AmoyDx
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CE IVD

AmoyDx® *ROS1* Gene Fusions Detection Kit

Instructions for Use

REF 8.01.0165 12 tests/kit For Stratagene Mx3000P™, ABI7500, LightCycler480 II, SLAN-96S

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Background

ROS1 is a receptor tyrosine kinase of insulin receptor family. *ROS1* gene fusions define a unique molecular subset of non–small-cell lung cancer (NSCLC). The *ROS1* fusion partners include *SLC34A2, CD74, SDC4, EZR* etc. These fusions lead to constitutive kinase activity and activation of downstream pathways, such as JAK/STAT, PI3K/AKT, RAS/MAPK etc., leading to carcinogenesis. It has been reported that the presence of the *ROS1* rearrangement is correlated with the efficacy of TKI therapy.

Based on analysis of tumor messenger RNA, *ROS1* gene fusions can be detected by real-time PCR method.

Intended Use

The AmoyDx® ROS1 Gene Fusions Detection Kit is an *in vitro* nucleic acid amplification test intended for qualitative detection of 14 *ROS1* gene fusions in human formalin-fixed paraffin-embedded (FFPE) tumor tissue RNA. The kit is intended to assess *ROS1* gene status in NSCLC patients and to aid the clinician in identifying NSCLC patients who may response to *ROS1* inhibitor therapy, such as Xalkori® (crizotinib).

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 *ROS1* variant cDNA, and mutant amplicon is detected by fluorescent probes labeled with FAM, while reference gene amplicon is detected by fluorescent probe labeled with HEX.

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

1) The **ROS1 RT Reaction Mix** contains primers specific for reverse transcription of both *ROS1* RNA and reference gene RNA into complementary DNA (cDNA).
2) **The ROS1 Reaction Mix** ①~④ contains primers and FAM-labeled probes specific for *ROS1* gene fusions. The **ROS1 Reaction Mix** ④ contains primers and HEX–labeled probe for detection of reference gene to reveal the presence of PCR inhibitors or RNA integrality that may lead to false negative results.
3) The **ROS1 Positive Control (PC)** contains recombinant gene with *ROS1* gene fusions.
4) The **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.

Kit Contents

This kit contains the following materials.

Table 1 Kit Contents

Tube No.	Content	Main Ingredients	Quantity	Fluorescent signal
①	ROS1 Reaction Mix ①	Primers, Probes, Mg^{2+}, dNTPs,	550 µL/tube ×1	FAM
②	ROS1 Reaction Mix ②	Primers, Probes, Mg^{2+}, dNTPs,	550 µL/tube ×1	FAM
③	ROS1 Reaction Mix ③	Primers, Probes, Mg^{2+}, dNTPs,	550 µL/tube ×1	FAM
④	ROS1 Reaction Mix ④	Primers, Probes, Mg^{2+}, dNTPs,	550 µL/tube ×1	FAM, HEX/VIC
⑤	ROS1 RT Reaction Mix	Primers, Mg^{2+}, dNTPs	300 µL/tube ×1	/
/	ROS1 Reverse Transcriptase	Reverse Transcriptase	10 µL/tube ×1	/
/	ROS1 Enzyme Mix	Taq DNA Polymerase, Uracil-N-Glycosylase	30 µL/tube ×1	/
/	ROS1 Positive Control	Plasmid DNA	150 µL/tube ×1	/

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 maximal freeze-thaw cycles is five.

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 to use AmoyDx RNA extraction kit (AmoyDx® FFPE RNA Kit) for paraffin embedded tissue specimens.
3) Spectrophotometer for measuring 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 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. 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 contact of 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, pipettes and other materials used should be from pre-amplification to post-amplification, and never backwards.
- Gloves should be worn and changed frequently when handling samples and reagents to prevent contamination.
- Use separate, dedicated pipettes and filtered pipette tips when handling samples and reagents to prevent exogenous nucleic acid contamination to the reagents.
- 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 is a low net fluorescence signal (dR) but a high background signal (R), please reduce the signal gain setting of instrument properly.
- For ABI instrument, please set up as follows: Reporter Dye: FAM, VIC; 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, 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 operations manual of the real-time PCR instrument for detailed instructions.
- We recommend that for all PCR instruments in use, a fluorescence calibration should be conducted once a year.

Assay Procedure

1. RNA Extraction

The specimen material must be extracted RNA from NSCLC FFPE tissue samples. The RNA extraction kit is not included in the kit. Before RNA extraction, it is essential to use a standard pathology methodology to ensure tumor sample quality. Carry out the RNA extraction according to the instructions of RNA extraction kit.

The OD value of extracted RNA should be measured using the spectrophotometer after extraction. OD_{260}/OD_{280} value should be between 1.9~2.1 and total RNA concentration should be between 50~800 ng/μL.

Note:

- *The FFPE tissue should be handled and stored properly. 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 **ROS1 RT Reaction Mix** and **ROS1 Reverse Transcriptase** out of the kit from the freezer. Other reagents remained in freezer at -20±5℃.
2) Thaw **ROS1 RT Reaction Mix** 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 **ROS1 Reverse Transcriptase** for 5~10 seconds prior to use.
4) Prepare a sterile nuclease-free PCR tube for each sample: pipet 18.5 μL **ROS1 RT Reaction Mix**, 0.5 μL **ROS1 Reverse Transcriptase** and 6 μL sample RNA to the tube. Mix thoroughly by vortexing, and centrifuge for 5~10 seconds.
5) Incubate the tubes at 42℃ for one hour.
6) Heat the tubes at 95℃ for 5 minutes, then transfer them to ice. The resulting sample cDNA are used for subsequent PCR amplification.
   Note: S*ample 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 **ROS1 Reaction Mixes** ①~④, **ROS1 PC** and **ROS1 Enzyme Mix** out of the kit from the freezer.
2) Thaw **ROS1 Reaction Mixes** ①~④ and **ROS1 PC** at room temperature. When the reagents are completely thawed, mix each reagent thoroughly by vortexing and centrifuge for 5~10 seconds to collect all liquid at the bottom of the tube.
3) Centrifuge **ROS1 Enzyme Mix** for 5~10 seconds prior to use.
4) Prepare sufficient ROS1 Master Mix 1~4 containing ROS1 Enzyme Mix and each ROS1 Reaction Mix (ROS1 Reaction Mix ①~④, respectively) in separate sterile centrifuge tube according to the ratio in Table 2. Thoroughly mix each ROS1 Master Mix by vortexing, and centrifuge for 5~10 seconds.

Table 2 ROS1 Master Mix

Content	Volume per test
ROS1 Enzyme Mix	0.3 μL
Each ROS1 Reaction Mix	35 μL
Total	**35.3 μL**

Note:

- *Every PCR run must contain one PC (Positive control) and one NTC (No template control).*
- *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: Dispense 35.3 μL of ROS1 Master Mix 1~4 to each PCR tube respectively. Then add 5 μL of nuclease-free water to each PCR tube. Cap the PCR tubes.
7) Prepare 4 PCR tubes for each sample: Dispense 35.3 μL of ROS1 Master Mix 1~4 to each PCR tube respectively. Then add 5 μL of sample cDNA to each PCR tube. Cap the PCR tubes.
8) Prepare 4 PCR tubes for PC: Dispense 35.3 μL of ROS1 Master Mix 1~4 to each PCR tube respectively. Then add 5 μL of ROS1 Positive Control to each PCR tube. 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 Recommended Plate Layout

Tube No.	Signal	1	2	3	4	5	6	7
①	FAM	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	NTC
②	FAM	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	NTC
③	FAM	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	NTC
④	FAM & HEX/VIC	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	NTC
⑤	FAM	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	PC
⑥	FAM	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	PC
⑦	FAM	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	PC
⑧	FAM & HEX/VIC	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	PC

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

Table 4 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	/

12) Start the PCR run immediately.
13) When the PCR run is finished, analyze the data according to the “Results Interpretation” procedures.

4. Result Interpretation

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

1) For NTC: The FAM Ct values of Reaction Mixes ①~④ and HEX/VIC Ct values of Reaction Mix ④ should be ≥ 31. If not, the data is *INVALID*. The sample should be retested.
2) For PC: The FAM Ct values of Reaction Mixes ①~④ and HEX/VIC Ct values of Reaction Mix ④ should be < 24. If not, the data is *INVALID*. The sample should be retested.
3) For the reference gene assay (HEX/VIC signal) in Reaction Mix ④ for each sample:
   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) Record the FAM Ct value of Reaction Mixes ①~④ for each sample:
   a) If the FAM Ct value of Reaction Mix ①~④ ≥30, the sample is determined as Negative (No *ROS1* fusion detected) or under the LOD (limit of Detection) of the kit.
   b) If any FAM Ct value of Reaction Mix ①~④ <30, the sample is determined as Positive (*ROS1* 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 and SLAN-96S.

1) Limit of detection:
   The limit of detection was established by testing plasmid DNA with 14 *ROS1* fusions. The results show the limit of detection for each *ROS1* fusion was 25 copies/μL.
2) Specificity:
   Specificity of the kit was established by testing 8 *ROS1* negative reference controls which were prepared with wild-type DNA (4 controls) or with wild-type RNA (another 4 controls) , the tests gave negative results and with 100% concordance rate.
3) Accuracy:
   Accuracy of the kit was established by testing 14 *ROS1* gene fusion positive reference controls which were prepared with plasmid with *ROS1* fusions, 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 with the FAM and HEX Ct value < 24 and Ct's CV (%) ≤ 10%.
5) Interfering substance:
   Two common potential interfering substances were selected in this study: oxyhemoglobin and triglyceride. It is confirmed that the potential maximum concentrations: 4 g/L oxyhemoglobin and 74 mmol/L triglyceride would not interfere with the test result.

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, combined 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 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 *ROS1* fusions.
7) Samples with negative result (No *ROS1* Fusion Detected) may harbor *ROS1* fusions not detected by this assay.

References

1) Bergethon K *et al*. *J Clin Oncol*.2012;46:863-70.
2) Tan J *et al*. *Expert Rev Anticancer Ther*.2012;12:447-56.
3) Janne P Q *et al*. *Clin Oncol*. 2012;30:878-79.

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

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

Reagent	Spliced Gene & Exon			ROS1 Spliced Exon
ROS1 Reaction Mix ①	*SLC34A2* exon4	*SLC34A2* exon13del	*CD74* exon6	32
	SDC4 exon2	*SDC4* exon4		
ROS1 Reaction Mix ②	*SLC34A2* exon4	*SLC34A2* exon13del	*CD74* exon6	34
	SDC4 exon4	*EZR* exon10		
ROS1 Reaction Mix ③	*TPM3* exon8	*LRIG3* exon16	*GOPC* exon8	35
ROS1 Reaction Mix ④	*GOPC* exon4			36