AmoyDx® *FGFR1-4* NGS Panel

Instructions for Use

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

REF 8.06.0110 24 tests/kit For Illumina NextSeq 500, NextSeq 550

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Background

Fibroblast growth factor receptors (FGFRs) is a member of the human genome tyrosine receptor kinase (TRK) family, four FGFRs have been identified: *FGFR1, FGFR2, FGFR3* and *FGFR4*. *FGFR* genes mutations are present in almost all malignant tumors, and cancers with a higher incidence rate include urothelial carcinoma, cholangiocarcinoma, breast cancer, endometrial cancer, and squamous-cell epithelioma, etc. At the same time, abnormal activation of FGFR has also been found in tumors such as lung cancer, liver cancer, breast cancer and other tumors[1,2,3]. Recent studies have shown that the total incidence of abnormal FGFR gene mutations in the population is approximately 7.1%. Among them, SNVs and insertions and deletions (InDels) mutations account for about 26%, and gene fusion mutations account for about 8% [4,5,6,7,8].

Targeted therapy drugs targeting FGFR have become a new option for the treatment of several cancers [9,10].Growing evidences suggest that the fibroblast growth factor/FGF receptor (FGF/FGFR) signaling has crucial roles in a multitude of processes during embryonic development and adult homeostasis by regulating cellular lineage commitment, differentiation, proliferation, and apoptosis of various types of cells. FGFR inhibitors can block the FGF/FGFR signaling pathway by targeting the target region, thereby achieving the purpose of inhibiting tumor growth.

Intended Use

The AmoyDx® *FGFR1-4* NGS Panel is a next-generation sequencing (NGS) based assay intended for the qualitative detection of single nucleotide variants (SNVs), insertions and deletions (InDels), and gene fusions in *FGFR1/FGFR2/FGFR3/FGFR4* genes (see Table S1). The assay allows the detection of SNVs, InDels, fusions using DNA and RNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue specimens.

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

Principles of the Procedure

The test kit is based on dual-directional capture (ddCAP) technology which is a targeted next-generation sequencing method that uses biotinylated oligonucleotide baits (probes) to hybridize to the target regions. 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 uses both DNA and RNA extracted from FFPE samples. The extracted DNA should be fragmented to the optimal length and the desired DNA size can be achieved via bead-based size selection, and the extracted total RNA is fragmented to desired size via high temperature and Mg^{2+}, and then the single-stranded RNA is reverse transcribed into double-stranded complementary DNA (cDNA). Next, the fragmented DNA and cDNA are mixed to be incubated with end repair enzyme and reagents to get the blunt-ended fragments with dA-tails, then ligated to adapter with complementary dT-overhangs, then a purification step is carried out using AMPure beads, then the PCR amplification is performed to enrich the libraries and each library is marked with unique dual index. Next, the library is performed with target

enrichment, the process including denature double-stranded library, hybridize biotinylated probes to targeted regions, enrichment using streptavidin bead and elution captured DNA/cDNA from 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 identify 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	Quantity
1	R1	FGFR-1st Synthesis Buffer	108 μL/tube × 1
2	R2	FGFR -Random Primers	27 μL/tube × 1
3	R3	FGFR -1st Synthesis Enzyme	54 μL/tube × 1
4	R4	FGFR -2nd Synthesis Buffer	216 μL/tube × 1
5	R5	FGFR -2nd Synthesis Enzyme	108 μL/tube × 1
6	E1	FGFR -End Repair Buffer	189 μL/tube ×1
7	E2	FGFR -End Repair Enzyme	81 μL/tube ×1
8	L1	FGFR -Ligation Buffer	810 μL/tube ×1
9	L2	FGFR -Ligation Enhancer	27 μL/tube ×1
10	L3	FGFR -Adapter	53 μL/tube ×1
11	P1	FGFR -PCR Buffer ①	675 μL/tube ×1
12	513~522	FGFR -S5 Primer	8 μL/tube ×8
13	716~729	FGFR -N7 Primer	6 μL/tube ×12
14	H1	FGFR -Blocking Reagent	189 μL/tube ×1
15	H2	FGFR -Capture Probe	135 μL/tube ×1
16	H3	FGFR -Hybridization Buffer	270 μL/tube ×1
17	B1	FGFR -Beads Wash Buffer	1350 μL/tube ×1
18	W1	5×Wash Buffer ①	1188 μL/tube ×2
19	W2	5×Wash Buffer ②	891 μL/tube ×2
20	W3	5×Wash Buffer ③	1188 μL/tube ×1
21	W4	5×Wash Buffer ④	1188 μL/tube ×1
22	P2	FGFR -PCR Buffer ②	783 μL/tube ×1
23	P3	FGFR -DNA Polymerase	27 μL/tube ×1
24	PC-R	FGFR -Positive Control- RNA	63 μL/tube × 1
25	NC-R	FGFR -Negative Control- RNA	63 μL/tube × 1
26	PC-D	FGFR -Positive Control- DNA	96 μL/tube × 1
27	NC-D	FGFR -Negative Control- DNA	96 μL/tube × 1

Note:

1. For labeling and sequence information of the primers, refer to Appendix Table S2.

2. FGFR-Ligation Buffer should be operated on ice.

3. For positive variants in the PC/NC, refer to Appendix Table S3.

Storage and Stability

The kit needs to be shipped in the cold chain at -15℃ to -25℃, and the shipping time should be no more than one week. All contents of the kit should be stored immediately upon receipt at -15℃ to -25℃.

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

Materials Required but Not Supplied

1) DNA/RNA extraction kit: AmoyDx® FFPE DNA/RNA Kit (Amoy Diagnostics) for DNA/RNA extraction from FFPE tissue samples.
2) Enzymatic fragmentation: KAPA Frag Kit for Enzymatic Fragmentation (KK8602).
3) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter).
4) Fluorometer and related kit: Quantus™ Fluorometer (Promega) and related kits QuantiFluor dsDNA System (Promega), QuantiFluor RNA System (Promega), or Qubit® 2.0/3.0/4 Fluorometer (Thermo Fisher Scientific) and related kits Qubit dsDNA HS Assay Kit, Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific).
5) Streptavidin coupled magnetic beads: Dynabeads MyOne™ Streptavidin T1 (Thermo Fisher Scientific).
6) Capillary electrophoresis analyzer and related kit: Agilent 2100 Bioanalyzer system and RNA 6000 Nano/Pico kit (Agilent), DNA 1000 Kit (Agilent) and High Sensitivity DNA Kit (Agilent); or LabChip GX Touch and DNA High Sensitivity Reagent Kit (PerkinElmer) and RNA Reagent Kit (PerkinElmer).
7) Sequencer: Illumina NextSeq 500/550.
8) Sequencing reagent: Illumina 300 cycles (Paired-End Reads, 2×150 cycles).
9) Illumina PhiX Control V3.
10) Nuclease-free water .
11) Absolute ethanol (AR).
12) TE-low solution (10 mM Tris, 0.1 mM EDTA, pH 8.0).
13) Ultrasonicator: Covaris M220 Focused-ultrasonicator (Covaris) and microTUBE-130 AFA Fiber Screwcap (Covaris).
14) Vacuum Concentrator: Concentrator Plus™ complete system (Eppendorf) and vacuum lyophilizer (Biocool) or other instrument with the same function.
15) PCR instrument: Applied Biosystems™ 2720 Thermal Cycler or ABI MiniAmp™ Plus Thermal Cycler.
16) Magnetic Stand: DynaMag™-2 Magnet (Thermo Fisher Scientific) and DynaMag™-96 Side Magnet (Thermo Fisher Scientific).
17) Water bath or heating block: Bioer ThermoCell Mixing and Heating (Bioer Technology) or equivalent.
18) Vortex mixer.
19) Mini centrifug.

20) Ice box for 0.2 mL and 1.5 mL tubes.

21) 1.5 mL nuclease-free centrifuge tubes.

22) 0.2 mL nuclease-free PCR tubes.

23) Low-binding centrifuge tube: 0.2 mL and 1.5 mL colorless low-binding centrifuge tube (Axygen) is recommended to use in the hybrid capture process.

24) Nuclease-free filtered pipette tips.

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 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 single use. DO NOT reuse.
- The unused reagents, used reagents, and waste must be disposed of properly. Waste disposal shall follow local regulation.

Cleaning

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

Specimen Preparation

- Sample DNA and RNA should be extracted from FFPE tumor tissue specimens. The DNA will be used for the detection of SNVs and InDels, and the RNA will be used for the detection of gene fusions.
- The FFPE tissue sample should be fixed in 10% neutral buffered formalin for no more than 48 hours (recommend within 6 hours). It is recommended that the tumor cell content is no less than 20%. For samples with a tumor cell content between 5% and 20%, it is recommended to re-collect the samples or use the microdissection technology to enrich the tumor cells. It is recommended to use the central section of paraffin blocks. The freshly cut sections of FFPE tissue should be used for DNA and RNA extraction immediately. The storage time of FFPE tissue samples should be no more than 5 years (recommend within 18 months). It is recommended to use the fresh sectioned FFPE samples immediately.
- It is recommended to use the commercialized extraction kit to perform the DNA/RNA extraction from FFPE samples. After extraction, use Quantus™ or Qubit®Fluorometer and the related DNA/RNA quantification kits to quantify the concentration of DNA and RNA. RNA integrity also requires quality control by capillary electrophoresis analyzer.
- **DNA concentration criteria:** The total amount of DNA should be no less than 30 ng.
- **RNA concentration criteria:** The total amount of RNA required varies depending on the DV200 value of the samples. Agilent 2100 is used for RNA integrity quality control, when DV200 value is ≥30%, the total amount of RNA should be no less than 20 ng (optimal 100 ng); when DV200 is <30%, the total amount of RNA should be no less than 200 ng.
- For unqualified samples, re-collection or re-extraction is required.
- The quantified DNA and RNA should be used for library preparation immediately, if not, the DNA samples should be stored at -15℃ to -25℃ for no more than 12 months, and RNA samples should be stored at -60°C to -80°C for no more than 12 months, or at -15℃ to -25℃ for no more than 4 months. During storage, avoid repeated freezing and thawing.

Assay Procedure

Note:

- *It is recommended to include a FGFR DNA-Positive Control (PC-D), a FGFR DNA-Negative Control (NC-D), a FGFR RNA-Positive Control (PC-R) and a FGFR RNA-Negative Control (NC-R) in the process of DNA/cDNA co-Librariey preparation, sequencing and data analysis.*
- *The library preparation process consists of FFPE DNA/cDNA Co-Library Preparation and Hybrid Capture.*

A. FFPE DNA/cDNA Co-Library Preparation

1. RNA Reverse Transcription to cDNA

1.1 FFPE RNA Fragmentation and Priming

1.1.1 RNA fragmentation is only required for intact or partially degraded RNA. For RNA samples with DV200 ≥ 30%, the total RNA input amount should be 20 ng to 200 ng (100 ng is recommended). Prepare the reaction mix for fragmentation and priming on ice with nuclease-free tube according to Table 2:

Table 2. RNA Fragmentation and Priming Mixture (DV200 ≥ 30%)

Component	Volume
Total FFPE RNA/PC RNA/NC RNA	X μL
(R1) FGFR-First Strand Synthesis Buffer	4 μL
(R2) FGFR-Random Primers	1 μL
Nuclease-free water	（13-X）μL
Total volume	18 μL

Note:

- *For FFPE samples, "X" stands for the volume of 20 ng-200 ng RNA (100 ng is recommended). If the concentration of FFPE RNA is less than 7.7 ng/μL, a lyophilizer should be used to concentrate the sample volume into 13 μL.*
- *For (PC-R) PC RNA or (NC-R) NC RNA, "X" is 13 μL.*

Mix thoroughly by pipetting up and down. Place the sample in a thermocycler and incubate the sample at 94℃ following the recommendations in Table 3.

Table 3. Suggested Fragmentation Times

RNA Type	DV200	Tm. &Time
Intact RNA	≥ 70%	94℃ 15 min
Partially Degraded RNA	30%~70%	94℃ 8 min

Note: *PC RNA and NC RNA should follow the intact RNA fragmentation procedure.*

1.1.2 For RNA samples with DV200 < 30%, it is recommended to increase the RNA input to 200-1000 ng (500 ng is recommended). Prepare the priming reaction on ice with nuclease-free tube according to Table 4.

Table 4. RNA Priming Mixture (DV200 < 30%)

Component	Volume
Total FFPE RNA	X μL
(R2) FGFR -Random Primers	1 μL
Nuclease-free water	(13-X) μL
Total volume	14 μL

Note*:*

If the concentration of FFPE RNA is less than 38.5 ng/μL, a lyophilizer should be used to concentrate the sample volume into 13 ul.

Mix thoroughly by pipetting up and down. Place in a thermocycler, and run the following program:

65℃, 5 min; 4℃, ∞; set the heat lid at 105℃.

1.2 First Strand cDNA Synthesis

1.2.1 For RNA sample with DV200 ≥ 30%, assemble the first strand synthesis mixture on ice by adding the following components according to Table 5.

Table 5. First Strand cDNA Synthesis Mixture (DV200 ≥ 30%)

Component	Volume
Fragmented and Primed RNA	18 µL
(R3) FGFR-First Strand Synthesis Enzyme Mix	2 µL
Total volume	20 µL

1.2.2 For RNA sample with DV200 < 30%, assemble the first strand synthesis mixture on ice by adding the following components according to Table 6.

Table 6. First Strand cDNA Synthesis Mixture (DV200 < 30%)

Component	Volume
Primed RNA	14 µL
(R1) FGFR-First Strand Synthesis Buffer	4 µL
(R3) FGFR-First Strand Synthesis Enzyme Mix	2 µL
Total volume	20 µL

1.2.3 Mix thoroughly by pipetting up and down. Place in a thermocycler, set the heat-lid ≥ 80℃, and run the following program according to Table 7.

Table 7. First Strand cDNA Synthesis Program

Temperature	Time	Cycle
25℃	10 min	1
42℃	30 min	1
50℃	2 min	10
42℃	2 min	
70℃	15 min	1
4℃	∞	1

1.3 Second Strand cDNA Synthesis

Assemble the second strand synthesis mixture on ice by adding the following components to the first strand reaction product according to Table 8.

Table 8. Second Strand cDNA Synthesis Mixture

Component	Volume
The first strand reaction product	20 µL
(R4) FGFR-Second Strand Synthesis Buffer	8 µL
(R5) FGFR-Second Strand Synthesis Enzyme Mix	4 µL
Nuclease-free water	48 µL
Total volume	80 µL

Keep the tube on ice, mix thoroughly by pipetting up and down. Incubate in a thermocycler for 1 hour at 16℃ with the heat lid off or no higher than 40℃.

1.4 cDNA Purification

1.4.1 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. Aliquot 176 µL of resuspended AMPure XP beads (2.2×) into a new 1.5 mL centrifuge tube.

1.4.2 Transfer all the second strand synthesis products (~80 µL) to the above 1.5 mL centrifuge tubes that had been aliquoted with AMPure XP beads, mix well on a vortex mixer or by pipetting up and down at least 10 times, centrifuge briefly, and then incubate for 5 min at room temperature. Then place the tubes on a magnetic stand for 2~3 min to separate beads from the supernatant until the solution is clear.

1.4.3 After the solution is clear, carefully remove and discard the supernatant, do not touch the beads with pipette tip. Add 400 µL of freshly prepared 80% ethanol to the tubes, and briefly spin the tubes while in the magnetic stand, incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1.4.4 Repeat Step 1.4.3 once.

1.4.5 Gently remove and discard the supernatant. 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.

1.4.6 Take the tubes off from magnetic stand. Add 33 µL Nuclease-free water to resuspend the beads, and incubate at room temperature for 2 min.

1.4.7 Place the tubes on the magnetic stand for 3~5 min until the solution turns clear. Keep the tubes on the magnetic stand and carefully transfer 31 µL of the supernatant containing the eluted cDNA into a new 1.5 mL centrifuge tube.

1.4.8 Use fluorescent dye method (Quantus™ Fluorometer or Qubit® 2.0/3.0/4 Fluorometer) to quantify the concentration of the above purified fragmented cDNA samples, to determine the amount of adapter to be added in the subsequent step (Step 4.3).

Note: *If the cDNA is not used immediately for next step, store at -20 ± 5℃ for no more than 48 hours.*

2. FFPE DNA fragmentation

For genomic DNA (gDNA) derived from FFPE tissue samples, it is recommended to use ultrasonic fragmentation (Covaris M220) to shear the gDNA into short fragments (150-350 bp). If ultrasonic fragmentation instrument is not available, enzymatic fragmentation (not provided) can be used as an alternative method.

For FGFR-Positive Control-DNA (PC-D) and FGFR-Negative Control-DNA (NC-D), skipping DNA fragmentation process (Step 2.1 to 2.3), since they were derived from fragmented cell line DNA and can be directly used in the End Repair step (Step 3) for DNA library preparation.

2.1 Ultrasonic fragmentation

Add genomic DNA sample (100 ng DNA is recommended, add TE-low Buffer to a final volume of 130 μL) into the Covaris microtube. Place the microtube into ultrasonicator Covaris M220 and perform the shearing according to the parameters in Table 9.

Table 9. Covaris M220 Parameters for DNA Shearing

Parameter	Setting value
Duty Factor	20%
Peak Incident Power (W)	50
Cycles Burst	200
Time (seconds)	180

2.2 Enzymatic Fragmentation

2.2.1 If ultrasonic instrument is not available, enzymatic fragmentation is an alternative method. For enzymatic fragmentation, KAPA Frag Kit for Enzymatic Fragmentation (KK8602) is recommended. Prepare the enzymatic fragmentation mixture on ice by adding the following components according to Table 10.

Table 10. Enzymatic Fragmentation Mixture

Component	Volume
KAPA Frag Enzyme	10 μL
KAPA Frag Buffer	5 μL
Genomic DNA (100 ng DNA is recommended)	35 μL
Total volume	50 μL

Note:

- *The enzymatic reaction system is sensitive to EDTA, so it is recommended to use nuclease-free water to elute the genomic DNA during the DNA extraction process, avoid using TE solution for DNA elution. Otherwise, 2× the volume of AMPure XP Beads should be used to purify the genomic DNA before fragmentation.*

2.2.2 Mix the solution thoroughly by vortexing or pipetting, and centrifuge briefly, then place the sample in a thermocycler with the heated lid off, and perform the following program according to Table 11.

Table 11. Enzymatic Fragmentation Procedure (Heated lid off)

Temperature	Time
4℃	1 min
37℃	10 min
4℃	hold

2.2.3 After the procedure is finished, add 5 μL Stop Solution to stop the reaction immediately, and then add 110 μL AMPure XP Beads for purification.

2.3 Fragment DNA purification

2.3.1 Take out the AMPure XP beads (2.0×) 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.

2.3.2 If the genomic DNA was fragmented by ultrasonic fragmentation method, transfer the 125 μL fragmented DNA into a new 1.5 mL centrifuge tube that contains the aliquoted 250 μL of AMPure XP beads;

If the genomic DNA was fragmented by the enzymatic fragmentation method, transfer all the fragmented DNA to a new 1.5 mL centrifuge tube that had been aliquoted with 110 μL of AMPure XP beads. Mix well by pipetting up and down at least 10 times, then incubate the tube at room temperature for 5 min. Then place the tube on a magnetic stand to separate the beads from the supernatant.

2.3.3 After the liquid is clear, carefully remove and discard the supernatant, do not touch the Beads with pipette tip. Add 400 μL of freshly prepared 80% ethanol to the tube, and briefly spin the tube while in the magnetic stand, incubate at room temperature for 30 s, and then carefully remove and discard the supernatant.

2.3.4 Repeat Step 2.3.3 once.

2.3.5 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.

2.3.6 Move the centrifuge tube away from the magnetic stand. Add 23 μL Nuclease-free water into the tube to resuspend the Beads, and incubate at room temperature for 2 min.

2.3.7 Place the tubes on the magnetic stand for 3~5 min until the solution turns clear.

2.3.8 Carefully open the tube lid and pipette 22 μL of the above eluted product into a new 1.5 mL centrifuge tube, which is the fragmented DNA.

2.3.9 Use fluorescent dye method (Quantus™ Fluorometer or Qubit® 2.0/3.0/4 Fluorometer) to quantify the concentration of DNA, and the total amount of the above purified fragmented DNA should be no less than 30 ng.

Note: *If the fragmented DNA is not used immediately for next step, store at -15℃ to -25℃.*

3. End Repair

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 assemble the end repair reaction by adding the following components according to Table 12.

Table 12. End Repair Reaction

Component	Volume
Fragmented DNA (from Step 2.3.9)	X μL
Nuclease-free water	20-X μL
Reverse Transcribed cDNA (from Step 1.4.8)	30 μL
(E1) FGFR -End Repair Buffer	7 μL
(E2) FGFR -End Repair Enzyme	3 μL
Total Volume	60 μL

Note:

- *For FFPE samples, "X" stands for the volume of 20-60 ng fragmented DNA, optiaml imput amount is 50-60 ng.*
- *For (PC-D) DNA-Positive Control and (NC-D) DNA-Negative Control, "X" are both 20 μL.*

- *In this step, the fragmented DNA and cDNA are mixed together for co-library construction. For cDNA reversed from FFPE RNA (or PC/NC RNA), all of the cDNA product from Step 1.4.8 (30 μL) should be added, as shown in the table above.*

3.2 Mix well by pipetting up and down. Place the sample tubes in a thermocycler, and run the following program: 20℃ for 30 min, 65℃ for 30 min.

3.3 The end repair products should be stored at -15℃ to -25℃ if not proceed to the next step.

4. Adapter Ligation

4.1 Take out the adapter and the following reagents to thaw at room temperature. When the reagents are completely thawed, mix well on a vortex mixer and centrifuge briefly, then prepare the adapter ligation mixture according to Table 13.

Table 13. Adapter Ligation Mixture

Component	Volume
(L1) FGFR -Ligation Buffer	30 μL
(L2) FGFR -Ligation Enhancer	1 μL
Total Volume	31 μL

Note: (L1) *FGFR-Ligation Buffer should be kept on ice when use..*

4.2 Add 31 μL of the above adapter ligation mixture into the 200 μL end repair product tube (from step 3.3), and mix thoroughly by pipetting up and down.

4.3 (L3) FGFR-adapter should be added at the ratio of 0.15 μL per 10 ng total DNA input (including genomic DNA and cDNA) to the tube (from step 4.2), pipette up and down to mix thoroughly. Incubate at 20℃ for 30 min in a thermocycler with the heat lid off or no higher than 40℃.

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

5. Ligation Products Purification

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. Aliquot 86 μL of resuspended AMPure XP beads into 1.5 mL new centrifuge tubes.

5.2 Transfer all the ligation products to the above 1.5 mL centrifuge tubes that had been aliquoted with AMPure XP beads, mix well by pipetting up and down and incubate for 5 min at room temperature. Then place the tube on a magnetic stand to separate beads from the supernatant for 3~5 min.

5.3 After the liquid is clear, carefully remove and discard the supernatant, do not touch the Beads with pipette tip. Add 200 μL of freshly prepared 80% ethanol to the tube, and briefly spin the tube while in the magnetic stand, incubate at room temperature for 30 s, and then carefully remove and discard the supernatant.

5.4 Repeat Step 5.3 once.

5.5 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.

5.6 Move the centrifuge tube away from the magnetic stand. Add 23 μL Nuclease-free water into the tube to resuspend the Beads, and incubate at room temperature for 2 min.

5.7 Place the tubes on the magnetic stand for 3~5 min until the solution turns clear.

5.8 Carefully open the tube lid and pipette 21 μL of the above eluted product into a new 200 μL PCR tube.

6. Library Amplification

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 assemble the library amplification mixture by adding the following components according to Table 14.

Table 14. Library Amplification Mixture

Component	Volume
(P1)FGFR -PCR Buffer ①	25 μL
(513~522) FGFR -S5 Primer	2 μL
(716~729) FGFR -N7 Primer	2 μL
Total volume	29 μL

Note:

- *There are 8 tubes of FGFR -S5 Primer and 12 tubes of FGFR -N7 Primer. Each of the FGFR -S5 Primer or FGFR -N7 Primer has a different index sequence. Use a different combination of FGFR -S5 Primer and FGFR -N7 Primer for each library.* ***Do not*** *use the same combination of index in the same sequencing run. The detailed information for the index sequence is shown in Appendix Table S2.*

6.2 Add the library amplification mixture into the PCR tubes (from Step 5.8). Mix well by pipetting up and down, centrifuge briefly.

6.3 Perform the following PCR program in Table 15.

Table 15. PCR Program

Temperature	Time	Cycle
98℃	45 sec	1
98℃	15 sec	11-13*
60℃	30 sec	
72℃	30 sec	
72℃	1 min	1
4℃	∞	1

Note*:*

- *For PC/NC, apply 11 cycles.*
- *For FFPE sample, the amplification cycle number differs according to the fragmented DNA and RNA input:*

  1) If the fragmented DNA input is less than 30 ng, or the RNA input is less than the recommended amount of 100 ng (DV200≥

30%) or 500 ng (DV200 < 30%), apply 13 cycles for amplification.

2) If fragmented DNA input ≥ 30 ng, and RNA input is ≥ 100 ng (DV200 ≥ 30%) or ≥ 500 ng (DV200 < 30%), apply 11 cycles..

- *The library products should be stored at 2-8℃ for if not proceed to the next step.*

7. Library Purification

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

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. Aliquot 40 μL of resuspended AMPure XP beads into 1.5 mL new centrifuge tubes.

7.2 Transfer all the library products to the above 1.5 mL centrifuge tubes that had been aliquoted with AMPure XP beads, mix well by pipetting up and down and incubate for 5 min at room temperature. Then place the tube on a magnetic stand to separate beads from the supernatant for 3~5 min.

7.3 After the liquid is clear, carefully remove and discard the supernatant, do not touch the Beads with pipette tip. Add 200 μL of freshly prepared 80% ethanol to the tube, and briefly spin the tube while in the magnetic stand, incubate at room temperature for 30 s, and then carefully remove and discard the supernatant.

7.4 Repeat Step 5.3 once.

7.5 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.

7.6 Move the centrifuge tube away from the magnetic stand. Add 32 μL TE-low solution (pH8.0) into the tube to resuspend the Beads, and incubate at room temperature for 2 min.

7.7 Place the tubes on the magnetic stand for 3~5 min until the solution turns clear.

7.8 Carefully open the tube lid and pipette all of the above eluted product into a new 1.5 mL centrifuge tube.

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

8. Quality Control for Library (QC)

Quantify the library concentration using Quantus™ or Qubit™ Fluorometer, the library DNA amount should be no less than 450 ng. For unqualified samples, re-collection, re-extraction, or library re-construction are required.

B. Hybridization Capture

1. Reagent Preparation

1.1 Take low-binding 200 μL PCR tubes for later use.

1.2 Take out the following reagents and thaw at room temperature. When the reagents are completely thawed, mix well on a vortex mixer and centrifuge briefly, then add the reagents into the 200 μL PCR tubes according to Table 16, mix well by pipetting up and down and put the cap on the tube.

Table 16. Pre-hybridization Mixture

Component	Volume
Pooled Library	≤ 30 μL (450 ng-1250 ng each sample, For 2-4 sample libraries, total ≤4000 ng)
(H1) FGFR -Blocking Reagent	7 μL
Total	≤ 37 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. Do not use the libraries with the same combination of index in a single hybridization pool.*
- *For FFPE samples, individual hybridization (single library per pool) is recommended, and the amount of each sample library should be 450 ng-1250 ng (optimal 1000 ng).*
- *When there are too many FFPE samples in the same batch, it is recommend to pool 2-4 sample libraries together with different index combinations in equal amounts for hybridization capture. The amount of each library should be 450 ng-1250 ng, and the total amount of the 2-4 sample libraries should be no more than 4 μg.*
- *FFPE samples with poor quality require individual hybridization to improve capture specificity and effective sequencing depth.*
- *For PC library and NC library, it is recommended to pool PC/NC together (with 500 ng per library) for hybridization and hybridize them separately from the FFPE libraries.*

1.3 Keep the PCR tube open and place into Vacuum Concentrator at 60℃ for 30~60 min, until the solution in the tubes is completly evaporated, avoid over drying.

Note: *If Vacuum Concentrator is not available, AMPure XP Beads can also be used for concentration, and the procedures are demonstrated briefly here: To the hybridization mix from the above step (Section B, Step 1.2) which contains the libraries and the FGFR-Blocking Reagent, add AMPure XP Beads at twice the volume of the hybridization mix. Use 200 μL 80% freshly prepared ethanol for washing (a total of two washes), and 10 μL of the (H3) FGFR -Hybridization Buffer for DNA elution. Then transfer all DNA eluates to a clean nuclease-free 0.2 mL PCR tube and proceed to Step 2.2 below.*

2. Hybridization

2.1 Take the tubes out of Vacuum Concentrator, add 10 μL (H3) FGFR-Hybridization Buffer into each tube and cap the tubes. Mix well on a vortex mixer and centrifuge briefly.

2.2 Take out the (H2) FGFR Capture Probe and thaw at the room temperature, vortex to mix well and then centrifuge briefly. Add 5 μL (H2) FGFR Capture Probe into the above tubes, vortex to mix well and centrifuge briefly. Place the tubes into thermocycler, set the following parameters to perform the hybridization: 95℃ for 10 min, 48℃ for 12-20 hours (16 hours is recommended).

3. Capture

3.1 Take out the Dynabeads MyOne™ Streptavidin T1 Magnetic Beads at room temperature for at least 30 min and shake the bottle of the Beads to mix well. Transfer sufficient Beads (10 μL for each hybridization system) into new 1.5 mL centrifuge tubes, add the same

volume of (B1) FGFR-Bead Wash Buffer, mix well by pipetting up and down for 10~20 times.

3.2 Place the tubes on the magnetic stand for 1 min until the liquid turns clear.

3.3 Remove and discard the supernatant carefully, do not touch the Beads. Add two times of the Beads volume (B1) FGFR-Bead Wash Buffer, mix well by pipetting up and down for 10~20 times.

3.4 Place the tubes on the magnetic stand for 1 min until the liquid turns clear.

3.5 Repeat the above Step 3.3.

3.6 Aliquot the above bead suspension into 200 µL PCR tubes at 20 µL per tube.

3.7 Place the 200 µL PCR tubes onto the magnetic stand for 1 min until the liquid turns clear.

3.8 Remove and discard the supernatant carefully, do not touch the Beads. Transfer the hybridization solution in Step 2.2 into PCR tubes with Beads, mix well by pipetting up and down for 10~20 times.

3.9 Place the PCR tubes into thermocycler, incubate at 48℃ for 45 min, take out the tubes and vortex every 15 min.

4. Washing

4.1 Turn on the ThermoCell with shaking function, set the temperature at 48℃.

4.2 Take out the (W1~W4) 5×Wash Buffer ①~④ at room temperature. When the reagents completely thawed, shake the tubes to mix well. Dilute the buffers according to the ratio in Table 17.

Table 17 Dilution of Wash Buffer

5× Wash Buffer	Diluted Wash Buffer	Volume of 5× Wash Buffer	Volume of Water	Total Volume
(W1) 5×Wash Buffer ①	1×Wash Buffer ①	88 µL	352 µL	440 µL
(W2) 5×Wash Buffer ②	1×Wash Buffer ②	66 µL	264 µL	330 µL
(W3) 5×Wash Buffer ③	1×Wash Buffer ③	44 µL	176 µL	220 µL
(W4) 5×Wash Buffer ④	1×Wash Buffer ④	44 µL	176 µL	220 µL

4.3 Place 1×Wash Buffer ① and 1×Wash Buffer ② in the ThermoCell at 48℃ for at least 10 min. Other buffers remain at room temperature.

4.4 When Step 3.9 is finished, add 100 µL the preheated 1×Wash Buffer ② to the PCR tubes, mix well by pipetting up and down for 10 times, transfer all the solution into 1.5 mL centrifuge tube (low binding). Centrifuge briefly and place the tubes onto magnetic stand for 1 min until the solution turns clear.

4.5 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℃ and 500 rpm for 5 min. Then centrifuge briefly and place the tubes onto magnetic stand for 30 s until the solution turns clear.

4.6 Repeat the above Step 4.5 once.

4.7 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 ②, incubate at 48℃ and 500 rpm 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.

4.8 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.

4.9 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 30 s, centrifuge briefly and place the tubes onto magnet for 1 min until the solution turns clear.

4.10 Remove and discard the supernatant carefully, do not touch the Beads. Take the tubes away from the magnetic stand. Add 20 µL Nuclease-free water, shake the tubes to mix well, centrifuge briefly.

Note: *If the products are not used for next step immediately, store at 2-8℃ for no more than one week.*

5. Post-Capture PCR Amplification

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

5.2 Vortex the capture products with Beads in Step 4.10 to mix well. Assemble the post-capture PCR amplification mixture by adding the following components according to Table18.

Table 18. Post-Capture PCR Amplification Mixture

Component	Volume
(P2) FGFR -PCR Buffer ②	29 µL
(P3) FGFR-DNA Polymerase	1 µL
Captured Products with Beads	20 µL
Total volume	50 µL

5.3 Mix well and perform the following PCR program in Table19.

Table 19. PCR Program

Temperature	Time	Cycle
95℃	5 min	1
95℃	30 sec	16
60℃	45 sec	
60℃	2 min	1
4℃	∞	1

6. Purification after Amplification

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. Aliquot 50 µL of resuspended AMPure XP beads into 1.5 mL new centrifuge tubes.

6.2 Transfer the amplification products in Step 5.3 to the above 1.5 mL centrifuge tube that had been aliquoted with AMPure XP beads, mix well by pipetting up and down and incubate for 5 min at room temperature. Then place the tube on a magnetic stand to separate

beads from the supernatant for 3~5 min.

6.3 After the liquid is clear, carefully remove and discard the supernatant, do not touch the Beads with pipette tip. Add 200 μL of freshly prepared 80% ethanol to the tube, and briefly spin the tube while in the magnetic stand, incubate at room temperature for 30 s, and then carefully remove and discard the supernatant.

6.4 Repeat the above Step 6.3.

6.5 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.

6.6 Move the centrifuge tube away from the magnetic stand. Add 30 μL TE-low solution (pH8.0) into the tube to resuspend the Beads, and incubate at room temperature for 2 min.

6.7 Place the tubes on the magnetic stand for 5 min until the solution turns clear.

6.8 Carefully open the tube lid and pipette all of the above eluted product into a new 1.5 mL centrifuge tube.

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

7. QC of Captured Library

7.1 Quantify the captured library with DNA quantification kit (QuantiFluor dsDNA System or Qubit dsDNA HS Assay Kit), the concentration should be more than 2.5 ng/μL.

7.2 Library size distribution QC (recommended): Assess the library size distribution with a recommended capillary electrophoresis analyzer and related kit. The peak size distribution of the DNA library should be at 200-550 bp, without obvious peaks of small and big fragments, as shown in Figure 1. For unqualified samples, re-collection, re-extraction, or library re-construction are required.

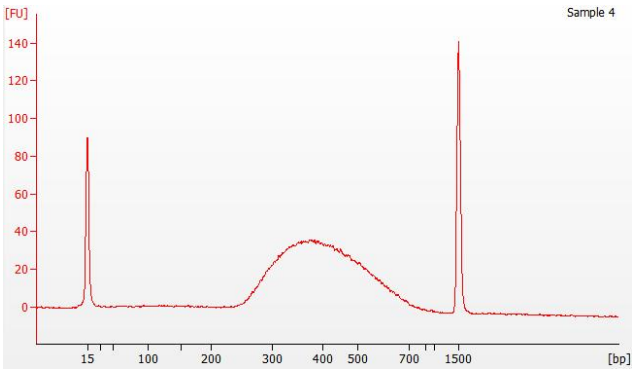

Figure 1. The fragment distribution of captured libraries

C. Sequencing

Illumina 300 cycles (paired end reads, 2 × 150 cycles) sequencing reagent and the matched Sequencers are recommended for sequencing. The recommended percentage of Illumina PhiX Control v3 is 1%. The sequencing data per sample should be no less than 0.3 Gb for FFPE tissue (or PC/NC) sample. All operations should be following Illumina standard process.The suggested sample quantity per run is listed in Table 20.

Table 20. Recommended Sequencers and Sample Quantity per Run

Sequencer	Flow Cell	Read Length	Sample Quantity/Run (0.3 Gb/sample)
NextSeq 500/550	Mid	2×150 bp	up to 96#
	High	2×150 bp	up to 96#

Maximum 96 indexes available.

Perform the denaturation and dilution of the libraries according to the sequencer's instructions. The final concentration of sequencing library is recommended in Table 21.

Table 21. Recommended Final Concentration of Sequencing Library

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

D. Data Analysis

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

Check Q30 value for the sequencing data:

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

Select the analysis module:

If the Q30 value is qualified, select the "ADXFGFR-tMut-Int" analysis module for data analysis.

Result Interpretation

The qualified criteria for data QC is shown in Table 22.

Table 22. Qualified Criteria for Data QC

Parameters	Qualified Criteria
Coverage	≥ 98%
SSBCDepth	≥ 500×
SSBC100	≥ 99%
DNADP	≥ 200×
RNADP	≥ 150×

Note: *The above qualified criteria need to be met at the same time, otherwise the testing is unqualified.*

The cut-off metrics are shown in Table 23.

Table 23.Cut-Off Metrics

Variant Type	Cut-Off Metrics
SNVs/InDels	Freq ≥1.80%, Var_DS≥ 2
Fusions	UniqReads ≥ 6

Note*:*

- *Q30: one base call in 1,000 is predicted to be incorrect meaning a base call accuracy of 99.9%.*

- *SSBCDepth: The average depth of all the individual base of target region, after single strand base calibration.*
- *SSBC100: The proportion of CDS regions with coverage depth > 100×.*
- *DNADP: The depth of DNA source reads.*
- *RNADP: The depth of RNA source reads.*
- *Freq: Frequency of mutant allele.*
- *Var_DS: The number of variant reads, after double strand base calibration.*
- *UniqReads: The number of fusion reads.*
- *The PC should be detected as positive result for the corresponding mutation as shown in the Table S3.*
- *The NC should be detected as negative within the detection range of this kit as shown in the note of Table S3. otherwise, the experiment is unqualified.*

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 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 or RNA degradation or the frequency under the limit of detection may also cause inaccurate 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 in the target region of the 4 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) Reduced amplification efficiency may occur in the *FGFR3* gene (NM_000142) Exon 2 region due to its complex secondary structure, and may result in under-representation of mutant molecules in the final sequencing library. This will results in reduction in the observed QC parameters and detection 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 for the Detection of SNV/InDel/Fusion

No.	Gene	Transcript	Alteration Type
1	*FGFR1*	NM_023110	SNV, InDel, Fusion
2	*FGFR2*	NM_000141	SNV, InDel, Fusion
3	*FGFR3*	NM_000142	SNV, InDel, Fusion
4	*FGFR4*	NM_213647	SNV, InDel, Fusion

Note: *The detection range of SNV/InDel is the whole CDS region and the ≤ 5bp of exon-intron boundaries region of FGFR1/FGFR2/FGFR3/FGFR4 genes; the detection range of fusion is the fusions including FGFR1/FGFR2/FGFR3/FGFR4 kinase domains.*

Table S2 Index Sequence Information for Primers

Primer Name	Sample Sheet Index Information (NextSeq)	Corresponding No. in Illumina Nextera XT v2 Prep Kits
FGFR-N716	TAGCGAGT	N716
FGFR-N718	GTAGCTCC	N718
FGFR-N719	TACTACGC	N719
FGFR-N720	AGGCTCCG	N720
FGFR-N721	GCAGCGTA	N721
FGFR-N722	CTGCGCAT	N722
FGFR-N723	GAGCGCTA	N723
FGFR-N724	CGCTCAGT	N724
FGFR-N726	GTCTTAGG	N726
FGFR-N727	ACTGATCG	N727
FGFR-N728	TAGCTGCA	N728
FGFR-N729	GACGTCGA	N729

Primer Name	Sample Sheet Index Information (NextSeq)	Corresponding No. in Illumina Nextera XT v2 Prep Kits
FGFR-S513	TCGACTAG	S513
FGFR-S515	TTCTAGCT	S515
FGFR-S516	CCTAGAGT	S516
FGFR-S517	GCGTAAGA	S517
FGFR-S518	CTATTAAG	S518
FGFR-S520	AAGGCTAT	S520
FGFR-S521	GAGCCTTA	S521
FGFR-S522	TTATGCGA	S522

Table S3 Positive Variants ((HotSpot mutations and fusions) in FGFR -Positive Control (PC)

No.	Gene	Alteration Type	Variants
1	*FGFR1*	SNV	NM_023110:exon11:c.1506G>T:p.(K502N):p.(Lys502Asn)
2	*FGFR1*	SNV	NM_023110:exon8:c.1028C>T:p.(A343V):p.(Ala343Val)
3	*FGFR2*	SNV	NM_000141:exon13:c.1745C>T:p.(P582L):p.(Pro582Leu)
4	*FGFR3*	SNV	NM_000142:exon3:c.270del:p.(Q92Sfs*6):p.(Gln92SerfsTer6)
5	*FGFR2*	Fusion	FGFR2(E17)-COL14A1(E34)
6	*FGFR3*	Fusion	FGFR3(E17)-TACC3(E11)

***Note**:*

1. *For the quality control of PC, all the variants listed in the above table must be detected, otherwise, the experiment is unqualified.*
2. *The FGFR3(E17)-TACC3(E10) fusion is an additional variant, which is not necessary for quality control and might be occasionally missed.*
3. *Please note that there are additional positive variants in PC/NC, but these variants are not necessary for quality control. These variants might be occasionally missed.*