IntelliPlex™ Lung Cancer Panel User Manual

REF 82028 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 Lung Cancer Panel, based on πCode™ technology and PlexBio's instrument platform, is an in vitro molecular assay intended for the qualitative identification of 36 DNA mutations in the KRAS, NRAS, PIK3CA, BRAF and EGFR genes as well as 19 gene rearrangements of the ALK, ROS1, RET, NTRK1 and MET genes. DNA and RNA are isolated from formalin-fixed paraffin-embedded (FFPE) tumor tissue from lung cancer patients. Results are intended to assist clinicians in identifying patients who may benefit from available targeted treatments.

2. INTRODUCTION

Studies of NSCLC has identified recurrent 'driver' mutations that occur in multiple oncogenes, including AKT1, ALK, BRAF, EGFR, HER2, KRAS, MEK1, MET, NRAS, PIK3CA, RET, and ROS1, and these markers serve as the basis for the molecular classification of NSCLC. Both DNA mutations and gene variants contribute to oncogenesis in NSCLC.

Targeted small molecule inhibitors are now available or being developed to benefit specific molecularly defined subsets of NSCLC patients, and assessment of a variety of mutation status of multiple oncogenes has become critical in the evaluation of cancer treatments. SelectAmp and πCode technology enables the multiplex, high sensitivity, single-well detection of mutations from specimens containing large amounts of wild-type genomic DNA with significantly reduced sample requirement compared to conventional methods. The **IntelliPlex Lung Cancer Panel** identifies 36 DNA single nucleotide mutations (Table 1) and 19 gene variants from RNA samples (Table 2).

Table 1. Mutations Detected (DNA Set)

Gene	Exon	Amino Acid Change	Nucleotide Change	COSMIC ID
KRAS	Exon2	p.G12A	c.35G>C	522
		p.G12D	c.35G>A	521
		p.G12V	c.35G>T	520
		p.G12C	c.34G>T	516
		p.G12R	c.34G>C	518
		p.G12S	c.34G>A	517
		p.G13D	c.38G>A	532
		p.G13C	c.37G>T	527
	Exon3	p.Q61K	c.181C>A	549
		p.Q61R	c.182A>G	552
		p.Q61L	c.182A>T	553
		p.Q61H	c.183A>C	554
		p.Q61H	c.183A>T	555
NRAS	Exon3	p.Q61H	c.183A>T	586
		p.Q61L	c.182A>T	583
		p.Q61K	c.181C>A	580
		p.Q61R	c.182A>G	584
PIK3CA	Exon9	p.E542K	c.1624G>A	760
		p.E545K	c.1633G>A	763
		p.E545Q	c.1633G>C	27133
	Exon20	p.H1047R	c.3140A>G	775
		p.H1047L	c.3140A>T	776
BRAF	Exon15	p.V600E1	c.1799T>A	476
EGFR	Exon18	p.G719S	c.2155G>A	6252
		p.G719A	c.2156G>C	6239
		p.G719C	c.2155G>T	6253

Gene	Exon	Amino Acid Change	Nucleotide Change	COSMIC ID
	Exon19	p.E746_A750del	c.2235_2249del15	6223
		p.E746_A750del	c.2236_2250del15	6225
		p.E746_S752>V	c.2237_2255>T	12384
		p.L747_P753>S	c.2240_2257del18	12370
		p.L747_A750>P	c.2239_2248TTAAGAGAAG>C	12382
		p.L747_T751delLREAT	c.2240_2254del15	6254
		p.L747_S752delLREATS	c.2239_2256del18	6255
	Exon20	p.T790M	c.2369C>T	6240
	Exon21	p.L858R	c.2573T>G	6224
		p.L861Q	c.2582T>A	6213

Table 2. Fusion Variants Detected (RNA Set)

Gene	Fusion Variant	Inferred Breakpoint
ALK	EML4-ALK V1	E13;A20
	EML4-ALK V2	E20;A20
	EML4-ALK V3a	E6a;A20
	EML4-ALK V3b	E6b;A20
	EML4-ALK V4	E14;A20
	EML4-ALK V5a	E2a;A20
	EML4-ALK V5b	E2b;A20
	EML4-ALK V"5"	E18;A20
ROS	SLC34A2-ROS1	S4;R32
		S4;R34
	CD74-ROS1	C6;R32
		C6;R34
RET	CCDC6-RET	C1;R12
	KIF5B-RET	K15;R11
		K15;R12
	TRIM33-RET	T14;R12
NTRK1	CD74-NTRK1	C8;N12
	MPRIP-NTRK1	M21;N14
MET	Exon14 skipping	-

3. TECHNOLOGICAL PRINCIPLES

The IntelliPlex kits utilizes three technologies, SelectAmp, πCode and one step RT-PCR, to achieve high sensitivity multiplex mutation detection.

SelectAmp Technology

SelectAmp technology enables mutation-specific multiplex PCR amplification by blocking amplification of wild-type sequences with Locked Nucleic Acid (LNA). The subsequent selective PCR amplification of mutated sequences increases assay sensitivity and specificity.

πCode MicroDisc

πCode MicroDisc is 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. πCodes tagged with different capture agents are pooled, enabling specific detection of multiple analytes in a one well reaction.

One-step RT-PCR (RNA Set)

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

Detection Principle

The test is based on five processes:

1. DNA or RNA extraction from specimens
2. Mutation-specific multiplex PCR or RT-PCR amplification
3. Hybridization of PCR amplicons with mutation-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 RNA 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 (RNA Set):**
  - **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 Lung Cancer Panel (DNA Set) includes sufficient reagent for up to 24 tests. Kit components include:

1. **LCP KIT-DNA Reaction Mix**
   Ref. No.:20356
   Quantity & Volume: 1 vials, 240 µL/vial
   Description: For PCR amplification
   Contents: 80% MyFi 5X Reaction Buffer, Magnesium chloride, dNTPs and Enhancer, 10% MyFi DNA polymerase (Microbial)

2. **LCP KIT-DNA Primer Mix**
   Ref. No.: 20355
   Quantity & Volume: 1 vial, 240 µL/vial
   Description: For PCR amplification
   Contents: 21% Forward Primer, 21% Reverse Primer (biotin labeled), 25% Locked Nucleic Acid

3. **LCP KIT-DNA πCode MicroDisc**
   Ref. No.: 20357
   Quantity & Volume: 1 vials, 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

4. **LCP KIT-DNA POS Control**
   Ref. No.: 20359
   Quantity & Volume: 1 vial, 120 µL/vial
   Description: Assay positive control
   Contents: Cell line DNA, Tris-EDTA Buffer

5. **LCP KIT-DNA NEG Control**
   Ref. No.: 20360
   Quantity & Volume: 1 vial, 120 µL/vial
   Description: Assay negative control
   Contents: ddH_2O

6. **LCP KIT-DNA Hy Buffer**
   Ref. No.: 20361
   Quantity & Volume: 1 bottle, 2.4 mL/bottle
   Description: For hybridization
   Contents: Saline-Sodium Phosphate-EDTA

The Lung Cancer Panel Kit (RNA Set) contains sufficient reagents for up to 24 tests. Kit components include:

1. **LCP KIT-RNA RT-PCR Buffer**
   Ref. No.: 20363
   Quantity & Volume: 1 vial, 300 uL/vial
   Description: For RT-PCR amplification
   Contents: 2X Reaction Mix, MgSO4 and dNTPs

2. **LCP KIT-RNA RT-PCR Enzyme**
   Ref. No.: 20364
   Quantity & Volume: 1 vial, 14.4 uL/vial
   Description: For RT-PCR amplification
   Contents: RT/Hot-Start Taq MIX, RNase Inhibitor

3. **LCP KIT-RNA Primer Mix**
   Ref. No.: 20362
   Quantity & Volume: 1 vial, 165.6 uL/vial
   Description: For RT-PCR amplification
   Contents: <20 % Forward Primer, <10 % Reverse Primer (biotin labeled)

4. **LCP KIT-RNA πCode MicroDisc**
   Ref. No.: 20365
   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. **LCP KIT-RNA POS Control**
   Ref. No.: 20366
   Quantity & Volume: 3 vials, lyophilized
   Description: Assay positive control; reconstituted with 25µL LCP-RNA ddH2O per vial prior to use.
   Contents: Cell line RNA and 80 % RNAstable® LD

6. **LCP KIT-RNA NEG Control**
   Ref. No.: 20367
   Quantity & Volume: 1 vial, 120 µL/vial
   Description: Assay negative control
   Contents: ddH_2O

7. **LCP KIT-RNA Hy Buffer**
   Ref. No.: 20369
   Quantity & Volume: 1 bottle, 2.4 mL/bottle
   Description: For hybridization
   Contents: Saline-Sodium Phosphate-EDTA

8. **LCP KIT-RNA ddH2O**
   Ref. No.: 20368
   Quantity & Volume: 1 vial, 100 µL /vial
   Description: for reconstitution of Lung Cancer Panel POS Control (RNA)
   Contents: Nuclease-free water

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

The Lung Cancer Panel Kit contains two buffers for πCode fluorescent labeling and πCode washing. Buffers are sufficient for up to 24 tests and include:

1. **SA-PE Solution**
   Ref. No.: 20007
   Quantity & Volume: 2 bottles, 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

2. **LCP KIT 10X Wash Buffer**
   Ref. No.: 20370
   Quantity & Volume: 2 bottles, 50 mL/bottle
   Description: For πCode washing
   Contents: Phosphate buffered saline, 1% Tween-20, <0.1% Sodium azide

6. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

Required products for compatibility with IntelliPlex kits:

- Greiner Bio-one 96-well plate (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 DNA extraction kit (Recommended: QIAamp DNA FFPE Tissue Kit, Qiagen; Cat. No. 56404 or equivalent)
- FFPE RNA extraction kit (Recommended: ReliaPrep™ FFPE Total RNA Miniprep System, Promega; Cat. No. Z1001/ 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

The **LCP-RNA RT-PCR Buffer and LCP- RNA RT-PCR Enzyme Mix** of the IntelliPlex Lung Cancer Kit should be **stored at -15°C to -25°C separately upon arrival**.

Other kit components of the IntelliPlex Lung Cancer Kit should be stored at 2°C to 8°C.

Any reconstituted RNA positive control could be stored at -20°C up to one month. Please use within one freeze-thaw cycle.

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 is incomplete, please contact PlexBio customer service (service@plexbio.com).

8. INSTRUMENTS AND SOFTWARE

Instruments

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

Software Installation

The Lung Cancer Panel 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 **Lung Cancer Panel 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 **Lung Cancer Panel** 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 Lung Cancer Panel** 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 FFPE sections of 10-µm thickness containing at least 10% tumor content are to be used in the Lung Cancer Panel 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 DNA

Extracted DNA can be stored at 2°C to 8°C for immediate use (≦ 24 hours), or at -15°C to -25°C for long-term (> 24 hours) storage. Do not subject the extracted DNA to repeated freeze/thaw cycles.

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 20 µL of extracted DNA (0.5 ng/µL) and 5 µL of extracted RNA (≥ 10 ng/µL) ready for analysis.

11. ASSAY PROCEDURE – DNA SET

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

11.1 DNA Quantification

1. Quantify the extracted DNA using a Qubit Fluorometer with dedicated quantitative reagents (or equivalent) according to the manufacturer's protocol.
2. The DNA stock concentration of the extracted sample must be 0.5 ng/µL to perform the IntelliPlex Lung Cancer Panel (DNA Set). Each PCR reaction uses 20 µL of a 0.5 ng/µL DNA stock (10 ng DNA input). DNA input amounts lower than 10 ng per reaction well are not recommended.

11.2 Multiplex PCR Amplification

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

For each PCR reaction:

LCP KIT-DNA Reaction Mix	10 µL
LCP KIT-DNA Primer Mix	10 µL
Sample/POS Control/NEG Control	20 µL
Total volume	40 µL

NOTE:

- The amount of Reaction Mix and Primer Mix 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.

4. Mix by tapping the PCR Reaction tubes and spin down before placing the tubes in the thermocycler. Set up the PCR program conditions as below:

PCR Program Conditions*

Temp. (˚C)	Time	Cycles
95	5 min	-
95	20 sec	36
70	20 sec	
60	60 sec	
4	Hold	-

NOTE: *Ramp rate: 20% (PlexBio; Cat. No. 80018). 3°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 LCP KIT-DNA πCode MicroDisc to 96 well plate:** Mix by vortexing the **LCP KIT-DNA πCode MicroDisc** tube for 10 seconds, then, without pipetting, add 20µL 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 LCP Kit-DNA 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 **seven** 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 5 runs of tests (including POS and NEG controls) for a maximum of 24 tests.

11.4 Image Decoding and Fluorescent Detection

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

NOTE:
- PlexBio 100 Fluorescent Analyzer must be calibrated regularly (once per month).
- Check that the correct ENC file has been imported.

2. Launch DeXipher to run the qualitative assay.
3. Mark the wells for sample, positive and negative controls.
4. Enter 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 mutation 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 Lung Cancer Panel Kits of the same lot will be required.
- The procedure described above must be followed to detect ≥ 0.1~2.11% mutant sequences in a background of wild-type DNA for the mutations listed in Table 1.

12. ASSAY PROCEDURE – RNA SET

12.1 RNA Quantification

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

12.2 Reconstitute LCP KIT-RNA POS Control:

1. Briefly centrifuge the LCP KIT- RNA POS Control tube.
2. Add 25μL of LCP KIT-RNA ddH2O to reconstitute by pipetting up and down.
3. Make sure the POS control is fully reconstituted and keep reconstituted tube 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.

12.3 Multiplex one-step RT-PCR Amplification

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

For each RT-PCR reaction:

LCP KIT RT-PCR buffer	12.5 μL
LCP KIT RT-PCR Enzyme	0.6 μL
LCP KIT Primer Mix	6.9 μL
Sample/ POS Control/ NEG Control	5 μL
Total	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.

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

RT-PCR Program Conditions#

Temp. (˚C)	Time	Cycles
55	15 min	-
95	2 min	-
95	15 sec	45
60	30 sec	
72	30 sec	
4	Hold	-

NOTE: *Ramp rate: 100% (PlexBio; Cat. No. 80018). 3°C/sec (ABI MiniAmp™; Cat. No. A37834).*

12.4 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 to prepare the 1X Wash Buffer.
2. **Add LCP KIT-RNA πCode MicroDisc to 96 well plate:** Mix the tube of πCode by vortexing for 10 seconds, then, without pipetting, add 20 µL 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 Lung Cancer Panel Hybridization Buffer-RNA** to each well.
4. Spin down the PCR products.
5. **Denature the PCR products** on the thermocycler by heating at 95˚C for **seven** 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 and place the 96-well plate into the IntelliPlex 1000 πCode Processor.
7. **Pipet the desired volume of SA-PE solution** into the IntelliPlex 1000 πCode Processor SA-PE solution tank (the V-tray). Please note that the dead volume of the V-tray is **500 µL** and the minimum usage unit 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.

12.5 Image Decoding and Fluorescent Detection

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

NOTE:

- PlexBio 100 Fluorescent Analyzer must be calibrated regularly (once per month).
- Check that the correct ENC file has been imported.

2. Launch DeXipher to run the qualitative assay.
3. Mark the wells for sample, positive and negative controls.
4. Enter 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 Lung Cancer Panel Kits of the same lot will be required.

- The procedure described above must be followed to detect ≥ 10 - 89 RNA copies in a background of wild-type RNA for the RNA variants in Table 1.

13. DISCLAIMERS

Negative test result

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

14. INTERPRETATION OF RESULT

Table 3. Interpretation of Results

Test Result	Reported Result	Interpretation
Detected	Refer to Table 1 and 2	Targeted mutation/ variant detected
Not Detected	None	Targeted mutation/ variant not detected
Invalid Assay	Invalid	**Possible Causes:** 1. PCR Inhibition (presence of inhibitor in the sample) 2. Improper stored reagents 3. Low sample DNA/ 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.
- "Mutation Detected" or "Variant Detected" indicates that the signal for at least one mutation/ variant site is greater than the cutoff value of the corresponding target.

15. ANALYTICAL PERFORMANCE OF DNA MUTATIONS

Limit of Blank (LoB)

The limit of blank (LoB) values were determined by 2 operators performing four replicates of 37 wild-type FFPE specimens across three days on 2 reagent lots and 2 sets of instruments. Origins of the FFPE specimens include biobanks from France, US, and Taiwan.

The cut-off values for each mutation site were then determined by calculating up to 120% of the measured maximum fluorescent signal intensity values from the LoB study. Only "No Mutation and variant Detected" results were observed in these wild type samples.

Limit of Detection (LoD)

The limit of detection (LoD) was determined using a dilution series (ranging from 0.10 – 2.5 %) containing different levels of mutant DNA derived from mutant plasmid. All testing samples were blended in a background of wild type FFPE DNA. Each dilution was tested with 21 replicates across 3 days per reagent lot across three operators and two reagent lots. The LoDs were determined based on a positive hit rate at 95% in PriProbit analysis (Table 4). The LoDs for the DNA set ranged from 0.10~2.11%.

Table 4. Limit of Detection (LoD) of DNA Mutation

DNA Set		
Amino Acid Change	**Nucleotide Change**	**LoD (% Mutation)**
p.G12A	c.35G>C	0.69%
p.G12D	c.35G>A	1.26%
p.G12V	c.35G>T	0.76%
p.G12C	c.34G>T	0.45%
p.G12R	c.34G>C	1.13%
p.G12S	c.34G>A	0.10%
p.G13D	c.38G>A	0.10%
p.G13C	c.37G>T	1.36%
p.Q61K	c.181C>A	1.01%
p.Q61R	c.182A>G	1.90%
p.Q61L	c.182A>T	0.28%
p.Q61H	c.183A>C	0.38%
p.Q61H	c.183A>T	0.50%
p.Q61H	c.183A>T	1.85%
p.Q61L	c.182A>T	0.89%
p.Q61K	c.181C>A	1.00%
p.Q61R	c.182A>G	0.47%
p.E542K	c.1624G>A	2.11%
p.E545K	c.1633G>A	1.12%
p.E545Q	c.1633G>C	0.66%
p.H1047R	c.3140A>G	0.72%
p.H1047L	c.3140A>T	0.65%
p.V600E1	c.1799T>A	0.10%
p.G719S	c.2155G>A	0.83%
p.G719A	c.2156G>C	0.62%
p.G719C (G>T)	c.2155G>T	0.77%
p.E746_A750del	c.2235_2249del15	0.76%
p.E746_A750del	c.2236_2250del15	0.59%
p.L747_P753>S	c.2237_2255>T	0.28%
p.L747_A750>P	c.2240_2257del18	0.24%
p.E746_S752>V	c.2239_2248TTAAGAGAAG>C	0.12%
p.L747_T751del	c.2240_2254del15	1.27%
p.L747_S752del	c.2239_2256del18	0.40%
p.T790M	c.2369C>T	1.05%
p.L858R	c.2573T>G	1.26%
p.L861Q	c.2582T>A	0.33%

Repeatability and Reproducibility

The repeatability and reproducibility of IntelliPlex Lung Cancer Panel- DNA set was evaluated across three reagent lots, two sites, four operators, four sets of instrument and six non-consecutive testing days. One operator performed two run per reagent lot per day for a total of 24 runs at one site. Repeatability of IntelliPlex Lung Cancer Panel- DNA set was demonstrated with wild-type FFPE samples, low level mutant (2x LoD) and high level mutant (6x LoD). The correct call of the all testing level was at least 95.8% (46/48) across all variance combined (i.e., site/instrument, operator, and day). Across all variance components (i.e., site/instrument, operator, and day), the overall coefficient of variation is smaller than 5% across all panel members.

Table 5. Accuracy (%) of Each Mutation at

Gene	Amino Acid Change	Mutation (%)	Mutation Not Detected/ Detected	Accuracy (%)	Overall Coefficient
KRAS	p.G12A	4.15	0/48	100	0.00%
		1.38	0/48	100	0.00%
	p.G12D	7.54	1/47	97.9	3.03%
		2.51	0/48	100	0.00%
	p.G12V	4.58	0/48	100	0.00%
		1.53	0/48	100	0.00%
	p.G12C	2.69	0/48	100	0.00%
		0.9	0/48	100	0.00%
	p.G12R	6.79	0/48	100	0.00%
		2.26	1/47	97.9	3.03%
	p.G12S	0.6	0/48	100	0.00%
		0.2	0/48	100	0.00%
	p.G13D	0.6	0/48	100	0.00%
		0.2	0/48	100	0.00%

Gene	Amino Acid Change	Mutation (%)	Mutation Not Detected/ Detected	Accuracy (%)	Overall Coefficient
	p.G13C	8.17	0/48	100	0.00%
		2.72	0/48	100	0.00%
	p.Q61K	6.09	0/48	100	0.00%
		2.03	0/48	100	0.00%
	p.Q61R	11.4	0/48	100	0.00%
		3.8	0/48	100	0.00%
	p.Q61L	1.69	0/48	100	0.00%
		0.56	0/48	100	0.00%
	p.Q61H(A>C)	2.3	0/48	100	0.00%
		0.77	0/48	100	0.00%
	p.Q61H(A>T)	3	0/48	100	0.00%
		1	0/48	100	0.00%
NRAS	p.Q61H(A>T)	11.1	0/48	100	0.00%
		3.7	0/48	100	0.00%
	p.Q61L	5.33	0/48	100	0.00%
		1.78	0/48	100	0.00%
	p.Q61K	6	0/48	100	0.00%
		2	0/48	100	0.00%
	p.Q61R	2.84	0/48	100	0.00%
		0.95	0/48	100	0.00%
PIK3CA	p.E542K	12.64	0/48	100	0.00%
		4.21	0/48	100	0.00%
	p.E545K	6.72	0/48	100	0.00%
		2.24	2/46	95.8	0.00%
	p.E545Q	3.98	0/48	100	0.00%
		1.33	1/47	97.9	3.03%
	p.H1047R	4.3	0/48	100	0.00%
		1.43	1/47	97.9	3.03%
	p.H1047L	3.91	0/48	100	0.00%
		1.3	0/48	100	0.00%
BRAF	p.V600E1	0.6	0/48	100	0.00%
		0.2	0/48	100	0.00%

Gene	Amino Acid Change	Mutation (%)	Mutation Not Detected/ Detected	Accuracy (%)	Overall Coefficient
EGFR	p.G719S	5.01	0/48	100	0.00%
		1.67	0/48	100	0.00%
	p.G719A	3.73	0/48	100	0.00%
		1.24	0/48	100	0.00%
	p.G719C	4.61	0/48	100	0.00%
		1.54	0/48	100	0.00%
	p.E746_A750del (6223)	4.56	0/48	100	0.00%
		1.52	0/48	100	0.00%
	p.E746_A750del (6225)	3.55	0/48	100	0.00%
		1.18	0/48	100	0.00%
	p.L747_P753>S	1.66	0/48	100	0.00%
		0.55	0/48	100	0.00%
	p.L747_A750>P	1.43	0/48	100	0.00%
		0.48	0/48	100	0.00%
	p.E746_S752>V	0.72	0/48	100	0.00%
		0.24	0/48	100	0.00%
	p.L747_T751del	7.59	0/48	100	0.00%
		2.53	0/48	100	0.00%
	p.L747_S752del	2.42	0/48	100	0.00%
		0.81	0/48	100	0.00%
	p.T790M	6.29	0/48	100	0.00%
		2.1	0/48	100	0.00%
	p.L858R	7.55	0/48	100	0.00%
		2.52	0/48	100	0.00%
	p.L861Q	1.99	0/48	100	0.00%
		0.66	0/48	100	0.00%
WT		-	192/0	100	0.00%

Cross-Contamination

This test is designed to assess cross-contamination during the washing experiment, which may lead to false positive results. Wild-type and BRAF V600E1 mutation samples were arranged in alternating order during PCR reaction and sample hybridization to test for carryover of mutation 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 QIAamp DNA FFPE Tissue Kit. EGFR L858R was selected as a representative mutation. Triplicate testing of EGFR L858R mutation DNA blended samples along 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 Interference Residual Volume (µl / 20µl DNA)
Xylene	$4*10^{-5}$
Ethanol	$2.7*10^{-4}$
Buffer ATL	$1.08*10^{-4}$
Proteinase K	$2.64*10^{-6}$
Buffer AL	$2.66*10^{-4}$
Wash Buffer AW1	0.1
Wash Buffer AW2	1

16. ANALYTICAL PERFORMANCE OF RNA VARIANTS

Limit of Blank (LoB)

The limit of blank (LoB) values were determined by performing eight replicates of 47 wild-type FFPE specimens on 2 reagent lots and one set of instruments. Origins of the FFPE specimens include biobanks from France, US, and Taiwan. The cutoff values of each targeted variants were determined by the measured maximum analytical signal intensity values, respectively. Only “No Mutation and variant Detected” results were observed in these wild type samples.

Limit of Detection (LoD)

The limit of detection (LoD) was determined using a dilution series (ranging from 5-200 copies) containing different copies of variant RNA. All testing samples were blended in a background of HEK293 cell line RNA. Each dilution was tested with 21 replicates across 3 days per reagent lot across three operators and two reagent lots. The LoDs were determined based on a positive hit rate at 95% in PriProbit analysis (Table 7). The LoDs for the RNA set ranged from 10 to 89 copies.

Table 7. Limit of Detection (LoD) of RNA Variant

RNA Set		
Target	**Inferred Breakpoint**	**LoD (copies)**
ALK V1	E13;A20	10
ALK V2	E20;A20	10
ALK V3a	E6a;A20	10
ALK V3b	E6b;A20	10
ALK V4	E14;A20	10
ALK V5a	E2a;A20	10
ALK V5b	E2b;A20	10
ALK V"5"	E18;A20	10
SLC34A2-ROS1(SL4R32)	S4;R32	50
SLC34A2-ROS1(SL4R34)	S4;R34	10
CD74-ROS1(C6R32)	C6;R32	89
CD74-ROS1(C6R34)	C6;R34	14
CCDC6-RET	C1;R12	10
KIF5B-RET(K15R11)	K15;R11	10
KIF5B-RET(K15R12)	K15;R12	10
TRIM33-RET	T14;R12	10
CD74-NTRK1	C8;N12	46
MPRIP-NTRK1	M21;N14	10
MET Exon 14 Skipping	-	40

Repeatability and Reproducibility

The repeatability and reproducibility of IntelliPlex Lung Cancer Panel- RNA set was evaluated across three reagent lots, two sites, four operators, two sets of instrument and five non-consecutive testing days. One operator performed two run per reagent lot per day for a total of 20 runs at one site. Repeatability of IntelliPlex Lung Cancer Panel- RNA set was demonstrated with HEK293 cell line RNA, low variant level (2x LoD) and high variant level (6x LoD). The correct call of the all testing level was at least 95% (38/40) across all variance combined (i.e., site/instrument, operator, and day). Across all variance components (i.e., site/instrument, operator, and day), the overall coefficient of variation is smaller than 10% across all panel members.

Table 8. Accuracy of Each RNA Variant

Variant	Variant Level (Copies)	Variant Not Detected/ Detected	Accuracy (%)	Overall Coefficient
EML4-ALK V1	60	0/40	100	0.00%
	20	0/40	100	0.00%
EML4-ALK V2	60	0/40	100	0.00%
	20	0/40	100	0.00%
EML4-ALK V3a	60	0/40	100	0.00%
	20	1/39	97.5	2.56%
EML4-ALK V3b	60	0/40	100	0.00%
	20	0/40	100	0.00%
EML4-ALK V4	60	0/40	100	0.00%
	20	2/38	95	5.26%
EML4-ALK V5a	60	0/40	100	0.00%
	20	0/40	100	0.00%
EML4-ALK V5b	60	0/40	100	0.00%
	20	0/40	100	0.00%
EML4-ALK V"5"	60	0/40	100	0.00%
	20	0/40	100	0.00%
CD74-ROS1(C6R32)	534	0/40	100	0.00%
	178	0/40	100	0.00%
CD74-ROS1(C6R34)	84	0/40	100	0.00%
	28	1/39	97.5	2.56%
SLC34A2-ROS1(SL4R32)	300	1/39	97.5	2.56%
	100	0/40	100	0.00%
SLC34A2-ROS1(SL4R34)	60	0/40	100	0.00%
	20	0/40	100	0.00%
KIF5B-RET(K15R11)	60	0/40	100	0.00%
	20	0/40	100	0.00%
KIF5B-RET(K15R12)	60	0/40	100	0.00%
	20	0/40	100	0.00%
TRIM33-RET	60	0/40	100	0.00%
	20	0/40	100	0.00%
CCDC6-RET	60	0/40	100	0.00%
	20	0/40	100	0.00%
CD74-NTRK1	276	0/40	100	0.00%
	92	0/40	100	0.00%
MPRIP-NTRK1	60	0/40	100	0.00%
	20	1/39	97.5	2.56%
MET Exon 14 Skipping	240	1/39	97.5	2.56%
	80	2/38	95	5.26%
WT	-	40/0	100	0.00%

Cross-Contamination

This test is designed to assess cross-contamination during the washing steps, which may lead to false positive results. Wild-type and EML4-ALK V3a 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 ReliaPrep™ FFPE Total RNA Miniprep System. CD74-NTRK1 (C8N12) was selected as a representative variant. Triplicate testing of CD74-NTRK1 (C8N12) variant and wild type blend cell line (HEK293) RNA extract samples with each potential interfering substance (as listed in Table 9), added before the PCR step, showed no interference on kit performance.

Table 9. Interfering Substances Tested

Interfering Substance	Assumed Interfering Residual Volume (% 30µl RNA)
isopropanol	0.5%
Lysis Buffer (LBA)	0.5%
BL Buffer	0.5%
Wash Solution	0.5%

17. 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. DNase/ RNase contamination.	2. Ensure all operating procedures are followed correctly. Ensure work environment is free of DNase/ 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.
πCode MicroDiscs Count Fail	DeXipher is unable to detect sufficient πCode MicroDiscs numbers for mutation/ variant 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. DNase/ RNase contamination.	3. Ensure all the operating procedures are followed correctly. Ensure work environment is free of DNase/ 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 DNA/ RNA.	1. Ensure human-derived DNA/ RNA samples are added. Do not use artificial DNA/ 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 quality. 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.

18. SYMBOLS

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

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