AmoyDx® HANDLE Classic NGS Panel

Instructions for Use

REF 8.06.0020 24 tests/kit For Illumina MiSeqDx, NextSeq 550Dx

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Background

Lung cancer is one of the most common malignant tumors, 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 is 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]; HER2 mutated patients will benefit from afatinib and the presence of KRAS, NRAS or PIK3CA gene mutation is correlated with primary resistance to EGFR-TKI [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].

Colorectal cancer (CRC) is the third most common cancer worldwide with 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 that colorectal cancer patients with KRAS/NRAS/PIK3CA/BRAF mutation 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 the treatment.

With the development of genetic testing, especially the development of Next Generation Sequencing (NGS), more and more biomarkers have been discovered to develop better targeted therapy of pan cancer patients therapy. Recently, several TRK inhibitors have been proved to be very effective for various cancers, and patients with *NTRK1*, *NTRK2, NTRK3* rearrangements could benefit from TRK inhibitors [16-17]. There are also some very important biomarkers for pan cancer target therapy. Together with several important biomarkers guiding pan cancer target therapy, combined detection of multiple gene mutations in cancer patients is extremely important for effective precise treatment.

Please note that this kit has not been combined with drugs for clinical trials. It is only used for detection of common mutations in 40 genes listed in Table S1. The test results are for clinical reference only. 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® HANDLE Classic 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, copy number amplifications (CNAs) and microsatellite instability (MSI) in 40 key solid tumour genes (see Table S1 and S2), using DNA and RNA isolated from formalin-fixed paraffin embedded (FFPE) tumour tissue specimens. 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 solid malignant neoplasms.

This assay is not automated and is for laboratory professional use only.

Principles of the Procedure

The test kit is based on Halo-shape ANnealing and Defer-Ligation Enrichment system (HANDLE system) technology which is an improved Molecular Inversion Probe (MIP) technology to capture the target gene region (Figure 1). 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, and it offers a time saving protocol that can be completed within 6 hours, and requires just about 1 hour of hands-on time. Firstly, the RNA is reverse transcribed into cDNA with help of the Reverse Transcriptase and the RT primers. Secondly, the cDNA product and the genomic DNA are combined together in one tube for hybridization. The probe contains an extension arm and a ligation arm which are complementary to the target gene region, and the probe anneals onto the DNA or cDNA template of the target region. Thirdly, the DNA is extended from the extension arm to the ligation arm with help of the DNA polymerase, then the nicks are repaired to generate the circular products with help of the DNA ligase. Next, the remaining linear probes, single-strand and double-strand DNA are digested with help of the exonuclease, and only the target circular DNA will be kept for PCR amplification. Finally, the universal PCR amplification is performed to enrich the target libraries, and the magnetic bead-based purification is performed to obtain the final library.

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.

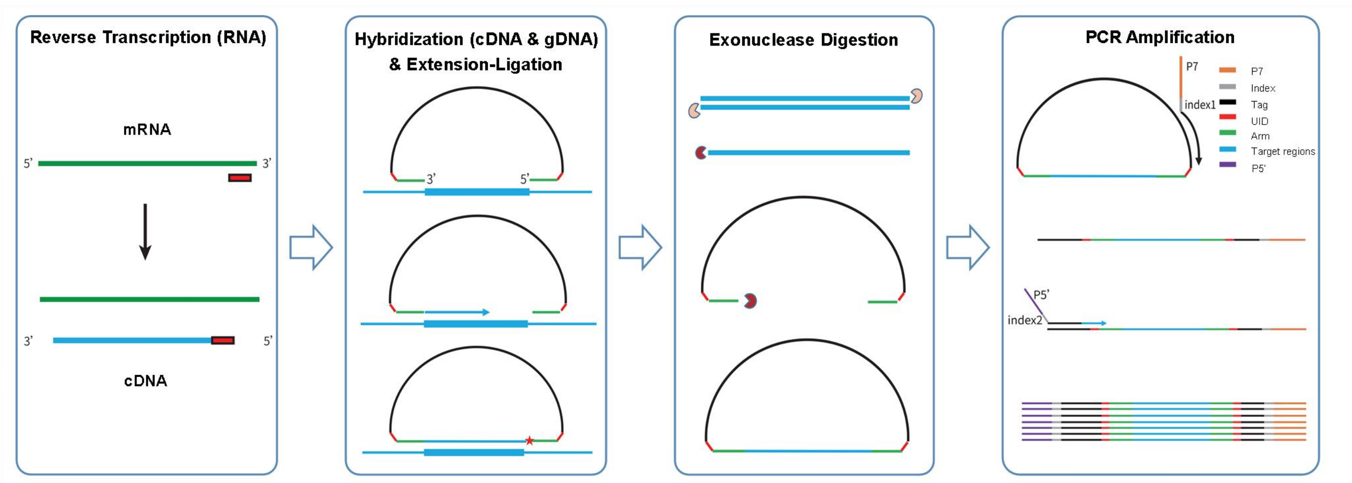

Figure 1 Principle of library construction (HANDLE system)

Kit Contents

This kit contains library construction reagents and positive controls (Table 1).

Table 1. Kit contents

No.	Content	Main Ingredient	Quantity
1-RT	**CP-RT Primers**	Oligonucleotides	28 μL/tube × 1
2-RT	**CP-RT Reaction Mix**	Tris-HCl, K^+, Mg^{2+}, dNTPs,	35 μL/tube × 1
3-RT	**CP-Reverse Transcriptase**	Reverse transcriptase	7 μL/tube × 1
4-Hyb	**CP-Probe**	Oligonucleotides	28 μL/tube × 1
5-Hyb	**CP-Hybridization Buffer**	Tris-HCl, Mg^{2+}	28 μL/tube × 1
6-EL	**CP-Extension Ligation Master Mix**	DNA polymerase, dNTPs, DNA Ligase, Ligation buffer	28 μL/tube × 1
7-ED	**CP-Exonuclease A**	DNA Exonuclease	14 μL/tube × 1
8-ED	**CP-Exonuclease B**	DNA Exonuclease	14 μL/tube × 1
9-Amp	**CP-PCR Master Mix**	Tris, Mg^{2+}, dNTPs, DNA polymerase	600 μL/tube × 1
502-522	**CP-S5 Primer ***	Oligonucleotides	5 μL/tube × 10
716-729	**CP-N7 Primer ***	Oligonucleotides	5 μL/tube × 12
PC-D	**CP-Positive Control-DNA**	DNA	70 μL/tube × 1
PC-R	**CP-Positive Control-RNA**	RNA	70 μL/tube × 1
NC-D	**CP-Negative Control-DNA**	DNA	70 μL/tube × 1
NC-R	**CP-Negative Control-RNA**	RNA	70 μL/tube × 1

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

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°C.

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

Additional Reagents and Equipment Required but Not Supplied

1) PCR instrument: Applied Biosystems™ 2720 Thermal Cycler or MiniAmp™ Thermal Cycler is recommended.
2) DNA quantification kit: QuantiFluor dsDNA System (Promega) or Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific) is recommended.
3) RNA quantification kit: QuantiFluor RNA System (Promega) or Qubit® RNA HS Assay Kit (Thermo Fisher Scientific) is recommended.
4) Fluorometer: Quantus™ Fluorometer (Promega), or Qubit 2.0/3/4 Fluorometer (Thermo Fisher Scientific) is recommended.
5) DNA/RNA extraction kit: AmoyDx® FFPE DNA/RNA Kit (AmoyDx) or AllPrep DNA/RNA FFPE Kit (Qiagen) is recommended for DNA and RNA co-extraction from FFPE sample. It is recommended to use RNase A (Thermo Fisher Scientific) to degrade RNA during the DNA extraction.
6) DNA purification kit: Agencourt AMPure XP Kit (Beckman Coulter) is recommended.
7) Sequencing reagent: Illumina 300 cycles (Paired-End Reads, 2×150 cycles) related reagents is recommended.
8) Sequencing instrument: Illumina MiSeqDx/NextSeq 550Dx is recommended.
9) Illumina PhiX Control V3.

10) 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 E-Gel™ Power Snap Electrophoresis System (Thermo Fisher Scientific) and E-Gel™ EX Agarose Gels, 2% (Thermo Fisher Scientific) is recommended.
11) Magnetic Stand: DynaMag™-2 Magnet (Thermo Fisher Scientific) or DynaMag™-96 Side Magnet (Thermo Fisher Scientific) is recommended.
12) Vortex mixer.
13) Mini centrifuge.
14) Nuclease-free centrifuge tubes.
15) Nuclease-free PCR tubes.
16) Nuclease-free filtered pipette tips.
17) PCR-grade water (nuclease-free)
18) TE-low solution (10 mM Tris, 0.1 mM EDTA, pH 8.0) or 10 mM Tris (pH 8.0).
19) Ethanol (AR).
20) Ice box for 0.2 mL and 1.5 mL tubes.

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 the expiry date.
- DO NOT use any other reagents from different lots.
- DO NOT use any other reagents from 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, pipets and other materials used should be from pre-amplification to post-amplification, and never backwards.

- Gloves should be worn and changed frequently when handling samples and reagents to prevent contamination.
- Use 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 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

- Sample DNA and RNA should be extracted from FFPE tissue sample. The DNA will be used for the detection of SNV, InDel, CNA and MSI, and the RNA will be used for the detection of gene fusion and MET exon14 skipping.
- The FFPE tissue sample should be fixed by 10% neutral buffered formalin for 6~24 hours (no more than 24 hours). The freshly cut sections of FFPE tissue should be used for DNA extraction immediately. The storage time for the FFPE tissue should be less than 2 years.
- It is recommended that the tumor cell content is no less than 20%. For MSI and CNA detection, the recommended tumor cell content is no less than 30%.
- For surgical tumor resection specimens, it is recommended to use 2~6 unstained slides (5~10μM thick) for DNA/RNA extraction. For tissue biopsy specimens, it is recommended to use 10~15 unstained slides (5μM thick) for DNA/RNA extraction.
- It is recommended to use a commercialized DNA/RNA co-extraction kit to perform the DNA and RNA extraction, and use RNase A to degrade RNA during the DNA extraction. After extraction, measure the concentration of extracted DNA and RNA using Quantus™ or Qubit®. The DNA concentration should be more than 6.25 ng/μL, and the total DNA should be more than 50 ng. The RNA concentration should be more than 4 ng/μL, and the total RNA should be more than 30 ng. For unqualified samples, re-collection or re-extraction are required.
- The qualified DNA and RNA should be used for library preparation immediately, if not, the DNA should be stored at -20±5℃ and the RNA should be stored at -80±5℃ for no more than 12 months, avoid repeated freezing and thawing.

Assay Procedure

Note:

- *It is recommended to include a Positive Control (PC, DNA concentration is 6.25 ng/μL, RNA concentration is 4 ng/μL) and a Negative Control (NC, DNA concentration is 6.25 ng/μL, RNA concentration is 4 ng/μL) in the process of library construction, sequencing, and data analysis.*
- *During the following DNA library preparation process, please use the corresponding adaptor in the PCR instrument to avoid PCR product evaporation.*

- *It is recommended to use fluorescent dye method (Qubit or Quantus Fluorometer) for all the DNA/RNA concentration measurement step.*

1. Reverse Transcription

1.1. Take out the (1-RT) **CP-RT Primers** and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer for 10-15 sec and centrifuge briefly, then keep the tube on ice.

1.2. Assemble the pre-reverse transcription reaction on ice in a nuclease-free 0.2mL PCR tube by adding the following components according to Table 2.

Table 2. Pre-reverse transcription reaction

Reagent	Volume
Nuclease-free water	7.5-χ μL
RNA	χ μL
(1-RT) CP-RT Primers	1 μL
Total	**8.5 μL**

Note:

- *For FFPE samples, "χ" stands for the volume of 30~400 ng RNA (400 ng is recommended).*
- *For PC/NC RNA, χ=7.5 μL, take 7.5 μL (PC-R) CP-Positive Control-RNA / (NC-R) CP-Negative Control-RNA to construct library.*

1.3. Mix the solution thoroughly by vortexing for 10-15 sec or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 9 μL and perform the following program: <u>65°C for 5 min, then immediately transfer the tubes to ice for at least 1 min. Then proceed immediately to step 1.4.</u>

1.4. Take out the (2-RT) **CP-RT Reaction Mix** and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer for 10-15 sec and centrifuge briefly, then keep the tube on ice. Take out the **CP-Reverse Transcriptase**, mix well on a vortex mixer for 10-15 sec and centrifuge briefly, then keep the tube on ice. Assemble the reverse transcription reaction on ice by adding the following components according to Table 3.

Table 3. Reverse transcription reaction

Reagent	Volume
(2-RT) CP-RT Reaction Mix	1.25 μL
(3-RT) CP-Reverse Transcriptase	0.25 μL
Pre-reverse transcription products (from step 1.3)	8.5 μL
Total	**10 μL**

Note: *It is recommended to prepare **freshly ready-to-use premix** of CP-RT Reaction Mix and CP-Reverse Transcriptase for precise pipetting when perform three or more samples simultaneously.*

1.5. Mix the solution thoroughly by vortexing for 10-15 sec or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 10 μL and perform the following program: <u>50°C for 50 min, 98°C for 10 min, 4°C hold.</u>

Note: *The reverse transcription products should be stored at 2~8°C for no more than 20 hours if not proceed to the next step.*

2. Hybridization

2.1. Take out the (4-Hyb) **CP-Probe** and (5-Hyb) **CP-Hybridization Buffer** and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer for 10-15 sec and centrifuge briefly, then keep the tube on ice.

2.2. Assemble the hybridization reaction on ice by adding the following components according to Table 4.

Table 4. Hybridization reaction (for DNA and RNA sample)

Reagent	Volume
Nuclease-free water	8-χ μL
DNA	χ μL
(4-Hyb) CP-Probe	1 μL
(5-Hyb) CP-Hybridization Buffer	1 μL
Reverse transcription product (from step 1.5)	10 μL
Total	**20 μL**

Note:

- *For FFPE samples, "χ" stands for the volume of 50~100 ng DNA (70 ng is recommended).*
- *For PC/NC DNA, χ=8 μL, take 8 μL (PC-D) CP-Positive Control-DNA / (NC-D) CP-Negative Control-DNA to construct library.*
- *It is recommended to prepare **freshly ready-to-use premix** of CP-Probe and CP-Hybridization Buffer for precise pipetting when perform three or more samples simultaneously.*

2.3. Mix the solution thoroughly by vortexing for 10-15 sec or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 20 μL and perform the following program: 98°C for 5 min, 60°C for 2 h, 4°C hold.

Note*: The hybridization products should be stored at 2~8°C for no more than 20 hours if not proceed to the next step.*

3. Extension-Ligation

3.1. Take out the (6-EL) **CP-Extension Ligation Master Mix** and thaw the reagent at room temperature. When the reagents are completely thawed, mix well on a vortex mixer for 10-15 sec and centrifuge briefly, then keep the tube on ice.

3.2. Take out the above hybridization product from the thermocycler and keep the tube on ice. Add 1 μL (6-EL) **CP-Extension Ligation Master Mix** into the PCR tubes, mix the solution thoroughly by vortexing for 10-15 sec or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 21 μL and perform the following program: 60°C for 10 min, 4°C hold. Then proceed directly to the exonuclease digestion.

Note:

- *Keep the tubes at low temperature after extension-ligation is finished, as high temperature like room temperature may increase the non-specificity. It is recommended to place the ice box besides the thermocycler, and when the extension-ligation program is finished, immediately transfer the tube on ice box.*
- *Perform the subsequent exonuclease digestion step immediately when the extension-ligation step is finished.*

4. Exonuclease Digestion

4.1. Take out the (7-ED) **CP-Exonuclease A** and (8-ED) **CP-Exonuclease B**, mix well on a vortex mixer for 10-15 sec and centrifuge briefly, then keep the tube on ice.

4.2. Assemble the exonuclease digestion reaction on ice by adding the following components according to Table 5.

Table 5. Exonuclease digestion reaction

Reagent	Volume
(7-ED) CP-Exonuclease A	0.5 µL
(8-ED) CP-Exonuclease B	0.5 µL
Extension-Ligation product (from step 3.2)	21 µL
Total	**22 µL**

4.3. Mix the solution thoroughly by vortexing for 10-15 sec or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 22 µL and perform the following program: 37°C for 30 min, 95°C for 10 min, 4°C hold.

Note:

- *It is recommended to prepare **freshly ready-to-use premix** of CP-Exonuclease A and CP-Exonuclease B for precise pipetting when perform three or more samples simultaneously.*
- *The products of exonuclease digestion should be stored at 2~8°C for no more than 20 hours if not proceed to the next step.*

5. PCR Amplification

5.1. Take out the **CP-S5 Primer, CP-N7 Primer** and (9-Amp) **CP-PCR Master Mix** and thaw the reagents at room temperature. When the reagents are completely thawed, mix well on a vortex mixer for 10-15 sec and centrifuge briefly, then keep the tube on ice.

5.2. Assemble the PCR amplification reaction on ice by adding the following components according to Table 6.

Table 6. PCR amplification reaction

Reagent	Volume
(9-Amp) CP-PCR Master Mix	25 µL
CP-S5 Primer	1.5 µL
CP-N7 Primer	1.5 µL
Exonuclease digestion product (from step 4.3)	22 µL
Total	**50 µL**

Note:

- *Each of the CP-S5 Primer or CP-N7 Primer has a different index sequence. Use different combination of CP-S5 Primer and CP-N7 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.*
- *Transfer the prepared tubes to the amplification room to perform PCR amplification and the following purification to avoid contamination.*

5.3. Mix the solution in each PCR tube thoroughly by vortexing for 10-15 sec or pipetting, and centrifuge briefly, then place the sample in a thermocycler, set the reaction volume as 50 µL, and then perform the following program according to Table 7.

Table 7. PCR program

Temperature	Time	Cycles
98°C	1 min	1
98°C	20 s	25
61°C	30 s	
72°C	20 s	
72°C	5 min	1
4°C	∞	1

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

6. Purification

6.1. Take out the AMPure XP beads and equilibrate them to room temperature for 30 min, and vortex the bottle of the beads for 10-15 sec to resuspend any magnetic particles that may have settled.

6.2. Add **40 µL resuspended beads** and **40 µL PCR products** into 1.5 mL centrifuge tubes, mix thoroughly by vortexing for 10-15 sec or pipetting, then incubate the mixture at room temperature for 5 min.

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.

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

6.5. Repeat step 6.4 once.

6.6. Briefly spin the tube, and put the tube back in the magnetic rack. 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.*

6.7. Remove the tube from the magnet. Elute DNA target from the beads by adding 30 µL TE-low solution or 10 mM Tris solution (not provided), mix thoroughly by vortexing for 10-15 sec or pipetting, and incubate for 3 min at room temperature.

6.8. Put the tube in the magnetic rack for 3~5 min until the solution turns clear. Without disturbing the bead pellet, transfer the supernatant into a clean 1.5 mL centrifuge tube to obtain the final library.

Note: *The purified library should be stored at -20°C for no more than two weeks if not proceed directly to sequencing.*

7. Library Quality Control (QC)

7.1. Library concentration QC: Quantify the library concentration by Quantus™ or Qubit® Fluorometer, the DNA concentration should be more than 10ng/µL, while 5~10 ng/µL was considered as risky (there is a risk of unqualified QC after data analysis).

7.2. Library fragment QC: Assess the library quality on an Agilent Bioanalyzer DNA chip, the main peak size of the DNA fragment should be at 150~400 bp, as shown in Figure 2.

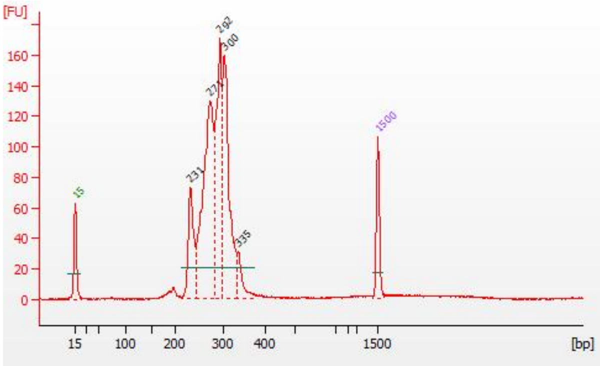

Figure 2. Example of library size distribution on a Bioanalyzer

Note:

- *If the library QC pass, then move to sequencing. If not, the library should be reconstructed.*
- *If the library concentration is less than 5 ng/μL, the original DNA or RNA may be of poor quality or the DNA or RNA concentration may be inaccurate or there may be operational errors during the experiment. The DNA concentration should be retested, and it is recommended to input 100 ng DNA and perform overnight hybridization (60 ℃ for 12~18 h) to rebuild the library.*

8. Sequencing

Illumina MiSeqDx or NextSeq 550Dx platform and corresponding Illumina 300 cycles (Paired-End Reads, 2×150 cycles) reagent is 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 and no more than 2 Gb when using NextSeq 550Dx platform, and no less than 375 Mb when using MiSeqDx platform. The suggested sample quantity per run is listed in Table 8.

Table 8. Recommended sequencing instruments and sample quantity per run

Sequencing Instrument	Flow Cell	Read Length	Sample Quantity/Run
MiSeqDx	V3	2× 150 bp	20
NextSeq 550Dx	High	2× 150 bp	up to 120#

Maximum 120 indexes available. The PhiX percentage must be adjusted to ensure over-sequencing is not performed.

Perform the denaturation and dilution of the libraries according to the instrument's instructions. The final concentration of sequencing library is recommended in Table 9.

Table 9. Recommended final concentration of sequencing library

Sequencing Instrument	Final Concentration
MiSeqDx	6~8 pM
NextSeq 550Dx	0.6~1 pM

Note: *The concentration converting formula:*

$$Library\ Concentration\ [nM] = \frac{Library\ Concentration\ [ng/\mu L] \times 10^6}{660 \times 275}$$

9. Data Analysis

When the sequencing is finished, adopt AmoyDx ANDAS Data Analyzer to analyze the sequencing data. Select the appropriate analysis module according to the sequencing platform, as shown in Table 10.

Table 10. Analysis module

Sequencing Instrument	Data Throughput	Analysis Module
MiSeqDx	375 Mb/sample	ADXHS-Classic-M
NextSeq 550Dx	1 Gb/sample	ADXHS-Classic

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.

Result Interpretation

The mutations are detected if meeting the following requirements.

- The "EffectiveDepth" of DNA should be no less than 400×, the "Depth" of each variant should be no less than 30×, and the inner RNA-Control should be no less than 20 copies.
- The Cut-off metrics are listed in Table 11.

Table 11. Cut-Off Metrics

Sample Type	Parameter	ADXHS-Classic (NextSeq 550Dx)	ADXHS-Classic-M (MiSeqDx)
DNA	Hot spot mutations of core regions (as shown in Table S4)	Freq ≥ 0.5%, ADP ≥ 4	Freq ≥ 0.5%, ADP ≥ 4
	Hot spot mutations of non-core regions, non-hot spot mutations	Freq ≥ 3%, ADP ≥ 4	Freq ≥ 3%, ADP ≥ 4
	CNA	≥ 3.5 copy number	≥ 4 copy number
	MSINum	≥ 15%	≥ 15%
RNA	Fusions	≥ 10 copies	≥ 10 copies
	MET exon14 skipping	≥ 40 copies	≥ 40 copies

- ***Important!*** *When using ADXHS-Classic-M analysis module，the detection of copy-number amplification (CNA) requires at least five samples to be extracted, library constructed, sequenced and analyzed simultaneously with reagents of the same lot. The CP-Positive Control and CP-Negative Control should be analyzed separately from the FFPE samples, otherwise it will affect the CNA detection of clinical samples.*
- ***Important!*** *If the detected MSINum value is between 12% and 26%, it is recommended to confirm the MSI status by PCR and capillary electrophoresis method.*

Note:

- *Q30: one base call in 1,000 is predicted to be incorrect meaning a base call accuracy of 99.9%.*
- *EffectiveDepth: The average depth of the target region after UMI calibration.*

- *Depth: The effective depth of the variant site after UMI calibration.*
- *Freq: Frequency of mutant allele.*
- *ADP: Depth of mutant allele.*
- *MSINum: Percentage of microsatellite instability sites.*
- *The cut-off metrics in the ADXHS-Classic and ADXHS-Classic-M analysis modules are the same, while the effective depth calibration algorithm is different.*
- *The PC should be detected as positive result for the corresponding mutation as shown in Table S5, and the NC should be detected as negative regarding the hotspot region. Otherwise, the Classic testing is unqualified, it is necessary to check if there is any operational error and the experiment should be repeated.*
- *If the RNA level test result is negative and the inner RNA-Control is less than 20 copies, it may be mainly due to poor RNA quality or insufficient amount of RNA input, or there may be some inhibitors in the RNA sample. It is recommended to re-extract RNA and reconstruct the library.*

Performances

1) Limit of Detection (LoD)

The LoD is listed in Table 12.

Table 12. The LoD of Classic panel

Analysis Module		**ADXHS-Classic (NextSeq 550Dx)**	**ADXHS-Classic-M (MiSeqDx)**
DNA	Hot spot mutations of core regions (Table S4)	Freq ≥ 1%	Freq ≥ 1%
	Hot spot mutations of non-core regions, Non-hot spot mutations	Freq ≥ 5%	Freq ≥ 5%
	CNA	≥ 4 copy number	≥ 5 copy number
	MSI Tumor Purity	≥ 10%	≥ 10%
RNA	Fusions	≥ 100 copies	≥ 200 copies
	MET exon14 skipping	≥ 250 copies	≥ 500 copies

2) Accuracy

Positive clinical FFPE specimens and positive reference standard were tested, and the positive percent agreement (PPA) for SNVs, Indels, fusions and CNAs detection was 100%, and the PPA for MSI detection was 96.9%.

3) Specificity

The negative percent agreement (NPA) for SNVs, Indels, fusions, CNAs and MSI detection was 100%.

4) Precision

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

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/RNA degradation or the frequency under the limit of detection may also cause a false negative result.

5) This kit only detects gene variants within the target region (as shown in Table S1). A negative detection result does not rule out the possible presence of other genetic variants outside the targeted region.

6) Sampling of different sites of tumor tissue may lead to different test results due to tumor heterogeneity.

7) The test results of this kit are for clinical reference only and should not be used as the sole basis for individualized treatment of patients. Clinicians should make comprehensive judgments on the test results based on factors such as the patient's condition, drug indications, treatment response and other laboratory test indicators.

8) False negative results may occur when InDels occurs on the probe binding regions.

9) The detection of MSI is only validated in FFPE samples from colorectal cancer and gastric cancer. MSI results from other cancers (eg. endometrial cancer, etc.) are for reference only.

10) The MSI status will be output as MSS or MSI. It does not distinguish between MSI-L and MSS.

11) Please strictly follow the sequence data volume recommended in the IFU, otherwise, it may lead to false negative or false positive results.

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Symbols

EU 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
i	Consult Instructions For Use		Keep Dry
	This Way Up		Fragile, Handle With Care

Appendix

Table S1. Gene Lists

No.	Gene	Transcripts	Mutation	Target Regions	Partially Covered Exons#
1	*AKT1*	NM_001014431	SNV, InDel	Exon 3,4	Exon 3,4
2	*ALK*	NM_004304	Fusion, SNV, InDel	Exon 21-25	Exon 21-25
3	*BRAF*	NM_004333	SNV, InDel	Exon 11,12,15,18	Exon 11,12,15,18
4	*CDK4*	NM_000075	CNA	/	/
5	*CTNNB1*	NM_001904	SNV, InDel	Exon 3	/
6	*DDR2*	NM_006182	SNV, InDel	Exon 5,8,13-18	Exon 5,8,13-18
7	*DPYD**	NM_000110	SNPs	rs3918290, rs55886062, rs67376798, rs75017182	/
8	*EGFR*	NM_005228	SNV, InDel	Exon 3,7,12,14,18-22	Exon 3,7,12,14,18-22
9	*ERBB2*	NM_004448	SNV, InDel, CNA	Exon 1-27	Exon 1-6,8-14,16-24,26-27
10	*ESR1*	NM_001122740	SNV, InDel	Exon 6,9	Exon 6,9
11	*FGFR1*	NM_001174067	Fusion, SNV, InDel	Exon 5,6,13,15	Exon 5,6,13,15
12	*FGFR2*	NM_000141	Fusion, SNV, InDel	Exon 3,5-9,12-16,18	Exon 3,5-9,12-16,18
13	*FGFR3*	NM_000142	Fusion, SNV, InDel	Exon 3,7-15,17	Exon 3,7-15,17
14	*FGFR4*	NM_213647	SNV, InDel	Exon 3,6,9,10,12,13,15,16	/
15	*HRAS*	NM_001130442	SNV, InDel	Exon 2-4	Exon 2-4
16	*IDH1*	NM_005896	SNV, InDel	Exon 4	Exon 4
17	*IDH2*	NM_002168	SNV, InDel	Exon 4	/
18	*KEAP1*	NM_203500	SNV, InDel	Exon 2-6	/
19	*KIT*	NM_000222	SNV, InDel	Exon 9,11,13,14,17,18	Exon 9,17
20	*KRAS*	NM_033360	SNV, InDel	Exon 2-4	Exon 2-4
21	*MAP2K1*	NM_002755	SNV, InDel	Exon 2,3,6	Exon 2,3,6
22	*MET*	NM_000245	SNV, InDel, Exon14 Skipping, CNA	Exon 3,9,12,14,15,20,21	Exon 12,15,21
23	*MYC*	NM_002467	CNA	/	/
24	*NFE2L2*	NM_006164	SNV, InDel	Exon 2	Exon 2
25	*NKX2-1*	NM_001079668	CNA	/	/
26	*NRAS*	NM_002524	SNV, InDel	Exon 2-4	Exon 2-4
27	*NRG1*	NM_013956	Fusion	/	/
28	*NTRK1*	NM_001007792	Fusion, SNV, InDel	Exon 14,15	Exon 14-15
29	*NTRK2*	NM_006180	Fusion, SNV, InDel	Exon 18,19	Exon 18-19
30	*NTRK3*	NM_002530	Fusion, SNV, InDel	Exon 16,17	Exon 16-17
31	*PDGFRA*	NM_006206	SNV, InDel	Exon 12,14,18	Exon 12
32	*PIK3CA*	NM_006218	SNV, InDel	Exon 2,3,5,6,8-10,14,21	Exon 2,3,5,6,8-10,14,21
33	*POLE*	NM_006231	SNV, InDel	Exon 3-14,19	Exon 4,5,12,19
34	*PTEN*	NM_000314	SNV, InDel	Whole CDS	/
35	*RB1*	NM_000321	SNV, InDel	Exon 1-27	Exon 1,7
36	*RET*	NM_020975	Fusion, SNV, InDel	Exon 5,6,8,10,11,13-16	Exon 5,6,8,10,11,13,14
37	*ROS1*	NM_002944	Fusion, SNV, InDel	Exon 36-38,40,41	Exon 36-38,40,41
38	*STK11*	NM_000455	SNV, InDel	Exon 1-9	Exon 3,6,9
39	*TP53*	NM_000546	SNV, InDel	Exon 2-11	Exon 2-4,11
40	*UGT1A1**	NM_000463	SNPs	rs10929302, rs8175347, rs4148323	/
41	MSI	/	55 MSI Sites	/	/

Note:

** The DPYD and UGT1A1 genes were covered to detect SNPs which are associated with cancer chemotherapy response.*

 # For certain genes, only specific regions or "hotspots" within the exons are covered, rather than the entire exonic sequence.

Table S2. Gene fusions

No.	Fusions	Genes
1	BCL11A exon4-del15059ins14-ALK exon20	*ALK*
2	BIRC6 exon10-ALK exon20	
3	CEBPZ exon2-ALK exon20	
4	CLIP1 exon22-ALK exon20	
5	COL25A1 exon3-ALK exon20	
6	DCTN1 exon26-ALK exon20	
7	EIF2AK3 exon2-ALK exon20	
8	EML4 exon13-ALK exon20	
9	EML4 exon13-ins69-ALK exon20	
10	EML4 exon14-del14-ALK exon20	
11	EML4 exon14-del38-ALK exon20	
12	EML4 exon14-del49ins11-ALK exon20	
13	EML4 exon15-del131-ALK exon20	
14	EML4 exon17-ALK exon20	
15	EML4 exon17-del46ins23-ALK exon20	
16	EML4 exon17-del46ins6-ALK exon20	
17	EML4 exon17-del58ins39-ALK exon20	
18	EML4 exon17-ins30-ALK exon20	
19	EML4 exon17-ins65-ALK exon20	
20	EML4 exon17-ins68-ALK exon20	
21	EML4 exon17-ins95-ALK exon20	
22	EML4 exon18-ALK exon20	
23	EML4 exon20-ALK exon20	
24	EML4 exon20-ins18-ALK exon20	
25	EML4 exon2-ALK exon20	
26	EML4 exon2-ins117-ALK exon20	
27	EML4 exon3-ins53-ALK exon20	
28	EML4 exon6-ALK exon19	
29	EML4 exon6-ALK exon20	
30	EML4 exon6-ins18-ALK exon20	
31	EML4 exon6-ins33-ALK exon20	
32	GCC2 exon13-ALK exon20	
33	GCC2 exon19-ALK exon20	
34	HIP1 exon21-ALK exon20	
35	HIP1 exon28-ALK exon20	
36	HIP1 exon30-ALK exon20	
37	KIF5B exon15-del14-ALK exon20	
38	KIF5B exon17-ALK exon20	
39	KIF5B exon24-ALK exon20	
40	KLC1 exon9-ALK exon20	
41	LMO7 exon15-ALK exon20	
42	MPRIP exon21-ALK exon20	
43	NBAS exon35-ALK exon20	
44	PHACTR1 exon6-ALK exon20	
45	PICALM exon19-ALK exon20	
46	PPM1B exon1-ALK exon20	
47	PRKAR1A exon10-ALK exon20	
48	PRKAR1A exon5-ALK exon20	
49	SQSTM1 exon5-ALK exon20	
50	STRN exon3-ALK exon20	
51	TFG exon4-ALK exon20	
52	TFG exon6-ALK exon20	
53	TNIP2 exon5-ALK exon20	
54	TPR exon15-ALK exon20	
55	CCDC6 exon5-ROS1 exon35	*ROS1*
56	CD74 exon6-ROS1 exon32	
57	CD74 exon6-ROS1 exon34	
58	CD74 exon6-ROS1 exon35	
59	CD74 exon8-ROS1 exon34	
60	CLTC exon31-ROS1 exon35	
61	EZR exon10-ROS1 exon34	
62	EZR exon10-ROS1 exon35	
63	GOPC exon4-ROS1 exon36	
64	GOPC exon8-ROS1 exon35	
65	KDELR2 exon5-ins8-ROS1 exon35	
66	LRIG3 exon16-ROS1 exon35	
67	MYO5A exon23-ROS1 exon35	
68	PPFIBP1 exon9-ROS1 exon35	
69	SDC4 exon2-ROS1 exon32	
70	SDC4 exon4-ROS1 exon32	
71	SDC4 exon4-ROS1 exon34	
72	SLC34A2 exon13-del47-ROS1 exon32	
73	SLC34A2 exon13-del47-ROS1 exon34	
74	SLC34A2 exon4-ROS1 exon32	
75	SLC34A2 exon4-ROS1 exon34	
76	TFG exon4-ROS1 exon35	
77	TMEM106B exon3-ROS1 exon35	
78	TPM3 exon7-ROS1 exon35	
79	TPM3 exon8-ROS1 exon35	
80	AKAP13 exon35-RET exon12	*RET*
81	CCDC186 exon10-RET exon12	
82	CCDC186 exon7-RET exon12	
83	CCDC186 exon9-RET exon12	
84	CCDC6 exon1-del107-RET exon11	
85	CCDC6 exon1-del199-RET exon11	
86	CCDC6 exon1-ins132del125-RET exon11	
87	CCDC6 exon1-RET exon10	
88	CCDC6 exon1-RET exon12	
89	CCDC6 exon1-RET exon2	
90	CCDC6 exon2-RET exon11	
91	CCDC6 exon2-RET exon12	
92	CCDC6 exon8-RET exon12	
93	CUX1 exon10-RET exon12	
94	DLG5 exon13-RET exon12	
95	ERC1 exon10-RET exon12	
96	ERC1 exon12-RET exon12	
97	ERC1 exon17-RET exon12	
98	ERC1 exon7-RET exon12	
99	FKBP15 exon25-RET exon12	
100	GOLGA5 exon7-RET exon12	
101	HOOK3 exon11-RET exon12	
102	RELCH exon10-RET exon12	
103	KIF13A exon18-RET exon12	
104	KIF5Bexon15-del107-RET exon11	
105	KIF5B exon15-RET exon12	
106	KIF5B exon16-RET exon12	
107	KIF5B exon18-RET exon12	
108	KIF5B exon22-RET exon12	

No.	Fusions	Genes
109	KIF5B exon23-RET exon12	*RET*
110	KIF5B exon24-RET exon11	
111	KIF5B exon24-RET exon8	
112	KTN1 exon29-RET exon12	
113	MPRIP exon19-RET exon12	
114	MYO5C exon25-RET exon12	
115	NCOA4 exon7-RET exon12	
116	NCOA4 exon8-del18-RET exon12	
117	NCOA4 exon8-del199-RET exon11	
118	NCOA4 exon8-RET exon12	
119	NCOA4 exon9-RET exon12	
120	PCM1 exon29-RET exon12	
121	PICALM exon19-RET exon12	
122	PRKAR1A exon7-RET exon12	
123	RUFY2 exon9-RET exon12	
124	RUFY3 exon12-RET exon12	
125	SPECC1L exon9-RET exon12	
126	TBL1XR1 exon9-RET exon12	
127	TNIP2 exon5-RET exon12	
128	TRIM24 exon9-RET exon12	
129	TRIM27 exon3-RET exon12	
130	TRIM33 exon11-RET exon12	
131	TRIM33 exon14-RET exon12	
132	TRIM33 exon16-RET exon12	
133	WAC exon3-RET exon12	
134	FGFR1 exon18-TACC1 exon7	*FGFR1*
135	BAG4 exon1-FGFR1 exon3	
136	BAG4 exon1-FGFR1 exon9	
137	BAG4 exon2-FGFR1 exon7	
138	ERLIN2 exon8-FGFR1 exon3	
139	FN1 exon22-FGFR1 exon4	
140	FN1 exon22-FGFR1 exon5	
141	FN1 exon23-FGFR1 exon4	
142	FN1 exon23-FGFR1 exon5	
143	FN1 exon28-FGFR1 exon6	
144	NSD3 exon1-FGFR1 exon3	
145	FGFR2 exon17-AFF3 exon7	*FGFR2*
146	FGFR2 exon17-AHCYL1 exon2	
147	FGFR2 exon17-AMOT exon3	
148	FGFR2 exon17-ATP6V1D exon3	
149	FGFR2 exon17-BICC1 exon10	
150	FGFR2 exon17-BICC1 exon16	
151	FGFR2 exon17-BICC1 exon18	
152	FGFR2 exon17-BICC1 exon2	
153	FGFR2 exon17-BICC1 exon3	
154	FGFR2 exon17-BICC1 exon9	
155	FGFR2 exon17-CASP7 exon3	
156	FGFR2 exon17-CCAR2 exon4	
157	FGFR2 exon17-CCDC186 exon4	
158	FGFR2 exon17-CCDC6 exon2	
159	FGFR2 exon17-CIT exon23	
160	FGFR2 exon17-COL14A1 exon34	
161	FGFR2 exon17-CREB5 exon8	
162	FGFR2 exon17-CTNNA3 exon14	
163	FGFR2 exon17-DDX21 exon2	

No.	Fusions	Genes
164	FGFR2 exon17-DZANK1 exon11	*FGFR2*
165	FGFR2 exon17-EIF4A2 exon8	
166	FGFR2 exon17-ERC1 exon7	
167	FGFR2 exon17-GAB2 exon2	
168	FGFR2 exon17-KIAA1217 exon3	
169	FGFR2 exon17-SHTN1 exon7	
170	FGFR2 exon17-SHTN1 exon9	
171	FGFR2 exon17-LZTFL1 exon8	
172	FGFR2 exon17-OGA exon12	
173	FGFR2 exon17-NOL4 exon7	
174	FGFR2 exon17-NRAP exon24	
175	FGFR2 exon17-NRBF2 exon4	
176	FGFR2 exon17-OFD1 exon3	
177	FGFR2 exon17-PAWR exon3	
178	FGFR2 exon17-PCM1 exon7	
179	FGFR2 exon17-POC1B exon11	
180	FGFR2 exon17-PPHLN1 exon3	
181	FGFR2 exon17-PPP1R21 exon16	
182	FGFR2 exon17-ROCK1 exon2	
183	FGFR2 exon17-SEPTIN10 exon6	
184	FGFR2 exon17-SLMAP exon2	
185	FGFR2 exon17-SORBS1 exon5	
186	FGFR2 exon17-STK26 exon3	
187	FGFR2 exon17-TACC2 exon11	
188	FGFR2 exon17-TACC3 exon11	
189	FGFR2 exon17-TBC1D1 exon9	
190	FGFR2 exon17-TP73 exon2	
191	FGFR2 exon17-TXLNA exon5	
192	FGFR2 exon17-WAC exon5	
193	FGFR2 exon18-BICC1 exon2	
194	FGFR2 exon18-LAMC1 exon27	
195	FGFR2 exon18-RABGAP1L exon20	
196	APIP exon1-FGFR2 exon10	
197	APIP exon1-FGFR2 exon6	
198	KLK2 exon1-FGFR2 exon5	
199	SLC45A3 exon1-FGFR2 exon2	
200	FGFR3 exon17-TLE5 exon2	*FGFR3*
201	FGFR3 exon17-AMBRA1 exon16	
202	FGFR3 exon17-BAIAP2L1 exon2	
203	FGFR3 exon17-del49-TACC3 exon4	
204	FGFR3 exon17-ELAVL3 exon2	
205	FGFR3 exon17-FBXO28 exon4	
206	FGFR3 exon17-JAKMIP1 exon4	
207	FGFR3 exon17-PHLDB3 exon10	
208	FGFR3 exon17-TACC3 exon10	
209	FGFR3 exon17-TACC3 exon11	
210	FGFR3 exon17-TACC3 exon12	
211	FGFR3 exon17-TACC3 exon13	
212	FGFR3 exon17-TACC3 exon14	
213	FGFR3 exon17-TACC3 exon2	
214	FGFR3 exon17-TACC3 exon3	
215	FGFR3 exon17-TACC3 exon6	
216	FGFR3 exon17-TACC3 exon7	
217	FGFR3 exon17-TACC3 exon8	
218	FGFR3 exon18-del112-TACC3 exon10	

No.	Fusions	Genes
219	FGFR3 exon18-del117-TACC3 exon8	*FGFR3*
220	FGFR3 exon18-del124-TACC3 exon9	
221	FGFR3 exon18-del125-TACC3 exon11	
222	FGFR3 exon18-del147-TACC3 exon7	
223	FGFR3 exon18-del27-TACC3 exon11	
224	FGFR3 exon18-del37ins15-TACC3 exon9	
225	ADAM9 exon18-NRG1 exon2	*NRG1*
226	AKAP13 exon5-NRG1 exon2	
227	ATP1B1 exon2-NRG1 exon2	
228	CD44 exon5-NRG1 exon2	
229	CD74 exon6-NRG1 exon3	
230	CD74 exon6-NRG1 exon6	
231	CD74 exon7-NRG1 exon2	
232	CD74 exon7-NRG1 exon6	
233	CD74 exon8-NRG1 exon6	
234	CDH1 exon2-NRG1 exon2	
235	CLU exon2-NRG1 exon6	
236	COX10-AS1 exon1-NRG1 exon2	
237	DIP2B exon1-NRG1 exon2	
238	DPYSL2 exon7-NRG1 exon6	
239	GDF15 exon1-NRG1 exon2	
240	HMBOX1 exon1-NRG1 exon6	
241	KIF13B exon2-NRG1 exon2	
242	MCPH1 exon10-NRG1 exon2	
243	MDK exon4-NRG1 exon6	
244	MRPL13 exon2-NRG1 exon2	
245	MTSS1 exon3-NRG1 exon2	
246	NOTCH2 exon4-NRG1 exon6	
247	PARP8 exon3-NRG1 exon2	
248	PCM1 exon2-NRG1 exon6	
249	PDE7A exon3-NRG1 exon6	
250	POMK exon2-NRG1 exon2	
251	RAB3IL1 exon9-NRG1 exon6	
252	RALGAPA1 exon20-NRG1 exon6	
253	RBPMS exon5-NRG1 exon2	
254	RBPMS exon5-NRG1 exon6	
255	ROCK1 exon1-NRG1 exon2	
256	SDC4 exon2-NRG1 exon4	
257	SDC4 exon2-NRG1 exon6	
258	SDC4 exon4-NRG1 exon6	
259	SETD4 exon2-NRG1 exon2	
260	SLC3A2 exon4-NRG1 exon6	
261	SLC3A2 exon5-NRG1 exon6	
262	SLC4A4 exon14-NRG1 exon2	
263	SMAD4 exon1-NRG1 exon6	
264	TENM4 exon12-NRG1 exon2	
265	THAP7 exon3-NRG1 exon2	
266	THBS1 exon6-NRG1 exon6	
267	TNC exon10-NRG1 exon6	
268	TNKS exon3-NRG1 exon2	
269	TSHZ2 exon1-NRG1 exon6	
270	VAMP2 exon4-NRG1 exon4	
271	VTCN1 exon2-NRG1 exon4	
272	NSD3 exon1-NRG1 exon2	
273	WRN exon30-NRG1 exon2	
274	ZMYM2 exon2-NRG1 exon2	*NRG1*
275	AFAP1 exon4-NTRK1 exon9	*NTRK1*
276	AMOTL2 exon6-NTRK1 exon12	
277	ARHGEF2 exon21-NTRK1 exon10	
278	ATP1B1 exon2-NTRK1 exon8	
279	ARHGEF11 exon40-NTRK1 exon12	
280	BCAN exon12-NTRK1 exon10	
281	BCAN exon13-NTRK1 exon11	
282	CD74 exon8-NTRK1 exon10	
283	CD74 exon8-NTRK1 exon12	
284	CEL exon7-NTRK1 exon8	
285	CHTOP exon5-NTRK1 exon10	
286	CHTOP exon5-NTRK1 exon11	
287	CTRC exon1-NTRK1 exon8	
288	DIAPH1 exon26-NTRK1 exon10	
289	EPHB2 exon3-NTRK1 exon8	
290	EPS15 exon21-NTRK1 exon10	
291	F11 exon4-NTRK1 exon10	
292	F11R exon4-NTRK1 exon10	
293	GRIPAP1 exon22-NTRK1 exon10	
294	GRIPAP1 exon22-NTRK1 exon11	
295	GRIPAP1 exon22-NTRK1 exon12	
296	GON4L exon21-NTRK1 exon8	
297	IRF2BP2 exon1-del48-NTRK1 exon10	
298	IRF2BP2 exon1-NTRK1 exon10	
299	IRF2BP2 exon1-NTRK1 exon8	
300	KIF21B exon14-NTRK1 exon10	
301	LMNA exon2-NTRK1 exon10	
302	LMNA exon4-NTRK1 exon10	
303	LMNA exon9-NTRK1 exon10	
304	LMNA exon9-NTRK1 exon12	
305	LMNA exon10-NTRK1 exon10	
306	LMNA exon10-NTRK1 exon11	
307	LMNA exon10-NTRK1 exon12	
308	LMNA exon11-NTRK1 exon12	
309	LMNA exon11-del150-NTRK1 exon11	
310	LMNA exon12-NTRK1 exon12	
311	LMNA exon2-NTRK1 exon11	
312	LMNA exon2-NTRK1 exon12	
313	LMNA exon2-NTRK1 exon16	
314	LMNA exon3-NTRK1 exon11	
315	LMNA exon4-NTRK1 exon12	
316	LMNA exon5-NTRK1 exon11	
317	LMNA exon6-del172-NTRK1 exon12	
318	LMNA exon8-NTRK1 exon12	
319	LMNA UTR3-NTRK1 exon12	
320	LRRC71 exon1-NTRK1 exon10	
321	MEF2D exon9-NTRK1 exon12	
322	MPRIP exon14-NTRK1 exon12	
323	MPRIP exon18-NTRK1 exon12	
324	MPRIP exon21-NTRK1 exon12	
325	MPRIP exon21-NTRK1 exon14	
326	MTMR6 exon1-NTRK1 exon8	
327	NFASC exon20-NTRK1 exon10	
328	NFASC exon21-NTRK1 exon10	

No.	Fusions	Genes
329	PEAR1 exon15-NTRK1 exon10	
330	PLEKHA6 exon14-NTRK1 exon10	
331	PLEKHA6 exon21-NTRK1 exon10	
332	PLEKHA6 exon21-NTRK1 exon9	
333	PPL exon11-NTRK1 exon13	
334	PPL exon21-NTRK1 exon10	
335	PPL exon21-NTRK1 exon11	
336	PPL exon22-del3058-NTRK1 exon11	
337	PRDX1 exon5-NTRK1 exon12	
338	RPL7A exon2-NTRK1 exon10	
339	SCYL3 exon11-NTRK1 exon12	
340	SQSTM1 exon2-NTRK1 exon10	
341	SQSTM1 exon5-NTRK1 exon10	
342	SQSTM1 exon6-NTRK1 exon10	
343	SSBP2 exon12-NTRK1 exon12	
344	TFG exon4-NTRK1 exon9	
345	TFG exon5-del258-NTRK1 exon8	
346	TFG exon5-NTRK1 exon10	
347	TFG exon5-NTRK1 exon9	
348	TFG exon6-NTRK1 exon10	
349	TNFSF15 exon1-NTRK1 exon11	*NTRK1*
350	TPM3 exon1-del132-NTRK1 exon12	
351	TPM3 exon5-NTRK1 exon11	
352	TPM3 exon5-NTRK1 exon12	
353	TPM3 exon7-NTRK1 exon10	
354	TPM3 exon7-del39-NTRK1 exon10	
355	TPM3 exon8-NTRK1 exon10	
356	TPM3 exon8-NTRK1 exon12	
357	TPM3 exon9-NTRK1 exon10	
358	TPM3 exon10-NTRK1 exon8	
359	TPM3 exon10-NTRK1 exon9	
360	TPM3 UTR3-NTRK1 exon9	
361	TPR exon10-NTRK1 exon10	
362	TPR exon16-del54ins13-NTRK1 exon10	
363	TPR exon21-NTRK1 exon10	
364	TPR exon21-NTRK1 exon9	
365	TPR exon22-NTRK1 exon10	
366	TPR exon6-NTRK1 exon12	
367	TRIM33 exon12-NTRK1 exon12	
368	TRIM63 exon8-NTRK1 exon9	
369	VANGL2 exon1-NTRK1 exon12	
370	ZBTB7B exon4-NTRK1 exon12	
371	AFAP1 exon13-NTRK2 exon12	
372	AGBL4 exon5-NTRK2 exon16	
373	AGBL4 exon6-NTRK2 exon16	
374	AGBL4 exon7-NTRK2 exon16	
375	BCR exon1-NTRK2 exon17	
376	ETV6 exon5-NTRK2 exon15	
377	GKAP1 exon9-NTRK2 exon16	*NTRK2*
378	KANK1 exon11-NTRK2 exon14	
379	KCTD8 exon1-NTRK2 exon16	
380	NACC2 exon5-NTRK2 exon13	
381	NOS1AP exon9-NTRK2 exon13	
382	PAN3 exon1-NTRK2 exon17	
383	PRKAR2A exon2-NTRK2 exon16	

No.	Fusions	Genes
384	QKI exon6-NTRK2 exon16	
385	SPECC1L exon10-NTRK2 exon15	
386	SPECC1L exon11-NTRK2 exon15	
387	SQSTM1 exon4-NTRK2 exon15	
388	SQSTM1 exon5-NTRK2 exon16	
389	SQSTM1 exon5-NTRK2 exon17	
390	STRN exon3-NTRK2 exon16	
391	STRN3 exon7-NTRK2 exon16	*NTRK2*
392	TBC1D2 exon6-NTRK2 exon14	
393	TLE4 exon7-NTRK2 exon15	
394	TRAF2 exon9-NTRK2 exon15	
395	TRIM24 exon12-NTRK2 exon15	
396	TRIM24 exon12-NTRK2 exon16	
397	VCAN exon6-NTRK2 exon12	
398	VCL exon16-NTRK2 exon12	
399	WNK2 exon24-NTRK2 exon16	
400	AKAP13 exon3-NTRK3 exon14	
401	AKAP13 exon14-NTRK3 exon14	
402	AKAP13 exon16-NTRK3 exon14	
403	BTBD1 exon4-NTRK3 exon14	
404	EEF1A1 exon8-NTRK3 exon14	
405	EML4 exon2-NTRK3 exon14	
406	EML4 exon6-NTRK3 exon14	
407	ETV6 exon4-NTRK3 exon12	
408	ETV6 exon4-NTRK3 exon13	
409	ETV6 exon4-NTRK3 exon14	
410	ETV6 exon4-NTRK3 exon15	
411	ETV6 exon5-NTRK3 exon13	
412	ETV6 exon5-NTRK3 exon14	
413	ETV6 exon5-NTRK3 exon15	
414	ETV6 exon5-NTRK3 exon16	
415	ETV6 exon6-NTRK3 exon13	
416	ETV6 exon6-NTRK3 exon15	
417	KANK1 exon2-NTRK3 exon14	
418	LYN exon8-NTRK3 exon14	*NTRK3*
419	MYO5A exon23-NTRK3 exon11	
420	MYO5A exon23-NTRK3 exon12	
421	MYO5A exon32-NTRK3 exon13	
422	PHACTR1 exon5-NTRK3 exon14	
423	PHACTR1 exon7-NTRK3 exon14	
424	RBPMS exon5-NTRK3 exon14	
425	SNHG26 exon3-NTRK3 exon16	
426	SPECC1L exon5-NTRK3 exon14	
427	SPECC1L exon8-NTRK3 exon13	
428	SQSTM1 exon4-NTRK3 exon14	
429	SQSTM1 exon5-NTRK3 exon14	
430	SQSTM1 exon6-NTRK3 exon15	
431	SQSTM1 exon7-NTRK3 exon14	
432	STRN exon3-NTRK3 exon14	
433	STRN3 exon3-NTRK3 exon14	
434	TFG exon6-NTRK3 exon14	
435	TMTC2 exon9-NTRK3 exon15	
436	VIM exon8-NTRK3 exon14	
437	WDR72 exon18-NTRK3 exon15	
/	/	/

Table S3. Index sequence information for primers

Name	Primer Index Information	Illumina Nextera XT v2Set D No.
CP-N716	TAGCGAGT	N716
CP-N718	GTAGCTCC	N718
CP-N719	TACTACGC	N719
CP-N720	AGGCTCCG	N720
CP-N721	GCAGCGTA	N721
CP-N722	CTGCGCAT	N722
CP-N723	GAGCGCTA	N723
CP-N724	CGCTCAGT	N724
CP-N726	GTCTTAGG	N726
CP-N727	ACTGATCG	N727
CP-N728	TAGCTGCA	N728
CP-N729	GACGTCGA	N729
CP-S502	CTCTCTAT	S502
CP-S503	TATCCTCT	S503
CP-S513	TCGACTAG	S513
CP-S515	TTCTAGCT	S515
CP-S516	CCTAGAGT	S516
CP-S517	GCGTAAGA	S517
CP-S518	CTATTAAG	S518
CP-S520	AAGGCTAT	S520
CP-S521	GAGCCTTA	S521
CP-S522	TTATGCGA	S522

Table S4. Core regions (sensitivity of 1%, tagged as VIP in the ANDAS output file)

No.	Gene	Core Regions
01	*BRAF*	NM_004333: exon15
02	*EGFR*	NM_005228: Exon18
03	*EGFR*	NM_005228: Exon19
04	*EGFR*	NM_005228: Exon20
05	*EGFR*	NM_005228:Exon21
06	*ERBB2*	NM_004448:Exon20
07	*KRAS*	NM_033360:Exon2
08	*KRAS*	NM_033360:Exon3
09	*NRAS*	NM_002524:Exon2
10	*NRAS*	NM_002524:Exon3
11	*PIK3CA*	NM_006218:Exon10
12	*PIK3CA*	NM_006218:Exon21

Table S5. Hot spot mutations (tagged as VIP in the ANDAS output file) and fusions of CP-Positive Control (DNA&RNA)

No.	Gene	Mutation
01	*EGFR*	NM_005228:exon20:c.2369C>T:p.(T790M)
02	*EGFR*	NM_005228:exon21:c.2573T>G:p.(L858R)
03	*EGFR*	NM_005228:exon19:c.2235_2249del15:p.(E746_A750del)
04	*KRAS*	NM_033360:exon2:c.38G>A:p.(G13D)
05	*PIK3CA*	NM_006218:exon10:c.1633G>A:p.(E545K)
06	*PIK3CA*	NM_006218:exon10:c.1645G>A:p.(D549N)
07	*ROS1*	SLC34A2:NM_006424:exon4-ROS1:NM_002944:exon32
08	*ROS1*	SLC34A2:NM_006424:exon4-ROS1:NM_002944:exon34
09	/	MSI-H

Note: Please note that there is an additional positive variant in PC, PIK3CA Exon3: c.353G>A:p.(G118D), but this variant is not necessary for quality control.

It will be detected under normal circumstances, but occasionally they may be missed.