IntelliPlex™ Lung Cancer Panel – cfDNA

REF **82030** **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 – cfDNA**, based on πCode™ technology and PlexBio's instrument platform, is an in vitro molecular assay intended for the qualitative identification of 55 DNA mutations in the KRAS, NRAS, PIK3CA, BRAF, EGFR, HER2, MEK1 and AKT1 genes using cell-free DNA derived from plasma of patients with non-small cell lung cancer (NSCLC). The kit is to be used by trained personnel in a professional laboratory environment and 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. Furthermore, copy number variations (CNVs) signatures have been developed to differentiate between NSCLC and SCLC, in particular for poorly differentiated NSCLC, to complement current pathology practices using small biopsies or cytology specimens. 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 – cfDNA** identifies 55 DNA mutations (Table 1).

Table 1. Mutations Detected

Gene	Exon	Amino Acid Change	Nucleotide Change	COSMIC ID
KRAS	2	p.G12S	c.34G>A (GGT>AGT)	517
		p.G12R	c.34G>C (GGT>CGT)	518
		p.G12C	c.34G>T (GGT>TGT)	516
		p.G12D	c.35G>A (GGT>GAT)	521
		p.G12A	c.35G>C (GGT>GCT)	522
		p.G12V	c.35G>T (GGT>GTT)	520
		p.G13D	c.38G>A (GGC>GAC)	532
		p.G13C	c.37G>T (GGC>TGC)	527
	3	p.Q61H	c.183A>C (CAA>CAC)	554
		p.Q61H	c.183A>T (CAA>CAT)	555
		p.Q61L	c.182A>T (CAA>CTA)	553
		p.Q61K	c.181C>A (CAA>AAA)	549
		p.Q61R	c.182A>G (CAA>CGA)	552
NRAS	3	p.Q61K	c.181C>A (CAA>AAA)	580

Gene	Exon	Amino Acid Change	Nucleotide Change	COSMIC ID
NRAS	3	p.Q61R	c.182A>G (CAA>CGA)	584
		p.Q61L	c.182A>T (CAA>CTA)	583
		p.Q61H	c.183A>C (CAA>CAC)	586
		p.Q61H	c.183A>T (CAA>CAT)	585
PIK3CA	9	p.E542K	c.1624G>A	760
		p.E545K	c.1633G>A	763
		p.E545Q	c.1633G>C	27133
	20	p.H1047R	c.3140A>G	775
		p.H1047L	c.3140A>T	775
BRAF	15	p.V600E1	c.1799T>A	476
EGFR	18	p.G719A	c.2156G>C	6239
		p.G719S	c.2155G>A	6252
		p.G719C	c.2155G>T	6253
	19	p.L747_S752del	c.2239_2256del18	6255
		p.L747_P753>S	c.2240_2257del18	12370
		p.E746_S752>V	c.2237_2255>T	12384
		p.L747_T751del	c.2239_2253del15	6254
		p.L747_T751del	c.2240_2254del15	12369
		p.L747_T751del	c.2238_2252del15	23571
		p.E746_A750del	c.2235_2249del15	6223
		p.E746_A750del	c.2236_2250del15	6225
		p.L747_A750>P	c.2239_2248TTAAGAGAAG>C	12382
		p.E746_T751>A	c.2237_2251del15	12678
		p.S752_I759delSPKANKEI	c.2253_2276del24	13556
		p.S752_I759delSPKANKEI	c.2254_2277del24	6256
	20	p.T790M	c.2369C>T	6240
EGFR	20	p.C797S	c.2389T>A	6493937
		p.C797S	c.2390G>C	5945664
		p.S768I	c.2303G>T	6241
		p.V769_D770insASV	c.2307_2308ins9GCCAGCGTG	12376
		p.H773_V774insH	c.2319_2320insCAC	12377
		p.D770_N771insG	c.2310_2311insGGT	12378
		p.D770_N771insSVD	2311_2312ins9GCGTGGACA	13428
		p.V769_D770insASV	2309_2310AC>CCAGCGTGGAT	13558
	21	p.L858R	c.2573T>G	6224
		p.L861Q	c.2582T>A	6213
AKT1	4	p.E17K	c.49G>A	33765
MEK1	2	p.Q56P	c.167A>C	1235481
		p.K57N	c.171G>T	1235478
HER2	20	p.Ala775_Gly776insYVMA	c.2324_2325ins12	20959

3. TECHNOLOGICAL PRINCIPLES

The IntelliPlex kits utilizes two technologies, SelectAmp and πCode, to achieve high sensitivity multiplex mutation detection.

SelectAmp

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 more than 85,000 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/ variants-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.
- 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.
- Material Safety Data Sheets (MSDS) are available upon request from PlexBio Customer Service.

5. KIT COMPONENTS

The **IntelliPlex Lung Cancer Panel – cfDNA** includes sufficient reagent for up to 24 tests. Kit components include:

1. **cLCP - DNA Reaction Mix**
   Ref. No.:20484
   Quantity & Volume: 1 vial, 240 µL/vial
   Description: For PCR amplification
   Contents: 80% 5X Reaction Buffer, Magnesium chloride, dNTPs and Enhancer, 10% DNA polymerase (Microbial)

2. **cLCP - DNA Primer Mix**
   Ref. No.: 20485
   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. **cLCP - DNA πCode MicroDisc**
   Ref. No.: 20486
   Quantity & Volume: 1 vial, 480 µL/vial
   Description: For PCR amplicon capture
   Contents: Glycerol, πCode, Phosphate buffered saline, 0.1% Albumin from bovine (Biological), <0.1% EDTA, <0.1% Sodium azide

4. **cLCP - DNA POS Control**
   Ref. No.: 20487
   Quantity & Volume: 1 vial, 120 µL/vial
   Description: Assay positive control
   Contents: Cell line DNA, Tris-EDTA Buffer

5. **NEG Control**
   Ref. No.: 20549
   Quantity & Volume: 1 vial, 120 µL/vial
   Description: Assay negative control
   Contents: ddH_2O

6. **cLCP - DNA Hy Buffer**
   Ref. No.: 20489
   Quantity & Volume: 1 bottle, 2.4 mL/bottle
   Description: For hybridization

Contents: Saline-Sodium Phosphate-EDTA

7. SA-PE Solution

Ref. No.: 20007
Quantity & Volume: 1 bottle, 7 mL/bottle
Description: Streptavidin-phycoerythrin for fluorescent signal acquisition
Contents: Phosphate buffered saline, 0.5% Streptavidin-phycoerythrin, 1% Albumin from bovine (Biological), <0.1% Sodium azide

8. 10X Assay Wash Buffer

Ref. No.: 20598
Quantity & Volume: 1 bottle, 50 mL/bottle
Description: For πCode washing
Contents: Phosphate buffered saline, 1% Tween-20, 0.5% Proclin 950

6. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

Required products for compatibility with IntelliPlex kits:

- 96-well plate (PlexBio; Cat. No. 80025 or Greiner Bio-one; Cat. No. 655101)
- IntelliPlex™ 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:

- Cell-Free DNA BCT®(Streck, Cat. No. 218961, 218962, 218992) or Vactainer® Venous Blood Collection Tube (BD, Cat. No. 367525)
- Qubit™ Fluorometer with dedicated quantitative reagents Qubit™ dsDNA HS Assay Kit (Invitrogen; Cat. No. Q32854 or equivalent)
- Clean tubes for PCR reaction (Gunster; Cat. No. MB-P08A or equivalent)
- Dedicated micropipette
- Filter tips for micropipette
- ddH_2O for dilution of 10X Assay Wash Buffer
- Cell-free DNA extraction kit (Recommended: QIAmp Circulating Nucleic Acid Kit (50), Qiagen Cat. No. 55114; ConcertBio cfDNA Extraction Kit (96), ConcertBio Cat. No. RC1101-4, or equivalent)
- Vortex mixer
- Micro-centrifuge
- Thermocycler (Recommended: MiniAmp™ Thermal Cycler, Applied Biosystems™; Cat. No. A37834 or equivalent)
- Computer (Recommended: PlexBio; Cat. No. 80002)

7. STORAGE, STABILITY AND TRANSPORTATION

Storage

The kit components of the **IntelliPlex Lung Cancer Panel – cfDNA** should be stored at 2°C to 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 is incomplete, please contact PlexBio customer service (service@plexbio.com).

8. INSTRUMENTS AND SOFTWARE

Instruments

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

Software Installation

The **IntelliPlex Lung Cancer Panel – cfDNA** 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 **LCP-cfDNA-cfRNA 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 **LCP-cfDNA-cfRNA** 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.

9. SPECIMENS

Specimen Collection

The IntelliPlex Lung Cancer Panel – cfDNA **has been validated to be used for plasma.** It is recommended to collect whole blood specimen with Streck Cell-Free DNA BCT®(Cat. No. 218961, 218962, 218992) or BD Vactainer® Venous Blood Collection Tube(Cat. No. 367525). Heparin is not recommended as it may interfere with PCR.

Whole Blood Transportation and Storage

Blood specimens in Streck Cell-Free DNA BCT® can be transported at 15-30°C and stored within 2 days at 15-30°C according to the product specification.

Blood specimen in BD Vacutainer® Venous Blood Collection Tubes can be transported at 15–25°C or 4°C and process within 1 hour.

Plasma Isolation for cfDNA extraction

To separate plasma from whole blood (8~9 mL), centrifuge the Streck Cell-Free DNA BCT® or BD Vacutainer® Venous Blood Collection Tubes at 1,600 x g for 10 minutes at room temperature.

Transfer upper plasma layer (~4 mL) to a conical tube (not provided). Filtrate the plasma with a 0.8 µm filter (not provided) to a new conical tube, then store in -80 °C for up to 6 months. Plasma should be transported with dry ice that keep plasma freeze during the transportation. Once arrived, move plasma into -80°C immediately.

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.

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) ready for analysis.

11. ASSAY PROCEDURE

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 (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 – cfDNA**. Each PCR reaction uses 20 µL of a 0.5 ng/µL DNA working stock (10 ng DNA input). Please prepare working stock for samples before preparing PCR. DNA input amounts lower or higher than 10 ng per reaction 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:

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

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	9
70	20 sec	
60~52*	20 sec	
60	25 sec	
95	20 sec	29
70	20 sec	
55	20 sec	
60	25 sec	
4	Hold	-

NOTE:

- *Ramp rate: 3°C/sec (ABI MiniAmp™; Cat. No. A37834).*
- *Temp. Setting for 60~52 indicates decreasing 1°C per cycle.*

11.3 DNA Hybridization and SA-PE Reaction

1. **Prepare 1X Assay Wash Buffer:** Transfer 50mL of the 10X Assay Wash Buffer to the IntelliPlex 1000 πCode Processor 1L Assay Wash Buffer bottle and add 450 ml ddH_2O. Mix by swirling.

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

   IntelliPlex 1000 πCode Processor Assay Wash Buffer consumption:

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

2. **Add 20 µL cLCP - DNA πCode MicroDisc to 96 well plate:** Mix by vortexing the correct tube of πCode for 10 seconds, then, by pipetting, add 20 µL 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 lane wise, in order of A1, B1…H1 and followed by A2, B2…H2 and so on.

3. **Add 100 µL of cLCP - 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 **7** minutes, immediately cooled on ice/cooler or thermocycler to ensure the denatured status. Spin down before use. Use immediately (within 1 hour after denaturation).

   NOTE: Pay attention to the lid temperature of thermocycler while taking out the denatured PCR products.

6. **Add 20 µL of the denatured PCR products** to each well.

7. **Pipet the desired volume of SA-PE solution** into the V Tray in SA-PE solution tank. Please note that the dead volume of V Tray is **500 µL** for up to 6 selected lanes or **800 µL** if more than 6 lanes are selected. The minimum usage of SA-PE is **one lane (900 µL)**.

   Calculation Example:
   For a 3-lane reaction, the required SA-PE solution volume is at least:
   400 µL x 3 lanes + 500 µL(dead volume)= 1.7 mL
   It is recommended to add extra solution volume into the V Tray to ensure sufficient dispensing volume.

   NOTE:

   Required SA-PE Solution by Lane(s):

Number of Processed Lane(s)	Required SA-PE Solution (µL)
1	900
2	1300
3	1700
4	2100
5	2500
6	2900
7	3600
8	4000
9	4400
10	4800
11	5200
12	5600

- SA-PE solution should be kept in the dark.

- **Do not** reuse the leftover SA-PE solution and V Tray tank. Replace a new V Tray with every assay run.

8. **Run hybridization and wash:** This assay uses the **DNA/RNA program** in the **Molecular Assay** window of the IntelliPlex 1000 πCode Processor. Refer to the IntelliPlex 1000 πCode Processor operation manual and follow the instructions to run the built-in assay program (Homepage/ Molecular Assay/ Well Selection/ DNA/RNA/ Confirm procedure conditions/ Start Running). The plate will be ready for decoding once the program is finished.

   NOTE:
   - IntelliPlex 1000 πCode Processor must be maintained properly and regularly.
   - **Do not** open the door when the instrument is in operation.
   - The kit contains sufficient reagents for 6 runs of 4 samples (including POS and NEG controls) for a maximum of 24 tests. Please note that the included Assay Wash Buffer is only sufficient for up to two independent runs. Additional Assay Wash Buffer can be ordered from PlexBio (Ref. No.: 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 **Qualitative Assay**.
3. Mark the wells for sample, positive and negative controls.
4. Enter Assay name and 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 Lung Cancer Panel – cfDNA** of the same lot will be required.

12. DISCLAIMERS

Negative Test Result

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

13. INTERPRETATION OF RESULT

The report generated by DeXipher includes the results of controls and samples tested in the same run. The result of external controls (POS Control and NEG Control) must be “Pass”. Otherwise, failed POS or NEG Control renders the whole assay run invalid, and the result of tested samples will not be reported.
If the result POS Control and NEG Control are “Pass”, the result of each tested sample will be reported on separate sheets in detail. For each tested sample, its internal controls (Reference Gene Control, Internal Control, SA-PE Monitor Control, πCode MicroDiscs Count, πCode MicroDiscs Combination and Blank Control) must be “Pass”, or the test of that sample is invalid. The detection result of invalid samples will not be shown. However, failed Reference Gene Control and Internal Control do not negate samples with mutation detected. A positive sample with failed Reference Gene Control is considered valid. The detection result of target genes will be shown for each valid sample.

Refer to the chapter **“Troubleshooting”** for control failure issues.

Table 2. Interpretation of Results

Test Result	Explanation	Action
Mutation Detected	Refer to Table 1	Targeted mutation detected
Mutation Not Detected	None	Targeted mutation not detected
Result Not Shown	The test is **INVALID** because external controls failed OR at least one of internal controls failed	See the chapter **"Troubleshooting"** for instructions and retest.

NOTE:

- All run and specimen validation are performed by the dedicated KIT APP along with IntelliPlex 1000 πCode Processor and PlexBio 100 Fluorescent Analyzer.
- "Mutation Detected" indicates that the signal for at least one mutation site is greater than the cutoff value of the corresponding target.

14. ANALYTICAL PERFORMANCE

Limit of Blank (LoB)

The limit of blank (LoB) values were determined by 2 operators performing two replicates of 120 plasma specimens on 2 reagent lots and 2 sets of instruments. Origins of the plasma specimens is non cancer people.

The cut-off values for each mutation site were then determined by calculating maximum plus three standard deviation of fluorescent signal intensity values from the LoB study. Only "None" results were recorded in the LoB study.

Limit of Detection (LoD)

The limit of detection (LoD) was determined using a dilution series (ranging from 0.10 – 5 %) containing different levels of mutant amplicon DNA derived from mutant plasmid. All testing samples were blended in a background of fragmented wild type human genomic 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 3). The LoDs ranged from 0.10~2.32%.

Table 3. Limit of Detection

Gene	Amino Acid Change	Nucleotide Change	LoD (% Mutation)
KRAS	p.G12S	c.34G>A (GGT>AGT)	0.24%
	p.G12R	c.34G>C (GGT>CGT)	0.59%
	p.G12C	c.34G>T (GGT>TGT)	0.24%
	p.G12D	c.35G>A (GGT>GAT)	0.28%
	p.G12A	c.35G>C (GGT>GCT)	0.59%
	p.G12V	c.35G>T (GGT>GTT)	0.10%
	p.G13D	c.38G>A (GGC>GAC)	0.10%
	p.G13C	c.37G>T (GGC>TGC)	0.76%
	p.Q61H	c.183A>C (CAA>CAC)	0.24%
	p.Q61H	c.183A>T (CAA>CAT)	1.49%
	p.Q61L	c.182A>T (CAA>CTA)	1.35%
	p.Q61K	c.181C>A (CAA>AAA)	0.63%
	p.Q61R	c.182A>G (CAA>CGA)	2.32%
NRAS	p.Q61K	c.181C>A (CAA>AAA)	0.29%
	p.Q61R	c.182A>G (CAA>CGA)	0.60%
	p.Q61L	c.182A>T (CAA>CTA)	0.50%
	p.Q61H	c.183A>C (CAA>CAC)	0.57%
	p.Q61H	c.183A>T (CAA>CAT)	1.65%
PIK3CA	p.E542K	c.1624G>A	0.62%
	p.E545K	c.1633G>A	0.73%
	p.E545Q	c.1633G>C	0.23%
	p.H1047R	c.3140A>G	0.47%
	p.H1047L	c.3140A>T	0.25%
BRAF	p.V600E1	c.1799T>A	0.10%
EGFR	p.G719A	c.2156G>C	0.12%
	p.G719S	c.2155G>A	0.24%
	p.G719C	c.2155G>T	0.25%
	p.L747_S752del	c.2239_2256del18	0.32%
	p.L747_P753>S	c.2240_2257del18	0.33%
	p.E746_S752>V	c.2237_2255>T	0.25%
	p.L747_T751del	c.2239_2253del15	1.73%
	p.L747_T751del	c.2240_2254del15	

Gene	Amino Acid Change	Nucleotide Change	LoD (% Mutation)
EGFR	p.L747_T751del	c.2238_2252del15	1.73%
	p.E746_A750del	c.2235_2249del15	0.42%
	p.E746_A750del	c.2236_2250del15	0.64%
	p.L747_A750>P	c.2239_2248TTAAGAGAAG>C	0.24%
	p.E746_T751>A	c.2237_2251del15	0.5%
	p.S752_I759delSPKANKEI	c.2253_2276del24	1.19%
	p.S752_I759delSPKANKEI	c.2254_2277del24	1.19%
	p.T790M	c.2369C>T	1.00%
	p.C797S	c.2389T>A	1.45%
	p.C797S	c.2390G>C	1.29%
	p.S768I	c.2303G>T	0.49%
	p.V769_D770insASV	c.2307_2308ins9GCCAGCGTG	1.11%
	p.H773_V774insH	c.2319_2320insCAC	0.39%
	p.D770_N771insG	c.2310_2311insGGT	0.74%
	p.D770_N771insSVD	2311_2312ins9GCGTGGACA	1.11%
	p.V769_D770insASV	2309_2310AC>CCAGCGTGGAT	1.11%
	p.L858R	c.2573T>G	0.48%
	p.L861Q	c.2582T>A	0.24%
AKT1	p.E17K	c.49G>A	0.29%
MEK1	p.Q56P	c.167A>C	0.58%
	p.K57N	c.171G>T	0.23%
HER2	p.Ala775_Gly776insYVMA	c.2324_2325ins12	0.30%
	p.Ala775_Gly776insYVMA	c.2325_2326ins12	

Repeatability and Reproducibility

The repeatability and reproducibility of **IntelliPlex Lung Cancer Panel – cfDNA** was evaluated across two reagent lots, two sites, four operators, four sets of instruments and eight non-consecutive testing days. One operator performed two run per reagent lot per day for a total of 16 runs at one site. Repeatability of IntelliPlex Lung Cancer Panel – cfDNA was demonstrated with wild-type plasma samples, low level mutant (2x LoD) and high level mutant (6x LoD). The total correct call of all testing level mutants was 99.9% (3517/3520), and the overall correct all of wild-type samples was 100% (256/256). Across all variance combined (i.e., site/instrument, operator, and day).

Table 4. Accuracy (%) of Each Mutation

Gene	Mutation	Mutation Level	Mutation Percentage	Fractional Proportion of Valid Results	Correct Calls (%)
KRAS	p.G12A	2x LoD	1.46%	32/32	100
		6x LoD	0.49%	32/32	100
	p.G12D	2x LoD	3.56%	32/32	100
		6x LoD	1.19%	32/32	100
	p.G12V	2x LoD	1.45%	32/32	100
		6x LoD	0.48%	31/32	96.9
	p.G12C	2x LoD	1.69%	32/32	100
		6x LoD	0.56%	32/32	100
	p.G12R	2x LoD	3.56%	32/32	100
		6x LoD	1.19%	32/32	100
	p.G12S	2x LoD	0.60%	32/32	100
		6x LoD	0.20%	32/32	100
	p.G13D	2x LoD	0.60%	32/32	100
		6x LoD	0.20%	32/32	100
	p.G13C	2x LoD	4.56%	32/32	100
		6x LoD	1.52%	32/32	100
	p.Q61H (A>C)	2x LoD	1.46%	32/32	100
		6x LoD	0.49%	32/32	100
	p.Q61H (A>T)	2x LoD	8.95%	32/32	100
		6x LoD	2.98%	32/32	100
	p.Q61L	2x LoD	8.11%	32/32	100
		6x LoD	2.70%	32/32	100
	p.Q61K	2x LoD	3.80%	32/32	100
		6x LoD	1.27%	32/32	100
	p.Q61R	2x LoD	13.92%	32/32	100
		6x LoD	4.64%	32/32	100

Gene	Mutation	Mutation Level	Mutation Percentage	Fractional Proportion of Valid Results	Correct Calls (%)
NRAS	p.Q61K	2x LoD	1.74%	32/32	100
		6x LoD	0.58%	32/32	100
	p.Q61R	2x LoD	3.61%	32/32	100
		6x LoD	1.20%	32/32	100
	p.Q61L	2x LoD	2.98%	32/32	100
		6x LoD	0.99%	32/32	100
	p.Q61H(A>C)	2x LoD	3.40%	32/32	100
		6x LoD	1.13%	32/32	100
	p.Q61H (A>T)	2x LoD	9.88%	32/32	100
		6x LoD	3.29%	32/32	100
PIK3CA	p.E542K	2x LoD	3.70%	32/32	100
		6x LoD	1.23%	32/32	100
	p.E545K	2x LoD	4.36%	32/32	100
		6x LoD	1.45%	32/32	100
	p.E545Q	2x LoD	1.37%	32/32	100
		6x LoD	0.46%	32/32	96.9
	C797S G>C	2x LoD	7.73%	32/32	100
		6x LoD	2.58%	32/32	100
BRAF	p.V600E1	2x LoD	0.60%	32/32	100
		6x LoD	0.20%	32/32	100
EGFR	G719A	2x LoD	0.70%	32/32	100
		6x LoD	0.23%	32/32	100
	G719S	2x LoD	1.45%	32/32	100
		6x LoD	0.48%	31/32	96.9
	G719C (G>T)	2x LoD	1.50%	32/32	100
		6x LoD	0.50%	32/32	100
	L747_S752del	2x LoD	1.90%	32/32	100
		6x LoD	0.63%	32/32	100
	L747_P753>S	2x LoD	1.98%	32/32	100
		6x LoD	0.66%	32/32	100
	E746_S752>V	2x LoD	1.50%	32/32	100
		6x LoD	0.50%	32/32	100
	L747_T751del	2x LoD	10.37%	32/32	100
		6x LoD	3.46%	32/32	100
	L747_T751del	2x LoD	10.37%	32/32	100
		6x LoD	3.46%	32/32	100
	L747_T751del	2x LoD	10.37%	32/32	100
		6x LoD	3.46%	32/32	100
	E746_A750del	2x LoD	2.52%	32/32	100
		6x LoD	0.84%	32/32	100

Gene	Mutation	Mutation Level	Mutation Percentage	Fractional Proportion of Valid Results	Correct Calls (%)
EGFR	E746_A750del	2x LoD	3.85%	32/32	100
		6x LoD	1.28%	32/32	100
	L747_A750>P	2x LoD	1.46%	32/32	100
		6x LoD	0.49%	32/32	100
	S752_I759delSPKANKEI	2x LoD	7.13%	32/32	100
		6x LoD	2.38%	32/32	100
	S752_I759delSPKANKEI	2x LoD	7.13%	32/32	100
		6x LoD	2.38%	32/32	100
	E746_T751>A	2x LoD	3.00%	32/32	100
		6x LoD	1.00%	32/32	100
	T790M	2x LoD	5.99%	32/32	100
		6x LoD	2.00%	32/32	100
	C797S T>A	2x LoD	8.68%	32/32	100
		6x LoD	2.89%	32/32	100
	C797S G>C	2x LoD	7.73%	32/32	100
		6x LoD	2.58%	32/32	100
	S768I	2x LoD	2.96%	32/32	100
		6x LoD	0.99%	32/32	100
	V769_D770insASV	2x LoD	6.65%	32/32	100
		6x LoD	2.22%	32/32	100
	D770_N771insSVD	2x LoD	6.65%	32/32	100
		6x LoD	2.22%	32/32	100
	p.V769_D770insASV	2x LoD	6.65%	32/32	100
		6x LoD	2.22%	32/32	100
	H773_V774insH	2x LoD	2.32%	32/32	100
		6x LoD	0.77%	32/32	100
	D770_N771insG	2x LoD	4.44%	32/32	100
		6x LoD	1.48%	32/32	100
	p.L858R (T>G)	2x LoD	2.87%	32/32	100
		6x LoD	0.96%	32/32	100
	p.L861Q	2x LoD	1.42%	32/32	100
		6x LoD	0.47%	32/32	100
AKT1	E17K	2x LoD	1.73%	32/32	100
		6x LoD	0.58%	32/32	100
MEK1	Q56P	2x LoD	3.46%	32/32	100
		6x LoD	1.15%	32/32	100
	K57N	2x LoD	1.40%	32/32	100
		6x LoD	0.47%	32/32	100

Gene	Mutation	Mutation Level	Mutation Percentage	Fractional Proportion of Valid Results	Correct Calls (%)
HER2	p.Ala775_Gly776insYVMA	2x LoD	1.81%	32/32	100
		6x LoD	0.60%	32/32	100
	p.Ala775_Gly776insYVMA	2x LoD	1.81%	32/32	100
		6x LoD	0.60%	32/32	100
-	WT	-	-	256/256	100

Cross-Contamination

This test is designed to assess cross-contamination during assay, which may lead to false positive results. Wild-type and KRAS G12S 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 ConcertBio cfDNA extraction 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 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)
Proteinase K	$3*10^{-6}$
Binding buffer-2	$3*10^{-4}$
Elution buffer-2	0.1
Lysis buffer	$3*10^{-4}$
Binding buffer-1	$3*10^{-4}$
Wash Buffer-1	0.1
Wash Buffer-2	1
Elution buffer-1	1

15. TROUBLESHOOTING

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

Problem	Possible Cause	Recommendations
No Valid Assay Assigned	1. No plate inserted.	1. Confirm plate is inserted and repeat reading.
	2. Plate inserted in wrong orientation.	2. Confirm orientation of plate and repeat reading.
	3. No assay APP installed.	3. Install assay APP and repeat reading.
	4. No ENC file imported.	4. Import ENC file and repeat reading.
	5. Two or more lots of reagent used.	5. One reagent lot used at a time.
Positive Control Fail / Negative Control Fail	1. No POS Control or NEG Control added.	1. Ensure POS Control and NEG Control are added.
	2. DNase contamination.	2. Ensure all operating procedures are followed correctly. Ensure work environment is free of DNase.
	3. Assay did not work.	3. Make sure all the assay procedures are followed correctly.
	4. Cross contamination between samples.	4. Clean all surfaces and equipment. Operate pre-PCR and post-PCR in the dedicated area and separate the equipment for use.
	5. Wrong PC/NC wells chose.	5. Choose the correct PC/NC wells and repeat reading.

Problem	Possible Cause	Recommendations
πCode MicroDiscs Count Fail	DeXipher is unable to detect sufficient πCode MicroDiscs numbers for mutation determination.	
	1. πCode MicroDiscs are not proper dispersed in the well.	1. Re-disperse the microplate using IntelliPlex 1000 processor, and repeat reading.
	2. Not enough πCode MicroDiscs added to well.	2. Ensure πCode MicroDiscs are well-mixed with proper amount added.
	3. Microbes exist in buffers.	3. Use freshly prepared Assay 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 lanes of microplate selected for SA-PE solution dispensing.	3. Repeat assay and make sure lanes selected correctly.

Problem	Possible Cause	Recommendations
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 (Assay Wash buffer and ddH_2O) 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. If still remains failed, ensure the collected samples meet specimen requirements. Retest with new samples if needed.
	3. PCR inhibition exists.	3. Follow sample extraction instructions carefully. Ensure required temperature ranges and centrifugation needs are complied. Ensure complete removal of ethanol.
	4. PCR procedures are not performed correctly.	4. Make sure all PCR procedures are followed correctly. Do not to use expired materials or mixed lots of reagents. Ensure storage conditions are correct.

16. SYMBOLS

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

17. REFERENCES

1. https://www.mycancergenome.org/content/disease/lung-cancer/
2. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M, Varmus H. (2004) EGFR receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA. 101(36):13306-11
3. Lie`vre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Coˆte JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P. (2006) KRAS mutation status is predictive of response to cetuimab therapy in colorectal cancer. Cancer Res. 66(8):3992-5
4. http://www.uniprot.org/uniprot/P01116
5. Baynes RD, Gansert J. (2009) KRAS mutational status as a predictor of epidermal growth factor receptor inhibitor efficacy in colorectal cancer. Am J Ther. 16(6):554-61
6. Sakuma, M. (2000) PriProbit, ver. 1.63. Available from James E. Throne USDA-ARS GMPRC, Manhattan, KS (http:// bru.usgmrl.ksu.edu/throne/)
7. Neumann J, Zeindl-Eberhart E, Kirchner T, Jung A. (2009) Frequency and type of KRAS mutations in routine diagnostic analysis of metastatic colorectal cancer. Pathol Res Pract. 2009; 205:858–862.

8. Food and Drug Administration. Class labeling changes to anti-EGFR monoclonal antibodies, cetuximab (Erbitux) and panitumumab (Vectibix): KRAS mutations. http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucml172905.htm.
9. Loupakis F, Ruzzo A, Cremolini C, et al. KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer. Br J Cancer 2009 Aug 18;101(4):715-21.
10. Siena S, Sartore-Bianchi A, Di Nicolantonio F, et al. Biomarkers predicting clinical outcome of epidermal growth factor receptortargeted therapy in metastatic colorectal cancer. J Natl Cancer Inst 2009 Oct 7;101(19):1308-24.

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