AmoyDx® Essential NGS Panel

Instructions for Use

REF 8.06.0002 24 tests/kit For Illumina NextSeq 500, NextSeq 550Dx

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Version: B1.0
Mar 2022

Background

Lung cancer is one of the most common malignant tumor, and ~85% of lung cancers are non-small cell lung cancer (NSCLC). There are many driver mutations in NSCLC. The frequency of mutations in NSCLC for *EGFR*, *KRAS*, *NRAS*, *PIK3CA, BRAF*, *HER2* and *MET* gene are 10-35%, 5-30%, 1%, 3-5%, 1-4.9%, 2-4% and 1-5% respectively. The frequency of gene fusion in NSCLC for *ALK*, *ROS1* and *RET* gene are 3-7%, 2% and 1-2% [1-8]. A large amount of clinical studies showed that gene mutation status is an important efficacy predictor for targeted therapy. For instance, *EGFR*-TKI would show better efficacy on patients with *EGFR* sensitizing mutation than wild-type gene [1], the presence of the *ALK* and *ROS1* gene fusions are correlated with the efficacy of ALK/MET inhibitor therapy [2-3], patients with *RET* fusion could benefit from MET/RET/VEGFR inhibitor [4], *BRAF* mutated patients will benefit from BRAF inhibitor treatment [5], and *KRAS*/*NRAS*/*HER2*/*PIK3CA* mutation status is associated with prognosis of some targeted drugs [6-8]. It is indicated in NCCN Guideline for NSCLC that gene mutation testing is required before targeted therapy, and it is strongly recommended to conduct multi-target test for the optimal precision oncology treatment [9]. Therefore, combined detection of multiple gene mutations in patients with NSCLC can provide more precise treatment.

Colorectal cancer (CRC) is the third most common cancer worldwide, and the metastatic disease accounts for 40-50% of newly diagnosed patients. In total, activating *KRAS*, *NRAS*, *PIK3CA* and *BRAF* mutations occur in 20-50%, 1-6%, 10-30% and 8-15% of colorectal cancers respectively [10-11]. Clinical studies have shown the *KRAS/NRAS/PIK3CA/BRAF* mutation positive colorectal cancer patients have poor response rate to anti-*EGFR* monoclonal antibodies [12-15]. Analysis of the mutation status of these four genes in patients with colorectal cancer helps to improve the objective response rate of treatment.

This kit is designed for detection of common mutations in 10 genes including *EGFR, ALK, ROS1, RET, KRAS, NRAS, PIK3CA, BRAF, HER2* and *MET* in NSCLC and CRC patients. 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® Essential NGS Panel is a next-generation sequencing (NGS) based *in vitro* diagnostic assay intended for qualitative detection of single nucleotide variants (SNVs), insertions and deletions (InDels), gene fusions, and copy number variations (CNVs) in 10 oncogenic driver genes (see Table 1), using DNA isolated from formalin-fixed paraffin embedded (FFPE) tumour tissue specimens, or circulating cell-free DNA (cfDNA) isolated from plasma derived from anti-coagulated peripheral whole blood specimens. The detection of CNVs is available for tissue-derived DNA only. The assay is intended to provide tumor mutation profiling to be used by qualified health care professionals in accordance with professional guidelines in oncology for patients with non-small cell lung cancer (NSCLC) and colorectal cancer (CRC).
This assay is not automated and is for laboratory professional use only.

Table 1. Gene Lists

Gene	Transcripts	Target Regions	Variants Detected
EGFR	NM_005228.3	Exons 17-24	SNVs, InDels
ALK	NM_004304.4	Exon 19, 20, 22, 23, 25; Intron 19	SNVs, InDels, Fusions
ROS1	NM_002944.2	Exon 31~36, 38; Intron 31, 33~35	SNVs, InDels, Fusions
RET	NM_020975.4	Exon7, 8, 10, 11, 12; Intron 7, 10, 11	SNVs, InDels, Fusions
KRAS	NM_033360.3	Exon 2, 3, 4	SNVs, InDels
NRAS	NM_002524.4	Exon 2, 3, 4	SNVs, InDels
PIK3CA	NM_006218.2	Exon10, 14, 21	SNVs, InDels
BRAF	NM_004333.4	Exon 11, 15	SNVs, InDels
HER2	NM_004448.3	Exon 19, 20	SNVs, InDels
MET	NM_000245.2	Exon 13~20; Intron 13	SNVs, InDels, CNV

Note: *The detection of MET CNVs is only for FFPE tissue sample, not for plasma sample.*

For the intron 31 of the ROS1 gene, the kit only covers part of it, not completely covered.

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 gemomic 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 include 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 2.

Table 2. Kit Contents

No.	Components	Main Ingredient	Quantity
1	LC-End Repair Buffer	Tris, Mg^{2+}	105 µL/tube ×1
2	LC-End Repair Enzyme	Klenow Enzyme	45 µL/tube ×1
3	LC-Ligation Buffer	Tris, Mg^{2+}, ATP, DTT, Ligase	450 µL/tube ×1
4	LC-Ligation Enhancer	Small Molecular Ligation Enhancer	15 µL/tube ×1
5	LC-Adaptor	Oligonucleotides	28 µL/tube ×1
6	LC-PCR Buffer ①	Tris, Mg^{2+}, dNTPs, DNA Polymerase	750 µL/tube ×1
7	LC-D5 Primer *	Oligonucleotides	6 µL/tube ×8
8	LC-D7 Primer *	Oligonucleotides	4 µL/tube ×12
9	LC-Blocking Reagent	Oligonucleotides	70 µL/tube ×1
10	LC-Capture Probe	Oligonucleotides	50 µL/tube ×1
11	LC-Hybridization Buffer	Formamide, Na^{+}, Tween, Dextran Sulfate	100 µL/tube ×1
12	LC-Bead Wash Buffer	Tris, EDTA-2Na, NaCl	500 µL/tube ×1
13	5×Wash Buffer ①	MES, NaCl, Tween	880 µL/tube ×1
14	5×Wash Buffer ②	NaCl, SDS, DTT	660 µL/tube ×1
15	5×Wash Buffer ③	Na^{+}, DTT	440 µL/tube ×1
16	5×Wash Buffer ④	Na^{+}, DTT	440 µL/tube ×1
17	LC-PCR Buffer ②	Tris, Primers, Mg^{2+}, dNTPs	290 µL/tube ×1
18	LC-DNA Polymerase	DNA Polymerase	15 µL/tube ×1
19	LC10-Positive Control**	Positive DNA	100 µL/tube ×1
20	LC-Negative Control***	Wild-type DNA	100 µL/tube ×1

* For labeling and sequence information of the primers, refer to Appendix Table S1.

** The positive variants in the LC-Positive Control are listed in Appendix Table S2.

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

Storage and Stability

The kit requires shipment on frozen ice packs and the shipping time should be less than one week. All contents of the kit should be stored immediately upon receipt at -20±5℃.

The shelf-life of the kit is six 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) or MagPure FFPE DNA LQ Kit (Magen) is recommended for DNA extraction from FFPE tissue samples; 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) 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) are recommended.
8) Sequencing instrument: Illumina NextSeq 500/NextSeq 550Dx is recommended.
9) Sequencing reagent: Illumina 300 cycles (Paired-End Reads, 2×150 cycles) is recommended.
10) Illumina PhiX Control V3.
11) Magnetic Stand: DynaMag™-2 Magnet (Thermo Fisher Scientific) and DynaMag™-96 Side Magnet (Thermo Fisher Scientific) are recommended.
12) Ultrasonicator: Covaris M220 Focused-ultrasonicator (Covaris) and microTUBE-130 AFA Fiber Screwcap (Covaris) are recommended.
13) Water bath or heating block: Bioer ThermoCell Mixing and Heating (Bioer Technology) or equivalent.
14) Rotator: LCD Digital Rotator (Dragonlab) 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) Nuclease-free water.
24) TE-low solution (10 mM Tris, 0.1 mM EDTA, pH 8.0).

Precautions and Handling Requirements

For *in vitro* diagnostic use.

Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use, and strictly follow the instruction during operation.
- 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 NSCLC or CRC patients.
- The FFPE tissue sample should be fixed by 10% neutral buffered formalin within 6 hours (no more than 48 hours). 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 18 months.
- 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 AmoyDx cell-free DNA collecting tube 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 -20±5℃ 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. The FFPE DNA should be more than 100 ng, and the plasma cfDNA should be more than 10 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 -20±5℃, avoid repeated freezing and thawing.

Assay Procedure

Note:

- *It is recommended to include a LC-Positive Control (PC) and a LC-Negative Control (NC) in the process of library preparation, sequencing and data analysis.*
- *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 genomic DNA (gDNA) derived from FFPE tissue samples, ultrasonic shearing or enzymatic digestion (not provided) should be used to shear the gDNA into short fragments before proceeding with library preparation. For cfDNA derived from plasma samples, or PC/NC DNA, it can be directly used for library preparation (starting from step 1.3 End Repair) without DNA shearing.

1.1. DNA Shearing (Only for FFPE DNA)

It is recommended to use Covaris M220 Focused-ultrasonicator (Covaris, Cat. No. 500295) and microTUBE-130 AFA Fiber Screwcap (Covaris, Cat. No. 520216) for DNA shearing, and the procedure is as follows.

Note: *Other methods can be also used to shear DNA, e.g. enzymatic digestion, please follow the reagent's instructions.*

1.1.1. 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 3.

Table 3. Parameters for DNA Shearing (Covaris M220)

Parameter	Setting Value
Duty Factor	20%
Peak Incident Power (W)	50
Cycles Burst	200
Times	170~230

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

1.1.2. After DNA shearing, it's recommended to use capillary electrophoresis analyzer and related kit for quality control, the peak size of the DNA fragment should be at ~220 bp, if not qualified, the sample DNA should be re-sheared or re-extracted.

1.2. DNA Fragment Purification (Only for FFPE DNA)

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. Transfer 125 µL of the above DNA shearing product (from step 1.1.1) into a clean nuclease-free 1.5 mL centrifuge tube, then add 250 µL resuspended AMPure XP beads, mix thoroughly by vortexing, 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 for a total of 2 washing steps.

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 bead pellet, 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. If not qualified, the sample DNA should be re-sheared or re-extracted.

***Note**: The purified fragmented DNA should be stored at -20±5 ℃ 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.

Table 4. End Repair Reaction

Reagent	**Volume**
Fragmented DNA/cfDNA/PC/NC	χ μL
Nuclease-free Water	25-χ μL
LC-End Repair Buffer	3.5 μL
LC-End Repair Enzyme	1.5 μL
Total	**30 μL**

Note:

- *For FFPE samples, "χ" stands for the volume of 30~50 ng fragmented DNA (50 ng is recommended).*
- *For plasma samples, "χ" stands for the volume of 10~30 ng cfDNA (30 ng is recommended). For the plasma cfDNA with low concentration, amplify proportionally the End repair system and Adaptor Ligation system (maintain the amount of Adaptor and the elution volume after purification of adaptor Ligation), to increase the initial DNA amount for library construction.*
- *For PC and NC, the DNA concentration is 1.2 ng/μL, take 25 μL PC/NC to construct library (χ=25).*

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℃ for 30 min, 65℃ for 30 min, 4℃ hold.

***Note**: The end repair products should be stored at -20±5 ℃ 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 adapter ligation reaction on ice by adding the following components according to Table 5.

Table 5. Adaptor Ligation Reaction

Reagent	Volume
End Repair Product (from step 1.3.3)	30 μL
LC-Ligation Buffer	15 μL
LC-Ligation Enhancer	0.5 μL
LC-Adaptor	1 μL
Total	**46.5 μL**

1.4.3. 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℃ for 15 min, 4℃ 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 1.4.3) into a clean nuclease-free 1.5 mL centrifuge tube, then add 42 μL resuspended AMPure XP beads, mix thoroughly by vortexing or pipetting, then incubate for 10 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 for a total of 2 washing steps.

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 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.*

1.5.7. 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 bead pellet, transfer the supernatant into a clean 0.2 mL PCR tube to obtain the ligation product.

***Note**: The purified fragmented DNA should be stored at -20±5 ℃ for no more than 20 hours if not proceed to the next step.*

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 6.

Table 6. PCR Amplification Reaction

Reagent	Volume
Purified Ligation Product (from step 1.5.8)	21 μL
LC-PCR Buffer ①	25 μL
LC-D5 Primer	2 μL
LC-D7 Primer	2 μL
Total	**50 μL**

***Note:** There are 8 tubes of LC-D5 Primer (LC-D501~LC-D508) and 12 tubes of LC-D7 Primer (LC-D701~LC-D712). Each of the*

LC-D5 Primer or LC-D7 Primer has a different index sequence. Use a different combination of LC-D5 Primer and LC-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 S1.*

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 7.

Table 7. PCR Program

Temperature	Time	Cycles
98℃	45 s	1
98℃	15 s	9 or 11
60℃	30 s	
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 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 of samples, NC and PC 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 10 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 for a total of 2 washing steps.

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 30 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 bead pellet, 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 -20±5 ℃ for no more than one week if not proceed to the next step.*

1.8. DNA Library Quality Control (QC)

1.8.1. Library concentration QC: Quantify the DNA library concentration using Quantus™ or Qubit® Fluorometer, the DNA concentration should be no less than 30 ng/μL, the DNA amount should be no less than 900 ng.

1.8.2. 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/NC 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.

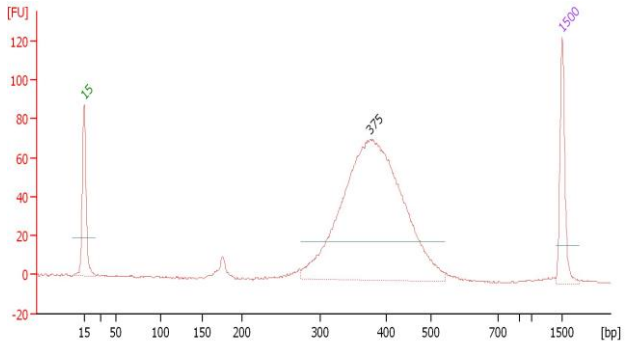

Figure 1. Example of FFPE DNA library size distribution on Agilent 2100 Bioanalyzer

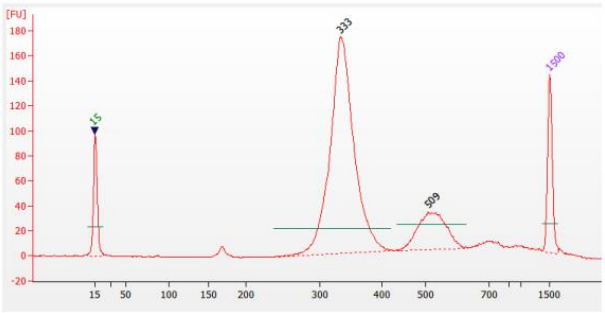

Figure 2. Example of plasma cfDNA library size distribution on Agilent 2100 Bioanalyzer

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 the hybridization capture. If not, the library should be reconstructed.*

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 8.

Table 8. Pre-hybridization Mix

Reagent	**Volume**
Library samples (1~4 samples)	≤ 60 μL
LC-Blocking Agent	7 μL
Total	**≤ 67 μL**

Note:

- *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 (LC-D5 and LC-D7).* ***Do not*** *use the same combination of index for two or more sample libraries in one hybridization mix.*
- *It is recommended to pool 4 sample libraries with different index combinations in equal amounts for hybridization capture. The amount of each library should be 500~1000 ng (1000 ng per library is recommended). If there are less than 4 sample libraries, reduce the number of samples for hybridization capture. The PC/NC library and the sample libraries can be mixed together for hybridization capture.*
- *For the sample library with poor DNA quality, it is recommended to input 500~1000 ng (1000 ng is recommended) per*

library and hybridize individually.

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 2 times volume of AMPure XP Beads for DNA purification, 80% freshly prepared ethanol for washing, and 10 μL LC-Hybridization Buffer for DNA elution. Then 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 LC-Hybridization Buffer and LC-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 LC-Hybridization Buffer into each sample tube and cap the tubes, vortex to mix well, then centrifuge briefly.

2.2.3. Add 5 μL LC-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℃ and perform the following program: 95℃ for 10 min, 48℃ for 16~20 hours, 48℃ 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 10 μL of streptavidin beads per capture into a clean nuclease-free 1.5 mL **low-binding** centrifuge tube. For example, for 1 capture, prepare 10 μL of beads and for 2 capture, prepare 20 μL of beads. Add the same volume of LC-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 LC-Bead Wash Buffer, the volume of which is twice the volume of the original Beads. 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 20 μL of resuspended beads into a clean 0.2 mL low-binding 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 (from Step 2.2.3) into the 0.2 mL tubes with beads, pipette or shake slightly to ensure the sample is fully resuspended, then place the tube on a thermocycler and perform the following program: 48℃ for 45 min, 48℃ hold. Set a timer for 45min, every 15min, remove the tube from the thermocycler and gently vortex to ensure the sample is fully resuspended. (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℃. Take out the 5× Wash Buffer ①~④ 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 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	5×Wash Buffer ①	88 μL	352 μL	440 μL
1×Wash Buffer ②	5×Wash Buffer ②	66 μL	264 μL	330 μL
1×Wash Buffer ③	5×Wash Buffer ③	44 μL	176 μL	220 μL
1×Wash Buffer ④	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℃ 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 the 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 1 min 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℃ for 5 min. Then centrifuge briefly and place the tubes onto the magnetic stand for 1 min 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 1×Wash Buffer ② , mix well by pipetting up and down for 10 times, then incubate the tubes at 48℃ for 5 min. Then centrifuge briefly and place the tubes onto the magnetic stand for 1 min 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 1 min 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 30s to mix well. Then centrifuge 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, do not touch the beads. Remove the tube from the magnetic stand, add 50 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 LC-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 LC-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 10.

Table 10. Post-hybridization PCR Amplification Reaction

Reagent	Volume
LC-PCR Buffer ②	29 μL
LC-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 11.

Table 11. Post-hybridization PCR Program

Temperature	Time	Cycles
95℃	5 min	1
95℃	30 s	17
60℃	45 s	
60℃	2 min	1
4℃	∞	1

Note: *The post-hybridization 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 post-hybridization 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 for a total of 2 washing steps.

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 30 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 3~5 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 -20±5 ℃ 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 DNA library concentration using Quantus™ or Qubit® Fluorometer, the DNA library concentration should be no less than 2.5 ng/μL and the DNA library amount should be no less than 75 ng.

2.7.2. Library fragment size QC: Assess the library quality with a recommended capillary electrophoresis analyzer and related kit. The captured library should show the same narrow distribution and the same peak size as the library before capture (The peak size of the library fragment should be at ~380 bp for FFPE DNA/PC/NC library and at ~330 bp for plasma cfDNA library).

Note: *If the library QC pass, then move to sequencing. If not qualified, the hybridization capture or the library preparation should be repeated.*

3. Sequencing

Illumina 300 cycles (Paired-End Reads, 2×150 cycles) related reagents and instruments are recommended for sequencing. The recommended percentage of Illumina PhiX Control v3 is 1%. The sequencing data per sample should be no less than 1 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 (1 Gb/sample)
NextSeq 500/550Dx	Mid	2×150 bp	~ 40
	High	2×150 bp	up to 96#

Maximum 96 indexes available.

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

Table 13. Recommended Final Concentration of Sequencing Library

Sequencing Instrument	Final Concentration
NextSeq 500/550Dx	0.8~1.3 pM

4. Data Analysis

When the sequencing is finished, adopt AmoyDx ANDAS Data Analyzer to analyze the sequencing data and detect the variants of the 10 genes mentioned above.

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 ADXLC10 analysis module and choose the sample type for data analysis.

Result Interpretation

The qualified criteria for data QC is shown in Table 14.

Table 14. Qualified Criteria for Data QC

Parameters	Qualified Criteria	
	FFPE DNA Library	Plasma cfDNA Library
Coverage	≥ 98%	≥ 98%
MeanDepth	≥ 10000×	≥ 10000×
SSBCDepth	≥ 500×	≥ 1500×

The filtering thresholds are shown in Table 15.

Table 15. Filtering Thresholds

Variants	Filtering Thresholds	
	FFPE DNA Library	Plasma cfDNA Library
SNVs/InDels	Freq ≥ 0.4%, Var_DS ≥ 2	Freq ≥ 0.1%, Var_SS ≥ 5
Fusions	Freq ≥ 0.4%, DSBC ≥ 2	Hotspot Fusion: Freq ≥ 0.1%, DSBC ≥ 3 Non-hotspot Fusion: Freq ≥ 0.1%, DSBC ≥ 5
CNV	≥ 3.5 copy number	NA (out of detection)

***Note**:*

- *Q30: one base call in 1,000 is predicted to be incorrect meaning a base call accuracy of 99.9%.*
- *MeanDepth: The average depth of the target region after de duplication calibration.*
- *SSBCDepth: The average depth of all the indivadual base of target region, after single strand base calibration.*
- *Freq: Frequency of mutant allele.*
- *Var_DS: The number of variant reads, after double strand base calibration.*
- *Var_SS: The number of variant reads, after single strand base calibration.*
- *DSBC: The number of fusion reads , after double strand base calibration.*
- *The PC should be detected as positive result for the corresponding mutation as shown in Table S2, and 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

1) Limit of Detection (LoD)

   For FFPE tissue sample, the LoD for SNV/InDel/Fusion detection is 1% allele frequency at 30 ng fragmented DNA input, and the LoD for MET CNV detection is 4 copy number.

   For plasma sample, the LoD for SNV/InDel/Fusion detection is 0.3% allele frequency at 30 ng cfDNA input.

2) Accuracy

   Positive cell lines, plasmid and clinical FFPE tissue samples were tested, and the positive percent agreement (PPA) for SNVs, InDels, fusions and CNVs detection was 100%.

   Negative clinical FFPE tissue samples were tested, and the negative percent agreement (NPA) for SNVs, InDels, fusions and CNVs detection was 100%.

3) Precision

   Repeatability studies demonstrated 100% PPA and 100% NPA for all variants assessed across operators, instruments, and days.

4) Cross-reactivity

   Cross-reactivity studies demonstrated 100% PPA and 100% NPA for all variants assessed, suggests that cross-reactivity poses a minimal risk to this assay.

5) Clinical data

   1563 FFPE tissue samples (1248 samples from NSCLC patients, 295 samples from CRC patients and 20 interference samples) were tested, and sanger sequencing was used as the reference method. For NSCLC samples, the positive concordance is 98.99%, the negative concordance is 82.49%, and overall concordance is 92.95%. For CRC samples, the positive concordance is 100%, the negative concordance is 94.36%, and overall concordance is 96.27%.

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.
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 and CNVs in the target region of the 10 genes. 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.
8) Improper sample processing, transport and storage may lead to false negative or false positive.

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Symbols

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

Appendix

Table S1. Index Sequence Information for Primers

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

Table S2. Positive variants in LC-Positive Control

No.	Gene	Variant Type	CDS Change
01	*EGFR*	SNV	NM_005228.3:exon20:c.2369C>T:p.T790M
02	*EGFR*	SNV	NM_005228.3:exon21:c.2573T>G:p.L858R
03	*KRAS*	SNV	NM_033360.3:exon2:c.35G>T:p.G12V
04	*ROS1*	Fusion	GOPC:NM_020399.3_exon8-ROS1:NM_002944.2_exon35
05	*MET*	Exon 14 Skipping	NM_000245.2:intron14:c.3028+1G>T:p.?
06	*MET*	CNV	Copy number amplification

Note: *The detection of MET CNV is only for FFPE tissue sample, not for plasma sample.*