GenVinSet

MTHFR C677T

Instructions for Use

Kit for detection of MTHFR C677T polymorphism

For In Vitro diagnostic use

Product code GVS-MTHFR-24 (24 tests)
GVS-MTHFR-48 (48 tests)

Store from –18 to –30ºC

CE

Rev 05 / 2020-02-07

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GenVinSet

MTHFR
C677T

Index

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GENVINSET

MTHFR C677T

Intended use

GENVINSET® MTHFR C677T is a kit for the C>T transition in the 677 position, located in the exon 4 of the *MTHFR* gen, using Real Time PCR technology with specific TaqMan® probes..

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Summary and explanation

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate metabolism. It catalyzes the intracellular conversion of 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 conversion of homocysteine to methionine. Althought the MTHFR enzyme does not participate in the clotting cascade as other proteins do, such as Factor II (FII) and Factor V (FV) (which belong to the called "coagulation factor" group), its involvement in the folate metabolic pathway may induce the development of thrombofilia, as well as increase the risk of Alzheimer's disease (1-4).

The mechanism through which the MTHFR protein is involved in thrombophilia development is not completely understood. It has been suggested that the inhibition of the folate cycle due to the lack of precursors leads to the accumulation of homocysteine in blood. The accumulation of this metabolite can cause a damage in the endothelial cells, which constitute the inner layer of blood vessels, thus triggering the formation of clots. These clots might then travel through circulation to other organs, which could lead to severe complicatios, such as a heart attack or a pulmonary embolism.

The *5,10-methylenetetrahydrofolate reductase* (*MTHFR*) gene is located on chromosome 1p36.3 and is composed of 2.0 kb, containing 11 exons. The product of the *MTHFR* gene is a 77 kDa protein (EC 1.5.1.20) (1,7). It have been described approximately 60 polymorphisms, as well as 41 rare yet deleterious mutations in the *MTHFR* gene (8). To date, the most studied and most common functional variant is the MTHFR C677T polymorphism (rs1801133). It consists of a cytosine (C) transition to a thymine (T) at nucleotide position 677 of exon 4, which results in the conversion of an alanine to a valine at position 222 of the aminoacid sequence (3,4). Heterozygous (CT) and mutated homozygous individuals (TT) for the C677T variant, have 65% and 30% reduced enzyme activity in comparison to wild type homozygous genotypes (CC), respectivey (9). As a result, TT homozygotes show reduced levels of serum folate and increased homocysteine levels, in comparison with their wild type counterparts (10-12).

The frequency of the MTHFR C677T variant differs among the different ethnias. The prevalence of the TT genotype ranges from 8–18% in Caucasians, 11–16% in Asians, 21-25% in latin populations and 0-6% in African-descending populations.

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Procedure principles

The detection method used by GENVINSET® MTHFR C677T is based on the Real Time PCR technology, using TaqMan® probes that specifically anneal to position 677 of the exon 4 of the *MTHFR* gene, monitoring the presence of C and/or T 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

In order to ensure a proper performance, reagents should be stored from -18ºC to -30ºC until their expiration date, indicated on the label of the vial. Do not perform more than 3 freeze/thaw cycles to the Primer Mix (GVS-MTHFR-PM) and Master Mix (GVS-MTHFR-MM) vials, as this could decrease the sensitivity of the assay and impair results.

Due to the photosensitivity nature of the reagents, 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
- q-PCR instrument specific reagents (in the case of using RotorGene Q, only 0.1 ml tubes are allowed)

Equipment

- q-PCR instrument. The following 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 mixer
- Pipettes (P1000, P200 & P10)

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Sample collection and preparation

The present 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, a validation assay with such extraction method should be done.

Caution

All biological and blood samples should be treated as possibly infectious. When manipulating them, the corresponding basic (universal) precautions should be taken. Samples should always be handled wearing the appropriate personal protection equipment.

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Usage procedures

A) PCR preparation

Precautions

- Thaw all of the kit components before starting the assay, mix and centrifuge them.
- Work on ice or over a cool block.
- The PCR should be setup 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 each C/T variant.

1. Thaw the samples. Prepare a mix with the Master Mix and the Primer Mix for n+1 samples:

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

2. Pipette 9 µL of this mix into the PCR tubes and add 1µL of DNA or Reaction Blank, in case of the contamination control well.

3. Seal the plate with convenient sealer, and centrifuge 1 min at 360 xg to ensure that all the volume settles to the bottom of the tube.

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

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B) Thermal cycler configuration

1. Set up the following amplification program:

	Cycle number	Temperature (ºC)	Time (mm:ss)	Ramp (%)	Analysis
Denaturalization	1	95	05:00	100	X
Cycles	50	95	00:15	100	X
		62	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. Set the "Reaction Volume" as 10 µl, and identify the operator and the samples.

d. Click on "Edit Profile" and set up the amplification program (see section 8.C.1). Select the step 60 sec at 62 ºC, and click on "Acquiring to Cycling A". Set "Green" and "Yellow" as the fluorescence acquisition channels. Press "OK". Click on "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. Scroll down the "Channel Settings" menu and select "Acquiring Channels". Then click on "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. Check the box "Perform Optimisation Before 1st Acquisition", and click on "Close".

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

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Results

Genvinset® MTHFR C677T constitutes a qualitative technique to detect the presence of a C and/or a T on the 677 position of the *MTHFR* gene.

It is not necessary to select any passive reference.

Using the present kit, the following results can be obtained:

Detection of T at position 677

On the FAM channel, the following amplification plot can be observed:

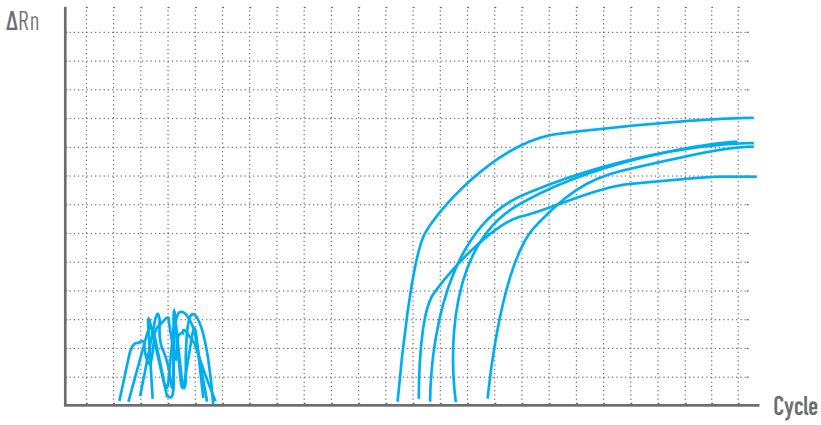

Figure 1. Positive and negative samples in the FAM channel.

Those samples that report an amplification curve can be considered as 677 T positive and they are identified by a numeric value called "Crossing Point" (Cp). This value corresponds to the cycle in which fluorescence is detected and, thereof, the amplification can be considered as positive.

Detection of C at position 677

On the VIC/HEX channel, the following amplification plot can be observed:

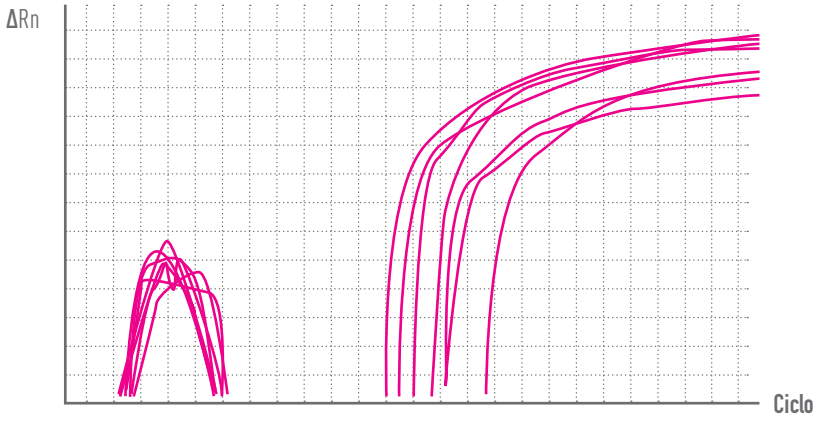

Figure 2. Positive and negative samples in the VIC/HEX channel

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Those samples that report an amplification curve can be considered as 677 C positive.

Genotyping analysis

In "Genotyping" or "Allelic Discrimination" analysis types, select FAM channel (mutation) on the Y axis, and HEX channel (wildtype) on the X axis. Results will appear similarly as in Figure 2, in which each dot consists of an x and y component. 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).

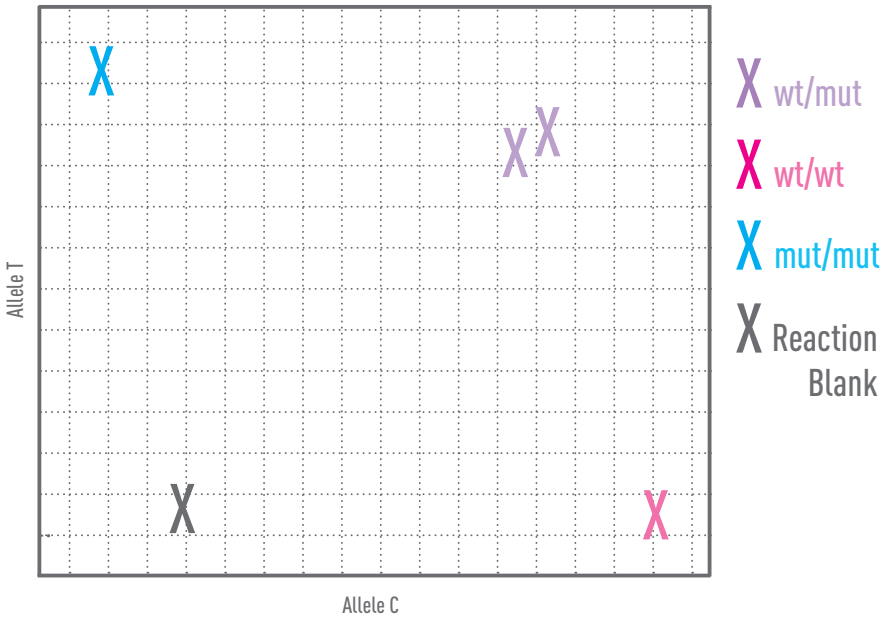

Figure 3. Plot showing one heterozygous sample (wt/mut), one normal sample (wt/wt) and one homozygous mutated sample (mut/mut) for C677T mutation, using Genvinset® MTHFR C677T kit.

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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 have to be taken into account in order to validate an assay:

- The Reaction Blank should report negative results in both FAM and VIC/HEX channels. An amplification curve with a Cp>35 value should be considered as negative. A Cp<35 value is associated with a contamination in the Reaction Blank and, therefore, all results from the experiment should be discarded.
- An heterozygous sample should report positive results in both FAM and VIC/HEX channels.

The assay must be performed 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 probes align specifically on the 677 position at the exon 4 of the *MTHFR* gene. No unspecific alignments have been detected. No cross-reaction phenomena with genomic DNA have been reported.

2. Analytical sensitivity

A dilution assay was performed, using 1:4 serial dilutions of a wild type sample for C677T polymorphism (no mutated alleles), an heterozygous sample (one mutated allele) and another homozygous one (two mutated alleles), obtained by a conventional extraction system, at an initial concentration of 27.8, 27.5 and 39.9 ng/µL, respectively. The following analytical sensitivity results were obtained:

- Detection Limit of wild type and mutated allele = 0.20 ng/µl (*)

(*) Cp < 35

3. Diagnostic sensitivity and specificity

In a human genomic DNA study, 89 samples obtained from a laboratory were analyzed. They were previously genotyped by another commercial kit.

All the tested samples were validated. The following results were obtained:

GENVINSET® MTHFR C677T		C/C	C/T	T/T
Previous method	C/C	29	0	0
	C/T	0	49	0
	T/T	0	0	11

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.
- Mutations or polymorphisms at annealing primer/probe sites are possible and may result of the lack of allele definition. Other technologies could be necessaries to resolve the typing.
- All the aforementioned conditions for the setup of the PCR should be carefully controlled. Any performances that do not meet such indications, 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 q-PCR thermal cycler must be calibrated according to the manufacturers´ recommendations and should be used in accordingly to the manufacturer's 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 any suspicions of possible loss of reactivity, contamination, external box deterioration or any other incidence that might affect the kits performance.
- Data and result interpretation should be revised by qualified personnel.
- Discard 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
  - Handle the kit components always according to accepted lab practices in oirder to avoid contamination.
  - Verify manipulation and storage conditions
  - Discard contaminated reagents
- **Pre-PCR area is contaminated**
  - Confirm that all necessary precautions in the pre-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**
  - Check that the sample added corresponds to the one indicated on the worksheet

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 the assay using diluted samples

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Fluorescence intensity too low

- **Kit degradation**
  - Check that the storage of the kit is correct, reviewing both proper temperature conditions and light exposure (which should be avoid)
  - Avoid more than 3 freeze/thaw cycles of the Primer Mix vial
  - Aliquote the reagents if necessary
  - Repeat the test with new reagents

Control C1 is positive

- **Cross contamination**
  - Always handle the kit components following all necessary practices to avoid contamination
- **Pipetting error**
  - Check that the sample added corresponds to the one indicated on the worksheet

Control C2 is negative

- **Pipetting error**
  - Check that the sample added corresponds to the one indicated on the worksheet.

Fluorescence intensity varies

- **The dirtiness on the outside of the tube walls interferes with the signal**
  - Handle all consumables wearing gloves
- **Volume is not settled to the bottom of the well or there are bubbles**
  - Perform a brief centrifugation to ensure that the volume settles to the bottom of the well and to remove all bubbles
- **Pipetting error**
  - Check that the correct volume has been added

There is no fluorescence signal

- **Incorrect reading channels selected**
  - Set the appropriate reading channels
- **Pipetting error or reagent absence**
  - Control the pipetting and the reaction setup
  - Repeat the PCR
- **No reading channel was selected in the thermal cycler program.**
  - Check and modify the thermal cycler program

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Changes to version 04

Version	Description of the modification
Rev. 04	Modification of PCR annealing temperature, from 64ºC to 62ºC.

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GenVinSet

MTHFR
C677T

Explanation of symbols used on the labels

For in vitro diagnostic use

This product fulfills the requirements of Directive 98/79/EC on in vitro diagnostic medical device

Catalogue number

Lot number

Expiration date

Contents sufficient for <n> tests

Manufactured by

Store at

Keep away from sunlight

Positive control

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