Mutector II

Warfarin

Genotyping kit

(Cat No. GP03)

User Manual V1.4

Version 1.4

Storage

Upon receipt of the kit, store at –20°C until use. At this temperature the reagents are stable for 1 year.

After first use, store all of the reagents at 2-8°C and keep them protected from direct light. At this condition the reagents are stable for 3 months.

For research use only, not for use in diagnostic procedures

Technical Support:1-888-825-6005

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Limited Product Warranty

It is imperative that the users strictly adhere to this manual. Failure to do so will void TrimGen's guarantee of this product. TrimGen Corporation makes no other warranties of any kind, expressed or implied, including without limitation, warranties of merchantability or fitness for a particular purpose.

Notice to Purchaser

The Mutector IITM kit is provided as a research use only product. The purchaser must determine the suitability of the product for their particular use. No claim or representation is intended for use of this product to identify any specific organism or for a specific clinical use (diagnostic, prognostic, therapeutic, or blood banking).

The purchase of Mutector IITM kit includes a limited, nonexclusive license to use the kit. This license does not grant rights to reproduce or modify the Mutector IITM kit for resale, or to use the Mutector IITM kit to manufacture commercial products without written approval of TrimGen Corporation. No other license, expressed, implied or by estoppels is granted.

Product Safety and Liabilities

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.

Introduction

The Mutector™ II Warfarin genotyping assay is a single tube test designed for identifying the following single nucleotide polymorphisms (SNPs):

CYP2C9*2	C430T
CYP2C9*3	A1075C
VKORC1	-1639 G>A

The assay uses TrimGen's proprietary technology called Shifted Termination Assay (STA). The STA technology accurately detects single nucleotide variation through multiple steps: (1) Sequence-specific amplification of target gene (2) Sequence-selective termination of target nucleotide and (3) Sequence-dependent primer extension.

The genotypes are easily differentiated by fragment size and colors to give clear-cut results.

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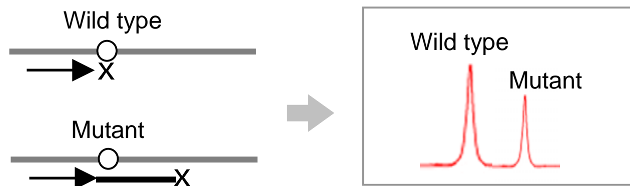
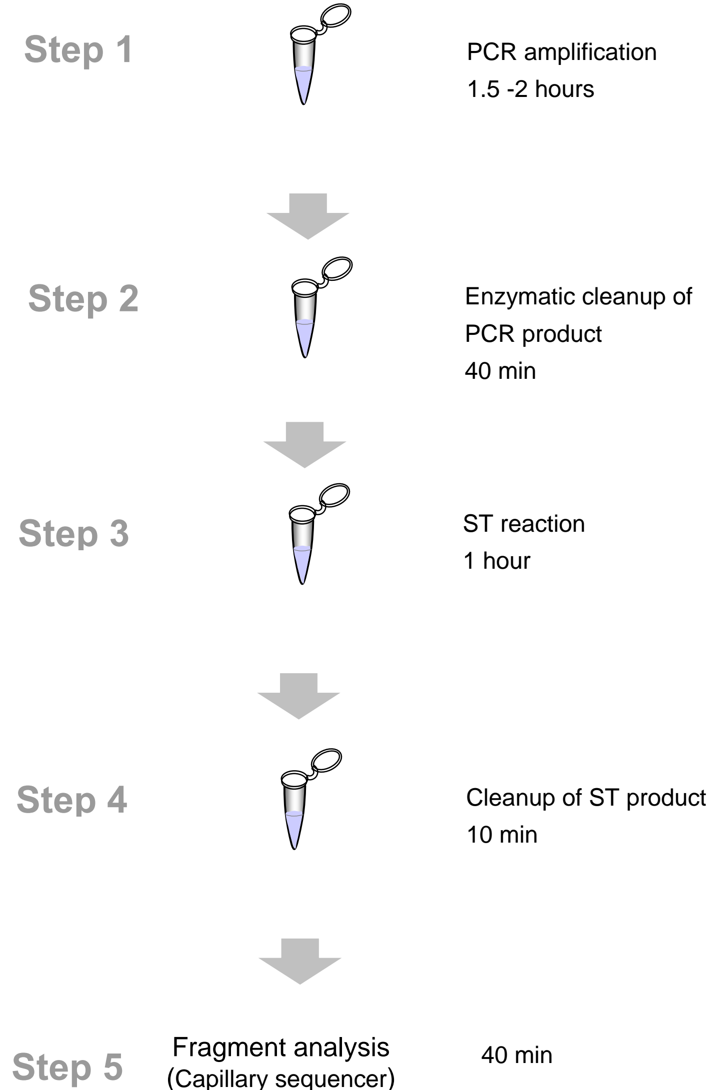
* Shifted Termination Assay (STA)

Shifted Termination Assay is a proprietary technology that uses a combination of uniquely designed primers, mixtures of modified enzymes and specially synthesized nucleotides. The STA reaction recognizes wild type or mutant target sequences and selectively terminates or extends the detection primers with 1 to 20 nucleotides. This extension is repeated 20 times with labeled nucleotides to enrich the detection signal. The enriched signals are then easily detected by fragment analysis.



Overview of Mutector II™ Detection



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Materials Provided:

The Mutector II™ Warfarin Kit contains reagents for 32 tests.

Materials	Cap label	Quantity
PCR Primer Mix	**PCR-P**	**50 µl**
Master Mix	**MM**	**1000 µl**
E1 Mix	**E1**	**430 µl**
ST- WR*	**ST**	**430 µl**
DP- WR	**DP**	**80 µl**
CTL- WR	**CTL**	**50 µl**
Loading Buffer*	**LB**	**1200 µl**
TF - Filters	N/A	**32**
Collection Tubes	N/A	**32**

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

Reagents Description:

PCR Primer Mix
PCR primer mix for amplification of VKORC1 and CYP2C9 gene.

Master Mix
Pre-mixed reagents for PCR amplification.

E1 Mix
Enzyme mix for cleanup of PCR products.

ST-WR (Light sensitive)
Pre-mixed reagents for signal enrichment and detection.

DP-WR
Pre-mixed detection primers.

CTL-WR
Pre-mixed genotype control DNA.

Loading Buffer (Light sensitive)
Contains the loading buffer for ABI capillary type sequencers and special fluorescent-labeled size standards.

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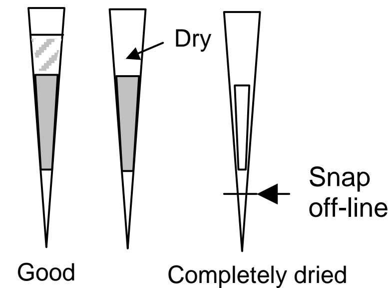
TF-50 Filter
TF-50 filter is a tip filter designed to remove free fluorescent dyes from the reaction mixture.



Store the tip filter in 2-8°C.
If the buffer on top of the gel evaporates (Dry, see picture on left), add 100-150µl deionized water to re-hydrate the gel. If the gel is completely dried (white in appearance), it is necessary to soak the gel overnight after adding water.

Before use, snap off the tip at the position of Snap off-line.

Materials required:

0.2 ml PCR tubes (8-well strip tube)

DS-32 Matrix Standard Kit for the 3100 and 3130 Series Systems (one time set up. Applied Biosystems Cat. No. 4345831)

Equipment required:

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

Sequencer:
Applied Biosystems capillary type Genetic/DNA Analyzer

Analysis Software:
Data Collection® software for ABI capillary sequencer
GeneMapper® for fragment analysis or GeneScan®

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DNA Sample Preparation:

Reagents for DNA preparation are not provided with the Mutector™ II kit.

Paraffin (FFPE) and fresh or frozen tissue samples
A kit specially designed for FFPE sample DNA extraction 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:
When using commercial DNA extraction kit for DNA extraction, adjust the final concentration of extracted DNA to **20-80 ng/µl**.
When using TrimGen's 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™ II tests for data analysis. Please choose either GeneMapper® or GeneScan® to analyze your data.

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

GeneScan® Analysis

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

Step II. GeneScan® Setup and Data Analysis
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 correct spectral channels to read 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	95°C 5 min
35 cycles	94°C 30 sec 52°C 45 sec 72°C 45 sec
1 cycle	72°C 5 min
	Hold at 4°C

Program 2 (E1 treatment)

	37°C 30 min 95°C 5 min
	Hold at 4°C

Program 3 (ST reaction)

1 cycle	94°C 4 minute
20 cycles	94°C 30 sec 50°C 45 sec 70°C 30 sec
	Hold at 4°C

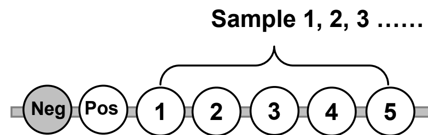
Mutector II™ Assay Protocol:

A. PCR Amplification

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

A blank control (water) and a positive control (your sample control) is suggested to run with samples each time.

A.1. Collect and label PCR tubes or a 96-well PCR plate as follows:



Neg: Negative control (water)
Pos: Positive control (CTL-WR)

A.2. Add **26**μl of Master mix into each tube.

A.3. Add **1**μl of PCR primer mix (PCR-P) to each tube.

A.4. Add **3**μl of nuclease-free water to the "**Neg**" tube for negative control.

A.5. Add **3**μl of CTL-WR to the "**Pos**" tube for positive control.

A.6. Add **3**μl of sample DNA (20-80 ng/μl) to correspondent tube.

A.7. Cap the tubes and place the tubes to thermal cycler

A.8. Run **Program 1**

Program 1	
1 cycle	95°C 5 min
35 cycles	94°C 30 sec 52°C 45 sec 72°C 45 sec
1 cycle	72°C 5 min
	Hold at 4°C

Note:

✓ Option: To verify the PCR product, load 5 μl of the PCR product onto 1-2% agarose gel and use 100 bp size markers to confirm the PCR products. The three PCR product sizes are:

VKORC1 = 167 bp
CYP 2C9*2 = 174 bp
CYP2C9*3 = 221 bp.

✓ The procedure can be temporarily stopped after **Program 1**. Store the PCR product in 4°C for next day testing.

B. PCR Product Clean-up

B.1. Collect **new** 0.2 ml tubes (One tube for each PCR reaction). Label the tubes the same as the PCR tubes.

B.2. Add **11 μl** of **E1 Mix** to each new tube.

B.3. Transfer **4μl** of PCR product to each tube. (The remaining PCR products can be stored at –20°C for re-testing).

PCR product may cause lab contamination. To avoid the PCR product contamination, use the filter tip for the pipetting, handle with care and perform this step in a designated area. After transfer, clean-up the work area and change gloves.

B.4. Cap the tubes, mix the contents and spin all tubes.

B.5. Incubate the tubes in thermal cycler using **Program 2**.

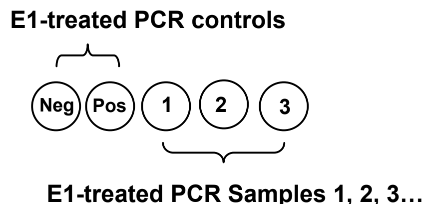
Program 2
37°C for 30 min
95°C for 5 min
Hold at 4°C

Note:

✓ The procedure can be temporarily stopped after Program 2. Store the reaction tube in 4°C for next day testing.

C. ST Reaction:

C.1. Collect 0.2ml strip tubes (1 tube per sample and 2 control tubes for each test run). Label the tubes as follows:



C.2. Add **11**µl of **ST-WR** into all tubes.

C.3. Add **2**µl of **DP-WR** into all tubes.

C.4. Add **2**µl of E1-treated Negative PCR control to the **"Neg"** tube.

C.5. Add **2**µ**l** of E1-treated Pos to the **"Pos"** tube.

C.6. Add **2**µ**l** of E1-treated Sample to each corresponding sample tube.

C.7. Cap the tubes, mix the contents and spin all tubes.

C.8. Place the tubes into thermal cycler and perform ST reaction using **Program 3**.

Program 3	
1 cycle	94°C 4 min
20 cycles	94°C 30 sec 50°C 45 sec 70°C 30 sec
Hold at 4°C	

✓The procedure can be temporarily stopped after Program 3. Store the reaction tube at 4°C for next day testing.

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D. ST Product Clean-up

Filter preparation

D.1. Collect the **TF-Filters** and **Collection Tubes** (one set for each ST reaction).

D.2. Snap off the bottom portion of the filter tip (ref. page 7 for snap off-line).

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

D.4. Discard the Collection Tubes and transfer the TF-Filters into a new Collection Tube. Label the Collection Tubes with sample ID. The TF-Filters are ready for use.

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D.5. After the ST reaction, load all ST reaction contents (15µl) onto the top of the gel in each pre-prepared **TF-Filter**.

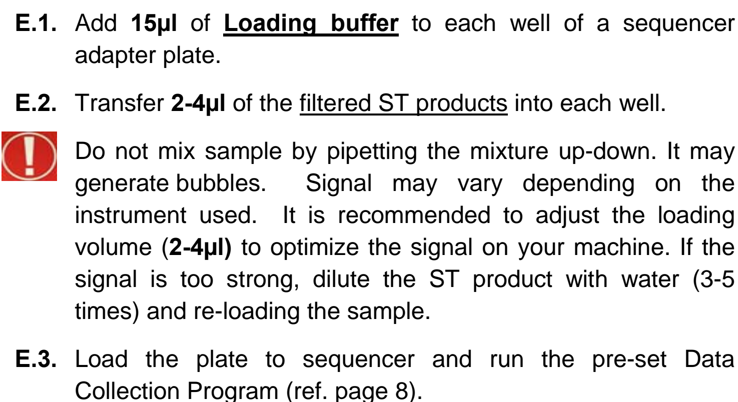
D.6. Centrifuge the **TF-Filters** at 1,000 x *g* (2,000-3000 rpm for most tabletop centrifuge) for 2-3 minutes.

D.7. Discard the **TF-Filters**. The solution in the tubes contains ST product and is ready for sample loading.

E. Sample Loading

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

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



Do not mix sample by pipetting the mixture up-down. It may generate bubbles. Signal may vary depending on the instrument used. It is recommended to adjust the loading volume (**2-4µl)** to optimize the signal on your machine. If the signal is too strong, dilute the ST product with water (3-5 times) and re-loading the sample.

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

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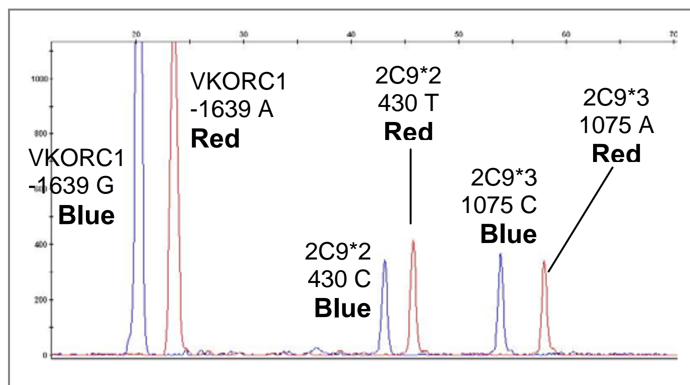
F. Data Analysis

The CTL-WR (Genotype controls) shows all genotypes (color and size). Use these controls as a standard to identify peak(s) present in the samples.

Results for mutation controls



#	Peak Color	Peak Size*	Interpretation
1	Blue	**20.27**	**VKORC1 –1639 G**
2	Red	**23.54**	**VKORC1 –1639 A**
3	Blue	**43.09**	**2C9*2 - 430C**
4	Red	**45.75**	**2C9*2 - 430T**
5	Blue	**53.86**	**2C9*3 - 1075C**
6	Red	**57.96**	**2C9*3 - 1075A**

*The data were produced using POP7 and 36 cm capillary. The peak size may vary slightly depending on instrument, polymer type and the length of capillary. **Customer can confirm the correct peak size using the CTL-WR.**

Notes: