CE IVD

AmoyDx® *EML4-ALK* Fusion Gene Detection Kit

For qualitative detection of 21 *EML4-ALK* fusions

Instruction for Use

REF 8.01.22001X024H	24 tests	For Stratagene Mx3000P™, ABI7500, LightCycler480 II, Bio-Rad CFX 96, Rotor-Gene Q/6000 (72 wells), SLAN-96S

Amoy Diagnostics Co., Ltd.
39 Dingshan Road, Haicang District,
Xiamen 361027, P. R. China
Tel: +86 592 6806835
Fax: +86 592 6806839
E-mail: sales@amoydx.com
Website: http://www.amoydx.com

EC REP **Wellkang Ltd**
Suite B, 29 Harley Street,
London W1G 9QR United Kingdom

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Background

The anaplastic lymphoma kinase (*ALK*) gene is frequently involved in rearrangements that lead to gene fusions in lung cancer. Fusion partner of echinoderm microtubule-associated protein-like 4 (*EML4*) is frequently found in non-small-cell lung cancer (NSCLC). The recombinant *EML4-ALK* activates the receptor tyrosine kinase's downstream signaling pathway, which includes *PI3K/AKT*, leading to carcinogenesis. It has been reported that the presence of the *EML4-ALK* fusion is correlated with the efficacy of *ALK*-targeted therapy. Based on analysis of tumor messenger RNA, *EML4-ALK* fusions can be detected by real-time PCR method.

Intended Use

AmoyDx® *EML4-ALK* Fusion Gene Detection Kit is a real-time PCR assay for qualitative detection of 21 *EML4-ALK* fusions in total RNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue in 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 kit is based on two major processes: 1) **Reverse Transcription**: extracted RNA from FFPE 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 *EML4-ALK* variant cDNA, and fusion amplicon is detected by fluorescent probes labeled with FAM.

The kit is composed of RT Reaction Mix, Reverse Transcriptase, Reaction Mix, Enzyme Mix and Positive Control.

1) The **EA RT Reaction Mix** contains primers specific for both *ALK* RNA and reference gene RNA. The reaction mix has been developed to ensure reverse transcription of the *EML4-ALK* RNA and the reference gene RNA into cDNA.
2) The **EA Fusion Gene Reaction Mix** ①～③ contain primers and FAM-labeled probes specific for *EML4-ALK* fusions.
3) The **EA External Control Reaction Mix** contains primers and FAM-labeled probes to amplify reference gene to reveal the presence of PCR inhibitors or compromised RNA integrity that may lead to false negative results.
4) The **EA Enzyme Mix** contains the Taq DNA polymerase for PCR amplification and uracil-N-glycosylase which works at room temperature to prevent PCR amplicon carryover contamination.
5) **EA Positive Control** contains recombinant gene with *EML4-ALK* fusions.

Kit Contents

This kit contains the following materials:

Table 1 Kit Contents

Tube No.	Content	Main Ingredients	Quantity	Fluorescent Signal
①	**EA Fusion Gene Reaction Mix ①**	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM
②	**EA Fusion Gene Reaction Mix ②**	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM
③	**EA Fusion Gene Reaction Mix ③**	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM
④	**EA External Control Reaction Mix**	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM
⑤	**EA RT Reaction Mix**	Primers, Mg^{2+}, dNTPs	550 μL/tube ×1	/
/	**EA Reverse Transcriptase**	Reverse Transcriptase	20 μL/tube ×1	/
/	**EA Enzyme Mix**	Taq DNA Polymerase, Uracil-N-Glycosylase	50 μL/tube ×1	/
/	**EA Positive Control**	Plasmid DNA	250 μL/tube ×1	/

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 recommend maximum freeze-thaw cycle is five cycles.

Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments:
   Stratagene Mx3000P™, ABI7500, LightCycler480 II, Bio-Rad CFX 96, Rotor-Gene Q/6000 (72 wells), or SLAN-96S.
2) RNA extraction kit: we recommend use of AmoyDx® FFPE RNA Kit, Cat No.: 8.02.24101X036G.
3) Spectrophotometer for measuring RNA concentration.
4) Mini centrifuge with rotor for centrifuge tubes.
5) Mini centrifuge with rotor for PCR tubes.
6) Nuclease-free centrifuge tubes.
7) Nuclease-free PCR tubes and caps.
8) Adjustable pipettors and filtered pipette tips for handling RNA.
9) Tube racks.
10) Disposable powder-free gloves.
11) Sterile, nuclease-free water.

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 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.
- 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 nucleic acid 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 signal (R), please reduce the signal gain setting of instrument properly.
- For ABI instruments please set up as follows: Reporter Dye: FAM; Quencher Dye: TAMRA; Passive Reference: NONE.
- For LightCycler480 II, if there is fluorescence crossover on instrument, fluorescence calibration is also required. To run the assays on a LightCycler machine, please use the Roche 480 adaptor, available from BIOplastics, Cat No. B79480.
- For SLAN-96S, please set up as follows: Probe mode: FAM. 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 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. RNA Extraction

The specimen material must be human total RNA extracted from NSCLC FFPE tissue samples. RNA extraction kit is not included in the kit. Carry out the RNA extraction according to the instructions of RNA extraction kit.

Before RNA extraction, it's essential to use standard pathology methodology to ensure tumor sample quality. Tumor samples are non-homogeneous, may also contain non-tumor tissue. Data from different tissue sections of the same tumor may be inconsistent. RNA from non-tumor tissue may not be detected with *EML4-ALK* fusion. It's better to use tumor tissue samples with more than 30% tumor cells.

The OD value of extracted RNA should be measured using the spectrophotometer after extraction. The OD_{260}/OD_{280} value should be between 1.9~2.1 and total RNA concentration should be between 20~800 ng/μL.

Note:

- *The FFPE tissue should be handled and stored properly, and the storage time should preferably be less than 2 years.*
- *The extracted RNA should be used immediately, if not, it should be stored at -20±5℃ for no more than one week.*

2. Reverse Transcription

1) Take **EA RT Reaction Mix** and **EA Reverse Transcriptase** out of the kit from the freezer, and other reagents remained in freezer at -20±5℃.
2) Thaw **EA RT Reaction Mix** at room temperature. When the reagents completely thawed, invert each tube for 10 times and briefly centrifuge to collect all liquid at the bottom of the tube.
3) Briefly centrifuge **EA Reverse Transcriptase** prior to use.
4) For each RNA sample, transfer 18.5 μL **EA RT Reaction Mix** and 0.5 μL **EA Reverse Transcriptase** and 6 μL sample RNA to a sterile centrifuge tube. Thoroughly mix the reagents by gently pipetting up and down more than 10 times, and then centrifuge briefly.
5) Incubate the tubes at 42℃ for one hour.
6) Heat the tubes at 95℃ for 5 minutes, then transfer the tubes on the ice. The cDNA solutions are obtained.

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

3. PCR amplification

1) Take **EA Fusion Gene Reaction Mix ①~③**, **EA External Control Reaction Mix**, **EA Enzyme Mix** and **EA Positive Control** out of the kit from the freezer.
2) Thaw **EA Fusion Gene Reaction Mix ①~③**, **EA External Control Reaction Mix** and **EA Positive Control** at room temperature. When the reagents completely thawed, invert the tube for 10 times and briefly centrifuge to collect all liquid at the bottom of the tube.
3) Briefly centrifuge **EA Enzyme Mix** prior to use.
4) Prepare sufficient EA Mater Mix containing EA Enzyme Mix and EA Fusion Gene Reaction Mix (①~③) or EA External Control Reaction Mix respectively in separate sterile centrifuge tube according to the ratio in Table 2. Thoroughly mix each EA Master Mix by gently pipetting up and down more than 10 times.

Table 2 EA Master Mix

Content	Volume per test
EA Enzyme Mix	0.3 μL
EA Fusion Gene Reaction Mix ①~③/ EA External Control Reaction Mix	35 μL
Total	**35.3 μL**

Note:

- *Every PCR run must contain one PC (Positive control) and one NTC (No template control).*
- *Do not vortex enzyme mix or any mixture with enzyme mix.*
- *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.*

5) Take out the sample cDNA and nuclease-free water for NTC.
6) Prepare 4 PCR tubes for NTC, transfer 35 μL of each of the 4 EA Master Mixes to the corresponding tubes. Then add 5 μL of nuclease-free water to each PCR tube, and cap the PCR tubes.
7) Prepare 4 PCR tubes for PC, transfer 35 μL of each of the 4 EA Master Mixes to the corresponding tubes. Then add 5 μL of Positive Control to each PCR tube, and cap the PCR tubes.
8) Prepare 4 PCR tubes for each sample, transfer 35 μL of each of the 4 EA Master Mixes to the corresponding tubes. Then add 5 μL of sample cDNA to each PCR tube, and cap the PCR tubes.
9) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.
10) Place the PCR tubes into the appropriate positions of the real-time PCR instrument. A recommended plate layout is shown in Table 3.

Table 3 Plate Layout

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12
①	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	NTC
②	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	NTC
③	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	NTC
④	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	NTC
①	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	PC
②	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	PC
③	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	PC
④	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	PC

11) Setup the PCR protocol using the cycling parameters in Table 4.

Table 4 PCR Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	5min	/
2	15	95℃	25s	/
		64℃	20s	/
		72℃	20s	/
3	31	93℃	25s	/
		60℃	35s	FAM
		72℃	20s	/

12) Start the PCR run immediately.

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

4. Results Interpretation

Before the data analysis, the following items should be checked:

1) For NTC: The FAM Ct values of EA Fusion Gene Reaction Mix ①~③ and EA External Control Reaction Mix should be ≥ 31. If not, the data is *INVALID*. The sample should be retested.

2) For Positive Control: The FAM Ct values of EA Fusion Gene Reaction Mix ①~③ and EA External Control Reaction Mix should be < 24. If not, the data is *INVALID*. The sample should be retested.

3) For the reference gene assay in EA External Control Reaction Mix:
   a) The FAM Ct value should be < 31.
   b) If the FAM Ct value ≥ 31, this indicates RNA degradation or presence of PCR inhibitors. The sample should be retested with increased or re-extracted RNA.

Analyze the fusion assay result for each sample:

4) Record the FAM Ct values of EA Fusion Gene Reaction Mix ①~③ for each sample:
   a) If the FAM Ct value of EA Fusion Gene Reaction Mix ①~③ ≥30, the sample is determined as negative (no *EML4-ALK* fusion detected) or under the LOD (limit of Detection) of the kit.
   b) If any FAM Ct value of EA Fusion Gene Reaction Mix ①~③ <30, the sample is determined as positive (*EML4-ALK* fusion detected).

5) The sample may contain two or more fusion patterns simultaneously.

Performance Characteristics

The performance characteristics of this kit were validated on Stratagene Mx3000P™, ABI7500, LightCycler480 II, Bio-Rad CFX 96, Rotor-Gene Q/6000 (72 wells), and SLAN-96S.

1) Limit of detection:
   The limit of detection of the kit was established by test the diluted *EML4-ALK* fusion plasmids. The kit allows detection of 25 copies/μL *EML4-ALK* fusion plasmid DNA.

2) Specificity:
   The kit was tested by 9 negative reference controls, which were prepared from 9 cases of FFPE tissue samples without *EML4-ALK* fusion confirmed by Sanger Sequencing. The test gave negative results and with 100% concordance rate.

3) Accuracy:
   The kit also was tested by 9 *EML4-ALK* fusion positive reference controls, which were prepared from 9 cases of lung cancer FFPE tissue samples with *EML4-ALK* fusion confirmed by Sanger Sequencing. The test gave corresponding positive results and with 100% concordance rate.

4) Precision:

Precision of the kit was established by testing of the precision reference control for 10 repeats; the test gave positive results, analyzed the FAM Ct values, CV (%) ≤5%.

Limitations

1) The kit is to be used only by personnel specially trained with PCR techniques.
2) The results can be used to assist clinical diagnosis, combining with other clinical and laboratory findings.
3) The kit has been validated for use with extracted RNA from NSCLC patient FFPE tissue.
4) The kit can only detect 21 *EML4-ALK* gene fusions listed in the appendix.
5) Reliable results are dependent on proper sample processing, transport, and storage.
6) The sample containing degraded RNA may affect the ability of the test to detect 21 *EML4-ALK* fusions.
7) Samples with negative result (No *EML4-ALK* Fusion Detected) may harbor *EML4-ALK* fusions not detected by this assay.

References

1) Sasaki T, *et al.* 2010. *Eur J Cancer*. 46:1773-80.
2) Kwak EL, *et al*, 2010. *N Engl J Med*. 363:1693-703.
3) Horn L. and Pao W, 2009. *J Clin Oncol*. 27:4232-5.

Symbols

1)		Authorized Representative in the European Community
2)		In Vitro Diagnostic Medical Device
3)		Manufacturer
4)		Catalogue Number
5)		Batch Code
6)		Use By
7)		Contains Sufficient for <n> Tests
8)		Temperature Limitation
9)		Consult Instructions For Use
10)		Keep Dry
11)		This Way Up
12)		Fragile, Handle With Care

Appendix

***EML4-ALK* Fusions Detected by the Kit**

Reagent	*EML4-ALK* Fusion Types			
EA Fusion Gene Reaction Mix ①	E6;A19	E6;A20	E6ins33;A20	E6;ins18A20
	E13;A20	E13;ins69A20	E20;A20	E20;ins18A20
EA Fusion Gene Reaction Mix ②	E14 ins11;del49A20	E14;del14A20	E14;del38A20	E15del60;del71A20
EA Fusion Gene Reaction Mix ③	E2;A20	E2;ins117A20	E3;ins53A20	E17;ins30A20
	E17ins61;ins34A20	E17ins65;A20	E17;ins68A20	E17del58;ins39A20
	E18;A20			