AmoyDx® HRR Liquid NGS Panel

Instructions for Use

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

REF	8.06.0188	24 reactions/kit	For Illumina NovaSeq 6000, NextSeq 500, NextSeq 550, NextSeq 550Dx (RUO mode), MiSeq, MiSeqDx (RUO mode), and MiniSeq

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Background

Liquid biopsies are increasingly being recognized as a viable alternative to traditional tissue biopsies, particularly for cancer types where obtaining tumor tissue is difficult or invasive. The Homologous Recombination Repair (HRR) pathway is essential for the repair of double-strand DNA breaks, a key factor in cancer development. Research indicates that various cancers are associated with an increased frequency of loss-of-function mutations in HRR pathway genes. Testing for HRR gene mutations may help identify cancer patients who could benefit from treatment with PARP inhibitors (PARPi) or platinum-based therapies. [1-5]

Intended Use

The AmoyDx® HRR Liquid NGS Panel is a next-generation sequencing (NGS)-based assay intended for the qualitative detection of single nucleotide variants (SNVs) and insertions and deletions (InDels) in the protein-coding regions and intron/exon boundaries of 24 genes, including *ATM, ATR, BARD1, BRCA1, BRCA2, BRIP1, CDK12, CHEK1, CHEK2, FANCA, FANCL, HDAC2, MLH1, MRE11, NBN, PALB2, RAD51B, RAD51C, RAD51D, RAD54L, PIK3CA, PTEN, AKT1,* and *AR,* using circulating cell-free DNA (cfDNA) isolated from plasma derived from anti-coagulated peripheral whole blood specimens. In addition, the assay also allows the detection of *PTEN* copy number losses.

The kit is intended to be used by trained professionals in a laboratory environment. The test results are for research use only, not for use in diagnostic procedures.

Principles of the Procedure

The test kit is based on dual-directional capture (ddCAP) technology which is a targeted next-generation sequencing method that employs biotinylated oligonucleotide baits (probes) to hybridize with specific target regions. Designed for use with cell-free DNA (cfDNA), the kit ensures high sensitivity in variant detection. During library construction, each DNA molecule is tagged with a unique molecular index (UMI) at both ends, enabling the elimination of amplification and sequencing biases, thereby enhancing the accuracy of variant detection.

The kit contains the reagents and enzymes needed for library preparation. First, the extracted cfDNA is incubated with end repair enzyme and reagents to get the blunt-ended DNA, then followed by two ligation steps to add adapters and form the double-stranded DNA products tagged with UMI. After ligation, the adapter-ligated DNA fragments undergo size selection using magnetic beads. PCR amplification is then performed to enrich the libraries, with each library being tagged with unique dual index. Subsequently, target enrichment is carried out. This involves denaturing the double-stranded library, hybridizing biotinylated probes to the complementary target DNA, and capturing the target DNA using streptavidin beads. Finally, the universal PCR amplification is performed to enrich the target libraries.

After quality control (QC), the qualified libraries are ready for sequencing on the Illumina platform. The sequencing data can be analyzed using the AmoyDx NGS data analysis system (ANDAS) to detect the genomic variants in the target regions.

Kit Contents

This kit contains the following components in Table 1.

Table 1. Kit Contents

No.	Component Number	Components	Quantity
1	E1	**HLB-End Repair Buffer**	72 μL/tube ×1
2	E2	**HLB-End Repair Enzyme Mix**	36 μL/tube ×1
3	L1	**HLB-Ligation 1 Buffer**	300 μL/tube ×1
4	L2	**HLB-Ligation 1 Adapter**	24 μL/tube ×1
5	L3	**HLB-Ligation 1 Enzyme**	36 μL/tube ×1
6	L4	**HLB-Ligation 2 Buffer**	54 μL/tube ×1
7	L5	**HLB-Ligation 2 Adapter**	48 μL/tube ×1
8	L6	**HLB-Ligation 2 Enzyme A**	6 μL/tube ×1
9	L7	**HLB-Ligation 2 Enzyme B**	12 μL/tube ×1
10	L8	**HLB-Purification Buffer**	1200 μL/tube ×1
11	P1	**HLB-Amplification Buffer ①**	600 μL/tube ×1
12	501~512	**ADx-C501-C512**	4 μL/tube ×12
13	701~712	**ADx-C701-C712**	4 μL/tube ×12
14	H1	**HLB-Blocker**	105 μL/tube ×1
15	H2	**HLB-Probe**	75 μL/tube ×1
16	H3	**HLB-Hyb Buffer**	150 μL/tube ×1
17	B1	**HLB-Beads Wash Buffer**	938 μL/tube ×2
18	W1	**HLB-5×Wash Buffer ①**	1320 μL/tube ×1
19	W2	**HLB-5×Wash Buffer ②**	990 μL/tube ×1
20	W3	**HLB-5×Wash Buffer ③**	660 μL/tube ×1
21	W4	**HLB-5×Wash Buffer ④**	660 μL/tube ×1
22	P2	**HLB-Amplification Buffer ②**	435 μL/tube ×1
23	P3	**HLB-Polymerase**	15 μL/tube ×1
24	PC	**HLB-Positive Control**	200 μL/tube ×1
25	NC	**HLB-Negative Control**	200 μL/tube ×1

Note:

1. *For labeling and sequence information of the primers, refer to Appendix Table S2.*
2. *For positive variants in the positive control (PC), refer to Appendix Table S3.*
3. *The enzymes provided in this kit are temperature-sensitive and should be kept on ice during handling.*

Storage and Stability

The kit requires shipment with cold-chain (recommended) or 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 -25℃ to -15℃.

The shelf-life of the kit is twelve months. Repeated thawing and freezing should be avoided. Do not exceed a maximum of five freeze–thaw cycles.

Materials Required but Not Supplied

1) Vacutainer Blood Collection Tube (cfDNA preservation) (Amoy Diagnostics) or other brand with equivalent performance.
2) PCR instrument: Applied Biosystems™ 2720 Thermal Cycler or Applied Biosystems™ miniamp or equivalent.
3) DNA quantification kit: QuantiFluor dsDNA System (Promega) or Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific).

4) Fluorometer: Quantus™ Fluorometer (Promega) or Qubit® 2.0/3.0/4 Fluorometer (Thermo Fisher Scientific).
5) DNA extraction kit: AmoyDx® Circulating DNA Kit (Amoy Diagnostics) or QIAamp Circulating Nucleic Acid Kit (Qiagen) or equivalent for cfDNA extraction from blood plasma samples.
6) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter) or equivalent.
7) Streptavidin coupled magnetic beads: Dynabeads MyOne™ Streptavidin T1 (Thermo Fisher Scientific)
8) 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).
9) Sequencer: Illumina NovaSeq 6000, NextSeq 500, NextSeq 550, NextSeq 550Dx (RUO mode), Miseq, MiSeqDx (RUO mode), or MiniSeq.
10) Sequencing reagent: Illumina 300 cycles (paired-end reads, 2×150 cycles).
11) Illumina PhiX Control V3.
12) Vacuum concentrator: Concentrator Plus™ complete system (Eppendorf) or equivalent.
13) Magnetic Stand: DynaMag™-2 Magnet (Thermo Fisher Scientific) and DynaMag™-96 Side Magnet (Thermo Fisher Scientific) or equivalent.
14) Water bath or heating block: Bioer ThermoCell Mixing and Heating (Bioer Technology) or equivalent.
15) Vortex mixer.
16) Mini centrifuge.
17) Ice box for 0.2 mL and 1.5 mL tubes.
18) Nuclease-free 0.5 mL and 1.5 mL centrifuge tubes .
19) Low-binding centrifuge tube: 1.5 mL colorless low-binding centrifuge tube (Axygen) is recommended to use in the hybrid capture process.
20) Nuclease-free 0.2 mL PCR tubes .
21) Nuclease-free filtered pipette tips.
22) Absolute ethanol (AR).
23) Nuclease-Free Water (RNase-free, DNase-free).
24) 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, and strictly follow the instruction during operation.
- Please check the compatible PCR instruments prior to use.

- DO NOT use the kit or any kit component after their expiry date.
- DO NOT mix or combine reagents from different lots in the tests.
- DO NOT use reagents from 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.

Decontamination and Disposal

- The kit includes positive control. It is essential to clearly differentiate the positive control from other reagents to prevent contamination, which may cause false positive results.
- PCR amplification is highly susceptible to cross-contamination. Therefore, all materials, including tubes, racks, and pipettes, should be handled in a strictly unidirectional flow from pre-amplification to post-amplification areas, ensuring that no reverse movement occurs.
- 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 consumables should be disposable. DO NOT reuse.
- The unused reagents, used reagents, and waste must be disposed properly.

Cleaning

- Upon completion of the experiment, thoroughly clean the work area and disinfect pipettes and equipment using 75% ethanol or a 10% hypochlorous acid solution.

Specimen Preparation

- The specimen material should be cfDNA isolated from plasma derived from anti-coagulated peripheral whole blood specimens.
- The peripheral whole blood should be no less than 10 mL. It is recommended to use EDTA as the anticoagulant; the use of heparin anticoagulant should be avoided. The plasma should be separated from the whole blood within 2 hours (no more than 4 hours) upon blood collection. If separation cannot be performed within this timeframe, it is advised to utilize a commercial cell-free DNA blood collection tube (such as those from AmoyDx, Streck, or equivalent) to collect the peripheral whole blood with no less than 10 mL of total volume. These tubes should be stored at room temperature for no longer than one week prior to plasma separation. The separated plasma should be used for cfDNA extraction immediately. If not, the plasma should be stored at -85℃ to -75℃ for no more than 18 months.
- Suggested plasma separation protocol for reference: Centrifuge the peripheral whole blood sample at 2000×g for 10 minutes, retain the supernatant, then centrifuge the supernatant at 8000×g for another 10 minutes and collect the final supernatant.

- If shipment is needed, the separated plasma requires shipment on cold chain or dry ice and the shipping time should be less than one week.
- It is recommended to use a commercialized extraction kit to perform the cfDNA extraction. After extraction, measure the concentration of extracted cfDNA using Quantus™ or Qubit® Fluorometer. The total cfDNA amount should be no less than 5 ng, optimal no less than 30 ng (Vacuum concentrator or magnetic beads can be used to increase the cfDNA concentration). For unqualified samples, re-collection or re-extraction is required.
- It is recommended to use the cfDNA immediately after extraction and quantification, if not, the cfDNA should be stored at -25°C to -15°C and avoid repeatedly freeze-thaw.

Assay Procedure

Note:

- *It is recommended to include an HLB-Positive Control (PC) and HLB-Negative Control (NC) together with the testing samples in the following 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 can be used as the quality control for the library construction process, and no need to run the sequencing or data analysis process.*
- *During the following cfDNA library preparation process, please use the corresponding adapter in the thermocycler to avoid PCR product evaporation.*
- *It is recommended to use fluorescent dye method (Quantus™ or Qubit® Fluorometer) for all the DNA concentration measurement steps.*
- *The library preparation process includes cfDNA library preparation and hybridization capture.*

1. cfDNA Library Preparation

1.1 cfDNA End Repair

1.1.1 Take out the reagents listed in Table 2 from -25℃ to -15℃, thaw the buffer at room temperature, mix well with vortex and spin down, place on ice until use. For E2 HLB-End Repair Enzyme Mix, invert several times, centrifuge briefly and place on ice until use. Prepare the reaction mix according to Table 2.

Table 2. Reaction Mix for cfDNA End Repair

No.	Reagent	Volume per Test
	cfDNA/PC/NC	χ µL
E1	HLB-End Repair Buffer	3 µL
E2	HLB-End Repair Enzyme Mix	1.5 µL
	Nuclease-Free Water	50-χ µL
	Total	**54.5 µL**

Note:

- *"χ" represents the volume of input cfDNA, with an optimal total amount of 30 ng and a minimum requirement of 5 ng. It is considered as risky if the total input amount falls within the range of 5 ng to 30 ng, in which case the entire available cfDNA is* *suggested to be taken as the input.*

- *The HLB-Positive Control (PC) and HLB-Negative Control (NC) input amount should be 25 µL.*

1.1.2 Vortex the reaction tubes to mix thoroughly and spin down, then place the reaction tubes in a thermocycler to perform the following program (**set the heated lid off or at 40℃**): 20℃ for 30 min, 4℃ hold.

While the end repair program runs, take out the reagents listed in Table 3 from -25℃ to -15℃, thaw the buffer and Adapter on ice box, mix well with vortex and spin down, place on ice until use. For L3 HLB-Ligation 1 Enzyme, invert several times, centrifuge briefly and place on ice until use.

Prepare the Ligation 1 reaction mix as Table 3 before the end repair cleanup steps.

Table 3. Reaction Mix for Ligation 1 Step

No.	Reagent	Volume per Test
L1	HLB-Ligation 1 Buffer	12.5 µL
L2	HLB-Ligation 1 Adapter	1 µL
L3	HLB-Ligation 1 Enzyme	1.5 µL
	Total	**15 µL**

Note: Immediately proceed to the Cleanup step when incubation is finished.

1.2 Cleanup after End Repair

Note: The same reaction tubes should be used for the following steps; no tube changes are necessary.

Before starting, the AMPure XP beads (or equivalent) should be equilibrated to room temperature, and vortexed for around 1 min to unsure the magnetic particles are resuspended evenly.

1.2.1 Take 137 µL AMPure XP beads to add to the product from step 1.1.2, mix well by pipetting.

1.2.2 Incubate at room temperature for 10 min.

1.2.3 Place the mix from previous step (spin down briefly after incubation) onto the magnetic stand for 3~5 min until the solution turns clear.

1.2.4 Gently remove and discard the supernatant while the reaction tubes are still on the magnetic stand. **Do not** touch the beads with pipette tips. Then keep the tubes on the magnetic stand, add 200 µL of freshly prepared 80% ethanol to incubate for 30 seconds.

1.2.5 Repeat step 1.2.4 once.

1.2.6 Carefully remove and discard the ethanol, air-dry for 3-5 min to evaporate the residual ethanol, until the beads lose their luster. (**Do not** over-dry the beads, otherwise it might cause decrease of the library yields)

Note: Immediately proceed to the Ligation 1 step.

1.3 Ligation 1

Note: The same reaction tubes should be used for the following steps; no tube changes are necessary.

1.3.1 Take the product from Step 1.2.6 off from the magnetic stand, add 15 µL Ligation 1 reaction mix prepared previously (from Step 1.1.2 Table 3).

1.3.2 Mix well by pipetting up and down for around 10 times, ensure the magnetic beads are resuspended evenly and then close the tube lids.

1.3.3 Place the reaction tubes in a thermocycler to perform the following program:

Set the heated lid to 70°C; if not adjustable, set it to 105°C.

20℃ for 15 min, 65℃ for 15 min, 4℃ hold.

Note: Do not hold at 4 ℃ for more than 2 h!

1.4 Ligation 2

Note: The same reaction tubes should be used for the following steps; no tube changes are necessary.

1.4.1 Take out the reagents listed in Table 4 from -25℃ to -15℃, thaw the buffer and Adapter at room temperature, mix well with vortex and spin down, place on ice until use. For L6 HLB-Ligation 2 Enzyme A and L7 HLB-Ligation 2 Enzyme B, invert several times, centrifuge briefly and place on ice until use. Prepare the Ligation 2 reaction mix according to Table 4.

Table 4. Reaction Mix for Ligation 2 Step

No.	Reagent	Volume per Test
L4	HLB-Ligation 2 Buffer	2.25 μL
L5	HLB-Ligation 2 Adapter	2 μL
L6	HLB-Ligation 2 Enzyme A	0.25 μL
L7	HLB-Ligation 2 Enzyme B	0.5 μL
	Total	**5 μL**

1.4.2 Add 5 μL Ligation 2 reaction mix to each sample, mix well by pipetting up and down for around 10 times, ensure the magnetic beads are resuspended evenly and then close the tube lids.

1.4.3 Place the reaction tubes in a thermocycler to perform the following program:

Set the heated lid to 70°C; if not adjustable, set it to 105°C.

65℃ for 30 min, 4℃ hold.

Note: Immediately proceed to the Cleanup step.

1.5 Cleanup after Ligation 2

Note: The same reaction tubes should be used for the following steps; no tube changes are necessary.

Before starting, the HLB-Purification Buffer (L8) should be equilibrated to room temperature.

1.5.1 Add 50 μL HLB-Purification Buffer to each sample, mix well by pipetting.

1.5.2 Incubate at room temperature for 10 min.

1.5.3 Place the mix from previous step (spin down briefly after incubation) onto the magnetic stand for 3~5 min until the solution turns clear.

1.5.4 Gently remove and discard the supernatant while the reaction tubes are still on the magnetic stand. **Do not** touch the beads with pipette tips. Then keep the tubes on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to incubate for 30 seconds.

1.5.5 Repeat step 1.5.4 once.

1.5.6 Carefully remove and discard the ethanol, air-dry for 3~5 min to evaporate the residual ethanol, until the beads lose their luster. (do not over-dry the beads, otherwise it might cause decrease of the library yields).

1.5.7 Take the tubes off from magnetic stand, add 23 μL Nuclease-free water to resuspend the magnetic beads and incubate at room temperature for 2 min.

1.5.8 Place the mix from previous step (spin down briefly after incubation) onto the magnetic stand for 3~5 min until the solution turns clear.

1.5.9 Keep the tubes on the magnetic stand and carefully transfer 21 μL supernatant containing the eluted cfDNA into new reaction tubes to proceed the amplification step.

Note: *The cleaned-up products could be temporally stored at -25℃ to -15℃ if not proceeding to the next step immediately.*

1.6 PCR Amplification

1.6.1 Take out the reagents listed in Table 5 from -25℃ to -15℃ to thaw at room temperature, mix well with vortex and spin down. Place them on ice box and prepare the reaction mix according to Table 5.

Table 5. PCR Amplification Reaction

No.	Reagent	Volume per Test
P1	HLB-Amplification Buffer ①	25 μL
501~512	ADx-C501-C512	2 μL
701~712	ADx-C701-C712	2 μL
/	Cleaned-up product (from step 1.5.9)	21 μL
	Total	**50 μL**

Note:

Please use different combination of the 501-512 and 701-712 primers for each sample.

1.6.2 Vortex the reaction tubes to mix thoroughly and spin down, then place the tubes in a thermocycler to perform the following program, set the heated lid to 105℃:

Table 6. PCR Amplification Program

Temperature	Time	Cycles
98℃	45 sec	1
98℃	15 sec	**N**
60℃	30 sec	
72℃	30 sec	
72℃	1 min	1
4℃	hold	1

Note:

N refers to cycling numbers.

For samples with input amount around 5~10 ng (including 10 ng), N =11;

For PC/NC and samples with input amount around 10~30 ng (including 30 ng), N =8;

1.7 Library Purification

1.7.1 Vortex the AMPure XP beads (or equivalent) that has been equilibrated to room temperature at maximum speed for 1 min to ensure even resuspension. After vortexing, add 65 µL of AMPure XP beads to the product from step 1.6.2, vortex briefly and spin down, incubate at room temperature for 5 min.

1.7.2 Place the mix from previous step onto the magnetic stand for 3~5 min until the solution turns clear. Gently remove and discard the supernatant while the tubes are still on the magnetic stand. **Do not** touch the beads with pipette tip.

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

1.7.4 Repeat step 1.7.3 once.

1.7.5 Briefly spin the tube and gently discard the residual liquid, then air dry the magnetic beads at room temperature till there is no moist luster can be observed. **Do not** over-dry the beads.

1.7.6 Remove the tubes from the magnet stand. Elute DNA target from the beads by adding 33 µL Low TE, mix thoroughly by vortexing or pipetting to resuspend the beads evenly, and incubate for 5 min at room temperature.

1.7.7 Place the tubes back on the magnetic stand for 3~5 min until the solution turns clear. Without disturbing the beads, carefully transfer 31 µL supernatant into a clean 1.5 mL PCR tube.

Note: *The purification products should be stored at -25 ℃ to -15 ℃ for no more than 6 months if not proceeding to the next step immediately. Repeatedly freezing and thawing should be avoided.*

1.8 DNA Pre-hyb Library Quality Control (QC)

1.8.1 Quantify the DNA pre-hyb library concentration with a recommended fluorescence-based method (eg. Quantus™ or Qubit® Fluorometer), the concentration of the DNA pre-hyb library should be no less than 16.7 ng/ µL, with a total amount of at least 500 ng. If not, the library is unqualified and it is recommended to re-sampling or re-constructing the library.

Note: *For No-template Control (NTC), the library output should be less than 75 ng. Otherwise, there may be contamination during the experiment and the experiment should be repeated.*

1.8.2 Pre-hyb library fragment size QC (Optional): Assess the quality of the pre-hyb library with a recommended capillary electrophoresis analyzer and associated kit. The peak size of the library fragment should fall within the range of 300 to 400 bp, as illustrated in Figure 1.

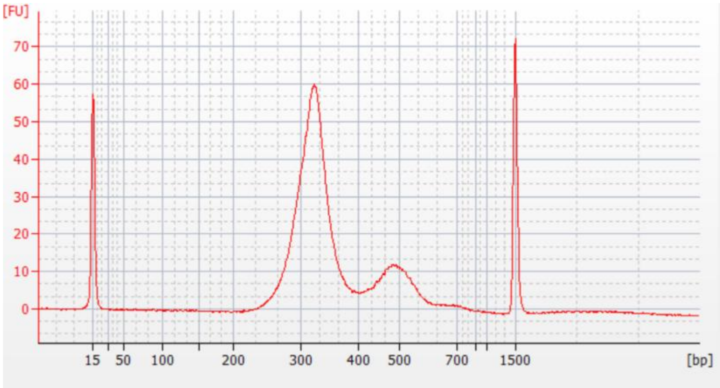

Figure 1. Example of cfDNA pre-hyb library size distribution on Agilent 2100 Bioanalyzer

2. Hybridization Capture

2.1 Reagent Preparation

2.1.1 Pool the pre-hyb libraries into a clean nuclease-free 0.2 mL PCR tube according to Table 7. The PC/NC library should be pooled separately from the cfDNA sample library.

Table 7. Suggested Pooling Amount of the Pre-hyb Libraries

Library pooling with n samples	n =1	n =2	n =3	n =4	n =5	n =6
Input amount of each pre-hyb cfDNA	750 ng	500 ng	500 ng	500 ng	500 ng	500 ng

Note:

- *It is recommended to perform hybridization capture with 1 to 6 samples, each utilizing different index combinations. Equal amounts of libraries from the same sample type should be mixed for hybridization, ensuring that each library has a unique index combination. Libraries with identical index combinations should not be included in the same hybridization pool.*
- *It is recommended to pool the PC DNA library and NC DNA library together, with 500 ng per library (n=2), and they should be pooled separately from the cf DNA libraries.*
- *For low-quality samples, it is recommended to process hybridization capture independently to improve capturing specificity and the effective depth of sequence results.*

2.1.2 Take out the reagents listed in Table 8 from -25℃ to -15℃ to thaw at room temperature, mix well with vortex and spin down. Place them on ice box and prepare the mix according to Table 8, mix thoroughly by vortexing or pipetting, and centrifuge briefly.

Table 8. Hybridization Preparation Mix

No.	Reagent	Volume per Test
Sample	Pre-hyb libraries (1 ~ 6 samples)	/
H1	HLB-Blocker	7 μL

2.1.3 Put the tubes in a vacuum concentrator with the tube lid open. incubate at 60°C until the solution in the tubes evaporates completely. Avoid over drying (do not leave the dried sample in vacuum concentrator for more than 10 min).

Note*: If a vacuum concentrator is no available, AMPure XP Beads can be used for this step as an alternative: Add AMPure XP Beads to the Hybridization Preparation Mix (from step 2.1.2) at twice its volume. After bead binding, remove the supernatant and wash the beads twice with 200 μL freshly prepared 80% ethanol. Elute with 10 μL (H3) HLB-Hyb Buffer. Transfer the entire eluate to a clean, nuclease-free 0.2 mL PCR tube and proceed to Step 2.2.3 below.*

2.2 Hybridization

2.2.1 Take out the (H3) HLB-Hyb Buffer and hybridization capture probe (H2) from -25℃ to -15℃, thaw the reagents at room temperature. Mix well with vortex and spin down then place the tubes on ice box.

2.2.2 Carefully remove the sample tubes from the Vacuum Concentrator, add 10 µL of the (H3) HLB-Hyb Buffer into each sample tube, vortex to mix well, then centrifuge briefly.

2.2.3 Add 5 µL (H2) HLB-Probe into each sample tube, mix thoroughly by vortexing and centrifuge briefly.

2.2.4 Place the tube on a thermocycler to perform the following program (Set the heated lid to 105℃): <u>95℃ for 10 min, 52℃ for 12~20 hours (16 hours is recommended).</u>

2.3 Capture

2.3.1 Take out the Dynabeads MyOne™ Streptavidin T1 Magnetic Beads and equilibrate to room temperature for 30 min. Vortex to resuspend the beads evenly. Aliquot 25 µL of streptavidin T1 beads per capture pools into a clean nuclease-free 1.5 mL low-binding centrifuge tube (e.g, for 1 capture, prepare 25 µL of streptavidin beads, and for 2 captures, prepare 50 µL of streptavidin beads accordingly). Then add the same volume of (B1) HLB-Beads Wash Buffer per capture, mix well by gently pipetting up and down for 10~20 times.

2.3.2 Place the mix onto the magnetic stand for 1 min until the solution turns clear.

2.3.3 Gently remove and discard the supernatant while the tubes are still on the magnetic stand. Do not touch the beads with pipette tip. Add (B1) HLB-Beads Wash Buffer at twice the volume of the beads added (based on the volume of beads in Step 2.3.1) to the tube containing beads, mix well by vortexing and spin down.

2.3.4 Place the mix onto the magnetic stand for 1 min until the solution turns clear.

2.3.5 Repeat step 2.3.3 once.

2.3.6 Aliquot 50 µL of the resuspended beads (Step 2.3.5) into a new 0.2 mL low-binding tube for each capture reaction, then place the tubes onto the magnetic stand for 1 min until the solution turns clear.

2.3.7 Gently remove and discard the supernatant while the tubes are kept on the magnetic stand. **Do not** touch the beads with pipette tip. When the hybridization program (step 2.2.4) is finished, quickly transfer all the hybridization product (~ 15 µL, from Step 2.2.4) into the 0.2 mL tubes with magnetic beads. Resuspend the beads and mix well by gently pipetting up and down quickly (avoid temperature drop during resuspension).

2.3.8 Place the tube on a thermocycler and perform the following program (Set the heated lid to 105℃): incubate at <u>52℃ for 45 min, 52℃ hold.</u> Setup the timer, during the incubation program, repeat the following steps every 15 min: take out the tubes from the thermocycler and vortex gently to resuspend the beads, and then quickly put it back in the thermocycler for incubation. (**This process requires rapid operation to avoid the temperature drop**)

Note: At the end of the 45 min program, remove the sample from the thermocycler, proceed immediately to the washing step.

2.4 Washing

Important! It is critical to complete the following three steps (from Step 2.4.1 to Step 2.4.3) ***before*** finishing the above Step 2.3.8.

2.4.1 Turn on the water bath or heating block with shaking function in advance and set the temperature at 52℃.

2.4.2 Take out the 5× Wash Buffer ①~④ (W1~W4) from -25°C to -15°C, thaw the reagents at room temperature. Mix well with vortex and spin down (all the wash buffers should be transparent). Dilute the following buffers to create the 1× working solutions according to Table 9.

Table 9. Dilution of Wash Buffer (per capture reaction)

1× Working Solution	**Component**	**Volume of 5× Wash Buffer**	**Volume of Nuclease-free Water**	**Total Volume**
1×Wash Buffer ①	(W1) 5×Wash Buffer ①	88 μL	352 μL	440 μL
1×Wash Buffer ②	(W2) 5×Wash Buffer ②	66 μL	264 μL	330 μL
1×Wash Buffer ③	(W3) 5×Wash Buffer ③	44 μL	176 μL	220 μL
1×Wash Buffer ④	(W4) 5×Wash Buffer ④	44 μL	176 μL	220 μL

2.4.3 After dilution, take sufficient volume of 1×Wash Buffer ① and 1×Wash Buffer ②, place it on the water bath or heating block to heat-up for at least 10 min to be ready to use. Leave the other diluted buffer at room temperature for use in next steps.

2.4.4 When the step 2.3.8 is finished, add 100 μL preheated 1×Wash Buffer ② to each sample tube, mix well by pipetting up and down for 10 times. Spin down briefly and place the tubes onto the magnetic stand for 1 min until the solution turns clear.

2.4.5 Remove and discard the supernatant carefully without touching the beads. Remove the tubes from the magnetic stand, add 200 μL preheated 1×Wash Buffer ①, mix well rapidly by pipetting up and down for 10 times (to avoid temperature drop). Incubate the tubes in a thermomixer at 52℃, 500 rpm for 5 min. Then spin down briefly and place the tubes onto the magnetic stand for 30 seconds until the solution turns clear.

Note：If such shaking condition (500 rpm) is not available, please mix manually every 2 minutes (quickly take out the tube, mix well gently by pipetting up and down, and then put it back into the 52℃ heating block). Each mixing process must be carried out quickly to prevent a sudden drop in temperature.

2.4.6 Repeat step 2.4.5 once.

2.4.7 Remove and discard the supernatant carefully without touching the beads. Remove the tube from the magnetic stand, add 200 μL preheated 1×Wash Buffer ②, mix well by pipetting up and down, then incubate the tubes in a thermomixer at 52℃, 500 rpm for 5 min (to improve the beads-binding specificity). Then spin down briefly and place the tubes onto the magnetic stand for 1 min until the solution turns clear.

Note：If such shaking condition (500 rpm) is not available, please mix manually every 2 minutes (quickly take out the tube, mix well gently by pipetting up and down, and then put it back into the 52 ℃ heating block). Each mixing process must be carried out quickly to prevent a sudden drop in temperature.

2.4.8 Remove and discard the supernatant carefully without touching the beads. Remove the tube from the magnetic stand, add 200 μL 1×Wash Buffer ③, shake or vortex at 2000 rpm for 1 min to mix well. Then spin down briefly and place the tubes onto the magnetic stand for 1 min until the solution turns clear.

2.4.9 Remove and discard the supernatant carefully without touching the beads. Remove the tube from the magnetic stand, add 200 μL 1×Wash Buffer ④, shake or vortex at 2000 rpm for 30 seconds to mix well. Then spin down briefly and place the tubes onto the magnetic stand for 1 min until the solution turns clear.

2.4.10 Remove and discard the supernatant carefully without touching the beads. Remove the tube from the magnetic stand, add 20 μL nuclease-free water, vortex to mix well and spin down. (Do not discard the beads).

2.5 Post-hybridization PCR Amplification

2.5.1 Take out the reagents listed in Table 10 from -25℃ to -15℃ to thaw the buffer at room temperature, mix well with vortex and spin down, place on ice until use. For P3 HLB-Polymerase, invert several times, centrifuge briefly and place on ice until use. Shake the captured products containing beads (from step 2.4.10) to resuspend the beads. Prepare the reaction mix according to Table 10.

Table 10. Post-hybridization PCR Amplification Reaction

No.	**Reagent**	**Volume**
P2	HLB-Amplification Buffer ②	29 μL
P3	HLB-Polymerase	1 μL
	Capture Product with Beads (from step 2.4.10)	20 μL
	Total	**50 μL**

2.5.2 Vortex the reaction tubes to mix thoroughly and spin down, then place the tubes in a thermocycler to perform the following program, set the Heat-lid to 105℃:

Table 11. Post-hybridization PCR Program

Temperature	**Time**	**Cycles**
95℃	5 min	1
95℃	30 sec	14
60℃	45 sec	
60℃	2 min	1
4℃	∞	1

Note: *The PCR products should be stored at -25℃ to -15℃ for no more than 20 hours if not proceeding to the next step immediately.*

2.6 Purification after Amplification

2.6.1 Take out the AMPure XP beads and equilibrate them to room temperature for at least 30 min. Vortex the AMPure XP beads with the maximum speed for 1 min, ensure the beads are resuspended evenly. After vortexing, add 50 μL AMPure XP beads to the PCR product from step 2.5.2, vortex briefly and spin down, incubate at room temperature for 5 min.

2.6.2 Place the tubes onto the magnetic stand for 3~5 min until the solution turns clear. Gently remove and discard the supernatant while the PCR tubes are still on the magnetic stand. **Do not** touch the beads with pipette tip.

2.6.3 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, then carefully remove and discard the supernatant.

2.6.4 Repeat step 2.6.3 once.

2.6.5 Briefly spin the tube and gently discard the residual liquid, then air dry the magnetic beads at room temperature till there is no moist luster can be observed. Do not over-dry the beads.

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

2.6.6 Remove the tubes from the magnet stand. Elute DNA target from the beads by adding 33 μL Low TE, mix thoroughly by vortexing or pipetting to resuspend the beads evenly, and incubate for 3 min at room temperature.

2.6.7 Place the tubes on the magnetic stand for 3~5 min until the solution turns clear. Without disturbing the beads, carefully transfer 31 μL supernatant into a clean nuclease-free 1.5 mL centrifuge tube.

***Note**: The purified DNA library should be stored at -25 ℃ to -15 ℃ for no more than 6 months if not proceed to the next step. Repeated thawing and freezing should be avoided.*

2.7 QC of the Captured Library

2.7.1 Quantify the library concentration with a recommended fluorescence-based method (eg. Quantus™ or Qubit® Fluorometer), the concentration of the captured library should be no less than 2.5 ng/ μL, with a total amount of at least 75 ng.

2.7.2 Library fragment size QC (Optional): Assess the library quality with a recommended capillary electrophoresis analyzer and associated kit. The peak size of the library fragment should be at 300-350 bp for plasma cfDNA library, without obvious peaks of small and big fragments, as shown in Figure 2.

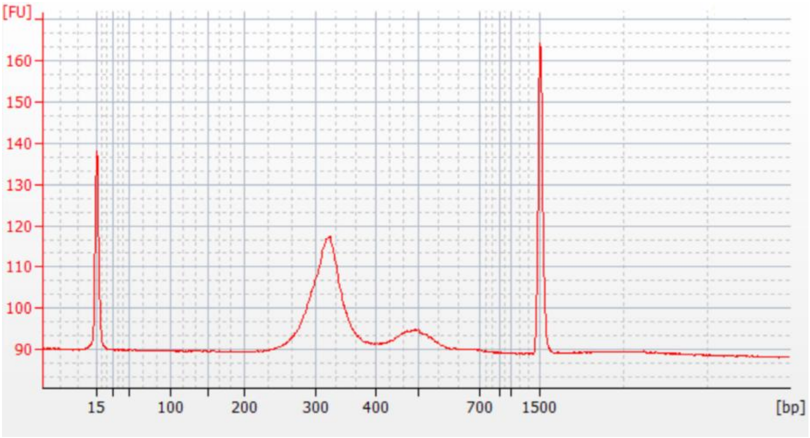

Figure 2. Example of plasma cfDNA captured library size distribution on Agilent 2100 Bioanalyzer

Note:

- *If the library QC pass, then move to sequencing. If not, re-collection, re-extraction, or library re-construction shall be conducted.*
- *The no template control (NTC) library should have a concentration below 5 ng/μL, and no peak between the targeted library size (300~350 bp). If not, there may be contamination during the experiment process, the test is unqualified and should be repeated.*

3. Sequencing

Illumina 300 cycles (Paired-End Reads, 2×150 cycles) related reagents and the matched Sequencers are recommended for sequencing. The recommended percentage of Illumina PhiX Control v3 is 1%. For the high-performance version, the sequencing data per sample library should be no less than 8 Gb, while for the standard-performance version, it should be no less than 1.5 Gb. The recommended sequencing data per PC/NC library should be at least 1.5 Gb. The suggested sample quantity per run is listed in Table 12.

Table 12. Recommended Sequencing Instruments and Sample Quantity per Run

Sequencer	Flow Cell	Read Length	High Performance: Sample Quantity/Run	Standard Performance: Sample Quantity/Run
MiSeq/MiSeqDx	v2	2×150 bp	N/A	1
	v3	2×150 bp	N/A	4
MiniSeq	High output	2×150 bp	N/A	4
NextSeq500/550/550Dx	Mid output	2×150 bp	4	23
	High output	2×150 bp	14	73
NovaSeq 6000	SP	2×150 bp	30	Up to 144*
	S1	2×150 bp	60	Up to 144*
	S2	2×150 bp	150	Up to 144*
	S4	2×150 bp	Up to 144*	Up to 144*

* *Maximum 144 index combinations 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 13.

Table 13. Recommended Final Concentration of Sequencing Library

Sequencing Instrument	Final Concentration
Miseq / MiseqDx	5~9 pM
MiniSeq	0.6~1.3 pM
NextSeq 500/550/550Dx	1.2~1.8 pM
NovaSeq 6000	0.75~1.0 nM

Note:

- *The concentration converting formula is as follows.*

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

- *It is recommended to perform the concentration conversion based on each library size obtained by quality control. If the library size of each library is not available, a fixed value of 400 bp can also be used for concentration conversion. Please note that there may be a risk of affecting the data output (higher or lower data output than expected) when using fixed values.*

Data Analysis

After sequencing is completed, select the "ADXHRR24-bMut" module for data analysis. Click on "Optional Parameters", and in the "Performance" option, select either "High" or "Standard" based on the performance version requirements. Once the settings are configured, click "Next" to enter the sample selection page. On the sample selection page, first select the sequencing batch that contains the samples to be analyzed under the "Select RUN" entry, then check the samples to be analyzed in the sample dialog box and add them to the analysis list.

Once the sample selection is complete, click "Create Analysis" to enter the analysis preview page. After confirming that everything is correct, click “START” to begin the automated analysis process.

Criteria of data QC:

The qualified criteria for cfDNA (and PC/NC) library data QC are shown in Table 14.

Table 14. cfDNA (and PC/NC) Library Data QC Qualified Criteria

Parameters	Qualified
cleanQ30	⩾ 75%
CoverageRatioUNIQ1000	⩾ 90%
CNVNoise	⩽ 0.2

***Note**:*

- *cleanQ30: the percentage of bases with Phred quality scores ⩾30 in clean data, indicating a base call accuracy of 99.9%.*
- *CoverageRatioUNIQ1000: The proportion of target regions that have unique depth more than 1000×.*
- *CNVNoise: Background depth noise in CNV calling. This parameter is used solely for CNV quality control and is not applicable to the quality assessment of other variant types.*

Result Interpretation

The cut-off metrics are shown in Table 15.

Table 15. Cut-Off Metrics

Variant Type	Performance Version (Data Amount)	Freq_US	Var_US	Freq_SS	Var_SS	Z-Score
SNV/InDel	High (8 Gb/sample)	0.09% MAF	5	0.06% MAF	4	/
	Standard (1.5 Gb/sample)	0.21% MAF	7	/	/	/
CNV Loss	High or Standard (8 Gb or 1.5 Gb/sample)	/	/	/	/	⩽-3
Polymer or STR		⩾5%	⩾20	⩾5%	⩾20	/

***Note**:*

- *Freq_US: Frequency of mutant allele, after de-duplication calibration.*
- *Var_US: The number of variant reads, after de-duplication calibration.*
- *Freq_SS: Frequency of mutant allele after single strand calibration.*
- *Var_SS: Depth of mutant allele after single strand calibration.*
- *For SNV/InDel, the above cut-off values are applicable to most of the variants, while may not be applicable to some individual variants due to the difference in background mutation abundance.*
- *Z-Score: copy number deviation from per-sample diploid baseline.*

Performance Characteristics

Limit of Detection (LoD)

For plasma samples with 30ng cfDNA input, the LoD for SNV/InDel is 0.2% (High Performance Version) and 0.5% (Standard Performance Version).

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 blood plasma samples.
3) The test results from this kit are intended for research purposes only.
4) Reliable results are dependent on proper sample processing, transport, and storage.
5) Users must follow the instructions strictly, any changes in operation may affect the testing results.
6) The ANDAS database is updated on a regular basis, however, data representing the latest research, including literature evidences, related databases, and etc, could take additional time to be incorporated into the database. The ANDAS software classification results might need to be further verified by representatives from medical services, genetic counselors, or other trained professionals, to evaluate the variants' classifications referring to updated databases and latest literatures, according to ENIGMA, ACMG or applicable local guidelines. Interpretation guideline from AmoyDx can be provided as reference if requested, please send such request to sales@amoydx.com for a copy
7) Negative results can not completely exclude the existence of mutated genes. Low cfDNA content, severe cfDNA degradation or the frequency under the limit of detection may also cause a false negative result.
8) This kit only detects gene variants in the target region (as shown in Table S1). If the detection result is negative, other variants out of the target regions of these genes cannot be excluded.
9) Mutations identified in cfDNA may originate from various sources, including circulating tumor DNA fragments, germline mutations, or non-tumor somatic alterations, such as clonal hematopoiesis of indeterminate potential (CHIP). Since this assay does not utilize paired white blood cell detection as a reference, mutations from different origins cannot be definitively distinguished.
10) Inaccurate results may occur if the mutation occurs at homologous regions.
11) PTEN CNV loss analysis is limited to detecting deletions involving the entire PTEN gene.

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.

[2] Walsh CS (2015) Two decades beyond BRCA1/2: Homologous recombination, hereditary cancer risk and a target for ovarian cancer therapy. Gynecol Oncol 137: 343-350.

[3] Lord CJ, Ashworth A (2016) BRCAness revisited. Nat Rev Cancer 16: 110-120.

[4] Nicholas Turner ATaAA (2004) Hallmarks of 'BRCAness' in sporadic cancers. PERSPECTIVES 4.5.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.

[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

🏭	Manufacturer	REF	Catalogue Number
LOT	Batch Code	⧗	Use By
Σ	Contains Sufficient for <n> Tests	🌡	Temperature Limitation
i	Consult Instructions For Use	☂	Keep Dry
⇈	This Way Up	🍷	Fragile, Handle With Care

Appendix

Table S1. Gene Lists

Gene	Transcripts	Target Regions	Variation Type
ATM	NM_000051	whole CDS (Exon2-63)	SNV; InDel
ATR	NM_001184	whole CDS (Exon1-47)	SNV; InDel
BARD1	NM_000465	whole CDS (Exon1-11)	SNV; InDel
BRCA1	NM_007294	whole CDS (Exon2, 3, 5-24)	SNV; InDel
BRCA2	NM_000059	whole CDS (Exon2-27)	SNV; InDel
BRIP1	NM_032043	whole CDS (Exon2-20)	SNV; InDel
CDK12	NM_016507	whole CDS (Exon1-14)	SNV; InDel
CHEK1	NM_001114122	whole CDS (Exon2-13)	SNV; InDel
CHEK2	NM_007194	whole CDS (Exon2-15)	SNV; InDel
FANCA	NM_000135	whole CDS (Exon1-43)	SNV; InDel
FANCL	NM_018062	whole CDS (Exon1-14)	SNV; InDel
HDAC2	NM_001527	whole CDS (Exon1-14)	SNV; InDel
MLH1	NM_000249	whole CDS (Exon1-19)	SNV; InDel
MRE11	NM_005591	whole CDS (Exon2-20)	SNV; InDel
NBN	NM_002485	whole CDS (Exon1-16)	SNV; InDel
PALB2	NM_024675	whole CDS (Exon1-13)	SNV; InDel
PIK3CA	NM_006218	whole CDS (Exon2-21)	SNV; InDel
RAD51B	NM_133509	whole CDS (Exon2-10, 11*)	SNV; InDel
RAD51C	NM_058216	whole CDS (Exon1-9)	SNV; InDel
RAD51D	NM_002878	whole CDS (Exon1-10)	SNV; InDel
RAD54L	NM_001142548	whole CDS (Exon2-19)	SNV; InDel
PTEN	NM_000314	whole CDS (Exon1-9)	SNV; InDel; CNV Loss
AKT1	NM_001382430	whole CDS (Exon3-15)	SNV; InDel
AR	NM_000044	whole CDS (Exon1*, 2-8)	SNV; InDel

*Note: The exons marked with * indicate that the exons listed in the genes are not completely covered.*

Table S2. Index Sequence Information for Primers

Primer Name	NextSeq/NovaSeq
ADx-C701	TAGCAGAA
ADx-C702	CAAGATCT
ADx-C703	GCAAGAGC
ADx-C704	CGTGCTTG
ADx-C705	GATTGCCG
ADx-C706	ATCCTGAT
ADx-C707	TGGAATGA
ADx-C708	CCAGCATC
ADx-C709	GTCCTCTA
ADx-C710	TCGCTAGG
ADx-C711	ATGACTAC
ADx-C712	AGCTCAGC

Primer Name	MiniSeq/MiSeq/ NextSeq/NovaSeq V1.5	NovaSeq V1.0
ADx-C501	ATCGTTGC	GCAACGAT
ADx-C502	AACGATTA	TAATCGTT
ADx-C503	GAGCGAAC	GTTCGCTC
ADx-C504	GTGTGAGA	TCTCACAC
ADx-C505	CCTAACAG	CTGTTAGG
ADx-C506	CGTCTGCG	CGCAGACG
ADx-C507	TGATCCTT	AAGGATCA
ADx-C508	TCAACGCT	AGCGTTGA
ADx-C509	TCACTCAC	GTGAGTGA
ADx-C510	GCTGACTC	GAGTCAGC
ADx-C511	CGCAGACA	TGTCTGCG
ADx-C512	GTACCAAT	ATTGGTAC

Table S3 Variants in HLB-Positive Control (PC) and HLB-Negative Control (NC)

Control Name	Gene	Variant
HLB -PC	*BRCA1*	NM_007294: Exon20:c.5251C>T:p.(R1751*)
	BRCA2	NM_000059: Exon11:c.4777G>T:p.(E1593*)
HLB -NC	Negative	Negative

Note:

- *The PC library should pass QC and should be detected as positive results for the corresponding mutations as shown in Table S3. Otherwise, the testing is unqualified, it is necessary to investigate the cause and re-test.*
- *The NC library should pass QC and should not contain any pathogenic or likely pathogenic mutations with a MAF of greater than or equal to 0.2%. Otherwise, the testing is unqualified, it is necessary to investigate the cause and re-test.*
- *Detecting mutations of non-pathogenic / non-likely-pathogenic variants in NC is acceptable and will not serve as a criterion for quality control failure.*