# Folate Metabolism Panel SNP

## Handbook

Real Time PCR kit for detection of MTHFR 677 C>T , MTHFR 1298 A>C, MTR 2756 A>G, MTRR 66 A>G

**REF T01002-96-S**

**Σ 24**

## NAME

**Folate Metabolism Panel SNP**

## INTRODUCTION

A single nucleotide polymorphism (SNP pronounced "snip") is a DNA polymorphisms at the level of a single nucleotide, a single base mutation in DNA. SNPs are ‘conserved’ across the genome and represent the most simple form and most common source of genetic polymorphism in the human genome: 90% of all human DNA polymorphisms are associated with SNPs and variation frequency is about 1 every 1000bp in the human genome (Sachidanandam et al.,2001).

The SNPs in the genome can affect gene functions, protein structure or expression and for these reasons they are used as markers in genetic disease studies (Kim & Mishra, 2007).

It’s sometimes possible to correlate a SNP with a particular trait or disease: susceptibility to disease may also be described as an ‘unfortunate trait that can be assessed checking if the mutated (unfortunate) polymorphism is carried in both alleles.

SNPs testing can be applied to:

- Diagnostics / risk profiling
- Drug response prediction
- Gene function identification

Several SNPs have been associated to genetic susceptibility to different diseases and disorders like for example:

- Hypertension
- Fibrinolysis
- Myocardial infarction
- Ischemic stroke
- Cancer
- Metabolic disorders

In order to perform SNP genotyping, two specific probes labeled with different dyes are used, the first for the wild type allele and the second for the mutant allele. If the assay results in the emission of only the first fluorescent color, then the individual is homozygous wild type at that locus. If the assay results in the emission of only the second fluorescent color, then the individual is homozygous mutant. And finally, if both fluorescent colors are produced, then the individual is heterozygous.

## INTENDED USE

**Folate Metabolism Panel SNP** Kit is intended for detection and allelic discrimination of genetic polymorphisms MTHFR 677 C>T , MTHFR 1298 A>C, MTR 2756 A>G, MTRR 66 A>G associated with methylenetetrahydrofolate reductase deficiency.

## PRINCIPLE OF ASSAY

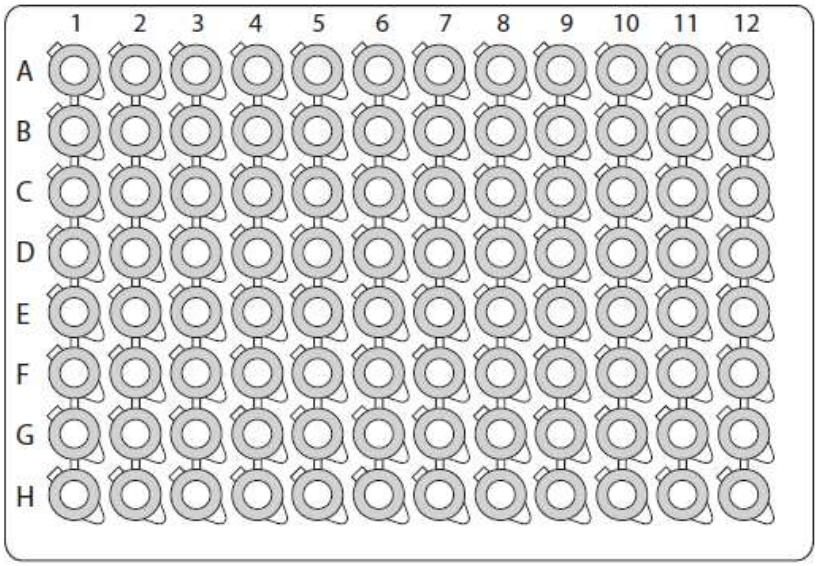
**Folate Metabolism Panel SNP** Kit is a qualitative test that allows the detection by Real Time PCR based on the amplification of the genome specific region using specific primers. In Real Time PCR the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescence intensities during the reaction allows the detection of accumulating product without re-opening of the reaction tubes after the PCR run.

## MATERIALS PROVIDED

**Ready to use 12x8 strip format**

- **12 x 8 strip ready to use** (each PCR tube contains 15 µl of PCR mix)
- **Taq polymerase,** 0,5 ml (1 vial)
- **Negative control C-**, 0,1 mL (1 vial)
- **C+ Homozygous Wild Type (allele 1-1)**, 50 µL (4 vials, 1 for each SNP)
- **C+ Heterozygous (allele 1-2)**, 50 µL (4 vials, 1 for each SNP)
- **C+ Homozygous Mutant (allele 2-2)**, 50 µL (4 vials, 1 for each SNP)

Contains reagents for 24 tests.




A- **MTHFR** 677 C>T

B- **MTHFR** 1298 A>C

C- **MTR** 2756 A>G

D- **MTRR** 66 A>G

E- **MTHFR** 677 C>T

F- **MTHFR** 1298 A>C

G- **MTR** 2756 A>G

H- **MTRR** 66 A>G

## MATERIALS REQUIRED BUT NOT PROVIDED

### Zone 1: sample preparation

- DNA extraction kit
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, Freezer

### Zone 2: Real Time amplification

- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Freezer, refrigerator
- Tube racks

## STORAGE INSTRUCTIONS

**Folate Metabolism Panel SNP** kit must be stored at 2-8°C. The kits can be shipped at 2-8°C and stored as indicated immediately on receipt.

## STABILITY

**Folate Metabolism Panel SNP** kit is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

## QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

## WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

## PRODUCT USE LIMITATIONS

Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

## SAMPLE COLLECTION, STORAGE AND TRANSPORT

**Folate Metabolism Panel SNP** kit can analyze genomic DNA extracted from:

- *whole blood* collected in EDTA tubes;
- *Buccal swab*: insert the swab into the nuclease-free 1,5 ml tube and add 0,2 ml of Transport medium. Vigorously agitate swabs in medium for 15-20 sec.

Specimens can be stored at +2-8°C for no longer than 24 hours, or freeze at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

## DNA ISOLATION

- DNA isolation kit. The following isolation kits are recommended:
  - ⇒ **Genomic column DNA Express** – spin column extraction kit (Sacace, REF K-1-1/E);
  - ⇒ **SaMag Blood DNA extraction kit** (Sacace, REF SM001) ;
  - ⇒ **QIAamp DNA Blood mini kit** (Qiagen, REF 51104);
  - ⇒ **DNA-Sorb-A** (Sacace, REF K-1-1/A) for buccal swab;

Please carry out DNA extraction according to the manufacturer's instruction.

## PROTOCOL

**Folate Metabolism Panel SNP kit** does not include reagents required for sample preparation and DNA extraction. Blood samples and biological materials must be processed by using the recommended kits or those with similar performances of quality and quantity of extracted DNA. Use of blood samples collected in tubes containing heparin is not recommended.

The analysis of the genomic DNA specimens using **Folate Metabolism Panel SNP** kit includes the following stages:

1. Preparing the Real Time PCR;
2. Real Time PCR analysis;
3. Data analysis with the software of Real Time PCR instrument;
4. Results analysis and conclusions.

## EXPERIMENTAL PROTOCOL

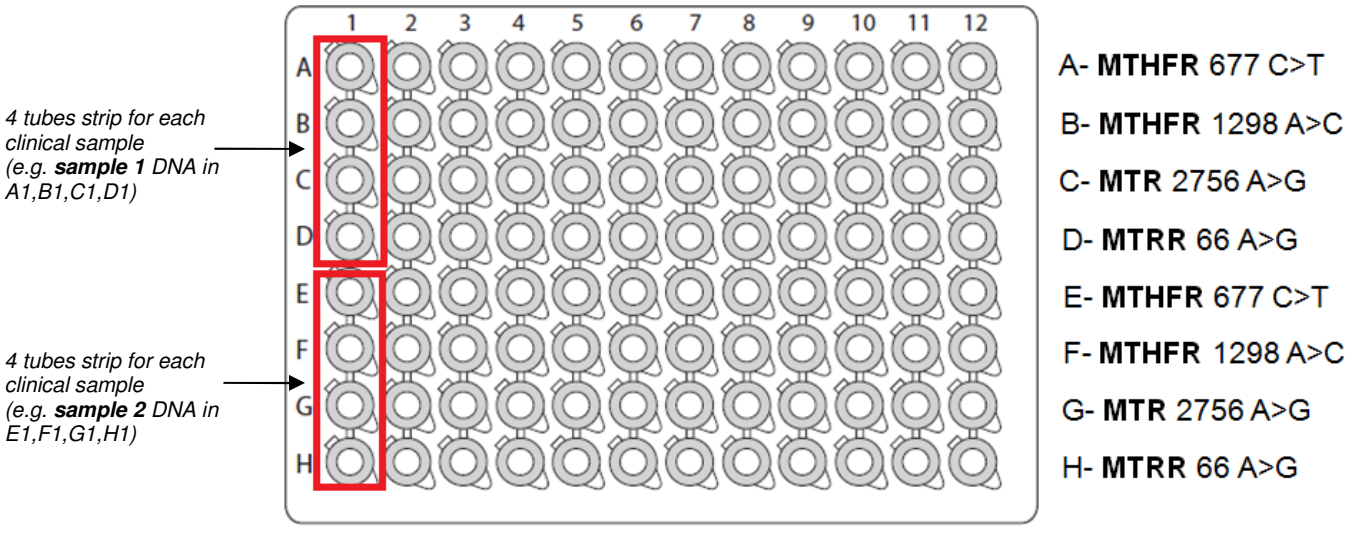
**Total reaction volume: 25 µl**

1. Prepare the necessary number of ready-to-use strips (one 4-tubes strip for each clinical sample) and mark them.
2. Spin for 3-5 sec the **Taq polymerase**, mix by pipetting and **add 5 µl** to each PCR tube.
3. Add into the corresponding PCR tubes **5.0 µl** of extracted DNA from sample:
   - **DNA** sample

   Add into the corresponding PCR tubes **5.0 µl** of controls* according to the related amplification mix (for example, C+ controls for MTFR 677 C>T must be added in the "A" line tubes, C+ controls for MTR 2656 A>G must be added in the "C" line tubes and so on, see below picture):
   - **C+ Homozygous Wild Type (allele 1-1)**
   - **C+ Heterozygous (allele 1-2)**
   - **C+ Homozygous Mutant (allele 2-2)**

** it's suggested to use at least 1 Positive control (for example C+ Heterozygous) for each run.*

4. Spin the tubes for 3–5 seconds to collect the drops.
5. Insert the tubes in the Real-time PCR instrument.




***NOTE:*** *use 4 tubes strip for each clinical sample. Add 5 µl of Taq polymerase to all tubes and then 5 µl of extracted DNA to each 4 tubes strip*

**Amplification**

Create a temperature profile on your instrument as follows:

| | **Rotor-type Instruments**[1] | | |
|---|---|---|---|
| **Step** | ***Temperature, °C*** | ***Time*** | **Repeats** |
| Hold | **80** | 2 min | 1 |
| Hold | **95** | 3 min | 1 |
| Cycling | **95** | 10 s | 40 |
| | **60** | **40 s fluorescence detection** | |

| ***Step*** | **Plate type instruments**[2] | | |
|---|---|---|---|
| | ***Temperature, °C*** | ***Time*** | ***Cycles*** |
| Hold | 80 | 2 min | 1 |
| Hold | 94 | 3 min | 1 |
| Cycling | 94 | 15 s | 5 |
| | 64 | 40 s | |
| Cycling 2 | 94 | 15 s | 35 |
| | 64 | **40 s fluorescence detection** | |

*[1] For example like RotorGene 6000/Q (Qiagen) NOTE: If the test is performed on Rotor Gene 6000/Q (Qiagen) instrument the tube caps may be marked and it's recommended to cut the strip into two equal parts (4 tubes), but it is necessary to strictly observe the order of the tubes in the rotor.*

[2] *For example, SaCycler-96™ (Sacace); CFX-96 / iQ5™ (BioRad); Mx3005P™ (Agilent); ABI® 7500 Real Time PCR (Applied)*; LightCycler® 96 (Roche).*

** To perform the test with ABI 7500 (Applied) a disposable plate adapter provided with the kit has to be used. Additional adapters can be purchased separately.*

Fluorescence is detected in FAM/Green, JOE/Yellow/HEX fluorescence channels.

## DATA ANALYSIS

**The fluorescent signal intensity is detected in 2 channels as shown in the table below:**

| FAM | HEX |
|---|---|
| **Allele 2 (mutant)** | **Allele 1 (wild type)** |

### Interpretation of results for RotorGene 6000/Q (Qiagen):

Principle of interpretation:

- **Signal only in allele 1 (channel Yellow) : homozygous wild type**
- **Signal only in allele 2 (channel Green) : homozygous mutated**
- **Signal in both allele 1 and allele 2 (channels Yellow and Green) : heterozygous**

**Analysis settings (RotorGene 6000/Q)**

| *Gene* | *Polymorphism* | *Channel / Allele* | *Threshold* | *Slope correct* | *Outlier Removal* | *Ignore first* |
|---|---|---|---|---|---|---|
| *MTHFR* | 677 C>T | Yellow: Ala (C) | 0,03 | on | 10% | 10 |
| | | Green: Val (T) | | | | |
| *MTHFR* | 1298 A>C | Yellow: Glu (A | 0,03 | on | 10% | 5 |
| | | Green: Ala (C) | | | | |
| *MTR* | 2756 A>G | Yellow:Asp (A) | 0,03 | on | 15% | 5 |
| | | Green: Gly (G) | | | | |
| *MTRR* | 66 A>G | Yellow: Ile (A) | 0,03 | on | 15% | 5 |
| | | Green: Met (G) | | | | |

**NOTE FOR RotorGene 6000/Q (Qiagen):** if a Ct value is higher than 32 the sample is considered invalid and must be repeated. If there is no Ct value in both channels the sample is invalid and must be repeated starting from the extraction.

### Interpretation of results for CFX-96/iQ5 (Bio-rad):

Principle of interpretation:

- **Signal only in allele 1 (channel HEX) : homozygous wild type**
- **Signal only in allele 2 (channel FAM) : homozygous mutated**
- **Signal in both allele 1 and allele 2 (channels HEX and FAM) : heterozygous**

Set **Baseline Cycles** at 5-15 and **Crossing Threshold** value as 100.

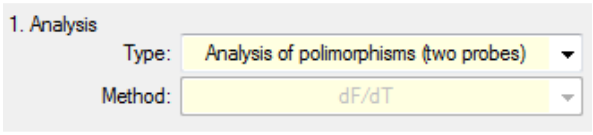
**NOTE FOR CFX-96/iQ5 (Bio-rad):** if a Ct value is higher than 32 the sample is considered invalid and must be repeated. If there is no Ct value in both channels the sample is invalid and must be repeated starting from the extraction.

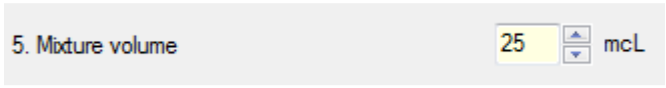
**Interpretation of results for SaCycler-96 (Sacace Biotechnologies):**

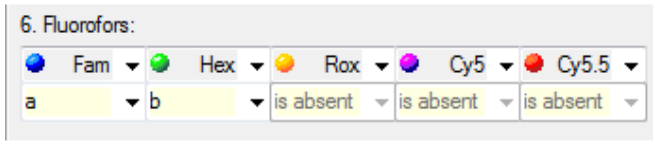
Principle of interpretation:

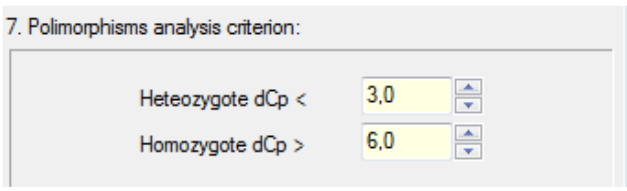
- **Signal only in allele 1 (channel HEX) : homozygous wild type**
- **Signal only in allele 2 (channel FAM) : homozygous mutated**
- **Signal in both allele 1 and allele 2 (channels HEX and FAM) : heterozygous**

**NOTE:** when creating new test for Folate Metabolism Panel SNP, select **"Analysis of polimorphisms (two probes)"**, name "a" on FAM channel and name "b" on HEX channel. Set **Heterozygote dCp < 3,0** and **Homozygote dCp > 6** (see pictures below).

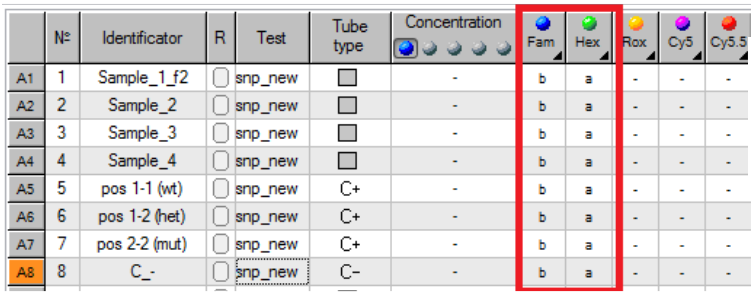


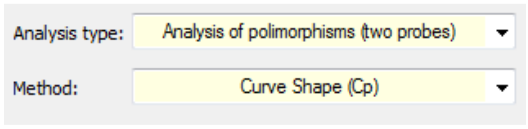



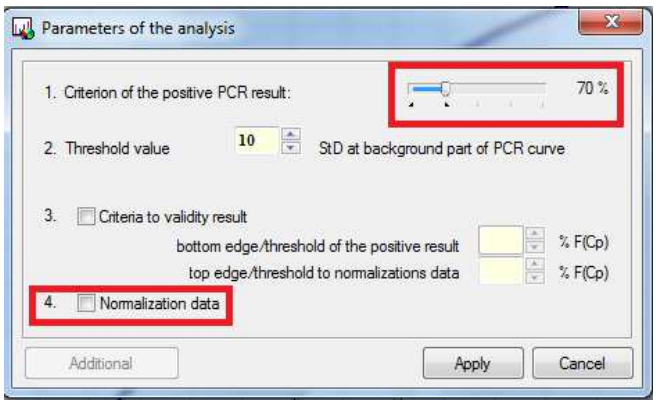

To analyze results, be sure to select **"Analysis of polimorphisms (two probes)"** as Analysis type and **"Curve Shape (Cp)"** as Method.



| | № | Identificator | R | Test | Tube type | Concentration | Fam | Hex | Rox | Cy5 | Cy5.5 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | 1 | Sample_1_f2 | | snp_new | | - | b | a | - | - | - |
| A2 | 2 | Sample_2 | | snp_new | | - | b | a | - | - | - |
| A3 | 3 | Sample_3 | | snp_new | | - | b | a | - | - | - |
| A4 | 4 | Sample_4 | | snp_new | | - | b | a | - | - | - |
| A5 | 5 | pos 1-1 (wt) | | snp_new | C+ | - | b | a | - | - | - |
| A6 | 6 | pos 1-2 (het) | | snp_new | C+ | - | b | a | - | - | - |
| A7 | 7 | pos 2-2 (mut) | | snp_new | C+ | - | b | a | - | - | - |
| A8 | 8 | C_- | | snp_new | C- | - | b | a | - | - | - |




Click on the icon for changing the parameter of data analysis , a new window will show up. The settings must be precisely as in the following picture, then click "**Apply**":




Set **70%** as "Criterion of the positive PCR result"; "Normalization data" checkbox must be **deselected.**

The results will be displayed in the table on the right (see below pictures as reference).

**Example of results:**

Results | Statistics

| N | Identificator | Polimorphism | | dCp | Cp Fam | Cp Hex |
|---|---|---|---|---|---|---|
| A1 | Sample_1_f2 | a | b | 0,2 | 19,2 | 19,1 |
| A2 | Sample_2 | b | b | >17 | | 17,7 |
| A3 | Sample_3 | b | b | >16 | | 18,3 |
| A4 | Sample_4 | b | b | >16 | | 18,8 |
| A5 | K+ | b | b | >15 | | 19,4 |
| A6 | K+ | a | b | 0,1 | 18,9 | 19,0 |
| A7 | K+ | a | a | >16 | 18,4 | |
| A8 | C_- | - | - | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| D1 | Sample_1_mthfr | a | b | 0,1 | 18,6 | 18,7 |
| D2 | Sample_2 | a | a | >18 | 17,0 | |
| D3 | Sample_3 | b | b | >17 | | 17,5 |
| D4 | Sample_4 | b | b | >16 | | 18,1 |
| D5 | K+ | b | b | >19 | | 15,7 |
| D6 | K+ | a | b | 0,4 | 13,9 | 13,5 |
| D7 | K+ | a | a | >21 | 13,0 | |
| D8 | C_- | - | - | | | |

**a = FAM (mutant, allele2)  b = HEX (wild type, allele1)**

| a | b | **= sample eterozygous (both alleles present)** |
|---|---|---|

| b | b | **= sample homozygous wild type (only allele 1 present)** |
|---|---|---|

| a | a | **= sample homozygous mutant (only allele 2 present)** |
|---|---|---|

**NOTE FOR SaCycler-96 (Sacace Biotechnologies):** if a Ct value is higher than 32 the sample is considered invalid and must be repeated. If there is no Ct value in both channels the sample is invalid and must be repeated starting from the extraction.

## KEY TO SYMBOLS USED

| | | | |
|---|---|---|---|
| REF | List Number |  | Caution! |
| LOT | Lot Number |  | Contains sufficient for <n> tests |
|  | Expiration Date | VER | Version |
|  | Store at | **NCA** | Negative Control of Amplification |
|  | Manufacturer | **NCE** | Negative control of Extraction |
|  | Consult instructions for use | **IC** | Internal Control |



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