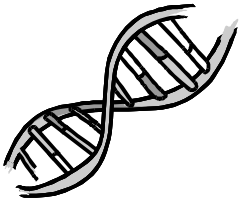
MutaPLATE® Laktase

real time PCR Kit



PCR test for analysis of **-13910 T/C** polymorphism in the regulatory region of the lactase phlorizin hydrolase (LPH) gene (genetic **lactose intolerance** due to primary lactase deficiency) in open *real time* PCR systems (e. g. RotorGene, Stratagene, ECO, ABI, SmartCycler).

REF KF1907132 Σ 32

REF KF1907196 Σ 96

-20°C

For research use only

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CE

CONTENT

1. INTENDED USE

MutaPLATE® Laktase *real time* PCR kit is a molecular biological test for analysis of -13910 T/C - polymorphism in the regulatory region of lactase-phlorizin-hydrolase gene (LPH) in open *real time* PCR systems e. g. RotorGene (Corbett Research), MX-3000P (Stratagene), ECO (Amplifa) or SmartCycler (Cepheid). Only the **clinical relevant C/C -** genotype causes a primary lactase deficiency often leading to (genetic) lactose intolerance.

2. INTRODUCTION

Patients with **lactose intolerance** can not digest milk sugar and suffer after ingestion of milk-products from **dyspepsia, nausia and bellyache**. Further symptoms like vertigo, sleep disorders, akne or depressions can also be triggered by lactose intolerance. A Therapy for affected persons is very simple and can be done by **lactose-free diet**. In Germany, about 15 million people are affected from primary lactase deficiency [1].

The main reason for lactose intolerance is a **genetically based deficiency** of the enzyme lactase phlorizin hydrolase (LPH), which is responsible for the disassembly of milk sugar. This widely distributed genetic disorder is a T/C polymorphism located at position **–13910** in the regulatory region of this gene [2]. Person **homozygous for C/C**-genotype are consequently deficient for enzyme lactase and posses higher **risk for lactose intolerance**. These results are in excellent accordance with results obtained by the **lactose hydrogen breath test** for the diagnosis of lactase non-persistence [3]. Nevertheless, not all C/C-carriers must show typical symptoms because a fall short of individual level is necessary.
Furthermore, in some cases lactose intolerance can be due to **secondary** causes like mal-resorption problems (e. g. Morbus Crohn patients), infections or chemotherapy [4].

In **babyhood** and **infancy** the lactase production is very high but it decreases with **higher age** resulting in manifestation of primary lactase deficiency. Also a **North-/ South gradient** is visible: In Scandinavia the homozygous C/C-constellation is very rare whereas in Germany prevalence is about 15-20%. In Southern European countries up to 30% of all adults carry the C-allels homozygous.

Patients suffering from lactose intolerance have also a higher **risk for osteoporosis** due to the reduced calcium-intake via milk products [5]. In consequence, the C/C-genotype associated with primary lactose intolerance is a genetic risk factor for bone fractures for elderly people [6].

[1] Buning C, Ockenga J, Kruger S, Jurga J, Baier P, Dignass A, Vogel A, Strassburg C, Weltrich R, Genschel J, Lochs H, Schmitt H. (2003) The C/C (-13910) and G/G (-22018) genotypes for adult-type hypolactasia are not associated with IBD. Scand J Gastroenterol **38:** 538-542.

[2] Enattah NS, Sahi T, Savilahti E, Terwilliger JD, Peltonen L, Jarvela I. (2002). Identification of a variant associated with adult-type hypolactasia. Nat Genet **30:** 233-237.

[3] Högenauer C, Hammer HF, Mellitzer K, Renner W, Krejs GJ, Toplak H (2005). Evaluation of a new DNA test compared with the lactose hydrogen breath test for the diagnosis fo lactase non-persistence. Eur J Gastroenterol Hepatol **17**: 371-376.

[4] Sibley E (2004) Genetic Variation/ Lactose Intolerance. Detection Methods/ Clinical Implications. Am J Pharmacogenomics **4** (4): 239-245.

[5] Obermeyer-Pietsch BM, Bonelli CM, Walter DE, Kuhn RJ, Fahrleitner-P A, Berghold A, Goessler W, Stepan V, Dobnig H, Leb G, Renner W (2004) Genetic predisposition for adult lactose intolerance and relation to diet, bone density, bone fractures. J Bone a. Mineral Research **19** (1): 42-47.

[6] Ennattah NS, Sulkava R, Halonen P, Kontula K, Järvelä I (2005) Genetic variant of lactase-persistent C/T- -13910 is associated with bone fractures in very old age. JAGS **53**: 79-82.

3. PRINCIPLE OF THE TEST

MutaPLATE® Laktase *real time* PCR Kit contains specific primers and additional material for the detection of the T/C (-13910) polymorphism in the regulatory region of the lactase-phlorizin-hydrolase gene. The variable area of the regulatory region from lactase gene is amplified by PCR using **genomic DNA-template**. The specific primers used in the kit flank the variable area of lactase gene (LCT) and generate an **amplificate of 222 bp**.

The standard PCR contains also **sequence specific oligonucleotides** marked with fluorescence dye. This specific hybridisation probe binds within the amplification product including the single nucleotide polymorphism (SNP) of target-DNA. Due to this, a **fluorescence signal** is generated (after **excitation with 530 nm** or if limited by instrument with 470 nm) and detected at **610 nm** through the **optical unit** of the *real time* PCR instrument.

Genotyping is performed by subsequent **melting curve analysis** of arised amplificates leading to unequivocal identification of **C/C-genotype** associated with lactose intolerance and respectively the clinical unobtrusive **CT- and T/T-variants**. This is due to the different melting points of the complexes formed by DNA template and "SNP-probes". The included "SNP-probe" is 100% homologous to the **C-allel**. Therefore the hybridisation probe needs a higher temperature for complex-dissociation from C-allel than from the T-allel (containing a mismatch destabilizing the complex). Consequently, samples with **heterozygous** genotype generate **both peaks** at different temperatures during the melting curve analysis.

4. KIT CONTENT

Each kit contains enough reagents to perform **32** respectively **96** tests. Each kit also contains a package insert.

Reference	**Type of reagent**	**Volume** KF1907132 (**32** det)	**Volume** KF1907196 (**96** det.)
Blue	**Enzyme-/ Buffer Mix**	**440 µl**	**3 x 440 µl**
Yellow	**Primer-/ Probe Mix**	**370 µl**	**3 x 370 µl**
Red	**Positive Control** (LPH, -13910 T/C)	**15 µl**	**3 x 15 µl**
Green	**Negative Control**	**200 µl**	**200 µl**

5. REQUIRED MATERIALS

Provided:

- Reagents for *real time* PCR
- Package insert

Not provided:

- *real time* PCR system (e. g. RotorGene, MX300P, ECO, SmartCycler, or others)
- PCR reaction tubes
- Cryo-container for PCR reaction tubes
- DNA extraktion kit for isolation of genomic DNA (ca. 10 ng/µl)
- Pipetts (0,5 – 200 µl)
- sterile filter tipps for micro-pipets
- sterile microtubes
- gloves (powder free)

6. STORAGE AND HANDLING

- All reagents should be **stored at <-20°C till immediate use**. Spin down kit components in their vials before long-term storage.
- **Avoid several freeze / thaw** cycles for the reagents; if necessary prepare suited aliquots and freeze reagents again **immediately**.
- During preparation of PCR perform all working steps in a cryo-container or **cool all reagents** in suited manner.
- Store Primer-/ Probe-Mix (**610 nm** detection mix) **in the dark** (**light protection**).
- All reagents can be used until the expiration date (printed on the labels).

7. WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- This assay needs to be carried out by especially in molecular biology skilled personnel.
- This assay needs to be run according to GLP (Good Laboratory Practice).
- Clinical samples should be regarded as potentially infectious materials.
- Mix all reagents carefully before use by pipetting (15-20x), but do not vortex.
- Do not use the kit after its expiration date.

8. TEST PERFORMANCE

Before start, **decontaminate** all working areas and used instruments. Thaw kit components **immediately before use** and handle detection mix (yellow) in the dark. Prepare the necessary amount of PCR reaction tubes in a pre-cooled cooling block and consider additional 2 tubes for controls (red, green). Keep DNA samples ready and mix again well before the direct use.

Master Mix preparation

Following table shows the composition for **one reaction** (final volume: 25 µl). For analysis of several samples in parallel, a **master mix** should be prepared in a sterile vial **multiplying** each single volume by number **N** of samples (inclusive controls). *Additionally, 10% more volume should be calculated for reasons of inaccuracy*. The reagents should be pipetted in same order as indicated in the table:

Reagent	Volume	Master Mix Volume
Detection Mix (yellow)	**10,5 µl**	**10,5 µl** x (N + 10%)
Enzyme Mix *ready to use* (blue)	**12,5 µl**	**12,5 µl** x (N + 10%)

Mix prepared master mix well by gently pipetting (about **15 – 20 x, do not vortex**) and aliquot **23 µl** into each PCR reaction tube (corresponding to patient-samples and controls).

Samples (+ Controls)

Add **2 µl** of each sample DNA in the corresponding PCR reaction tube; prepare first **both controls** (1. negative and 2. positive). Close all tubes (but negative control last as "true contamination control") and transfer them into the *real time* PCR instrument (retain same order).

Protocol

Activate following **PCR-protocol** and perform subsequently the *real time* PCR:

Experimental Protocol

Program:	**Denaturation**			Type:	None	Cycles	1
Segment Number	Temperature Target (°C)	Hold Time	Step (C'/sec)	2° Target Temp. (°C)	Step Size	Step Delay (Cycles)	Acquisition Mode
1	95	120	20	0	0	0	None

Program:	**Amplification**			Type:	Quantification	Cycles	45
Segment Number	Temperature Target (°C)	Hold Time	Step (C'/sec)	2° Target Temp. (°C)	Step Size	Step Delay (Cycles)	Acquisition Mode
1	95	30	20	0	0	0	None
2	50	30	20	0	0	0	Single
3	72	30	20	0	0	0	None

Program:	**Melting Curve**			Type:	Melting Curves	Cycles	1
Segment Number	Temperature Target (°C)	Hold Time	Step (C'/sec)	2° Target Temp. (°C)	Step Size	Step Delay (Cycles)	Acquisition Mode
1	95	20	20	0	0	0	None
2	40	20	20	0	0	0	None
3	75	0	0.2	0	0	0	Continuous

Program:	Cooling			Type:	None	Cycles	1
Segment Number	Temperature Target (°C)	Hold Time	Step (C'/sec)	2° Target Temp. (°C)	Step Size	Step Delay (Cycles)	Acquisition Mode
1	40	30	20	0	0	0	None

9. ANALYSIS OF GENOTYPES AND INTERPRETATION OF RESULTS

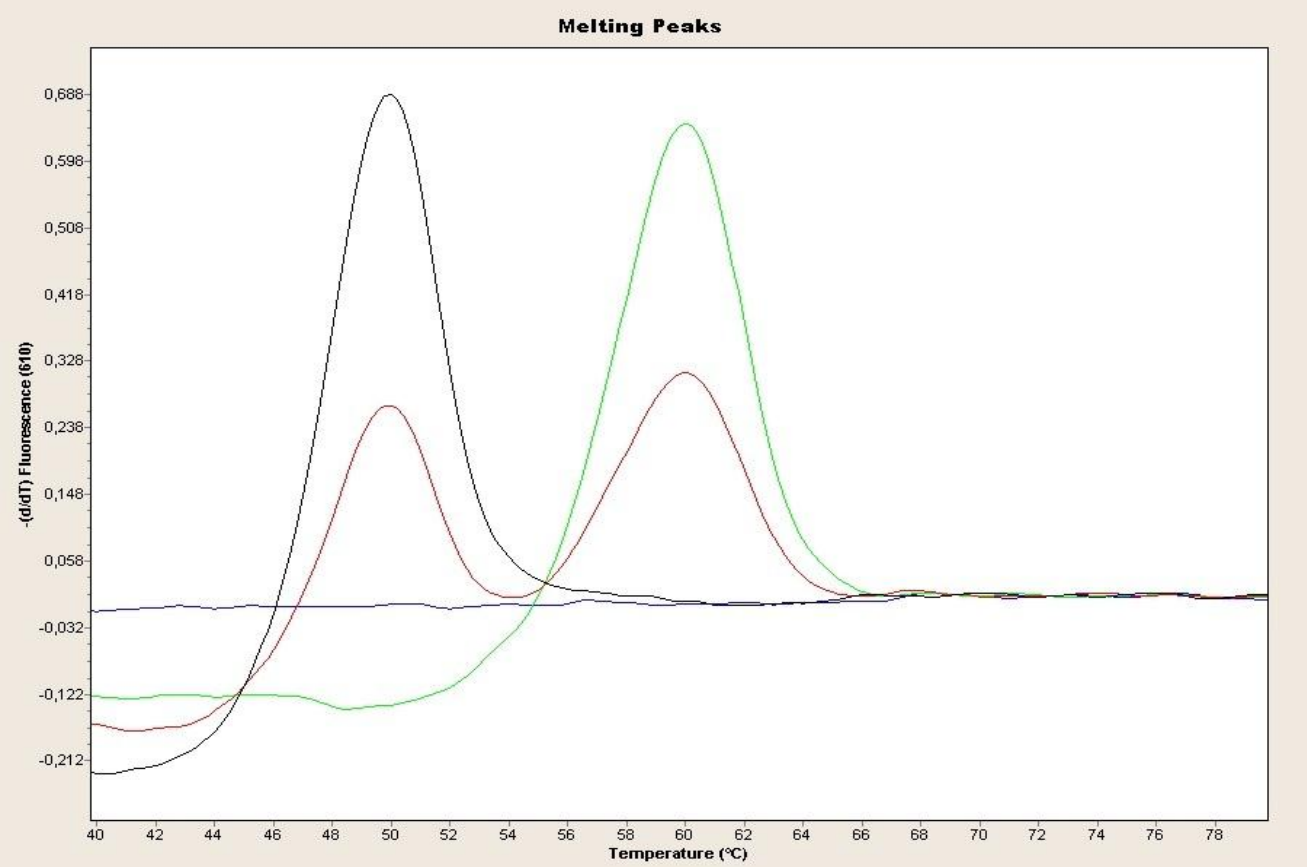
Results of **melting curve** analysis for the T/C (-13910) polymorphism are shown **at 610 nm** Excision is done at 530 nm (resp. optional 470 nm in some instruments, e.g. RotorGene); choose corresponding channel of your *real time* PCR instrument.

(In case requested by the instrument, use following pre-settings for the melting curve analysis: Calculation mode: polynominal, Digital filter: enabled, Degrees to average: 9)

The **positive control** contains a template **heterozygous** for T/C (-13910) polymorphism (one allel carries mutation (T-allel) and the other the wildtype (C-allel).

Following **figure** shows a typical **example** for **homozygous** (black: T/T, green: C/C) as well as **heterozygous** (red: C/T) samples. Indicated temperatures should be found again within +/- 1°C:

T/C (-13910) polymorphism



Temperature **T**-allel: 52,0 °C Temperature **C**-allel: 61,0 °C

10. TROUBLESHOOTING

No fluorescence peak with positive control or samples at **610 nm** :

- Proof PCR-program of the real time PCR instrument in use:

⇒ repeat analysis with corrected protocol.

- MutaPLATE® Laktase kit was thawed/ frozen more than twice or stored longer than four days at 2-8 °C:

⇒ consider storage recommendations. Repeat analysis with new MutaPLATE® Laktase reagents.

- low quality of DNA -template:

⇒ exactly follow the manufacturer`s manual for DNA extraction.

Low fluorescence peak at **610 nm**:

- mix single components carfully before use (only by pipetting several times - do not vortex!).
- cool all stock solutions during the working steps in suited manner and protect the detection mix from light.
- Working on ice or with cooled (4°C) Block is recommended.

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For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.

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