artus® TPMT LC PCR Kit

Handbook

24 (catalog no. 4622063)

Qualitative in vitro Diagnostics

For use with the *LightCycler*® Instrument

April 2007 – Version 1

CE

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QIAGEN GmbH, QIAGEN Strasse 1, D-40724 Hilden

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artus TPMT LC PCR Kit

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The *artus* TPMT LC PCR Kit is a CE-marked diagnostic kit according to the European In Vitro Diagnostic Directive 98/79/EC. Not available in all countries.

The QIAamp Kits are intended for general laboratory use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Purchase of *artus* PCR Kits is accompanied by a limited license to use them in the polymerase chain reaction (PCR) process for human and veterinary in vitro diagnostics in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Applied Biosystems or as purchased, i.e. an authorized thermal cycler. The PCR process is covered by the foreign counterparts of U.S. Patents Nos. 5,219,727 and 5,322,770 and 5,210,015 and 5,176,995 and 6,040,166 and 6,197,563 and 5,994,056 and 6,171,785 and 5,487,972 and 5,804,375 and 5,407,800 and 5,310,652 and 5,994,056 owned by F. Hoffmann-La Roche Ltd.

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Table of Contents

artus® TPMT LC PCR Kit*

For use with the *LightCycler*® Instrument.

1. Contents

	Labelling and contents	Art. No. 4622063 24 reactions
Blue	*TPMT LC Master A*	2 x 12 rxns
Blue	*TPMT LC Master B*	2 x 12 rxns
Yellow	*TPMT LC Mg-Sol*¤	1 x 400 µl
Red	*TPMT LC Control Aw*	1 x 200 µl
Red	*TPMT LC Control Av*	1 x 200 µl
Red	*TPMT LC Control B*	1 x 200 µl
White	*Water (PCR grade)*	1 x 1,000 µl

¤ *Mg-Sol* = *Magnesium Solution*

2. Storage

The components of the *artus* TPMT 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.

* TPMT = Thiopurine S-methyltransferase.

3. Additionally Required Materials and Devices

- Disposable powder-free gloves
- DNA isolation kit (see **8.1 DNA Isolation**)
- Pipettes (adjustable)
- Sterile pipette 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)
- *LightCycler*® Cooling Block
- *LightCycler*® Instrument
- *LightCycler*® Capping Tool

4. General Precautions

The user should always pay attention to the following:

- Please analyse a **maximum of twelve samples** per PCR run. If more than twelve samples are analysed in parallel, untypical melting curve behaviour might be observed for the heterozygous variant nt 238.
- Use sterile pipette 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 no or reduced activity of the enzyme thiopurine S-methyltransferase (TPMT) due to a genetic modification bear an elevated risk for the occurrence of side-effects (1 - 3) under the therapy with 6-thioguanine, 6-mercaptopurine or azathioprine (e.g. hemic toxicity, hepatic toxicity). An examination of the TPMT gene allows the estimation of the genetically caused risk of therapy-induced side-effects. Carriers of a genetic variant of the TPMT 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 thiopurine S-methyltransferase enzyme activity is determined among others by modifications in the TPMT 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 TPMT gene are located at nucleotides (nt) 238, 460 and 719. Further genetic variants have been described in the literature, which, however, were rarely or only once observed in a population. About 10 % of the white population show a reduced TPMT activity of approx. 75 %. No measurable enzyme activity can be observed in 0.3 % of these human beings. Comparative investigations of genotype and phenotype observed a correlation of 87 % between genotype and enzyme activity.

Genotyping may help to individually optimize medication. Particularly, in the case of tumour treatment using thiopurines, treatment of chronic inflammatory bowel diseases or after transplantations the analysis of the TPMT gene is useful. Adverse drug reactions and increased costs (prolonged stay in hospital etc.) may be reduced.

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* TPMT LC PCR Kit allows a simple, fast and safe testing of human DNA for the presence of a clinically relevant genetic variant in the TPMT gene. This analysis allows the assessment of therapy-induced risks, for example as a result of thiopurine treatment.

The analysis is performed by the detection of the genetic variants within the TPMT gene using the *LightCycler*® Instrument. The reagents contain primers for the amplification of particular regions of the human TPMT gene as well as fluorescence labelled probes for the detection of genetic variants at nucleotide positions nt 238 in exon 5, nt 460 in exon 7 and nt 719 in exon 10. In addition, positive controls *A* (*Aw* / *Av*) and *B* are 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* TPMT 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	Catalogue number	Manufacturer
Blood	QIAamp DNA Blood Mini Kit (50)	51 104	QIAGEN

- The *artus* TPMT 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

Attention: Please analyse a **maximum of twelve samples** per PCR run. If more than twelve samples are analysed in parallel, untypical melting curve behaviour might be observed for the heterozygous variant nt 238.

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, the positive controls (*TPMT LC Control Aw*, *TPMT LC Control Av* and *TPMT LC Control B*) and a negative control (*Water*, *PCR grade*) included in the *artus* TPMT 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**
1. Preparation of Master Mix	*TPMT LC Master A* *or* *TPMT LC Master B*	16 µl
	TPMT LC Mg-Sol	2 µl
	Total Volume	**18 µl**
2. Preparation of PCR assay	Master Mix	18 µl
	Sample	2 µl
	Total Volume	**20 µl**

Pipette 18 µl of the Master Mix into the plastic reservoir of each capillary. Subsequently add 2 µl of the eluate from the DNA isolation. Correspondingly, 2 µl of the *TPMT LC Control A* (*Aw* / *Av*) or *TPMT LC Control B* 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 (2,000 rpm).

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

Preparing the PCR

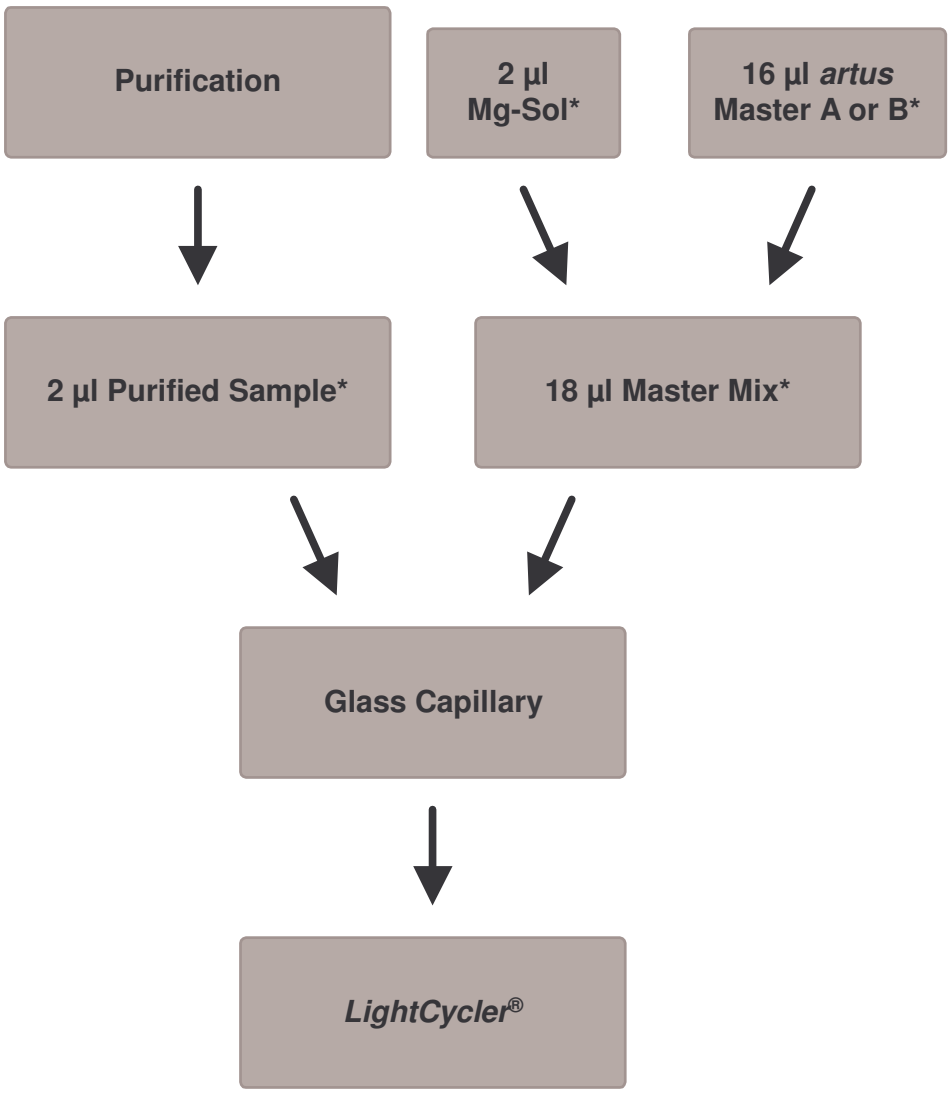

Fig. 1: Schematic workflow.

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

8.3 Programming of the *LightCycler*® Instrument

For the detection of the genetic variants in the TPMT gene, create a temperature profile on your *LightCycler*® 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*® Instrument in the *LightCycler Operator's Manual*.

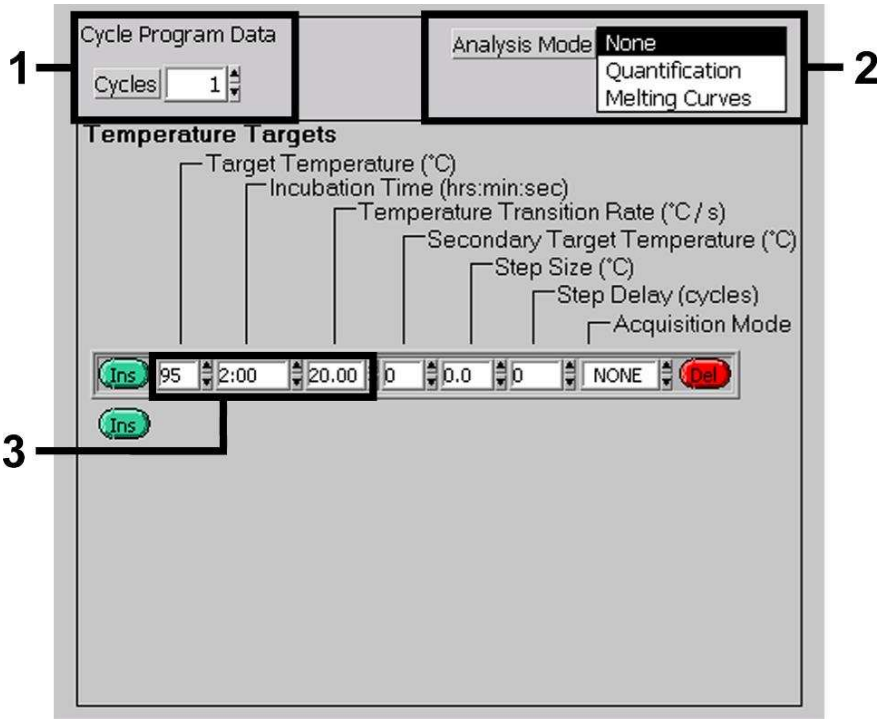

Fig. 2: Initial Activation of the Hot Start Enzyme.

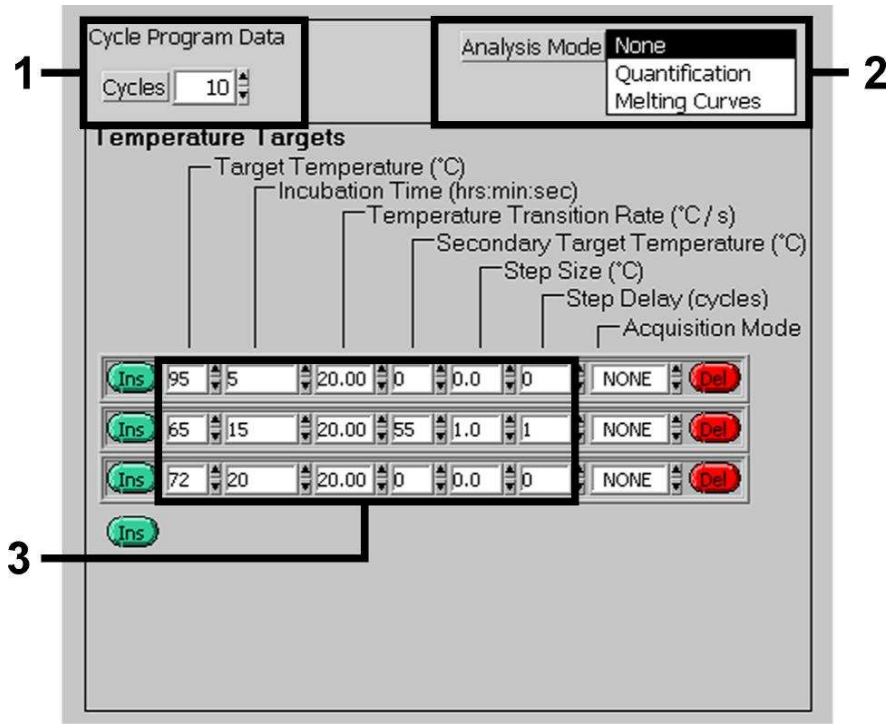

Fig. 3: Touch Down Step.

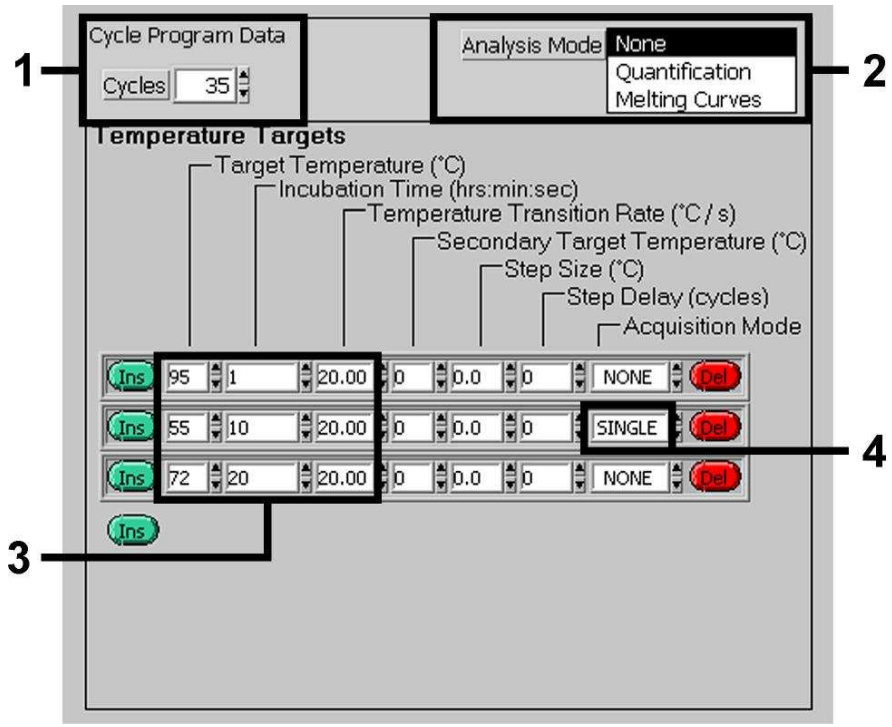

Fig. 4: Amplification of the DNA.

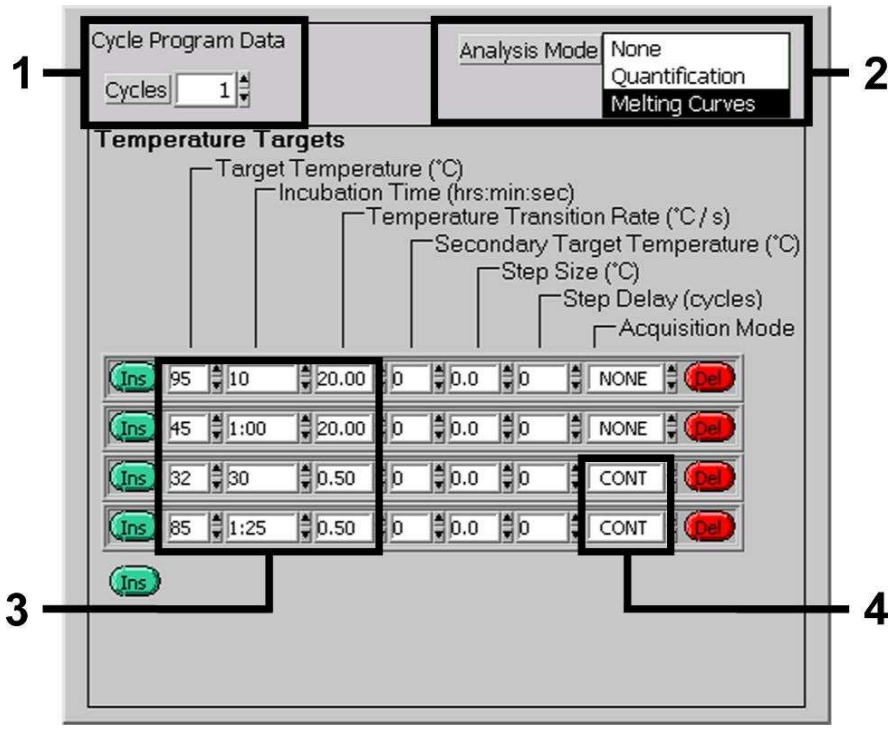

Fig. 5: Melting Curve.

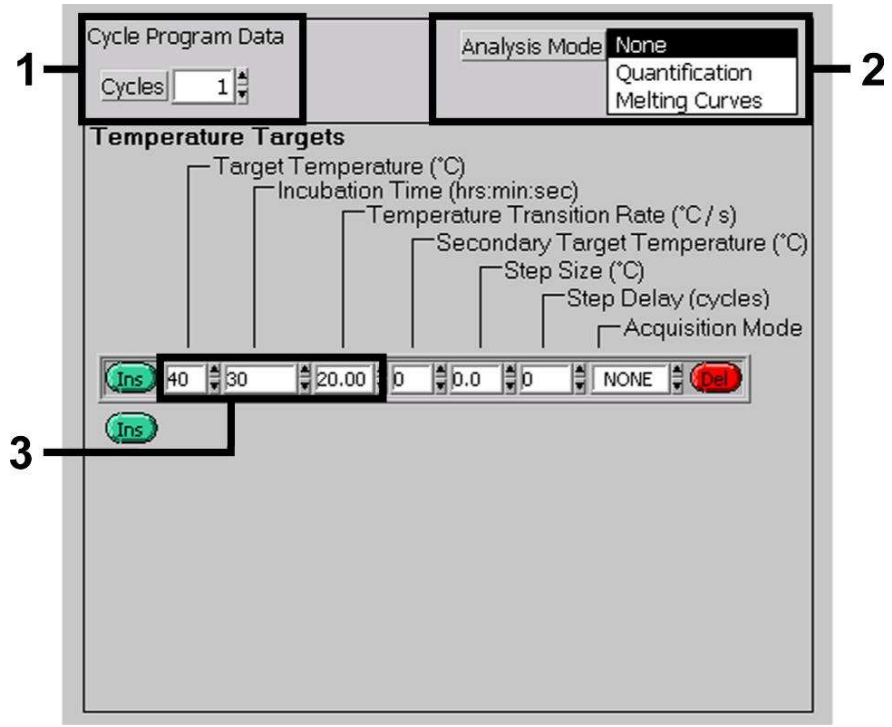

Fig. 6: Cooling.

9. Data Analysis

In multicolour 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* TPMT LC PCR Kit please select fluorescence display options F2/Back-F1 and F3/Back-F1 for the TPMT PCR. In most cases a quantitative amplification during the *LightCycler*® run cannot be observed using the *artus* TPMT LC PCR Kit.

The components of the *artus* TPMT LC PCR Kit allow the detection of two genetic variants (wild-type = wt; variant = var) at nucleotide positions (nt) 238, 460 and 719 in the TPMT gene. The determination of the genetic variants is performed by means of the *Melting Curve* programme. 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).

Master	Channel F2			Channel F3		
	nt	wt	var	nt	wt	var
A	238	54 °C	64 °C	-	-	-
B	460	57 °C	47 °C	719	42 °C	54 °C

Please note that the melting points may differ from the indicated temperatures by ± 2 °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 to Fig. 9) show the fluorograms for the detection of the polymorphisms at nucleotide positions nt 238, nt 460 and

nt 719 in the homozygous wild-type form as well as in the heterozygous or the homozygous variant form. The positive controls (*TPMT LC Control A* (*Aw* / *Av*) and *B*) included in the *artus* TPMT LC PCR Kit represents the heterozygous allelic state.

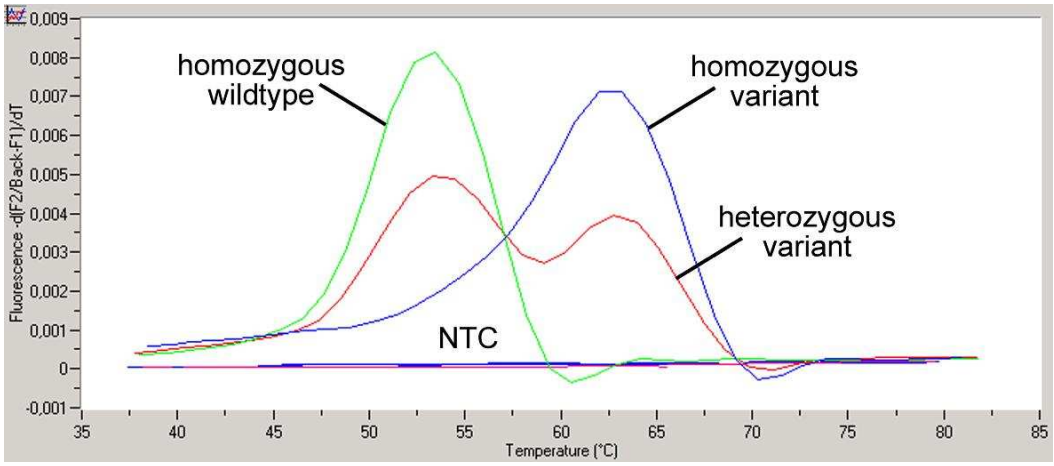

Fig. 7: Fluorogram for the detection of the nucleotide exchange at nt 238 by means of the *artus* TPMT LC PCR Kit (***Master A***) in fluorimeter channel F2/Back-F1. NTC: non-template control (negative control).

Attention: Please analyze **at maximum twelve samples** per PCR run, as untypical melting curve behaviour might occur for the heterozygous variant nt 238, if more than twelve samples are analyzed in parallel.

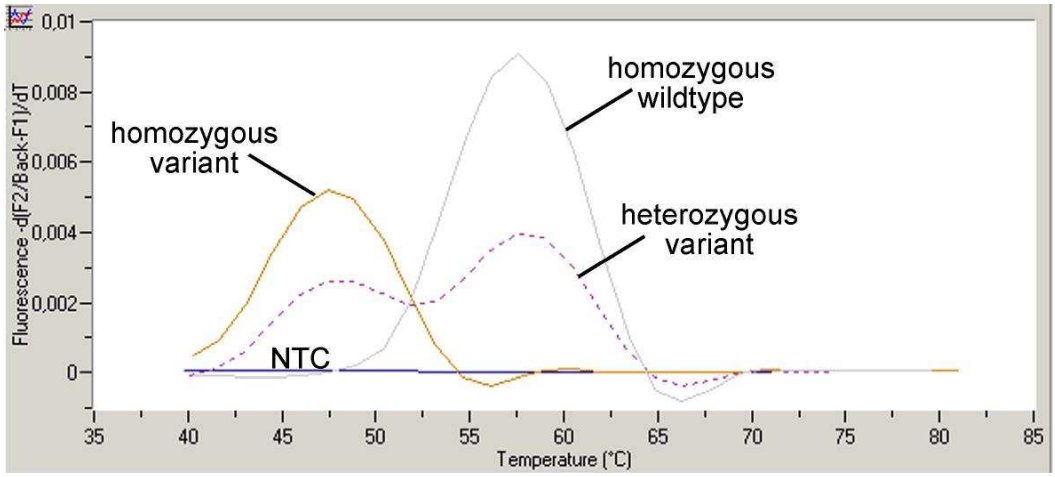

Fig. 8: Fluorogram for the detection of the nucleotide exchange at nt 460 by means of the *artus* TPMT LC PCR Kit (***Master B***) in fluorimeter channel F2/Back-F1. NTC: non-template control (negative control).

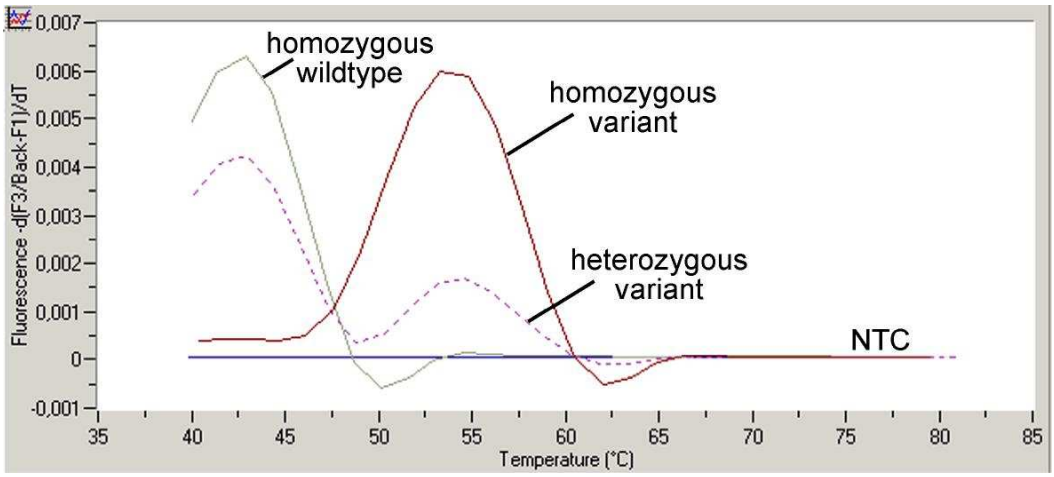

Fig. 9: Fluorogram for the detection of the nucleotide exchange at nt 719 by means of the *artus* TPMT LC PCR Kit (***Master B***) in fluorimeter channel F3/Back-F1. NTC: non-template control (negative control).

The genotype is determined by combining the allelic variants (see Table 2). 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. A reduction of the TPMT 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 are expected to exhibit a reduced enzyme activity. For example the presence of heterozygosity at position nt 460 **and** nt 719, lead to the genotype TPMT*3A/*1 as well as TPMT*3B/*3C. If both alleles are affected, the risk of genetically induced side-effects is increased. Using the *artus* TPMT LC PCR Kit three genetic variants within the TPMT gene are examined. This allows the detection of the following alleles: TPMT*1, TPMT*2, TPMT*3A, TPMT*3B and TMPT*3C.

Table 2: Genetic variants in the TPMT gene.

Allele	nt 238 F2	nt 460 F2	nt 719 F3	Enzyme activity
TPMT*1				normal
TPMT*2	x			reduced
TPMT*3A		x	x	no activity
TPMT*3B		x		reduced
TPMT*3C			x	reduced

The alleles described above result in different possible genotypes. These are listed in the following table (see Table 3).

Table 3: Influence of the genotype on the TPMT enzyme activity.

Homozygous wild-type genotype	Heterozygous or homozygous variant genotype	Homozygous variant genotype
TPMT*1/*1	TPMT*1/*2	TPMT*3A/*3A
	TPMT*1/*3A	
	TPMT*1/*3B	
	TPMT*1/*3C	
	TPMT*2/*2	
	TPMT*2/*3A	
	TPMT*2/*3B	
	TPMT*2/*3C	
	TPMT*3A/*3B	
	TPMT*3A/*3C	
	TPMT*3B/*3B	
	TPMT*3B/*3C	
	TPMT*3C/*3C	
normal enzyme activity	**reduced enzyme activity**	**no enzyme activity**

10. Troubleshooting

No signal with the positive controls (*TPMT LC Control Aw*, *Av* or *B*) or the samples in fluorimeter channel F2/Back-F1 or F3/Back-F1, respectively:

- Incorrect programming of the temperature profile of the *LightCycler*® Instrument.
  - → Compare the temperature profile with the protocol (see **8.3 Programming of the *LightCycler*® 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* TPMT 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.

No signal in the *TPMT LC Master A* sample in fluorimeter channel F3/Back-F1:

- → The *TPMT LC Master A* generates only one signal in fluorimeter channel F2/Back-F1.

Untypical melting curve behaviour at the detection of the nucleotid substitution nt 238 in fluorimeter channel F2/Back-F1:

- More than twelve samples have been analysed in parallel.

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* TPMT LC PCR Kit allows the detection of the individual genetic constitution with respect to the genetic variants nt 238, nt 460 and nt 719 in the human TPMT 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* TPMT 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 in gene banks published sequences 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 in the literature 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 (EN375) 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.

13. Safety Information

For safety information of the *artus* TPMT LC PCR Kit, please consult the appropriate material safety data sheet (MSDS). The MSDS are available online in convenient and compact PDF format at **www.qiagen.com/support/msds.aspx**.

14. Quality Control

In accordance with QIAGEN's ISO 9001 and ISO 13485-certified Total Quality Management System, each lot of *artus* TPMT LC PCR Kit is tested against predetermined specifications to ensure consistent product quality.

15. References

(1) Andersen JB, Szumlanski C, Weinshilboum RM, Schmiegelow K. Pharmacokinetics, dose adjustments, and 6-mercaptopurine/ methotrexate drug interactions in two patients with thiopurine methyltransferase deficiency. Acta Paediatr. 1998 Jan; 87 (1): 108 - 111.

(2) Krynetski EY, Schuetz JD, Galpin AJ, Pui CH, Relling MV, Evans WE. A single point mutation leading to loss of catalytic activity in human thiopurine S-methyltransferase. Proc Natl Acad Sci U S A. 1995 Feb 14; 92 (4): 949 - 953.

(3) Mackay IM. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. 2004; 10 (3): 190 - 212.

(4) Schwab M, Schaffeler E, Marx C, Fischer C, Lang T, Behrens C, Gregor M, Eichelbaum M, Zanger UM, Kaskas BA. Azathioprine therapy and adverse drug reactions in patients with inflammatory bowel disease: impact of thiopurine S-methyltransferase polymorphism. Pharmacogenetics. 2002 Aug; 12 (6): 429 - 436.

16. Explanation of Symbols

Use by

Batch code

Manufacturer

Catalogue number

Material number

Handbook

In vitro diagnostic medical device

<N>

Contains sufficient for <N> tests

Temperature limitation

Mg-Sol *Magnesium Solution*

Austria ■ **QIAGEN Vertriebs GmbH** ■ Löwengasse 47/6 ■ 1030 Wien
Orders 0800/28-10-10 ■ Fax 0800/28-10-19 ■ Technical 0800/28-10-11

Canada ■ **QIAGEN Inc.** ■ 2800 Argentia Road ■ Unit 7 ■ Mississauga ■ Ontario ■ L5N 8L2
Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

France ■ **QIAGEN S.A.** ■ 3 avenue du Canada ■ LP 809 ■ 91974 COURTABOEUF CEDEX
Orders 01-60-920-920 ■ Fax 01-60-920-925 ■ Technical 01-60-920-930

Germany ■ **QIAGEN GmbH** ■ QIAGEN Strasse 1 ■ 40724 Hilden
Orders 02103-29-12000 ■ Fax 02103-29-22000 ■ Technical 02103-29-12400

Italy ■ **QIAGEN S.p.A**. ■ Via Grosio, 10/10 ■ 20151 Milano
Orders 02-33430-411 ■ Fax 02-33430-426 ■ Technical 800-787980

Japan ■ **QIAGEN K.K.** ■ Forefront Tower II ■ 13-1, Kachidoki 3 Chome ■ Chuo-ku, Tokyo 104-0054
Telephone 03-5547-0811 ■ Fax 03-5547-0818 ■ Technical 03-5547-0811

Switzerland ■ **QIAGEN AG** ■ Garstligweg 8 ■ 8634 Hombrechtikon
Orders 055-254-22-11 ■ Fax 055-254-22-13 ■ Technical 055-254-22-12

USA ■ **QIAGEN Inc.** ■ 27220 Turnberry Lane ■ Valencia ■ CA 91355
Orders 800-426-8157 ■ Fax 800-718-2056 ■ Technical 800-DNA-PREP (800-362-7737)

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