IntelliPlex™ ALK Rearrangement Kit User Manual

REF 82023 24 Reactions

RUO For Research Use Only

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IMPORTANT:
Read the instructions carefully prior to use

1. INTENDED USE

The IntelliPlex ALK Rearrangement Kit, based on πCode™ technology and PlexBio's instrument platform, is an in vitro molecular assay intended for the qualitative detection of 24 gene rearrangements of the ALK gene using RNA samples derived from formalin-fixed paraffin-embedded (FFPE) tumor tissues from patients with non-small cell lung cancer (NSCLC). Results are intended to assist clinicians in identifying patients with NSCLC who may benefit from ALK inhibitors.

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 πCode 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 of the ALK gene

Variants	Exon Note
V1	E13:A20
V2	E20:A20
V3a	E6:A20
V3b	E6ins33:A20
V3c	E6ins18:A20
V4	E14:ins11del49 A20
"V4"	E15del60:del71 A20
V5a	E2:A20
V5b	E2:ins117 A20
"V5"	E18:A20
V6	E13:ins69 A20
V7	E14:del12 A20
V8a	E17:ins30 A20
V8b	E17ins61:ins34 A20
EML4-ALK	E6:A19
	E20:ins18 A20
	E3:ins53 A20
	E14ins2: ins56 A20
	E17:ins68 A20
KIF5B-ALK	K15:A20
	K24: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 πCode - for detection of 24 ALK gene rearrangements in one reaction.

πCode MicroDisc

πCode MicroDisc is designed to generate up to 16,000 distinct circular image patterns for multiplexing applications. Each πCode has a distinct circular image pattern, which corresponds to a specific capture agent conjugated to the surface of the disc. All capture agent tagged πCode are pooled, enabling capturing and detection of specific analytes in one reaction.

Detection Principle

The test is based on five processes listed as follows:

I. RNA extraction from FFPE samples
II. Multiplex one-step RT-PCR amplification
III. cDNA hybridization of PCR amplicons with fusion-specific probes tagged on πCode in a single well reaction
IV. Incubation for SA-PE and fluorescence conjugation
V. Image pattern decoding and fluorescent signal detection by the PlexBio™ 100 Fluorescent Analyzer

4. WARNINGS AND PRECAUTIONS

- For research use only.
- This assay kit should be used by qualified laboratory personnel only.
- Do not freeze-thaw the ALK POS Control more than 3 times.
- Do not use a kit or reagent past its expiration date.
- Note that tumor samples are non-homogeneous and may also contain non-tumor sections from a same sample to cause false-negative results.
- Component reagents have been diluted optimally. Further dilution of the component reagents is not recommended.
- Specimens should be handled as infectious material. Follow universal precaution 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 would interfere with decoding and signal acquisition.
- 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 surface, pipettes and anything else present in experiment.
- 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.
- 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.
- 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. The kit components supplied are listed as follows.

1. **ALK KIT RT-PCR Buffer**
   Ref. No.: 20171-R
   Quantity & Volume: 1 vial, 300 uL/vial
   Description: For RT-PCR amplification
   Contents: 2X Reaction Mix
   MgSO4 and dNTPs

2. **ALK KIT RT-PCR Enzyme**
   Ref. No.: 20172-R
   Quantity & Volume: 1 vial, 14.4 uL/vial
   Description: For RT-PCR amplification
   Contents: RT/HotStar Taq MIX
   Rnase Inhibitor (Ribolock)

3. **ALK KIT RT-PCR Primer Mix**
   Ref. No.: 20170-R
   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 πCode MicroDisc**
   Ref. No.: 20174-R
   Quantity & Volume: 1 vial, 480 µL/vial
   Description: For PCR amplicon capture
   Contents: Glycerol
   Phosphate buffered saline
   0.1% Albumin, from bovine (Biological)
   <0.1% EDTA
   <0.1% Sodium azide

5. **ALK KIT POS Control**
   Ref. No.: 20168-R
   Quantity & Volume: 3 vials, lyophilized
   Description: Assay positive control; reconstituted with 25µL ddH2O per vial prior to use.
   Contents: 20 % EML4-ALK v1 RNA
   80 % RNAstable®

6. **ALK KIT NEG Control**
   Ref. No.: 20173-R
   Quantity & Volume: 1 vial, 120 µL/vial
   Description: Assay negative control
   Contents: ddH2O

7. **SA-PE Solution**
   Ref. No.: 20302
   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
   <0.1% Sodium azide

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

9. **ALK KIT 10X Wash Buffer**
   Ref. No.: 20175-R
   Quantity & Volume: 1 bottle, 50 mL/bottle
   Description: For πCode washing
   Contents: Phosphate buffered saline
   1% Tween-20
   <0.1% Sodium azide

10. **ALK ddH2O**
    Ref. No.: 20167-R
    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 stand for positive control, negative control and hybridization buffer, respectively.

6. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- 96-well plate (Greiner Bio-one; Cat. No. 655101)
- Clean tubes for RT-PCR reaction
- Dedicated micropipette
- Filter tips for micropipette
- ddH2O for dilution of 10X Wash Buffer
- FFPE RNA extraction kit (RNeasy® FFPE Kit; Qiagen; Cat. No. 73504)
- Vortex mixer
- Microcentrifuge
- U Tray (PlexBio; Cat. No. 80023)
- V Tray (PlexBio; Cat. No. 80024)
- DigiPlex™ Thermocycler (PlexBio; Cat. No. 80018)
- IntelliPlex 1000 πCode Processor (PlexBio; Cat. No. 80033)
- PlexBio 100 Fluorescent Analyzer (PlexBio; Cat. No. 80000)
- Industrial Computer (PlexBio; Cat. No. 80002)
- DeXipher™ RU (PlexBio; Cat. No. 80050)

7. STORAGE, STABILITY AND TRANSPORTATION

Storage

All kit components of the IntelliPlex ALK Rearrangement Kit should be stored at 2°C to 8°C. Once opened, the reagent components are stable for 8 months or until the expiration date, whichever comes first.

Stability

Do not use the IntelliPlex ALK Rearrangement Kit when it is 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 IntelliPlex ALK Rearrangement Kit is at 2-8°C. If the kit is broken or has components missing, please contact the PlexBio Customer Service.

8. INSTRUMENT AND SOFTWARE

Instrument

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

Software Installation

NOTE:

- ***For the first time assay operation, please make sure the KIT APP is installed into DeXipher.***
- ***The ENC file contains the information of kit lot no. and expiration date.***

KIT APP Installation

1. Open the ALK KIT APP from the USB drive provided and run "Installer.exe".
2. Click on "Install" from the pop up window and finish the APP installation.

Note: The KIT APP only needs to be installed for the first time running until the new version release.

ENC File Installation

1. Download the corresponding ENC File from PlexBio's website (Homepage/ Download/ ENC Files/ ALK Kit/ Corresponding Kit Lot no.)
2. Save to the computer and make sure the ENC file matches the lot no. of the assay kit.
3. Click on the button as shown below on the DeXipher homepage.

4. Click on the button as shown below to import kit.

5. Select and import the corresponding ENC file into the software.

9. SPECIMENS

Specimen Collection

The non-small-cell lung cancer (NSCLC) formalin-fixed paraffin embedded (FFPE) tissue have been validated to use with the IntelliPlex ALK Rearrangement kit. It is recommended to use the RNeasy® FFPE Kit (Qiagen, Cat. No. 73504 for 50 Rxn) for RNA extraction.

Specimen Transportation and Storage

FFPE specimens can be transported and stored at 15-30°C for over 12 months.

Storage of Extracted RNA

Extracted RNA can be stored at -20°C for immediately use, or at -80°C for long-term storage. Do not repeatedly freeze and thaw the extracted RNA.

10. ASSAY PROCEDURE

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

10.1 RNA Extraction

1. Follow the instructions provided by the RNA extraction kit manufacturer. It is recommended to use RNeasy® FFPE Kit (50) for FFPE specimens with the elution volume for 14-30µL of 5-20µm FFPE sections
2. Quantify the RNA using a Nanodrop UV-Vis Spectrophotometer according to the manufacturer's protocol.
3. The RNA Stock concentration from the specimens must be ≥ 10 ng/µL to perform the IntelliPlex ALK Rearrangement Kit. Each RT-PCR reaction per specimen is run by using 5 µL of a 10 ng/µL RNA Stock (total RNA ≥ 50 ng).

10.2 Multiplex one-step RT-PCR Amplification

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

Table 2: RT-PCR Reaction Mix Preparation*

Material	Vol. (µL) per reaction
ALK RT-PCR Buffer	12.5
ALK RT-PCR Enzyme	0.6
ALK RT-PCR Primer Mix	6.9
Extracted RNA/PC/NC	5
Total	25

***Note:**

- *The amount of one-step RT-PCR reagent required depends on the number of reactions.*
- *Both POS Control and NEG Control reactions should be included in every run of the assay.*

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 shown in Table 3:

Table 3: RT-PCR Program Conditions[#]

Temp. (˚C)	Time	Cycles
45	15 min	1
95	2 min	1
95	15 sec	50
60	30 sec	
72	30 sec	
4	Hold	1

[#]*Note: Ramp rate: 5°C/sec*

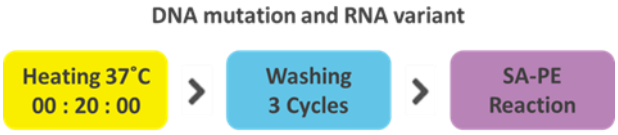
10.3 cDNA Hybridization and SA-PE Reaction

1. Transfer the 10X Wash Buffer to 1L Wash Buffer bottle of the IntelliPlex 1000 πCode Processor supplied and add 450 ml ddH_2O to prepare 1X Wash Buffer for use.
2. Pipet the desired volume of SA-PE solution into 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**.

Calculation Example:
For 3 rows reaction, the SA-PE solution needed is **400 µL x 3 rows + 500 µL = 1.7mL (at least)**.
In order to ensure the instrument has sufficient solution to dispense, pipet extra volume into the solution tank is recommended.

***Note:**
- *SA-PE solution should be kept in the dark.*
- ***Do not** reuse the leftover of SAPE solution and the V-tray tank.*

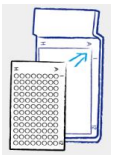
3. Mix by vortexing the ALK πCode MicroDisc for 10 seconds, and add 20 µL of ALK πCode MicroDisc to each well directly without further pipetting. Vortex the ALK πCode MicroDisc after loading every 4 wells to ensure homogeneous suspension of πCode MicroDisc.
4. Add 100 µL of ALK Hy Buffer to each well.
5. Spin down the PCR products.
6. Denature the PCR products on the thermocycler by heating up to 95˚C for 5 minutes then cooling down to 4˚C without delay.
7. Spin down the PCR products, and keep PCR products on the ice before adding to wells.
8. Add 10 µL of the denatured PCR products to each well.
9. Refer to IntelliPlex 1000 πCode Processor operation manual and follow the instructions for built-in assay program as described (Homepage/ Molecular Assay/ Select well rows/ DNA&RNA/ Confirm procedure conditions as figure shown below/ Start Running). The plate will be ready for decoding once program finished.



***Note:**
- ***Do not** open the door during the instrument operation.*

10.4 Image Decoding and Fluorescent Detection

1. Place the plate on the PlexBio 100 Fluorescent Analyzer as shown below.



2. Click on the button as shown below on the DeXipher homepage.

3. Select the well for detection and import the needed information of each detection samples. Click next step as figure shown as below.

4. Enter the “Assay Name” for the assay and click icon as shown below for image pattern decoding and fluorescent signal acquisition.

5. Export the results for data analysis or calculation or the PDF report.

11. DISCLAIMERS

Negative test result

A negative test result means that 24 variants of the ALK gene was not detectd by IntelliPlex ALK Rearrangement Kit. It does not preclude the gene rearrangements on the ALK gene. Moreover, false negative test results may be due to experimental errors or other causes. Clinical interpretation of the results should be made in combination with all other clinical findings.

Positive test result

A positive test result means that 24 variants of the ALK gene are detected by IntelliPlex ALK Rearrangement Kit. It does not preclude the possibility that the specimen did not have the gene rearrangements on the ALK gene. False positive test results may be caused by experimental errors or other causes. Clinical interpretation of the results should be made in combination with all other clinical findings.

12. INTERPRETATION OF RESULTS

Table 4: Interpretation of Result

Test Result	Report Result	Interpretation
Fusion Detected	Refer to Table. 1	Fusion detected on the specified targeted ALK region
Fusion Not Detected	None	Fusion not detected on the targeted ALK regions
Invalid Assay	Invalid	**Possible Cause:** 1. PCR Inhibition 2. SAPE Reaction Failed 3. Low Input or Low Quality of Sample RNA 4. Low πCode Disc Count 5. No πCode Detected 6. Blank πCode Control Failed

13. ANALYTICAL PERFORMANCE

Limit of Blank (LoB)

The limit of blank (LoB) values were determined by testing RNA derived from wild-type ALK cell line (HEK293) and from 17 wild-type lung cancer FFPE tissue samples with 60 replicates each. Based on the results, the maximum analytical signal intensity values for each variants were used as the cutoff values for each targeted variants of the assays.

"No Fusion Detected" results were only observed in the samples presence of ALK wild-type RNA.

Limit of Detection (LoD)

The limit of detection (LoD) of IntelliPlex ALK Rearrangement Kit was determined for 24 gene rearrangements on the ALK gene. RNA tested included the following specimen types:

- RNA copies: RNA extracted from cells and calculated the copy number of target RNA based on mutant plasmid.

Testing was performed using two lots of the IntelliPlex ALK Rearrangement Kit. RNA samples detection dynamic range is diluted from 750 copies to 5 copies. Each level of RNA was tested with 21 replicates across 3 days per reagent lot. The LoDs of each lot were determined based on a positive hit rate at 95% in PriProbit analysis as shown in table 5.

Table 5. Limit of Detection (LoD) of each variants

Type	LoD (RNA Copies/ Reaction)
E13;A20	19
E20;A20	46
E6;A20	11
E6ins33;A20	11
E6ins18;A20	16
E14;ins11del49 A20	34
E15del60;del71 A20	118
E2;A20	35
E2;ins117 A20	5
E18;A20	269
E13;ins69 A20	53
E14;del12 A20	41
E17;ins30 A20	808
E17ins61;ins34 A20	236
E6;A19	33
E20;ins18 A20	248
E3;ins53 A20	31
E14ins2; ins56 A20	461
E17;ins68 A20	1209
K15;A20	12
K24;A20	29
T4;A20	5
T5;A20	77
T6;A20	35

Repeatability and Reproducibility

The repeatability and reproducibility of IntelliPlex ALK Rearrangement Kit was evaluated across two reagent lots, 4 operators, 2 sets of instrument and 10 non-consecutive testing days. Four replicate runs were performed per reagent lot per day for a total of 40 valid runs at one site. Results of IntelliPlex ALK Rearrangement Kit was demonstrated with low level mutant (2x LoD) and high level mutant (6x LoD). The accuracy of the all testing level was at least 98% (39/40) across all variance combined (i.e., site/instrument, operator, and day).

Table 6. Repeatability and Reproducibility Accuracy across All Variance Combined

Variants	Level	Mutation Detected	Mutation Not Detected	Accuracy (%)
E13;A20	6X	40	0	100%
	2X	40	0	100%
E20;A20	6X	40	0	100%
	2X	40	0	100%
E6;A20	6X	39	1	98%
	2X	40	0	100%
E6ins33;A20	6X	39	1	98%
	2X	40	0	100%
E6ins18;A20	6X	40	0	100%
	2X	40	0	100%
E14;ins11del49 A20	6X	40	0	100%
	2X	40	0	100%
E15del60;del71 A20	6X	40	0	100%
	2X	40	0	100%
E2;A20	6X	40	0	100%
	2X	40	0	100%
E2;ins117 A20	6X	39	1	98%
	2X	40	0	100%
E18;A20	6X	39	1	98%
	2X	39	1	98%
E13;ins69 A20	6X	40	0	100%
	2X	40	0	100%
E14;del12 A20	6X	40	0	100%
	2X	40	0	100%
E17;ins30 A20	6X	40	0	100%
	2X	39	1	98%
E17ins61:ins34 A20	6X	40	0	100%
	2X	40	0	100%
E6;A19	6X	40	0	100%
	2X	40	0	100%
E20;ins18 A20	6X	40	0	100%
	2X	40	0	100%
E3;ins53 A20	6X	40	0	100%
	2X	40	0	100%
E14ins2; ins56 A20	6X	40	0	100%
	2X	39	1	98%
E17;ins68 A20	6X	40	0	100%
	2X	39	1	98%
K15;A20	6X	40	0	100%
	2X	39	1	98%
K24;A20	6X	40	0	100%
	2X	40	0	100%
T4;A20	6X	40	0	100%
	2X	39	1	98%
T5;A20	6X	40	0	100%
	2X	40	0	100%
T6;A20	6X	40	0	100%
	2X	39	1	98%

Cross-Contamination

The test is designed to access the cross-contamination during all assay operation steps which may lead to the false positive results. The tested wild-type samples and V1 (E13:A20) variant sample of ALK gene were placed in rotated order between wells in the 96-well plate. The results demonstrated that there was no cross-contamination during all assay operation steps.

Interference

The test is designed to evaluate the impact of potentially carrying over substances from Qiagen's RNeasy® FFPE Kit. The V1 (E13:A20) variant sample of ALK gene and each potential interference substances (Listed as below) were tested in three replicates. The result indicated the interference substances will not interfere with the performance of the Intelliplex ALK Rearrangement Kit.

Table 7: The tested interfering substances

Interfering Substance	Assumed Interference Residue Volume (ul / 30ul 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%

14. SYMBOLS

Table 8: Symbols

Symbol	Explanation	Symbol	Explanation
RUO	For research use only	REF	Catalog number
LOT	Batch number		Consult instructions for use
	Manufacturer		Use by Date
	Temperature limitation		Caution
	Contains sufficient for <n> tests		Date of Manufacture

15. REFERENCES

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