IntelliPlex™ RET/NTRK1 Rearrangement Kit User Manual

82025 24 Reactions

For In-Vitro Diagnostic Use

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IMPORTANT:
Read the instructions carefully prior to use

1. INTENDED USE

The IntelliPlex RET/NTRK1 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 RET and NTRK1 genes using RNA samples derived from formalin-fixed paraffin- embedded of non-small cell lung cancer (NSCLC) tissue.

2. INTRODUCTION

The *RET* gene encodes a receptor tyrosine kinase belonging to the RET family of receptor tyrosine kinases. It is normally expressed in tissues derived from the neural crest. Activated RET causes activation of multiple downstream cellular pathways. Although genomic alterations in RET are found in several different types of cancer, oncogenic kinase fusions involving the RET gene are found in ~1% of non-small cell lung cancers. NTRK1 (neurotrophic tyrosine kinase, receptor, type 1) is a receptor tyrosine kinase that is part of the TRK (tropomyosin-related kinases) superfamily of receptor tyrosine kinases. NTRK1 acts in control of cell growth and differentiation via the MAPK, PI3K and PLC-γ pathways. CD74 and MPRIP are two 5' fusion gene partners of NTRK1 with established roles in NSCLC.

Studies indicate efficacy of RET-tyrosine kinase inhibitors in the treatment of RET fusion-positive NSCLC, and several TRK inhibitors are under clinical development. The IntelliPlex RET/NTRK1 Rearrnagement 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 RET/NTRK1 Rearrangement Kit** identifies 14 rearrangements of the RET and NTRK1 genes (Table 1).

Table 1. Variants Detected

Gene	Fusion Variant	Inferred Breakpoint
RET	KIF5B-RET	K15;R11
		K15;R12
		K16;R12
		K22;R12
		K23;R12
		K24;R8
		K24;R11
	CCDC6-RET	C1;R12
	NCOA4-RET	N6;R12
		N8;R12
	TRIM33-RET	T14;R12
	CUX1-RET	CX10;R12
NTRK1	CD74-NTRK1	C8;N12
	MPRIP-NTRK1	M21;N14

3. TECHNOLOGICAL PRINCIPLES

The **IntelliPlex RET/NTRK1 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 while yielding highly sensitive results.

πCode MicroDisc

πCode MicroDisc are manufactured to generate up to 16,000 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 would be 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 (MSDS) are available upon request from PlexBio Customer Service.

5. KIT COMPONENTS

The **IntelliPlex RET/NTRK1 Rearrangement Kit** contains sufficient reagents for up to 24 tests. Kit components include:

1. **RET/NTRK1 RT-PCR Buffer**
   Ref. No.: 20229
   Quantity & Volume: 1 vial, 300 uL/vial
   Description: For RT-PCR amplification
   Contents: 2X Reaction Mix, MgSO4 and dNTPs

2. **RET/NTRK1 RT-PCR Enzyme**
   Ref. No.: 20230
   Quantity & Volume: 1 vial, 14.4 uL/vial
   Description: For RT-PCR amplification
   Contents: RT/Hot-Start Taq MIX,
   RNase Inhibitor

3. **RET/NTRK1 RT-PCR Primer Mix**
   Ref. No.: 20228
   Quantity & Volume: 1 vial, 165.6 uL/vial
   Description: For RT-PCR amplification
   Contents: <20 % Forward Primer,
   <10 % Reverse Primer (biotin labeled)

4. **RET/NTRK1 πCode MicroDisc**
   Ref. No.: 20232
   Quantity & Volume: 1 vial, 480 µL/vial
   Description: For PCR amplicon capture

Contents: πCode MicroDisc, Glycerol,
Phosphate buffered saline,
0.1% Albumin- from bovine (Biological),
<0.1% EDTA and <0.1% Sodium azide

5. **RET/NTRK1 POS Control**
Ref. No.: 20226
Quantity & Volume: 3 vials, lyophilized
Description: Assay positive control; each vial should be reconstituted with 25μL RET/NTRK1 ddH2O prior to use.
Contents: 20 % RNA of K15; R12 cell line,
80 % RNAstable® LD

6. **RET/NTRK1 NEG Control**
Ref. No.: 20231
Quantity & Volume: 1 vial, 120 μL/vial
Description: Assay negative control
Contents: ddH2O

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. **RET/NTRK1 Hy Buffer**
Ref. No.: 20224
Quantity & Volume: 1 bottle, 2.4 mL/bottle
Description: For hybridization
Contents: Saline-Sodium Phosphate-EDTA

9. **RET/NTRK1 10X Wash Buffer**
Ref. No.: 20233
Quantity & Volume: 1 bottle, 50 mL/bottle
Description: For πCode washing
Contents: Phosphate buffered saline,
1% Tween-20, and <0.1% Sodium azide

10. **RET/NTRK1 ddH2O**
Ref. No.: 20225
Quantity & Volume: 1 vial, 1.5 mL/vial
Description: For reconstitution of RET/NTRK1 POS Control
Contents: Nuclease-free water

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

6. 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)

Required components:

- 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
- ddH2O for dilution of 10X Wash Buffer
- FFPE RNA extraction kit (Recommended: RNeasy® FFPE Kit; Qiagen; Cat. No. 73504 or equivalent)
- Vortex mixer
- Micro-centrifuge
- Thermocycler (Recommended: DigiPlex™ Thermocycler, PlexBio; Cat. No. 80018/ MiniAmp™ Thermal Cycler, Applied Biosystems™; Cat. No. A37834 or equivalent)
- Industrial Computer (Recommended: PlexBio; Cat. No. 80002)

7. STORAGE, STABILITY AND TRANSPORTATION

Storage

All kit components should be stored at 2-8°C.

Stability

Do not use any kit that has expired. All 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).

8. INSTRUMENT AND SOFTWARE

Instrument

Please refer to the instrument user manual for complete operation instructions (Thermocycler, IntelliPlex 1000 πCode Processor and PlexBio 100 Fluorescent Analyzer).

Software Installation

The RET/NTRK1 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. Log into www.plexbio.com and download the **RET/NTRK1 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. Log into www.plexbio.com and download the **RET/NTRK1 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.

9. SPECIMENS

Specimen Collection

The **IntelliPlex RET/NTRK1 Rearrangement Kit** has been validated to be used for formalin-fixed paraffin embedded (FFPE) tissues from 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 RET/NTRK1 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.

10. 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.

11. ASSAY PROCEDURE

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

11.1 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).

11.2 Multiplex one-step RT-PCR Amplification

1. Briefly centrifuge the POS Control tube and reconstitute with 25µL RET/NTRK1 ddH2O per vial by pipetting up and down. Keep reconstituted POS control on ice prior to use.

   NOTE: Do not repeatedly freeze-thaw the reconstituted POS control. Use within one freeze-thaw cycle and store the leftover at -20°C if needed.
2. Vortex to mix each sample before use.
3. Spin down and keep samples on ice.
4. Prepare the one-step RT-PCR Reaction:

   For each RT-PCR reaction:

Component	Volume
RET/NTRK1 RT-PCR Buffer	13 µL
RET/NTRK1 RT-PCR Enzyme	0.6 µL
RET/NTRK1 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 depends on the number of reactions.
- Both POS Control and NEG Control are required for test validity and report generation and must be included in each assay run.

5. 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: 100% (PlexBio; Cat. No. 80018). 3.0°C/sec (ABI MiniAmp™; Cat. No. A37834).*

11.3 DNA Hybridization and SA-PE Reaction

1. **Prepare 1X Wash Buffer:** Transfer 50mL of the 10X Wash Buffer to the IntelliPlex 1000 πCode Processor 1L Wash Buffer bottle and add 450 ml ddH_2O.
2. **Add πCode MicroDisc to 96 well plate:** Mix by vortexing the **RET/NTRK1 πCode** for 10 seconds, then, without 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 respectively in order of A1, B1…H1 and followed by A2, B2…H2 and so on.
3. **Add 100 µL of RET/NTRK1 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 five minutes, immediately cooled on ice/ cooler or thermocycler to ensure the denatured status. Within an hour following the denaturation, the PCR products should be spun down immediately before adding to wells for hybridization.

   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 SA-PE solution tank (V-tray). Please note that the dead volume of V-tray is **500 µL** and the minimum usage of SA-PE is **one row (900 µL)**.

   Calculation Example:
   For a 3-row reaction, the required SA-PE solution volume is at least:
   400 µL x 3 rows + 500 µL(dead volume)= 1.7 mL
   It is recommended to add extra solution volume into the V-tray to ensure sufficient dispensing volume.
8. **Run hybridization and wash:** Refer to the IntelliPlex 1000 πCode Processor operation manual and follow the instructions to set up the built-in assay program (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:

- SA-PE solution should be kept in the dark.
- **Do not** reuse the leftover SA-PE solution and V-tray tank.
- **Do not** open the door when the instrument is in operation.
- The kit contains sufficient reagents for 6 runs of tests (including POS and NEG controls) for a maximum of 24 tests.

11.4 Image Decoding and Fluorescence 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 sample information and assay name. 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 1 to 96 tests (including POS and NEG controls) per 96 well Microwell plate. When running more than 24 specimens, multiple IntelliPlex RET/NTRK1 Rearrangement Kits of the same lot will be required.
- The procedure described above must be followed to detect ≥ 5~120 RNA copies in a background of wild-type RNA for the RET/NTRK1 fusion variants in Table 1.

12. 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.

13. INTERPRETATION OF RESULTS

Table 2. Interpretation of Results

Test Result	Reported Result	Interpretation
Fusion Detected	Refer to Table 1	Targeted fusion detected
Fusion Not Detected	None	Targeted fusion not detected
Invalid Assay	Invalid	**Possible Causes:** 1. PCR Inhibition (presence of inhibitor in the sample) 2. Improper stored reagents 3. Low sample RNA input or quality 4. Low πCode Disc Count (the πCode tube was not well-vortexed before pipetting) 5. Reagent not added 6. Failed Blank πCode Control 7. Sample quality due to improper fixation process or storage condition

NOTE:

- All run and specimen validation is performed by the dedicated KIT APP along with IntelliPlex 1000 πCode Processor and PlexBio 100 Fluorescent Analyzer.
- In case of heterogeneity of samples or multiple fusion variants, only the dominantly detected fusion variant is reported. “Fusion Detected” indicates that the signal for at least one fusion site is greater than the cutoff value of the corresponding target. When multiple variants are detected in a sample, only the one that exhibits the highest signal is reported.

14. 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 12~486 copies.

Table 3. Limit of Detection (LoD)

Variant Breakpoint	LoD (RNA Copies/ Reaction)
K15;R11	30
K15;R12	26
K16;R12	12
K22;R12	486
K23;R12	372
K24;R8	13
K24;R11	152
C1;R12	112
N6;R12	22
N8;R12	71
T14;R12	47
CX10;R12	56
C8;N12	37
M21;N14	45

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 instrument, 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 92.5% (37/40) across all variance combined (i.e., site/instrument, operator, and day) (Table 4). Reproducibility coefficient was 0~7.44% (Table 5).

Table 4. Accuracy

Variant Breakpoint	Level (x LOD)	Variant Not Detected/ Detected	Accuracy (%)
K15;R11	6X	0/40	100
	2X	3/37	92.5
K15;R12	6X	0/40	100
	2X	1/39	97.5
K16;R12	6X	0/40	100
	2X	2/38	95
K22;R12	6X	0/40	100
	2X	3/37	92.5
K23;R12	6X	1/39	97.5
	2X	1/39	97.5
K24;R8	6X	0/40	100
	2X	2/38	95
K24;R11	6X	0/40	100
	2X	0/40	100
C1;R12	6X	0/40	100
	2X	1/39	97.5
N6;R12	6X	0/40	100
	2X	0/40	100
N8;R12	6X	1/39	97.5
	2X	0/40	100
T14;R12	6X	0/40	100
	2X	0/40	100
CX10;R12	6X	0/40	100
	2X	0/40	100
C8;N12	6X	0/40	100
	2X	0/40	100
M21;N14	6X	0/40	100
	2X	1/39	97.5
WT (HEK293)	-	0/40	100
WT (HuT78)	-	0/40	100

Table 5. Reproducibility Coefficient

Variant Breakpoint	Level (x LOD)	Overall Coefficient
K15;R11	6X	0.00%
	2X	3.82%
K15;R12	6X	0.00%
	2X	3.63%
K16;R12	6X	0.00%
	2X	7.44%
K22;R12	6X	0.00%
	2X	3.82%
K23;R12	6X	3.63%
	2X	3.63%

Variant Breakpoint	Level (x LOD)	Overall Coefficient
K24;R8	6X	0.00%
	2X	7.44%
K24;R11	6X	0.00%
	2X	0.00%
C1;R12	6X	0.00%
	2X	3.63%
N6;R12	6X	0.00%
	2X	0.00%
N8;R12	6X	3.63%
	2X	0.00%
T14;R12	6X	0.00%
	2X	0.00%
CX10;R12	6X	0.00%
	2X	0.00%
C8;N12	6X	0.00%
	2X	0.00%
M21;N14	6X	0.00%
	2X	3.63%

Cross-Contamination

This test is designed to assess cross-contamination during the washing steps, which may lead to false positive results. Wild-type and KIF5B-RET (K15; R12) 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. KIF5B-RET (K15;R12) was selected as a representative variant. Triplicate testing of 6xLOD of KIF5B-RET (K15;R12) 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%

15. 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 or NEG Control added. 2. RNase contamination. 3. Assay did not work. 4. Cross contamination between samples. 5. Wrong PC/NC wells chose.	1. Ensure POS Control 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.

Problem	Possible Cause	Recommendations
πCode MicroDiscs Count Fail	DeXipher is unable to detect sufficient πCode MicroDiscs numbers for fusion variants determination.	
	1. πCode MicroDiscs are not proper dispersed in the well.	1. Re-vortex the microplate and repeat reading.
	2. Not enough πCode MicroDiscs added to well.	2. Ensure πCode MicroDiscs are well-mixed with proper amount added.
	3. Microbes exist in buffers.	3. Use freshly prepared wash buffer and ddH_2O for hybridization to reduce πCode MicroDiscs loss rate.
	4. Instruments error or malfunction.	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.	1. Make sure all the assay procedures are followed correctly. Calculate sufficient SA-PE solution volume for dispensing. Repeat test.
	2. SA-PE solution inactivation.	2. Ensure correct storage condition and minimize the light exposure. Do not use SA-PE past its expiration date.
	3. Incorrect tested rows of microplate selected for SA-PE solution dispensing.	3. Repeat assay and make sure rows selected correctly.
Blank Control Fail	"Background" is determined by measuring MFI of an internal control that should not give a signal.	
	1. Wrong hybridization conditions.	1. Check correct hybridization program is selected.
	2. Residues of SA-PE solution in wells after hybridization.	2. Ensure all buffers (Wash buffer and ddH2O) on IntelliPlex 1000 Processor are fresh-made and sufficient for washing procedures.
	3. PlexBio 100 Fluorescent Analyzer is not calibrated.	3. Perform calibration on PlexBio 100 Fluorescent Analyzer.
	4. Markings on plates.	4. Do not make any marking on plate.

Problem	Possible Cause	Recommendations
Internal Control Fail	Internal Control monitors all steps in the procedure and must be positive.	
	1. PCR inhibition exists.	1. Follow instructions of sample extraction carefully. Ensure required temperature ranges and centrifugation needs are complied. Ensure complete removal of ethanol.
	2. PCR procedures are not performed correctly.	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. RNase contamination.	3. Ensure all the operating procedures are followed correctly. Ensure work environment is free of RNase.
	4. Hybridization did not work.	4. Make sure all the assay procedures are followed correctly. Ensure samples are freshly heat-denatured.

Problem	Possible Cause	Recommendations
Reference Gene Fail	Reference Gene monitors quality of tested sample and must be positive.	
	1. No Sample added or absence of human-derived RNA.	1. Ensure human-derived RNA samples are added. Do not use artificial RNA as samples which may generate invalid results.
	2. Insufficient sample input for assays or poor sample quality.	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. PCR inhibition exists.	3. Follow sample extraction instructions carefully. Ensure required temperature ranges and centrifugation needs are complied. Ensure complete removal of ethanol.
	4. PCR procedures are not performed correctly.	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.

16. SYMBOLS

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

17. REFERENCES

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4. Takeuchi, K. et al. (2012)RET, RET/NTRK1 and ALK fusions in lung cancer Nature Medicine 2012 18, 378–381.
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