IntelliPlex™ EGFR Mutation Kit User Manual

REF **82006 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 EGFR Mutation Kit, based on πCode™ technology and PlexBio's instrument platform, is an in-vitro molecular assay intended for qualitative identification of 40 nucleotide changes in exons 12, 18, 19, 20 and 21 of the *EGFR* gene using DNA samples derived from formalin-fixed paraffin-embedded (FFPE) of non-small cell lung cancer (NSCLC) tissue. Results are intended to assist clinician in identifying NSCLC patients who may benefit from treatment with receptor tyrosine kinase inhibitors like Erlotinib.

2. INTRODUCTION

Epidermal growth factor receptor (EGFR) is a cell surface receptor tyrosine kinase involved in the intracellular signaling cascades including the PI3K-AKT-mTOR and RAS-RAF-MEK-ERK pathways. Many types of cancers are associated with mutations in the EGFR gene. Mutations are often found in exons 18, 19, 20 and 21, which encode several regions of the kinase domain. Some mutations cause gene amplification, while others cause increased kinase activity. In any case, these mutations upregulate EGFR activity, leading to enhanced cell survival and proliferation.
Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) such as Erlotinib (orally active) has commonly been used as a therapeutic option in patients with non-small-cell lung cancer (NSCLC). Efficacy of Erlotinib has been demonstrated in NSCLC patients, in particular those harboring activating mutations of the EGFR gene. Some mutations in EGFR are associated with increased response to EGFR-TKI's, while other mutations are associated with resistance to certain treatments. Assessment of EGFR mutation status is therefore crucial for the treatment evaluation of patients with NSCLC. SelectAmp and πCode technology enables the multiplex, single-well detection of single nucleotide mutations of the EGFR gene from specimens containing large amounts of wild-type genomic DNA with significantly reduced sample requirement compared to conventional methods. The IntelliPlex EGFR Mutation Kit identifies 40 nucleotide changes in exons 12, 18, 19, 20 and 21 of the EGFR gene (Table 1).

Table 1. Mutations Detected

Gene	Exon	Amino Acid Change	Nucleotide Change	COSMIC ID
EGFR	Exon 12	p.S492R	c.1476C>A	236670
	Exon 18	p.G719S	c.2155G>A	6252
		p.G719C	c.2155G>T	6253
		p.G719A	c.2156G>C	6239
	Exon 19	p.L747_T751>S	c.2240_2251del12	6210
		p.L747_E749delLRE	c.2239_2247delTTAAGAGAA	6218
		p.E746_S752>D	c.2238_2255del18	6220
		p.E746_A750delELREA	c.2235_2249del15	6223
		p.E746_A750delELREA	c.2236_2250del15	6225
		p.L747_T751delLREAT	c.2239_2253del15	6254
		p.L747_S752delLREATS	c.2239_2256del18	6255
		p.E746_S752>A	c.2237_2254del18	12367
		p.L747_T751delLREAT	c.2240_2254del15	12369
		p.L747_P753>S	c.2240_2257del18	12370
		p.L747_A750>P	c.2239_2248TTAAGAGAAG>C	12382
		p.L747_T751>P	c.2239_2251>C	12383
		p.E746_S752>V	c.2237_2255>T	12384
		p.E746_S752>I	c.2235_2255>AAT	12385
		p.E746_T751>V	c.2237_2252>T	12386
		p.L747_P753>Q	c.2239_2258>CA	12387
		p.L747_S752>Q	c.2239_2256>CAA	12403
		p.E746_T751>VA	c.2237_2253>TTGCT	12416
		p.L747_T751>Q	c.2238_2252>GCA	12419
		p.L747_A750>P	c.2238_2248>GC	12422
		p.E746_T751>A	c.2237_2251del15	12678
		p.E746_T751delELREAT	c.2236_2253del18	12728
		p.E746_A750>IP	c.2235_2248>AATTC	13550
		p.E746_T751>I	c.2235_2252>AAT	13551
		p.E746_T751>IP	c.2235_2251>AATTC	13552
		p.E746_P753>VS	c.2237_2257>TCT	18427
		p.L747_T751delLREAT	c.2238_2252del15	23571
		p.K745_E749del	c.2233_2247del15	26038
	Exon 20	p.T790M	c.2369C>T	6240
		p.S768I	c.2303G>T	6241
		p.V769_D770insASV	c.2307_2308insGCCAGCGTG	12376
		p.H773_V774insH	c.2319_2320insCAC	12377
		p.D770_N771insG	c.2310_2311insGGT	12378
	Exon 21	p.L861Q	c.2582T>A	6213
		p.L858R	c.2573T>G	6224
		p.L858R	c.2573_2574TG>GT	12429

3. TECHNOLOGICAL PRINCIPLES

The **IntelliPlex EGFR Mutation Kit** utilizes two technologies, SelectAmp and πCode, 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 are manufactured to generate up to 16,000 distinct circular image patterns for multiplexing applications. Each πCode MicroDisc has a distinct circular image pattern, which corresponds to a specific capture agent conjugated to the surface of the disc. πCode tagged with different capture agents are pooled, enabling specific detection of multiple analytes in a one-well reaction.

Detection Principle

The test is based on five processes:

1. DNA extraction from specimens
2. Mutation-specific multiplex 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.
- 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.
- 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.
- Safety Data Sheets (SDS) are available upon request from PlexBio Customer Service.

5. QUALITY CONTROL

The IntelliPlex EGFR Mutation Kit contains a series of internal control πCode MicroDiscs that monitor the PCR amplification, SA-PE incubation procedure and background noise. Those controls must meet the specification in each test well with intensities above the cutoffs from the same run, or the run will be considered failed. The external controls (positive control and negative control) monitor the whole testing procedure to prevent false positive and false negative results. The test is considered invalid if any of the controls fail.

6. KIT COMPONENTS

The **IntelliPlex EGFR Mutation Kit** contains sufficient reagents for up to 24 tests. The kit components include:

1. **EGFR KIT Reaction Mix**
   Ref. No.: 20080
   Quantity & Volume: 1 vial, 264 µL/vial
   Description: For PCR amplification
   Contents: MyFi 5X Reaction Buffer, MyFi DNA polymerase (Microbial), buffered solution containing $MgSO_4$ and dNTPs
2. **EGFR KIT Primer Mix**
   Ref. No.: 20081
   Quantity & Volume: 1 vial, 120 µL/vial
   Description: For PCR amplification
   Contents: ~4 µM Primer (including biotin-labeled primers)
3. **EGFR KIT POS Control**
   Ref. No.: 20082
   Quantity & Volume: 1 vial, 20 µL/vial
   Description: Assay positive control
   Contents: EGFR L858R plasmid DNA (Microbial), Tris-EDTA Buffer
4. **NEG Control**
   Ref. No.: 20642
   Quantity & Volume: 1 vial, 2 mL/vial
   Description: Assay negative control
   Contents: Nuclease-free water
5. **EGFR KIT Assay Plate**
   Ref. No.: 20084
   a. **πCode MicroDisc**
      Position& Volume: Column 1-3 (A1-H3); air-dried
      Description: Control πCode and πCode conjugated with DNA probes for detection
      Contents: πCode MicroDisc, Glycerol, Phosphate buffered saline, 0.1% Albumin- from bovine (Biological), <0.1% EDTA and <0.1% Sodium azide, Sucrose
   b. **Hy Buffer**
      Position& Volume: Column 5-7 (A5-H7); 100 µL/well
      Description: For hybridization
      Contents: Saline-Sodium Phosphate-EDTA
   c. **SA-PE Solution**
      Position& Volume: Column 9-11 (A9-H11); 175 µL/well
      Description: Streptavidin-phycoerythrin for fluorescent signal acquisition
      Contents: Phosphate buffered saline, 0.5% Streptavidin-phycoerythrin, 1% Albumin- from bovine (Biological), <0.1% Sodium azide

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

7. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

Required products for compatibility with IntelliPlex kits:

- 96-well plate (PlexBio; Cat. No. 80025 or Greiner Bio-one; Cat. No. 655101)
- IntelliPlex 1000™ πCode Processor (PlexBio; Cat. No. 80033)
- PlexBio 100 Fluorescent Analyzer (PlexBio; Cat. No. 80000)
- U Tray (PlexBio; Cat. No. 80023)
- V Tray (PlexBio; Cat. No. 80024)
- DeXipher™ MD (Required: PlexBio; Cat. No. 80051)
- IntelliPrep TRIO 240 (PlexBio; Cat. No. 80042)
- IntelliPrep TRIO FFPE DNA Nucleic Acid Extraction Kit (PlexBio; Cat. No. 83015)
- (Optional) Qubit™ Fluorometer with dedicated quantitative reagents (Invitrogen; any models) or equivalent
- (Optional) FFPE DNA extraction kit (Recommended: QIAamp DNA FFPE Tissue Kit, Qiagen; Cat. No. 56404 or equivalent)
- Clean tubes for PCR reaction (Gunster; Cat. No. MB-P08A or equivalent)
- 10X Assay Wash Buffer (PlexBio; Cat. No. 80220)
- Deionized water for dilution of 10X Wash Buffer
- Dedicated micropipette
- Filter tips for micropipette
- Vortex mixer
- Micro-centrifuge
- Thermocycler (Recommended: MiniAmp™ Thermal Cycler, Applied Biosystems™; Cat. No. A37834 or equivalent)
- Industrial Computer (Recommended: PlexBio; Cat. No. 80002)

8. 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).

9. INSTRUMENT AND SOFTWARE

Instrument

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

Software Installation

The EGFR Mutation kit has a designated Kit App and ENC file. The Kit App contains the πCode target assignments and the ENC file includes the lot number and expiration date. Please make sure you have the Kit App installed and the ENC file imported into DeXipher before your first assay run.

Kit App Installation

1. Visit www.plexbio.com and download the **EGFR Mutation 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. Visit www.plexbio.com and download the **EGFR Mutation 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.

10. SPECIMENS

Specimen Collection

The **IntelliPlex EGFR Mutation Kit** has been validated to be used for formalin-fixed paraffin embedded tissues (FFPET) of NSCLC.

NOTE:

- FFPET specimens may be stored ≤ 30°C for up to 12 months after the date of tissue collection and processing. The optimal tissue fixation time for test should be less than 72 hr.
- Only FFPET sections of 10-µm thickness containing at least 10% tumor content are to be used in the EGFR Mutation 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.

11. BEFORE YOU START

Samples are recommended to purify by using IntelliPrep TRIO 240 system or QIAamp DNA FFPE Tissue Kit. Please follow the operation procedures carefully according to the manufacturer's instructions. Refer to the user manuals of instruments and extraction kits for more operation details.

For using **IntelliPrep TRIO 240**:

Please follow the **Section 12.1a** for operating extraction, quantitation and PCR preset automatically on IntelliPrep TRIO 240. The specimen volume needed for IntelliPrep TRIO 240 purification processing is 400 µL, and eluted in 100 µL elution buffer. Note that the extracted DNA concentration should be in between 2.5~ 50 ng/µL to ensure optimal assay performance.

For using **QIAamp DNA FFPE Tissue Kit**:

Please follow the **Section 12.1b** for operating extraction, quantitation, and PCR preparation manually. Check that you have 4 µL of extracted DNA (≥ 2.5 ng/µL) ready for analysis.

12. ASSAY PROCEDURE

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

12.1a (Auto) DNA Extraction, Quantitation and PCR Preparation on IntelliPrep TRIO 240

1. Follow IntelliPrep TRIO 240 and IntelliPrep TRIO FFPE Nucleic Acid Extraction Kit 's operating instructions carefully. The DNA extraction, quantitation and PCR preset are done automatically on IntelliPrep TRIO 240.

2. Once all processes complete, transfer the PCR preset plate for following procedures (Section 12.2) and collect the Elution Tubes for purified nucleic acid to store the elutes properly.

12.1b (Manual) DNA Extraction, Quantitation and PCR Preparation

1. Follow QIAamp DNA FFPE Tissue Kit's operating instructions for DNA extraction. The DNA stock concentration should be ≥ 2.5 ng/µL to ensure optimal assay performance.
2. Quantify the extracted DNA using Qubit Fluorometer with dedicated quantitative reagents (or equivalent) according to the manufacturer's protocol.
3. Each PCR reaction uses 4 µL of a 2.5 ng/µL DNA working stock (10 ng DNA input). Please prepare working stock for all samples before preparing PCR reactions. DNA input amounts lower or higher than 10 ng per reaction are not recommended.
4. Vortex to mix each sample before use.
5. Spin down and keep samples on ice.
6. Prepare the PCR Reaction:

For each PCR reaction:

EGFR Reaction Mix	11 µL
EGFR Primer Mix	5 µL
Sample/POS Control/NEG Control	4 µL
Total volume	20 µL

NOTE:

- The amount of Reaction Mix and Primer Mix 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.

12.2 PCR Amplification Conditions

1. Place the PCR tubes/ PCR plate on 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: 3°C/sec (ABI MiniAmp™; Cat. No. A37834).*

12.3 DNA Hybridization and SA-PE Reaction

1. **Prepare 1X Wash Buffer:** Transfer 100mL of the 10X Assay Wash Buffer (PlexBio; Ref: 80220) to the IntelliPlex 1000 πCode Processor 1L Wash Buffer bottle and add 900 ml deionized water. Mix by swirling.

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

IntelliPlex 1000 πCode 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. **Resuspend πCode MicroDisc** – Take out the **EGFR KIT Assay Plate.** Use filter tips to pierce the aluminum membrane carefully and **dispense 100 µL of Hy Buffer (Hy Buffer; Column 5-7)** to each πCode wells **(πCode; Column 1-3)** directly. Pipetting mixing and place the plate for 15 minutes to dissolved πCode completely.
3. Pipetting mixing each well for at least three times to ensure homogeneous suspension of πCode MicroDisc and transfer all dissolved πCode to a new 96-well plate.
4. Spin down the PCR products.
5. **Denature the PCR products** on the thermocycler by heating at 95˚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:

   - 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.
   - 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. Pierce the membrane of **EGFR KIT Assay Plate (SA-PE Solution; Column 9-11)** to **pipet 175 µL for each well of SA-PE solution** into the V Tray in SA-PE tank. Please note that the dead volume of the V Tray is **500 µL** and the minimum usage of SA-PE is **one lane (900 µL)**.

   NOTE:
   Required SA-PE Solution by Lane(s):

Number of Processed Lane(s)	Required SA-PE Solution (µL)	Number of SA-PE Lane(s) Required
1	900	1
2	1300	2
3	1700	3

   - 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 user 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.
- Additional 10X Assay Wash Buffer can be ordered from PlexBio (PlexBio; Ref: 80220).

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 2 to 96 tests (including POS and NEG controls) per 96 well Microwell plate. When running more than 24 specimens, multiple IntelliPlex EGFR Mutation Kits of the same lot will be required.
- The procedure described above must be followed to detect ≥ 0.5~2.9% mutant sequences in a background of wild-type DNA for the EGFR mutations in Table 1.

13. DISCLAIMERS

Negative Test Result

A negative test result means that the targeted mutation 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 mutation 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.

14. INTERPRETATION OF RESULTS

Test Result	Reported Result	Interpretation
Mutation Detected	Ex. T790M (Refer to Table 1 for details)	Targeted mutation detected
Mutation Not Detected	None	Targeted mutation not detected
Invalid Assay	Invalid	**Possible Causes:** 1. PCR Inhibition (presence of inhibitor in the sample) 2. Improper stored reagents 3. Low sample DNA input or quality 4. Low πCode Disc Count (the πCode tube was not 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 runs and specimen validation were performed by the dedicated KIT APP along with IntelliPlex 1000 πCode Processor and PlexBio 100 Fluorescent Analyzer.
- In case of heterogeneity of samples or multiple mutations, only the dominantly detected mutation is reported. “Mutation Detected” indicates that the signal for at least one mutation site is greater than the cutoff value of the corresponding target. When multiple mutations are detected in a sample, only the one that exhibits the highest signal is reported.

15. ANALYTICAL PERFORMANCE

Limit of Blank (LoB)

The cutoff value of each targeted mutation was determined based on limit of blank (LoB) testing that performed on 66 tissue FFPE which were obtained from NSCLC patients from biobank of France or US and IRB approval of hospitals. The EGFR mutation status of each specimens were confirmed by commercial kit before testing. Each specimen was extracted following the procedure according to user manual of extraction kit and tested in duplicates by two reagent lots. The cutoff values of each targeted mutations were then determined by 1.4-folds of observed maximum analytical signal intensity values. Only “No Mutation Detected” results were observed in these wild type samples.

Limit of Detection (LoD)

The limit of detection (LoD) values were determined by three operators each using two reagent lots. The limit of detection (LoD) was determined using a dilution series (ranging from 0.5-10%) containing different levels of mutant DNA (either from cell lines or mutant plasmid) blended in a background of wild type cell line (K562) DNA. Each mutation level was tested with seven replicates across three days per reagent lot. The LoDs were determined based on a positive hit at 95% in PriProbit analysis (Table 3). LoDs ranged from 0.5~2.9%.

Table 3. Limit of Detection (LoD)

Amino Acid Change	Nucleotide Change	LoD (% Mutation)
p.S492R	c.1476C>A	0.5
p.G719S	c.2155G>A	1.0
p.G719C	c.2155G>T	0.58
p.G719A	c.2156G>C	2.3
p.L747_T751>S	c.2240_2251del12	0.5
p.L747_E749delLRE	c.2239_2247delTTAAGAGAA	1.6
p.E746_S752>D	c.2238_2255del18	0.57
p.E746_A750delELREA	c.2235_2249del15	0.5
p.E746_A750delELREA	c.2236_2250del15	0.53
p.L747_T751delLREAT	c.2239_2253del15	0.5
p.L747_S752delLREATS	c.2239_2256del18	0.5
p.E746_S752>A	c.2237_2254del18	0.5
p.L747_T751delLREAT	c.2240_2254del15	0.5
p.L747_P753>S	c.2240_2257del18	0.5
p.L747_A750>P	c.2239_2248TTAAGAGAAG>C	0.5
p.L747_T751>P	c.2239_2251>C	0.5
p.E746_S752>V	c.2237_2255>T	0.5
p.E746_S752>I	c.2235_2255>AAT	0.5
p.E746_T751>V	c.2237_2252>T	0.5
p.L747_P753>Q	c.2239_2258>CA	0.5
p.L747_S752>Q	c.2239_2256>CAA	0.5
p.E746_T751>VA	c.2237_2253>TTGCT	0.5
p.L747_T751>Q	c.2238_2252>GCA	1.0
p.L747_A750>P	c.2238_2248>GC	0.5
p.E746_T751>A	c.2237_2251del15	0.5

Amino Acid Change	Nucleotide Change	LoD (% Mutation)
p.E746_T751delELREAT	c.2236_2253del18	0.5
p.E746_A750>IP	c.2235_2248>AATTC	0.5
p.E746_T751>I	c.2235_2252>AAT	1.5
p.E746_T751>IP	c.2235_2251>AATTC	0.53
p.E746_P753>VS	c.2237_2257>TCT	0.5
p.L747_T751delLREAT	c.2238_2252del15	0.5
p.K745_E749del	c.2233_2247del15	0.5
p.T790M	c.2369C>T	0.8
p.S768I	c.2303G>T	2.9
p.V769_D770insASV	c.2307_2308insGCCAGCGTG	1.4
p.H773_V774insH	c.2319_2320insCAC	2.0
p.D770_N771insG	c.2310_2311insGGT	2.4
p.L861Q	c.2582T>A	0.5
p.L858R	c.2573T>G	2.5
p.L858R	c.2573_2574TG>GT	1.1

Repeatability and Reproducibility

The repeatability and reproducibility of each mutation in IntelliPlex EGFR Mutation Kit was evaluated across two reagent lots, two operators, two sets of instruments and five non-consecutive testing days. Four replicate runs were performed per reagent lot per day for a total of 40 valid runs at one site. Repeatability of IntelliPlex EGFR Mutation Kit was demonstrated with low-level mutant (2x LoD) and high-level mutant (6x LoD). The accuracy was at least 92.5% (37/40) across all variances combined (i.e., mutation level, site/instrument, operator, and day).

Table 4. Accuracy

Amino Acid Change	Mutation (%)	Mutation Not Detected/ Detected	Accuracy (%)
p.S492R	1.00	0/40	100
	3.00	0/40	100
p.G719S	1.14	1/39	97.5
	3.42	0/40	100
p.G719C	1.16	1/39	97.5
	3.48	0/40	100
p.G719A	4.60	0/40	100
	13.8	0/40	100
p.L747_T751>S	1.00	0/40	100
	3.00	0/40	100
p.L747_E749delLRE	3.2	0/40	100
	9.6	0/40	100
p.E746_S752>D	1.14	1/39	97.5
	3.42	0/40	100
p.E746_A750delELREA	1.00	1/39	97.5
	3.00	0/40	100
p.E746_A750delELREA	1.06	1/39	97.5
	3.18	1/39	97.5
p.L747_T751delLREAT	1.00	1/39	97.5

Amino Acid Change	Mutation (%)	Mutation Not Detected/ Detected	Accuracy (%)
	3.00	0/40	100
p.L747_S752delLREATS	1.00	0/40	100
	3.00	0/40	100
p.E746_S752>A	1.00	0/40	100
	3.00	0/40	100
p.L747_P753>S	1.00	1/39	97.5
	3.00	0/40	100
p.L747_A750>P	1.00	0/40	100
	3.00	0/40	100
p.L747_T751>P	1.00	0/40	100
	3.00	0/40	100
p.E746_S752>V	1.00	0/40	100
	3.00	0/40	100
p.E746_S752>I	1.00	0/40	100
	3.00	0/40	100
p.E746_T751>V	1.00	0/40	100
	3.00	0/40	100
p.L747_P753>Q	1.00	1/39	97.5
	3.00	0/40	100
p.L747_S752>Q	1.00	0/40	100
	3.00	0/40	100
p.E746_T751>VA	1.00	0/40	100
	3.00	0/40	100
p.L747_T751>Q	1.00	0/40	100
	3.00	0/40	100
p.L747_A750>P	1.00	1/39	97.5
	3.00	0/40	100
p.E746_T751>A	1.00	0/40	100
	3.00	0/40	100
p.E746_T751delELREAT	1.00	1/39	97.5
	3.00	0/40	100
p.E746_A750>IP	1.00	0/40	100
	3.00	0/40	100
p.E746_T751>I	4.50	3/37	92.5
	9.00	0/40	100
p.E746_T751>IP	1.06	0/40	100
	3.18	0/40	100
p.E746_P753>VS	1.00	1/39	97.5
	3.00	0/40	100
p.K745_E749del	1.00	0/40	100
	3.00	0/40	100
p.T790M	1.60	0/40	100
	4.80	0/40	100
p.S768I	5.80	1/39	97.5
	17.4	0/40	100
p.V769_D770insASV	2.80	1/39	97.5
	8.40	0/40	100
p.H773_V774insH	4.00	2/38	95

Amino Acid Change	Mutation (%)	Mutation Not Detected/ Detected	Accuracy (%)
	12.00	1/39	97.5
p.D770_N771insG	4.80	3/37	92.5
	14.40	0/40	100
p.L861Q	1.00	0/40	100
	3.00	0/40	100
p.L858R	5.00	0/40	100
	15.00	0/40	100
p.L858R	2.20	0/40	100
	6.60	0/40	100

Cross-reactivity

Cross-reactivity was evaluated with EGFR homolog (HER2) plasmids to test for false positives. Two test groups included a DNA blend of 5% of EGFR mutant (COSM6224) in a background of 95% of HER2 DNA and 100% HER2 DNA. Each group was performed in duplicate per day across two days. No cross-reactivity was observed.

Cross-Contamination

The test is designed to assess cross-contamination during the assay operation steps, which may lead to false positives. One operator tested one reagent lot in replicate across three days. EGFR wild-type and L858R mutation FFPE 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

The test is designed to evaluate the impact of potential substances carried over from the QIAamp DNA FFPE Tissue Kit. EGFR mutant (COSM6224) was selected as a representative mutation. Triplicate testing of EGFR mutant samples (plasmid blend in wild type background) with each potential interfering substance (Table 5) added before the PCR step showed no interference on kit performance.

Table 5. Interfering Substances Tested

Interfering Substance	Assumed Interfering Residual Volume (µL / 20 µL 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

Method Comparison

The performance of IntelliPlex EGFR Mutation Kit was compared to Sanger sequencing, which is considered to be the gold standard. A total of 40 FFPE lung cancer specimens were analyzed; the results are summarized in Table 6. Concordance between IntelliPlex EGFR Mutation Kit and Sanger sequencing was 100% positive agreement (sensitivity) and 96.6% negative agreement (specificity). The overall agreement was 97.5%.

Table 6. Comparison of IntelliPlex EGFR Mutation Kit and Sanger Sequencing

		Sanger Sequencing	
		Mutation Detected	Mutation Not Detected
IntelliPlex EGFR Mutation Kit	Mutation Detected	11	1
	Mutation Not Detected	0	28
Positive agreement = 100% Negative agreement = 96.6% Overall agreement = 97.5%			

16. TROUBLESHOOTING

The troubleshooting listed below addresses possible problem causes and solutions provided during assay procedures.

Problem	Possible Cause	Recommendations
No Valid Assay Assigned	1. No plate inserted. 2. Plate inserted in wrong orientation. 3. No assay APP installed. 4. No ENC file imported. 5. Two or more lots of reagent used.	1. Confirm plate is inserted and repeat reading. 2. Confirm orientation of plate and repeat reading. 3. Install assay APP and repeat reading. 4. Import ENC file and repeat reading. 5. One reagent lot used at a time.
Positive Control Fail / Negative Control Fail	1. No POS Control or NEG Control added. 2. DNase contamination. 3. Assay did not work. 4. Cross contamination between samples. 5. Wrong PC/NC wells chose.	1. Ensure POS Control and NEG Control are added. 2. Ensure all operating procedures are followed correctly. Ensure work environment is free of DNase. 3. Make sure all the assay procedures are followed correctly. 4. Clean all surfaces and equipment. Operate pre-PCR and post-PCR in the dedicated area and separate the equipment for use. 5. Choose the correct PC/NC wells and repeat reading.
πCode MicroDiscs Count Fail	DeXipher is unable to detect sufficient πCode MicroDiscs numbers for mutation determination.	
	1. πCode MicroDiscs are not proper dispersed in the well. 2. Not enough πCode MicroDiscs added to well. 3. Microbes exist in buffers. 4. Instruments error or malfunction.	1. Re-disperse the microplate using IntelliPlex 1000 Processor, and repeat reading. 2. Ensure πCode MicroDiscs are well-mixed with proper amount added. 3. Use freshly prepared wash buffer and deionized water for hybridization to reduce πCode MicroDiscs loss rate. 4. Contact PlexBio Customer Service.

Problem	Possible Cause	Recommendations
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 deionized water) 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.
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 contamination.	3. Ensure all the operating procedures are followed correctly. Ensure work environment is free of DNase.
	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.	1. Ensure human-derived DNA samples are added. Do not use artificial DNA 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.

17. SYMBOLS

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

18. REFERENCES

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