IntelliPlex® ROS1 Rearrangement Kit
User Manual

REF **82024** **24 Reactions**

CE IVD **For In-Vitro Diagnostic Use**

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IMPORTANT:
Read the instructions carefully prior to use

1. INTENDED USE

The IntelliPlex ROS1 Rearrangement Kit, based on πCode® technology and PlexBio's instrument platform, is an in vitro RT-PCR assay intended for qualitative detection of 14 gene rearrangements involving in *ROS1* gene using RNA samples derived from formalin-fixed paraffin- embedded of non-small cell lung cancer (NSCLC) tissue. The IntelliPlex ROS1 Rearrangement Kit is for in vitro diagnostic use and intended to be used by trained laboratory professionals.

2. INTRODUCTION

ROS1 (also known as *ROS, MCF3 or c-ros-1*) is a proto-oncogene highly expressed in a variety of tumor cell lines, and a member of the sevenless subfamily of tyrosine kinase insulin receptor genes. ROS1 fusions contain an intact tyrosine kinase domain that activates downstream signaling pathways, such as JAK/STAT, PI3K/AKT, and RAS/MAPK, resulting in increased cell proliferation. ROS1 rearrangement-positive NSCLC can be treated effectively with ROS1 inhibitors such as crizotinib. Therefore, assessment of *ROS1* rearrangement may be beneficial in the treatment evaluation of patients with NSCLC. The IntelliPlex ROS1 Rearrangement kit combines one step RT-PCR with πCode technology to enable multiplex, single-well detection of gene rearrangements from RNA specimens containing large amounts of wild-type RNA. The **IntelliPlex ROS1 Rearrangement Kit** identifies 14 rearrangements of the *ROS1* gene (Table 1).

Table 1: Variants Detected

Gene	Fusion Variant	Inferred Breakpoint
ROS1	CD74-ROS1	C6;R32
		C6;R34
	EZR-ROS1	E10;R34
	SLC34A2-ROS1	S4;R32
		S4;R34

Gene	Fusion Variant	Inferred Breakpoint
		S13del;R32
		S13del;R34
	SDC4-ROS1	SD2;R32
		SD4;R32
		SD4;R34
	TPM3-ROS1	T8;R35
	FIG-ROS1	F3;R36
		F7;R35
	LRIG3-ROS1	L16;R35

3. TECHNOLOGICAL PRINCIPLES

The **IntelliPlex ROS1 Rearrangement Kit** utilizes two technologies, one-step RT-PCR and πCode, to achieve high sensitivity multiplex variant detection.

One-step RT-PCR

One-step RT-PCR combines cDNA synthesis and PCR amplification in a single tube, reducing operation time and contamination risk while yielding highly sensitive results.

πCode MicroDisc

πCode MicroDisc are manufactured to generate up to 85,000 distinct circular image patterns for multiplexing applications. Each πCode MicroDisc has a distinct circular image pattern, which corresponds to a specific capture agent conjugated to the surface of the disc. πCode tagged with different capture agents are pooled, enabling specific detection of multiple analytes in a one-well reaction.

Detection Principle

The test is based on five processes:

1. RNA extraction from specimens
2. Multiplex one step RT-PCR amplification
3. Hybridization of PCR amplicons with variant-specific probe tagged πCode in a one-well reaction
4. Fluorescent labeling with streptavidin-phycoerythrin
5. Image pattern decoding and fluorescent signal detection by the PlexBio® 100 Fluorescent Analyzer

4. WARNINGS AND PRECAUTIONS

- For in vitro diagnostic use.
- This assay kit should only be used by qualified laboratory personnel.
- **Do not repeatedly freeze-thaw the reconstituted positive control. Use within one freeze-thaw cycle.**
- Separate, dedicated rooms and equipment for pre- and post- PCR process with unidirectional manner to avoid any contaminations are required.
- Pre-PCR process preparation should be operated in laminar flow hood to avoid contamination.
- Do not use a kit or reagent past its expiration date.
- Note that tumor samples are non-homogeneous in terms of genotype, and may contain non-tumor sections, which can cause false negative results.
- Reagent components have been diluted optimally. Further dilution of the component reagents is not

recommended.

- Specimens should be handled as infectious material. Please follow universal precautions for safe use.
- Store assay kits and reagents according to the product label and instructions.
- Do not mix reagents from different lots.
- Dispose of unused reagents, specimens and waste according to applicable central/federal, state, and local regulations.
- Wear powderless gloves and do not touch and make any markings on the bottom of the plate at any time, as fingerprints and markings may interfere with decoding and signal acquisition.
- General laboratory precautions should be taken:
  - Do not pipette by mouth.
  - Wear protective clothing (e.g., disposable powderless gloves and laboratory coats) and eye protection.
  - Do not eat, drink or smoke in the laboratory.
  - Wash hands thoroughly after handling samples and reagents.
- **Avoid RNase contamination:**
  - **Create an RNase-free working environment.**
  - **Wear gloves during all steps of the procedure.**
  - **Change gloves frequently.**
  - **Use sterile, disposable polypropylene tubes and filter strips.**
  - **Keep tubes closed whenever possible during the preparation.**
  - **Use RNase removing product to clean bench surfaces, pipettes and other components used in the experiment.**
- The workspace, including racks and pipettes, should be thoroughly cleaned and wiped with 0.5% sodium hypochlorite solution followed by wiping with a 70% ethanol solution. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution.
- Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.
- Material Safety Data Sheets (SDS) are available upon request from PlexBio Customer Service.

5. QUALITY CONTROL

The IntelliPlex ROS1 Rearrangement Kit contains a series of internal control πCode MicroDiscs that monitor the PCR amplification, SA-PE incubation procedure and background noise. Those controls must meet the specification in each test well with intensities above the cutoffs from the same run, or the run will be considered failed. The external controls (positive control and negative control) monitor the whole testing procedure to prevent false positive and false negative results. The test is considered invalid if any of the controls fail.

6. KIT COMPONENTS

The **IntelliPlex ROS1 Rearrangement Kit** contains sufficient reagents for up to 24 tests. Kit components include:

(1) ROS1 RT-PCR Buffer
Ref. No.: 20200
Quantity & Volume: 1 vial, 300 µL/ vial
Description: For RT-PCR amplification
Contents: 2X Reaction Mix, MgSO4 and dNTPs

(2) ROS1 RT-PCR Enzyme
Ref. No.: 20201
Quantity & Volume: 1 vial, 14.4 µL/ vial
Description: For RT-PCR amplification
Contents: RT/HotStar Taq MIX, RNase Inhibitor (Ribolock)

(3) ROS1 RT-PCR Primer Mix
Ref. No.: 20199
Quantity & Volume: 1 vial, 165.6 µL/ vial
Description: For RT-PCR amplification
Contents: <20 % Forward Primer, <10 % Reverse Primer (biotin labeled)

(4) ROS1 πCode MicroDisc
Ref. No.: 20203
Quantity & Volume: 1 vial, 480 µL/vial
Description: For PCR amplicon capture
Contents: Glycerol, πCode, Phosphate buffered saline, 0.1% Albumin from bovine (Biological), <0.1% EDTA, <0.1% Sodium azide

(5) ROS1 POS Control
Ref. No.: 20197
Quantity & Volume: 3 vials, lyophilized
Description: Assay positive control; each vial should be reconstituted with 25 µL nuclease-free water per vial prior to use.
Contents: RNA of C6; R34 cell line, RNAstable® LD

(6) NEG Control
Ref. No.: 20549
Quantity & Volume: 1 vial, 120 µL/vial
Description: Assay negative control
Contents: Nuclease-free water

(7) SA-PE Solution
Ref. No.: 20007
Quantity & Volume: 1 bottle, 7 mL/bottle
Description: Streptavidin-phycoerythrin for fluorescent signal acquisition
Contents: Phosphate buffered saline, 0.5% Streptavidin-phycoerythrin, 1% Albumin from bovine (Biological), <0.1% Sodium azide

(8) ROS1 Hy Buffer
Ref. No.: 20195
Quantity & Volume: 1 vial, 2.4 mL/vial
Description: For hybridization
Contents: Saline-Sodium Phosphate-EDTA

(9) ddH_2O
Ref. No.: 20548
Quantity & Volume: 1 vial, 1.5 mL/vial
Description: For reconstitution of ROS1 POS Control
Contents: Nuclease-free water

NOTE: POS Control, NEG Control and Hy Buffer refer to positive control, negative control and hybridization buffer, respectively.

7. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

Required products for compatibility with IntelliPlex kits:

- 96-well plate (PlexBio; Cat. No. 80025 or Greiner Bio-one; Cat. No. 655101)
- IntelliPlex® 1000 πCode Processor (PlexBio; Cat. No. 80033)
- PlexBio® 100 Fluorescent Analyzer (PlexBio; Cat. No. 80000)

- U Tray (PlexBio; Cat. No. 80023)
- V Tray (PlexBio; Cat. No. 80024)
- DeXipher™ MD (Required: PlexBio; Cat. No. 80051)
- 10X Assay Wash Buffer (PlexBio; Cat. No. 80220)
- Deionized water for dilution of 10X Assay Wash Buffer

Required components:

- (Recommended) FFPE RNA extraction kit (RNeasy® FFPE Kit; Qiagen; Cat. No. 73504 or equivalent)
- Qubit™ Fluorometer with dedicated quantitative reagents (Invitrogen; any models) or equivalent
- Clean tubes for PCR reaction (Gunster; Cat. No. MB-P08A or equivalent)
- Dedicated micropipette
- Filter tips for micropipette
- Disposable powderless gloves
- Vortex mixer
- Micro-centrifuge
- Thermocycler (Recommended: MiniAmp™ Thermal Cycler, Applied Biosystems™; Cat. No. A37834 or equivalent)
- Computer (Recommended: PlexBio; Cat. No. 80002)

8. STORAGE, STABILITY AND TRANSPORTATION

Storage

All kit components of the **IntelliPlex ROS1 Rearrangement Kit** should be stored at 2°C to 8°C.

Reconstituted ROS1 POS Control should be single use. Do not repeatedly freeze-thaw the RNA POS Control.

Stability

Do not use any kit that has expired. All unopened components are stable up to the expiration date on the label if handled and stored under the recommended conditions.

Transportation

The shipping temperature for the kit is 2-8°C. If the kit package or components are incomplete, please contact PlexBio customer service (service@plexbio.com).

9. INSTRUMENT AND SOFTWARE

Instrument

Refer to the instrument user manuals for complete installation and operation instructions (Thermocycler, IntelliPlex 1000 πCode Processor and PlexBio 100 Fluorescent Analyzer).

Software Installation

The ROS1 Rearrangement Kit has a designated Kit App and ENC file. The Kit App contains the πCode target assignments and the ENC file includes the lot number and expiration date. Please make sure you have the Kit App installed and the ENC file imported into DeXipher before your first assay run.

Kit App Installation

1. Visit www.plexbio.com and download the **ROS1 Rearrangement Kit** App.
2. Click on the “Installer” in the APP folder and follow the instructions to complete Kit App installation.

NOTE:
The Kit App only needs to be installed once. Version updates will be notified by customer service.

ENC File Installation

1. Visit www.plexbio.com and download the **ROS1 Kit** ENC file. Each kit lot number will have a unique ENC file, so you will need to download a new ENC file each time you purchase a kit with a different lot number. Make sure to select the ENC file with the lot number that corresponds to your kit.
2. Save the ENC file to your computer.
3. Follow the PlexBio 100 Fluorescent Analyzer User Manual to import the ENC file.

10. SPECIMENS

Specimen Collection

The **IntelliPlex ROS1 Rearrangement Kit** has been validated to be used for formalin-fixed paraffin embedded tissues (FFPET) of NSCLC.

NOTE:

- FFPET specimens may be stored ≤ 30°C for up to 12 months after the date of tissue collection and processing. The optimal tissue fixation time for test should be less than 72 hr.
- Only FFPET sections of 10-µm thickness containing at least 10% tumor content are to be used in the ROS1 Rearrangement Test. Any specimen containing less than 10% tumor content should be macro-dissected prior to deparaffinization.
- Do not use stained FFPE specimens which could generate invalid and/or incorrect results.

Specimen Transportation

FFPE specimens can be transported at room temperature.

Storage of Extracted RNA

Extracted RNA can be stored at **-20°C** for immediate use (≤ 24 hours), or at -80°C for long-term (1 – 14 days) storage. Do not subject the extracted RNA to repeated freeze/thaw cycles.

11. BEFORE YOU START

1. Check that the Kit App has been installed and the lot specific ENC file has been imported to DeXipher.
2. Check that you have 5 µL of extracted RNA (≥ 10 ng/µL) ready for analysis.

12. ASSAY PROCEDURE

Warning:
Read the instructions carefully and follow every step of the assay protocol correctly.

12.1 RNA Quantification

1. Quantify the extracted RNA using a Qubit Fluorometer with dedicated quantitative reagents (or equivalent) according to the manufacturer's protocol.
2. The RNA Stock concentration should be ≥ 10 ng/µL to ensure optimal performance. Each RT-PCR reaction uses 5 µL of a ≥10 ng/µL RNA Stock (at least 50 ng of total RNA input are recommended).

12.2 Reconstitute ROS1 POS Control

1. Briefly centrifuge the ROS1 POS Control tube.
2. Add 25 µL of ddH_2O to each required vial of ROS1 POS Control.
3. Make sure the material is fully reconstituted by pipetting up and down several times.

NOTE: Reconstituted POS Control should be single use. Do not repeatedly freeze-thaw the reconstituted POS control.

12.3 Multiplex one-step RT-PCR Amplification

1. Vortex to mix each sample before use.
2. Spin down and keep samples on ice.
3. Prepare the one-step RT-PCR Reaction:

For each RT-PCR reaction:

ROS1 RT-PCR Buffer	13 µL
ROS1 RT-PCR Enzyme	0.6 µL
ROS1 RT-PCR Primer Mix	6.4 µL
Sample/POS Control/NEG Control	5 µL
Total volume	25 µL

NOTE:

- The amount of one-step RT-PCR reagent required for a Master Mix depends on the number of reactions. Always prepare a surplus.
- Both POS Control and NEG Control are required for test validity and report generation and must be included in each assay run.

4. Mix by tapping the tubes and spin down before placing the tubes on the thermocycler. Set up the one-step RT-PCR program conditions as below:

PCR Program Conditions*

Temp. (˚C)	Time	Cycles
45	15min	-
95	2 min	-
95	15 sec	50
60	30 sec	
72	30 sec	
4	Hold	-

NOTE: *Ramp rate: 3.0°C/sec (ABI MiniAmp™; Cat. No. A37834).*

12.4 Hybridization and SA-PE Reaction

1. **Prepare 1X Assay Wash Buffer:** Transfer 100mL of the 10X Assay Wash Buffer (PlexBio; Ref: 80220) to the IntelliPlex 1000 πCode Processor 1L Wash Buffer bottle and add 900 ml deionized water. Mix by swirling.

NOTE: The prepared 1X Assay Wash Buffer can be used for up to one week. Please always check the Wash Buffer is sufficient for assay runs. Additional 10X Assay Wash Buffer can be ordered from PlexBio (Ref. No: 80220).

IntelliPlex 1000 πCode Processor Assay Wash Buffer consumption:

Procedure	Assay Wash Buffer Consumption (mL)
Self-test	50
DNA & RNA program (1 lane, up to 8 tests)	150
DNA & RNA program (3 lanes, up to 24 tests)	220

2. **Add 20 µLπCode MicroDisc to 96 well plate:** Mix by vortexing the **ROS1 πCode** for 10 seconds, then, by pipetting, add 20 µL of the πCode to each well directly. Vortex the tube of πCode every four wells in between dispensing to ensure homogeneous suspension.

NOTE: Each amplified PCR products (including samples, POS and NEG control) should be added into wells lane wise, in order of A1, B1…H1 and followed by A2, B2…H2 and so on.

3. **Add 100 µL of ROS1 Hy Buffer** to each well.

4. Spin down the PCR products.

5. **Denature the PCR products** on the thermocycler by heating at 95˚C for 5 minutes, immediately cooled on ice/ cooler or thermocycler to ensure the denatured status. Spin down before use. Use immediately (within 1 hour after denaturation).

NOTE: Pay attention to the lid temperature of thermocycler while taking out the denatured PCR products.

6. **Add 10 µL of the denatured PCR products** to each well.

7. **Pipet the desired volume of SA-PE solution** into the V Tray in SA-PE tank. Please note that the dead volume of the V Tray is 500 µL for up to 6 selected lanes or 800 µL if more than 6 lanes are selected. The minimum usage unit of SA-PE is one lane (900 µL).

> **Calculation Example:**
> For a 3-lane reaction, the required SA-PE solution volume is at least:
> **400 µL x 3 lanes + 500 µL(dead volume)= 1.7 mL**
> It is recommended to add extra solution volume into the V Tray to ensure sufficient dispensing volume.

NOTE: Required SA-PE Solution by Lane(s):

Number of Processed Lane(s)	Required SA-PE Solution (µL)
1	900
2	1300
3	1700
4	2100
5	2500
6	2900
7	3600
8	4000
9	4400
10	4800
11	5200
12	5600

- SA-PE solution should be kept in the dark.
- **Do not** reuse the leftover SA-PE solution and V Tray tank. Replace a new V Tray with every assay run.

8. **Run hybridization and wash:** This assay uses the **DNA/RNA program** in the **Molecular Assay** window of the IntelliPlex 1000 πCode Processor. Refer to the IntelliPlex 1000 πCode Processor operation manual and follow the instructions to run the built-in assay program as described (Homepage/ Molecular Assay/ Well Selection/ DNA/RNA / Confirm procedure conditions/ Start Running). The plate will be ready for decoding once the program is finished.

NOTE:

- IntelliPlex 1000 πCode Processor must be maintained properly and regularly.
- **Do not** open the door when the instrument is in operation.
- The kit contains sufficient reagents for 3 runs of 8 samples (including POS and NEG controls) for a maximum of 24 tests.

12.5 Image Decoding and Fluorescent Detection

1. Follow the PlexBio 100 Fluorescent Analyzer User Manual to set up the read.

   NOTE:

   - PlexBio 100 Fluorescent Analyzer must be calibrated regularly (once per month).
   - Check that the correct ENC file has been imported.
2. Launch DeXipher to run the **Qualitative Assay**.
3. Mark the wells for sample, positive and negative controls.
4. Enter Assay name and place the plate into the device with the correct orientation as shown on the screen.
5. The raw data will be analyzed through the kit ENC to generate the variant call report.

NOTE:

- A single run can include from 2 to 96 tests (including POS and NEG controls) per 96 well Microwell plate. When running more than 24 specimens, multiple IntelliPlex ROS1 Rearrangement Kits of the same lot will be required.

13. DISCLAIMERS

Negative Test Result

A negative test result means that the targeted variant was not detected by the kit. Experimental errors or other causes may lead to false negative results. Interpretation of the results should consider these possibilities and be made in combination with other clinical findings.

Positive Test Result

A positive test result means that the targeted variant was detected by the kit. Experimental errors or other causes may lead to false positive results. Interpretation of the results should consider these possibilities and be made in combination with other clinical findings.

14. INTERPRETATION OF RESULTS

The report generated by DeXipher includes the results of controls and samples tested in the same run. The result of external controls (POS Control and NEG Control) must be “Pass”. Otherwise, failed POS or NEG Control renders the whole assay run invalid, and the result of tested samples will not be reported.
If the result POS Control and NEG Control are “Pass”, the result of each tested sample will be reported on separate sheets in detail. For each tested sample, its internal controls (Reference Gene Control, Internal Control, SA-PE Monitor Control, πCode MicroDiscs Count, πCode MicroDiscs Combination and Blank Control) must be “Pass”, or the test of that sample is invalid. The detection result of invalid samples will not be shown. However, failed Reference Gene Control and Internal Control do not negate samples with mutation detected. A positive sample with failed Reference Gene Control is considered valid. The detection result of target genes will be shown for each valid sample.

Refer to the chapter **“Troubleshooting”** for control failure issues.

Table 2. Interpretation of Results

Test Result	Explanation	Action
Variant Detected	Refer to Table 1	Targeted variant detected
Variant Not Detected	None	Targeted variant not detected
Result Not Shown	The test is **INVALID** because external controls failed OR at least one of internal controls failed	See the chapter **“Troubleshooting”** for instructions and retest.

NOTE:

- All runs and specimen validation were performed by the dedicated KIT APP along with IntelliPlex 1000 πCode Processor and PlexBio 100 Fluorescent Analyzer.
- "Variant Detected" indicates that the signal for at least one variant site is greater than the cutoff value of the corresponding target.

15. ANALYTICAL PERFORMANCE

Limit of Blank (LoB)

The limit of blank (LoB) values were determined by one operator performing replicates of two wild-type cell lines (HEK293 and HuT78) and 15 wild-type FFPE specimens across three days on two reagent lots. Duplicates of another 20 wild-type FFPE specimens from different biobank and procurement year were also tested. Only "No Fusion Detected" results were observed in these wild type RNA samples.

The cutoff values of each targeted variants were determined by the measured maximum analytical signal intensity values, respectively.

Limit of Detection (LoD)

The limit of detection (LoD) was determined using a dilution series (ranging from 0-750 copies) of RNA extracted from cell line transiently transfected with each variant. Copy number for each RNA stock was determined by Droplet Digital PCR. Each variant was tested at five copy levels, each with 21 replicates performed by three operators across three days on two reagent lots. The LoDs were determined based on a positive hit rate at 95% in PriProbit analysis (Table 3). The LoDs ranged from ≤ 5~120 copies.

Table 3. Limit of Detection (LoD) of each variants

Variant Breakpoint	LoD (RNA Copies/ Reaction)
C6;R32	84
C6;R34	17
E10;R34	≤ 5
S4;R32	51
S4;R34	10
S13del;R32	10
S13del;R34	≤ 5
SD2;R32	20
SD4;R32	120
SD4;R34	36
T8;R35	102
F3;R36	57
F7;R35	62
L16;R35	10

Repeatability and Reproducibility

The repeatability and reproducibility for variants in the assay was evaluated with low (2x LoD) or high (6x LoD) variant levels of variant RNA blended in wild type RNA background, across two operators, two sets of instruments, two sites, five testing days, on three reagent lots. Four replicate runs were performed per reagent lot per day for a total of 20 valid runs at one site. RNA from two wild type (WT) cell lines were included as negative controls. The accuracy across all tested levels was at least 95% (38/40) across all variances combined (i.e., site/instrument, operator, and day) (Table 4). Reproducibility coefficient was 0~3.54% (Table 5).

Table 4. Accuracy

Variant Breakpoint	Level (x LOD)	Variant Not Detected/ Detected	Accuracy (%)
C6;R32	6X	0/40	100
	2X	0/40	100
C6;R34	6X	0/40	100
	2X	2/38	95
E10;R34	6X	0/40	100
	2X	0/40	100
S4;R32	6X	1/39	97.5
	2X	1/39	97.5
S4;R34	6X	0/40	100
	2X	1/39	97.5
S13del;R32	6X	2/38	95
	2X	0/40	100
S13del;R34	6X	0/40	100
	2X	2/38	95
SD2;R32	6X	0/40	100
	2X	0/40	100
SD4;R32	6X	1/39	97.5
	2X	1/39	97.5
SD4;R34	6X	0/40	100
	2X	1/39	97.5
T8;R35	6X	0/40	100
	2X	0/40	100
F3;R36	6X	1/39	97.5
	2X	1/39	97.5
F7;R35	6X	0/40	100
	2X	0/40	100
L16;R35	6X	0/40	100
	2X	0/40	100
WT (HEK293)	-	40/0	100
WT (HuT78)	-	40/0	100

Table 5. Reproducibility Coefficient

Variant Breakpoint	Level (x LOD)	Overall Coefficient
C6;R32	6X	0.00%
	2X	0.00%
C6;R34	6X	0.00%
	2X	0.00%
E10;R34	6X	0.00%
	2X	0.00%
S4;R32	6X	3.54%
	2X	3.54%
S4;R34	6X	0.00%
	2X	3.54%
S13del;R32	6X	0.00%
	2X	0.00%
S13del;R34	6X	0.00%
	2X	0.00%
SD2;R32	6X	0.00%
	2X	0.00%
SD4;R32	6X	3.54%
	2X	3.54%
SD4;R34	6X	0.00%
	2X	3.54%
T8;R35	6X	0.00%
	2X	0.00%
F3;R36	6X	3.54%
	2X	3.54%
F7;R35	6X	0.00%
	2X	0.00%
L16;R35	6X	0.00%
	2X	0.00%

Cross-Contamination

This test is designed to assess cross-contamination during the washing steps, which may lead to false positive results. Wild-type and CD74-ROS1 (C6; R34) samples were arranged in alternating order during PCR reaction and sample hybridization to test for carryover of variant signals to wild type wells. No cross-contamination was observed.

Carryover Interference

This test is designed to evaluate the impact of potential substances carried over from the RNA FFPE extraction kit. CD74-ROS1 (C6; R34) was selected as a representative variant. Triplicate testing of CD74-ROS1 (C6; R34) variant and wild type blend cell line (HEK293) RNA extract samples with each potential interfering substance (as listed in Table 6), added before the PCR step, showed no interference on kit performance.

Table 6. Interfering substances Tested

Interfering Substance	Assumed Interfering Residual Volume (% 30µl RNA)
Xylene	0.5%
Buffer PKD	0.5%
DNase Booster Buffer	0.5%
Ethanol	0.5%
Buffer RPE	0.5%
RNase-Free DNase I	0.25%

16. TROUBLESHOOTING

The troubleshooting listed below addresses possible problem causes and solutions provided during assay procedures.

Problem	Possible Cause	Recommendations
No Valid Assay Assigned	1. No plate inserted. 2. Plate inserted in wrong orientation. 3. No assay APP installed. 4. No ENC file imported. 5. Two or more lots of reagent used.	1. Confirm plate is inserted and repeat reading. 2. Confirm orientation of plate and repeat reading. 3. Install assay APP and repeat reading. 4. Import ENC file and repeat reading. 5. One reagent lot used at a time.
Positive Control Fail / Negative Control Fail	1. No POS Control/ NEG Control added. Or POS Control not reconstituted properly. 2. RNase contamination. 3. Assay did not work. 4. Cross contamination between samples. 5. Wrong PC/NC wells chose.	1. Ensure POS Control reconstituted properly and do not repeatedly freeze-thaw POS control. Ensure POS and NEG Control are added. 2. Ensure all operating procedures are followed correctly. Ensure work environment is free of RNase. 3. Make sure all the assay procedures are followed correctly. 4. Clean all surfaces and equipment. Operate pre-PCR and post-PCR in the dedicated area and separate the equipment for use. 5. Choose the correct PC/NC wells and repeat reading.
πCode MicroDiscs Count Fail	DeXipher is unable to detect sufficient πCode MicroDiscs numbers for analysis.	
	1. πCode MicroDiscs are not proper dispersed in the well. 2. Not enough πCode MicroDiscs added to well. 3. Microbes exist in buffers. 4. Instruments error or malfunction.	1. Re-disperse the microplate using IntelliPlex 1000 Processor, and repeat reading. 2. Ensure πCode MicroDiscs are well-mixed with proper amount added. 3. Use freshly prepared assay wash buffer and deionized water for hybridization to reduce πCode MicroDiscs loss rate. 4. Contact PlexBio Customer Service.
SA-PE Monitor Control Fail	Performance of SA-PE is assessed by the SAPE Monitor Control.	
	1. No SA-PE was added or insufficient SA-PE solution for dispensing. 2. SA-PE solution inactivation. 3. Incorrect tested lanes of microplate selected for SA-PE solution dispensing.	1. Make sure all the assay procedures are followed correctly. Calculate sufficient SA-PE solution volume for dispensing. Repeat test. 2. Ensure correct storage condition and minimize the light exposure. Do not use SA-PE past its expiration date. 3. Repeat assay and make sure lanes selected correctly.

Problem	Possible Cause	Recommendations
Blank Control Fail	"Background" is determined by measuring MFI of an internal control that should not give a signal.	
	1. Wrong hybridization conditions. 2. Residues of SA-PE solution in wells after hybridization. 3. PlexBio 100 Fluorescent Analyzer is not calibrated. 4. Markings on plates.	1. Check correct hybridization program is selected. 2. Ensure all buffers (Assay Wash Buffer and deionized water) on IntelliPlex 1000 Processor are fresh-made and sufficient for washing procedures. 3. Perform calibration on PlexBio 100 Fluorescent Analyzer. 4. Do not make any marking on plate.
Internal Control Fail	Internal Control monitors all steps in the procedure and must be positive.	
	1. PCR inhibition exists. 2. PCR procedures are not performed correctly. 3. RNase contamination. 4. Hybridization did not work.	1. Follow instructions of sample extraction carefully. Ensure required temperature ranges and centrifugation needs are complied. Ensure complete removal of ethanol. 2. Make sure all PCR procedures are followed correctly. Do not to use expired materials or mixed lots of reagents. Ensure storage conditions are correct. 3. Ensure all the operating procedures are followed correctly. Ensure work environment is free of RNase. 4. Make sure all the assay procedures are followed correctly. Ensure samples are freshly heat-denatured.
Reference Gene Fail	Reference Gene monitors quality of tested sample and must be positive.	
	1. No Sample added or absence of human-derived RNA. 2. Insufficient sample input for assays or poor sample quality. 3. PCR inhibition exists. 4. PCR procedures are not performed correctly.	1. Ensure human-derived RNA samples are added. Do not use artificial RNA as samples which may generate invalid results. 2. Quantify samples and check the sample input and RIN (RNA integrity number) value. If still remains failed, ensure the collected samples meet specimen requirements. Retest with new samples if needed. 3. Follow sample extraction instructions carefully. Ensure required temperature ranges and centrifugation needs are complied. Ensure complete removal of ethanol. 4. Make sure all PCR procedures are followed correctly. Do not to use expired materials or mixed lots of reagents. Ensure storage conditions are correct.

17. SYMBOLS

Symbol	Explanation	Symbol	Explanation
IVD	In-vitro diagnostic use	REF	Catalog number
LOT	Batch number		Consult instructions for use
	Manufacturer		Use by Date
CE	European Union Conformity	EC REP	European Authorized Representative
Σ	Contains sufficient for <n> tests		Date of Manufacture
	Temperature limitation		

18. REFERENCES

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4. Sakuma, M. (2000) PriProbit, ver. 1.63. Available from James E. Throne USDA-ARS GMPRC, Manhattan, KS (http://bru.usgmrl.ksu.edu/throne/)
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