AmoyDx
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AmoyDx® Comprehensive Panel

Instructions for Use

For Research Use Only.Not for Use in Diagnostic Procedures.

REF 8.06.0113 24 tests/kit For Illumina NovaSeq 6000, NextSeq 500, NextSeq 550

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Background

Lung cancer is one of the most common cancer in the world. Targeted therapy has emerged as an important approach that has successfully prolonged overall survival for cancer patients. The National Comprehensive Cancer Network (NCCN) guidelines for patients with metastatic non-small cell lung cancer (NSCLC) recommend testing for *EGFR, BRAF, ERBB2* mutations and *MET* exon 14 skipping; *ALK, ROS1, RET* and *NTRK1/2/3* rearrangements; and *MET* amplification. [1] Colorectal cancer (CRC) ranks as the second most lethal cancer and the third most prevalent malignant tumor worldwide. The NCCN guidelines for patients with colon cancer recommend testing for *KRAS, NRAS* and *BRAF* mutation; *HER2* amplification; tumor microsatellite instability (MSI) or mismatch repair (MMR) status. Approximately 20% of cases of colon cancer are associated with familial clustering, and first-degree relatives of patients with colorectal adenomas or invasive CRC are at increased risk for CRC. Lynch syndrome is the most common form of genetically determined colon cancer predisposition, accounting for 2% to 4% of all CRC cases. NCCN guidelines for patients with colon cancer recommend genetic testing for lynch syndrome for family risk assessment. [2] Breast cancer and ovarian cancer are the most common cancer in women, and are the leading cause of cancer deaths. Patients who carry the *BRCA1* and *BRCA2* gene mutations have an underlying genetic predisposition for breast and ovarian cancers. *BRCA1* mutation carriers' estimated lifetime risk for breast cancer is up to 87% and 54% for ovarian cancer; *BRCA2* mutation carriers' lifetime risk for breast cancer is up to 56% and 21% for ovarian cancer. [3] NCCN guidelines for patients with breast cancer and ovarian cancer recommend testing for *BRCA1/2* mutations to identify candidates for PARP inhibitor therapy. [4,5] Polymorphisms in genes coding for metabolising enzymes and drug transporters can affect drug efficacy and toxicity. The detection of genetic polymorphisms can help to identify individuals predisposed to a high risk of toxicity and low response from standard doses of anti-cancer drugs. [6,7]

The AmoyDx® Comprehensive Panel is a next-generation sequencing based testing which can detect a broad range of alterations. The test results are for clinical reference only. The clinician should judge the test results based on the patient's condition, drug indications, treatment response and other laboratory test indicators comprehensively.

Intended Use

The AmoyDx® Comprehensive Panel is a next-generation sequencing (NGS) based assay intended for the qualitative detection of single nucleotide variants (SNVs), insertions and deletions (InDels), gene fusions, and copy number variations (CNVs) in 110 genes (see Table S1), single nucleotide polymorphisms (SNPs) in 19 genes (see Table S2), and tumor MSI status. The assay allows the detection of SNVs, InDels, fusions, CNVs, SNPs and MSI using DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue specimens, and the detection of SNVs, InDels, fusions and SNPs using circulating cell-free DNA (cfDNA) isolated from plasma derived from anti-coagulated peripheral whole blood specimens.

The kit is intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

The test kit is based on dual-directional capture (ddCAP) technology which is a targeted next-generation sequencing method that uses biotinylated oligonucleotide baits (probes) to hybridize to the target regions. The test kit is designed for use with fragmented genomic DNA

(gDNA) or cfDNA. During the library construction process, each individual DNA molecule is tagged with a unique molecular index (UMI) at both ends, which allows high sensitivity in variant detection by eliminating any library amplification and sequencing bias.

For FFPE tissue samples, the extracted DNA should be sheared into short fragments, using either mechanical methods (e.g., ultrasonication shearing) or enzymatic digestion, then the purified fragmented DNA can be used for downstream library preparation. For plasma samples, the extracted cfDNA can be used directly to downstream library preparation.

The test kit includes the reagents and enzymes needed for library preparation. First, the fragmented DNA or cfDNA are incubated with end repair enzyme and reagents to get the blunt-ended fragments with dA-tails, then the DNA fragments are ligated to adaptors with complementary dT-overhangs, then the adaptor-ligated DNA fragments are size-selected through AMPure beads, then the PCR amplification is performed to enrich the libraries and each library is tagged with unique dual index. Next, the library is performed with target enrichment, the process including denature the double-strand library, hybridize biotinylated probes to the complementary target DNA, and enrich the captured target DNA using streptavidin beads. Finally, the universal PCR amplification is performed to enrich the target libraries.

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

No.	Component number	Components	Main Ingredient	Quantity
1	E1	**CH-End Repair Buffer**	Tris, Mg^{2+}	105 µL/tube ×1
2	E2	**CH-End Repair Enzyme**	Klenow Enzyme	45 µL/tube ×1
3	L1	**CH-Ligation Buffer**	Tris, Mg^{2+}, ATP, DTT, Ligase	450 µL/tube ×1
4	L2	**CH-Ligation Enhancer**	Small Molecular Ligation Enhancer	15 µL/tube ×1
5	L3	**CH-Adaptor**	Oligonucleotides	28 µL/tube ×1
6	P1	**CH-PCR Buffer ①**	Tris, Mg^{2+}, dNTPs, DNA Polymerase	750 µL/tube ×1
7	501~508	**CH-D5 Primer ***	Oligonucleotides	6 µL/tube ×8
8	701~712	**CH-D7 Primer ***	Oligonucleotides	4 µL/tube ×12
9	H1	**CH-Blocking Reagent**	Oligonucleotides	84 µL/tube ×1
10	H2	**CH-Capture Probe**	Oligonucleotides	75 µL/tube ×1
11	H3	**CH-Hybridization Buffer**	Formamide, Na^{+}, Tween, Dextran Sulfate	120 µL/tube ×1
12	B1	**CH-Bead Wash Buffer**	Tris, EDTA-2Na, NaCl	1200 µL/tube ×2
13	W1	**5×Wash Buffer ①**	MES, NaCl, Tween	1056 µL/tube ×1
14	W2	**5×Wash Buffer ②**	NaCl, SDS, DTT	792 µL/tube ×1
15	W3	**5×Wash Buffer ③**	Na^{+}, DTT	528 µL/tube ×1
16	W4	**5×Wash Buffer ④**	Na^{+}, DTT	528 µL/tube ×1
17	P2	**CH-PCR Buffer ②**	Tris, Primers, Mg^{2+}, dNTPs	348 µL/tube ×1
18	P3	**CH-DNA Polymerase**	DNA Polymerase	18 µL/tube ×1
19	PC	**CH-Positive Control****	Positive DNA	100 µL/tube ×1

** For labeling and sequence information of the primers, refer to Appendix Table S3.*

*** The positive variants in the (PC) CH-Positive Control are listed in Appendix Table S4.*

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 -25℃ to -15℃.

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

Additional Reagents and Equipment Required but Not Supplied

1) PCR instrument: Applied Biosystems™ 2720 Thermal Cycler (or equivalent) 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® DNA/RNA Extraction Kit (Amoy Diagnostics), AmoyDx® Magnetic FFPE DNA Extraction Kit or MagPure FFPE DNA LQ Kit (Magen) is recommended for DNA extraction from FFPE tissue samples, It is recommended to use RNase A (Thermo Fisher Scientific, or equivalent) to degrade RNA during the FFPE DNA extraction; AmoyDx® Circulating DNA Kit (Amoy Diagnostics) or QIAamp Circulating Nucleic Acid Kit (Qiagen) is recommended for cfDNA extraction from blood plasma samples..
5) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter) or CleanNGS magnetic beads (Vdobiotech) is recommended.
6) Streptavidin coupled magnetic beads: Dynabeads MyOne™ Streptavidin T1 (Thermo Fisher Scientific) is recommended.
7) 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.
8) Sequencing instrument: Illumina NovaSeq 6000 or NextSeq 500, NextSeq 550 is recommended.
9) Sequencing reagent: Illumina 300 cycles (paired-end reads, 2×150 cycles) is recommended.
10) Illumina PhiX Control V3.
11) Vacuum concentrator: Concentrator Plus™ complete system (Eppendorf) is recommended.
12) Magnetic Stand: DynaMag™-2 Magnet (Thermo Fisher Scientific) and DynaMag™-96 Side Magnet (Thermo Fisher Scientific) are recommended.
13) Ultrasonicator: Covaris M220 Focused-ultrasonicator (Covaris) and microTUBE-130 AFA Fiber Screwcap (Covaris) are recommended.
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 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) PCR-grade 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 use any other reagents from different lots in the tests.
- DO NOT use any other reagents in the other test kits.

Safety Information

- Handle all specimens and components of the kit as potentially infectious material using safe laboratory procedures.
- Only trained professionals can use this kit. Please wear suitable lab coat and disposable gloves while handling the reagents.
- Avoid skin, eyes and mucous membranes contact with the chemicals. In case of contact, flush with water immediately.
- DO NOT pipette 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.
- 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. The work area for post-amplification operation should be separated from the area for pre-amplification.
- Gloves should be worn and changed frequently when handling samples and reagents to prevent contamination.
- Using 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 reagents, and waste must be disposed of properly.

Cleaning

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

Specimen Preparation

- The specimen material should be genomic DNA isolated from FFPE tissue samples or cfDNA isolated from blood plasma samples of cancer patients.
- The FFPE tissue sample should be fixed by 10% neutral buffered formalin. It is recommended to use the central section of paraffin blocks. The tumor cell content should be no less than 20%. It is recommended to use freshly cut sections for FFPE DNA extraction. The storage time for the FFPE tissue should be less than 2 years.
- The peripheral whole blood should be more than 10 mL. The EDTA anticoagulant is recommended during the blood collection, avoid using heparin anticoagulant. The plasma should be separate from the whole blood within 2 hours (no more than 4 hours) after blood collection. If not, it is recommended to use a commercialized cell-free DNA blood collection tube (AmoyDx or Streck) to collect the peripheral whole blood and store the tube at room temperature for no more than one week before plasma separation. If shipment is needed, the separated plasma requires shipment on frozen ice packs and the shipping time should be less than one week. The separated plasma should be used for cfDNA extraction immediately, if not, the plasma should be stored at -25℃ to -15℃ for no more than 18 months.
- It is recommended to use a commercialized DNA extraction kit to perform the DNA extraction according to the sample type. After extraction, measure the concentration of extracted DNA using Quantus™ or Qubit® Fluorometer. For FFPE samples, the DNA concentration should be no less than 1.0 ng/μL, and the DNA amount should be no less 50 ng (optimal should be no less 100 ng). For plasma samples, the cfDNA concentration should be no less 0.2 ng/μL, and the cfDNA amount should be no less 10 ng (optimal should be no less 20 ng). For unqualified samples, re-collection or re-extraction is required.
- The qualified FFPE DNA or cfDNA should be used for library preparation immediately, if not, the DNA should be stored at -25℃ to -15℃ for no more than 8 months, avoid repeated freezing and thawing.

Assay Procedure

Note:

- *It is recommended to include a CH-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 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 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 consists of FFPE DNA or cfDNA library preparation and hybridization capture .*

1. FFPE DNA or cfDNA Library preparation

For FFPE tissue samples, ultrasonic shearing or enzymatic digestion (not provided) should be used to shear the extracted genomic DNA (gDNA) into short fragments before proceeding with library preparation. For plasma samples, the extracted cfDNA can be directly used for library preparation (starting from step 1.3 End Repair) without DNA shearing.

1.1. DNA Shearing (for FFPE DNA only)

1.1.1. Ultrasonic shearing

It is recommended to use Covaris M220 Focused-ultrasonicator (Covaris) and microTUBE-130 AFA Fiber Screwcap (Covaris) for DNA shearing, and the procedure is as follows.

Add 130 μL FFPE gDNA sample (take 100 ng FFPE gDNA and add TE-low solution to a final volume of 130 μL) into the Covaris microTUBE-130. Place the tube into the Covaris M220, and perform the shearing according to Table 2.

Table 2. Parameters for DNA Shearing (Covaris M220)

Parameter	Setting Value
Duty Factor	20%
Peak Incident Power (W)	50
Cycles Burst	200
Times	180

Note*: The shearing time might be adjusted according to the sample quality.*

1.1.2. Enzymatic digestion

It is recommended to use KAPA Frag Kit for Enzymatic Fragmentation (KAPA Biosystems, Cat. No. KK8602) for DNA shearing, and the procedure is as follows.

1.1.2.1. Take 100 ng genomic DNA sample (FFPE DNA) into the PCR tube. Assemble the enzymatic fragmentation reaction mix on ice according to Table 3.

Table 3. Enzymatic Fragmentation Reaction

Reagent	Volume per Test
KAPA Frag Enzyme	10 μL
KAPA Frag Buffer	5 μL
Genomic DNA (100ng)	χ μL
PCR-grade water	35-χ μL
Total	**50 μL**

Note:

- *For FFPE samples, "χ" stands for the volume of 100 ng DNA (minimum 50 ng).*
- *The enzymatic fragmentation reaction system was sensitive to EDTA. When use enzymatic fragmentation, the extracted genomic DNA should be eluted with PCR-grade water rather than TE solution. If not, it is recommend to purify the DNA with 2× AMPure XP Beads and re-elute DNA with PCR-grade water before performing the enzymatic fragmentation.*

1.1.2.2. Mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler with the heated lid set off, and perform the following program: 4℃ for 1 min, 37℃ for 10 min, 4℃ hold.

1.1.2.3. After the enzymatic fragmentation reaction is finished, add 5 μL Stop Solution to stop the reaction immediately. Then proceed immediately to the purification step (step 1.2.2) with 110 μL resuspended AMPure XP beads.

1.2. DNA Fragment Purification (for FFPE DNA only)

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

1.2.2. For ultrasonic shearing, transfer 125 μL the above fragmented DNA product (from step 1.1.1) into a clean nuclease-free 1.5 mL centrifuge tube, add 250 μL resuspended AMPure XP beads and mix well on a vortex mixer; for enzymatic digestion, transfer 55 μL the above fragmented DNA product (from step 1.1.2.3) into a clean nuclease-free 1.5 mL centrifuge tube, add 110 μL resuspended AMPure XP beads and mix well on a vortex mixer. Then incubate for 5 min at room temperature.

1.2.3. 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.

1.2.4. Keep the tubes on the magnetic stand, add 400 μL of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for at least 30 seconds, and then carefully remove and discard the supernatant.

1.2.5. Repeat step 1.2.4 once.

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

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

1.2.7. Remove the tube from the magnet. Elute DNA target from the beads by adding 27 μL nuclease-free water (not provided), mix thoroughly by vortexing or pipetting, and incubate for 2 min at room temperature.

1.2.8. Put the tube in the magnetic stand for 3~5 min until the solution turns clear. Without disturbing the beads, carefully transfer the supernatant into a clean nuclease-free 1.5 mL centrifuge tube to obtain the fragmented DNA.

1.2.9. Quantify the fragmented DNA concentration with a recommended kit. For the Quantus™ or Qubit® Fluorometer, the DNA amount should be more than 30 ng.

***Note**: The purified fragmented DNA should be stored at -25℃ to -15℃ for no more than one week if not proceed to the next step.*

1.3. End Repair

1.3.1. Take out the following reagents and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer and centrifuge briefly, then keep the tube on ice.

1.3.2. Assemble the end repair reaction on ice in a clean nuclease-free 0.2 mL PCR tube by adding the following components according to Table 4 (The reaction should be adjusted according to the sample type).

Table 4. End Repair Reaction

Reagent	Volume for Genomic DNA/PC	Volume for cfDNA
Fragmented DNA	χ µL	χ µL
PCR-grade Water	25-χ µL	50-χ µL
(E1) CH-End Repair Buffer	3.5 µL	7 µL
(E2) CH-End Repair Enzyme	1.5 µL	3 µL
Total	**30 µL**	**60 µL**

Note:

- *For FFPE samples, "χ" stands for the volume of 30 ng fragmented DNA. If the total fragmented DNA is less than 30 ng, take 25 µL fragmented DNA for library construction.*
- *For plasma samples, "χ" stands for the volume of 30 ng cfDNA. If the total cfDNA is less than 30 ng, take 50 µL cfDNA for library construction.*
- *For PC (fragmented genomic DNA), the DNA concentration is 1.2 ng/µL, take 25 µL PC for library construction (χ=25).*
- *For No-template Control (NTC), take 25 µL PCR-grade Water for library construction (χ=0).*

1.3.3. Mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler. Perform the following program: 20°C for 30 min, 65°C for 30 min, 4°C hold.

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

1.4. Adaptor Ligation

1.4.1. Take out the following reagents and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer and centrifuge briefly, then keep the tube on ice.

1.4.2. Assemble the ligation mixture on ice by adding the following components according to Table 5 (The reaction should be adjusted according to the sample type).

Table 5. Ligation Mixture

Reagent	Volume for Genomic DNA/PC	Volume for cfDNA
(L1) CH-Ligation Buffer	15 µL	30 µL
(L2) CH-Ligation Enhancer	0.5 µL	1 µL
Total	**15.5 µL**	**31 µL**

1.4.3. Mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly, then add the ligation mixture into the End Repair Product tube (from step 1.3.3), and mix thoroughly.

1.4.4. Add appropriate volume of (L3) **CH-Adaptor** to the product tube (from step 1.4.3) according to the sample type: for FFPE DNA samples or PC, the (L3) **CH-Adaptor** should be added at the ratio of 0.25 µL per 10 ng fragmented DNA input (eg. with 30 ng fragmented DNA input, you need to add 0.75 µL (L3) CH-Adaptor); for plasma cfDNA, the (L3) **CH-Adaptor** should be adjusted according to the original cfDNA input, as shown in Table 6.

Table 6. Adaptor Dosage for Plasma cfDNA

cfDNA Input	Adaptor Volume
10 ng ≤ cfDNA ≤ 15 ng	0.83 μL
15 ng < cfDNA ≤ 20 ng	1.11 μL
20 ng < cfDNA ≤ 30 ng	1.67 μL

1.4.5. Mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler. Perform the following program with the heated lid off: 20°C for 15 min, 4°C hold.

***Note**: Perform the subsequent purification step immediately when the adaptor ligation step is finished.*

1.5. Purification after Adaptor Ligation

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

1.5.2. Transfer all of the above ligation product (from step 1.4.5) into a clean nuclease-free 1.5 mL centrifuge tube. For FFPE DNA samples or PC, add 42 μL resuspended AMPure XP beads, mix thoroughly by vortexing or pipetting, then incubate for 5 min at room temperature; for plasma cfDNA, add 83 μL resuspended AMPure XP beads, mix thoroughly by vortexing or pipetting, then incubate for 5 min at room temperature.

1.5.3. Place the centrifuge tubes onto the magnetic stand for 3~5 min until the solution turns clear. Gently remove and discard the supernatant while the centrifuge tube is on the magnetic stand. **Do not** touch the beads with pipette tip.

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

1.5.5. Repeat step 1.5.4 once.

1.5.6. Briefly spin the tube, and put the tube back in the magnetic stand. Completely remove the residual ethanol, and air dry the beads for 3~5 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.*

1.5.7. Remove the tube from the magnet. Elute DNA target from the beads by adding 23 μL nuclease-free water (not provided), mix thoroughly by vortexing or pipetting, and incubate for 2 min at room temperature.

1.5.8. Put the tube in the magnetic stand for 3~5 min until the solution turns clear. Without disturbing the beads, carefully transfer 21 μL supernatant into a clean 0.2 mL PCR tube.

1.6. Library Amplification

1.6.1. Take out the following reagents and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer and centrifuge briefly, then keep the tube on ice.

1.6.2. Assemble the PCR amplification reaction on ice in a nuclease-free 0.2 mL PCR tube by adding the following components according to Table 7.

Table 7. PCR Amplification Reaction

Reagent	Volume
Purified Ligation Product (from step 1.5.8)	21 μL
(P1) CH-PCR Buffer ①	25 μL
(501~508) CH-D5 Primer	2 μL
(701~712) CH-D7 Primer	2 μL
Total	**50 μL**

Note: *There are 8 tubes of CH-D5 Primer (CH-D501~CH-D508) and 12 tubes of CH-D7 Primer (CH-D701~CH-D712). Each of the CH-D5 Primer or CH-D7 Primer has a different index sequence. Use a different combination of CH-D5 Primer and CH-D7 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 S3.*

1.6.3. Mix the solution in each PCR tube thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler, and perform the following program according to Table 8.

Table 8. PCR Program

Temperature	Time	Cycles
98℃	45 s	1
98℃	15 s	
60℃	30 s	9 or 11
72℃	30 s	
72℃	1 min	1
4℃	∞	1

Note*:*

- *Amplification cycle number differs according to different sample type. 11 cycles for FFPE DNA (or PC DNA), and 9 cycles for plasma cfDNA is recommended.*
- *The PCR products should be stored at 2~8 ℃ for no more than 20 hours if not proceed to the next step.*

1.7. Library Purification

Note: *Each* ***library*** *should be purified individually.*

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

1.7.2. Transfer all of the above PCR product (from step 1.6.3) into a clean nuclease-free 1.5 mL centrifuge tube, then add 40 μL resuspended AMPure XP beads, mix thoroughly by vortexing or pipetting, then incubate for 5 min at room temperature.

1.7.3. 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.

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

1.7.5. Repeat step 1.7.4 once.

1.7.6. Briefly spin the tube, and put the tube back in the magnetic stand. Completely remove the residual ethanol, and air dry the beads for

2~3 min while the tube is on the magnetic stand with the lid open.

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

1.7.7. Remove the tube from the magnet. Elute DNA target from the beads by adding 32 μL nuclease-free water (not provided), mix thoroughly by vortexing or pipetting, and incubate for 2 min at room temperature.

1.7.8. Put the tube in the magnetic stand for 3~5 min until the solution turns clear. Without disturbing the beads, carefully transfer the supernatant into a clean nuclease-free 1.5 mL centrifuge tube to obtain the library product.

***Note**: The purified DNA library should be stored at -25 ℃ to -15 ℃ for no more than one week if not proceed to the next step.*

1.8. DNA Library Quality Control (QC)

Quantify the DNA library concentration with a recommended fluorescence-based method (eg. Quantus™ or Qubit® Fluorometer), the DNA concentration should be more than 30 ng/μL, and the DNA amount should be more than 900 ng. If not, the library is unqualified, the library should be reconstructed.

***Note:** For No-template Control (NTC), the library output should be less than 60 ng. Otherwise, there may be contamination during the experiment and the experiment should be repeated.*

2. Hybridization Capture

2.1. Reagent Preparation

2.1.1. Take out the following reagents 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.

2.1.2. Assemble the pre-hybridization mix on ice in a clean nuclease-free 0.2 mL PCR tube by adding the following components according to Table 9.

Table 9. Pre-hybridization Mix

Reagent	Volume
Library samples (1 ~ 6 samples)	≤ 120 μL (0.75 μg ~ 4 μg)
(H1) CH-Blocking Reagent	7 μL
Total	**≤ 127 μL**

Note:

- *It is recommended to pool 2~6 sample libraries with different index combinations in equal amounts for hybridization capture. The amount of each library should be 500 ng~1000 ng (1000 ng per library is recommended), and the total amount of the 2~6 sample libraries should be 1 ug ~ 4 ug.*
- *For a single sample library which requires individual hybridization, the amount should be 750 ng ~ 4000 ng (1000 ng is recommended).*
- *It is recommended to mix equal amounts of libraries of the same sample type in one hybridization pool. If it is necessary to mix different types of samples in one hybridization pool, the library input should be adjusted according to the data volume ratio of different libraries. Each library should have a different index combination (CH-D5 and CH-D7). **Do not** use the same*

combination of index for two or more sample libraries in one hybridization pool.

2.1.3. Mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly. Put the tubes into a vacuum concentrator with the tube lid open. Set the temperature at 60℃ and dry down the mixture. Avoid over drying.

***Note**: AMPure XP Beads can also be used for DNA concentration (optional): using AMPure XP Beads with a volume twice the sample library volume for DNA purification, 200 µL freshly prepared 80% ethanol for washing (2 times), and 10 µL* (H3) *CH-Hybridization Buffer for final DNA elution. Transfer all DNA eluate to a clean nuclease-free 0.2 mL PCR tube and proceed to step 2.2.3.*

2.2. Hybridization

2.2.1. Take out the (H3) CH-Hybridization Buffer and (H2) CH-Capture Probe, 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.

2.2.2. Carefully remove the sample tubes from the vacuum concentrator, then add 10 µL (H3) CH-Hybridization Buffer into each sample tube and cap the tubes, vortex to mix well, then centrifuge briefly.

2.2.3. Add 5 µL (H2) CH-Capture Probe into each sample tube, mix well on a vortex mixer and centrifuge briefly. Place the tube on a thermocycler with the heated lid set to 105°C and perform the following program: 95°C for 10 min, 48°C for 12~20 hours (16 hours is recommended), 48°C hold.

2.3. Capture

2.3.1. Take out the Dynabeads MyOne™ Streptavidin T1 Magnetic Beads and equilibrate them to room temperature for 30 min. Shake the bottle of the beads to resuspend any magnetic particles that may have settled.

2.3.2. Aliquot 40 µL of streptavidin beads per capture into a clean nuclease-free 1.5 mL **low-binding** centrifuge tube (For example, for 1 capture, prepare 40 µL of streptavidin beads, and for 2 capture, prepare 80 µL of streptavidin beads). Then add the same volume of (B1) CH-Bead Wash Buffer per capture, mix well by gently pipetting up and down for 10~20 times.

2.3.3. Place the centrifuge tube onto the magnetic stand for 1 min until the solution turns clear.

2.3.4. Gently remove and discard the supernatant while the centrifuge tube is on the magnetic stand. **Do not** touch the beads with pipette tip. Add (B1) CH-Bead Wash Buffer with a volume twice the the original streptavidin beads volume, mix well by pipetting up and down for 10~20 times.

2.3.5. Place the centrifuge tube onto the magnetic stand for 1 min until the solution turns clear.

2.3.6. Repeat step 2.3.4 once.

2.3.7. Aliquot 80 µL of resuspended beads into a clean nuclease-free 0.2 mL PCR tube for each capture reaction. Place the PCR tube onto the magnetic stand (DynaMag™-96 Side Magnet is recommended) for 1 min until the solution turns clear.

2.3.8. Gently remove and discard the supernatant while the tube is on the magnetic stand. **Do not** touch the beads with pipette tip. When the hybridization program (step 2.2.3) is finished, quickly transfer all of the hybridization product (~ 15 µL, from Step 2.2.3) into the 0.2 mL tubes with beads.

2.3.9. Mix well by gently pipetting up and down for 10~20 times, then place the tube on a thermocycler and perform the following program: 48°C for 45 min, 48°C hold. Set the timer to 45 min, during the incubation, repeat the following steps every 15 min:Remove the tube

from the thermalcycler and vortex gently to ensure that the sample is completely resuspended, and then quickly put it back in the thermalcycler for incubation. (This process requires rapid operation to avoid the temperature drop of the hybridization solution)

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

2.4. Washing

2.4.1. Turn on the water bath or heating block in advance and set the temperature at 48°C. Take out the 5× Wash Buffer ①~④（W1-W4）and thaw the reagents at room temperature. When the reagents are completely thawed, shake the tubes to mix well (all the wash buffers should be transparent). Dilute the following buffers to create the 1× working solutions according to Table 10.

Table 10. 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

2.4.2. After dilution, take sufficient of 1×Wash Buffer ① (440 μL for each capture reaction) and 1×Wash Buffer ② (330 μL for each capture reaction), and heat the tubes to 48°C for at least 10 min in a water bath or heating block. The 1×Wash Buffer ③ and the 1×Wash Buffer ④ should be kept at room temperature.

2.4.3. When the step 2.3.9 is finished, add 100 μL preheated 1×Wash Buffer ② to each sample tube, mix well by pipetting up and down for 10 times, transfer all of the solution into a clean 1.5 mL **low-binding** centrifuge tube. Centrifuge briefly and place the tubes onto the magnetic stand for 10 seconds until the solution turns clear.

2.4.4. Remove and discard the supernatant carefully, do not touch the beads. Remove the tube from the magnetic stand, add 200 μL preheated 1×Wash Buffer ①, mix well by pipetting up and down for 10 times, then incubate the tubes at 48°C for 5 min. Then centrifuge briefly and place the tubes onto the magnetic stand for 10 seconds until the solution turns clear.

2.4.5. Repeat step 2.4.4 once.

2.4.6. Remove and discard the supernatant carefully, do not touch the beads. Remove the tube from the magnetic stand, add 200 μL preheated 1×Wash Buffer ② , mix well by pipetting up and down for 10 times, then incubate the tubes at 48°C for 5 min. Then centrifuge briefly and place the tubes onto the magnetic stand for 10 seconds until the solution turns clear.

2.4.7. Remove and discard the supernatant carefully, do not touch the beads. Remove the tube from the magnetic stand, add 200 μL 1×Wash Buffer ③, vortex for 1min to mix well. Then centrifuge briefly and place the tubes onto the magnetic stand for 10 seconds until the solution turns clear.

2.4.8. Remove and discard the supernatant carefully, do not touch the beads. Remove the tube from the magnetic stand, add 200 μL 1×Wash Buffer ④, vortex for 30 seconds to mix well. Then centrifuge briefly and place the tubes onto the magnetic stand for 30 seconds until the solution turns clear.

2.4.9. Remove and discard the supernatant carefully, do not touch the beads. Remove the tube from the magnetic stand, add 40 μL

nuclease-free water (not provided), vortex to mix well, then centrifuge briefly. (**Do not** discard the beads)

Note: *The captured products should be stored at 2~8*℃ *for no more than one week if not proceeding to the next step.*

2.5. Post-hybridization PCR Amplification

2.5.1. Take out the (P2) CH-PCR Buffer ② and thaw at room temperature. When the reagents completely thawed, vortex to mix well and centrifuge briefly, then keep the tube on ice. Take out the (P3) CH-DNA Polymerase, centrifuge briefly, then keep the tube on ice.

2.5.2. Assemble the post-hybridization PCR amplification reaction on ice in a clean nuclease-free 0.2 mL PCR tube by adding the following components according to Table 11.

Table 11. Post-hybridization PCR Amplification Reaction

Reagent	**Volume**
(P2) CH-PCR Buffer ②	29 μL
(P3) CH-DNA Polymerase	1 μL
Capture Product with Beads (from step 2.4.9)	20 μL
Total	**50 μL**

2.5.3. Mix the solution in each PCR tube thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler, and perform the following program according to Table 12.

Table 12. Post-hybridization PCR Program

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

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

2.6. Purification after Amplification

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

2.6.2. Transfer 50 μL of the PCR product (from step 2.5.3) into a clean nuclease-free 1.5 mL centrifuge tube, then add 50 μL resuspended AMPure XP beads, mix thoroughly by vortexing or pipetting, then incubate for 5 min at room temperature.

2.6.3. 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.

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

2.6.5. Repeat step 2.6.4 once.

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

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

2.6.7. Remove the tube from the magnet. Elute DNA target from the beads by adding 32 μL TE-low solution (10 mM Tris, 0.1 mM EDTA, pH 8.0) (not provided), mix thoroughly by vortexing or pipetting, and incubate for 2 min at room temperature.

2.6.8. Put the tube in the magnetic stand for 5 min until the solution turns clear. Without disturbing the bead pellet, transfer the supernatant into a clean nuclease-free 1.5 mL centrifuge tube to obtain the captured library product.

Note*: The purified DNA library should be stored at -25 ℃ to -15 ℃ for no more than one week if not proceed to the next step.*

2.7. Captured Library QC

2.7.1. Library concentration QC: Quantify the library concentration with Quantus™ or Qubit® Fluorometer, the DNA concentration should be more than 2.5 ng/μL, and the DNA amount should be more than 75 ng.

2.7.2. Library fragment size QC: Assess the library quality with a recommended capillary electrophoresis analyzer and related kit. The peak size of the library fragment should be at ~380 bp for FFPE DNA/PC library and at ~330 bp for plasma cfDNA library, without obvious peaks of small and big fragments, as shown in Figure 1 and Figure 2.

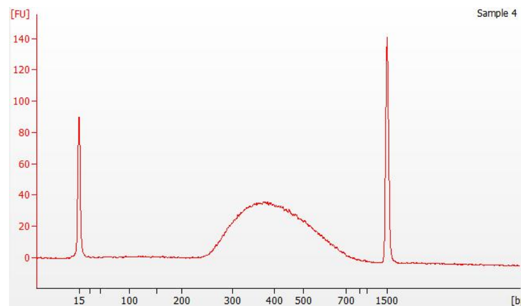

Figure 1. Example of FFPE DNA library size distribution on Agilent 2100 Bioanalyzer

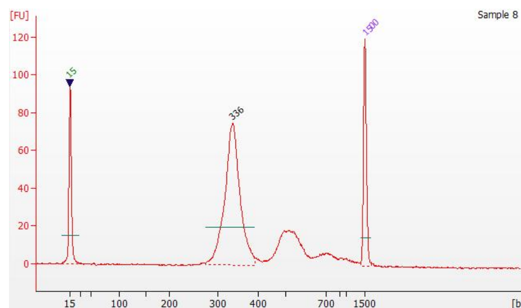

Figure 2. Example of plasma cfDNA library size distribution on Agilent 2100 Bioanalyzer

Note:

- *The library distribution shown in the figures 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.*
- *If the library QC pass, then move to sequencing. If not, the library is unqualified, the library should be reconstructed.*
- *If the NTC library detects the target length fragment, there may be contamination during the experiment process, the test is unqualified and the test 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 FFPE DNA or PC, the sequencing data per sample should be no less than 1.5 Gb; for plasma cfDNA, the sequencing data per sample should be no less than 8.0 Gb. The suggested sample quantity per run is listed in Table 13.

Table 13. Recommended Sequencing Instruments and Sample Quantity per Run

Sequencer	**Flow Cell**	**Read Length**	**Sample Quantity/Run**	
			FFPE DNA or PC (1.5 Gb/sample)	**Plasma cfDNA (8 Gb/sample)**
NextSeq500/550	Mid output	2×150 bp	26	5
	High output	2×150 bp	80	15
NovaSeq 6000	SP	2×150 bp	Up to 96*	31
	S1	2×150 bp	Up to 96*	62

** As there are 8 tubes of CH-D5 Primer (CH-D501~CH-D508) and 12 tubes of CH-D7 Primer (CH-D701~CH-D712), the maximum combinations of index is 96 kinds when using this kit, so in order to ensure that each sample has a unique index combination, the maximum number of the loading samples is 96 samples (FFPE DNA library) per flow cell on NovaSeq 6000.*

Perform the denaturation and dilution of the libraries according to the instrument's instructions. The final concentration of sequencing library is recommended in Table 14.

Table 14. Recommended Final Concentration of Sequencing Library

Sequencing Instrument	**Final Concentration**
NextSeq 500/550	0.8~1.3 pM
NovaSeq 6000	1~1.3 nM

4. Data Analysis

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

Check Q30 value for the sequencing data:

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

Select the analysis module:

If the Q30 value is qualified, select the "ADXPAN116" analysis module and choose the sample type (Tissue or Plasma) for data analysis.

Result Interpretation

The qualified criteria for data QC is shown in Table 15.

Table 15. Qualified and Risky Criteria for Data QC

Sample Type	**Parameters**	**Qualified**	**Risky**
Tissue DNA/PC	Coverage	≥ 95%	/
	CoverageRatioUNIQ180	≥ 95%	85%~95%
	CNV-CV and CNV-UNI	CNV-CV < 0.4 and CNV-UNI < 1.5	/
Plasma cfDNA/PC	Coverage	≥ 95%	/
	CoverageRatioUNIQ1550	≥ 90%	85%~90%

***Note**:*

- *Coverage: The proportion of the sequencing data mapped region to the designed target region.*
- *CoverageRatioUNIQ180: The proportion of hotspot regions with coverage depth ≥ 180×.*
- *CoverageRatioUNIQ1550: The proportion of hotspot regions with coverage depth ≥ 1550×.*
- *CNV-CV: The coefficient of Variation (CV) of the intra-gene normalized depth.*
- *CNV-UNI: The uniformity of the inter-gene normalized depth.*
- *The parameters CNV-CV and CNV-UNI are only applicable to the quality control of CNV testing.*
- *If the results of data QC are at risk level, it is recommended to re-extract and re-test the samples. The risky data can only be used for analysis when the remaining samples are not enough, but there may be risks of missing mutations, affecting the limit of detection or decreasing the accuracy of CNV detection. In this case, all the positive variants detected should be checked manually and indicated in the test report.*

The mutations are detected if meeting the following requirements.

The Cut-off metrics are shown in Table 16.

Table 16. Cut-Off Metrics

Alteration Type	**Filtering Thresholds**	
	FFPE DNA Library	**Plasma cfDNA Library**
SNVs/InDels	Hotspots: Freq_SS ≥ 1%, Var_US ≥ 5, Var_SS ≥ 2 Non-hotspots: Freq_SS ≥ 1%, Var_SS ≥ 8, Var_DS ≥ 3	Hotspots: Freq_SS ≥ 0.17%, Var_SS ≥ 3 Non-hotspots: Freq_SS ≥ 0.3%, Var_SS ≥ 8, Var_DS ≥ 4
Fusions	Hotspots: ssbcAD ≥ 14 Non-hotspots: ssbcAD ≥ 16	Hotspots: ssbcAD ≥ 14 Non-hotspots: ssbcAD ≥ 16
CNVs	Amplification ≥ 3.5 copy number	NA (out of detection)
MSI	MSINum ≥ 15%	NA (out of detection)

***Note**:*

- *Freq_SS: Frequency of mutant allele, after single strand base calibration.*
- *Var_US: The number of variant reads, after de duplication calibration.*
- *Var_DS: The number of variant reads, after double strand base calibration.*
- *Var_SS: The number of variant reads, after single strand base calibration.*
- *ssbcAD: The number of fusion reads, after single strand base calibration.*
- *MSINum: Percentage of microsatellite instability sites.*
- *The PC should be detected as positive results for the corresponding mutations as shown in Table S4. Otherwise, the testing is unqualified, it is necessary to check if there is any operational error and the experiment should be repeated.*

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 and plasma samples.
3) Reliable results are dependent on proper sample processing, transport, and storage. Improper sample processing, transport and storage may lead to false negative or false positive results.

4) Negative results can not completely exclude the existence of mutated genes. Low tumor cell content, severe DNA degradation or the frequency under the limit of detection may also cause a false negative result.
5) Different parts of the tumor tissue or different sampling times may cause different mutation results due to tumor heterogeneity.
6) This kit only detects SNVs, InDels, fusions, CNVs, SNPs and MSI in the target region (as shown in Table S1 and Table S2). If the detection result is negative, other variants out of the target regions of these genes cannot be excluded.
7) False positive may occur if the fusions happen in highly repetitive regions.

References

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Symbols

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. Gene Lists for the Detection of SNV/InDel/Fusion/CNV

No.	Gene	Transcript	Alteration Type	Target Region	Remark
1	*AKT1*	NM_001014431	SNV, InDel	Exon3,4	#
2	*AKT2*	NM_001626	SNV, InDel, CNV	Exon2,4,6,8,10,12,14	#
3	*AKT3*	NM_005465	SNV, InDel, CNV	Exon2,4,6,8,10,12,14	#
4	*ALK*	NM_004304	SNV, InDel, Fusion	Exon18-28; IVS18,19. Fusion partner gene coverage: EML4 (NM_019063) Exon6,13,20; IVS6,13,20	
5	*APC*	NM_000038	SNV, InDel	Exon16	#
6	*AR*	NM_000044	SNV, InDel	Exon4,5,8	#
7	*ARAF*	NM_001654	SNV, InDel	Exon7,14	#
8	*ARID1A*	NM_006015	SNV, InDel	Exon1-20	
9	*ATM*	NM_000051	SNV, InDel	Exon17,47,59,63	#
10	*ATR*	NM_001184	SNV, InDel	Exon10	#
11	*AURKA*	NM_003600	SNV, InDel, CNV	Exon2-9	#
12	*BAP1*	NM_004656	SNV, InDel	Whole CDS	
13	*BCL2L11*	NM_138621	SNV, InDel	Exon2,3,4, IVS2	
14	*BRAF*	NM_004333	SNV, InDel, Fusion	Exon8,9,11,15; IVS8	
15	*BRCA1*	NM_007294	SNV, InDel	Whole CDS	
16	*BRCA2*	NM_000059	SNV, InDel	Whole CDS	
17	*CCND1*	NM_053056	SNV, InDel, CNV	Exon1-5	#
18	*CCNE1*	NM_001238	SNV, InDel, CNV	Exon2,4,6,8,10,12	#
19	*CD274*	NM_014143	SNV, InDel, CNV	Exon2-7	#
20	*CDK12*	NM_016507	SNV, InDel	Exon1-14	
21	*CDK4*	NM_000075	SNV, InDel, CNV	Exon2-8	#
22	*CDK6*	NM_001145306	SNV, InDel, CNV	Exon2-8	#
23	*CDKN2A*	NM_000077	SNV, InDel	Exon1-3	#
24	*CDKN2B*	NM_004936	SNV, InDel	Exon1-2	
25	*CREBBP*	NM_004380	SNV, InDel	Exon1-31	
26	*CTNNB1*	NM_001904	SNV, InDel	Exon3	
27	*DDR2*	NM_006182	SNV, InDel	Exon5,8,13-18	
28	*EGFR*	NM_005228	SNV, InDel, CNV	Exon1-21	
29	*EIF1AX*	NM_001412	SNV, InDel	Exon1-7	#
30	*EPAS1*	NM_001430	SNV, InDel, CNV	Exon1,3,5,7,9,11,13,15	#
31	*EPCAM*	NM_002354	SNV, InDel	Whole CDS	Lynch syndrome related genes
32	*ERBB2*	NM_004448	SNV, InDel, CNV	Exon1-27	#
33	*ERBB3*	NM_001982	SNV, InDel	Exon18-24	
34	*ERBB4*	NM_005235	SNV, InDel	Exon18-24	
35	*ESR1*	NM_001122740	SNV, InDel	Exon6,8,9	#
36	*ETS2*	NM_005239	SNV;InDel	Exon8	#
37	*FANCA*	NM_000135	SNV, InDel	Exon33	#
38	*FBXW7*	NM_033632	SNV, InDel	Whole CDS	
39	*FGF19*	NM_005117	SNV, InDel, CNV	Whole CDS,IVS2	

No.	Gene	Transcript	Alteration Type	Target Region	Remark
40	*FGF3*	NM_005247	SNV, InDel, CNV	Exon1-3,IVS2	#
41	*FGFR1*	NM_023110	SNV, InDel, CNV	Exon3,5,7,9,11,13,15,17,18	
42	*FGFR2*	NM_000141	SNV, InDel, Fusion, CNV	Exon2,4,6,8,10,12,14,16-18; IVS17; 3' UTR	#
43	*FGFR3*	NM_000142	SNV, InDel, Fusion, CNV	Whole CDS; IVS17; 3' UTR	
44	*FGFR4*	NM_213647	SNV, InDel	Whole CDS	
45	*FLCN*	NM_144997	SNV, InDel	Exon4-14	
46	*FLT3*	NM_004119	SNV, InDel	Exon6	
47	*GNAS*	NM_000516	SNV, InDel	Exon1,8,9	#
48	*HIF1A*	NM_001530	SNV, InDel, CNV	Exon1,3,5,7,9,11,13,15	#
49	*HRAS*	NM_001130442	SNV, InDel	Exon2,3	#
50	*IDH1*	NM_005896	SNV, InDel	Exon4	#
51	*IDH2*	NM_002168	SNV, InDel	Exon4	#
52	*IGF1R*	NM_000875	SNV, InDel, CNV	Exon1,3,5,7,9,11,13,15,17,19,21	#
53	*JAK1*	NM_002227	SNV, InDel	Exon12-25	
54	*JAK2*	NM_004972	SNV, InDel	Exon12-25	
55	*JAK3*	NM_000215	SNV, InDel	Exon11-24	
56	*KDM5C*	NM_004187	SNV, InDel	Whole CDS	
57	*KDR*	NM_002253	SNV, InDel	Exon21	#
58	*KIT*	NM_000222	SNV, InDel	Exon9-14,17	
59	*KRAS*	NM_033360	SNV, InDel	Exon2,3,4	
60	*MAP2K1*	NM_002755	SNV, InDel	Exon1,2,6	#
61	*MAPK1*	NM_002745	SNV, InDel, CNV	Exon1-8	#
62	*MET*	NM_000245	SNV, InDel, CNV	Whole CDS; IVS13-14	
63	*MLH1*	NM_000249	SNV, InDel	Whole CDS	Lynch syndrome related genes
64	*MRE11*	NM_005591	SNV, InDel	Exon14	
65	*MSH2*	NM_000251	SNV, InDel	Whole CDS	Lynch syndrome related genes
66	*MSH6*	NM_000179	SNV, InDel	Whole CDS	Lynch syndrome related genes
67	*MTOR*	NM_004958	SNV, InDel	Exon29-58	#
68	*MYC*	NM_002467	SNV, InDel, CNV	Exon1-3	#
69	*NF1*	NM_001042492	SNV, InDel	Whole CDS	
70	*NF2*	NM_000268	SNV, InDel	Whole CDS	
71	*NOTCH1*	NM_017617	SNV, InDel	Exon26,34	
72	*NRAS*	NM_002524	SNV, InDel	Exon2,3	
73	*NRG1*	NM_013964	Fusion	Fusion partner gene coverage: CD74 (NM_001025159) Exon7,8,9; IVS6,7,8; SDC4 (NM_002999) Exon2,3; IVS2.	
74	*NTRK1*	NM_002529	SNV, InDel, Fusion	Exon8,9,10,13; IVS8,9	
75	*NTRK2*	NM_006180	SNV, InDel, Fusion	Exon12,13,15,16; IVS12,15	
76	*NTRK3*	NM_002530	SNV, InDel, Fusion, CNV	Exon3,5,7,9,11,13,15,17,19. Fusion partner gene coverage: ETV6 (NM_001987) Exon4,5,6; IVS4,5	#
77	*PALB2*	NM_024675	SNV, InDel	Whole CDS	
78	*PAX8*	NM_003466	SNV, InDel, Fusion	Exon8,9,10,11; IVS8,10	

No.	Gene	Transcript	Alteration Type	Target Region	Remark
79	*PDCD1*	NM_005018	SNV, InDel	Exon1-5	#
80	*PDGFRA*	NM_006206	SNV, InDel, CNV	Exon2,4,6,8,10,12-14,16,18,19,21,23	#
81	*PGR*	NM_000926	SNV, InDel, CNV	Exon1-8	#
82	*PIK3CA*	NM_006218	SNV, InDel, CNV	Whole CDS	
83	*PIK3R1*	NM_181523	SNV, InDel	Exon9-16	
84	*PMS2*	NM_000535	SNV, InDel	Whole CDS	Lynch syndrome related genes
85	*POLD1*	NM_002691	SNV, InDel	Exon7,8,9,23	
86	*POLE*	NM_006231	SNV, InDel	Exon1-14	
87	*PSMD4*	NM_002810	SNV, InDel, CNV	Exon1,3,5,7,9	#
88	*PTCH1*	NM_000264	SNV, InDel	Exon1,3,14,15	#
89	*PTEN*	NM_000314	SNV, InDel	Whole CDS	
90	*RAF1*	NM_002880	SNV, InDel	Exon7,10-17	
91	*RASA1*	NM_002890	SNV, InDel	Exon1-25	
92	*RASAL1*	NM_004658	SNV, InDel	Exon11-19	
93	*RB1*	NM_000321	SNV, InDel	Whole CDS	
94	*RET*	NM_020975	SNV, InDel, Fusion, CNV	Exon6,7,8,10-18; IVS6,7,10,11	
95	*RICTOR*	NM_152756	SNV, InDel, CNV	Exon1,3,5,7,9,11,13,15,17,19,21,23,25,27,29 ,31,33,35,37	#
96	*RIT1*	NM_006912	SNV, InDel	Whole CDS	
97	*ROS1*	NM_002944	SNV, InDel, Fusion	Exon31-42; IVS31,33,34,35. Fusion partner gene coverage: CD74 (NM_001025159) Exon7-9; IVS6,7,8; SLC34A2 (NM_006424) Exon4,14; IVS4; SDC4 (NM_002999) Exon2,3; IVS2.	
98	*RSF1*	NM_016578	SNV, InDel, CNV	Exon1,3,5,7,9,11,13,15	#
99	*SF3B1*	NM_012433	SNV, InDel	Exon14,15	
100	*SMAD4*	NM_005359	SNV, InDel	Whole CDS	
101	*SMARCA4*	NM_003072	SNV, InDel	Whole CDS	
102	*SMO*	NM_005631	SNV, InDel, CNV	Whole CDS	
103	*STK11*	NM_000455	SNV, InDel	Whole CDS	
104	*TERT*	NM_198253	SNV, InDel	Promoter region	#
105	*TOP2A*	NM_001067	SNV, InDel, CNV	Exon1,3,5,7,9,11,13,15,17,19,21,23,25,27,29 ,31,33,35	#
106	*TP53*	NM_000546	SNV, InDel	Whole CDS	
107	*TSC1*	NM_000368	SNV, InDel	Whole CDS	
108	*TSC2*	NM_000548	SNV, InDel	Whole CDS	
109	*TSHR*	NM_000369	SNV, InDel	Exon10	#
110	*VHL*	NM_000551	SNV, InDel	Whole CDS	

Note: The genes marked with # indicate that the exons listed in these genes are not completely covered, but only the hotspots in the exons.

Table S2. Gene Lists for the Detection of Polymorphisms (19 genes)

NO.	Gene	SNP ID	NO.	Gene	SNP ID
1	*ABCB1*	rs1045642	23	*DPYD*	rs80081766
2	*ABCB1*	rs2032582	24	*DPYD*	rs2297595
3	*C8orf34*	rs1517114	25	*DPYD*	rs61622928
4	*CDA*	rs2072671	26	*DPYD*	rs17376848
5	*CDA*	rs60369023	27	*DPYD*	rs3918290
6	*CYP19A1*	rs4646	28	*DYNC2H1*	rs716274
7	*CYP2D6*	rs1065852	29	*ERCC1*	rs11615
8	*DPYD*	rs72549303	30	*GSTP1*	rs1695
9	*DPYD*	rs1801158	31	*MTHFR*	rs1801133
10	*DPYD*	rs1801159	32	*MTRR*	rs1801394
11	*DPYD*	rs1801160	33	*SEMA3C*	rs7779029
12	*DPYD*	rs72549309	34	*SLC28A3*	rs885004
13	*DPYD*	rs1801266	35	*SLC28A3*	rs7853758
14	*DPYD*	rs1801265	36	*SOD2*	rs4880
15	*DPYD*	rs1801267	37	*TP53*	rs1042522
16	*DPYD*	rs1801268	38	*UGT1A1*	rs10929302
17	*DPYD*	rs72549306	39	*UGT1A1*	rs4148323
18	*DPYD*	rs78060119	40	*UGT1A1*	rs8175347
19	*DPYD*	rs55886062	41	*UMPS*	rs1801019
20	*DPYD*	rs75017182	42	*XPC*	rs2228001
21	*DPYD*	rs67376798	43	*XRCC1*	rs25487
22	*DPYD*	rs115232898	44	*XRCC1*	rs1799782

Table S3. Index Sequence Information for Primers

Name	Sample Sheet Index Information (NextSeq 500/550)	Corresponding No. in TruSeq HT Sample Prep Kits
CH-D701	ATTACTCG	D701
CH-D702	TCCGGAGA	D702
CH-D703	CGCTCATT	D703
CH-D704	GAGATTCC	D704
CH-D705	ATTCAGAA	D705
CH-D706	GAATTCGT	D706
CH-D707	CTGAAGCT	D707
CH-D708	TAATGCGC	D708
CH-D709	CGGCTATG	D709
CH-D710	TCCGCGAA	D710
CH-D711	TCTCGCGC	D711
CH-D712	AGCGATAG	D712
CH-D501	AGGCTATA	D501
CH-D502	GCCTCTAT	D502
CH-D503	AGGATAGG	D503
CH-D504	TCAGAGCC	D504
CH-D505	CTTCGCCT	D505
CH-D506	TAAGATTA	D506
CH-D507	ACGTCCTG	D507
CH-D508	GTCAGTAC	D508

Table S4. Positive Variants in CH-Positive Control (PC)

No.	Gene	Alteration Type	CDS change
1	*APC*	SNV	NM_000038:exon16:c.4012C>T:p.(Q1338*):p.(Gln1338Ter)
2	*EGFR*	SNV	NM_005228:exon20:c.2369C>T:p.(T790M):p.(Thr790Met)
3	*EGFR*	SNV	NM_005228:exon21:c.2573T>G:p.(L858R):p.(Leu858Arg)
4	*KRAS*	SNV	NM_033360:exon2:c.35G>T:p.(G12V):p.(Gly12Val)
5	*MET*	MET exon 14 skipping	NM_000245:intron14:c.3028+1G>T:p.?:p.?
6	*TP53*	SNV	NM_000546:exon8:c.916C>T:p.(R306*):p.(Arg306Ter)
7	*TP53*	SNV	NM_000546:exon8:c.818G>A:p.(R273H):p.(Arg273His)
8	*TP53*	SNV	NM_000546:exon6:c.638G>A:p.(R213Q):p.(Arg213Gln)
9	*ROS1*	Fusion	GOPC:NM_020399_exon8--ROS1:NM_002944_exon35
10	*MET*	CNV	MET Amplification
11	*MYC*	CNV	MYC Amplification

Note:

- *The detection of CNV is available for FFPE tissue sample only, not for plasma sample.*
- *For the quality control of PC, all the variants listed in the above table must be detected, otherwise, the experiment is unqualified.*
- *Please note that there are two additional positive variants in PC, one is a SNV in PIK3CA (NM_006218: exon3: c.353G>A:p.(G118D):p.(Gly118Asp)), and the other is a SNV in TERT (NM_198253:FlankingRegion5:c.-124C>T:p.?:p.?), but these two variants are not necessary for quality control. These two variants will be detected under normal circumstances, but occasionally they may be missed.*