CE IVD

AmoyDx® Essential NGS Panel

（Reversible Terminator Sequencing）

Instruction for Use

REF 8.06.27401X024I 24 tests For Illumina NextSeq, MiSeq

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Background

Lung cancer is one of the most common malignant tumor, and 80-85% 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 has not been combined with drugs for clinical trials. It is only used for detection of common mutations in 10 genes including *EGFR*, *ALK*, *ROS1*, *RET*, *KRAS*, *NRAS*, *PIK3CA*, *BRAF*, *HER2* and *MET* in lung adenocarcinoma and colorectal cancer 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 (Reversible Terminator Sequencing), based on Next Generation Sequencing (NGS) technology, is intended for qualitative detection of single nucleotide variants (SNVs), indels and fusions in 10 driver genes (see Table 1) in DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue or plasma sample in patients with NSCLC or CRC by converting sample nucleic acids to sequenceable libraries. The kit is intended to be used to aid clinician to identify multi-target status for NSCLC and CRC patients.

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

Table 1 Gene Alterations

Gene	Region	Alteration Type
EGFR	Exons 17-24	SNV, Indels
ALK	Exon 20	SNV, Indels, Fusions
ROS1	Exon 32/34/35/36	SNV, Indels, Fusions
RET	Exon 8/11/12	SNV, Indels, Fusions
KRAS	Exon 2/3/4	SNV, Indels
NRAS	Exon 2/3/4	SNV, Indels
PIK3CA	Exon 10/14/21	SNV, Indels
BRAF	Exon 15	SNV, Indels
HER2	Exon 20	SNV, Indels
MET	Intron 14	SNV, Indels

Principles of the Procedure

The kit is developed based on constructing DNA sequencing library, enriching the target regions in the library with specific probes and the Next Generation Sequencing technology to accomplish the one-time detection of multiple mutations in various genes.

For FFPE samples, the extracted DNA is sheared into fragments firstly, then the fragments are selected by AMPure XP magnetic beads (for plasma samples, the extracted DNA fragments can be used directly). The selected DNA fragments are performed with end repair, dA-tailing. The Adaptor is attached to the ends of end-repaired and dA-tailing reaction product by DNA ligase. Then the PCR is performed with the primers with Index and the polymerase to obtain the amplified library. The amplified library and the biotinylated DNA probe undergo solution hybridization, and then the streptavidin coupled magnetic beads is used to enrich the library combined with the probe. Then the PCR is performed to obtain the captured library. The captured library undergoes high-throughput sequencing to obtain sequencing data from which the gene variant information is obtained by analyzing the data with matched software.

Kit Contents

This kit contains library construction reagents, hybridization capture reagents, and positive control (Table 2).

Table 2 Kit Contents

No.	Content	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	LC-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.

Storage and Stability

The kit requires shipment on frozen ice packs. All contents of the kit should be stored immediately upon receipt at -20±5℃.

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

Additional Reagents and Equipment Required but Not Supplied

1) PCR Instrument: ABI 2720 PCR Instrument or other PCR instrument.
2) DNA quantification kit: QuantiFluor dsDNA System (Promega, Cat. No. E2670) or Qubit dsDNA HS Assay Kit (Thermo

Fisher Scientific, Cat. No. Q32851/Q32854) are recommended.

3) Fluorometer: Quantus™ Fluorometer (Promega, Cat. No. E6150) or Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Cat. No. Q32866) are recommended.
4) DNA extraction kit: for FFPE sample, the AmoyDx® DNA Extraction Kit (AmoyDx, Cat. No.8.02.23501X036G) or AmoyDx® DNA/RNA Extraction Kit (AmoyDx, Cat. No. 8.02.23601X036G) are recommended, for plasma sample, the AmoyDx® Circulating DNA Kit (AmoyDx, Cat. No.8.02.26201X024G) or QIAamp Circulating Nucleic Acid Kit (Qaigen, Cat. No. 55114) are recommended.
5) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter, Cat. No. A63880, A63881 or A63882) is recommended. The kit contains AMPure XP Beads which should be balanced to room temperature before use.
6) Streptavidin coupled magnetic beads: Dynabeads MyOne™ Streptavidin T1 (Thermo Fisher Scientific, Cat. No. 65601/65602) is recommended.
7) qPCR library quantification kit: KAPA SYBR FAST qPCR Kits (Kapa Biosystems, Cat. No. KK4824) is recommended.
8) Sequencing Instrument: Illumina NextSeq series or Illumina 300 cycles (Paired-End Reads, 2×150 cycles) related instruments.
9) Sequencing reagent: Illumina NextSeq 500 Mid Output Reagent kit V2 (300 cycles) (Illumina, Cat. No. FC-404-2003) and Illumina NextSeq 500 High Output Reagent kit V2 (300 cycles) (Illumina, Cat. No. FC-404-2004), or Illumina 300 cycles (Paired-End Reads, 2×150 cycles) related reagents.
10) Illumina PhiX Control V3, Cat. No. FC-110-3002.
11) Vacuum concentrator: Concentrator Plus™ complete system (Eppendorf, Cat. No. 5305000.304) is recommended.
12) Capillary electrophoresis analyzer and the related kit: Agilent 2100 Bioanalyzer system and the related DNA Kit (Agilent Technologies, Cat. No. 5067-1504), Agilent 2200 TapeStation and the related DNA kit or LabChip GX Touch and the related kit (PerkinElmer) are recommended.
13) Magnetic Stand: DynaMag™-2 Magnet (Thermo Fisher Scientific, Cat. No. 12321D) and DynaMag™-96 Side Magnet (Thermo Fisher Scientific, Cat. No. 12331D) are recommended.
14) DNA shearing instrument: M220 Focused-ultrasonicator (Covaris, 4482277) is recommended.
15) Low adsorption centrifuge tube used in hybrid capture process: 1.5 mL colorless low-binding centrifuge tube (Axygen, Cat. No. MCT-150-L-C) is recommended.
16) Dry bath with oscillation function or Electro-Thermostatic Water Cabinet.
17) Vortex mixer.
18) Mini centrifuge.
19) Nuclease-free centrifuge tubes.
20) Nuclease-free PCR tubes.
21) Nuclease-free filtered pipette tips.
22) PCR-grade water (Nuclease-free), TE-low solution (10 mM Tris, 0.1 mM EDTA, pH 8.0)
23) Ethanol (AR).

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 reagent 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, pipets and other materials used should be from pre-PCR to post-PCR, and never backwards. The work area for post-PCR operation should be separated from the area for pre-PCR.
- 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 must be human genomic DNA extracted from FFPE tissue or circulating DNA extracted from plasma samples. DNA extraction reagents are not included in the kit. Before DNA extraction, it's essential to use standard pathology methodology to ensure tumor sample quality. Carry out the DNA extraction according to the instructions of DNA extraction kit.

***Note**: during the extraction, denaturation of double stranded DNA should be avoided, e.g. processing at 95 ℃.*

For FFPE tissue samples

- The tumor tissue sample should be fixed by 10% neutral buffered formalin for better within 6 hours or no more than 48 hours.
- It's better to use tumor tissue samples with more than 20% tumor cells.
- The storage time for the FFPE tissue should be less than 18 months.
- The amount of DNA extracted from FFPE tissue should be more than 100 ng by Quantus or Qubit.

For plasma samples

- The plasma sample should be separated from EDTA anti-coagulated peripheral blood. It is required to separate the plasma from the blood after being collected within 2 hours.
- The recommended volume of plasma is no less than 4 mL.
- The amount of DNA extracted from plasma sample should be more than 10 ng by Quantus or Qubit.

***Note:** The extracted DNA should be used immediately, if not, it should be stored at -20±5℃, avoiding freezing and thawing.*

Assay Procedure

It is recommended to include a Positive Control (PC) and a Negative Control (NC) in the process of Library Construction, Hybrid Capture, and Sequencing and Data Analysis.

1. Library Construction

For FFPE tissue sample, the extracted DNA should be sheared before the end repair. For the plasma sample, the extracted DNA can be used directly for end repair.

1.1. DNA Shearing

1) Add 130 μL sample DNA into the reaction tube matched with DNA Shearing Instrument. If the total amount of DNA is more than 500 ng, take 500 ng for shearing; if the total amount of DNA is between 100~500 ng, take 100 ng for shearing. Place the tube in DNA shearing instrument, and perform the shearing according to the parameters in Table 3.

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

Table 3 Parameters for DNA Shearing

Item	Parameter
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.*

2) The sample DNA should be mainly sheared into fragments of about 220 bp, the quality control can be conducted by capillary electrophoresis. If unqualified, the sample DNA should be re-sheared or re-extracted.

1.2. DNA Fragment Purification

1) AMPure XP Beads is recommended for DNA fragment purification. Take out the AMPure XP Beads (Beads) at the room temperature for 30 min, and shake the bottle of the Beads to resuspend any magnetic particles that may have settled.
2) a. If 500 ng DNA is used for shearing

   Transfer 120 μL the above fragmented DNA into 1.5 mL centrifuge tube, add 96 μL Beads into this centrifuge tube and mix well, then incubate the tube at room temperature for 5 min. Place the tube onto Magnetic Stand for 3~5 min until the solution turns clear. Carefully transfer the solution into a 1.5 mL centrifuge tube and discard the Beads. Add 48 μL Beads into the above centrifuge tube and mix well, then let it stand for 5 min. Place the tube onto Magnetic Stand for 3~5 min until the solution turns clear.

   b. If 100 ng DNA is used for shearing

   Transfer 120 μL the above fragmented DNA into 1.5 mL centrifuge tube, add 144 μL Beads into this centrifuge tube and mix well, then incubate the tube at room temperature for 5 min. Place the tube onto Magnetic Stand for 3~5 min until the solution turns clear.
3) Remove and discard the supernatant carefully, do not touch the Beads. Add 200 μL newly-prepared 80% ethanol, let it stand for 30 s.
4) Repeat the above step 3).
5) Remove and discard the ethanol. Allow the Beads to dry at room temperature with lid open for 2~3 min until the Beads show matt surface. Over drying of the Beads may decrease elution efficiency.
6) Move the centrifuge tube away from the Magnetic Stand. Add 25 μL PCR-grade water into the tube to resuspend the Beads, and incubate for 2 min.
7) Place the tube onto the Magnetic Stand for 3~5 min until the solution turns clear.
8) Open the tube, transfer all solution into a centrifuge tube, the solution is the purified fragmented DNA.
9) Test the concentration of DNA with Nucleic acid quantification kit. The final total DNA amount should be no less than 30 ng.

   Note*: if the DNA is not used immediately for next step, store it at -20±5 ℃.*

1.3. End Repair

1) Take out the following reagents at room temperature, when the reagents completely thawed, vortex the tubes to mix well. Prepare the end repair system solution for each sample according to the ratio in Table 4.

Table 4 End Repair System Solution

Reagent	Volume per test
Fragmented DNA/PC/NC	χ μL
PCR-grade Water	25-χ μL
LC-End Repair Buffer	3.5 μL
LC-End Repair Enzyme	1.5 μL
Total	**30 μL**

Note:

- *χ stands for the volume of 10~30 ng plasma DNA (30 ng is recommended) or volume of 30~50 ng fragmented DNA (50 ng is recommended).*
- *For PC and NC, the DNA concentration is 1.2 ng/μL, χ is 25 μL*
- *For the plasma DNA 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.*

2) Mix well the solutions and transfer them into 0.2 mL PCR tube and then place them in PCR instrument, incubate at 20℃ for 30 min and then at 65℃ for 30 min.

***Note**: if the incubated solutions are not used immediately for next step, store them at -20±5 ℃.*

1.4. Adaptor Ligation

1) Take out the LC-Adaptor and the following reagents at the room temperature, when the reagents completely thawed, shake the tubes to mix well. Prepare the Adaptor Ligation system solution according to the ratio in Table 5.

Table 5 Adaptor Ligation System Solution

Reagent	Volume per test
LC-Ligation Buffer	15 μL
LC-Ligation Enhancer	0.5 μL
Total	**15.5 μL**

Note: the operation of LC-Ligation Buffer should be on the ice.

2) Add 15.5 μL of the above solution into the 0.2 mL PCR tube in Step 2) in section 1.3, and mix well by pipetting up and down.

3) Add 1 μL LC-Adaptor and mix well, then place in PCR instrument, incubate at 20℃ for 15 min (do not cover the heating lid).

1.5. Purification after Adaptor Ligation

1) AMPure XP Beads is recommended for purification after Adaptor Ligation. Take out the AMPure XP Beads (Beads) at the room temperature for 30 min. Shake the bottle of the Beads to resuspend any magnetic particles that may have settled

2) Transfer the above incubated solutions into 1.5 mL centrifuge tubes, add 42 μL Beads into each tube and mix well, let it stand for 10 min, then put the tubes onto Magnetic Stand for 3~5 min until the solution turns clear.

3) Remove and discard the supernatant carefully, do not touch the Beads. Add 200 μL newly-prepared 80% ethanol, and let it stand for 30 s.

4) Repeat the above Step 3).

5) Remove and discard the ethanol. Allow the Beads to dry at room temperature with lid open for 3~5 min until the Beads show matt surface. Over drying of the Beads may decrease elution efficiency.

6) Move the centrifuge tube away from the Magnetic Stand. Add 21 μL PCR-grade water into the tube to resuspend the Beads, and let it stand for 2 min.

7) Place the tubes onto the Magnetic Stand for 3~5 min until the solution turns clear.

8) Open the tube lid and pipette all supernatant into 0.2 mL PCR tubes.

1.6. Library Amplification

1) Take out the following reagents at room temperature. When the reagents completely thawed, shake the tubes to mix well. Prepare the Library Amplification System solution according to the ratio in Table 6.

Table 6 Library Construction PCR Mix

Reagent	Volume per test
LC-PCR Buffer ①	25 μL
LC-D5 Primer	2 μL
LC-D7 Primer	2 μL
Total	**29 μL**

Note: *there are 8 tubes of LC-D5 Primer with different index (LC-D501~LC-D508) and 12 tubes of LC-D7 Primer with different index (LC-D701~LC-D712), the sequence for each tube is different from others. Select different combination of LC-D5 Primer and LC-D7 Primer for each Library, and avoid the same combination in the same sequencing run. The sequences of LC-D5 Primer and LC-D7 Primer in this kit refer to the ones in TruSeq HT Sample Prep Kits, the details listed in Appendix.*

2) Add the prepared Library Construction PCR Mix into the 0.2 mL PCR tubes in Step 8) in section 1.5, mix well by pipetting up and down. Centrifuge briefly.

3) Perform the following PCR program in Table 7.

Table 7 PCR Program

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

Note*: Amplification cycle number differs according to sample type. 11 cycles for FFPE tissue sample DNA and 9 cycles for plasma DNA are recommended.*

1.7. Library Purification

Note: each library of samples, NC and PC should be purified individually.

1) Transfer the amplification product into 1.5 mL centrifuge tubes, add 20 μL AMPure XP Beads (room temperature) to each tube and mix well, let it stand for 10 min, then place the tubes onto Magnetic Stand for 3~5 min until the solution turns clear.

2) Carefully pipette the solution into 1.5 mL centrifuge tubes.

3) Add 25 μL Beads into the tubes and mix well, let it stand for 5 min. Place the tubes onto the Magnetic Stand for 3~5 min until the solution turns clear.

4) Remove and discard the supernatant carefully, do not touch the Bead. Add 200 μL newly-prepared 80% ethanol, wait for 30 s.

5) Repeat the above Step 4).

6) Remove and discard the ethanol. Allow the Beads to dry at room temperature with lid open for 2~3 min until the Beads show matt surface. Over drying of the Beads may decrease elution efficiency.

7) Move the centrifuge tube away from the Magnetic Stand. Add 15 μL PCR-grade water into the tube to resuspend the Beads, wait for 2 min.

9) Place the tubes onto the Magnetic Stand for 3~5 min until the solution turns clear.

10) Open the tube lid and pipette all supernatant into 1.5 mL centrifuge tubes.

***Note**: if the solutions are not used immediately for next step, store them at -20±5 ℃ for no more than one week.*

1.8. QC for Library

1) Test the concentration of DNA library by an DNA quantification kit (QuantiFluor dsDNA System or Qubit dsDNA HS Assay Kit) and calculate the DNA amount. The DNA amount of library should be more than 0.5 μg. If it's not qualified, repeat the library construction Perform capillary electrophoresis for sample DNA library or PC/NC DNA library, the main fragment size for DNA library should be 380 bp for FFPE tissue sample or 330 bp for plasma sample.

Note: DNA damage in FFPE tissue samples may decrease the size of DNA fragment to be smaller than 380 bp (e.g. 330 bp, which is much larger than 160 bp). The fragment of 160 bp (shown in Figure 1) will not affect the following steps).

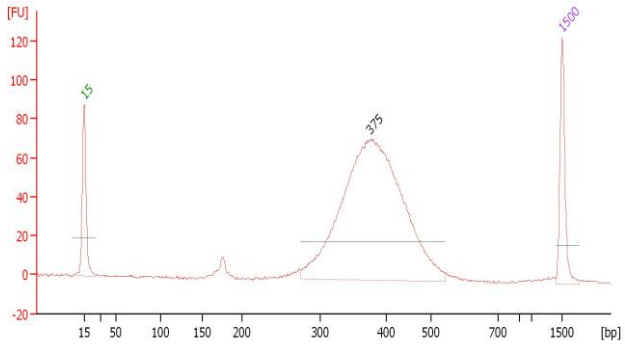

Figure 1 Example of a library QC.

***Note:** 15 bp stands for lower marker peak, 1500 bp stands for upper marker peak*

2. Hybrid Capture

2.1. Reagent Preparation

1) Take out the lids of 0.2 mL PCR tubes, and puncture a small hole in each lid.

2) Take out the following reagents in Table 8 at the room temperature, When the reagents completely thawed, vortex the tubes to mix well, and centrifuge briefly. Add the reagents respectively into the 0.2 mL PCR tubes according to the volume in Table 8, mix well by pipetting up and down, cap the tubes with the lid prepared in Step 1).

Note: we recommend to mix 4 sample libraries with different index combination (LC-D5 and LC-D7) for hybrid capture. The DNA amount for each library is 0.5~1 μg (1 μg is recommended), the DNA amount for the each of 4 libraries should be the same. If there are less than 4 libraries, reduce the number of samples for hybrid capture. PC/NC library and Sample Libraries can be mixed together for hybrid capture. For the sample library with low DNA quality, it's recommended to do hybrid capture individually.

Table 8 Hybridization Preparation System

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

3) Place the 0.2 mL PCR tubes with the above reagents into Vacuum Concentrator at 60℃ for 30~60 min, until the solution in the tubes evaporates to dryness.

Note: AMPure XP Beads can also be used for concentration instead of Vacuum Concentrator: 2 times volume of the Beads used for purification, 80% ethanol used for washing, 10 μL LC-Hybridization Buffer used for elution.

2.2. Hybridization

1) Take the tubes out of Vacuum Concentrator, carefully uncover the lid, add 10 μL LC-Hybridization Buffer into each tube and cap the tubes (with new lids instead of the lids with small hole), vortex to mix well, then centrifuge briefly.
2) Take out the LC-Capture Probe at the room temperature, shake to mix well and then centrifuge briefly. Add 5 μL LC-Capture Probe into the above tubes, shake to mix well and centrifuge briefly. Place the tubes into PCR instrument, set the following parameters to perform the hybridization: 95℃ for 10 min, 48℃ for 16~20 hours.

2.3. Capture

1) Take out the Dynabeads MyOne™ Streptavidin T1 Magnetic Beads at room temperature for 30 min, and shake the bottle of the Beads to mix well. Transfer sufficient Beads (10 μL for each hybridization system) into 1.5 mL centrifuge tube, add the same volume of LC-Bead Wash Buffer, mix well by pipetting up and down for 10~20 times.
2) Place the tubes onto Magnetic Stand for 1 min until the solution turns clear.
3) Remove and discard the supernatant carefully, do not touch the Beads. Add sufficient LC-Bead Wash Buffer of which volume is two times of the Beads volume, mix well by pipetting up and down for 10~20 times.
4) Place the tubes onto Magnetic Stand for 1 min until the solution turns clear.
5) Repeat the above Step 3).
6) Dispense 20 μL of the Beads suspension into each PCR tube, place the 0.2 mL PCR tubes onto the 96-well Magnetic Stand for 1 min until the solution turns clear.
7) Remove and discard the supernatant carefully, do not touch the Beads. Transfer the hybridization solution in Step 2) in Section 2.2 into these PCR tubes with Beads, pipette or shake slightly to mix well quickly to avoid the temperature drop). Place the PCR tubes into PCR instrument, incubate at 48℃ for 45 min, and take out the tubes and vortex every 15 min.

2.4. Washing

1) Turn on the Dry bath with oscillation function, set the temperature at 48℃.
2) Take out the 5×Wash Buffer ①~④ at room temperature. When the reagents completely thawed, shake the tubes to mix well. All 5×Wash Buffers should be transparent. Dilute the buffers according to the ratio in Table 9.

Table 9 Dilution of Wash Buffer

5× Wash Buffer	Diluted Wash Buffer	Volume of 5× Wash Buffer	Volume of water	Total volume
5×Wash Buffer ①	1×Wash Buffer ①	88 μL	352 μL	440 μL
5×Wash Buffer ②	1×Wash Buffer ②	66 μL	264 μL	330 μL
5×Wash Buffer ③	1×Wash Buffer ③	44 μL	176 μL	220 μL
5×Wash Buffer ④	1×Wash Buffer ④	44 μL	176 μL	220 μL

3) Place 1×Wash Buffer ① and 110 μL of 1×Wash Buffer ② (transferred into a new tube) in the Dry bath at 48℃ for at least 10 min. Other buffers remain at room temperature.
4) When the Step 7) in Section 2.3 is finished, add 100 μL the preheated 1×Wash Buffer ② to the PCR tubes with hybridization system and Beads, mix well by pipetting up and down for 10 times, transfer the solution into 1.5 mL centrifuge tube (low adsorption). Centrifuge briefly and place the tubes onto Magnetic Stand for 1 min until the solution turns clear.
5) Remove and discard the supernatant carefully, do not touch the Beads. Take the tubes away from Magnetic Stand. Add 200 μL the preheated 1×Wash Buffer ①, pipette up and down to mix well quickly to avoid the temperature drop. Place the tubes to 48℃ for 5 min. Then centrifuge briefly and place the tubes onto Magnetic Stand for 30 s until the solution turns clear.
6) Repeat the above Step 5) once.
7) Remove and discard the supernatant carefully, do not touch the Beads. Take the tubes away from Magnetic Stand. Add

200 μL 1×Wash Buffer ②, incubate at 48℃ for 5 min to improve the specificity. Then centrifuge briefly and place the tubes onto Magnetic Stand for 1 min until the solution turns clear.

8) Remove and discard the supernatant carefully, do not touch the Beads. Take the tubes away from Magnetic Stand. Add 200 μL 1×Wash Buffer ③, shake the tubes to mix well for 1 min, centrifuge briefly and place the tubes onto Magnetic Stand for 1 min until the solution turns clear.

9) Remove and discard the supernatant carefully, do not touch the Beads. Take the tubes away from Magnetic Stand. Add 200 μL 1×Wash Buffer ④, shake the tubes to mix well for 30s, centrifuge briefly and place the tubes onto Magnetic Stand for 1 min until the solution turns clear.

10) Remove and discard the supernatant carefully, do not touch the Beads. Take the tubes away from the Magnetic Stand. Add 50 μL PCR-grade water, shake the tubes to mix well, centrifuge briefly.

***Note**: if the solution is not used for next step immediately, store it at 2~8 ℃ for no more than one week.*

2.5. Amplification of Capture product

1) Take out LC-PCR Buffer ② at room temperature. When the reagents completely thawed, shake the tubes to mix well. Take out LC-DNA Polymerase and vortex the tubes to mix well, centrifuge briefly, put it onto ice box for use.

2) Take the capture product with Beads in Step 10) in Section 2.4, shake to mix well. Prepare the Hybrid Capture PCR Mix according to the ratio in Table 10.

Table 10 Hybrid Capture PCR Mix

Reagent	**Volume**
LC-PCR Buffer ②	29 μL
LC-DNA Polymerase	1 μL
Capture product with Beads	20 μL
Total	**50 μL**

3) Perform the following PCR program in Table 11.

Table 11 PCR Program

Temperature	**Time**	**Cycle**
95℃	5 min	1
95℃	30 s	16~18
60℃	45 s	
60℃	2 min	1
4℃	∞	1

***Note:** the amplification cycle number differs according to sample type. 17 cycles is recommended here.*

2.6. Purification after Amplification

1) Take out the AMPure XP Beads at room temperature for 30 min, and shake the bottle of the Beads to mix well.

2) Transfer the amplification product in Step 3) in section 2.5 to 1.5 mL centrifuge tube, add 20 μL AMPure XP Beads, mix well by pipetting up and down, let it stand for 5 min. Place the tubes onto Magnetic Stand for 3~5 min until the solution turns clear.

3) Pipette the solution into a new 1.5 mL centrifuge tube.

4) Add 30 μL AMPure XP Beads into the above tube, mix well by pipetting up and down. Let it stand for 5 min. Put the tubes on Magnetic Stand for 3~5 min until the solution turns clear.

5) Carefully remove and discard the supernatant, do not touch the Beads with pipette tip. Add 200 μL newly-prepared 80% ethanol, wait for 30 s.

6) Repeat the above Step 5).

7) Remove and discard the ethanol. Allow the Beads to dry at room temperature with lid open for 2~3 min until the Beads

show matt surface. Over drying of the Beads may decrease elution efficiency.

8) Move the centrifuge tube away from the Magnetic Stand. Add 20 μL TE-low solution (10 mM Tris and 0.1 mM EDTA, pH8.0) into the tube to resuspend the Beads, wait for 2 min.

9) Place the tubes onto the Magnetic Stand for 5 min until the solution turns clear.

11) Open the tube lid and pipette all supernatant into 1.5 mL centrifuge tube.

***Note**: if the solutions are not used immediately for next step, store them at -20±5 ℃ for no more than one week.*

2.7. Quality Control

The quality control is performed with capillary electrophoresis. The distribution of library should be consistent to the one before hybrid capture without obvious peaks of small fragments and big fragments. The library should be tested by nucleic acid quantification kit (the library concentration should be more than 2.5 ng/μL) or qPCR library quantification kit (the concentration of diluted library for sequencing should be more than 2 nM) for quality control of library concentration.

3. Sequencing

Illumina 300 cycles (Paired-End Reads, 2×150 cycles) related reagents and instruments are recommended for sequencing. The sequencing data for each sample library is recommended not less than 1 Gb, and the percentage of PhiX for each run is 1%.

Perform the denaturation and dilution of the libraries according to the instrument's instructions.

1) If the sample quantity is less than 35, it's recommended to use Illumina NextSeq 500 Mid Output V2 Reagent kit (300 cycles).

2) If the sample quantity is between 35~96, it's recommended to use Illumina NextSeq 500 High Output V2 Reagent kit (300 cycles).

3) The final concentration of sequencing library is recommended in Table 12.

Table 12 Recommended Final Concentration of Sequencing Library

Sequencing Instrument	**Final Concentration**
NextSeq Series	0.8~1.2 pM
MiSeq	8~9 pM

4. Sequencing Data Analysis

The sequencing data analysis is performed by AmoyDx NGS data analysis system software - ANDAS Data Analyzer to obtain the related gene variant information.

Result Interpretation

The result is qualified only when the sample coverage is more than 98%, the average original depth (MeanDepth) is more than 10000× and the average effective depth (SSBCDepth) is more than 500×. If not, the sequencing data is insufficient, repeat the sequencing.

If the result meets all of the following criteria (a-b), the sample is interpreted as POSITIVE, if not, the sample is interpreted as NEGATIVE or under the Limit of Detection.

a. Allele effective depth of mutation site is not less than 500×. Mutant Frequency is not less than 0.4%.

b. The Mutant Copy Number (DSBCDepth) is not less than 2.

***Note**:*

- *The constructed DNA library should be more than 0.5 μg. The main size of fragment DNA should be 380 bp for FFPE tissue sample or 330 bp for plasma sample. DNA damage in FFPE tissue samples may decrease the size of DNA fragment to be smaller than 380 bp (e.g. 330 bp, which is much larger than 160 bp). The fragment of 160 bp (shown in Figure 1) will not affect the following steps). If it's not qualified, repeat the library construction.*
- *The DNA amount after capture should be more than 50 ng, the DNA fragment distribution of library should be consistent with the one before hybrid capture, if not, the hybrid capture should be repeated.*

- *The obtained Coverage in sequencing should be more than 98%, if not, the experiment should be repeated*.
- *The PC should be detected as positive result for the corresponding mutation.*
- *The NC should be detected as negative.*

Performance Characteristics

The analytical performance characteristics were established using clinical specimens and cell line/plasmids with specific variants listed in Tables 13-14.

Table 13 Gene Mutation

Exon	AA Change	CDS Change	Cosmic ID
EGFR Exon 18	G719A	2156G>C	6239
	G719S	2155G>A	6252
	G719C	2155G>T	6253
EGFR Exon 19	E746_A750del	2235_2249del15	6223
	E746_A750del	2236_2250del15	6225
	L747_P753delinsS	2240_2257del18	12370
	E746_S752delinsV	2237_2255delinsT	12384
	L747_A750delinsP	2239_2248delinsC	12382
	L747_T751del	2240_2254del15	12369
EGFR Exon 20	T790M	2369C>T	6240
	S768I	2303G>T	6241
	D770_N771insG	2310_2311insGGT	12378
EGFR Exon 21	L858R	2573T>G	6224
	L861Q	2582T>A	6213
KRAS Exon 2	G12D	35G>A	521
	G12A	35G>C	522
	G12V	35G>T	520
	G12S	34G>A	517
	G12C	34G>T	516
KRAS Exon 3	Q61H	183A>C	554
NRAS Exon 2	G12D	35G>A	564
NRAS Exon 3	Q61R	182A>G	584
	Q61K	181C>A	580
PIK3CA Exon 21	H1047R	3140A>G	775
BRAF Exon 15	V600E	1799T>A	476
HER2 Exon 20	A775_G776insYVMA	2324_2325ins12*	20959
MET Exon 14	-	3082+1G>T	24687

*The 12 inserted bases in *HER2* Exon20 are ATACGTGATGGC.

Table 14 Gene Fusion

Gene	Fusion	Cosmic ID
ALK	*EML4* exon 13; *ALK* exon 20	463
	EML4 exon 6; *ALK* exon 20	474
	EML4 exon 20; *ALK* exon 20	465
ROS1	*CD74* exon6; *ROS1* exon 34	1201
	GOPC exon 8; *ROS1* exon 35	1251
RET	*KIF5B* exon 15; *RET* exon 12	1233

1) Limit of Detection

10 ng, 30 ng and 50 ng fragmented DNA of 0.5%, 1% and 5% mutant content were tested for 20 repeats, and the positive rates for 10 ng, 30 ng and 50 ng fragmented DNA of 1% and 5% mutant content were more than 95%. For verification, 10 ng fragmented DNA of 1% mutant content were tested for 20 repeats using three batches of AmoyDx® Essential NGS Panel and come out a positive rate of at least 95%. Therefore, the kit allows detection of 1% mutant DNA in a background of 99% normal

DNA at 10 ng fragmented DNA amount.

2) Accuracy

Test the positive reference controls which were prepared from positive cell line, plasmid or clinical FFPE tissue samples with positive mutations, and the test showed corresponding positive results with 100% concordance rate. Test the negative reference controls which were prepared from clinical FFPE tissue samples with wild-type DNA and normal human genomic DNA, and the test showed negative results with 100% concordance rate.

3) Precision

Precision reference controls of high or mid mutant content and negative precision reference controls were tested 5 repeats by 3 batches of the kits, the concordance rate was 100%.

Precision reference controls of high, mid, or low mutant content and negative precision reference controls were tested once a day by 3 batches of the kits for 20 days, the concordance rate was 100%.

4) Cross-reactivity

Test non-human genomic DNA (Escherichia Coli), and the result showed negative result. Test 10 positive reference controls with 33 mutations covered by this kit, the result showed the corresponding positive and, no cross-reactivity.

5) Clinical data

1586 NSCLC FFPE tissue samples, 499 colorectal cancer (CRC) FFPE tissue samples and 20 interference FFPE tissue samples were tested; Sanger Sequencing was used as 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 results can be used to assist clinical diagnosis, combining with other clinical and laboratory findings.
3) The kit has been only validated for use with FFPE and plasma samples.
4) Samples with negative result (no mutation detected) may harbor other mutations not listed in Tables 13-14.
5) False positive may occur if the fusions happen in highly repetitive region.
6) 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

Sequence Information

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