~10
15	<i>PTEN</i>	NM_000314	Exon 1~9
16	<i>RAD51B</i>	NM_133509	Exon 2~11
17	<i>RAD51C</i>	NM_058216	Exon 1~9
18	<i>RAD51D</i>	NM_002878	Exon 1~10
19	<i>RAD54L</i>	NM_001142548	Exon 2~19
20	<i>TP53</i>	NM_000546	Exon 2~11

Table S2. Index Sequence Information for Primers

Name	Primer Index Information	Illumina Nextera XT v2 Set B No.	Name	Primer Index Information	Illumina Nextera XT v2 Set B No.
HCOM-N716	TAGCGAGT	N716	HCOM-S502	CTCTCTAT	S502
HCOM-N718	GTAGCTCC	N718	HCOM-S503	TATCCTCT	S503
HCOM-N719	TACTACGC	N719	HCOM-S505	GTAAGGAG	S505
HCOM-N720	AGGCTCCG	N720	HCOM-S506	ACTGCATA	S506
HCOM-N721	GCAGCGTA	N721	HCOM-S507	AAGGAGTA	S507
HCOM-N722	CTGCGCAT	N722	HCOM-S508	CTAAGCCT	S508
HCOM-N723	GAGCGCTA	N723	HCOM-S510	CGTCTAAT	S510
HCOM-N724	CGCTCAGT	N724	HCOM-S511	TCTCTCCG	S511
HCOM-N726	GTCTTAGG	N726			
HCOM-N727	ACTGATCG	N727			
HCOM-N728	TAGCTGCA	N728			
HCOM-N729	GACGTCGA	N729			

Table S3. The Positive Variants (pathogenic/likely pathogenic), HD and GSS status in HCOM-Positive Control

No	Gene	Pathogenic/likely pathogenic variants, HD and GSS status
1	<i>BRCA1</i>	NM_007294:exon20:c.5251C>T:p.(R1751*)
2	<i>BRCA2</i>	NM_000059:exon11:c.4777G>T:p.(E1593*)
3	<i>TP53</i>	NM_000546:exon5:c.524G>A:p.(R175H)
4	<i>PTEN</i>	HD status: <i>PTEN</i> HD positive
5	/	GSS status: GSS positive