Mutector™

Mutation Detection Kit

KRAS

Mutation Analysis Reagents
(Codons 12 and 13)

User Manual V1.1

Cat No. GP05-C3

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.

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Notice to Purchaser

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When working with the kit reagents, always wear a lab coat, disposable gloves, and protective goggles. TrimGen Corporation shall not be liable for any direct, indirect, consequential or incidental damages arising out of the misuse, the results of use, or the inability to use this product.

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Information in this document is subject to change without notice.

Introduction

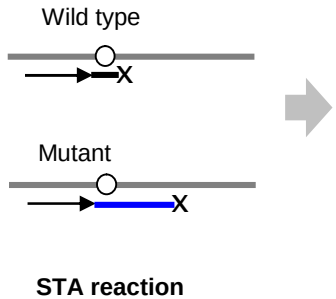
Mutector™ KRAS kit is designed to detect 12 mutations occurring in codons 12 and 13 of KRAS gene.

Codon 12	Codon 13
Gly12Ser (GGT>AGT)	Gly13Ser (GGC>AGC)
Gly12Arg (GGT>CGT)	Gly13Arg (GGC>CGC)
Gly12Cys (GGT>TGT)	Gly13Cys (GGC>TGC)
Gly12Asp (GGT>GAT)	Gly13Asp (GGC>GAC)
Gly12Ala (GGT>GCT)	Gly13Ala (GGC>GCC)
Gly12Val (GGT>GTT)	Gly13Val (GGC>GTC)

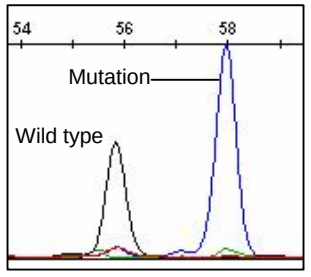
The kit uses Shifted Termination Assay* (STA) technology to enrich the mutation signal and is able to accurately detect low-level somatic mutations.

* 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



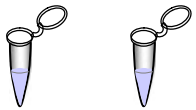
Fragment analysis

Overview of Mutector™ Assay

PCR amplification
1.5 hours*

** Time varies by thermal cycler used*

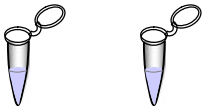
PCR product clean up
30 min



(codon 12) (codon 13)

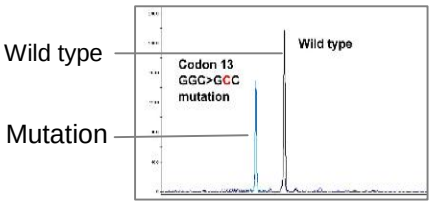
EM reaction
Mutation enrichment and detection
45 min*

** Time varies by thermal cycler used*



(codon 12) (codon 13)

EM products cleanup
5 min



Capillary Electrophoresis
Fragment analysis
30-40 min

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Materials Provided:

The Mutector™ *KRAS* Mutation Differentiation kit contains reagents for 32 tests.

Materials	Quantity	Description
Master Mix	650µl	Reagents for DNA amplification
KRAS PCR Primers	50µl	PCR primer mix for amplification of *KRAS* gene
Clean-up Enzymes	430µl	Enzyme mix for cleanup of PCR products
KRAS EM-12*	430µl	Pre-mixed reagents for enrichment and detection of codon 12mutations
KRAS EM-13*	430µl	Pre-mixed reagents for enrichment and detection of codon 13mutations
KRAS DP-12	80µl	Pre-mixed detection primers for codon 12mutations
KRAS DP-13	80µl	Pre-mixed detection primers for codon 13mutations
KRAS Control Codon 12	50µl	Mutation controls for codon 12
KRAS Control Codon 13	50µl	Mutation controls for codon 13
Loading Buffer*	1000µl x 2	Sample loading buffer with size standards
TF-50 Filters	64	Filter tip for reaction clean up
Collection Tubes	64	Collection tube for the TF-50 filter

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

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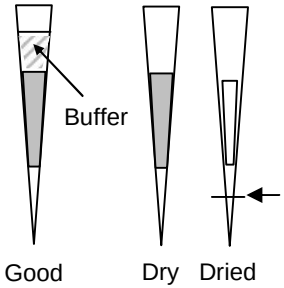
TF-50 Filter

Filter tip to remove free fluorescent dyes.



Store the filters at 2-8°C. If the buffer inside the tip evaporated, or completely dried, add 200µl deionized water, soak the gel overnight. The tip is ready to use.

Snap off-line. Before spin the filter tip, snap off bottom of the tip.

Materials required:

0.2 ml PCR tubes (8-well strip tube)

DS-32 Matrix Standard Kit for calibration of sequencer (Applied Biosystems Pat No. 4345831)

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
A kit specially designed for FFPE samples is available at TrimGen (WaxFree™ DNA, Cat. WF-50 for 50 samples, WF-100 for 100 samples).

Blood
Any commercially available DNA extraction kit is acceptable.

DNA concentration adjustment:
When using a column or bead DNA extraction method, adjust the final concentration of extracted DNA to **20-80 ng/«l**.

The DNA concentration calculated using classical UV method (measure OD) may give incorrect DNA concentration.
A fluorescent method that directly measure intact DNA, such as pico green method, is recommended.

When using TrimGen's FFPE DNA preparation kit, follow the 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 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.

KRAS Controls Codon 12 (C12) and **KRAS Controls Codon 13 (C13)** are required for each test run. A negative control (water) is recommended to run with the samples each time.

A.1. Prepare PCR Reaction Mix:

Master Mix = 18 x (_____ + 3*) x 1.1 = _____ «l**
# of Samples

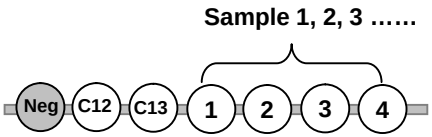
KRAS PCR Primers = 1 x (_____ + 3*) x 1.1 = _____ «l**
# of Samples

*For negative and KRAS controls (C12 & C13).

** 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 (water)
C12: KRAS Controls Codon 12
C13: KRAS Controls Codon 13

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

A.4. Add **2«l** of nuclease-free water to the "**Neg"** tube.

A.5. Add **2«l** of **KRAS Controls Codon 12** to the "C12**"** tube.

A.6. Add **2**«**l** of **KRAS Controls Codon 13** to the "C13**"** tube.

A.7. Add **1-2**«**l*** 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**«**l*** final extract to each sample tube.

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

A.8. 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 product can be verify by agarose gel (5 μl loading). The correct band size is **120 bp**.

STOP 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, prepare step B1-B2.

B. PCR Products Clean Up

B.1. Collect 0.2 ml strip tubes, one tube for each PCR reaction. Label the tubes the same as the PCR tubes.

B.2. Add **11**«**l** of **Clean-up Enzymes (C-UP)** to each new tube.

B.3. Transfer **4**«**l** of PCR products to each tube (the remaining PCR products can be stored at –20°C for re-test).

B.4. Mix the contents and spin all tubes.

B.5. 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 incubation, prepare step C1-C4.

C. Mutation Enrichment and Detection (EM reaction)

C.1. Collect two 2 ml tubes and label one tube with "**EM12**" and the other tube with "**EM13**." Prepare EM mix as follows:

Tube EM12 - **EM12 mix** for codon 12:

KRAS EM-12 = 11 x (_____ + 2*) x 1.1 = _____«l
# of Samples

KRAS DP-12 = 2 x (_____ + 2*) x 1.1 = _____ «l
# of Samples

* For Controls
Add the reagents to the tube and mix gently.

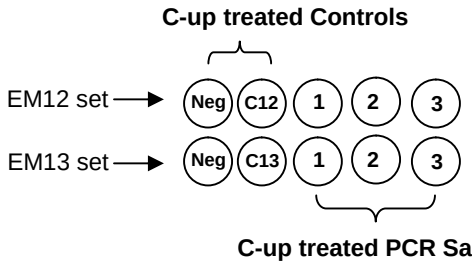
Tube EM13 - **EM13 mix** for codon 13:

KRAS EM-13 = 11 x (_____ + 2*) x 1.1 = _____«l
# of Samples

KRAS DP-13 = 2 x (_____ + 2*) x 1.1 = _____ «l
# of Samples

* For Controls
Add the reagents to the tube and mix gently.

C.2. Collect 0.2 ml strip tubes (2 tubes per sample and 4 control tubes for each test run). Label the tubes as follows: One set for codon 12 (EM12 set) and another set for codon 13 (EM13 set).



C.3. Transfer **13**µl of **EM12 mix** (from step C.1) into all tubes in EM12 set.

C.4. Transfer **13**µl of **EM13 mix** (from step C.1) into all tubes in EM13 set.

C.5. Add **2«l** of C-up treated Negative PCR control to the **"Neg"** tube in **both** EM12 and EM13 sets.

C.6. Add **2«l** of C-up treated **C12** (from step B.5) to the **"C12"** tube.

C.7. Add **2«l** of C-up treated **C13** (from step B.5) to the **"C13"** tube.

C.8. Add **2«l** of C-up treated Sample (from step B.5) to each corresponding sample tube in **both** EM12 and EM13 sets.

C.9. Mix the contents and spin all tubes.

C.10. Place the tubes into the thermal cycler and perform EM 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 EM reaction, prepare step D1-D3.

D. EM Product Clean-up

Filter Tip Preparation

D.1. Collect the **TF-50 Filter Tips** and **Collection Tubes** (one set for each EM reaction).

D.2. Snap off the bottom portion of the filter tip (ref. page 7 for snap off-line) and put tip back to collection tube.

D.3. Centrifuge the **TF-50 Filter Tips** at 1,000 x *g* (3000 rpm for most tabletop centrifuge) for 2-3 minutes to remove the buffer from the filters.

D.4. Discard the Collection Tubes and move the **TF-50 Filter Tips** into a new Collection Tube. Label the Collection Tubes with sample ID. The **TF-50 Filter Tips** are ready for use.

D.5. After the EM reaction, load entire 15µl reaction mix onto the top of gel in each of pre-prepared **TF-50 Filter Tips**.

D.6. Centrifuge the **TF-50 Filter Tips** at 1,000 x *g* (3000 rpm for most tabletop centrifuge) for 2-3 minutes.

D.7. Discard the **TF-50 Filter Tips**. The solution in the collection tubes contains cleaned EM reaction products and is ready for sample loading.

E. Sample Loading

E.1. Add **15µl** of the **Loading buffer** to each well of a sequencer adapter plate.

E.2. Transfer **2-4µl** of the filtered EM products into each well.

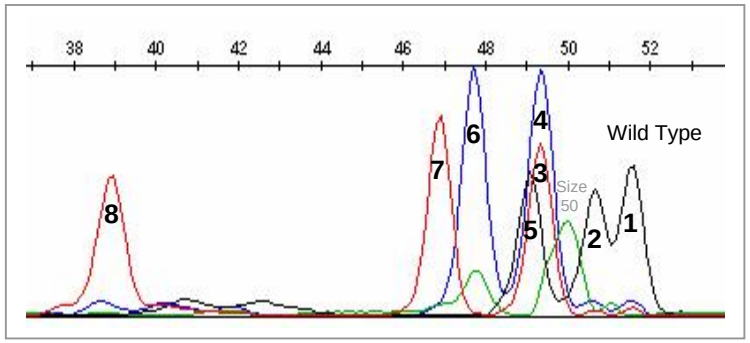
E.3. Load the plate to sequencer and run the pre-set Data Collection Program (ref. page 8).

F. Data Analysis

The KRAS 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 KRAS Controls Codon 12

8 peaks will be presented as follows:



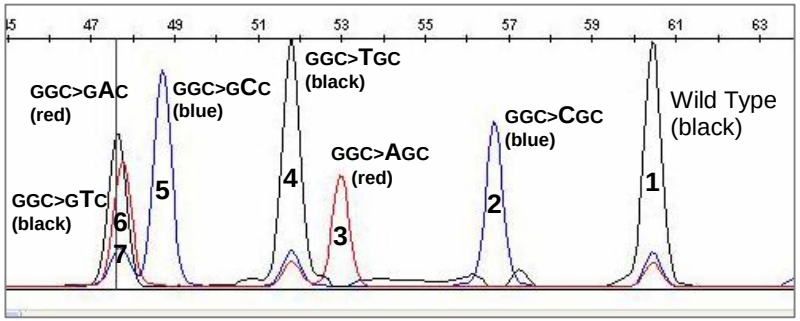
#	Peak Color	Interpretation
1	Black	Wild type
2	Black	Mutation GGT>GTT
3	Red	Mutation GGT>GAT
4	Blue	Mutation GGT>GCT
5	Black	Mutation GGT>TGT
6	Blue	Mutation GGT>CGT
7	Red	Mutation GGT>AGT
8	Red	Mutation GGT>GTT

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

Two GTT mutation peaks are shown in this panel: one peak (#2) is next to and has the same color as the wild type peak. When the resolution of capillary or polymer is low, the mutant peak may merge with the wild type peak, which makes it difficult to identify the mutation. To overcome this issue, a special primer is designed to generate the other peak (#8) with a different size and color.

Results for KRAS Controls Codon 13

7 peaks will be presented as follows:



Read the peaks from right, and write the peak size in table below:

#	Peak Color	Interpretation
1	Black	Wild type
2	Blue	Mutation GGC>CGC
3	Red	Mutation GGC>AGC
4	Black	Mutation GGC>TGC
5	Blue	Mutation GGC>GCC
6	Red	Mutation GGC>GAC
7	Black	Mutation GGC>GTC

Any peaks that not match the peaks in this panel are 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 KRAS Controls Codon 12 and 13.**

Sample Analysis

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

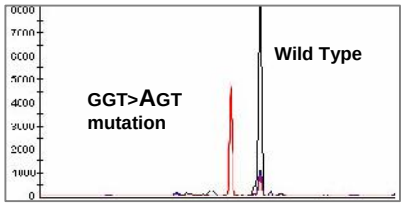
Any peaks that do not match the correct size and color are not considered as mutations.

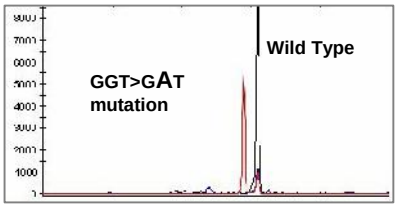
The peak size between the control and the sample panel may slightly shifted due to migration differences between individual capillary tubes.

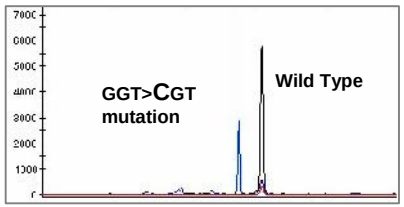
Example

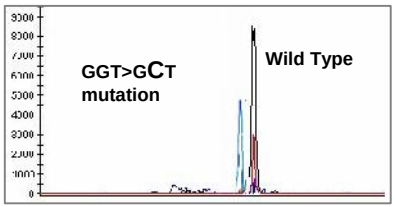
The following data derived from Genetic Analyzer 3130 using 36-cm capillary array and POP-7 polymer.

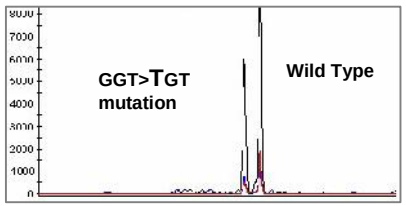
Mutations in Codon 12

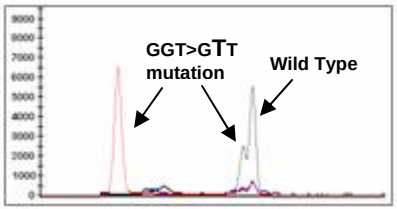












Mutations in Codon 13