GenVinSet

MTHFR C677T

Instructions for Use

Kit for detection of MTHFR C677T polymorphism

For In Vitro diagnostic use

Reference GVS-MTHFR-24 (24 tests)
GVS-MTHFR-48 (48 tests)

Store from –18 to –30ºC

CE

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Intended use

GENVINSET MTHFR C677T is a kit for the determination of the C->T change at position 677 of the exon 4 of the MTHFR gene by Real Time PCR using specific TaqMan® probes technology.

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Summary and explanation

Methylenetetrahydrofolate reductase is a key enzyme in folate metabolism that catalyzes the conversion of intracellular 5,10-methylenetetrahydrofolate (5,10-methylene THF) to 5-methyltetrahydrofolate (5-methyl THF), the predominant circulatory form of folate and primary methyl donor for the remethylation of homocysteine to methionine. Although MTHFR protein does not participate in the clotting cascade as other proteins such as FII and FV (and therefore belonging to the well-known group of coagulation factors), the involvement of MTHFR in the folate metabolic pathway may affect thrombophilia development, as well as increased cancer risk or Alzheimer's disease appearance (1 – 4).

The mechanism through which MTHFR protein is involved in thrombophilia development is not completely understood. It has been suggested that the inability of the folate metabolism to continue due to the lack of precursors leads to the accumulation of homocysteine in blood. This metabolite could develop damage in the innermost layer of blood vessels, the endothelial cells, and thus triggering the clot formation. Performed studies show that there is an increased likelihood of forming blood clots when having an elevated homocysteine level. These clots might break away from the wall of the vessels and travel to main organs, leading to potentially fatal complications such us a heart attack or a pulmonary embolism (5,6).

The 5,10-methylenetetrahydrofolate reductase (MTHFR) gene contains a 2.0 kb coding region with 11 exons, and is located on chromosome 1p36.3. The gene product of MTHFR is a 77-kd protein of the same name (EC 1.5.1.20) (1,7). Approximately 60 polymorphisms, as well as 41 rare but deleterious mutations have been described in the MTHFR gene (8). The most common functional variant and most studied to date is the thermolabile MTHFR C677T polymorphism (rs1801133). The C677T variant is a C to T transition in exon 4 at nucleotide 677, resulting in the conversion of alanine to valine at position 222 of the MTHFR amino acid sequence (3,4). Heterozygotes (CT) and homozygotes (TT) for the C677T variant have respectively 65% and 30% of the MTHFR enzyme activity observed in homozygous wild type subjects (CC) (9). As a result, TT homozygotes have been associated with lower serum folate levels and higher homocysteine levels than their wild type homozygous counterparts (10-12).

The frequency of MTHFR C677T varies by ethnicity. The prevalence of the TT genotype ranges from 8–18% in Caucasians, 11–16% in Asians, 21–25% in Latinos, and 0–6% for those of African descent (13).

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Procedure principles

The detection method used by GENVINSET MTHFR C677T is based in a primer specific PCR, which anneal to position 677 of the exon 4 of the MTHFR gene monitored with TaqMan® probes for A and G nucleotides.

This technique provides high resolution, high sensitivity, specificity and reproducibility.

(*) See Section "Procedure Limitations"

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Kit contents

GVS-MTHFR-24 (24 tests)

- GVS-MTHFR-PM: Primer Mix (PM).
- GVS-MTHFR-MM: Master Mix (MM).
- GVS-MTHFR-C1: Control WT/WT (C1).
- GVS-MTHFR-C2: Control MUT/MUT (C2)
- GVS-RB: Reaction Blank (RB).

GVS-MTHFR-48 (48 tests)

- GVS-MTHFR-PM: Primer Mix (PM).
- GVS-MTHFR-MM: Master Mix (MM).
- GVS-MTHFR-C1: Control WT/WT (C1).
- GVS-MTHFR-C2: Control MUT/MUT (C2)
- GVS-RB: Reaction Blank (RB).

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Kit storage

All of the kit's reagents should be stored from -18ºC to -30ºC and they are stable at this temperature until its expiration date, as indicated in the vials. Do not perform more than 3 freezing/thawing cycles to the Primer Mix (GVS-MTHFR-PM) and Master Mix (GVS-MTHFR-MM) vials as this could reduce the assays sensitivity and affect results.

Due to the reagent's photo sensitivity nature, avoid continuous exposure to light.

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Materials required but not supplied

General

- Gloves
- Lab coat

Consumables

- Filter tips (P1000, P200 & P10).
- 1.5 ml autoclaved tubes
- RT-PCR instrument specific reagents (in the case of using RotorGene Q, only 0.1 ml tubes allowed).

Equipment

- RT-PCR instrument. The next devices have been validated:
  - StepOne™, Applied Biosystems™
  - 7500 Real-Time PCR System, Applied Biosystems™
  - LightCycler® 96 System, Roche
  - LightCycler® 480, Roche
  - Rotor-Gene® Q, Qiagen®
- Vortex
- Pipettes (P1000, P200 & P10)

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Sample collection and preparation

This test should only be performed with complete blood samples treated with EDTA anti coagulation agents or citrate. Heparin can interfere with the PCR process and should not be used in this procedure.

The technique is compatible with several DNA extraction methods. Before delivering results with a diagnostic purpose, perform a validation assay with such extraction method.

Caution

All biological and blood samples should be treated as possibly infectious. When manipulating them, observe all basic (universal) precautions. All sample manipulation should be done with gloves and appropriate protection.

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Procedures

A) PCR preparation

Precautions

- Thaw all of the kit components before starting the assay, mix and centrifuge them.
- Work over ice or a cold block.
- The PCR should be assembled in the Pre-PCR area.
- Use only filtered tips and 1.5 ml autoclaved tubes.
- Use gloves and lab coat at all times.
- In each test performed it is recommended to use one control sample for G/A variants.

1. Take the samples out of the freezer. Prepare the master mix and primer mix to n+1 samples:

	Vol. per sample (µL)
Master Mix	5
Primer Mix	4

2. Pipette 9 µL of this mix on the capillaries over the cold holder y add 1µL of DNA or Reaction Blank in the case of the control of contamination well.

3. Seal the plate with convenient sealer, spin down the volume by centrifuging 1 min 360xg.

4. Place the plate in the thermal cycler and start the following cycle.

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B) Thermal cycler configuration

1. Set up the next amplification program:

	Cycle number	Temperature (ºC)	Time (mm:ss)	Ramp (%)	Analysis
Desnaturalizatión	1	95	05:00	100	X
Cycles		95	00:15	100	X
		64	1:00	100	Single
Cooling	1	15	∞	100	X

2. Set up the reading channels

The emitted fluorescence must be read in FAM (495-520 nm) and HEX (535-554 nm) channels.

NOTE – Special settings for Rotor Gene Q:

a. Open the Rotor-Gene Q – Pure Detection software. Select the tab "Advanced" in the window New Run, and click "New".

b. Select the type of rotor used (only 0.1 ml tubes accepted, see section 6). Select the "Locking Ring Attached" box and continue by clicking "Next".

c. Type the "Reaction Volume" as 10 ul, and identify the operator and the sample.

d. Click "Edit Profile" and set up the amplification program (see section 8.C.1). Select the step 60 sec at 64 ºC, and click "Acquiring to Cycling A". Select the channels for fluorescence acquisition "Green" and "Yellow". Then "OK". Click "OK" to accept and close the "Edit Profile" window.

e. Click "Gain Optimisation" in the "Run New Wizard" dialog box to open the "Auto-Gain Optimization Setup" window. In the scroll menu of "Channel Settings" select "Acquiring Channels" and then "Add". In the window "Auto-Gain Optimisation Channel Settings", set the following parameters for each channel ("Green" and "Yellow"):

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Tube position = 1

Target Sample Range: 5 FI up to 10 FI

Acceptable Gain Range: -10 to 10

f. Activate the option "Perform Optimisation Before 1st Acquisition", and click "Close".

g. Select "Next" and then "Start Run" in the "New Run Wizard" window.

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Results

GENVINSET C677T MTHFR is a qualitative technique to identify presence of C and/or T at position 677 of the exon 4 of the MTHFR gene.

It is not necessary to select any passive reference.

The results of this technique can be obtained as follows:

Detection of T at position 677

Selecting FAM channel in Amplification Plot, we can see next graphic:

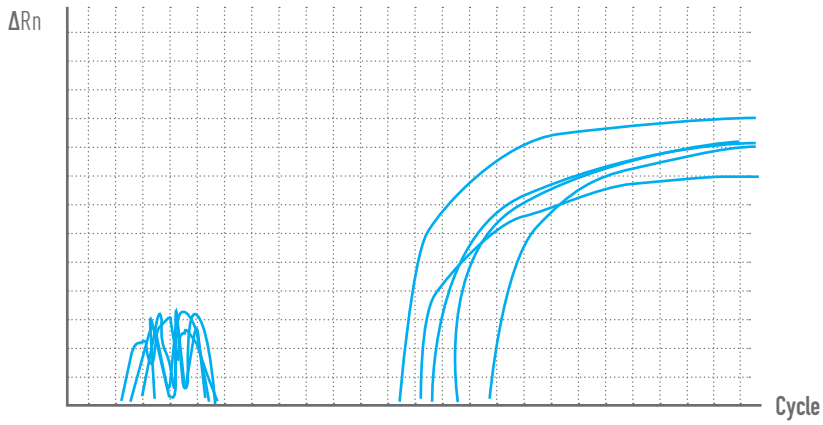

Figure 1. FAM channel results of positive and negative samples.

Samples generating an amplification curve are 677 T positive and they are identified by a numeric value called Crossing Point (Cp) corresponding with the cycle in that fluorescence can be detected.

Detection of C at position 677

Selecting VIC/HEX channel in Amplification Plot, we can see the next graphic:

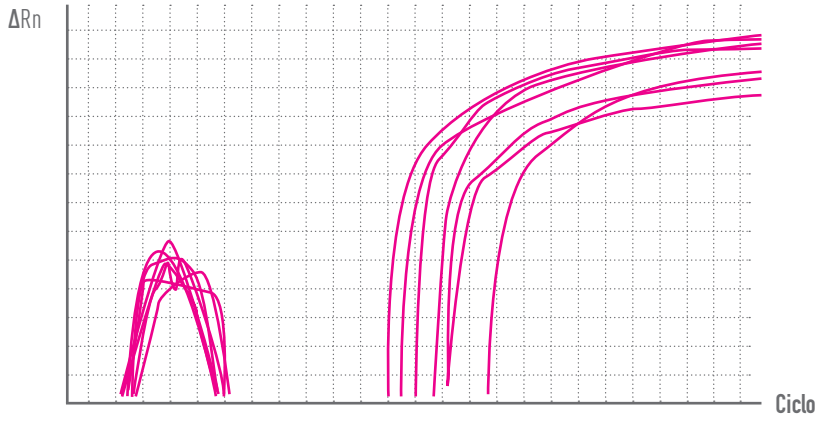

Figure 2. HEX channel results of positive and negative samples.

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Samples generating an amplification curve are 677 C positive and they are identified by a numeric value called Crossing Point (Cp) corresponding with the cycle in that fluorescence can be detected.

Genotyping analysis

In Genotyping or Allelic Discrimination analysis types, select FAM channel (mutation) in Y axis, and HEX channel (wildtype) in X axis. The results will be shown similarly to those in Figure 2, as dots with different X and Y components. The different kind of samples, wt/wt, wt/mut and mut/mut, will be distributed into 3 groups within the plot. The Reaction Blank will be placed near the origin of coordinates (0,0).

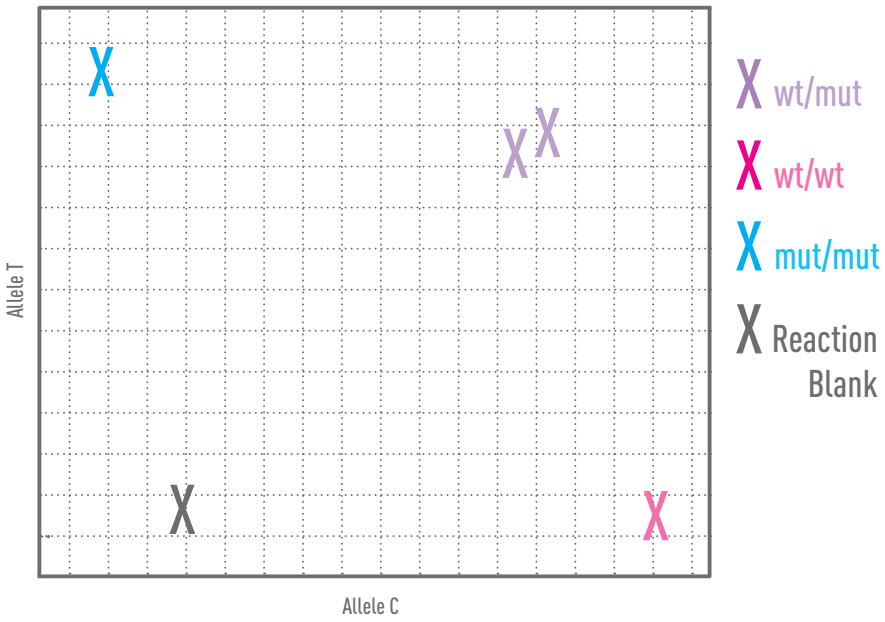

Figure 3. Plot showing one heterozygous sample (wt/mut), one normal sample (wt/wt) and one homozygous mutated sample (mut/mut) for C677T mutation, using Genvinset MTHFR C677T kit. Graphic distribution according to detected alleles.

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Quality control

Due to the qualitative nature of this test, it will not be necessary to perform a calibration.

The following criteria should be known for the assay to be considered valid:

- The Reaction Blank must perform negative results both for C and T at position 677. Cp (Crossing Point) values>35 would be considered as negative result. Cp values <35 inform about a contamination in the session so results should be discarded.
- A heterozygous sample must perform positive results for both C/T.

The assay must be made according to the kit recommendations, as well as other quality control procedures that comply with local, federal and/or certifying agencies specifications.

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Specific operation data

1. Analytical specificity

The alignment of primers and probes in the region surrounding position 677 of the exon 4 of the MTHFR gene has revealed the absence of non-specific bindings. No cross-reaction phenomena with genomic DNA have been reported.

2. Analytical sensitivity

A dilution assay was performed, using 1:4 serialized dilutions of a DNA sample normal for C677T mutation (no mutated alleles) and other one heterozygous for C677T (one mutated allele), obtained by a conventional extraction system, at an initial concentration of 32.80 and 50.00 ng/µL respectively. The following results were obtained for analytical sensitivity:

- Detection Limit of normal and mutated allele = 0.19 ng/µl (*)

(*) Cp < 35

3. Diagnostic sensitivity and specificity.

In a study of human genomic DNA, 138 samples obtained from a laboratory were analyzed. They were previously genotyped by another commercial kit.

Of the 138 samples tested, all of them were validated:

GENVINSET MTHFR C677T		C/C	C/T	T/T
Previous method	C/C	27	0	0
	C/T	0	79	0
	T/T	0	0	32

There is a 100% match in the results obtained with GENVINSET MTHFR C677T and the genotyping previously obtained with another commercial kit.

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Procedure limitations

- The method detects the C/T SNP at position 677 of the exon 4 of the MTHFR gene.
- The described conditions for the PCR should be precisely controlled. Deviations from these parameters can lead to poor results.
- All GENVINSET components manipulation must be done according to general lab best practices and be adjusted to local regulations.
- The RT-PCR thermal cycler must be calibrated according to the manufacturers´ recommendations y should be used in accordance to manufacturers' instructions.
- Do not mix components from other kits or lot numbers.
- Do not use the kit after its expiration date.
- Do not use the kit if there are suspicions of possible loss of reactivity, contamination, container deterioration or any other incidence that might affect the kits performance.
- Data and result interpretation should be revised by qualified personnel.
- Eliminate expired reagents according to applicable regulations.

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Troubleshooting guide

Problem

- **Probable cause(s)**
  - Suggested corrective measure(s)

Reaction Blank (H_2O) is positive

- **Primer Mix/Master Mix/Reaction Blank contamination**
  - Repeat the experiment with new Primer Mix/Master Mix/ Reaction Blank aliquots
  - Perform the kit components manipulation always according to accepted practices to avoid contamination.
  - Verify manipulation and storage conditions.
  - Discard contaminated reagents
- **Pre-PCR area is contaminated**
  - Confirm that all necessary precautions in the PCR area have been followed
  - Check for possible contamination problems in other PCR techniques
  - Confirm suitability of the used reagents (1.5 ml tubes, pipette tips)
- **Pipetting error**
  - Always check that the added sample matches the sample sheet

Low or no signal in all samples. Control samples are OK

- **Bad quality of DNA**
  - Repeat the sample extraction verifying each step (Hemoglobin can interfere with the PCR)
- **Blood processed without previous frozen step**
  - Repeat extraction with a new blood aliquot previously frozen.
- **Samples with very low DNA concentration**
  - Check DNA concentration.
- **DNA samples with high concentration**
  - Perform a DNA extraction method validation assay testing some dilution of the DNA samples.

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Fluorescence intensity too low

- **Kit degradation**
  - Confirm the kits correct storage (Primer Mix vial stored in darkness)
  - Avoid more than 3 freeze/unfreeze cycles of the primer mix vial
  - Aliquote the reagents if necessary
  - Repeat the series with new reagents

Control C1 is positive

- **Cross contamination**
  - Handle the kit's components always with all currently contamination avoidance practices
- **Pipetting error**
  - Always check that the added sample matches the sample sheet

Control C2 is negative

- **Pipetting error**
  - Always check that the added sample matches the sample sheet

Fluorescence intensity varies

- **There is dirt on the outside which interferes with the signal**
  - Manipulate the plates always wearing gloves
- **The volume is not at the bottom of the well or there is an air bubble**
  - Centrifuge to make sure the sample is at the bottom of the well and there are no air bubbles, according to the technics protocol.
- **Pipetting error**
  - Verify the volume added in each well is correct

There is no fluorescence signal

- **Incorrect reading channels selected**
  - Configure the correct reading channels.
- **Pipetting error or reagent absence**
  - Control the pipetting and the reactions configuration
  - Repeat the PCR
- **No reading channel was selected in the thermal cycler's program.**
  - Revise and modify the thermal cycler program

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