AmoyDx
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CE IVD

AmoyDx® Multi-Gene Mutations Detection Kit

Instructions for Use

REF	8.01.26301W006A	6 tests/kit	For Stratagene Mx3000P™
REF	8.01.26301W006B	6 tests/kit	For LightCycler480 II, cobas® z480
REF	8.01.26301W006D	6 tests/kit	For SLAN-96S
REF	8.01.26301W006J	6 tests/kit	For ABI7500

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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 patients with NSCLC for the *EGFR* gene is 10~35%, for *KRAS* 15~25%, for *BRAF 1~4%*, for *NRAS* is 1%, for *HER2* 2~4%, and for *PIK3CA* 1~3%. About 3~7% of NSCLC patients have gene fusion in *ALK,* 2% in ROS1, and 1% in RET [1]. A large amount of clinical studies showed that gene mutation status is important efficacy predictor for targeted therapy. For instance, *EGFR*-TKI would show better efficacy on patient with *EGFR* sensitizing mutation than the patient with wild-type gene [2-3], the presence of the *ALK*, *ROS1* and *RET* gene fusions are correlated with the efficacy of TKI therapy [4-6], and *KRAS*, *BRAF*, *NRAS*, *HER2* and *PIK3CA* are associated with prognosis of some targeted drugs [7-11]. 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 to determine the optimal precision oncology treatment [12].

Intended Use

The AmoyDx® Multi-Gene Mutations Detection Kit is a real-time PCR assay for qualitative detection of 118 hotspot mutations/fusions in *EGFR*, *KRAS*, *BRAF*, *NRAS*, *HER2*, *PIK3CA*, *ALK*, *ROS1* and *RET* genes in human genomic DNA and total RNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue. The kit is intended to be used to aid clinician to identify the multi-target status of NSCLC patients.

The kit is for *in vitro* diagnostic use, and intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

The RNA gene fusion detection system uses specific primers and fluorescent probes which are designed for both side genes of the fusion, combining a one-step RT-PCR procedure to detect *ALK*, *ROS1* and *RET* gene fusions based on tumor message RNA. The protocol comprises reverse transcription of target RNA and reference gene RNA to generate complementary DNA (cDNA) and simultaneous PCR amplification of resulting cDNA.

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 the 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 occurring.

The kit contains reverse transcriptase for reverse transcription of target RNA and reference gene RNA into cDNA, and Taq DNA polymerase for PCR amplification and uracil-N-glycosylase which works at room temperature to prevent PCR amplicon carryover contamination.

This kit contains RNA gene fusion and DNA gene mutation detection systems.

1. Tubes ①~④ of **LMG Reaction Mix** strip are designed for RNA gene fusion and internal control detection. The reaction mixes include primers and FAM-labeled probes specific for *ALK* gene fusion (Reaction Mix ①), *ROS1* gene fusion (Reaction Mix ②~③) and *RET* gene fusion (Reaction Mix ④). The internal control system of tubes ①~④ contains primers and HEX/VIC-labeled probes for reference gene *HPRT1*, to assess the RNA quality and monitor the accuracy of experimental operation.
2. Tubes ⑤~⑫ of **LMG Reaction Mix** strip are designed for DNA gene mutation and external control detection. The Reaction Mixes ⑤~⑪ include primer, FAM-labeled probes and HEX/VIC-labeled probes specific for *EGFR*, *KRAS*, *BRAF*, *NRAS*, *HER2* and *PIK3CA* gene mutations. The External Control Reaction Mix contains primers and FAM/HEX/VIC-labeled probes for a region of genomic DNA that has no known mutations or SNPs, to assess the DNA quality.
3. The **LMG Enzyme Mix A** contains the Transcriptase, Taq DNA polymerase for PCR amplification and uracil-N-glycosylase which works at room temperature to prevent PCR amplicon carryover contamination.
4. The **LMG 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.

Kit Contents

This kit contains the following materials (see Table 1):

Table 1 Kit Contents

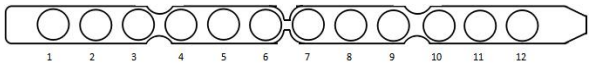
Content	Main Ingredients	Quantity
LMG Reaction Mix	12-tube strip*	8 strips*
LMG Enzyme Mix A	Taq DNA Polymerase, Uracil-N-Glycosylase, Transcriptase	50 µL/tube ×1
LMG Enzyme Mix B	Taq DNA Polymerase, Uracil-N-Glycosylase	30 µL/tube ×1
LMG Positive Control	Plasmid DNA	350 µL/tube ×1

* Each 12-tube strip includes the following contents for testing one sample or one control (see Table 2).

Table 2 Information of the 12-tube strip

Tube No.	Content	Main Ingredients	Quantity	Florescence Signal
①	LMG Reaction Mix ①	Primers, Probes, Mg^{2+}, dNTPs	30 µL	FAM, HEX/VIC
②	LMG Reaction Mix ②	Primers, Probes, Mg^{2+}, dNTPs	30 µL	FAM, HEX/VIC
③	LMG Reaction Mix ③	Primers, Probes, Mg^{2+}, dNTPs	30 µL	FAM, HEX/VIC
④	LMG Reaction Mix ④	Primers, Probes, Mg^{2+}, dNTPs	30 µL	FAM, HEX/VIC
⑤	LMG Reaction Mix ⑤	Primers, Probes, Mg^{2+}, dNTPs	35 µL	FAM, HEX/VIC
⑥	LMG Reaction Mix ⑥	Primers, Probes, Mg^{2+}, dNTPs	35 µL	FAM, HEX/VIC
⑦	LMG Reaction Mix ⑦	Primers, Probes, Mg^{2+}, dNTPs	35 µL	FAM, HEX/VIC
⑧	LMG Reaction Mix ⑧	Primers, Probes, Mg^{2+}, dNTPs	35 µL	FAM, HEX/VIC
⑨	LMG Reaction Mix ⑨	Primers, Probes, Mg^{2+}, dNTPs	35 µL	FAM, HEX/VIC
⑩	LMG Reaction Mix ⑩	Primers, Probes, Mg^{2+}, dNTPs	35 µL	FAM, HEX/VIC
⑪	LMG Reaction Mix ⑪	Primers, Probes, Mg^{2+}, dNTPs	35 µL	FAM, HEX/VIC
⑫	LMG External Control Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	35 µL	FAM, HEX/VIC

Note: *Distinguish Tube ⑫ from Tube ① according to the trapezoid end of strip edge, described as follows:*



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 eight months. The maximal number of freeze-thaw cycle is five.

Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments:

   Stratagene Mx3000P™, ABI7500, LightCycler480 II, cobas® z480, or SLAN-96S.

2) DNA/RNA extraction kit: we recommend to use AmoyDx® FFPE DNA/RNA Kit for paraffin embedded tissue specimens.
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 centrifuge tubes.
8) Adjustable pipettors and filtered pipette tips for handling DNA/RNA.
9) Tube racks.
10) Disposable powder-free gloves.
11) Sterile, nuclease-free water.
12) 1×TE buffer (pH 8.0).

Precautions and Handling Requirements

For *in vitro* diagnostic use.

Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use. Strictly follow the instruction during operation.
- Please check the compatible real-time PCR instruments with the test kit 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 could use this kit. Please wear suitable lab coat and disposable gloves while handling the reagents.
- Avoid contact of the skin, eyes and mucous membranes 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 results.
- 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 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 experiment, 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 Stratagene Mx3000P™, if there's low net fluorescence signal (dR) but high background signals (R), please reduce the signal gain setting of instrument properly.
- For ABI 7500, please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.
- For LightCycler480 II and cobas® z480 instrument, if there is fluorescence crossover, fluorescence calibration is required. To run the assays on a Roche PCR machine, please use the Roche 480 adaptor, available from BIOplastics, Cat No. B79480. It's essential to place a 12-tube strip on the first row (Row A) of the adaptor.
- For SLAN-96S, please set up as follows: Probe mode: FAM, VIC. During the result analysis, open the "Preference" window, in "Chart Options" section; select "Selected Wells" for "Y-Axis Scaling Auto-adjust By" and "Absolute Fluorescence Value Normalization" for "Amplification Curve".
- Refer to the operation manual of the real-time PCR instrument for detailed instructions.
- We recommend that for all PCR instruments in use, fluorescence calibration should be conducted once a year.

Assay Procedure

1. DNA/RNA Extraction

The specimen material must be human genomic DNA and total RNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples. DNA/RNA extraction reagents are not included in the kit. Before the extraction of DNA and RNA, it is very important to make sure that there is at least 30% tumor cells in the FFPE tissue samples.

The $OD_{260/280}$ value of extracted DNA and RNA should be between 1.8~2.1, and total RNA concentration should be between 10~500 ng/μL (measured by the spectrophotometer, the NanoDrop 1000 /2000 spectrophotometer is recommended).

The amount of extracted DNA for EGFR detection is shown in Table 3.

Table 3 Recommended DNA concentration

FFPE tissue storage time	DNA concentration	DNA amount
≤ 3 months	1.5 ng/μL	7.05 ng
> 3 months & ≤ 1 year	2 ng/μL	9.4 ng
> 1 year & ≤ 2 years	2.5~3 ng/μL	11.75~14.1 ng

Note:

- *The FFPE tissue should be handled and stored properly. The storage time should preferably be less than 2 years.*
- *The extracted DNA and RNA should be used immediately. If not, it should be stored at -20±5℃. Avoid using extracted RNA stored for more than 2 weeks.*
- *Before detection, dilute the extracted DNA with 1×TE buffer (pH 8.0) to designated concentrations. We recommend using at least 5 μL DNA for 10 times dilution, to ensure the validity of final concentration.*

2. Detection Procedure

1) Take out **LMG Enzyme Mix A**, **LMG Enzyme Mix B** and **LMG Positive Control (PC)** out of the kit from the freezer, and other reagents remained in freezer at -20±5℃.
2) Thaw **LMG Positive Control (PC)** at room temperature. When the reagent is completely thawed, mix the reagent thoroughly by vortexing and centrifuge for 5~10 seconds to collect all liquid at the bottom of the tube.
3) Centrifuge **LMG Enzyme Mix A** and **LMG Enzyme Mix B** for 5~10 seconds prior to use.
4) Take out the sample DNA (see Table 3 for DNA concentration) or RNA and nuclease-free water for No-template Control (NTC).
5) Prepare LMG Master Mix A: Add 5 μL **LMG Enzyme Mix A** into the following solutions respectively: 45 μL sample RNA, 45 μL **LMG Positive Control** and 45 μL nuclease-free water. Mark the solutions as S-Mix A (if more samples, name as S1-Mix A, S2-Mix A…), P-Mix A, N-Mix A. Mix each solution thoroughly by vortexing and centrifuge for 5~10 seconds.
6) Prepare LMG Master Mix B: Add 2.7 μL **LMG Enzyme Mix B** into the following solutions respectively: 42.3 μL sample DNA, 42.3 μL **LMG Positive Control** and 42.3 μL nuclease-free water. Mark the solutions as S-Mix B (if more samples, name S1-Mix B, S2-Mix B…), P-Mix B, N-Mix B. Mix each solution thoroughly by vortexing and centrifuge for 5~10 seconds.

   Note:

   - *Each PCR run must contain one PC and one 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.*
7) Take out sufficient **LMG Reaction Mix** strips and centrifuge the strips if there are any reagent droplets in the caps of the PCR tubes. Then briefly uncover the caps prior to use.
8) Prepare one 12-tube strip for NTC: Transfer 10 μL N-Mix A to tubes ①~④, and transfer 5 μL N-Mix B to tubes ⑤~⑫. Cap the PCR tubes.
9) Prepare one 12-tube strip for each sample: Transfer 10 μL S-Mix A to tubes ①~④, and transfer 5 μL S-Mix B to tubes ⑤~⑫. Cap the PCR tubes.
10) Prepare one 12-tube strip for PC: Transfer 10 μL P-Mix A to tubes ①~④, and transfer 5 μL P-Mix B to tubes ⑤~⑫. Cap the PCR tubes.

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

12) Place the PCR tubes into the appropriate positions of the real-time PCR instrument. A recommended plate layout is shown in Table 4.

Table 4 Suggested PCR Plate Layout

96 well layout												
Tube	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1
B	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2
C	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3
D	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4
E	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5
F	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6
G	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
H	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

13) Setup the PCR Protocol using the cycling parameters in Table 5.

Table 5 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	42℃	5 min	/
		95℃	5 min	/
2	10	95℃	25 s	/
		64℃	20 s	/
		72℃	20 s	/
3	36	93℃	25 s	/
		60℃	35 s	FAM and HEX/VIC
		72℃	20 s	/

14) Start the PCR run immediately.

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

3. Result Interpretation

Before data analysis, the following items should be checked:

1) For the negative control (NTC): The FAM Ct values of Tubes ①~⑪ and HEX/VIC Ct values of Tubes ⑤~⑪should be ≥36. If not, the data is *INVALID*. The sample should be retested.

2) For Positive Control: The FAM Ct values and HEX/VIC Ct values of Tubes ①~⑫ should be < 30. If not, the data is *INVALID*. The sample should be retested.

Analyze the mutation/fusion assay for each sample:

3) For Tubes ①~④, analyze *ALK*, *ROS1* and *RET* gene fusions status:

   a. Check Internal control HEX/VIC signals of Tubes ①~④ for each sample:

      i. If HEX/VIC Ct values of Tubes ①~④ are ≤30, continue with the analysis.

      ii. If any HEX/VIC Ct value of Tubes ①~④ is >30, which indicates the partial fragmentation or degradation of RNA, or the presence of PCR inhibitors, or any error with experimental operation. The sample should be retested with increased or re-extracted RNA. But if the FAM Ct value is <35, the sample is determined as positive.

   b. Check FAM signals in Tubes ①~④ for RNA gene fusion for each sample:

      i. If FAM Ct value of Tube ① is <35, the samples is determined as *ALK* positive (*ALK* gene fusion detected). If FAM Ct value of Tube ① is ≥35, the sample is determined as *ALK* negative (no *ALK* gene fusion detected).

      ii. If any FAM Ct value of Tubes ②~③ is <35, the samples is determined as *ROS1* positive (*ROS1* gene fusion detected). If all the FAM Ct values of Tube ②~③ are ≥35, the sample is determined as *ROS1* negative (no *ROS1* gene fusion detected).

      iii. If FAM Ct value of Tube ④ is <35, the samples is determined as *RET* positive (*RET* gene fusion detected). If FAM Ct value of Tube ④ is ≥35, the sample is determined as *RET* negative (no *RET* gene fusion detected).

4) For Tubes ⑤~⑫, analyze DNA gene mutations status:

a. Check FAM and HEX/VIC signals of Tube ⑫ for each sample:

i. If FAM and HEX/VIC Ct values of FAM and HEX/VIC signals of Tube ⑫ are ≥20 and ≤26, continue with the analysis.

ii. If FAM and HEX/VIC Ct values of Tube ⑫ is <20, it indicates that the DNA is overloaded, the DNA amount should be reduced. If the mutation signals of Tubes ⑤~⑪ are negative, the result are plausible.

iii. If FAM and HEX/VIC Ct values of tube ⑫ >26, it indicates the partial fragmentation or degradation of DNA or the presence of PCR inhibitors, or any error with experimental operation. The sample should be retested with increased or re-extracted DNA.

b. Check FAM and HEX/VIC signals of Tubes ⑤~⑪ for each sample. Based on different mutant Ct values, the detection results are divided into strong positive, weak positive or negative (Table 6).

i. If any FAM or HEX/VIC Ct values of Tube ⑤~⑪ is in Optimal Ct range, the sample is determined as corresponding mutation positive.

ii. If any FAM or HEX/VIC Ct values of Tubes ⑤~⑪ is in Acceptable Ct range, calculate the ΔCt value for each mutation showing positive amplification.

a) **ΔCt = Mutant FAM (HEX/VIC) Ct value - External Control FAM (HEX/VIC) Ct value**. The Mutant Ct value refers to FAM or HEX/VIC Ct value of sample mutant signal, External Control Ct value refers to FAM or HEX/VIC Ct value of the sample signal of 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 HEX/VIC Ct values of Tubes ⑤~⑪ are in Negative Ct range, the sample is determined as negative (no mutation detected) or under the LOD of the kit.

Table 6 Result Determination

Tube No.		⑤	⑥	⑦	⑧	⑨	⑩	⑪	Results
FAM	Optimal Ct range	Ct<31	Ct<31	Ct<31	Ct<31	Ct<31	Ct<31	Ct<31	Positive
	Acceptable Ct range	31≤Ct <34	31≤Ct <34	31≤Ct <33	31≤Ct <33	31≤Ct <34	31≤Ct <33	31≤Ct <34	Interpret the results according to the ΔCt value
	ΔCt Cut-off value	11	11	7	9	9	9	10	
	Negative Ct range	Ct≥34	Ct≥34	Ct≥33	Ct≥33	Ct≥34	Ct≥33	Ct≥34	Negative or under the LOD*.
HEX /VIC	Optimal Ct range	Ct<31	Ct<31	Ct<31	Ct<31	Ct<31	Ct<30	Ct<30	Positive
	Acceptable Ct range	31≤Ct <34	31≤Ct <33	31≤Ct <34	31≤Ct <34	31≤Ct <33	30≤Ct <33	30≤Ct <33	Interpret the results according to the ΔCt value
	ΔCt Cut-off value	8	9	9	10	9	9	8	
	Negative Ct range	Ct≥34	Ct≥33	Ct≥34	Ct≥34	Ct≥33	Ct≥33	Ct≥33	Negative or under the LOD*.

* LOD: limit of detection

5) The sample may contain two or more fusions or mutations simultaneously.

Performance Characteristics

The performance characteristics of this kit were validated on Stratagene Mx3000P™, ABI7500, LightCycler480 II, cobas® z480, and SLAN-96S.

1) Analytical sensitivity: The kit allows detection of 1% gene mutation in 7.05~14.1 ng FFPE sample DNA and detection of 450 copies gene fusion armored RNA in 0.09~4.5μg FFPE sample RNA.

2) Specificity: The specificity of the kit was established by testing negative reference controls. The test gave negative results and negative concordance rate was 100%.

3) Accuracy: accuracy of the kit was established by testing internal positive references control, the test gave positive results and positive concordance rate was 100%.

4) 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 5%.

5) Interfering substance: two common potential interfering substances hemoglobin and triglyceride were evaluated in this study. It is confirmed that the potential maximum concentrations: 2 g/L hemoglobin and 37 mmol/L triglyceride would not interfere with the test result.

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 results can be used to assist clinical diagnosis, combined with other clinical and laboratory findings.
3) The kit has been validated for use with FFPE tissue DNA.
4) The kit can only detect the 118 hotspot mutations or fusions listed in the appendix.
5) Reliable results are dependent on proper sample processing, transport, and storage.
6) If the sample only contains degraded DNA or RNA, this may affect the ability of the test to detect the intended mutations or fusions.
7) Samples with negative result (no mutation detected) may not mean that there are no mutations or fusions, but are just not detected by this assay.

References

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Symbols

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

Appendix 1

Gene Fusions Detected by this Kit

Tube & Signal	Target	Fusion Type	Cosmic ID	Internal Name
① FAM	*ALK*	*EML4* exon 13; *ALK* exon 20	/	EML4-ALK-1
		EML4 exon 6 ins 33; *ALK* exon 20		EML4-ALK-2
		EML4 exon 20; *ALK* exon 20		EML4-ALK-3
		EML4 exon 18; *ALK* exon 20		EML4-ALK-6
		EML4 exon 2; *ALK* exon 20		EML4-ALK-7
		EML4 exon 17; ins68 *ALK* exon 20		EML4-ALK-8
		EML4 exon 2; ins117 *ALK* exon 20		EML4-ALK-9
		EML4 exon 13; ins69 *ALK* exon 20		EML4-ALK-10
		EML4 exon 6; *ALK* exon 20		EML4-ALK-11
		EML4 exon 6; *ALK* exon 19		EML4-ALK-12
		EML4 exon 6; ins18 *ALK* exon 20		EML4-ALK-13
		EML4 exon 20; ins18 *ALK* exon 20		EML4-ALK-14
		EML4 exon 17del58; ins39 *ALK* exon 20		EML4-ALK-17
		EML4 exon 17 ins65; *ALK* exon 20		EML4-ALK-18
		EML4 exon 17; ins30 *ALK* exon 20		EML4-ALK-19
		EML4 exon 17 ins61; ins34 *ALK* exon 20		EML4-ALK-20
		EML4 exon 3; ins53 *ALK* exon 20		EML4-ALK-21
		KIF5B exon 24; *ALK* exon 20		KIF5B-ALK-1
		KIF5B exon 17; *ALK* exon 20		KIF5B-ALK-2
		KLC1 exon9; *ALK* exon20		KLC1-ALK
		TFG exon4; *ALK* exon20		TFG-ALK
② FAM	*ROS1*	*SLC34A2* exon4; *ROS1* exon 32	1197	ROS1-M1
		SLC34A2 exon14 del ; *ROS1* exon 32	1260	ROS1-M2
		CD74 exon6; *ROS1* exon 32	1203	ROS1-M3
		SDC4 exon2; *ROS1* exon 32	1266	ROS1-M4
		SDC4 exon4; *ROS1* exon 32	1279	ROS1-M5
		SLC34A2 exon4; *ROS1* exon 34	/	ROS1-M6
		SLC34A2 exon14 del; *ROS1* exon 34	/	ROS1-M7
		CD74 exon6; *ROS1* exon 34	1201	ROS1-M8
		SDC4 exon4; *ROS1* exon 34	/	ROS1-M9
		EZR exon10; *ROS1* exon 34	1268	ROS1-M10
③ FAM	*ROS1*	*TPM3* exon 8; *ROS1* exon 35	1274	ROS1-M11
		LRIG3 exon 16; *ROS1* exon 35	1270	ROS1-M12
		GOPC exon 8; *ROS1* exon 35	1251	ROS1-M13
④ FAM	*RET*	*CCDC6* exon 1; *RET* exon 12	5918	RET–M2
		NCOA4 exon6; *RET* exon 12	/	RET–M5
		KIF5B exon 15; *RET* exon 12	60431	RET-M15
		KIF5B exon 16; *RET* exon 12	60431	RET–M16
		KIF5B exon 23; *RET* exon 12	60431	RET–M17
		KIF5B exon 22; *RET* exon 12	60431	RET–M19

Appendix 2

Gene Mutations Detected by this Kit

Tube & Signal	Mutation Name	Exon	Base Change	Cosmic ID	Name
⑤ FAM	E746_A750del (1)	*EGFR* Exon 19	2235_2249del15	6223	Ex19-M1
	E746_A750del (2)		2236_2250del15	6225	Ex19-M2
	L747_P753>S		2240_2257del18	12370	Ex19-M3
	E746_T751>I		2235_2252>AAT(complex)	13551	Ex19-M4
	E746_T751del		2236_2253del18	12728	Ex19-M5
	E746_T751>A		2237_2251del15	12678	Ex19-M6
	E746_S752>A		2237_2254del18	12367	Ex19-M7
	E746_S752>V		2237_2255>T(complex)	12384	Ex19-M8
	E746_S752>D		2238_2255del18	6220	Ex19-M9
	L747_A750>P		2238_2248>GC(complex)	12422	Ex19-M10
	L747_T751>Q		2238_2252>GCA(complex)	12419	Ex19-M11
	L747_E749del		2239_2247delTTAAGAGAA	6218	Ex19-M12
	L747_T751del		2239_2253del15	/	Ex19-M13
	L747_S752del		2239_2256del18	6255	Ex19-M14
	L747_A750>P		2239_2248TTAAGAGAAG>C(complex)	12382	Ex19-M15

	L747_P753>Q		2239_2258>CA(complex)	12387	Ex19-M16
	L747_T751>S		2240_2251del12	6210	Ex19-M17
	L747_T751del		2240_2254del15	12369	Ex19-M18
	L747_T751>P		2239_2251>C(complex)	12383	Ex19-M19
	L747_T751del		2238_2252del15	23571	Ex19-M20
	L747_S752>Q		2239_2256>CAA(Complex)	12403	Ex19-M21
	L747_A750>P		2239_2250>CCC(Complex)	/	Ex19-M24
	L747_K754>QL		2239_2261>CAATT(Complex)	/	Ex19-M25
	E746_K754>EQHL		2238_2261>GCAACATCT(Complex)	/	Ex19-M26
	L747_S752>Q		2238_2256>GCAA (Complex)	26441	Ex19-M27
⑤ HEX /VIC	S768I	*EGFR* Exon 20	2303G>T	6241	Ex20-M2
⑥ FAM	L858R	*EGFR* Exon 21	2573T>G	6224	Ex21-M1
⑥ HEX/VIC	G719A	*EGFR* Exon 18	2156G>C	6239	Ex18-M1
	G719S		2155G>A	6252	Ex18-M2
	G719C		2155G>T	6253	Ex18-M3
⑦ FAM	T790M	*EGFR* Exon 20	2369C>T	6240	Ex20-M1
⑦ HEX/VIC	L861Q	*EGFR* Exon 21	2582T>A	6213	Ex21-M2
⑧ FAM	G12D	*KRAS* Exon 2	35G>A	521	KRAS-M1
	G12S		34G>A	517	KRAS-M4
⑧ HEX/VIC	V600E	*BRAF* Exon 15	1799T>A	476	BRAF-M1
	V600K		1798_1799GT>AA(complex)	473	BRAF-M2
	V600E2		1799_1800TG>AA (complex)	475	BRAF-M3
	V600R		1798_1799GT>AG(complex)	474	BRAF-M4
	V600D1		1799_1800TG>AC(complex)	/	BRAF-M5
	V600D2		1799_1800TG>AT(complex)	477	BRAF-M6
⑨ FAM	G12A	*KRAS* Exon 2	35G>C	522	KRAS-M2
	G12V		35G>T	520	KRAS-M3
	G12R		34G>C	518	KRAS-M5
	G12C		34G>T	516	KRAS-M6
	G13C		37G>T	527	KRAS-M14
⑨ HEX/VIC	A775_G776insYVMA	*HER2* Exon 20	2325_2326 ins12 (TACGTGATGGCT)	12558	HER2-M1
	A775_G776insYVMA		2324_2325 ins12 (ATACGTGATGGC)	20959	HER2-M2
	M774_A775insAYVM		2322_2323ins12 (GCATACGTGATG)	682	HER2-M5
⑩ FAM	Q61R	*NRAS* Exon 3	182A>G	584	NRAS-M1
	Q61K		181C>A	580	NRAS-M2
	Q61L		182A>T	583	NRAS-M5
	Q61H		183A>C	586	NRAS-M8
⑩ HEX/VIC	G776>VC	*HER2* Exon 20	2326_2327ins3 (TGT)	12553	HER2-M3
	P780_Y781insGSP		2339_2340 ins9 (TGGCTCCCC)	303948	HER2-M4
	P780_Y781insGSP		2339_2340ins9 (GGGCTCCCC)	12555	HER2-M6
	P780_Y781insGSP		2340_2341ins9 (GGCTCCCCA)	12556	HER2-M7
	G776>VC		2326_2327ins3 (TTT)	12552	HER2-M8
	P780_Y781insGSP		2339_2340ins9 (CGGCTCCCC)	/	HER2-M10
	G776>VC		2326_2327insTAT	/	HER2-M15
	G776>VC		2326_2327insTCT	85995	HER2-M16
	G776>LC		2326G>TTAT	20895	HER2-M19
⑪ FAM	H773_V774insH	*EGFR* Exon 20	2319_2320insCAC	12377	E-20-M3
	D770_N771insG		2310_2311insGGT	12378	E-20-M4
	V769_D770insASV		2307_2308insGCCAGCGTG	12376	E-20-M5
	D770_N771insSVD		2311_2312insGCGTGGACA	13428	E-20-M8
	V769_D770insASV		2309_2310AC>CCAGCGTGGAT	13558	E-20-M9
	H773_V774insNPH		2319_2320insAACCCCCAC	12381	E-20-M10
	H773_V774insQ		2319_2320insCAG	131552	E-20-M12
	N771_P772insT		2313_2314insACC	/	E-20-M15
	N771_P772insH		2314_2315insACC	1238031	E-20-M16
	P772_H773insQ		2318_2319insACA	/	E-20-M17
	H773_V774insY		2319_2320insTAC	/	E-20-M18
	V769_D770insGSV		2308_2309insGCAGCGTGG	18429	E-20-M21
	D770_N771insG		2310_2311insGGG	/	E-20-M23
	D770_N771insG		2310_2311insGGC	13004	E-20-M24
	P772_H773insDNP		2307_2308insGACAACCCC	6962050	E-20-M26
⑪ HEX/VIC	H1047R	*PIK3CA* Exon 20	3140A＞G	775	PI-M1
	E545K	*PIK3CA* Exon 9	1633G>A	763	PI-M4