AmoyDx® OncoPro Liquid NGS Panel

Instructions for Use

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

REF	8.06.0150	24 reactions/kit	For Illumina NovaSeq 6000, NextSeq 500, NextSeq 550 and NextSeq 550Dx (RUO mode)

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Background

Invasive techniques for sampling and diagnosing have been progressively replaced by non-invasive methods over the past decade due to their innate limitations. Non-invasive diagnostic method that based on cell-free DNA (cfDNA) or plasma circulating tumor DNA (ctDNA) (so-called “liquid biopsy”) can be successfully used as biomarkers for diagnosis and monitoring of treatment response. For instance, lung cancer, the most fatal cancer globally, can be assessed using plasma ctDNA testing. Guidelines for patients with metastatic non-small cell lung cancer (NSCLC) suggest that plasma ctDNA testing is a useful minimally invasive method to identify ALK, BRAF, EGFR, HER2, MET exon 14 skipping, RET, ROS1, and other oncogenic biomarkers. Currently, tumour biopsy specimens represent the gold-standard biological tissue to identify those efficient biomarkers identified that allows precise therapeutic guidance. However, technical feasibility, tumour heterogeneity and cancer evolution are major limitations of this single-snapshot approach. Genotyping circulating tumour DNA (ctDNA) has been addressed as potentially overcoming such limitations.

NGS coupled with liquid biopsy samples enables the detection of homologous recombination repair (HRR) genes, key HRR genes include *AKT1, AR, ATM, ATR, BARD1, BRCA1, BRCA2, BRIP1, CDK12, CHEK1, CHEK2, FANCA, FANCL, HDAC2, MLH1, MRE11, NBN, PALB2, PTEN, RAD51B, RAD51C, RAD51D* and *RAD54L*. HRR pathways detects with these genes are particularly associated with breast, ovarian, and prostate cancers that are characterized by high variability in outcomes. There is therefore an urgent need to use new tools to enable improving risk stratification as well as select the most effective treatment that maximize cure and extend life expectancy. Meanwhile, efficacy appears to be driven by the cohort of tumors with at least one alteration in *BRCA2, BRCA1, or ATM*, and in particular, by tumors with *BRCA2* or *BRCA1* mutations based on exploratory gene-by-gene analysis. There may be heterogeneity of response to olaparib for non-BRCA mutations based on which gene has a the specific gene mutation.

The low allelic fractions in liquid biopsy samples can be challenging to detect accurately due to the background noise from normal DNA for copy number variations (CNVs). Also, differentiating true microsatellite instability (MSI) events from sequencing errors and other artifacts is crucial for accurate detection by liquid biopsy samples. However, by breaking such challenges, liquid biopsy-based NGS for CNV and MSI detection enables real-time monitoring of treatment response in cancer patients. Changes in CNVs and MSI detected in ctDNA over time can indicate response or resistance to medication, guiding medical decisions and allowing for timely adjustments.

Overall, the current use of liquid biopsy samples in NGS holds promise for improving cancer detection, medical selection, and monitoring, with ongoing research and validation efforts expected to further enhance its utility and adoption..[1-9]

Intended Use

The AmoyDx® OncoPro Liquid NGS 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, copy number variations (CNVs) in 152 genes (see Table S1), as well as tumor MSI status. The assay allows the detection of SNVs, InDels, fusions, CNVs and MSI 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. 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 uses biotinylated oligonucleotide baits (probes) to hybridize to the target regions. The test kit is designed for use with 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.

The test kit includes 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 unique molecular identifiers (UMI), then PCR amplification is performed to enrich the libraries and to label each library with unique dual indexes. Next, the library is performed with 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	**OCB-End Repair Buffer**	Tris, Mg^{2+}	72 µL/tube ×1
2	E2	**OCB-End Repair Enzyme Mix**	Klenow Enzyme	36 µL/tube ×1
3	L1	**OCB-Ligation 1 Buffer**	Tris, Mg^{2+}, ATP, DTT	300 µL/tube ×1
4	L2	**OCB-Ligation 1 Adapter**	Oligonucleotides	24 µL/tube ×1
5	L3	**OCB-Ligation 1 Enzyme**	Ligase	36 µL/tube ×1
6	L4	**OCB-Ligation 2 Buffer**	Tris, Mg^{2+}, ATP, DTT	54 µL/tube ×1
7	L5	**OCB-Ligation 2 Adapter**	Oligonucleotides	48 µL/tube ×1
8	L6	**OCB-Ligation 2 Enzyme A**	Ligase	6 µL/tube ×1
9	L7	**OCB-Ligation 2 Enzyme B**	Ligase	12 µL/tube ×1
10	L8	**OCB-Purification Buffer**	PEG/NaCl	1200 µL/tube ×1
11	P1	**OCB-Amplification Buffer ①**	Tris, Mg^{2+} , dNTPs, DNA Polymerase	600 µL/tube ×1
12	501~512	**OCB-C501-C512**	Oligonucleotides	4 µL/tube ×12
13	701~712	**OCB-C701-C712**	Oligonucleotides	4 µL/tube ×12
14	H1	**OCB-Blocker**	Oligonucleotides	84 µL/tube ×1
15	H2	**OCB-Probe**	Oligonucleotides	60 µL/tube ×1
16	H3	**OCB-Hyb Buffer**	Formamide, Na^+, Tween, Dextran Sulfate	120 µL/tube ×1
17	B1	**OCB-Beads Wash Buffer**	Tris, EDTA-2Na, NaCl	1500 µL/tube ×1
18	W1	**OCB-5×Wash Buffer ①**	MES, NaCl, Tween	1056 µL/tube ×1

19	W2	**OCB-5×Wash Buffer ②**	NaCl, SDS, DTT	792 µL/tube ×1
20	W3	**OCB-5×Wash Buffer ③**	Na^+, DTT	528 µL/tube ×1
21	W4	**OCB-5×Wash Buffer ④**	Na^+, DTT	528 µL/tube ×1
22	P2	**OCB-Amplification Buffer ②**	Tris, Primers, Mg^{2+}, dNTPs	348 µL/tube ×1
23	P3	**OCB-Polymerase**	DNA Polymerase	12 µL/tube ×1
24	PC	**OCB-Positive Control**	Fragmented DNA	200 µL/tube ×1
25	NC	**OCB-Negative Control**	Fragmented DNA	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 cold-chain shipment, and the shipping time should be less than one week and shipping temperature should be no more than -15℃. All contents of the kit should be stored immediately upon receipt at -15℃ to -25℃.

The shelf-life of the kit is twelve months. It is recommended to freeze-thaw for no more than five cycles.

Materials Required but Not Supplied

1) Cell-free DNA Protection Vacuum Tube (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) for cfDNA extraction from blood plasma samples.
6) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter) or CleanNGS magnetic beads (Vdobiotech).
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 or NextSeq 500, NextSeq 550 or NextSeq 550Dx (RUO mode).
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).
13) Magnetic Stand: DynaMag™-2 Magnet (Thermo Fisher Scientific) and DynaMag™-96 Side Magnet (Thermo Fisher Scientific).
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 (Axygen).

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 (Axygen).

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 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.

Decontamination and Disposal

- The kit contains positive control, strictly distinguish the positive control from other reagents to avoid contamination which may cause inaccurate results.
- PCR amplification is extremely sensitive to cross-contamination. The flow of tubes, racks, pipettes and other materials used should be from pre-amplification to post-amplification, and never backwards. The working 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 cfDNA isolated from plasma derived from anti-coagulated peripheral whole blood specimens.
- The peripheral whole blood should be no less 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 (in total should be no less than 10 mL) and store the tube at room temperature for less than one week before plasma separation. The plasma after separation should be more than 4 mL (minimum 3 mL).
- The plasma separation protocol for reference: centrifuge the peripheral whole blood sample at 2000× g for 10 min, keep the supernatant then centrifuge again at 8000× g for 10 min, keep the 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. 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.
- 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 cfDNA concentration should be no less than 0.1 ng/μL and 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 -15℃ to -25℃ and avoid repeatedly freeze-thaw.

Assay Procedure

Note:

- *It is recommended to include a OCB-Positive Control (PC) and OCB-Negative Control (NC) together with the testing samples 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 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 -15℃ to -25℃, thaw the buffer at room temperature, mix well with vortex and spin down, place on ice until use. For E2 OCB-End Repair Enzyme Mix, upside down 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	OCB-End Repair Buffer	3 μL
E2	OCB-End Repair Enzyme Mix	1.5 μL
	Nuclease-Free Water	50-χ μL
	Total	**54.5 μL**

***Note**:*

- *"χ" stands for the volume of cfDNA input amount, which in total should be optimally 30 ng, minimum 5 ng, 5≤ χ <30 ng is considered as risky.*
- *The OCB-Positive Control (PC) and OCB-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: 20℃ for 30 min (set the heat-lid off or at 40℃), 4℃ hold.

While the end repair program runs, take out the reagents listed in Table 3 from -15℃ to -25℃, thaw the buffer and Adapter on ice box, mix well with vortex and spin down, place on ice until use. For L3 OCB-Ligation 1 Enzyme, upside down 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	OCB-Ligation 1 Buffer	12.5 μL
L2	OCB-Ligation 1 Adapter	1 μL
L3	OCB-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: no need to change the reaction tubes in the following steps.

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 lustre. (**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: no need to change the reaction tubes in the following steps.

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:

(Heat-lid set to 70℃, for those unadjustable heat-lid can be set at 105℃)

20℃ for 15 min, 65℃ for 15 min, 4℃ hold. **Do not hold at 4℃ for more than 2 h!**

1.4 Ligation 2

Note: no need to change the reaction tubes in the following steps.

1.4.1 Take out the reagents listed in Table 4 from -15℃ to -25℃, thaw the buffer and Adapter at room temperature, mix well with vortex and spin down, place on ice until use. For L6 OCB-Ligation 2 Enzyme A and L7 OCB-Ligation 2 Enzyme B, upside down 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	OCB-Ligation 2 Buffer	2.25 µL
L5	OCB-Ligation 2 Adapter	2 µL
L6	OCB-Ligation 2 Enzyme A	0.25 µL
L7	OCB-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:

(Heat-lid set to 70℃, for those unadjustable heat-lid can be set at 105℃)

65℃ for 30 min, 4℃ hold.

Note: Immediately proceed to the Ligation 2 Cleanup step.

1.5 Cleanup after Ligation 2

Note: no need to change the reaction tubes in the following steps.

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

1.5.1 Add 50 μL OCB-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 lusture. (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 should be stored at -15℃ to -25℃ for no more than one week if not proceeding to the next step immediately.*

1.6 PCR Amplification

1.6.1 Take out the reagents listed in Table 5 from -15℃ to -25℃ 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	OCB-Amplification Buffer ①	25 μL
501~512	OCB-C501~C512	2 μL
701~712	OCB-C701~C712	2 μL
/	Cleaned-up product (from step 1.5.9)	21 μL
	Total	**50 μL**

Note:

Please use different combination of the C501-C512 and C701-C712 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 Heat-lid set to 105℃:

Table 6. PCR Amplification Program

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

Note: χ refers to cycling numbers.

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

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

1.7 Library Purification

It is required to perform library purification step separately for each one of the testing sample, positive control and negative control PCR amplification products.

1.7.1 Vortex the AMPure XP beads (or equivalent) that has been equilibrated to room temperature with the maximum speed for 1 min, ensure the beads are resuspended evenly. After vortexing, take 65 μL AMPure XP beads to add 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 at least 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 -15℃ to -25℃ for no more than 6 months if not proceeding to the next step immediately and avoid repeatedly freeze-thaw.*

1.8 DNA Library Quality Control (QC)

1.8.1 Quantify the DNA library concentration with a recommended fluorescence-based method (eg. Quantus™ or Qubit® Fluorometer), the total DNA amount should be no less than 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 Library fragment size QC (Optional): Assess the library quality with a recommended capillary electrophoresis analyzer and related kit. The peak size of the library fragment should be at 300~400 bp, as shown in Figure 1.

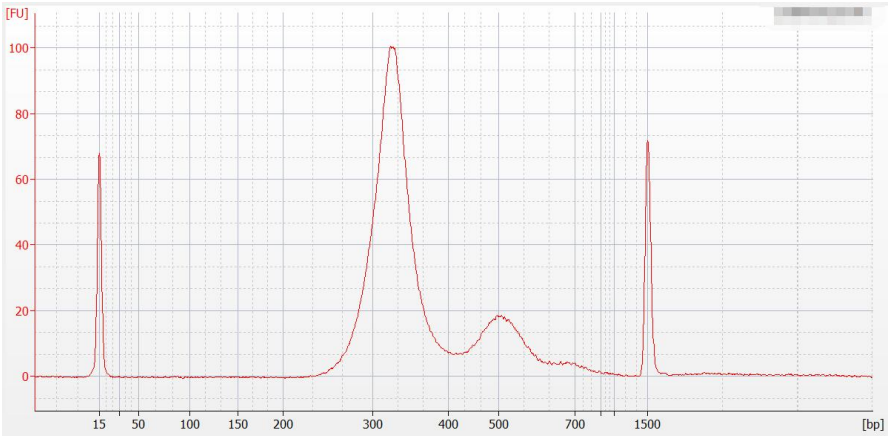

Figure 1. Example of cfDNA library size distribution on Agilent 2100 Bioanalyzer

2. Hybridization Capture

2.1 Reagent Preparation

2.1.1 Pool the 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 Library Pooling Amount

Library pooling with n samples	n =1	n =2	n =3	n =4	n =5	n =6
cfDNA input amount per sample	750 ng	500 ng	500 ng	500 ng	500 ng	500 ng

Note:

- *It is recommended to proceed hybridization capturing with 1~6 samples with different Index combination. It is recommended to mix equal amounts of libraries of the same sample type for hybridization, and each library should have a different index combination. Do not use the libraries with the same combination of index in a single 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 capturing alone to improve capturing specificity and effective depth of its sequence results.*

2.1.2 Take out the reagents listed in Table 8 from -15℃ to -25℃ 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.

Table 8. Pre-Hybridization Mix

No.	Reagent	Volume per Test
Sample	Library samples (1 ~ 6 samples)	/
H1	OCB-Blocker	7 μL

2.1.3 Mix the pre-hybridization mixure thoroughly by vortexing or pipetting, and centrifuge briefly. Put the tubes in a vacuum concentrator with the tube lid open. Set the temperature at 60℃ to proceed the drying process until liquid will be completely evaporated. Avoid over drying (do not leave the dried sample in vacuum concentrator for more than 10 min).

***Note**: AMPure XP Beads can also be used for this step (optional): using AMPure XP Beads with a twice volume of the Hybridization Capture Mix, wash with 200 μL freshly prepared 80% ethanol (twice), then elute target DNA with 10 μL (H3) OCB-Hyb Buffer. Then transfer all DNA eluates 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) OCB-Hyb Buffer and hybridization capture probe (H2) from -15℃ to -25℃, 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) OCB-Hyb Buffer into each sample tube, vortex to mix well, then centrifuge briefly.

2.2.3 Add 5 μL (H2) OCB-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: 95℃ for 10 min, 52℃ for 12~20 hours (16 hours is recommended).

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) OCB-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) OCB-Beads Wash Buffer at twice the volume of the beads added (based on the volume of beads in Step 2.3.2) 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 of the hybridization product (~ 15 μL, from Step 2.2.4) into the 0.2 mL tubes with aliquoted T1 magnetic beads (to avoid temperature drop). Mix well by gently pipetting up and down for 10~20 times.

2.3.8 Place the tube on a thermocycler and perform the following program: incubate at 52℃ for 45 min.

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 ensure the samples are evenly resuspended, 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

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

2.4.2 Take out the 5× Wash Buffer ①~④ (W1~W4) from -15℃ to -25℃, 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, transfer all of the solution into a clean 1.5 mL low-binding centrifuge tube. Spin down briefly and place the tubes onto the magnetic stand for 10 seconds 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), then 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 specificity). Then spin down briefly and place the tubes onto the magnetic stand for 1 min until the solution turns clear.

2.4.8 Remove and discard the supernatant carefully without touching the beads. Remove the tube from the magnetic stand, add 200 µL preheated 1×Wash Buffer ③, 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, 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)

Note: *The captured products should be stored at* -15℃ to -25℃ *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 reagents listed in Table 10 from -15℃ to -25℃ to thaw the buffer at room temperature, , mix well with vortex and spin down, place on ice until use. For P3 OCB-Polymerase, upside down several times, centrifuge briefly and place on ice until use. Prepare the reaction mix according to Table 10.

Table 10. Post-hybridization PCR Amplification Reaction

No.	Reagent	Volume
P2	OCB-Amplification Buffer ②	29 µL
P3	OCB-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 set to 105℃:

Table 11. Post-hybridization PCR Program

Temperature	Time	Cycles
95℃	5 min	1
95℃ 60℃	30 sec 45 sec	13
60℃	2 min	1
4℃	∞	1

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

2.6 Purification after Amplification

2.6.1 Vortex the AMPure XP beads that has been equilibrated to room temperature with the maximum speed for 1 min, ensure the beads are resuspended evenly. After vortexing, take 50 μL AMPure XP beads to add to the 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.

2.6.6 Remove the tubes from the magnet stand. Elute DNA target from the beads by adding 33 μL Nuclease-free water or Low TE (for long term storage), mix thoroughly by vortexing or pipetting to resuspend the beads evenly, and incubate for 5 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 PCR tube.

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

2.7 Captured Library QC

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

2.7.2 Library fragment size QC (Optional): Assess the library quality with a recommended capillary electrophoresis analyzer and related kit. The peak size of the library fragment should be at 300-400 bp for plasma cfDNA library, without obvious peaks of small and big fragments, as shown in Figure 2.

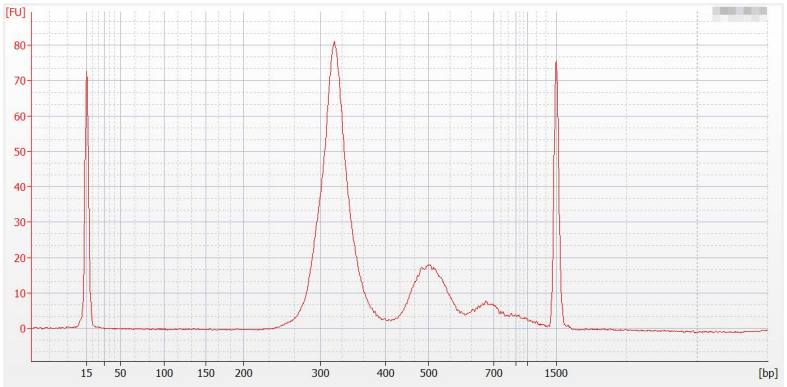

Figure 2. Example of plasma cfDNA captured library size distribution on Agilent 2100 Bioanalyzer

Note:

- *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 each cfDNA sample and PC/NC, the sequencing data should be no less than 16 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	Sample Quantity/Run
NextSeq500/550/550Dx	Mid output	2×150 bp	2
	High output	2×150 bp	7
NovaSeq 6000	SP	2×150 bp	15
	S1	2×150 bp	31
	S2	2×150 bp	78
	S4	2×150 bp	Up to 144*

* *Maximum 144 indexes available.*

Perform the denaturation and dilution of the libraries according to the instrument's instructions. The final concentration of sequencing library is recommended in Table 13.

Table 13. Recommended Final Concentration of Sequencing Library

Sequencing Instrument	Final Concentration
NextSeq 500/550/550Dx	1.2-1.8 pM
NovaSeq 6000	0.75-1 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

When the sequencing is finished, adopt AmoyDx ANDAS Data Analyzer to analyze the sequencing data. Select the "ADXOncoProPlus-bDNA-Int" analysis module for data analysis.

Criteria of data QC:

The qualified criteria and risky 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%
CoverageUniq1000	≥ 80%
RAWreadsOnTarget	≥ 60%
UNIQMedianDepth	≥ 2000×
CNVNoise	≤ 0.2

***Note**:*

- *cleanQ30: One base call in 1,000 is predicted to be incorrect, meaning a base call accuracy of 99.9% in clean data.*
- *CoverageUniq1000: The proportion of target regions that have unique depth more than 1000×.*
- *RAWreadsOnTarget: The proportion of reads mapping to the target region.*
- *UNIQMedianDepth: Median unique depth of the targeted regions.*
- *CNVNoise: The background depth noise of the CNV calling. Please note that this parameter is used as quality control for CNV calling only, not relevant to the quality control of other variants calling.*

Result Interpretation

The cut-off metrics are shown in Table 15.

Table 15. Cut-Off Metrics

Variant Type	Variant Tags/Type	Freq_US	Var_US	Z-Score	MSI-Score
		Median (min-max)	Median (min-max)	/	/
SNV/InDel	SNV	0.2% (0.05%-0.4%)	6 (5-7)	/	/
	InDel (≥ 5 bp)	0.03% (0.03%-0.2%)	5 (5-5)	/	/
	InDel (< 5 bp)	0.125% (0.05%-0.2%)	5 (5-5)	/	/
Fusion	HotFusion	/	≥ 6	/	/
	HotRevFusion	/	≥ 10	/	/
	HotGene	/	≥ 12	/	/
	HotRevGene	/	≥ 16	/	/
	others	/	≥ 24	/	/
CNV	amplification	/	/	≥ 3	/
	loss	/	/	≤ -3	/
MSI	MSI	/	/	/	≥100

***Note**:*

- *For SNV/InDel variant calling, the cut-off metrics are adjusted based on the background noise of each specific region covered by the panel. The median and minimum-maximum cut-off values for SNV/InDel variant calling are shown in the table above.*
- *Freq_US: Frequency of mutant allele, after de-duplication calibration.*
- *Var_US: The number of variant reads, after de-duplication calibration.*
- *HotFusion: Hot gene fusions, the breakpoints occurs at a typical region.*
- *HotRevFusion: Hot gene fusions, the breakpoints occurs at the typical region but in reversed direction.*

- *HotGene: Hot gene fusions, the breakpoints is not occurred at a typical region.*
- *HotRevGene: Hot gene fusions, the breakpoints is not occurred at a typical region and in reversed direction.*
- *Z-Score: copy number deviation from per-sample diploid baseline.*
- *MSI-Score: Overall rating score for level of microsatellite instability.*
- *The testing results of MSI status is for reference only, it is recommended to confirm the MSI status with other method such as PCR and capillary electrophoresis method.*
- *The PC should be detected as positive results for the corresponding mutations as shown in Table S3. Otherwise, the testing is unqualified, it is necessary to check if there is any operational error and the experiment should be repeated.*
- *The NC should be detected as negative regarding the hotspot regions of the detection range of this kit. Otherwise, the testing is unqualified, it is necessary to check if there is any operational error and the experiment should be repeated.*

Performance Characteristics

Limit of Detection (LoD)

For plasma samples with 30ng cfDNA input, the LoD for hotspot SNV/InDel is 0.3% allele frequency, the LoD for non-hotspot SNV/InDel is 0.5% allele frequency, the LoD of fusion detection is 0.5% allele frequency, the LoD of CNV amplification detection is 2.3 copy number, and the LoD of MSI is 0.5% tumor content.

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) 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) 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.
6) The kit does not require paired white blood cells detection as reference, inaccurate results may occur on non-hotspot variants, and it is recommended to be noted for further applications of the testing results.

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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

No.	Gene	Transcripts	Target Regions	Variation Type
1	ABRAXAS1	NM_139076	whole CDS (Exon1-9)	SNV; InDel
2	AKT1	NM_001382430	whole CDS (Exon3-15)	SNV; InDel
3	AKT2	NM_001626	whole CDS (Exon2-14)	SNV; InDel
4	AKT3	NM_005465	whole CDS (Exon2-14)	SNV; InDel
5	ALK	NM_004304	Exon2*, Exon3*, Exon18-29	SNV; InDel; Fusion
6	APC	NM_000038	whole CDS (Exon2-16)	SNV; InDel
7	AR	NM_000044	whole CDS (Exon1-8)	SNV; InDel; Fusion
8	ARAF	NM_001654	Exon7,14	SNV;InDel
9	ARID1A	NM_006015	whole CDS (Exon1-20)	SNV; InDel
10	ATM	NM_000051	whole CDS (Exon2-63)	SNV; InDel
11	ATR	NM_001184	whole CDS (Exon1-47)	SNV; InDel
12	AURKA	NM_198437	whole CDS (Exon2-9)	SNV; InDel
13	B2M	NM_004048	whole CDS (Exon1-3)	SNV; InDel
14	BAP1	NM_004656	whole CDS (Exon1-17)	SNV; InDel
15	BARD1	NM_000465	whole CDS (Exon1-11)	SNV; InDel
16	BCL2L11	NM_138621	Exon3*	SNV; InDel
17	BRAF	NM_004333	whole CDS (Exon1-18)	SNV; InDel; Fusion
18	BRCA1	NM_007294	whole CDS (Exon2-23)	SNV; InDel; CNV
19	BRCA2	NM_000059	whole CDS (Exon2-27)	SNV; InDel; CNV
20	BRIP1	NM_032043	whole CDS (Exon2-20)	SNV; InDel
21	CCND1	NM_053056	whole CDS (Exon1-5)	SNV; InDel
22	CCND2	NM_001759	whole CDS (Exon1-5)	SNV; InDel
23	CCNE1	NM_001238	whole CDS (Exon2-12)	SNV; InDel
24	CD274	NM_014143	whole CDS (Exon2-7)	SNV; InDel
25	CDH1	NM_004360	whole CDS (Exon1-16)	SNV; InDel
26	CDK12	NM_016507	whole CDS (Exon1-14)	SNV; InDel
27	CDK4	NM_000075	whole CDS (Exon2-8)	SNV; InDel
28	CDK6	NM_001145306	whole CDS (Exon2-8)	SNV; InDel
29	CDKN2A	NM_000077	whole CDS (Exon1-3)	SNV; InDel
30	CDKN2B	NM_004936	whole CDS (Exon1-2)	SNV; InDel
31	CHEK1	NM_001114122	whole CDS (Exon2-13)	SNV; InDel
32	CHEK2	NM_007194	whole CDS (Exon2-15)	SNV; InDel
33	CTNNB1	NM_001904	Exon3,7,8,10	SNV; InDel
34	CYP2D6	NM_000106	whole CDS (Exon1-9)	SNV; InDel
35	DDR2	NM_006182	Exon5,8,13-18	SNV; InDel
36	DPYD	NM_000110	Exon2*,4*,6,7*,10*,11*,13*,14*,18*,21*,	SNV; InDel
37	EGFR	NM_005228	whole CDS (Exon1-28)	SNV; InDel; Fusion
38	EPCAM	NM_002354	whole CDS (Exon1-9)	SNV; InDel
39	ERBB2	NM_004448	whole CDS (Exon1-27)	SNV; InDel; Fusion; CNV
40	ERBB3	NM_001982	Exon3,7,8,18-24,25*	SNV; InDel
41	ERBB4	NM_005235	Exon17*,18-24,25*,27*,28*	SNV; InDel; Fusion
42	ERCC1	NM_001983	whole CDS (Exon2-10)	SNV; InDel
43	ERCC2	NM_000400	whole CDS (Exon1-23)	SNV; InDel
44	ERCC3	NM_000122	whole CDS (Exon1-15)	SNV; InDel
45	ERCC4	NM_005236	whole CDS (Exon1-11)	SNV; InDel
46	ESR1	NM_000125	whole CDS (Exon1-8)	SNV; InDel; Fusion
47	EZH2	NM_004456	whole CDS (Exon2-20)	SNV; InDel
48	FANCA	NM_000135	whole CDS (Exon1-43)	SNV; InDel

49	FANCD2	NM_001018115	whole CDS (Exon2-44)	SNV; InDel
50	FANCL	NM_018062	whole CDS (Exon1-14)	SNV; InDel
51	FANCM	NM_020937	whole CDS (Exon1-23)	SNV; InDel
52	FBXW7	NM_001349798	whole CDS (Exon4-14)	SNV; InDel
53	FGF19	NM_005117	whole CDS (Exon1-3)	SNV; InDel
54	FGF3	NM_005247	whole CDS (Exon1-3)	SNV; InDel
55	FGFR1	NM_023110	whole CDS (Exon2-18)	SNV; InDel; Fusion
56	FGFR2	NM_000141	whole CDS (Exon2-18)	SNV; InDel; Fusion
57	FGFR3	NM_000142	whole CDS (Exon2-18)	SNV; InDel; Fusion
58	FGFR4	NM_213647	whole CDS (Exon2-10,12-18), Exon11*	SNV; InDel; Fusion
59	FH	NM_000143	whole CDS (Exon1-10)	SNV; InDel
60	GATA3	NM_002051	whole CDS (Exon2-6)	SNV; InDel
61	GEN1	NM_001130009	whole CDS (Exon2-14)	SNV; InDel
62	GNA11	NM_002067	whole CDS (Exon1-7)	SNV; InDel
63	GNAQ	NM_002072	whole CDS (Exon1-7)	SNV; InDel
64	GNAS	NM_000516	Exon6,8,9	SNV; InDel
65	HDAC2	NM_001527	whole CDS (Exon1-14)	SNV; InDel
66	HNF1A	NM_000545	whole CDS (Exon1-10)	SNV; InDel
67	HRAS	NM_005343	Exon2-4	SNV; InDel
68	IDH1	NM_005896	Exon4	SNV; InDel
69	IDH2	NM_002168	Exon4,11*	SNV; InDel
70	JAK1	NM_002227	Exon12-25	SNV; InDel
71	JAK2	NM_004972	Exon12-25	SNV; InDel
72	JAK3	NM_000215	Exon11-24	SNV; InDel
73	KDR	NM_002253	Exon11*,21,24	SNV; InDel
74	KEAP1	NM_203500	whole CDS (Exon2-6)	SNV; InDel
75	KIT	NM_000222	Exon7-9,11,13,14,16,17,18*	SNV; InDel; Fusion
76	KRAS	NM_004985	Exon2,3,4	SNV; InDel
77	MAP2K1	NM_002755	Exon2,3,6	SNV; InDel
78	MAP2K2	NM_030662	whole CDS (Exon1-11)	SNV; InDel
79	MAP2K4	NM_003010	Exon1-5,7-11	SNV; InDel
80	MAPK1	NM_002745	whole CDS (Exon1-8)	SNV; InDel
81	MAPK3	NM_002746	whole CDS (Exon1-8)	SNV; InDel
82	MDM2	NM_002392	whole CDS (Exon1-11)	SNV; InDel
83	MDM4	NM_002393	whole CDS (Exon2-11)	SNV; InDel
84	MET	NM_000245	whole CDS (Exon2-21)	SNV; InDel; Exon14 Skipping; Fusion; CNV
85	MLH1	NM_000249	whole CDS (Exon1-19)	SNV; InDel
86	MPL	NM_005373	whole CDS (Exon1-12)	SNV; InDel
87	MRE11	NM_005591	whole CDS (Exon2-20)	SNV; InDel
88	MSH2	NM_000251	whole CDS (Exon1-16)	SNV; InDel
89	MSH6	NM_000179	whole CDS (Exon1-10)	SNV; InDel
90	MTAP	NM_002451	whole CDS (Exon1-8)	SNV; InDel
91	MTOR	NM_004958	whole CDS (Exon2-58)	SNV; InDel
92	MUTYH	NM_001048174	whole CDS (Exon2-16)	SNV; InDel
93	MYC	NM_002467	whole CDS (Exon1-3)	SNV; InDel
94	MYCN	NM_005378	whole CDS (Exon2-3)	SNV; InDel
95	NBN	NM_002485	whole CDS (Exon1-16)	SNV; InDel
96	NF1	NM_001042492	whole CDS (Exon1-58)	SNV; InDel
97	NF2	NM_000268	whole CDS (Exon1-16)	SNV; InDel
98	NFE2L2	NM_006164	whole CDS (Exon1-5)	SNV; InDel
99	NOTCH1	NM_017617	whole CDS (Exon1-34)	SNV; InDel
100	NPM1	NM_002520	whole CDS (Exon1-11)	SNV; InDel
101	NRAS	NM_002524	whole CDS (Exon2-5)	SNV; InDel

102	NRG1	NM_013964	Exon1*,2*,3*,4*,5*,6*	SNV; InDel; Fusion
103	NTRK1	NM_002529	Exon2,4,6-17	SNV; InDel; Fusion;
104	NTRK2	NM_006180	Exon3,5,7,9-17	SNV; InDel; Fusion
105	NTRK3	NM_001012338	whole CDS (Exon3-20)	SNV; InDel; Fusion
106	PALB2	NM_024675	whole CDS (Exon1-13)	SNV; InDel
107	PDCD1	NM_005018	whole CDS (Exon1-5)	SNV; InDel
108	PDGFRA	NM_006206	whole CDS (Exon2-23)	SNV; InDel; Fusion
109	PIK3CA	NM_006218	whole CDS (Exon2-21)	SNV; InDel; CNV
110	PIK3CB	NM_006219	whole CDS (Exon3-24)	SNV; InDel
111	PIK3R1	NM_181523	whole CDS (Exon2-16)	SNV; InDel
112	PMS2	NM_000535	whole CDS (Exon1-15)	SNV; InDel
113	POLD1	NM_002691	whole CDS (Exon2-27)	SNV; InDel
114	POLE	NM_006231	whole CDS (Exon1-49)	SNV; InDel
115	PPARG	NM_138711	whole CDS (Exon3-8)	SNV; InDel
116	PTCH1	NM_000264	whole CDS (Exon1-23)	SNV; InDel
117	PTEN	NM_000314	whole CDS (Exon1-9)	SNV; InDel; CNV
118	PTPN11	NM_002834	whole CDS (Exon1-15)	SNV; InDel
119	RAD50	NM_005732	whole CDS (Exon1-25)	SNV; InDel
120	RAD51	NM_002875	whole CDS (Exon2-10)	SNV; InDel
121	RAD51B	NM_133510	whole CDS (Exon2-10), Exon11*	SNV; InDel
122	RAD51C	NM_058216	whole CDS (Exon1-9)	SNV; InDel
123	RAD51D	NM_002878	whole CDS (Exon1-10)	SNV; InDel
124	RAD52	NM_134424	whole CDS (Exon2-12)	SNV; InDel
125	RAD54L	NM_003579	whole CDS (Exon1-18)	SNV; InDel
126	RAF1	NM_002880	Exon4-17	SNV; InDel; Fusion
127	RASA1	NM_002890	whole CDS (Exon1-25)	SNV; InDel
128	RB1	NM_000321	whole CDS (Exon1-27)	SNV; InDel
129	RET	NM_020975	Exon2*,6-8, 9*,10-18	SNV; InDel; Fusion
130	RHEB	NM_005614	Exon2,8	SNV; InDel
131	RHOA	NM_001664	whole CDS (Exon2-5)	SNV; InDel
132	RICTOR	NM_152756	whole CDS (Exon1-38)	SNV; InDel
133	RIT1	NM_006912	whole CDS (Exon2-6)	SNV; InDel
134	RNF43	NM_017763	whole CDS (Exon2-10)	SNV; InDel
135	ROS1	NM_002944	Exon2,4,7,22*,23*,31-42	SNV; InDel; Fusion
136	SETD2	NM_014159	whole CDS (Exon1-21)	SNV; InDel
137	SF3B1	NM_012433	Exon14-15	SNV; InDel
138	SLX4	NM_032444	whole CDS (Exon2-15)	SNV; InDel
139	SMAD4	NM_005359	whole CDS (Exon2-12)	SNV; InDel
140	SMARCA4	NM_003072	whole CDS (Exon2-35)	SNV; InDel
141	SMO	NM_005631	whole CDS (Exon1-12)	SNV; InDel
142	STK11	NM_000455	whole CDS (Exon1-9)	SNV; InDel
143	TERT	NM_198253	Exon5	SNV; InDel; Fusion
144	TMPRSS2	NM_005656	whole CDS (Exon2-14)	SNV; InDel; Fusion
145	TP53	NM_000546	whole CDS (Exon2-11)	SNV; InDel
146	TSC1	NM_000368	whole CDS (Exon3-23)	SNV; InDel
147	TSC2	NM_000548	whole CDS (Exon2-42)	SNV; InDel
148	TSHR	NM_000369	Exon10*	SNV; InDel
149	UGT1A1	NM_000463	Exon1*	SNV; InDel
150	VHL	NM_000551	whole CDS (Exon1-3)	SNV; InDel
151	XRCC1	NM_006297	whole CDS (Exon1-11,13-17), Exon12*	SNV; InDel
152	XRCC2	NM_005431	whole CDS (Exon1-3)	SNV; InDel

*Note: The exons marked with * indicate that the exons listed in the genes are not completely covered, but only the hotspot regions.*

Table S2. Index Sequence Information for Primers

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

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

Table S3 Positive Variants (hotspot mutations, fusions, and CNV) in OCB-Positive Control (PC)

No.	Gene	Alteration Type	CDS change
1	*PIK3CA*	SNV	NM_006218:exon3:c.353G>A:p.(G118D)
2	*EGFR*	SNV	NM_005228:exon20:c.2369C>T:p.(T790M)
3	*EGFR*	SNV	NM_005228:exon21:c.2573T>G:p.(L858R)
4	*MET*	Exon 14 skipping	NM_000245:intron14:c.3028+1G>T:p.?
5	*EGFR*	InDel	NM_005228:exon19:c.2235_2249del:p.(E746_A750del)
6	*SLC34A2-ROS1*	Fusion	SLC34A2:NM_006424:exon4--ROS1:NM_002944:exon32
7	*MET*	CNV	amplification

***Note**:*

- *For the quality control of PC, all the variants listed in the above table must be detected, otherwise, the experiment is unqualified.*
- *The NC should be detected as negative regarding the hotspot regions of the detection range of this kit.*
- *Please note that there might be additional variants found in PC/NC, for example, there is a fusion variant: RNASEH2B:NM 024570.4:exon6--ALK:NM 004304.5:exon5, but such variant is not necessary for quality control and might be occasionally missed.*