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

REF KF1907196

-20°C

For in vitro diagnostic only
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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 malresorption 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].

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.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of reagent</th>
<th>Volume KF1907132 (32 det.)</th>
<th>Volume KF1907196 (96 det.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>Enzyme-/Buffer Mix</td>
<td>440 µl</td>
<td>3 x 440 µl</td>
</tr>
<tr>
<td>Yellow</td>
<td>Primer-/Probe Mix</td>
<td>370 µl</td>
<td>3 x 370 µl</td>
</tr>
<tr>
<td>Red</td>
<td>Positive Control (LPH, -13910 T/C)</td>
<td>15 µl</td>
<td>3 x 15 µl</td>
</tr>
<tr>
<td>Green</td>
<td>Negative Control</td>
<td>200 µl</td>
<td>200 µl</td>
</tr>
</tbody>
</table>
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 extraction kit for isolation of genomic DNA (ca. 10 ng/µl)
- Pipettes (0.5 – 200 µl)
- sterile filter tips for micro-pipets
- sterile microtubes
- gloves (powder free)

6. STORAGE AND HANDLING

- All reagents should be stored at \(-20^\circ\text{C}\) 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:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Master Mix Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection Mix (yellow)</td>
<td>10,5 µl</td>
<td>10,5 µl ( \times (N + 10%) )</td>
</tr>
<tr>
<td>Enzyme Mix ready to use (blue)</td>
<td>12,5 µl</td>
<td>12,5 µl ( \times (N + 10%) )</td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>Program:</th>
<th>Denaturation</th>
<th>Type:</th>
<th>Cycles</th>
<th>Acquisition Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temperature Target (°C)</td>
<td>Hold Time</td>
<td>2° Target Temp. (°C)</td>
<td>None</td>
</tr>
<tr>
<td>Segment Number</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
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<td>120</td>
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<table>
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<th>Quantification</th>
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<tr>
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<td>Temperature Target (°C)</td>
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<td>2° Target Temp. (°C)</td>
<td>Step Size</td>
<td>Step Delay (Cycles)</td>
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<tr>
<td>Segment Number</td>
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<td>30</td>
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</table>

<table>
<thead>
<tr>
<th>Program:</th>
<th>Melting Curve</th>
<th>Type:</th>
<th>Melting Curves</th>
<th>Cycles</th>
<th>Acquisition Mode</th>
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<td></td>
<td>Temperature Target (°C)</td>
<td>Hold Time</td>
<td>2° Target Temp. (°C)</td>
<td>Step Size</td>
<td>Step Delay (Cycles)</td>
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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 allele carries mutation (T-allele) and the other the wildtype (C-allele).

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
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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EAGLE BIOSCIENCES, INC.

20A NW Blvd, Suite 112 Nashua, NH  03063
Phone: 617-419-2019  •  FAX: 617-419-1110
www.EagleBio.com  •  info@eaglebio.com

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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