



# Mutector™

Mutation Detection Kit

## PIK3CA

Mutation Analysis Reagents

User Manual V1.5

Cat No. GP08

32 reactions



[www.trimgen.com](http://www.trimgen.com)

**Storage**

Upon receipt of the kit, store at  $-20^{\circ}\text{C}$  until use. At this temperature the reagents are stable for 6 months.

After first use, store all of the reagents at  $2-8^{\circ}\text{C}$  and keep them protected from direct light. At this condition the reagents are stable for 1 month.

For research use only, not for use in diagnostic procedures

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## Introduction

Phosphoinositide-3-kinase (PI3K) plays an important role in the EGFR signaling pathway. The PI3K activates the serine/threonine kinase AKT, which in turn regulates several signaling pathways controlling cell survival, growth and proliferation. The gene, PIK3CA, encoding the catalytic subunit of the enzyme, has been found to mutate frequently in human cancers. The studies found that the PI3KCA mutations in cancer cells associated with early recurrence, poor prognosis and drug resistance.

The Mutector™ PIK3CA Mutation Analysis Reagents uses TrimGen's proprietary technology called Shifted Termination Assay (STA) to detect the following 5 mutations:

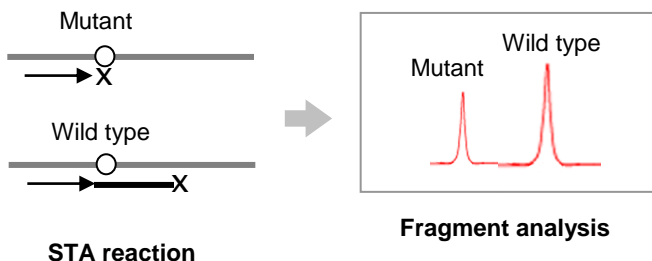
- E542K (G1624A, exon 9)
- E545K (G1633A, exon 9)
- E545G (A1634G, exon 9)
- H1047R (A3140G, exon 20)
- H1047L (A3140T, exon 20)

The kit contains reagents enough for 32 reactions.

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### \* Shifted Termination Assay (STA)

Shifted Termination Assay is a proprietary multi-base primer extension method. STA technology extends primers by multiple bases to increase signal strength and fragment size, creating mutation peaks that are easily distinguished from wild type. Mutations are confirmed by both peak color and size. The STA technology can detect low-level mutations often missed by sequencing.



## Overview of Mutector™ Assay

Total assay time: 2-3 hours; hands-on time: 10-20 min

### Step 1



PCR amplification  
1.5 hours\*

*\* Time varies depending on the type of thermal cycler used*



### Step 2



PCR product clean-up  
30 min



### Step 3



Mutation detection (ST reaction)  
40 min\*

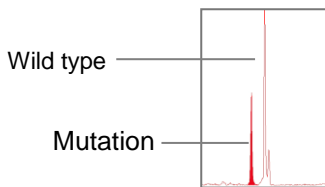
*\* Time varies depending on the type of thermal cycler used*



### Step 4

Load sample  
to sequencer

5 min



Capillary Electrophoresis  
Fragment analysis  
30-40 min\*

*\* Time varies depending on the type of sequencer*

## Materials Provided:

The Mutector™ PI3K Mutation Analysis Reagents can perform 32 reactions.

Materials	Quantity
<b>Master Mix</b>	<b>700 µL</b>
<b>PCR-P 08</b>	<b>50 µL</b>
<b>C-UP1</b>	<b>40 µL</b>
<b>C-UP2</b>	<b>40 µL</b>
<b>C-UP Buffer</b>	<b>430 µL</b>
<b>ST-D*</b>	<b>430 µL</b>
<b>DP-08</b>	<b>80 µL</b>
<b>M-08</b>	<b>50 µL</b>
<b>Loading Bfx*</b>	<b>1000 µL</b>

\* **Light sensitive:** Keep these reagents protected from direct light.

## Reagent Description:

### Master Mix

Reagents for DNA amplification.

### PCR-P 08

PCR primer mix for amplification of PIK3CA gene.

### C-UP1 and C-UP2

Enzymes for cleanup of PCR products.

### C-UP Buffer

Buffer for C-UP1 and C-UP2.

### ST-D (Light sensitive)

Pre-mixed STA reagents for mutation detection.

### DP-08

Detection primers for PIK3CA gene.

### M-08

Mutant and wild type control DNA for PIK3CA gene. The controls are sufficient for 32 test runs.

### Loading Bfx (Light sensitive)

Loading buffer for ABI capillary type sequencers and special fluorescence-labeled size standards.

## Materials Required:

0.2 ml PCR tubes (8-well strip tube)

DS-32 Matrix Standard Kit (Applied Biosystems Cat. No. 4345831). The calibration defines the color definition on the sequencer to read the test results correctly. This is a one-time set up procedure for all Mutector assays.

## Equipment Required:

**Thermal Cycler:** Any type of thermal cycler is acceptable for performing the assay.

**Sequencer:** Any type of Applied Biosystems's Genetic Analyzer or comparable capillary electrophoresis equipment.

**Analysis Software:** GeneMapper® or GeneScan® for fragment analysis



## DNA Sample Preparation:

Any commercially available DNA extraction kit is acceptable.

### Paraffin (FFPE) tissue samples

A kit specially designed for FFPE sample DNA extraction is available from TrimGen.

Product information:

WaxFree™ DNA for 50 samples (Cat. # WF-50)

WaxFree™ DNA for 100 samples (Cat. # WF-100)

### **DNA concentration:**

When using a column or bead DNA extraction method, adjust the final concentration of extracted DNA to **20-80 ng / $\mu$ L**

When using TrimGen's DNA preparation kit, follow the preparation kit protocol to perform the PCR reaction.

## Sequencer Setup:

First time users should set up the analysis program for the ABI sequencer (one time setup). After setup, the program can perform data analysis for all Mutector™ II tests. Please choose either GeneMapper® or GeneScan® to analyze your data.

### **GeneMapper® Analysis**

#### **Step I. GeneMapper® Setup**

[www.trimgen.com/docs/PartI-GeneMapper-Setup.pdf](http://www.trimgen.com/docs/PartI-GeneMapper-Setup.pdf)

#### **Step II. Data Collection® Software Setup**

[www.trimgen.com/docs/PartII-Data-Collection-Setup.pdf](http://www.trimgen.com/docs/PartII-Data-Collection-Setup.pdf)

#### **Step III. Data Analysis Using GeneMapper®**

[www.trimgen.com/docs/PartIII-Data-Analysis-GeneMapper.pdf](http://www.trimgen.com/docs/PartIII-Data-Analysis-GeneMapper.pdf)

### **GeneScan® Analysis**

#### **Step I. Data Collection® Software Setup**

[www.trimgen.com/docs/PartII-Data-Collection-Setup.pdf](http://www.trimgen.com/docs/PartII-Data-Collection-Setup.pdf)

#### **Step II. GeneScan® Setup and Data Analysis**

[www.trimgen.com/docs/PartIV-Genescan.pdf](http://www.trimgen.com/docs/PartIV-Genescan.pdf)



## Important

### **Spectral calibration is required before running the test**

To read the test results correctly, the sequencer needs to be calibrated with the DS-32 calibration kit (Applied Biosystems Cat. No. 4345831). This is a one-time calibration to set up the correct spectral channels. Refer to the DS-32 Matrix standards kit manual to perform spectral calibration.

## Thermal Cycling Programs:

### **Program 1** (PCR amplification)

1 cycle	94°C 5 min
35 cycles	94°C 30 sec
	52°C 30 sec
	72°C 30 sec
1 cycle	72°C 5 min
	Hold at 4°C

### **Program 2** (C-UP treatment)

	37°C 25 min
	95°C 5 min
	Hold at 4°C

### **Program 3** (STA reaction)

1 cycle	94°C 4 minute
20 cycles	94°C 20 sec
	55°C 30 sec
	70°C 20 sec
	Hold at 4°C

## Mutector™ Assay Protocol:

### A. PCR Amplification

Thaw all reagents and keep on ice. Spin down the reagents before use.

Negative and positive controls are recommended to be run each time.

#### A.1. Prepare PCR reaction mix (PM):

The PCR reaction mix can be prepared by using the following formula:

$$\text{Master Mix} = 18 \times \left( \frac{\quad}{\text{(Total \# of samples)}} + 2^* \right) \times 1.1^{**} = \quad \mu\text{L}$$

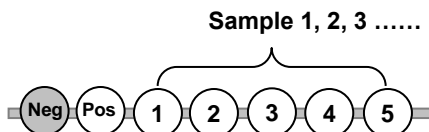
$$\text{PCR-P 08} = 1 \times \left( \frac{\quad}{\text{(Total \# of samples)}} + 2^* \right) \times 1.1^{**} = \quad \mu\text{L}$$

\*2 tubes for negative and positive sample controls.

\*\* Adjustment for pipetting error.

Collect one 2 mL tube and label with "**PM**" (PCR reaction mix). Transfer the above reagents to the tube and mix the contents. This is the master mix for PCR amplification.

**A.2.** Collect 0.2 mL PCR strip tubes and label the tubes as follows:



**Neg:** Negative control

**Pos:** Positive control

- A.3.** Add **19  $\mu\text{L}$**  of **PM** into each of the tubes.
- A.4.** Add **1  $\mu\text{L}$**  of negative control sample to the “**Neg**” tube.
- A.5.** Add **1  $\mu\text{L}$**  of positive control sample to the “**Pos**” tube.
- A.6.** Add **1-2  $\mu\text{L}$**  of sample DNA (20-80 ng/ $\mu\text{L}$ ) to each sample tube.  
When using TrimGen’s WaxFree kit for paraffin sample DNA extraction, add **1  $\mu\text{L}$**  of the final extract to each sample tube.
- A.7.** Place the PCR tubes in a thermal cycler and run **Program 1**.

<b><u>Program 1</u></b>	
1 cycle	94°C 5 min
35 cycles	94°C 30 sec
	52°C 30 sec
	72°C 30 sec
1 cycle	72°C 5 min
	Hold at 4°C

**Note:**

- ✓ Optional: To verify the PCR products, run 5 $\mu\text{L}$  of the PCR products on a 1-2% agarose gel. The correct band size is around **100 bp**.



The procedure can be temporarily stopped after **Program 1**.  
The PCR product can be stored at 4°C for next day test.

**During the PCR amplification process, prepare steps B1-B2.**

## B. PCR Product Clean-up

### B.1. Prepare **C-UP Mix**:

$$\text{C-Buffer} = 10 \mu\text{L} \times \left( \frac{\quad}{\# \text{ of PCR tubes}} \right) \times 1.1^{**} = \quad \mu\text{L}$$

$$\text{C-UP1} = 1.0 \mu\text{L} \times \left( \frac{\quad}{\# \text{ of PCR tubes}} \right) \times 1.1^{**} = \quad \mu\text{L}$$

$$\text{C-UP2} = 1.0 \mu\text{L} \times \left( \frac{\quad}{\# \text{ of PCR tubes}} \right) \times 1.1^{**} = \quad \mu\text{L}$$

Mix the reagents and spin down

\*\* For pipetting error.

**B.2.** Collect 0.2 mL strip tubes, one tube for each PCR reaction, for example, if you have 8 tubes for PCR, then collect 8 tubes.

Label the tubes with "C" and sample name in the same manner as the PCR tubes

**B.3.** Add 11  $\mu\text{L}$  of **C-UP mix** to each new tube.

**B.4.** Transfer 4  $\mu\text{L}$  of each sample's PCR product to corresponding C-UP tube.

**B.5.** Cap the tubes, mix the contents and spin all tubes.

**B.6.** Incubate the tubes in a thermal cycler using **Program 2**.

#### **Program 2**

37°C for 25 min

95°C for 5 min

Hold at 4°C

During the product clean-up incubation, prepare steps C1-C4.

## C. STA Mutation Detection

- C.1. Collect one 2 mL tube and label with “ST”. Mix the ST-D reagent and DP-08 (detection primer) to make the ST Mix.

The ST Mix can be prepared by using the following formula:

### ST Mix

$$\text{ST-D} = 11 \times \left( \frac{\quad}{\text{Total \# of C-UP samples}} + 1^* \right) \times 1.1^{**} = \quad \mu\text{L}$$

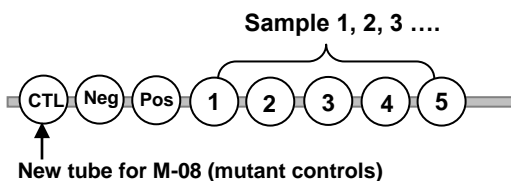
$$\text{DP-08} = 2 \times \left( \frac{\quad}{\text{Total \# of C-UP samples}} + 1^* \right) \times 1.1^{**} = \quad \mu\text{L}$$

\*One extra tube for M-08 (Mutant Controls)

\*\* Adjustment for pipetting error.

Add the reagents to the tube and mix gently.

- C.2. Collect 0.2 ml strip tubes, one tube for each C-UP treated sample. **Add one new tube for M-08 (Mutant Controls) and label the tube with CTL.**



**The M-08 (mutant controls) is designed for data analysis and must be run each time.**

- C.3. Add 13  $\mu\text{L}$  of **ST Mix** into each tube.
- C.4. Transfer 2  $\mu\text{L}$  of **C-UP treated PCR product** to each corresponding tube.
- C.5. Add 2  $\mu\text{L}$  of **M-08** into the **CTL tube**.
- C.6. Cap the tubes, mix the contents and spin down all tubes.
- C.7. Place the tubes into the thermal cycler and perform the ST reaction using **Program 3**.

**Program 3**

1 cycle	94°C 4 min
20 cycles	94°C 20 sec
	55°C 30 sec
	70°C 20 sec
	Hold at 4°C

**D. Sample Loading**

- D.1.** Add **15 µL** of the **Loading Buffer** to each well of a sequencer adapter plate.
- D.2.** Transfer **5 µL** of the ST products into each well. Confirm and remove any bubbles in the well.
- D.3.** Load the plate into the sequencer and run the preset Data Collection Program (GeneMapper or GeneScan).

## E. Data Analysis

E.1. Open the analysis software GeneMapper or Genescan.

E.2. Follow the instruction to add the data for analysis.

The instructions are provided online:

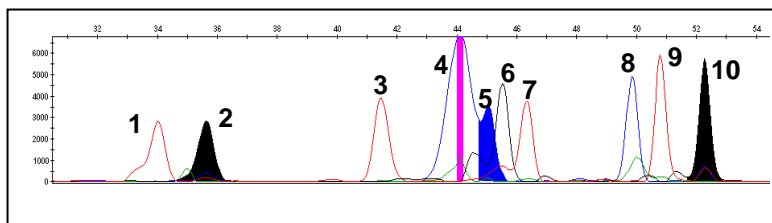
GeneMapper: [www.trimgen.com/docs/PartIII-Data-Analysis-GeneMapper.pdf](http://www.trimgen.com/docs/PartIII-Data-Analysis-GeneMapper.pdf)

GeneScan: [www.trimgen.com/docs/PartIV-Genescan.pdf](http://www.trimgen.com/docs/PartIV-Genescan.pdf).

E.3. Confirm results of mutant control M-08

In the sample plot window (shows graphic data), find the results of the mutant control (M-08). Read the peaks from left to right, and record peak size in Test Report Form (download the Form from <http://www.trimgen.com/docs/M2-report-form-GP08.xls>). The peak size of the Mutant Controls is the standard for sample analysis.

### Result of M-08 (mutant controls)



Peak #	Peak Color	Peak Size*	Interpretation
1	Red	34.04	Mutation - E542K (G1624A)
2	Black	35.62	<b>Wildtype - 542WT (1624WT)</b>
3	Red	41.44	Internal control
4	Blue	44.17	Mutation - E545G (A1634G)
5	Blue	45.06	<b>Wildtype - 545WT (1633 WT)</b>
6	Black	45.50	<b>Wildtype - 545WT (1634 WT)</b>
7	Red	46.34	Mutation - E545K (G1633A)
8	Blue	49.83	Mutation - H1047R (A3140G)
9	Red	50.79	Mutation - H1047L (A3140T)
10	Black	52.28	<b>Wildtype - 1047WT (3140WT)</b>

\*Peak size may vary slightly depending on instrument, polymer type and the length of capillary.



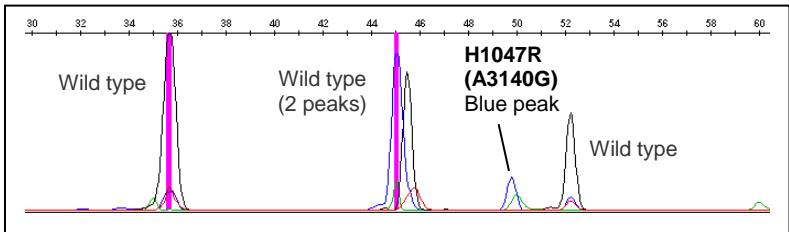
**E.4.** Data analysis for sample (ref <http://trimgen.com/docs/PI3K-data-analysis.pdf> )

Four wild type peaks are observed in every sample. If all four peaks are not observed, it indicates that the DNA amplification failed (see troubleshooting section) or the sample is 100% mutation(s), such as mutant cell lines. If sample contains mutant DNA, the mutation(s) will show as additional peak(s). **Compare the peak size and color with the mutant controls (M-08).** The peak size may be slightly shifted due to migration differences between individual capillary tubes (Comparing the wild type peak of the sample with the wild type peak of the Mutant Controls can identify the migration shift). Fill in the peak size to the Test Report Form. Any peak that does not match the Mutant Controls will not be considered as evidence of a mutation.

**Example of assay result**

Sample: FFPE sample, one section (1 x 0.5 cm, 10 µm).

DNA extraction: WaxFree DNA kit.



## F. Troubleshooting

### F.1. “Color leak-through”

When the sample DNA concentration is too high, the STA reaction generates a strong fluorescent signal (>5,000 rfu). Fluorescence spillover into other channels will occur, for example, the black peak of the wild type signal may be observed in the red and/or blue channels. The “leak-through” peaks are the same size as the original peak and should not be considered mutation peaks.

### F.2. The size standard is too high

The height of the size standard varies with STA reaction efficiency. Generally, the size standard intensity (green color) is 2,000-3,000 rfu. When the STA efficiency is too low (poor DNA quality, improper handling of STA reagents), the size standard may reach 5,000-7,000 rfu. Diluting the size standard with de-ionized formamide will reduce the size standard signal.

### F.3. The signal peak is too high

The assay chemistry is optimized to detect mutations in samples with low DNA concentration, such as DNA from a fine needle aspiration (FNA) sample. For regular FFPE samples, the assay signal may be too high to analyze (peak height >8000, the top of the peak is not visible or the peak is highlighted with pink color).

Diluting the final STA product with de-ionized water can effectively reduce the signal. Do not dilute the assay reagents as it will cause improper enzyme reactions and generate false results.

Each laboratory has different PCR instruments, and the signal strength may vary among the laboratories. The first time user should define the dilution factor (2-20 times dilution) during initial validation. Once the dilution factor is determined, most samples will have a consistent result.

### F.4. Data failed to be analyzed

- The size standard may be miscalculated by the sequencer. Check the size standard and manually correct it (see the sequencer’s instruction manual). Reanalyze the data after correction of size standard.

- Check the raw data, if the signals from the sample and size standard are too low, the capillary tube may be blocked by a bubble. The sample needs to be re-loaded.
- If the sample signal is too strong and the size standard is too low, the STA products will compete with the size standard DNA to enter the capillary tube. Diluting the final STA product with de-ionized water and reloading the sample will easily resolve this problem.

#### F.6. Graphic data does not automatically appear

- Check the raw data, if the signals from the sample and size standard are too low, the capillary tube is blocked by a bubble. The sample needs to be re-loaded. When adding a sample to the loading plate, do not use the pipettor to mix the sample, as this will generate bubbles. The sample DNA in the loading plate will automatically enter into the capillary tube by electric current.
- If the sample signal is too strong and the size standard is too low, the ST products will compete with the size standard DNA to enter the capillary tube. When the size standard is too low, the software can not detect the size standard correctly and the program will not show the graphic data. Diluting the final ST product with de-ionized water and reloading the sample will easily resolve this problem.
- The size standard may have miscalculated. Check the size standard and manually correct the size standard (see the sequencer's instruction manual). Reanalyze the data after correction of size standard.

#### F.7. Missing wild type peak(s)

The wild type peaks are built-in reaction controls for sample DNA amplification, these peaks should appear in all samples. If wild type peaks are not observed, it indicates that the PCR amplification failed. The possible causes could be poor DNA quality, low DNA concentration or PCR inhibitor in the DNA sample.

#### F.8. Background noise

The background of the assay is normally low. When the STA reaction is too strong (peak signal over 8000 rfu and highlighted

with pink color), background noise (also called “pull up”) can be seen. To resolve this issue, simply dilute the final ST product with de-ionized water and re-load the sample.

Overloading DNA is another cause of increased background noise. Reducing the input DNA amount will resolve this issue.

#### **F.8. Unexpected peak**

Presence of a peak that does not match any of the peaks in the Mutation Control

The Mutation Control contains 13 peaks (wild-type and mutant peaks). If you see a peak that does not match the Mutation Control, please contact Technical Support for further analysis.

#### **F.9. Mutation peak cut-off**

For some samples, there could be a small peak observed in one of the mutation positions. To verify the peak, you need to confirm the signal strength of the wild type peak. If the wild type peak is too high (cannot see the top of the peak and the peak is highlighted with pink color), your ST reaction is too strong and the small peak may be a “pull up” from background noise. Follow the trouble shooting F2 to dilute the final product of ST reaction with de-ionized formamide.

After dilution, reload the sample. If you can see the top of the wild type peak, then use the following calculation to identify the small peak:

$$\text{Ratio} = (\text{Height of mutant peak}) / (\text{Height of wild type peak})$$

If the ratio is larger than 0.08, the peak is considered to be a mutation peak (this ratio does not represent the percentage of the mutation present in the sample). Otherwise the peak is background pull up and does not indicate the presence of a mutation in the sample.