IntelliPlex™ RET/NTRK1 Rearrangement Kit User Manual

REF 82025 24 Reactions

RUO For Research Use Only

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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

RET gene encodes a receptor tyrosine kinase (RTK) belonging to the RET family of RTKs. It is normally expressed in tissues derived from the neural crest. Activated RET resulting in activation of multiple downstream cellular pathways. Although genomic alterations in RET are found in several different types of cancer, the in-frame rearrangements in NSCLC were only recently discovered in 2011. 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 the two fusion partners of NTRK1 that have been described in non-small cell lung cancer.

It has been reported that the rearrangements of *RET* and *NTRK1* are both sensitive to TKI therapy.

It is thus critical to assess the rearrangement status of RET and NTRK1 gene. Detection of 14 rearrangements of the RET and NTRK1 gene per reaction in the background of wild-type RNA in specimens is feasible based on the one-step RT-PCR assay design and πCode technology. The IntelliPlex RET/NTRK1 Rearrangement Kit identifies 14 rearrangements of the RET and NTRK1 gene, as listed in Table 1.

Table 1: Variants of the RET/NTRK1 gene

Gene	Variants	Exon Note
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 - for detection of 14 RET and NTRK1 genes 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 use a kit or reagent past its expiration date.
- Do not freeze-thaw the RET/NTRK1 POS Control more than 3 times
- 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. Please 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 RET/NTRK1 Rearrangement Kit contains sufficient reagents for up to 24 tests. The kit components supplied are listed as follows.

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/HotStar Taq MIX
   RNase Inhibitor (Ribolock)

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: Glycerol
   Phosphate buffered saline
   0.1% Albumin, from bovine (Biological)
   <0.1% EDTA
   <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 ddH_2O prior to use.
   Contents: 20 % RNA of K15;R12 cell line
   80 % RNAstable®

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
   <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 ; <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 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 RET/NTRK1 Rearrangement Kit should be stored at 2°C to 8°C.

Stability

Do not use the IntelliPlex RET/NTRK1 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 RET/NTRK1 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 RET/NTRK1 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/ “RET/NTRK1 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 RET/NTRK1 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 RET/NTRK1 Rearrangement Kit. Each RT-PCR reaction per specimen is run by using 5 µL of a 50 ng/µL RNA Stock (total ≥ 50 ng RNA).

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
RET/NTRK1 RT-PCR Buffer	13
RET/NTRK1 RT-PCR Enzyme	0.6
RET/NTRK1 RT-PCR Primer	6.4
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 ddH2O 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:

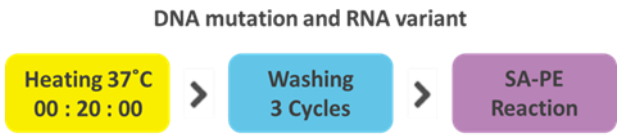
For 3 rows reaction, the SA-PE solution needed is **400 µL x 3 rows + 500 µL = 1.7mL (at least)**.

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 RET/NTRK1 πCode MicroDisc for 10 seconds, and add 20 µL of RET/NTRK1 πCode MicroDisc to each well directly without further pipetting. Vortex the RET/NTRK1 πCode MicroDisc after loading every 4 wells to ensure homogeneous suspension of πCode MicroDisc.
4. Add 100 µL of RET/NTRK1 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.

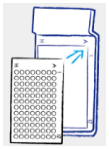


***Note:**

- ***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 14 variants of the RET or NTRK1 genes were not detected by IntelliPlex RET/NTRK1 Rearrangement Kit. It does not preclude the gene rearrangements on the RET or NTRK1 genes. 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 14 variants of the RET or NTRK1 genes are detected by IntelliPlex RET/NTRK1 Rearrangement Kit. It does not preclude the possibility that the specimen did not have the gene rearrangements on the RET or NTRK1 genes. 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	**KIF5B-RET:** K15;R11 K15;R12 K16;R12 K22;R12 K23;R12 K24;R8 K24;R11	Fusion detected on the specified targeted RET and NTRK1 region
	CCDC6-RET: C1;R12	
	NCOA4-RET: N6;R12	

	N8;R12	
	TRIM33-RET: T14;R12	
	CUX1-RET: CX10;R12	
	CD74-NTRK1: C8;N12	
	MPRIP-NTRK1: M21;N14	
Fusion Not Detected	None	Fusions not detected on the targeted RET and NTRK1 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 RET and NTRK1 cell line (HEK293 and HuT78) and from 15 wild-type lung cancer FFPE tissue samples with 108 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 RET and NTRK1 wild-type RNA.

Limit of Detection (LoD)

The limit of detection (LoD) of IntelliPlex RET/NTRK1 Rearrangement Kit was determined for 14 gene rearrangements on the RET and NTRK1 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 RET/NTRK1 Rearrangement Kit. RNA samples detection dynamic range is diluted varies from 1000 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)
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 of IntelliPlex RET/NTRK1 Rearrangement Kit was evaluated across two reagent lots, 4 operators, 2 sets of instrument and 5 non-consecutive testing days. Four replicate runs were performed per reagent lot per day for a total of 20 valid runs at one site. Results of IntelliPlex RET/NTRK1 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 92.5% (37/40) 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 (%)
K15;R11	6X	40	0	100
	2X	37	3	92.5
K15;R12	6X	40	0	100
	2X	39	1	97.5
K16;R12	6X	40	0	100

	2X	38	2	95
K22;R12	6X	40	0	100
	2X	37	3	92.5
K23;R12	6X	39	1	97.5
	2X	39	1	97.5
K24;R8	6X	40	0	100
	2X	38	2	95
K24;R11	6X	40	0	100
	2X	40	0	100
C1;R12	6X	40	0	100
	2X	39	1	97.5
N6;R12	6X	40	0	100
	2X	40	0	100
N8;R12	6X	39	1	97.5
	2X	40	0	100
T14;R12	6X	40	0	100
	2X	40	0	100
CX10;R12	6X	40	0	100
	2X	40	0	100
C8;N12	6X	40	0	100
	2X	40	0	100
M21;N14	6X	40	0	100
	2X	39	1	97.5
WT (HEK293)	-	0	40	100
WT (HuT78)	-	0	40	100

Table 7. Coefficient of Each RET/NTRK1 Variants

Variants	Mutation Level	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%
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

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 KIF5B-RET (K15:R12) variant sample of RET 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 KIF5B-RET (K15:R12) variant sample of RET 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 RET/NTRK1 Rearrangement Kit.

Table 8: 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 9: 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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