AmoyDx® Pan Lung Cancer PCR Panel

Instructions for Use

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

REF 8.01.0234 8 tests/kit For LightCycler480 II, cobas® z480, Bio-Rad CFX96

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Background

Lung cancer is one of the most common malignant tumor, and 80~85% of lung cancers are non-small cell lung cancer (NSCLC). There are many driver mutations in NSCLC. The frequency of mutations in NSCLC for *EGFR*, *HER2, KRAS* and *BRAF* genes are respectively 10~50% [1], 1~4% [2-3], 5~25% [4-6] and 1~2% [7-8]. About 3~7% [9-12], 1% [13-14], 1% [13, 15-17], 0.12% [18], 0.02% [18], 0.08% [18] of NSCLC patients have gene fusions in *ALK*, *ROS1*, *RET*, *NTRK1*, *NTRK2* and *NTRK3* genes, and approximately 1% of lung adenocarcinoma patients harbor *MET* exon 14 skipping mutations [19]. Targeted therapies have been developed and approved for use in patients whose tumors have some of the genomic alterations seen in NSCLC. For instance, there are approved *EGFR* inhibitors [20-21], *ALK* inhibitors [22-23], *ROS1* inhibitors [24-25], *NTRK* inhibitors [25-27] and *BRAF* inhibitors [7, 28] for patients with specific genomic alterations in these genes. Testing for genomic alterations is a requirement in order to identify patients that may benefit from these targeted therapies and testing of multiple genomic alterations is recommended by the NCCN guidelines [29]. Furthermore, there are many drugs in late stage development for other alterations (*RET* [30], *MET* [31], *HER2* [32], and *KRAS* [33]).

Intended Use

The AmoyDx® Pan Lung Cancer PCR Panel is a real-time PCR assay for qualitative detection of 167 hotspot alterations in *EGFR*, *ALK*, *ROS1*, *KRAS*, *BRAF*, *HER2*, *RET*, *MET*, *NTRK1*, *NTRK2* and *NTRK3* genes. The kit is intended to be used to aid clinician to identify multi-gene status for NSCLC patients.

The kit is for research use only, and intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

This kit contains RNA gene fusion detection system in LEG Reaction Mix A and DNA gene mutation detection system in LEG Reaction Mix B.

The RNA gene fusion detection includes two processes: 1) Reverse Transcription: extracted RNA from FFPE or fresh tumor tissue is employed in this step, reverse transcription of target RNA enables complementary DNA (cDNA) synthesis with the action of reverse transcriptase and specific primers. 2) PCR Amplification: the specific primers are designed for amplification of cDNA, and *ALK*, *ROS1*, *RET*, *MET*, *NTRK1*, *NTRK2* and *NTRK3* variant amplicon is detected by fluorescent probes.

The DNA gene mutation detection system uses ADx-ARMS technology, which comprises specific primers and fluorescent probes to detect gene mutations. During the amplification, the target mutant DNA is matched with the bases at 3' end of the primer, and amplified efficiently, then the mutant amplicon is detected by fluorescent-labeled probes. While the wild-type DNA cannot be matched with specific primers, there is no amplification occurs.

The kit contains LEG Reaction Mix A strips, LEG Reaction Mix B strips, LEG RT Reaction Mix, sufficient positive control and enzyme.

1) **LEG Reaction Mix A strips** are designed for RNA fusion detection and internal control detection. The LEG Reaction Mixes A1~A8 include primers and FAM-labeled probes specific for detection of *ALK*/*NTRK1*/*NTRK2*/*NTRK3*/*ROS1*/*RET* gene fusions and *MET* exon14 skipping mutation, and the LEG Reaction Mixes A4/A8 also contain primers and VIC-labeled probe for detection of housekeeping gene *HPRT1* as reference gene to assess the RNA quality.

2) **LEG Reaction Mix B strips** are designed for DNA mutation detection and external control detection. The Reaction Mixes B1~B7 include primers and FAM/VIC/ROX-labeled probes specific for detection of hotspot mutations in *EGFR*, *HER2*, *KRAS* and *BRAF* genes. And the LEG Reaction Mix B8 contains DNA external control reaction mix, which is composed of primer and FAM/VIC/ROX-labeled probes for detection of a region of genomic DNA that has no known mutations or polymorphisms, to assess the DNA quality.

3) The **LEG RT Reaction Mix I** contain primers specific for reverse transcription of mRNA of *ALK*, *NTRK1, NTRK2, NTRK3* gene and reference gene into cDNA.

4) The **LEG RT Reaction Mix II** contain primers specific for reverse transcription of mRNA of *ROS1*, *RET*, *MET* gene and reference gene into cDNA.

5) The **LEG Reverse Transcriptase** is for reverse transcription of mRNA of target genes and reference gene into cDNA.

6) The **LEG Enzyme Mix A** and **LEG Enzyme Mix B** contains the Taq DNA polymerase for PCR amplification and uracil-N-glycosylase which works at room temperature to prevent PCR amplicon carryover contamination.

7) The **LEG Positive Control** contains recombinant gene with *EGFR*, *KRAS*, *BRAF*, *HER2*, *ALK*, *ROS1*, *RET*, *MET*, *NTRK1*, *NTRK2* and *NTRK3* alternations.

Kit Contents

This kit contains the following materials:

Table 1 Kit Contents

Content	Main Ingredients	Quantity
LEG Reaction Mix A (FU)	Primers, probe, Mg^{2+}, dNTPs	8-tube strip* ×12
LEG Reaction Mix B (MU)	Primers, probe, Mg^{2+}, dNTPs	8-tube strip* ×12
LEG RT Reaction Mix I	Primers, Mg^{2+}, dNTPs	220 µL/tube ×1
LEG RT Reaction Mix II	Primers, Mg^{2+}, dNTPs	220 µL/tube ×1
LEG Reverse Transcriptase	Reverse Transcriptase	16 µL/tube ×1
LEG Enzyme Mix A	Taq DNA Polymerase, Uracil-N-Glycosylase	45 µL/tube ×1
LEG Enzyme Mix B	Taq DNA Polymerase, Uracil-N-Glycosylase	45 µL/tube ×1
LEG Positive Control	Plasmid DNA	500 µL/tube ×1

*Each strip (8-tube) includes the following contents (Tables 2~3):

Table 2 Information of **LEG Reaction Mix A**

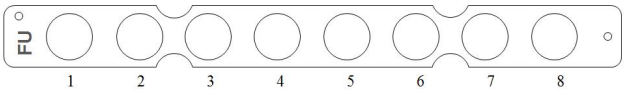
Tube No.	Reagent	Target to detect	Quantity	Florescence Signal
①	LEG Reaction Mix A1	*ALK* Fusions	35 µL	FAM
②	LEG Reaction Mix A2	*NTRK1* Fusions	35 µL	FAM
③	LEG Reaction Mix A3	*NTRK2* Fusions	35 µL	FAM
④	LEG Reaction Mix A4	*NTRK3* Fusions & *HPRT1*	35 µL	FAM, VIC
⑤	LEG Reaction Mix A5	*ROS1* Fusions	35 µL	FAM
⑥	LEG Reaction Mix A6	*ROS1* Fusions	35 µL	FAM
⑦	LEG Reaction Mix A7	*MET* exon 14 skipping mutation	35 µL	FAM
⑧	LEG Reaction Mix A8	*RET* Fusions & *HPRT1*	35 µL	FAM, VIC

Table 3 Information of **LEG Reaction Mix B**

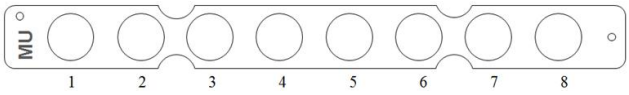
Tube No.	Reagent	Target to detect	Quantity	Florescence Signal
①	LEG Reaction Mix B1	*EGFR* Mutations	35 μL	FAM, VIC
②	LEG Reaction Mix B2	*EGFR* Mutations	35 μL	FAM, VIC
③	LEG Reaction Mix B3	*EGFR* Mutations	35 μL	FAM, VIC
④	LEG Reaction Mix B4	*EGFR/HER2* Mutations	35 μL	FAM, VIC
⑤	LEG Reaction Mix B5	*EGFR/KRAS* Mutations	35 μL	FAM, VIC
⑥	LEG Reaction Mix B6	*KRAS/HER2* Mutations	35 μL	FAM, VIC
⑦	LEG Reaction Mix B7	*KRAS/BRAF/EGFR* Mutations	35 μL	FAM, VIC, ROX
⑧	LEG Reaction Mix B8	External Control	35 μL	FAM, VIC, ROX

Note: *Distinguish Tube ⑧ from Tube ① according to the label and hole position at the strip edge, described as follows.*

For LEG Reaction Mix A:



For LEG Reaction Mix B:



Storage and Stability

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

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

Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instrument: LightCycler480 II, cobas® z480, and Bio-Rad CFX96.
2) DNA/RNA extraction kit: we recommend use of AmoyDx extraction kit (AmoyDx® FFPE DNA/RNA Kit for FFPE tumor tissue, or AmoyDx® Tissue DNA Kit, AmoyDx® Tissue RNA Kit for fresh tumor tissue).
3) Spectrophotometer for measuring DNA/RNA concentration.
4) Mini centrifuge with rotor for centrifuge tubes.
5) Mini centrifuge with rotor for PCR tubes.
6) Vortexer.
7) Nuclease-free PCR tubes and caps.
8) Nuclease-free centrifuge tubes.
9) Adjustable pipettors and filtered pipette tips for handling DNA/RNA.
10) Tube racks.
11) Disposable powder-free gloves.
12) Sterile, nuclease-free water.
13) 1×TE buffer (pH 8.0).

Precautions and Handling Requirements

Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use, and strictly follow the instruction during operation.
- Please check the compatibility of the real-time PCR instruments prior to use.
- DO NOT use the kit or any kit component after their expiry date.
- DO NOT use any other reagents from different lots in the tests.
- DO NOT use any other reagent in the other test kits.

Safety Information

- Handle all specimens and components of the kit as potentially infectious material using safe laboratory procedures.
- As all the chemicals have potential hazard, only trained professionals can use this kit. Please wear suitable lab coat and disposable gloves while handling the reagents.
- Avoid skin, eyes and mucous membranes contact with the chemicals. In case of contact, flush with water immediately.
- DO NOT pipet by mouth.

Decontamination and Disposal

- The kit contains positive control; strictly distinguish the positive control from other reagents to avoid contamination which may cause false positive.
- PCR amplification is extremely sensitive to cross-contamination. The flow of tubes, racks, pipets and other materials used should be from pre-amplification to post-amplification, and never backwards.
- 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 DNA/RNA contamination to the reagents.
- Please pack the post-amplification tubes with two disposable gloves and discard properly. DO NOT open the post- amplification PCR tubes.
- All disposable materials are for one time use. DO NOT reuse.
- The unused reagents, used kit, and waste must be disposed of properly.

Cleaning

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

Instrument Setup

- Setup the reaction volume as 40 μL.

- For LightCycler480 II and cobas® z480 instrument, it's necessary to conduct Color Compensation prior to the first use according to Color Compensation instructions. Please contact AmoyDx Technical Support or Account Manager to get the AmoyDx® Pan Lung Cancer Detection Reagent (V2) (for Color Compensation) and Color Compensation instructions.
- Refer to the real-time PCR instrument operator's manual for detailed instructions.
- We recommend that all PCR instruments in use should be conducted fluorescence calibration once a year.

Assay Procedure

1. DNA/RNA Extraction

The specimen material must be human genomic DNA and total RNA extracted from tumor tissue samples. DNA/RNA extraction reagents are not included in the kit. It's better to use tumor tissue samples with more than 20% tumor content.

The $OD_{260/280}$ value of extracted DNA and RNA should be between 1.7~2.1.

The total RNA concentration for gene fusion detection is shown in Table 4.

Table 4 Recommended RNA concentration

Sample type	Storage time	RNA concentration	Remark
FFPE tissue	≤ 2 years	10~100 ng/μL	• If RNA is between 10~100 ng/μL, use the original RNA without dilution; • If RNA is more than 100 ng/μL, dilute the RNA to 100 ng/μL.
Fresh tissue	/	2~30 ng/μL	• If RNA is between 2~30 ng/μL, use the original RNA without dilution; • If RNA is more than 30 ng/μL, dilute the RNA to 30 ng/μL.

The amount of extracted DNA for gene mutation detection is shown in Table 5.

Table 5 Recommended DNA concentration

Sample type	Storage time	DNA concentration	DNA amount/reaction
FFPE tissue	≤ 3 months	1.5 ng/μL	7.5 ng
	> 3 months & ≤ 1 year	2 ng/μL	10 ng
	> 1 year & ≤ 2 years	2.5~3 ng/μL	12.5~15 ng
Fresh tissue	/	0.5~1 ng/μL	2.5~5 ng

Note:

- *The FFPE tissue should be handled and stored properly, and the storage time should preferably be less than 2 years.*
- *The extracted DNA should be used immediately, if not, it should be stored appropriately, usually at -20±5℃ for no more than 6 months.*
- *The extracted RNA should be used immediately, if not, it should be stored appropriately, usually at -20±5℃ for no more than 3 months.*
- *The extracted DNA/RNA shall be measured by the spectrophotometer, the NanoDrop 1000 /2000 spectrophotometer is recommended.*
- *Before detection, dilute the extracted DNA with 1×TE buffer (pH 8.0) to designated concentration; dilute the extracted RNA with nuclease-free water to designated concentration. We recommend using at least 5 μL DNA for 10 times dilution, to ensure the validity of final concentration.*

2. RNA Reverse Transcription

1) Take **LEG RT Reaction Mix I, LEG RT Reaction Mix II** and **LEG Reverse Transcriptase** out of the kit from the freezer, and

other reagents remained in freezer at -20±5℃.

2) Thaw the **LEG RT Reaction Mix I** and **LEG RT Reaction Mix II** at room temperature. When the reagents completely thawed, mix each reagent by vortexing and centrifuge for 5~10 seconds to collect all liquid at the bottom of the tube.

3) Centrifuge **LEG Reverse Transcriptase** for 5~10 seconds prior to use.

4) For each RNA sample, prepare RNA reverse transcription solutions containing LEG Reverse Transcriptase, Sample RNA, and RT Reaction Mix (**LEG RT Reaction Mix I** or **LEG RT Reaction Mix II**, respectively) in separate 0.2 mL PCR tube according to the ratio in Table 6. Thoroughly mix each reverse transcription solution by vortexing, and centrifuge for 5~10 seconds.

Table 6 RNA Reverse Transcription Solutions

Reagent	Volume per test
LEG RT Reaction Mix	18.5 µL
LEG Reverse Transcriptase	0.5 µL
Sample RNA	6 µL
Total	**25 µL**

5) Incubate the tubes at 42℃ for one hour.

6) Heat the tubes at 95℃ for 5 minutes, then transfer the PCR tubes on the ice. The resulting **Sample cDNA** are ready for PCR amplification. Mark the solutions as S-cDNA 1 and S-cDNA 2, (if more samples, name as S1-cDNA 1, S2-cDNA 1, …, Sn-cDNA 1 and S1-cDNA 2, S2-cDNA 2, …, Sn-cDNA 2)

Note: *sample cDNA should be used immediately, if not, it should be stored at -20±5 ℃ for no more than 3 days after reverse transcription.*

3. RNA and DNA Mutations Detection

Note:

- *Each PCR run must contain one Positive Control (PC) and one Negative Control (NTC).*
- *The prepared mixtures should be used immediately, avoid prolonged storage.*
- *Due to the viscosity of the enzyme mix, pipet slowly to ensure all mix is completely dispensed from the tip.*
- *Pipet enzyme mix by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.*

1) Take out the **LEG Positive Control** (PC) and thaw the reagents at room temperature. When the reagents completely thawed, mix each reagent by vortexing and centrifuge for 5~10 seconds to collect all liquid at the bottom of the tube.

2) Take out the **LEG Enzyme Mix A** and **LEG Enzyme Mix B**, centrifuge for 5~10 seconds prior to use.

3) Take out the sample cDNA, sample DNA and nuclease-free water for NTC.

4) For **RNA detection**:

   a) Prepare S-Mix A1 and S-Mix A2: Add 1.3 µL **LEG Enzyme Mix** A into the above 25 µL **S cDNA 1** and **S cDNA 2** tube respectively. Mark the solutions as S-Mix A1 and S-Mix A2. Mix each solution thoroughly by vortexing, and centrifuge for 5~10 seconds.

   b) Prepare N-Mix A and P-Mix A: Add 2.34 µL **LEG Enzyme Mix** A into 45 µL nuclease-free water and 45 µL **LEG Positive Control**, respectively. Mark the solutions as N-Mix A and P-Mix A. Mix each solution thoroughly by vortexing, and centrifuge

for 5~10 seconds.

c) Take out **LEG Reaction Mix A** strips (sufficient for samples, PC and NTC) and centrifuge the strips. Then gently uncover the caps prior to use.

d) Prepare one **LEG Reaction Mix A** strip for NTC: Add 5 μL N-Mix A into Tube ①~⑧, cap the PCR tubes.

e) Prepare one **LEG Reaction Mix A** strip for each sample: Add 5 μL S-Mix A1 into Tube ①~④, 5 μL S-Mix A2 into Tube ⑤~⑧, Cap the PCR tubes.

f) Prepare one **LEG Reaction Mix A** strip for PC: Add 5 μL P-Mix A into Tube ①~⑧, cap the PCR tubes.

5) For **DNA detection**:

a) Prepare LEG Master Mix B: Add 2.7 μL **LEG Enzyme Mix B** into 45 μL sample DNA/45 μL nuclease-free water/45 μL **LEG Positive Control**, respectively. Mark the solutions as S-Mix B (if more samples, name as S1-Mix B, S2-Mix B, …, Sn-Mix B), N-Mix B, P-Mix B. Mix each solution thoroughly by vortexing and centrifuge for 5~10 seconds.

b) Take out **LEG Reaction Mix B** strips (sufficient for samples, PC and NTC) and centrifuge the strips. Then gently uncover the caps prior to use.

c) Prepare one **LEG Reaction Mix B** strip for NTC: Add 5 μL N-Mix B into Tube ①~⑧, and cap the PCR tubes.

d) Prepare one **LEG Reaction Mix B** strip for each sample: Add 5 μL S-Mix B into Tube ①~⑧, and cap the PCR tubes.

e) Prepare one **LEG Reaction Mix B** strip for PC: Add 5 μL P-Mix B to Tube ①~⑧, and cap the PCR tubes.

6) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.

7) Place the PCR tubes into the appropriate positions of the real-time PCR instrument. A recommended plate layout is shown in Table 7.

Table 7 Suggested PCR Plate Layout

	RNA Detection						DNA Detection					
Well	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Sample1	Sample2	Sample3	Sample4	NTC	PC	Sample1	Sample2	Sample3	Sample4	NTC	PC
B	Sample1	Sample2	Sample3	Sample4	NTC	PC	Sample1	Sample2	Sample3	Sample4	NTC	PC
C	Sample1	Sample2	Sample3	Sample4	NTC	PC	Sample1	Sample2	Sample3	Sample4	NTC	PC
D	Sample1	Sample2	Sample3	Sample4	NTC	PC	Sample1	Sample2	Sample3	Sample4	NTC	PC
E	Sample1	Sample2	Sample3	Sample4	NTC	PC	Sample1	Sample2	Sample3	Sample4	NTC	PC
F	Sample1	Sample2	Sample3	Sample4	NTC	PC	Sample1	Sample2	Sample3	Sample4	NTC	PC
G	Sample1	Sample2	Sample3	Sample4	NTC	PC	Sample1	Sample2	Sample3	Sample4	NTC	PC
H	Sample1	Sample2	Sample3	Sample4	NTC	PC	Sample1	Sample2	Sample3	Sample4	NTC	PC

8) Setup the PCR Protocol using the cycling parameters in Table 8.

Table 8 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	42℃	5min	/
		94℃	5min	/
2	10	94℃	25s	/
		63℃	20s	/
		71℃	20s	/
3	36	92℃	25s	/
		59℃	35s	FAM, VIC and ROX
		71℃	20s	/

9) Start the PCR run immediately.

10) When the PCR run is finished, analyze the data according to the "Results Interpretation" procedures.

4. Result Interpretation

General recommendation for threshold setting:

It's better to adjust the threshold value manually. Adjust the threshold value by each reaction mix:

For LightCycler480 II and cobas® z480 instrument, adjust the threshold value as follows:

a. For each reaction mix, choose Positive Control;

b. Display the amplification curve in "Noise Band" view;

c. Adjust the "Noise Band" value at the plateau range of the amplification curve, record the Noise Band value (Figure 1);

d. Set the Noise Band value as 6% × Noise Band value the plateau range, click Calculate to obtain and read the Ct values (Figure 2);

(e.g. The Noise Band value of the Positive Control plateau range is 35.8877, the threshold value = 6% ×35.887 = 2.1532.)

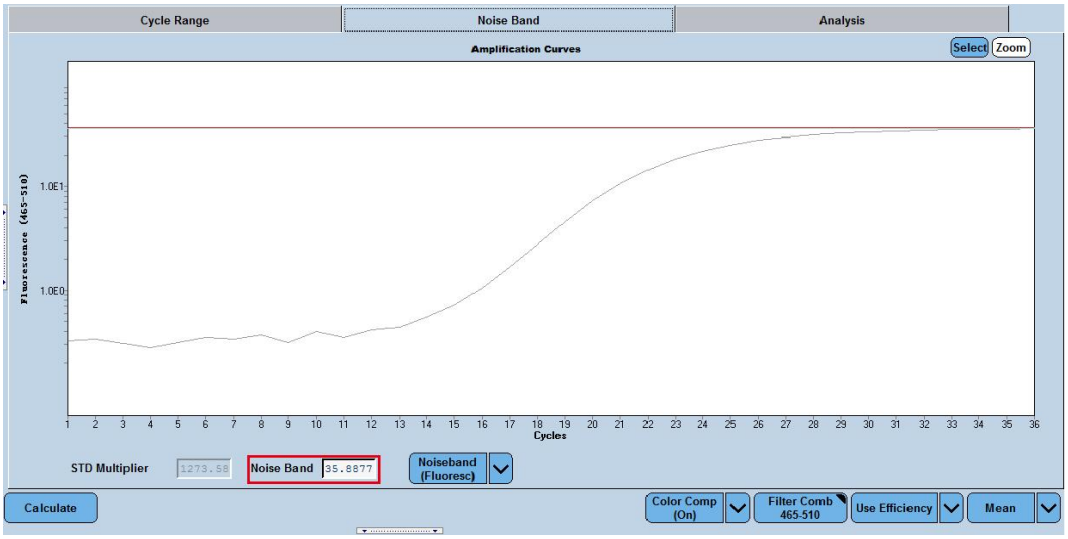


Figure 1

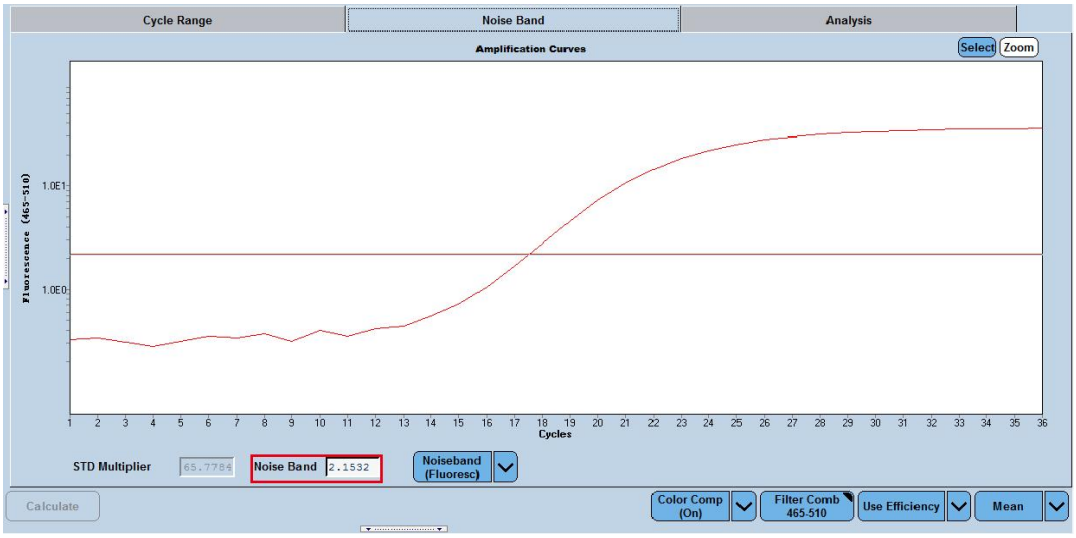


Figure 2

For Bio-Rad CFX96 instrument, adjust the threshold value as follows:

a. For each reaction mix, choose Positive Control;

b. Adjust the "Threshold" at the plateau range of the amplification curve, right click and select "Baseline Threshold…" to record the "Threshold" value (Figure 3);

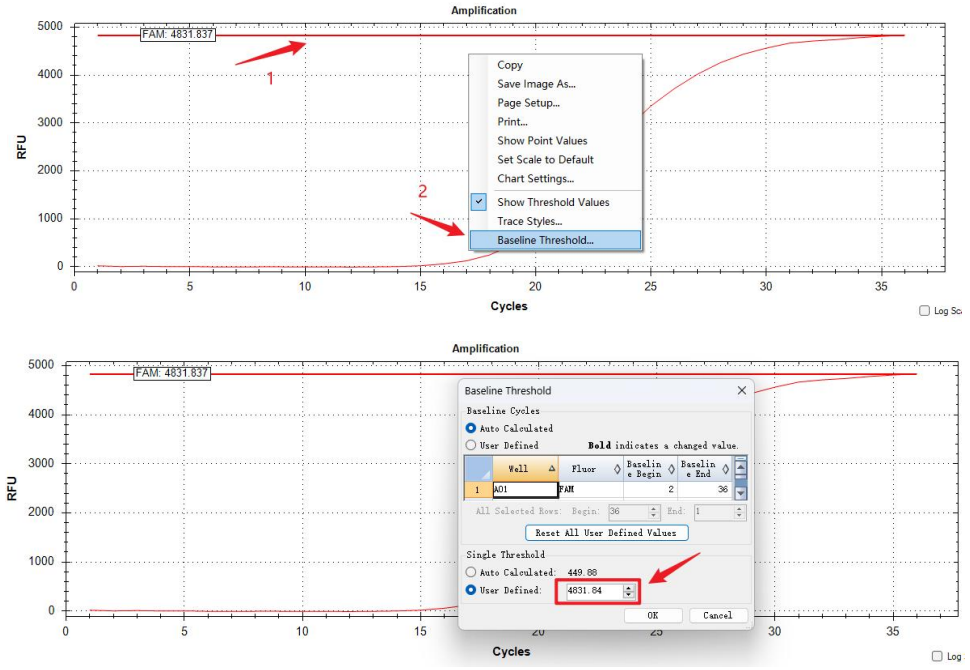


Figure 3

c. Set the "Threshold" value as 5% × "Threshold" value of the plateau range to obtain the Ct values (Figure 4);

(e.g. The "Threshold" value of the Positive Control plateau range is 4831.84, the threshold value = 5% ×4831.84 = 241.592)

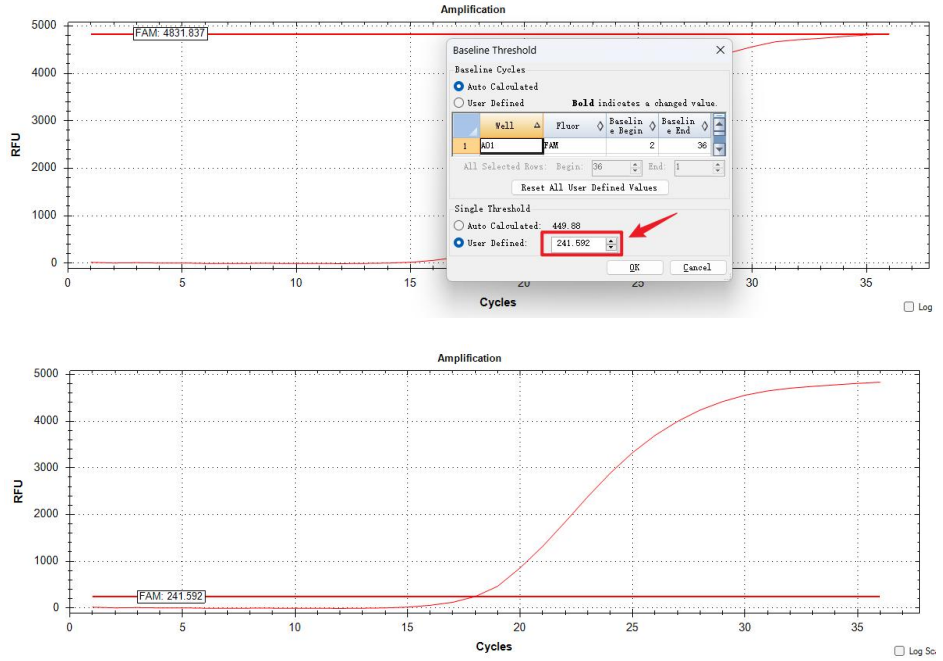


Figure 4

Before data analysis, the following items should be checked:

1) For the negative control (NTC): The FAM Ct values of LEG Reaction Mix A1~A8, the FAM and VIC Ct values of LEG Reaction

Mix B1~B7, and the ROX Ct value of LEG Reaction Mix B7 should be ≥36. If not, the data is *INVALID*. The sample should be retested.

2) For Positive Control: The FAM Ct values of LEG Reaction Mix A1~A8 and VIC Ct values of LEG Reaction Mix A4/A8, the FAM and VIC Ct values of LEG Reaction Mix B1~B8, and the ROX Ct values of LEG Reaction Mix B7/B8 should be < 25. If not, the data is *INVALID*. The sample should be retested.

Note:

- *Select one reaction mix and one fluorescence channel at a time for fusion / mutation analysis.*
- *If there is low fluorescent signal, please zoom in the amplification curve.*

Analyze RNA fusion assay for each sample:

3) For LEG Reaction Mix A1~A8, analyze *ALK*, *NTRK1*, *NTRK2*, *NTRK3*, *ROS1*, *MET* and *RET* gene fusions status:

a. Check the RNA Internal control VIC signals of LEG Reaction Mix A4/A8 for each sample:

i. If both VIC Ct values of LEG Reaction Mix A4/A8 are < 33 and either one is < 27, continue with the analysis.

ii. If both VIC Ct values of LEG Reaction Mix A4/A8 are ≥ 27 or either one is ≥ 33, which indicates the partial fragmentation or degradation of RNA, or the presence of PCR inhibitors. The sample should be retested with increased or re-extracted RNA.

b. Check FAM signals LEG Reaction Mix A1~A8 for RNA gene variants for each sample (see Table 9):

Table 9 Result Determination

LEG Reaction Mix A	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**Results**
Detected Target	*ALK*	*NTRK1*	*NTRK2*	*NTRK3*	*ROS1*	*ROS1*	*MET*	*RET*	
Positive Ct range	Ct<28	Ct<28	Ct<28	Ct<28	Ct<28	Ct<28	Ct<28	Ct<28	Positive
Negative Ct range	Ct≥28	Ct≥28	Ct≥28	Ct≥28	Ct≥28	Ct≥28	Ct≥28	Ct≥28	Negative or under the LOD*

* LOD: limit of detection

i. If any FAM Ct values of LEG Reaction Mix A1~A8 is in Positive Ct range, the sample is determined as corresponding fusion positive.

ii. If all the FAM Ct values of LEG Reaction Mix A1~A8 are in Negative Ct range, the sample is determined as negative (No fusion detected) or under the LOD of the kit.

Analyze DNA mutation assay for each sample:

4) For LEG Reaction Mix B1~B8, analyze DNA gene mutations status:

a. Check FAM signals of LEG Reaction Mix B8 for each sample:

i. If FAM Ct values of LEG Reaction Mix B8 are ≥17.5 and ≤24, continue with the analysis.

ii. If FAM Ct values of LEG Reaction Mix B8 is <17.5, it indicates the DNA is overloaded, the DNA amount should be reduced. If the mutation signals of LEG Reaction Mix B1~B7 are negative, the result is believable.

iii. If FAM Ct values of LEG Reaction Mix B8 >24, it indicates the partial fragmentation or degradation of DNA or the presence of PCR inhibitors. The sample should be retested with increased or re-extracted DNA.

b. Check FAM, VIC and ROX signals of LEG Reaction Mix B1~B7 for each sample (see Table 10):

Table 10 Result Determination

LEG Reaction Mix B		B1	B2	B3	B4	B5	B6	B7	Results
FAM	Optimal Ct range	Ct<30	Ct<30	Ct<30	Ct<30	Ct<30	Ct<30	Ct<30	Positive
	Acceptable Ct range	30≤Ct <33	30≤Ct <33	30≤Ct <33	30≤Ct <33	30≤Ct <33	30≤Ct <33	30≤Ct <33	Interpret the results according to the ΔCt value
	ΔCt Cut-off value	10	9	8	8	8	9	9	
	Negative Ct range	Ct≥33	Ct≥33	Ct≥33	Ct≥33	Ct≥33	Ct≥33	Ct≥33	Negative
VIC	Optimal Ct range	Ct<30	Ct<30	Ct<30	Ct<30	Ct<30	Ct<30	Ct<30	Positive
	Acceptable Ct range	30≤Ct <33	30≤Ct <33	30≤Ct <33	30≤Ct <33	30≤Ct <33	30≤Ct <33	30≤Ct <33	Interpret the results according to the ΔCt value
	ΔCt Cut-off value	8	8	9	8	8	8	9	
	Negative Ct range	Ct≥33	Ct≥33	Ct≥33	Ct≥33	Ct≥33	Ct≥33	Ct≥33	Negative
ROX	Optimal Ct range	/	/	/	/	/	/	Ct<30	Positive
	Acceptable Ct range	/	/	/	/	/	/	30≤Ct <33	Interpret the results according to the ΔCt value
	ΔCt Cut-off value	/	/	/	/	/	/	9	
	Negative Ct range	/	/	/	/	/	/	Ct≥33	Negative

i. If any FAM/VIC Ct value of LEG Reaction Mix B1~B7 or ROX Ct value of LEG Reaction Mix B7 is in Optimal Ct range, the sample is determined as corresponding mutation positive.

ii. If any FAM/VIC Ct value of LEG Reaction Mix B1~B7 or ROX Ct value of LEG Reaction Mix B7 is in Acceptable Ct range, calculate the ΔCt value for each mutation showing positive amplification.

a) **ΔCt = Mutant FAM (VIC/ROX) Ct value - External Control FAM (VIC/ROX) Ct value**. The Mutant Ct value refers to FAM/VIC/ROX Ct value of sample mutant signal, External Control Ct value refers to FAM/VIC/ROX Ct value of sample external control signal.

b) If the ΔCt value is less than the corresponding cut-off ΔCt value, the sample is determined as positive (Mutation detected).

c) If the ΔCt value is equal or more than the corresponding cut-off ΔCt value, the sample is determined as negative (No mutation detected) or under the LOD of the kit.

iii. If all the FAM and VIC Ct values of LEG Reaction Mix B1~B7, ROX Ct value of LEG Reaction Mix B7 are in Negative Ct range, the sample is determined as negative (No mutation detected) or under the LOD of the kit.

5) Some cross-reactivity may occur between *KRAS* mutation reactions. If VIC signal in LEG Reaction Mix B5 and FAM signal in LEG Reaction Mix B6 are both positive, the reaction mix with smaller Ct value is determined as true positive, while the other reaction mix with bigger Ct value needs to be determined according to the cross-reactivity cut-off ΔCt value criteria (see Table 11).

a) If the ΔCt value is less than the cross-reactivity cut-off value, the positive curve is determined as true positive.

b) If the ΔCt value is greater than or equal to the cross-reactivity cut-off value, the result is determined as negative.

Table 11 Cross-reactivity Cut-off ΔCt value

Reaction Mix / Signal / Mutation Name	B5/VIC	B6/FAM
KRAS-G12R (KRAS-M5)	5.58	
KRAS-G12C (KRAS-M6)		12.09

Note: *If VIC Ct value of LEG Reaction Mix B5 is equal to FAM Ct value of LEG Reaction Mix B6, the result should be KRAS mutation positive in both LEG Reaction Mixes B5/B6 (co-occurrence).*

6) The sample may contain two or more variants simultaneously.

Performance Characteristics

The performance characteristics of this kit were validated on LightCycler480 II, cobas® z480 and Bio-Rad CFX96.

1) Sensitivity:
   - For DNA mutation, the kit allows detection of 1~5% gene mutation in 10 ng DNA amount.
   - For RNA fusion, the kit allows detection of 25 copies/μL gene variant RNA.
2) Accuracy: accuracy of the kit was established by testing internal positive references and negative references, the detection rates are 100%.
3) Precision: precision of the kit was established by performing precision reference for 10 repeats, all results were positive, coefficient of variation for Ct values (CV, %) was less than 10%.

Limitations

1) The kit is to be used only by personnel specially trained in the techniques of PCR and the use of real-time PCR instruments.
2) The kit has been validated for use with tumor tissue samples.
3) The kit can only detect the 167 hotspot variants listed in the appendix.
4) Reliable results are dependent on proper sample processing, transport, and storage.
5) The sample containing degraded DNA or RNA may affect the ability of the test to detect the intended mutations or fusions.
6) Samples with negative result (No mutation detected) may harbor mutations or fusions not detected by this assay.

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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
	Keep Away from Sunlight		

Appendix 1

Gene Fusions Detected with LEG Reaction Mix A

Tube / Signal	Target to detect	Fusion Type	Name
① FAM	*ALK*	*EML4* exon13;*ALK* exon20	EML4-ALK-1
		EML4 exon6 ins33;*ALK* exon20	EML4-ALK-2
		EML4 exon20;*ALK* exon20	EML4-ALK-3
		EML4 exon18;*ALK* exon20	EML4-ALK-6
		EML4 exon2;*ALK* exon20	EML4-ALK-7
		EML4 exon17;ins68 *ALK* exon20	EML4-ALK-8
		EML4 exon2;ins117 *ALK* exon20	EML4-ALK-9
		EML4 exon13;ins69 *ALK* exon20	EML4-ALK-10
		EML4 exon6;*ALK* exon20	EML4-ALK-11
		EML4 exon6;*ALK* exon19	EML4-ALK-12
		EML4 exon6;ins18 *ALK* exon20	EML4-ALK-13
		EML4 exon20;ins18 *ALK* exon20	EML4-ALK-14
		EML4 exon17del58;ins39 *ALK* exon20	EML4-ALK-17
		EML4 exon17 ins65;*ALK* exon20	EML4-ALK-18
		EML4 exon17;ins30 *ALK* exon20	EML4-ALK-19
		EML4 exon17 ins61;ins34 *ALK* exon20	EML4-ALK-20
		EML4 exon3;ins53 *ALK* exon20	EML4-ALK-21
		KIF5B exon24;*ALK* exon20	KIF5B-ALK-1
		KIF5B exon17;*ALK* exon20	KIF5B-ALK-2
		KLC1 exon9;*ALK* exon20	KLC1-ALK
		TFG exon4;*ALK* exon20	TFG-ALK
② FAM	*NTRK1*	*TFG* exon5;*NTRK1* exon9	NTRK1-E9-M1
		TPM3 exon8;*NTRK1* exon10	NTRK1-E10-M1
		SQSTM1 exon5;*NTRK1* exon10	NTRK1-E10-M3
		TPR exon16 del54;*NTRK1* ins13 exon10	NTRK1-E10-M5
		TPR exon21;*NTRK1* exon10	NTRK1-E10-M6
		CD74 exon8;*NTRK1* exon10	NTRK1-E10-M7
		IRF2BP2 exon1;*NTRK1* exon10	NTRK1-E10-M8
		IRF2BP2 exon1 del48;*NTRK1* exon10	NTRK1-E10-M9
		TFG exon5;*NTRK1* exon10	NTRK1-E10-M12
		GRIPAP1 exon22;*NTRK1* exon10	NTRK1-E10-M14
		F11R exon4;*NTRK1* exon10	NTRK1-E10-M15
		SQSTM1 exon6;*NTRK1* exon10	NTRK1-E10-M17
		TPM3 exon8;*NTRK1* exon12	NTRK1-E12-M1
		MPRIP exon21;*NTRK1* exon12	NTRK1-E12-M3
		SSBP2 exon12;*NTRK1* exon12	NTRK1-E12-M4
		MPRIP exon14;*NTRK1* exon12	NTRK1-E12-M11
		MPRIP exon18;*NTRK1* exon12	NTRK1-E12-M12
		GRIPAP1 exon22;*NTRK1* exon12	NTRK1-E12-M14
③ FAM	*NTRK2*	*TRIM24* exon12;*NTRK2* exon15	NTRK2-E15-M1
		T*RIM24* exon12;*NTRK2* exon16	NTRK2-E16-M1
		SQSTM1 exon5;*NTRK2* exon16	NTRK2-E16-M3
		STRN exon3;*NTRK2* exon16	NTRK2-E16-M7
		SQSTM1 exon5;*NTRK2* exon17	NTRK2-E17-M2
④ FAM	*NTRK3*	*ETV6* exon4;*NTRK3* exon14	NTRK3-EX14-M1
		ETV6 exon5;*NTRK3* exon14	NTRK3-EX14-M2
		EML4 exon2;*NTRK3* exon14	NTRK3-EX14-M3
		SQSTM1 exon5;*NTRK3* exon14	NTRK3-EX14-M4
		RBPMS exon5;*NTRK3* exon14	NTRK3-EX14-M7
		ETV6 exon5;*NTRK3* exon15	NTRK3-EX15-M1
		ETV6 exon4;*NTRK3* exon15	NTRK3-EX15-M2
		SQSTM1 exon6;*NTRK3* exon15	NTRK3-EX15-M3
⑤ FAM	*ROS1*	*SLC34A2* exon4;*ROS1* exon32	ROS1-M1
		SLC34A2 exon13 del2046;*ROS1* exon32	ROS1-M2
		CD74 exon6;*ROS1* exon32	ROS1-M3

		SDC4 exon2;*ROS1* exon32	ROS1-M4
		SDC4 exon4;*ROS1* exon32	ROS1-M5
		SLC34A2 exon4;*ROS1* exon34	ROS1-M6
		SLC34A2 exon13 del2046;*ROS1* exon34	ROS1-M7
		CD74 exon6;*ROS1* exon34	ROS1-M8
		SDC4 exon4;*ROS1* exon34	ROS1-M9
		EZR exon10;*ROS1* exon34	ROS1-M10
⑥ FAM	*ROS1*	*TPM3* exon8;*ROS1* exon35	ROS1-M11
		LRIG3 exon16;*ROS1* exon35	ROS1-M12
		GOPC exon8;*ROS1* exon35	ROS1-M13
⑦ FAM	*MET*	*MET* Exon 14 skipping mutation	MET-M2
⑧ FAM	*RET*	*CCDC6* exon1;*RET* exon12	RET–M2
		NCOA4 exon6;*RET* exon12	RET–M5
		KIF5B exon15;*RET* exon12	RET-M15
		KIF5B exon16;*RET* exon12	RET–M16
		KIF5B exon23;*RET* exon12	RET–M17
		KIF5B exon22;*RET* exon12	RET–M19
		TRIM33 exon14;*RET* exon12	LRET-M22
		CUX1 exon10;*RET* exon12	LRET-M32
		KIAA1468 exon10;*RET* exon12	LRET-M40
		KIF13A exon18;*RET* exon12	LRET-M41
		MPRIP exon19;*RET* exon12	LRET-M42
		MYO5C exon25;*RET* exon12	LRET-M44
		PICALM exon19;*RET* exon12	LRET-M45
		RUFY2 exon9;*RET* exon12	LRET-M49
		TNIP2 exon5;*RET* exon12	LRET-M55
		WAC exon3;*RET* exon12	LRET-M57

Appendix 2

Gene Mutations Detected with LEG Reaction Mix B

Tube / Signal	Target to detect	Mutation	Base Change	Cosmic ID	Name	LOD
① FAM	*EGFR* Exon 19	E746_A750del (1)	2235_2249del15	6223	E-19-M1	1%
		E746_A750del (2)	2236_2250del15	6225	E-19-M2	1%
		L747_P753>S	2240_2257del18	12370	E-19-M3	1%
		E746_T751>I	2235_2252>AAT(complex)	13551	E-19-M4	1%
		E746_T751del	2236_2253del18	12728	E-19-M5	1%
		E746_T751>A	2237_2251del15	12678	E-19-M6	1%
		E746_S752>A	2237_2254del18	12367	E-19-M7	1%
		E746_S752>V	2237_2255>T(complex)	12384	E-19-M8	1%
		E746_S752>D	2238_2255del18	6220	E-19-M9	1%
		L747_A750>P	2238_2248>GC(complex)	12422	E-19-M10	1%
		L747_T751>Q	2238_2252>GCA(complex)	12419	E-19-M11	1%
		L747_E749del	2239_2247del9	6218	E-19-M12	1%
		L747_T751del	2239_2253del15	6254	E-19-M13	1%
		L747_S752del	2239_2256del18	6255	E-19-M14	1%
		L747_A750>P	2239_2248TTAAGAGAAG>C(complex)	12382	E-19-M15	1%
		L747_P753>Q	2239_2258>CA(complex)	12387	E-19-M16	1%
		L747_T751>S	2240_2251del12	6210	E-19-M17	1%
		L747_T751del	2240_2254del15	12369	E-19-M18	1%
		L747_T751>P	2239_2251>C(complex)	12383	E-19-M19	1%
		L747_T751del	2238_2252del15	23571	E-19-M20	1%
		L747_S752>Q	2239_2256>CAA(Complex)	12403	E-19-M21	1%
		L747_A750>P	2239_2250>CCC(Complex)	/	E-19-M24	1%
		L747_K754>QL	2239_2261>CAATT(Complex)	/	E-19-M25	1%
		E746_K754>EQHL	2238_2261>GCAACATCT(Complex)	/	E-19-M26	1%
		L747_S752>Q	2238_2256>GCAA (Complex)	26441	E-19-M27	1%
① VIC	*EGFR* Exon 20	S768I	2303G>T	6241	E-20-M2	1%
② FAM	*EGFR* Exon 21	L858R	2573T>G	6224	E-21-M1	1%

② VIC	*EGFR* Exon 18	G719A	2156G>C	6239	E-18-M1	1%
		G719S	2155G>A	6252	E-18-M2	2%
		G719C	2155G>T	6253	E-18-M3	1%
③ FAM	*EGFR* Exon 20	T790M	2369C>T	6240	E-20-M1	2%
③ VIC	*EGFR* Exon 21	L861Q	2582T>A	6213	E-21-M2	1%
④ FAM	*EGFR* Exon 20	H773_V774insH	2319_2320insCAC	12377	E-20-M3	1%
		D770_N771insG	2310_2311insGGT	12378	E-20-M4	1%
		V769_D770insASV	2307_2308insGCCAGCGTG	12376	E-20-M5	1%
		D770_N771insSVD	2311_2312insGCGTGGACA	13428	E-20-M8	1%
		V769_D770insASV	2309_2310AC>CCAGCGTGGAT	13558	E-20-M9	5%
		H773_V774insNPH	2319_2320insAACCCCCAC	12381	E-20-M10	1%
		.D770_N771insGF	2310_2311insGGGTTT	655155	E-20-M14	1%
		N771_P772insH	2311_2312insACC	6963572	E-20-M16	1%
		H773_V774insY	2319_2320insTAC	/	E-20-M18	1%
		H773_V774insPH	2319_2320insCCCCAC	12380	E-20-M19	1%
		V769_D770insGSV	2308_2309insGCAGCGTGG	18429	E-20-M21	1%
		N771_P772insHH	2311_2312insACCACC	6931207	E-20-M22	1%
		D770_N771insG	2310_2311insGGG	/	E-20-M23	1%
		D770_N771insG	2310_2311insGGC	13004	E-20-M24	1%
		P772_H773insDNP	2307_2308insGACAACCCC	6962050	E-20-M26	1%
		D770>GY	2308_2309insGTT	12427	E-20-M34	1%
		.D770_N771insGD	2310_2311insGGGGAC	85795	E-20-M36	1%
		D770_N771insGL	2310_2311insGGGTTA	48921	E-20-M37	1%
		N771>GF	2311_2312AA>GGGTT	18431	E-20-M38	1%
		N771_P772>SVDNR	2312_2315ACCC>13(GCGTGGACAACCG)	13554	E-20-M40	1%
		D770_N771insGT	2310_2311insGGCACA	1238029	E-20-M41	1%
		N771>KL	2312_2313insACT	6438147	E-20-M44	2%
		N771_P772insVDN	2307_2308insGACAACGTG	20885	E-20-M52	1%
		P772_H773insTP	2316C>AACCCCT	12388	E-20-M55	1%
		H773>PNPY	2317_2318insCTAACCCCT	1735761	E-20-M56	1%
④ VIC	*HER2* Exon 20	G776>VC	2326_2327insTGT	12553	HER2-M3	1%
		P780_Y781insGSP	2339_2340 insTGGCTCCCC	303948	HER2-M4	1%
		P780_Y781insGSP	2339_2340insGGGCTCCCC	12555	HER2-M6	1%
		P780_Y781insGSP	2340_2341insGGCTCCCCA	12556	HER2-M7	1%
		G776>VC	2326_2327insTTT	12552	HER2-M8	1%
		P780_Y781insGSP	2339_2340insCGGCTCCCC	6865893	HER2-M10	1%
		G776>VC	2326_2327insTAT	/	HER2-M15	1%
		G776>VC	2326_2327insTCT	85995	HER2-M16	1%
		G776>LC	2326G>TTAT	20895	HER2-M19	1%
		G776>LC	2326G>CTTT	12554	HER2-M20	1%
		G776>LC	2326G>TTGT	19875	HER2-M21	1%
		V777_G778insCG	2331_2332insTGTGGG	303939	HER2-M24	1%
⑤ FAM	*EGFR* Exon 20	V774_C775insHV	2321_2322insCCACGT	18432	E-20-M32	1%
		V774_C775insHV	2322_2323insCACGTG	22948	E-20-M33	1%
		H773_V774insAH	2320_2321insCCCACG	1238028	E-20-M35	1%
		P772_H773insV	2316_2317insGTT	255205	E-20-M42	1%
⑤ VIC	*KRAS* Exon 2	G12C	34G>T	516	KRAS-M6	1%
⑥ FAM	*KRAS* Exon 2	G12A	35G>C	522	KRAS-M2	5%
		G12V	35G>T	520	KRAS-M3	2%
		G12R	34G>C	518	KRAS-M5	2%
		G13C	37G>T	527	KRAS-M14	1%
⑥ VIC	*HER2* Exon 20	A775_G776insYVMA	2325_2326 ins12 (TACGTGATGGCT)	12558	HER2-M1	1%
		A775_G776insYVMA	2324_2325 ins12 (ATACGTGATGGC)	20959	HER2-M2	1%
⑦ FAM	*KRAS* Exon 2	G12D	35G>A	521	KRAS-M1	5%
		G12S	34G>A	517	KRAS-M4	5%
⑦ VIC	*BRAF* Exon 15	V600E	1799T>A	476	BRAF-M1	1%
⑦ ROX	*EGFR* Exon 20	C797S	2389T>A	6493937	E-20-M6	1%
			2390G>C	5945664	E-20-M7	1%