# AmoyDx® *ALK* Gene Fusions and *ROS1* Gene Fusions Detection Kit

**For qualitative detection of 26 *ALK* gene fusions and 14 *ROS1* gene fusions**

Instruction for Use

| | | | |
|---|---|---|---|
| REF | 8.01.24401W008A | 8 tests | For Stratagene Mx3000P™, ABI 7500 |
| REF | 8.01.24401W008B | 8 tests | For LightCycler 480 II |
| REF | 8.01.24401W008D | 8 tests | For SLAN-96S |

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## Background

*ALK* gene fusions and *ROS1* gene fusions define unique molecular subsets of non–small-cell lung cancer (NSCLC). *EML4*, *KIF5B*, *TFG*, *KLC1* etc., are identified as fusion partners of *ALK*; *SLC34A2*, *CD74*, *SDC4*, *EZR* etc., are identified as fusion partners of *ROS1*. These fusions lead to constitutive kinase activity and activation of downstream pathways, leading to carcinogenesis. It has been reported that the presence of the *ALK* gene fusions and *ROS1* gene fusions are correlated with the efficacy of TKI therapy. Based on analysis of tumor messenger RNA, *ALK* gene fusions and *ROS1* gene fusions can be detected by real-time PCR method.

## Intended Use

The AmoyDx® *ALK* Gene Fusions and *ROS1* Gene Fusions Detection Kit is a real-time PCR assay for qualitative detection of 26 *ALK* gene fusions and 14 *ROS1* gene fusions in human total RNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue.

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 cDNA of *ALK* and *ROS1* fusions, and the fusion amplicon is detected by fluorescent probes.

The kit is composed of ALK RT Reaction Mix, ROS1 RT Reaction Mix, ALK&ROS1 Reaction Mix strips, sufficient positive control and enzyme.

1) **ALK&ROS1 RT Reaction Mix** contains primers specific for reverse transcription of *ALK*/*ROS1* RNA and reference gene RNA into cDNA.
2) **ALK&ROS1 Reaction Mix** ①-④ contain primers and FAM-labeled probes specific for *ALK* gene fusions. **ALK&ROS1 Reaction Mix** ⑤-⑧ contain primers and FAM-labeled probes specific for *ROS1* gene fusions.
3) **ALK&ROS1 Reaction Mix** ⑧ also contains primers and HEX(VIC)-labeled probe for detection of housekeeping gene *HPRT1* as reference gene to reveal the presence of PCR inhibitors or compromised RNA integrity that may lead to false negative results.
4) **ALK&ROS1 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) **ALK&ROS1 Positive Control** contains recombinant gene with *ALK* gene fusions and *ROS1* fusions.

## Kit Contents

This kit contains the following materials:

Table 1 Kit Contents

| Content | Main Ingredients | Quantity |
|---|---|---|
| **ALK&ROS1 Reaction Mix** | 8-tube strip* | 12 strips |
| **ALK&ROS1 RT Reaction Mix** | Primers, $Mg^{2+}$, dNTPs | 37 μL/tube ×8 |
| **ALK&ROS1 Reverse Transcriptase** | Reverse Transcriptase | 12 μL/tube ×1 |
| **ALK&ROS1 Enzyme Mix** | Taq DNA Polymerase, Uracil-N-Glycosylase | 45 μL/tube ×1 |
| **ALK&ROS1 Positive Control** | Plasmid DNA | 250 μL/tube ×1 |

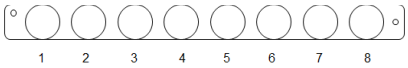
*Each strip (8-tube) includes the following contents for testing one sample or one control. Tubes ①~④ is for testing *ALK* gene fusions, Tubes ⑤~⑧ is for testing *ROS1* gene fusions (Table 2).

Table 2 Information of the 8-tube Strip

| Tube No. | Reagent | Detection Target | Main ingredients | Quantity | Fluorescent Signal |
|---|---|---|---|---|---|
| ① | ALK&ROS1 Reaction Mix ① | *ALK*-1 | Primers, Probes, $Mg^{2+}$, dNTPs | 35 μL | FAM |
| ② | ALK&ROS1 Reaction Mix ② | *ALK*-2 | Primers, Probes, $Mg^{2+}$, dNTPs | 35 μL | FAM |
| ③ | ALK&ROS1 Reaction Mix ③ | *ALK*-3 | Primers, Probes, $Mg^{2+}$, dNTPs | 35 μL | FAM |
| ④ | ALK&ROS1 Reaction Mix ④ | *ALK*-4 | Primers, Probes, $Mg^{2+}$, dNTPs | 35 μL | FAM |
| ⑤ | ALK&ROS1 Reaction Mix ⑤ | *ROS1*-1 | Primers, Probes, $Mg^{2+}$, dNTPs | 35 μL | FAM |
| ⑥ | ALK&ROS1 Reaction Mix ⑥ | *ROS1*-2 | Primers, Probes, $Mg^{2+}$, dNTPs | 35 μL | FAM |
| ⑦ | ALK&ROS1 Reaction Mix ⑦ | *ROS1*-3 | Primers, Probes, $Mg^{2+}$, dNTPs | 35 μL | FAM |
| ⑧ | ALK&ROS1 Reaction Mix ⑧ | *ROS1*-4 & HPRT1 | Primers, Probes, $Mg^{2+}$, dNTPs | 35 μL | FAM & HEX/VIC |

***Note:***

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




## Storage and Stability

The kit requires shipment on frozen ice packs. All contents 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, or SLAN-96S.
2) RNA extraction kit. We recommend use of AmoyDx RNA extraction kit (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) Adjustable pipettors and filtered pipette tips for handling RNA.
8) Tube racks.
9) Disposable powder-free gloves.
10) 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 signals (R), please reduce the signal gain setting of instrument properly.
- For ABI7500, please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.
- For LightCycler480 II, if there is fluorescence crossover on the 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, 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 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 extracted RNA 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. The OD value of RNA samples should be measured using the spectrophotometer after extraction.

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.

Total RNA from non-tumor tissue may not be detected with *ALK* or *ROS1* fusions. 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 1.8~2.1 and total RNA concentration should be between 50~800 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 **ALK&ROS1 RT Reaction Mix** and **ALK&ROS1 Reverse Transcriptase** as need out of the kit from the freezer, and other reagents remained in freezer at -20±5℃.
2) Thaw **ALK&ROS1 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 **ALK&ROS1 Reverse Transcriptase** prior to use.
4) For each RNA sample, transfer 1.0 μL ALK&ROS1 Reverse Transcriptase and 12 μL sample RNA into an **ALK&ROS1 RT Reaction Mix** tube. Mix well 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 **ALK&ROS1 cDNA** 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 **ALK&ROS1 Positive Control** and **ALK&ROS1 Enzyme Mix** out of the kit from the freezer.
2) Thaw **ALK&ROS1 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 **ALK&ROS1 Enzyme Mix** prior to use.
4) Take out the sample cDNA and nuclease-free water for NTC (No template control).
5) Transfer 3μL **ALK&ROS1 Enzyme Mix** into 50 μL **ALK&ROS1 cDNA**, 50 μL **ALK&ROS1 Positive Control** (PC) and 50 μL NTC respectively to obtain **ALK&ROS1 cDNA Mix**, **PC Mix** and **NTC Mix**.
6) Thoroughly mix each Mix by gently pipetting up and down more than 10 times.

   ***Note:***

   - *Each PCR run must contain one PC and one NTC.*
   - *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.*
7) Take out the **ALK&ROS1 Reaction Mix** strips (sufficient for samples, PC and NTC), 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) Add 5 μL of prepared **NTC Mix** into each PCR tube of NTC strip, and cap the PCR tubes.
9) Add 5 μL sample **ALK&ROS1 cDNA Mix** into each PCR tube of sample strip, and cap the PCR tubes.
10) Add 5 μL **PC Mix** into each PCR tube of PC strip, and 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 3.

Table 3 Plate Layout

| Tube No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| ① | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Sample 8 | PC | NTC |
| ② | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Sample 8 | PC | NTC |
| ③ | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Sample 8 | PC | NTC |
| ④ | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Sample 8 | PC | NTC |
| ⑤ | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Sample 8 | PC | NTC |
| ⑥ | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Sample 8 | PC | NTC |
| ⑦ | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Sample 8 | PC | NTC |
| ⑧ | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Sample 8 | PC | NTC |

13) 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 and HEX(VIC) |
| | | 72℃ | 20s | / |

14) Start the PCR run immediately.

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

## 4. Results Interpretation

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

1) For NTC: The FAM Ct values of Reaction Mixes ①~⑧ and HEX/VIC Ct value of Reaction Mix ⑧ should be ≥31. If not, the data is *INVALID*. The sample should be retested.

2) For Positive Control: The FAM Ct values of Reaction Mixes ①~⑧ and HEX/VIC Ct value of Reaction Mix ⑧ should be < 24. If not, the data is *INVALID*. The sample should be retested.

3) Analyze the sample reference gene assay (HEX/VIC) in Reaction Mix ⑧:
   a) The HEX/VIC Ct value should be ≤20.
   b) If the HEX/VIC Ct value >20, this indicates RNA degradation or presence of PCR inhibitors. The sample should be retested with increased or re-extracted RNA, as there may be false negative results.

***Analyze the fusion assay for each sample:***

4) Check the FAM Ct value for each sample:
   a) If the sample FAM Ct values of Reaction Mixes ①~⑧ ≥30, the sample is determined as negative (no *ALK&ROS1* fusion detected) or under the LOD (limit of Detection) of the kit.
   b) If any FAM Ct value of Reaction Mixes ①~⑧ <30, the sample is determined as positive (*ALK* fusion or *ROS1* fusion detected):
      - If any FAM Ct value of Reaction Mix ①~④ <30, the sample contains *ALK* gene fusion;
      - If any FAM Ct value of Reaction Mix ⑤~⑧ <30, the sample contains *ROS1* gene fusion.

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 and SLAN-96S.

1) Limit of detection:
   The kit allows detection of 25 copies/μL *ALK* gene fusion plasmid DNA and *ROS1* gene fusion plasmid DNA.
2) Specificity:
   The kits have been validated of non-specificity amplification at a background of 500 ng wild-type DNA or 9600 ng wild-type RNA.
3) Accuracy:
   Accuracy of the kit was established by testing ALK&ROS1 fusion-positive reference controls and negative reference controls, the detection concordance rate are 100%.
4) Precision:
   Precision of the kit was established by performing a certain ALK&ROS1 fusion-positive reference control for 10 repeats, CV (coefficient of variation) of Ct values ≤10%.

## 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 FFPE tissue.
4) The kit can only detect 26 *ALK* and 14 *ROS1* 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 26 *ALK* and 14 *ROS1* fusions.
7) Samples with negative result (No *ALK* or *ROS1* Fusion Detected) may harbor *ALK or ROS1* fusions not detected by this assay.

## References

1) Sasaki T *et al*., *Eur J Cancer*. 2010;46:1773-80.
2) OU SH *et al*., *Oncologist*. 2012;17:1351-1375.
3) Bergethon K *et al*., *J Clin Oncol*.2012;46:863-70.
4) Tan J *et al*., *Expert Rev Anticancer Ther*.2012;12:447-56.

## 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 |

# Appendix

***ALK* Gene Fusions and *ROS1* Gene Fusions Detected by the Kit**

| Reagent | Detection Target | Fusion patterns | Abbreviation of Fusion patterns | |
|---|---|---|---|---|
| ALK&ROS1 Reaction Mix ① | *ALK*-1 | *EML4-ALK* | E6ins33;A20;<br>E20;A20<br>E6;A19<br>E6;A20 | E13;A20<br>E6;ins18A20<br>E13;ins69A20<br>E20;ins18A20 |
| ALK&ROS1 Reaction Mix ② | *ALK*-2 | *EML4-ALK*<br>*KIF5B-ALK* | E14ins11;del49A20<br>E14;del14A20<br>KI15;A20 | E15del60;del71A20<br>E14;del38A20 |
| ALK&ROS1 Reaction Mix ③ | *ALK*-3 | *EML4-ALK* | E2;A20<br>E2;ins117A20<br>E3;ins53A20<br>E17ins61;ins34A20<br>E17del58;ins39A20 | E17;ins68A20<br>E18;A20<br>E17;ins30A20<br>E17ins65;A20 |
| ALK&ROS1 Reaction Mix ④ | *ALK*-4 | *KIF5B-ALK*<br>*KLCL1-ALK*<br>*TFG-ALK* | KI17;A20<br>KI24;A20<br>T4;A20 | KL9;A20 |
| ALK&ROS1 Reaction Mix ⑤ | *ROS1*-1 | *SLC34A2-ROS1*<br>*CD74-ROS1*<br>*SDC4* | SL4;R32<br>SL14del;R32<br>CD6;R32 | SD2;R32<br>SD4;R32 |
| ALK&ROS1 Reaction Mix ⑥ | *ROS1*-2 | *SLC34A2-ROS1*<br>*CD74-ROS1*<br>*SDC4-ROS1*<br>*EZR-ROS1* | SL4;R34<br>SL14del;R34<br>CD6;R34 | SD4;R34<br>EZ10;R34 |
| ALK&ROS1 Reaction Mix ⑦ | *ROS1*-3 | *TPM3-ROS1*<br>*LRIG3-ROS1*<br>*GOPC-ROS1* | TP8;R35<br>LR16;R35<br>GO8;R35 | |
| ALK&ROS1 Reaction Mix ⑧ | *ROS1*-4 & *HPRT1* | *GOPC-ROS1* | GO4;R36 | |