



# IntelliPlex™ ALK Rearrangement Kit User Manual

**REF** 82023 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 ALK Rearrangement Kit, based on  $\pi$ Code™ technology and PlexBio's instrument platform, is an in vitro RT-PCR assay intended for qualitative detection of 24 gene rearrangements in *ALK* gene using RNA samples derived from formalin-fixed paraffin-embedded non-small cell lung cancer (NSCLC) tissue. The IntelliPlex ALK Rearrangement Kit is intended to assist clinicians in identifying NSCLC patients who may benefit from the Crizotinib treatment.

## 2. INTRODUCTION

The *anaplastic lymphoma kinase (ALK)* gene is an oncogene in NSCLC and other cancers. Chimeric proteins, such as EML4-ALK, contain an N-terminus derived from EML4 fused to the C-terminal kinase domain of ALK, which permits the ligand-independent dimerization and constitutive activation of ALK. Other fusion partners of ALK include KIF5B and TFG. Several ALK inhibitors have been developed for the treatment of ALK-positive NSCLC. The IntelliPlex ALK Rearrangement kit combines one step RT-PCR with  $\pi$ Code technology to enable multiplex,

single-well detection of gene rearrangements from RNA specimens containing large amounts of wild-type RNA. The IntelliPlex ALK Rearrangement Kit identifies 24 rearrangements of the *ALK* gene (Table 1).

**Table 1. Variants Detected**

Gene	Fusion Variant	Inferred Breakpoint
ALK	EML4-ALK A19	E6;A19
	EML4-ALK V1	E13;A20
	EML4-ALK V2	E20;A20
	EML4-ALK V3a	E6;A20
	EML4-ALK V3b	E6ins33;A20
	EML4-ALK V3c	E6ins18;A20
	EML4-ALK V4	E14;ins11del49A20
	EML4-ALK V"4"	E15del60;del71A20
	EML4-ALK V5a	E2b;A20
	EML4-ALK V5b	E2;ins117A20
	EML4-ALK V"5"	E18;A20
	EML4-ALK V6	E13;ins69A20
	EML4-ALK V7	E14;del12A20
	EML4-ALK V8a	E17;isn30A20
	EML4-ALK V8b	E17ins61;ins34A20
	EML4-ALK i2	E14isn2;isn56A20
	EML4-ALK i18	E20;isn18A20
	EML4-ALK i53	E3;ins53A20
	EML4-ALK i68	E17;isn68A20
	KIF5B-ALK	
		K15;A20
TFG-ALK		T4;A20
		T5;A20
		T6;A20

## 3. TECHNOLOGICAL PRINCIPLES

The IntelliPlex ALK Rearrangement Kit utilizes two technologies, one-step RT-PCR and  $\pi$ Code, 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.

## $\pi$ Code MicroDisc

$\pi$ Code MicroDisc are manufactured to generate up to 16,000 distinct circular image patterns for multiplexing applications. Each  $\pi$ Code MicroDisc has a distinct circular image pattern, which corresponds to a specific capture agent conjugated to the surface of the disc.  $\pi$ Code 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  $\pi$ Code 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 (MSDS) are available upon request from PlexBio Customer Service.

## 5. KIT COMPONENTS

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

1. **ALK KIT RT-PCR Buffer**  
**Ref. No.:** 20171  
**Quantity & Volume:** 1 vial, 300 uL/vial  
**Description:** For RT-PCR amplification  
**Contents:** 2X Reaction Mix, MgSO<sub>4</sub> and dNTPs
2. **ALK KIT RT-PCR Enzyme**  
**Ref. No.:** 20172  
**Quantity & Volume:** 1 vial, 14.4 uL/vial  
**Description:** For RT-PCR amplification  
**Contents:** RT/Hot-Start Taq MIX, RNase Inhibitor

**3. ALK KIT RT-PCR Primer Mix**  
**Ref. No.:** 20170  
**Quantity & Volume:** 1 vial, 165.6 uL/vial  
**Description:** For RT-PCR amplification  
**Contents:** <20 % Forward Primer,  
 <10 % Reverse Primer (biotin labeled)

**4. ALK KIT  $\pi$ Code MicroDisc**  
**Ref. No.:** 20174  
**Quantity & Volume:** 1 vial, 480  $\mu$ L/vial  
**Description:** For PCR amplicon capture  
**Contents:**  $\pi$ Code MicroDisc, Glycerol,  
 Phosphate buffered saline,  
 0.1% Albumin- from bovine (Biological),  
 <0.1% EDTA and <0.1% Sodium azide

**5. ALK KIT POS Control**  
**Ref. No.:** 20168  
**Quantity & Volume:** 3 vials, lyophilized  
**Description:** Assay positive control; reconstituted  
 with 25 $\mu$ L ddH<sub>2</sub>O per vial prior to use.  
**Contents:** 20 % EML4-ALK v1 RNA,  
 80 % RNastable<sup>®</sup> LD

**6. NEG Control**  
**Ref. No.:** 20549  
**Quantity & Volume:** 1 vial, 120  $\mu$ L/vial  
**Description:** Assay negative control  
**Contents:** ddH<sub>2</sub>O

**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. ALK KIT Hy Buffer**  
**Ref. No.:** 20166  
**Quantity & Volume:** 1 bottle, 2.4 mL/bottle  
**Description:** For hybridization  
**Contents:** ALK Detector,  
 Saline-Sodium Phosphate-EDTA

**9. 10X Wash Buffer**  
**Ref. No.:** 20546  
**Quantity & Volume:** 1 bottle, 50 mL/bottle  
**Description:** For  $\pi$ Code washing  
**Contents:** Phosphate buffered saline,  
 1% Tween-20 and <0.1% Sodium azide

**10. ddH<sub>2</sub>O**  
**Ref. No.:** 20548  
**Quantity & Volume:** 1 vial, 1.5 mL/vial  
**Description:** For reconstitution of ALK 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<sup>™</sup> 1000  $\pi$ Code 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<sup>™</sup> MD (Required: PlexBio; Cat. No. 80051)

### Required components:

- Qubit<sup>™</sup> 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
- ddH<sub>2</sub>O for dilution of 10X Wash Buffer
- FFPE RNA extraction kit (Recommended: RNeasy<sup>®</sup> FFPE Kit; Qiagen; Cat. No. 73504 or equivalent)
- Vortex mixer
- Micro-centrifuge
- Thermocycler (Recommended: DigiPlex<sup>™</sup> Thermocycler, PlexBio; Cat. No. 80018/ MiniAmp<sup>™</sup> Thermal Cycler, Applied Biosystems<sup>™</sup>; 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  $\pi$ Code Processor and PlexBio 100 Fluorescent Analyzer).

### Software Installation

The ALK Rearrangement Kit has a designated Kit App and ENC file. The Kit App contains the  $\pi$ Code 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](http://www.plexbio.com) and download the **ALK 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](http://www.plexbio.com) and download the **ALK 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 ALK Rearrangement Kit** has been validated to be used for formalin-fixed paraffin embedded tissues (FFPET) of NSCLC.

#### NOTE:

- FFPET specimens may be stored  $\leq 30^{\circ}\text{C}$  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- $\mu\text{m}$  thickness containing at least 10% tumor content are to be used in the ALK 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  $-20^{\circ}\text{C}$  for immediate use ( $\leq 24$  hours), or at  $-80^{\circ}\text{C}$  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  $\mu\text{L}$  of extracted RNA ( $\geq 10$  ng/ $\mu\text{L}$ ) 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  $\geq 10$  ng/ $\mu\text{L}$  to ensure optimal performance. Each RT-PCR reaction uses 5  $\mu\text{L}$  of a  $\geq 10$  ng/ $\mu\text{L}$  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 25 $\mu\text{L}$  ddH<sub>2</sub>O 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  $-20^{\circ}\text{C}$  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:**

ALK RT-PCR Buffer	12.5 $\mu$ L
ALK RT-PCR Enzyme	0.6 $\mu$ L
ALK RT-PCR Primer Mix	6.9 $\mu$ L
Sample/POS Control/NEG Control	5 $\mu$ L
<b>Total volume</b>	<b>25 <math>\mu</math>L</b>

**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.

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. ( $^{\circ}$ C)	Time	Cycles
45	15 min	-
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 $^{\circ}$ C/sec (ABI MiniAmp<sup>TM</sup>; Cat. No. A37834).

**11.3 Hybridization and SA-PE Reaction**

1. **Prepare 1X Wash Buffer:** Transfer 50mL of the 10X Wash Buffer to the IntelliPlex 1000  $\pi$ Code Processor 1L Wash Buffer bottle and add 450 ml ddH<sub>2</sub>O. Mix by swirling.

**NOTE:** The prepared 1X Wash Buffer can be used for up to one week.

IntelliPlex 1000  $\pi$ Code Processor Wash Buffer consumption:

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

2. **Add 20  $\mu$ L  $\pi$ Code MicroDisc to 96 well plate:** Mix by vortexing the **ALK  $\pi$ Code** for 10 seconds, then, by pipetting, add 20  $\mu$ L of the  $\pi$ Code to each well directly. Vortex the tube of  $\pi$ Code 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  $\mu$ L of ALK Hy Buffer** to each well.

4. Spin down the PCR products.

5. **Denature the PCR products** on the thermocycler by heating at 95 $^{\circ}$ 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  $\mu$ L 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  $\mu$ L** for up to 6 selected lanes or **800  $\mu$ L** if more than 6 lanes are selected. The minimum usage of SA-PE is **one lane (900  $\mu$ L)**.

**Calculation Example:**

For a 3-lane reaction, the required SA-PE solution volume is at least:  
**400  $\mu$ L x 3 lanes + 500  $\mu$ L (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 ( $\mu$ L)
1	900
2	1300
3	1700
4	2100
5	2500
6	2900
7	3600
8	4000

Number of Processed Lane(s)	Required SA-PE Solution (µL)
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 (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 6 runs of tests (including POS and NEG controls) for a maximum of 24 tests. Please note that the included Wash Buffer is only sufficient for up to two independent runs. Additional Wash buffer can be ordered from PlexBio (Ref. No: 80210).

**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 2 to 96 tests (including POS and NEG controls) per 96 well Microwell plate. When running more than 24 specimens, multiple IntelliPlex ALK Rearrangement Kits of the same lot will be required.
- The procedure described above must be followed to detect  $\geq 5 \sim 1209$  RNA copies in a background of wild-type RNA for the ALK 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	<b>Possible Causes:</b> <ol style="list-style-type: none"> <li>1. PCR Inhibition (presence of inhibitor in the sample)</li> <li>2. Improper stored reagents</li> <li>3. Low sample RNA input or quality</li> </ol>

Test Result	Reported Result	Interpretation
Invalid Assay	Invalid	<p><b>Possible Causes:</b></p> <ol style="list-style-type: none"> <li>4. Low <math>\pi</math>Code Disc Count (the <math>\pi</math>Code tube was not well-vortexed before pipetting)</li> <li>5. Reagent not added</li> <li>6. Failed Blank <math>\pi</math>Code Control</li> <li>7. Sample quality due to improper fixation process or storage condition</li> </ol>

**NOTE:**

- All run and specimen validation were performed by the dedicated KIT APP along with IntelliPlex 1000  $\pi$ Code 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 17 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 5~1209 copies.

**Table 3. Limit of Detection (LoD)**

Variant	LoD (RNA Copies/ Reaction)
EML4-ALK (E6;A19)	33
EML4-ALK V1	19
EML4-ALK V2	46
EML4-ALK V3a	11
EML4-ALK V3b	11
EML4-ALK V3c	16
EML4-ALK V4	34
EML4-ALK V"4"	118
EML4-ALK V5a	35
EML4-ALK V5b	5
EML4-ALK V"5"	269
EML4-ALK V6	53
EML4-ALK V7	41
EML4-ALK V8a	808
EML4-ALK V8b	236
EML4-ALK i2	248
EML4-ALK i18	31
EML4-ALK i53	461
EML4-ALK i68	1209
KIF5B-ALK (K24;A20)	29
KIF5B-ALK (K15;A20)	12
TFG-ALK (T4;A20)	5
TFG-ALK (T5;A20)	77
TFG-ALK (T6;A20)	35

## Repeatability and Reproducibility

The repeatability and reproducibility of variants in the assay was evaluated at low (2x LoD) and high (6x LoD) variant levels of variant RNA blended in wild type RNA background, across two operators, two sets of instrument, two sites, and ten non-consecutive testing days. Four replicate runs were performed per reagent lot per day for a total of 40 valid runs at one site. The accuracy across all tested levels was at least 98% (39/40) across all variance combined (i.e., site/instrument, operator, and day) (Table 4).

**Table 4. Accuracy**

Variant	Level (x LOD)	Variant Not Detected/ Detected	Accuracy (%)
EML4-ALK V1	6X	0/40	100%
	2X	0/40	100%
EML4-ALK V2	6X	0/40	100%
	2X	0/40	100%
EML4-ALK V3a	6X	1/39	98%
	2X	0/40	100%
EML4-ALK V3b	6X	1/39	98%
	2X	0/40	100%
EML4-ALK V3c	6X	0/40	100%
	2X	0/40	100%
EML4-ALK V4	6X	0/40	100%
	2X	0/40	100%
EML4-ALK V"4"	6X	0/40	100%
	2X	0/40	100%
EML4-ALK V5a	6X	0/40	100%
	2X	0/40	100%
EML4-ALK V5b	6X	1/39	98%
	2X	0/40	100%
EML4-ALK V"5"	6X	1/39	98%
	2X	1/39	98%
EML4-ALK V6	6X	0/40	100%
	2X	0/40	100%
EML4-ALK V7	6X	0/40	100%
	2X	0/40	100%
EML4-ALK V8a	6X	0/40	100%
	2X	1/39	98%
EML4-ALK V8b	6X	0/40	100%

Variant	Level (x LOD)	Variant Not Detected/ Detected	Accuracy (%)
EML4-ALK A19	2X	0/40	100%
	6X	0/40	100%
	2X	0/40	100%
EML4-ALK i2	6X	0/40	100%
	2X	1/39	98%
EML4-ALK i18	6X	0/40	100%
	2X	0/40	100%
EML4-ALK i53	6X	0/40	100%
	2X	0/40	100%
EML4-ALK i68	6X	0/40	100%
	2X	1/39	98%
KIF5B-ALK (K24;A20)	6X	0/40	100%
	2X	1/39	98%
KIF5B-ALK (K15;A20)	6X	0/40	100%
	2X	0/40	100%
TFG-ALK (T4;A20)	6X	0/40	100%
	2X	1/39	98%
TFG-ALK (T5;A20)	6X	0/40	100%
	2X	0/40	100%
TFG-ALK (T6;A20)	6X	0/40	100%
	2X	1/39	98%

## Cross-Contamination

This test is designed to assess cross-contamination during the washing steps, which may lead to false positive results. Wild-type and EML4-ALK V1 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. EML4-ALK V1 was selected as a representative variant. Triplicate testing of EML4-ALK V1 variant and wild type blend cell line (HEK293) RNA extract samples with each potential interfering substance (as listed in Table 5), added before the PCR step, showed no interference on kit performance.



**Table 5. 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.	1. Confirm plate is inserted and repeat reading.
	2. Plate inserted in wrong orientation.	2. Confirm orientation of plate and repeat reading.
	3. No assay APP installed.	3. Install assay APP and repeat reading.
	4. No ENC file imported.	4. Import ENC file and repeat reading.
	5. Two or more lots of reagent used.	5. One reagent lot used at a time.
Positive Control Fail / Negative Control Fail	1. No POS Control or NEG Control added.	1. Ensure POS Control and NEG Control are added.
	2. RNase contamination.	2. Ensure all operating procedures are followed correctly. Ensure work environment is free of RNase.
	3. Assay did not work.	3. Make sure all the assay procedures are followed correctly.
	4. Cross contamination between samples.	4. Clean all surfaces and equipment. Operate pre-PCR and post-PCR in the dedicated area and separate the equipment for use.
	5. Wrong PC/NC wells chose.	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-disperse the microplate using IntelliPlex 1000 Processor, 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 <sub>2</sub> O 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 lanes of microplate selected for SA-PE solution dispensing.	3. Repeat assay and make sure lanes 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 ddH <sub>2</sub> O) 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
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
	In-vitro diagnostic use		Catalog number
	Batch number		Consult instructions for use
	Manufacturer		Use by Date
	Temperature limitation		Caution
	Contains sufficient for <n> tests		Date of Manufacture
	European Union Conformity		European Authorized Representative


### 17. REFERENCES

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