Mutector™

Mutation Detection Kit

NRAS

Mutation Analysis Reagents
(Codons 12 and 13)

User Manual V1.1

Cat No. GP18

32 reactions

TrimGen
GENETIC DIAGNOSTICS

www.trimgen.com

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Storage

Upon receipt of the kit, store at –20°C until use. At this temperature the reagents are stable for 6 months.

After first use, store all of reagents at 2-8°C and keep them protected from direct light. At this condition the reagents are stable for 1 month.

Notice to Purchaser

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Introduction

Mutector™ NRAS kit is designed to detect 12 mutations occurring in codons 12 and 13 of NRAS gene.

Codon 12	Codon 13
G12S (GGT>AGT)	G13S (GGT>AGT)
G12R (GGT>CGT)	G13R (GGT>CGT)
G12C (GGT>TGT)	G13C (GGT>TGT)
G12D (GGT>GAT)	G13D (GGT>GAT)
G12A (GGT>GCT)	G13A (GGT>GCT)
G12V (GGT>GTT)	G13V (GGT>GTT)

The kit uses Shifted Termination Assay* (STA) technology to enrich the mutation signal and is able to accurately detect low-level somatic mutations.

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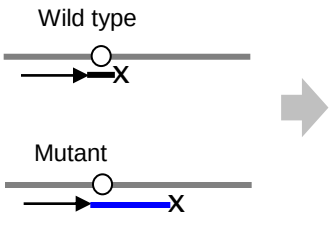
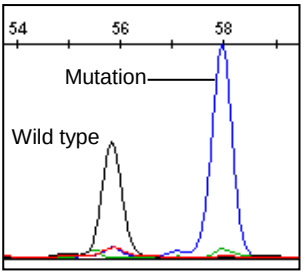
* Shifted Termination Assay (STA)

Shifted Termination Assay is a proprietary technology that uses uniquely designed primers, mixtures of modified enzymes and specially synthesized nucleotides. STA technology extends primers by multiple bases to increase signal strength and fragment size, creating mutation peaks that are easily distinguished from wild type. The enriched mutation signals are then detected by fragment analysis. The STA technology can detect low-level mutations often missed by sequencing.



STA reaction



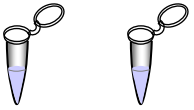
Fragment analysis

Overview of Mutector™ Assay

PCR Amplification
1.5 hours*

** Time varies by thermal cycler used*

PCR Product Clean-up
30 min

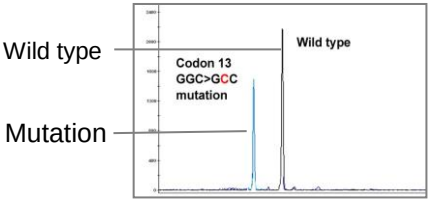


(Codon 12) (Codon 13)

STA reaction (Mutation detection)
40 min*

** Time varies by thermal cycler used*

Sample Loading
To Sequencer



Capillary Electrophoresis
Fragment analysis
25-40 min*

** Time varies depending on the type of sequencer*

Materials Provided:

The Mutector™ *NRAS* Mutation Detection kit contains reagents enough for 32 tests.

Reagents	Quantity	Description
Master Mix	650 µl	Master Mix Reagents for DNA amplification
NRAS PCR Primers	50 µl	PCR primer mix for amplification of *NRAS* gene codon12 and condon 13
C-UP1	40 µl	Enzyme 1 for cleanup of PCR products
C-UP2	40 µl	Enzyme 2 for cleanup of PCR products
C-UP Buffer	430 µl	Buffer for C-UP reaction
NRAS ST-12*	430 µl	Pre-mixed STA reagents for detection of NRAS codon 12 mutations
NRAS ST-13*	430 µl	Pre-mixed STA reagents for detection of NRAS codon 13 mutations
NRAS DP-12	80 µl	Pre-mixed detection primers for codon 12 mutations
NRAS DP-13	80 µl	Pre-mixed detection primers for codon 13 mutations
NRAS CTL-12	120 µl	Mutation controls for codon 12
NRAS CTL-13	120 µl	Mutation controls for codon 13
Loading Buffer*	1000 µl x 2	Sample loading buffer with size standards

* **Light Sensitive:** Keep these reagents protected from direct light.

Materials required:

0.2 ml PCR tubes (8-well strip tube)

DS-32 Matrix Standard kit (Applied Biosystems Cat. No. 4345831). This kit is a one-time calibration to set up the correct spectral channels. This is required for all Mutector II assays.

Equipment required:

Thermal Cycler:
Any type of thermal cycler with a 0.2 ml tube block is acceptable for performing the assay.

Sequencer:
Applied Biosystems Genetic Analyzer

Instrument	Data Collection	Data Analysis
Genetic analyzer 3100	Data Collection Software v3.0 or v3.1	GeneMapper® Software v4.0 or v4.1
Genetic analyzer 3700		
Genetic analyzer 3130		
Genetic analyzer 3500	3500 Data Collection Software v1.0	GeneMapper® Software v4.1

DNA Sample Preparation:

Reagents for DNA preparation are not provided with the kit.

Paraffin (FFPE) and fresh or frozen tissue samples

TrimGen has developed the WaxFree DNA extraction kit especially for FFPE samples. The kit uses special resins that bind and remove PCR inhibitors in the tissue extracts, leaving all DNA or RNA fragments in the extract. This method recovers more DNA in comparison with other extraction methods. The kit has been validated in many laboratories using a variety of FFPE samples as well as fresh and frozen tissue samples. WaxFree's simple procedure and high DNA yield ensures a PCR amplification success rate of > 95%.

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 /μl**
When using TrimGen's WaxFree DNA kit, follow the user manual to perform 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 apply to all Mutector™ tests for data analysis.

GeneMapper® Analysis

Step I. GeneMapper® Setup
www.trimgen.com/docs/PartI-GeneMapper-Setup.pdf

Step II. Data Collection® Software Setup
www.trimgen.com/docs/PartII-Data-Collection-Setup.pdf

Step III. Data Analysis Using GeneMapper®
www.trimgen.com/docs/PartIII-Data-Analysis-GeneMapper.pdf

Important

Spectral calibration is required before running the test

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 spectral channels to collect the test results. Refer to the DS-32 Matrix standards kit to prepare the DS-32 matrix standards. Run a Matrix Standard Set DS-32 (5FAM, JOE, NED, ROX) to perform a spectral calibration.

Thermal Cycling Programs:

Program 1 (PCR)

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 (Clean-up)

	37°C	25 min
	95°C	5 min
	Hold at 4°C	

Program 3 (EM reaction)

1 cycle	94°C	4 min
20 cycles	94°C	45 sec
	60°C	20 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.

A negative control (water) is recommended to run with samples each time.

A.1. Prepare PCR Reaction Mix:

Master Mix = 18 x (_____ + 2*) x 1.1 = _____ µl**
# of Samples

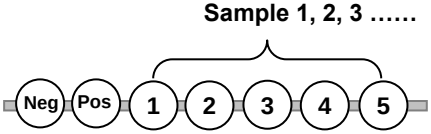
NRAS PCR Primers = 1 x (_____ + *2) x 1.1 = _____ µl**
# of Samples

* For negative and positive sample controls.

** For pipetting error.

Transfer entire volume of the reagents to one tube and gently mix (avoid bubble) the contents. This is the PCR Reaction Mix.

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



Neg: Negative Control
Pos: Positive Control

A.3. Transfer **19 µl** of PCR Reaction Mix into all of the tubes.

A.4. Add **1 µl** of nuclease-free water to the "**Neg"** tube.

A.5. Add **1 µl** of **NRAS Positive Control** to the **"Pos"** tube.

A.6. Add **1-2 µl*** of sample DNA (20-80 ng/µl) to each sample tube. When using TrimGen WaxFree kit for paraffin sample DNA extraction, add **0.5-1 µl*** final extract to each sample tube.

Add too much sample may cause an inhibition of PCR reaction.

A.7. Place the PCR tubes in a thermal cycler and run **Program 1**.

Program 1	
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

Optional: The PCR products can be verified by agarose gel electrophoresis (5 µl loading). The correct band size is **120 bp**.

The procedure can be temporarily stopped after **Program 1**. The PCR products can be stored at 4°C for 2-3 days.

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

B. PCR Products Clean Up

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

C-Buffer = 10 µL x (_____) x 1.1 = _____ µL**
# of PCR tubes

C-UP1 = 1.0 µL x (_____) x 1.1 = _____ µL**
# of PCR tubes

C-UP2 = 1.0 µL x (_____) x 1.1 = _____ µL**
# of PCR tubes

Mix the reagents and spin down
** For pipetting error

B.2. Collect 0.2 ml strip tubes, one tube for each PCR reaction. Label the tubes the same way as the PCR tubes.

B.3. Add **12 µl** of **C-UP Mix** to each new tube.

B.4. Transfer **6 µl** of PCR products to each tube (the remaining PCR products can be stored at –20°C for re-test).

B.5. 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 clean-up incubation, prepare steps C1-C4.

C. STA Reaction (Mutation Detection)

C.1. Collect two 2 ml tubes and label one tube with "**ST12**" and the other tube with "**ST13**." Prepare ST mixes as following:

Tube ST12 - **ST12 mix** for codon 12:

NRAS ST-12 = 11 x (_____ + 1*) x 1.1 = _____µl**
# of Samples

NRAS DP-12 = 2 x (_____ + 1*) x 1.1 = _____ µl**
# of Samples

* For CTL-12 ** Adjustment for pipetting error
Add the reagents to the tube and mix gently.

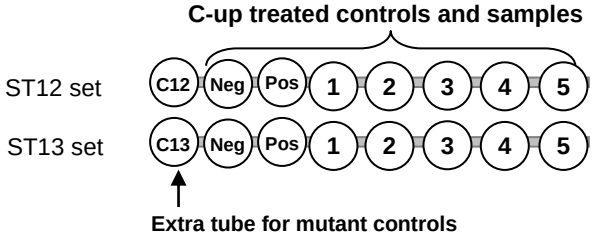
Tube ST13 - **ST13 mix** for codon 13:

NRAS ST-13 = 11 x (_____ + 1*) x 1.1 = _____µl**
# of Samples

NRAS DP-13 = 2 x (_____ + 1*) x 1.1 = _____ µl**
# of Samples

* For CTL-13 ** 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. One set for codon 12 (ST12 set) and another set for codon 13 (ST13 set). **Add an extra tube** for each set (ST12 for C12 and ST13 for 13) and label the tubes as follows:



The NRAS CTL-12 and CTL-13 must be run each time.

C.3. Transfer **13** μl of **ST12 mix** (from step C.1) into all tubes in ST12 set.

C.4. Transfer **13** μl of **ST13 mix** (from step C.1) into all tubes in ST13 set.

C.5. Add **5μl each** of C-up treated controls and samples to their corresponding tube in **both** ST12 and ST13 sets.

C.6. Add **5μl** of **CTL-12** to the **"C12"** tube.

C.7. Add **5μl** of **CTL-13** to the **"C13"** tube.

C.8. Mix the contents and spin all tubes.

C.9. Place the tubes into a thermal cycler and perform ST reaction using **Program 3**.

Program 3	
1 cycle	94°C 4 min
20 cycles	94°C 45 sec 60°C 20 sec 70°C 20 sec
	Hold at 4°C

During the STA reaction, prepare step D1-D3.

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 and remove any bubbles in the well.

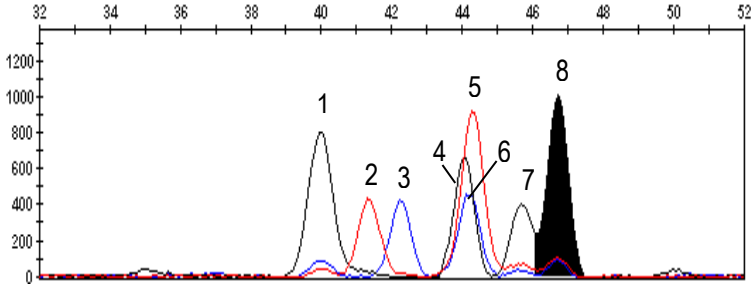
D.3. Load the plate to sequencer and run the pre-set Data Collection Program (ref. page 8).

E. Data Analysis

The NRAS Controls Codon 12 and Codon 13 represent the mutation patterns (color and size). Use these controls as standards to identify the peaks present in the test samples.

Results for NRAS Controls Codon 12

8 peaks will be presented as following:



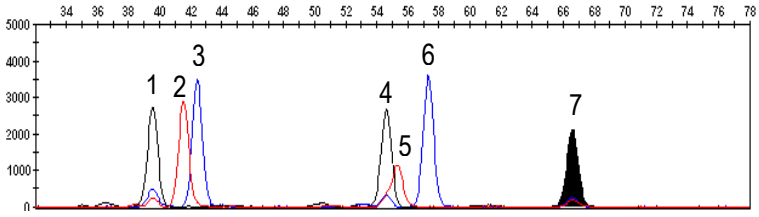
Peak #	Peak Color	Mutation	Nucleotide Change
1	Black	G12V	GGT > GTT
2	Red	G12S	GGT > AGT
3	Blue	G12R	GGT > CGT
4	Black	G12C	GGT > TGT
5	Red	G12D	GGT > GAT
6	Blue	G12A	GGT > GCT
7	Black	G12V	GGT > GTT
8	Black	Wild Type	GGT

Any peaks that do not match the peaks in this panel are to be disregarded.

Two GTT mutation peaks (#1 and #7) are shown in this panel.

Results for NRAS Controls Codon 13

7 peaks will be presented as following:



Peak #	Peak Color	Mutation	Nucleotide Change
1	Black	G13V	GGT > GTT
2	Red	G13D	GGT > GAT
3	Blue	G13A	GGT > GCT
4	Black	G13C	GGT > TGT
5	Red	G13S	GGT > AGT
6	Blue	G13R	GGT > CGT
7	Black	Wild Type	GGT

Any peaks that do not match the peaks in this panel are to be disregarded.

The pattern, size or position of the peaks may vary slightly depending on instrument, polymer type and the length of capillary. **Customer should validate the correct size for each peak by using the NRAS Controls Codon 12 and 13.**

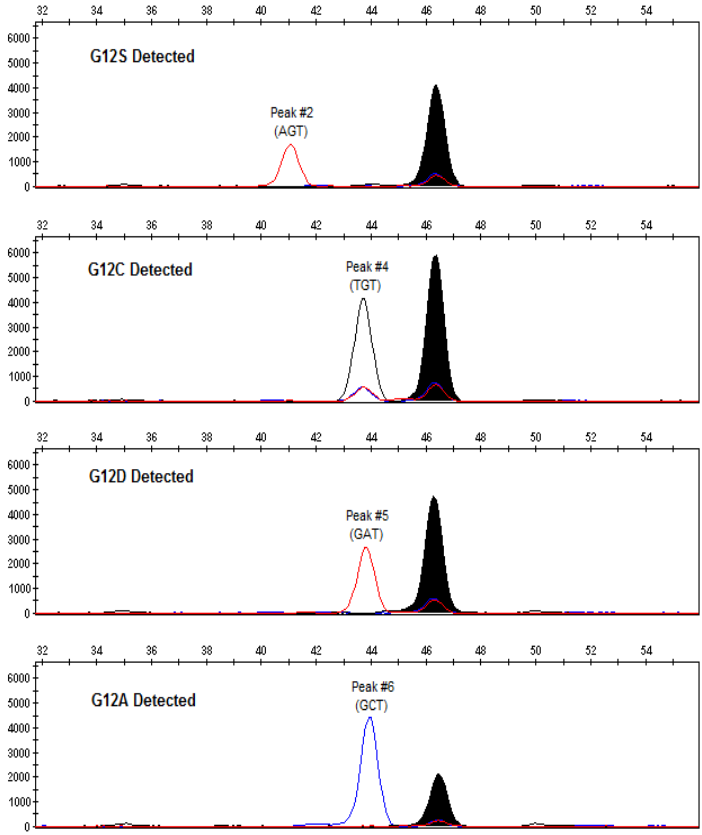
Sample Analysis

The wild type peak is a **black peak** on the right. The mutation(s) will show as additional peak(s). The peak size and color of the mutation peaks in the NRAS Controls Codon 12 and 13 are used as references to identify mutations in the sample.

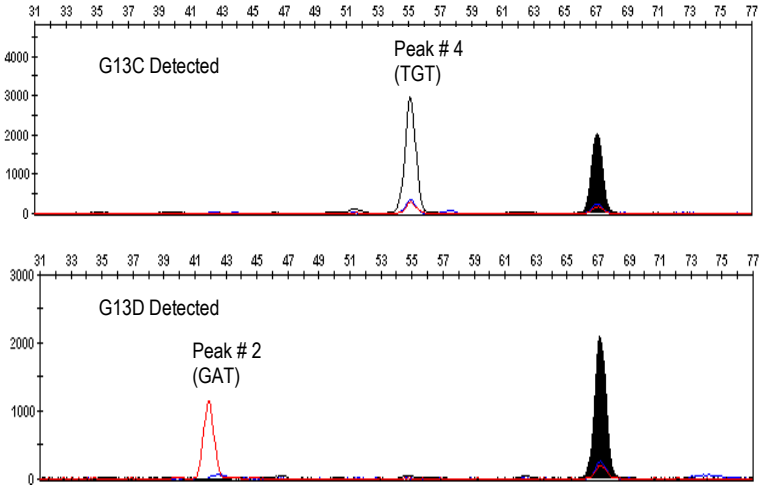
Any peaks that do not match the correct size and color are not considered as mutations.

The peak size between the control and the sample panel may slightly shifted due to migration differences between individual capillary tubes.

Example results of clinical FFPE samples for codon 12 analysis



Example results of clinical FFPE samples for codon 13 analysis



F. Troubleshooting

F.1. "Color leak-through"

When the sample DNA concentration is too high, the ST reaction generates a strong fluorescent signal >5,000 rfu. Fluorescence spillover will occur. For example, the black peak of the wild type signal may be observed in the red and/or blue channels. This color spillover is caused by limitation of the instrument. The "leak-through" peak will have the exact same peak size as the original peak. Because the mutation peaks have different peak size, leak-through will not affect data analysis.

F.2. The peak signal is too high

The assay is set at a condition to detect mutations in a small sample, such as DNA extracted from fine needle aspiration (FNA) sample. For regular FFPE sample, the assay signal may be too high to analyze (peak height >8000 rfu, cannot see the top of the peak or the peak is highlighted with pink color). Diluting the final STA product with de-ionized water can efficiently reduce the signal and optimize the peak height. Do not dilute the assay reagents, it will cause improper enzymatic reaction and generate a miss call. Each laboratory has different PCR instrument(s), the signal intensity may vary among the laboratories, first time users

should define the dilution factor (1-20 times dilution). Once the dilution factor is determined, the assay will have consistent results.

F.3. Graphic data will not automatically show

- Check the raw data. If the signals from the sample and size standards are too low, the capillary tube may be blocked by a bubble. The sample needs to be re-loaded. When adding a sample to the loading plate, carefully add the sample to avoid bubbles.
- The ST products will compete with the size standard DNA to enter the capillary tube. If the sample signal is too strong and the size standard is too low, the software cannot 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 be miscalculated. Check the size standard and manually correct the size standard (see the sequencer's instruction manual). Reanalyze the data after correction of the size standard.

F.4. No wild type peak

The wild type peak is an internal control for sample DNA amplification; this peak should show in all samples. If the peak is not observed, it indicates that the PCR amplification failed. The possible causes could be poor DNA quality, low DNA concentration and/or existence of PCR inhibitors in the DNA sample (see page 8 for DNA sample preparation section).

F.5. Background noise

Normally, the background of the assay is low. When the peak signal is too strong (over 8000 rfu and highlighted with pink color), background noise may pull-up as peak. To resolve this issue, simply dilute the final ST product with de-ionized water and re-load the sample.

F.6. A peak that does not match with any peak in Mutant Controls (CTL)

If such peaks is detected, please contact our tech support for further analysis. In some circumstances, when the sample DNA concentration is too low or the PCR did not amplify DNA properly

- an unusual peak will appear in a very different position (most of them are far from the wild type peak). Any peaks outside of the data interpretation zone (25-80 on x-axis) are not considered for analysis.

F.7. Mutation peak cut-off

For some samples, a small peak may be 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 "pull up" from background noise. Follow F.2. to dilute the final product of the ST reaction with de-ionized water. After dilution, reload the sample. If you can see the top of the wild type peak, use the following calculation to identify the small peak:

Ratio = (Area of mutant peak) / (Area of wild type peak)

If the ratio is larger than 0.06, the peak is determined to be a mutation peak (the ratio does not represent the percentage of the mutation present in the sample). Otherwise, the peak is a background pull-up and does not indicate the presence of a mutation in the sample.

F.8 "Bumper peak"

For some samples, there are peaks that show as a "bumper" (see figure below). Most of these peaks are background pull-up. The causes for the bumper peaks are over loading of the ST product. Refer to F.2. in the Troubleshooting to dilute the final ST product.