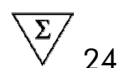


artus[®] MTHFR LC PCR Kit

Handbook



Qualitative in vitro Diagnostics

For use with the *LightCycler[®] 1.1/1.2/1.5* Instrument

December 2011 – Version 1



4621063, 4621065



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artus MTHFR LC PCR Kit*

For use with the *LightCycler 1.1/1.2/1.5* Instrument.

1. Contents

	Labeling and contents	Art. No. 4621063 24 reactions
Blue	<i>MTHFR LC Master</i>	2 x 12 reactions
Red	<i>MTHFR LC Control</i>	1 x 200 µl
White	<i>Water (PCR grade)</i>	1 x 1000 µl

2. Storage

The components of the *artus* MTHFR LC PCR Kit should be stored at –20°C and are stable until the expiry date stated on the label. Repeated thawing and freezing (> 2 x) should be avoided, as this may reduce the sensitivity. If the reagents are to be used only intermittently, they should be frozen in aliquots. Storage at +4°C should not exceed a period of five hours.

3. Additionally Required Materials and Devices

- Disposable powder-free gloves
- DNA isolation kit (see **8.1 DNA Isolation**)
- Pipettes (adjustable)
- Sterile pipet tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- *Color Compensation Set* (Roche Diagnostics, Cat. Nr. 2 158 850) for the installation of a *Crosstalk Color Compensation* file
- *LightCycler* Capillaries (20 µl)

* MTHFR = Methylentetrahydrofolate reductase.

- *LightCycler* Cooling Block
- *LightCycler* 1.1/1.2/1.5 Instrument
- *LightCycler* Capping Tool

4. General Precautions

The user should always pay attention to the following:

- Use sterile pipet tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice or in the *LightCycler* Cooling Block.

5. Background Information

In addition to age, sex, nutrition and comedication, especially genetic factors may influence enzyme activity. It is known that patients with an altered activity of the methylenetetrahydrofolate reductase enzyme (MTHFR) due to a genetic modification bear an elevated risk for the occurrence of side-effects under the therapy with methotrexate (e.g., severe blood count alterations, mucositis, hyperhomocysteinemia). Methotrexate is a folic acid antagonist and is used among others for the therapy of rheumatoid arthritis and tumor diseases. An examination of the MTHFR gene allows the estimation of the genetically caused risk of therapy-induced side-effects. Carriers of a genetic variant of the MTHFR gene may be identified before the start of a therapy and may consequently be treated with an alternative therapy or with a markedly reduced drug dosage.

The methylenetetrahydrofolate reductase enzyme activity is determined among others by modifications in the MTHFR gene. Genetic modifications may for example cause an amino acid exchange. The resulting altered conformation of the enzyme has an influence on its activity. The most frequent

genetic variants in the MTHFR gene are located at nucleotides nt 677 and nt 1298. Further genetic variants have been described in the literature, which, however, were rarely or only once observed in a population.

The MTHFR enzyme is one of the main components of folic acid metabolism. A reduced enzyme activity results in an influence on methionine synthesis from homocysteine. Since an enrichment of homocysteine may cause diverse diseases (thrombosis, arteriosclerosis), the presence of a genetic variant in the MTHFR gene results in an elevated risk for the occurrence of these diseases.

Genotyping may help to individually optimize medication and to lower therapy costs (prolonged stay in hospital, etc.) arising due to undesired side-effects as well as to recognize a genetically based risk for hyperhomocysteinemia at an early stage.

6. Principle of the Test Procedure

Specific regions of the human genome are amplified by means of the polymerase chain reaction (PCR) for genetic diagnostics. In real-time PCR the amplified product is detected via fluorescent dyes. These are usually linked to oligonucleotide probes which bind specifically to the amplified product. The PCR amplification is followed by a melting curve analysis which allows the identification and discrimination of gene variants. Since the reaction tubes do not have to be re-opened after the PCR run, the risk of contamination is significantly reduced (Mackay, 2004).

7. Product Description

The *artus* MTHFR LC PCR Kit represents a simple, fast and safe method of testing human DNA for the presence of clinically relevant genetic variants of the MTHFR gene. This analysis allows the assessment of therapy-induced risks, for example as a result of methotrexate treatment.

The analysis is performed by the detection of the genetic variants within the MTHFR gene using the *LightCycler* 1.1/1.2/1.5 Instrument. The reagents contain primers for the amplification of particular regions of the human MTHFR gene as well as fluorescence labeled probes for the detection of genetic variants at nucleotide positions nt 677C>T and nt 1298A>C. In addition, a positive control is run in parallel in a separate reaction.

Since the test is based on the amplification of human genomic DNA, fluorescence signals within the melting curve segment must be detectable independently of the presence of an allelic variant. The absence of a detectable signal is indicative for an inefficient DNA extraction or a PCR inhibition. Therefore, an additional internal control is not necessary in this genetic test.

Attention: The signals of the melting curve analysis are decisive for the data analysis. In most cases a quantitative amplification during the *LightCycler* run using the *artus* MTHFR LC PCR Kit cannot be observed. This, however, has no influence on the melting curve analysis.

8. Protocol

8.1 DNA Isolation

Kits for the isolation of DNA from blood are offered by a variety of manufacturers. Sample amounts for the DNA isolation procedure depend on the protocol used. Please carry out the DNA isolation according to the manufacturer's instructions. The following isolation kit is recommended:

Sample Material	Nucleic acid isolation kit	Catalog number	Manufacturer
Blood	QIAamp® DNA Blood Mini Kit (50)	51 104	QIAGEN

- The *artus* MTHFR LC PCR Kit should not be used with **phenol**-based isolation methods.
- When using isolation protocols with **ethanol**-containing washing buffers, please carry out an additional centrifugation step (three minutes, 13,000 rpm) before the elution to remove any remaining ethanol. This prevents possible inhibition of PCR through ethanol.

8.2 Preparing the PCR

Make sure that the Cooling Block as well as the capillary adapters (accessories of the *LightCycler* Instrument) are pre-cooled to +4°C. Place the desired number of *LightCycler* capillaries into the adapters of the Cooling Block. Before each use, all reagents need to be thawed completely, mixed (by repeated up and down pipetting or by quick vortexing), and centrifuged briefly.

For each application, a positive (*MTHFR LC Control*) and a negative control (*Water, PCR grade*) included in the *artus* MTHFR LC PCR Kit have to be considered.

Please use the following pipetting scheme for the preparation of the PCR reactions (for a schematic overview see Fig. 1):

	Number of samples	1
Preparation of PCR assay	<i>MTHFR LC Master</i>	18 µl
	Sample	2 µl
	Total Volume	20 µl

Pipet 18 µl of the *MTHFR LC Master* into the plastic reservoir of each capillary. Subsequently add 2 µl of the eluate from the DNA isolation. Correspondingly, 2 µl of the *MTHFR LC Control* as a positive control and 2 µl of water (*Water, PCR grade*) as a negative control are applied. Close the capillaries. In order to transfer the preparation from the plastic reservoir into the capillaries, centrifuge the adapters including the capillaries in a desktop centrifuge for ten seconds at a maximum of 400 x g (2000 rpm).

Attention: In order to avoid contaminations, the caps should be placed on the capillaries using the *LightCycler* Capping Tool.

Preparing the PCR

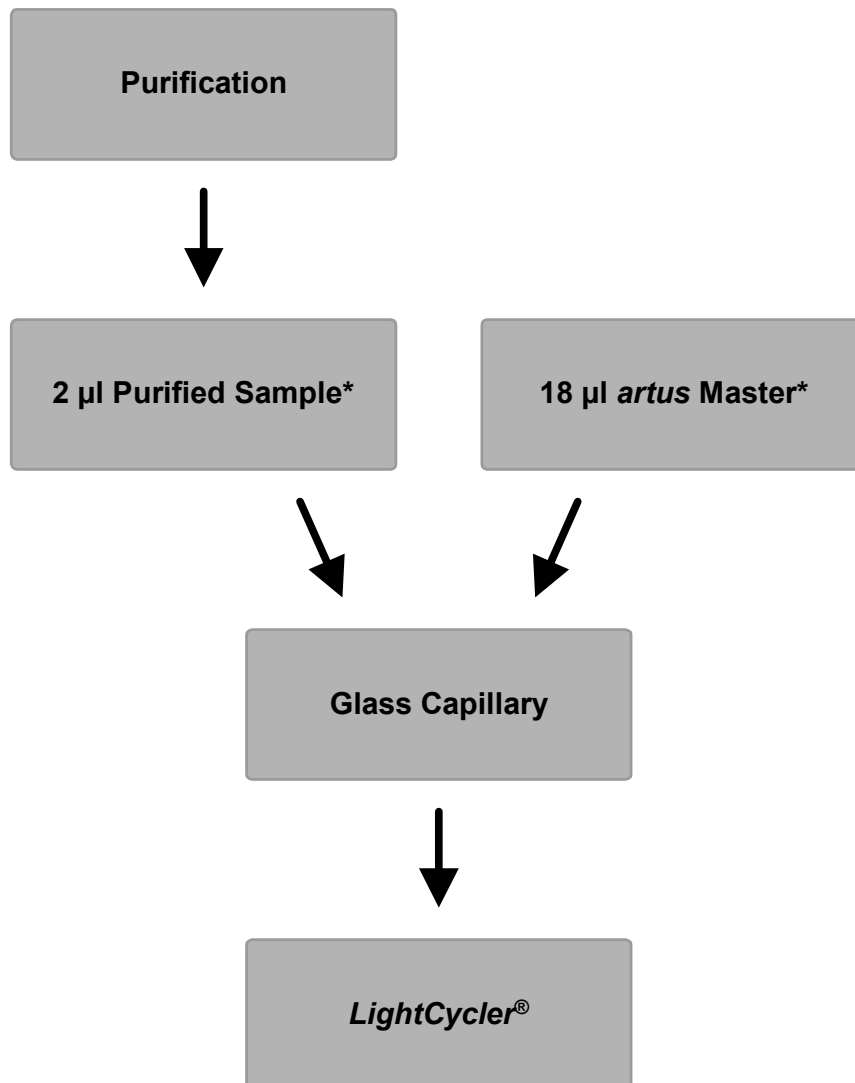


Fig. 1: Schematic workflow.

*Please make sure that the solutions are thawed completely, mixed well and centrifuged briefly.

8.3 Programming of the *LightCycler* 1.1/1.2/1.5 Instrument

For the detection of the genetic variants in the MTHFR gene, create a temperature profile on your *LightCycler* 1.1/1.2/1.5 Instrument according to the following five steps (see Fig. 2 – 6).

- | | |
|---|--------|
| A. Initial Activation of the Hot Start Enzyme | Fig. 2 |
| B. Touch Down Step | Fig. 3 |
| C. Amplification of the DNA | Fig. 4 |
| D. Melting Curve | Fig. 5 |
| E. Cooling | Fig. 6 |

Pay particular attention to the settings for *Analysis Mode*, *Cycle Program Data* and *Temperature Targets*. In the illustrations these settings are framed in bold black. Please find further information on programming the *LightCycler* 1.1/1.2/1.5 Instrument in the *LightCycler Operator's Manual*.

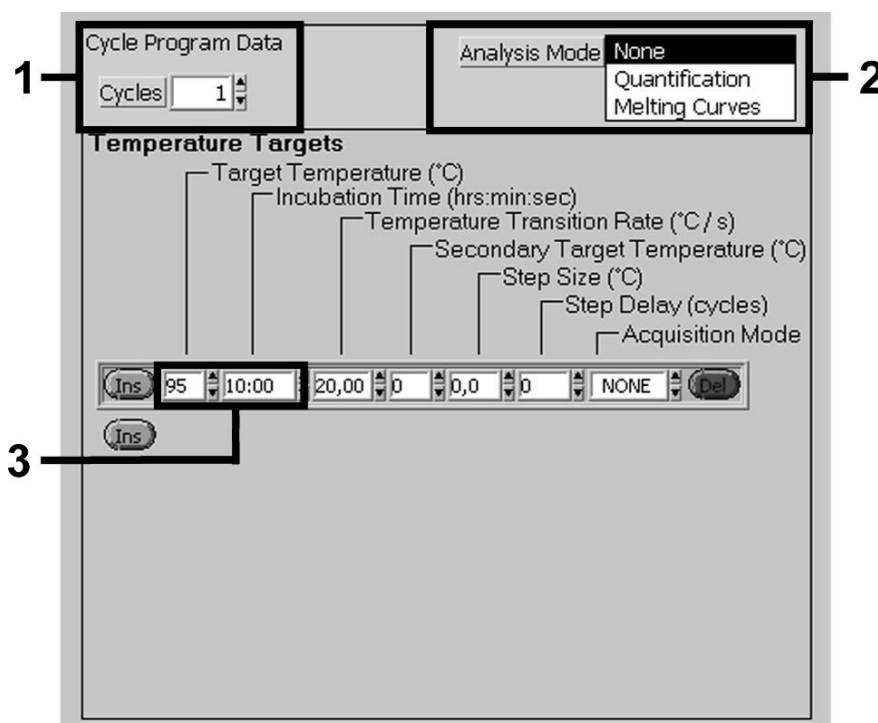


Fig. 2: Initial Activation of the Hot Start Enzyme.

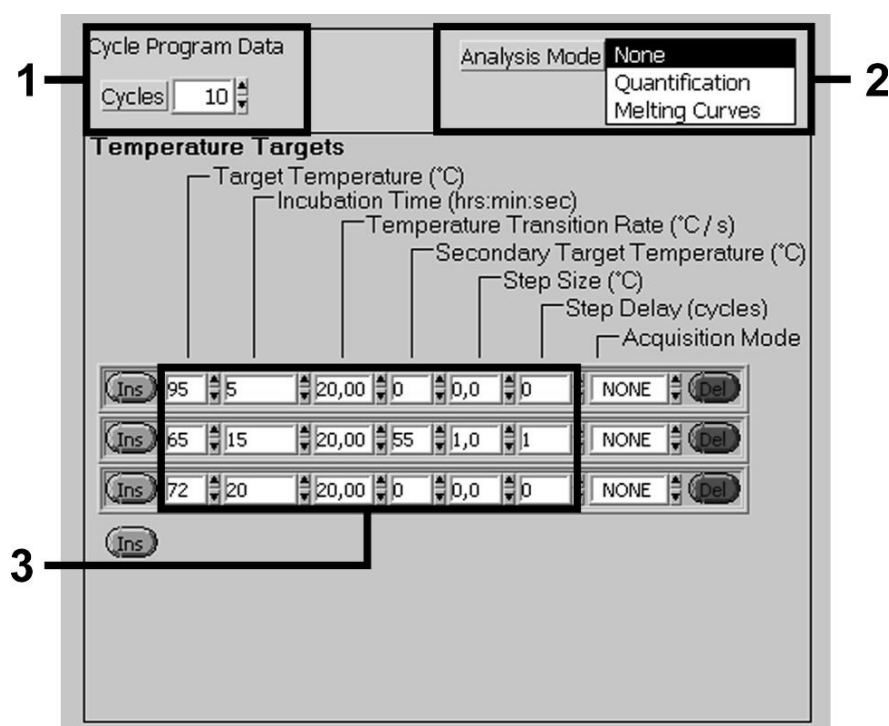


Fig. 3: Touch Down Step.

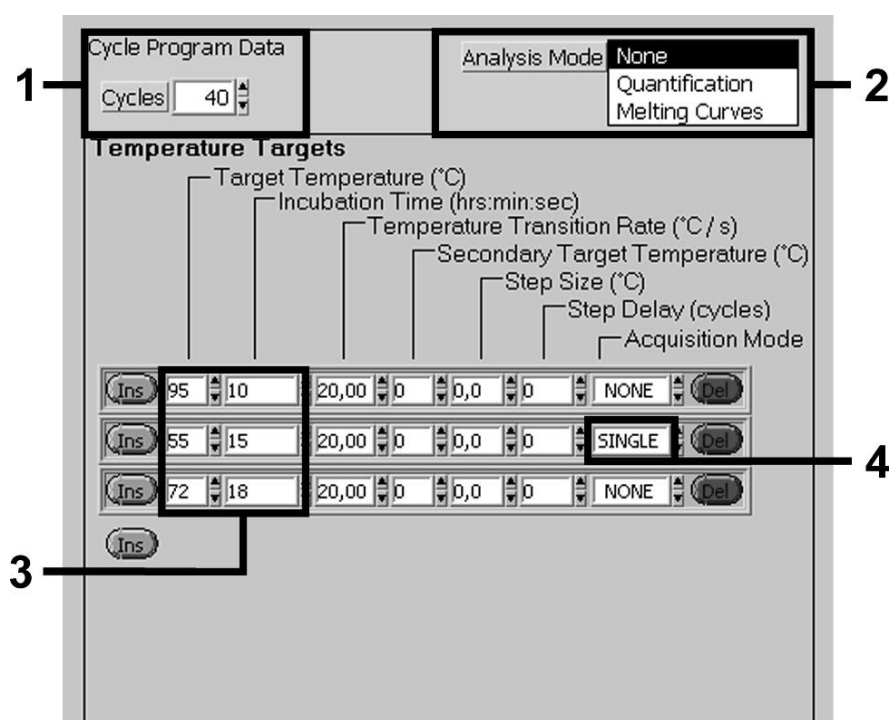


Fig. 4: Amplification of the DNA.

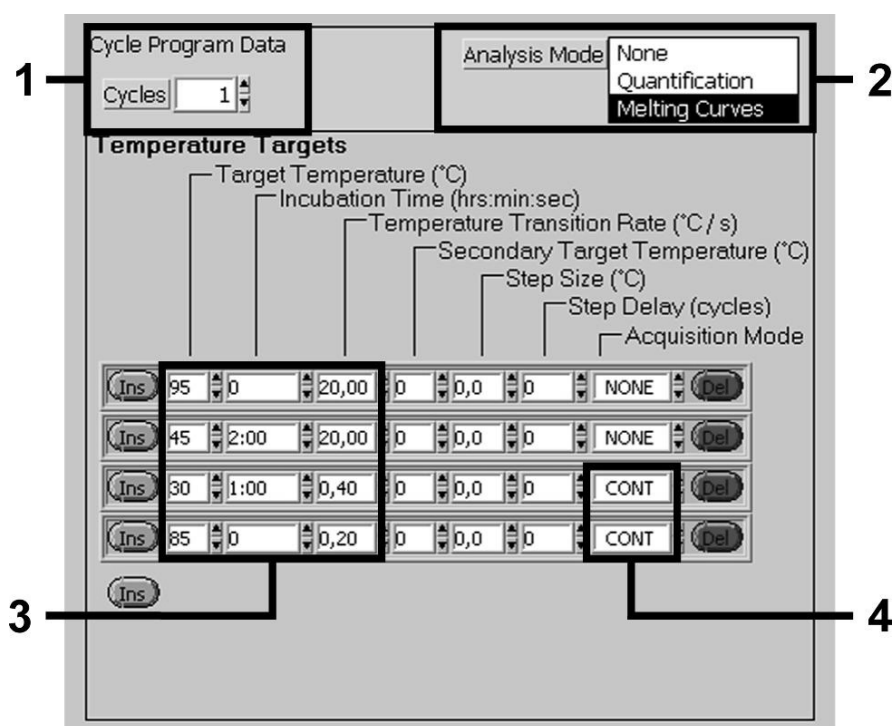


Fig. 5: Melting Curve.

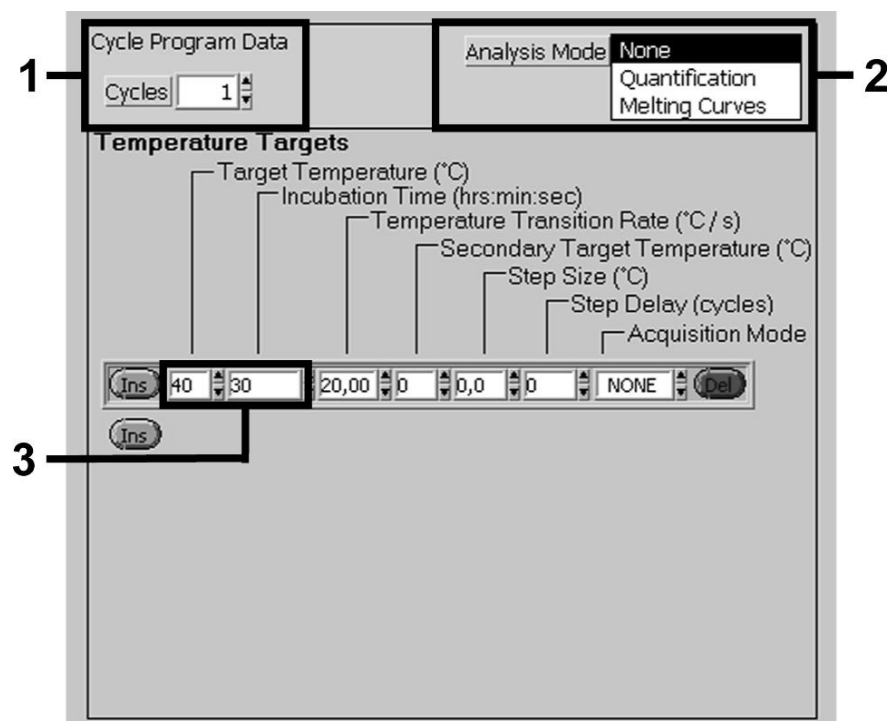


Fig. 6: Cooling.

9. Data Analysis

In multicolor analyses interferences occur between fluorimeter channels. The *LightCycler* Instrument's software contains a file termed *Color Compensation File*, which compensates for these interferences. Open this file before, during, or after the PCR run by activating the *Choose CCC File* or the *Select CC Data* button. If no *Color Compensation File* is installed, generate the file according to the instructions in the *LightCycler Operator's Manual*. After the *Color Compensation File* has been activated, separate signals appear in fluorimeter channels F1, F2, and F3. For analysis of the PCR results gained with the *artus* MTHFR LC PCR Kit please select fluorescence display options F2/Back-F1 and F3/Back-F1 for the MTHFR PCR. In most cases a quantitative amplification during the *LightCycler* run cannot be observed using the *artus* MTHFR LC PCR Kit.

The components of the *artus* MTHFR LC PCR Kit allow the detection of two genetic variants (wild-type = wt; variant = var) at nucleotide positions nt 677 and nt 1298 in the MTHFR gene. The determination of the genetic variants is performed by means of the *Melting Curve* program. The melting points in the fluorogram indicate the presence of the wild-type or the genetic variants at the temperatures given in the following table (see Table 1). In the case of heterozygosity, the curve displays two peaks.

Table 1: Melting points of the wild-type (wt) and the genetic variants (var).

channel F2			channel F3		
nt	wt	var	nt	wt	var
677	62°C	53°C	1298	46°C	57°C

Please note that the melting points may differ from the indicated temperatures by $\pm 2^{\circ}\text{C}$. In many cases it is advisable to switch off the *Digital Filter* for a better representation of the fluorogram.

The following figures (see Fig. 7 and 8) show the fluorograms for the detection of the polymorphisms at nucleotide positions nt 677 and nt 1298 in the homozygous wild-type form as well as in the heterozygous or the homozygous variant form.

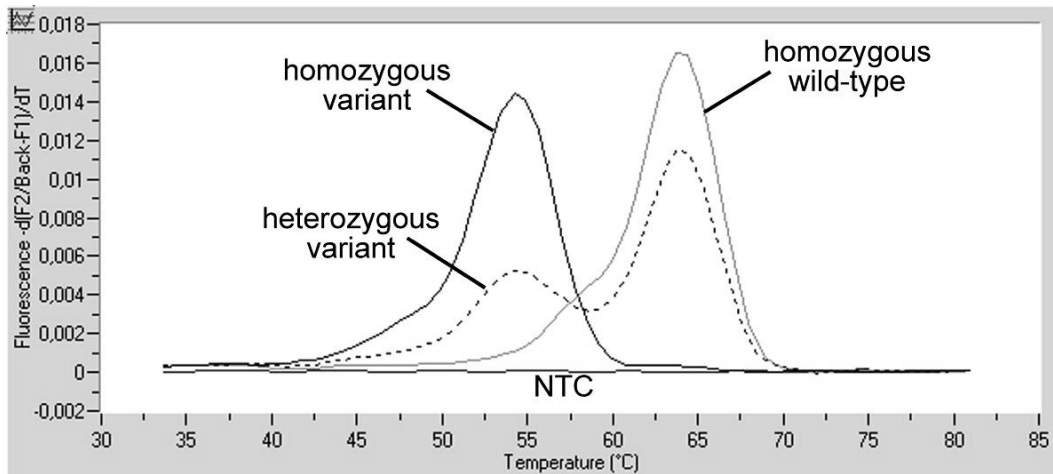


Fig. 7: Fluorogram for the detection of the nucleotide exchange at nt 677 by means of the *artus* MTHFR LC PCR Kit in fluorimeter channel F2/Back-F1. NTC: no template control (negative control).

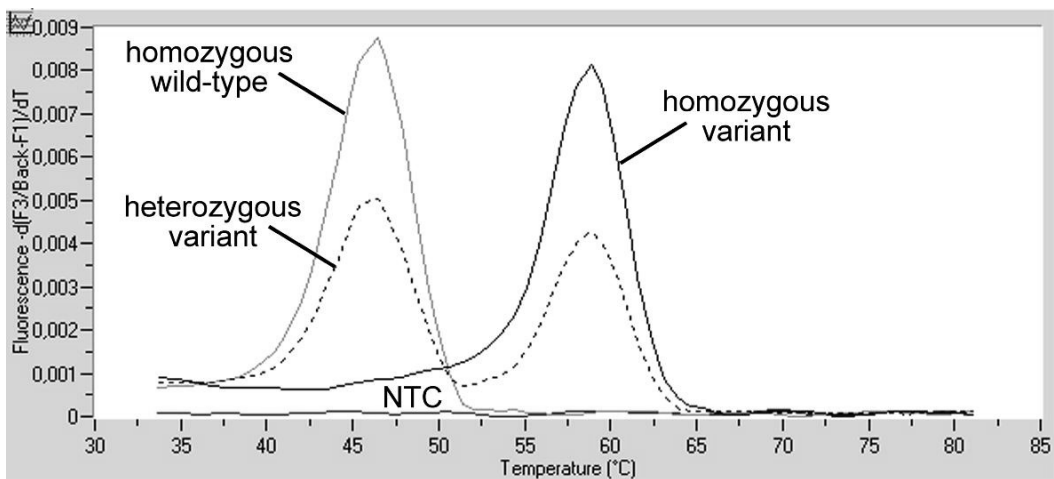


Fig. 8: Fluorogram for the detection of the nucleotide exchange at nt 1298 by means of the *artus* MTHFR LC PCR Kit in fluorimeter channel F3/Back-F1. NTC: no template control (negative control).

The genotype is determined by combining the allelic variants. It has to be pointed out that in the presence of two heterozygous variants these may be located on one allele as well as on two alleles. So far the combined presence of both genetic variants in the homozygous form has not been reported in the literature. A reduction of the MTHFR enzyme activity may be expected if at least one genetic variant is present.

Carriers of two wild-type alleles are expected to exhibit a normal enzyme activity, provided other non-genetic factors do not influence it. Carriers of at least one genetically modified allele (MTHFR wt677/var677, wt1298/var1298) are expected to exhibit a reduced enzyme activity. If both alleles are affected, the risk of genetically induced side-effects is increased.

The alleles described above result in different possible genotypes. These are listed in the following table (see Table 2).

Table 2: Influence of the genotype on the enzyme activity.

Homozygous wild-type genotype	Heterozygous variant genotype	Homozygous variant genotype
MTHFR wt677/wt677 wt1298/wt1298	MTHFR wt677/var677 wt1298/wt1298	MTHFR var677/var677 wt1298/wt1298
	MTHFR wt677/wt677 wt1298/var1298	MTHFR wt677/wt677 var1298/var1298
	MTHFR wt677/var677 wt1298/var1298	
normal enzyme activity	reduced enzyme activity	

The positive control (*MTHFR LC Control*) included in the *artus* MTHFR LC PCR Kit represents the heterozygous allelic states (see Fig. 7 and 8).

10. Troubleshooting

No signal with the positive control (*MTHFR LC Control*) or the samples in fluorimeter channels F2/Back-F1 or F3/Back-F1, respectively:

- Incorrect programming of the temperature profile of the *LightCycler 1.1/1.2/1.5* Instrument.
 - Compare the temperature profile with the protocol (see **8.3 Programming of the *LightCycler 1.1/1.2/1.5* Instrument**).
- Incorrect configuration of the PCR reaction.
 - Check your work steps by means of the pipetting scheme (see **8.2 Preparing the PCR**) and repeat the PCR, if necessary.
- The storage conditions for one or more kit components did not comply with the instructions given in **2. Storage** or the *artus* MTHFR LC PCR Kit had expired.
 - Please check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
- The PCR was inhibited.
 - Make sure that you use a recommended isolation method (see **8.1 DNA Isolation**) and stick closely to the manufacturer's instructions.
 - Make sure that during the DNA isolation the recommended additional centrifugation step has been carried out before the elution in order to remove any residual ethanol (see **8.1 DNA Isolation**).
- DNA was lost during extraction.
 - Make sure that you use a recommended isolation method (see **8.1 DNA Isolation**) and stick closely to the manufacturer's instructions.

Weak fluorescence peak

- Mix the components thoroughly before use.
- Check the amplification conditions.
- Pre-cool the Cooling Block including the adapters to approximately +4°C.
- Cool all reagents during pipetting.

If you have any further questions or problems, please contact our Technical Service.

11. Specifications

11.1 Analytical Sensitivity

The *artus* MTHFR LC PCR Kit allows the detection of the individual genetic constitution with respect to the genetic variants nt 677 and nt 1298 in the human MTHFR gene by means of the *LightCycler* technology. Human genomic DNA was purified from blood samples, quantified by spectrophotometry, and diluted in serial dilution steps. A minimum of 0.12 ng genomic DNA (20 copies) per PCR corresponding to 0.005 – 0.02 µl blood (depending on donor and purification) is sufficient for the detection of the genetic variant.

11.2 Analytical Specificity

The specificity of the *artus* MTHFR LC PCR Kit is first and foremost ensured by the selection of the primers and probes, as well as the selection of stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. Furthermore, the specificity for the detection of this genetic polymorphism was ensured by sequencing of the single allelic variants and subsequent sequence comparison in international gene data banks.

11.3 Diagnostic Sensitivity and Specificity

The frequency of polymorphisms in the Caucasian population, as described by Botto & Yang (2000), was confirmed using 300 DNA samples and the kit components.

12. Product Use Limitations

- All reagents may exclusively be used in in vitro diagnostics.
- The product is to be used by personnel specially instructed and trained in the in vitro diagnostics procedures only.
- 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 expired components.
- The MTHFR genotype should only be interpreted in combination with additional clinical information. Monitoring for side effects of methotrexate should be performed on all patients after genetic testing to minimize risks associated to false negative results.
- Although rare, new mutations within the regions of the MTHFR gene covered by the kit's primers and/or probe but at other locations than nucleotides nt 677 and nt 1298 may result in shifts of the melting curves.

13. Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

Discard sample and assay waste according to your local safety regulations.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

14. Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of *artus* MTHFR LC PCR Kit has been tested against predetermined specifications to ensure consistent product quality.

15. References

- (1) Botto LD, Yang Q. 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. *Am J Epidemiol.* 2000 May 1; 151 (9): 862 – 877.
- (2) Junker R, Nowak-Gööt U, Fobker M. The methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and other genetic causes of hyperhomocysteinemia in venous thrombosis. *J Lab Med.* 2001; 25 (7/8): 239 – 253.
- (3) Kang SS, Wong PW, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. *Am J Hum Genet.* 1991 Mar; 48 (3): 536 – 545.
- (4) Mackay IM. Real-time PCR in the microbiology laboratory. *Clin. Microbiol. Infect.* 2004; 10 (3): 190 – 212.
- (5) Toffoli G, Veronesi A, Boiocchi M, Crivellari D. MTHFR gene polymorphism and severe toxicity during adjuvant treatment of early breast cancer with cyclophosphamide, methotrexate, and fluorouracil (CMF). *Ann Oncol.* 2000 Mar; 11 (3): 373 – 374.

16. Explanation of Symbols



Use by



Batch code



Manufacturer



Catalog number



Material number



Handbook



In vitro diagnostic medical device



Components



Contains



Number



<N>

Contains sufficient for <N> tests



Temperature limitation



Consult instructions for use

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artus MTHFR LC PCR Kit

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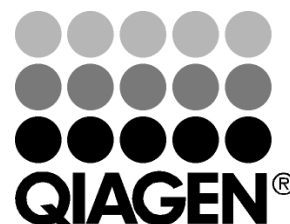
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