MutaREAL® 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 real time capillary systems (e. g. LightCycler®, Roche).

For in vitro diagnostic only

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1. INTENDED USE

MutaREAL® Laktase real time PCR kit is a molecular biological test for analysis of the -13910 T/C - polymorphism in the regulatory region of lactase phlorizin hydrolase gene (LPH) in capillary systems (e. g. LightCycler®, Roche). The clinical relevant C/C - genotype causes 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].

3. PRINCIPLE OF THE TEST

**MutaREAL® 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 with the LightCycler® (Roche). The variable area of the regulatory region from lactase gene is amplified by PCR using LightCycler®-capillaries and **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 additionally **two sequence specific oligonucleotides** marked with fluorescence dye (FRET-hybridization probes) – called „anchor probe“ and „SNP-probe“. Both probes bind closely together at the amplified target-DNA which includes the single nucleotide polymorphism (SNP). Due to this, a fluorescence signal is generated and detected by the **optical unit** of the **real time** PCR instrument (e. g. Light Cycler®, Roche).

**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 24 respectively 96 tests. Each kit also contains a package insert.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of reagent</th>
<th>Volume KF2907124 (24 det.)</th>
<th>Volume KF2907196 (96 det.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue A1a</td>
<td>enzyme mix</td>
<td>ca. 10 µl</td>
<td>3 x ca. 10 µl</td>
</tr>
<tr>
<td>Blue A1b</td>
<td>enzyme buffer</td>
<td>ca. 60 µl</td>
<td>3 x ca. 60 µl</td>
</tr>
<tr>
<td>Yellow A2</td>
<td>primer / probe mix</td>
<td>450 µl</td>
<td>4 x 450 µl</td>
</tr>
<tr>
<td>Red A3</td>
<td>positive control (LPH, -13919 T/C)</td>
<td>10 µl</td>
<td>3 x 10 µl</td>
</tr>
<tr>
<td>Green A4</td>
<td>negative control</td>
<td>200 µl</td>
<td>200 µl</td>
</tr>
</tbody>
</table>
5. REQUIRED MATERIALS

Provided:
- PCR reagents
- Package insert

Not provided:
- real time PCR capillary system (e.g. LightCycler® instrument, Roche)
- PCR reaction tubes (e.g. LightCycler® capillaries, Roche)
- Tabel centrifuge (e.g. LightCycler® capillary centrifuge, Roche)
- Cryo container for PCR reaction tubes (e.g. LightCycler® Cooling Block, Roche)
- Color Compensation Kit
- DNA extraction kit for isolation of genomic DNA (ca. 10 ng/µl)
- Pipets (0.5 – 200 µl)
- sterile filter Tipps for micro pipets
- sterile microtubes
- gloves (powder free)

6. STORAGE AND HANDLING

- All reagents (A1 to A4) should be stored at <-20°C till immediate use and then thawed carefully (at 8°C in refrigerator). Spin down kit components in their vials before long-term storage.
- Avoid several freeze / thaw cycles for the reagents A1, A2 and A3 (if necessary prepare suited aliquots and freeze again immediately).
- During preparation of PCR perform all working steps in a cryo-container (e.g. LightCycler® Cooling block) or cool all reagents in suited manner.
- Primer-/ Probe-Mix (A2) should be stored 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.
- Clinical samples should be regarded as potentially infectious materials.
- 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, 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 gently at 8°C and handle detection mix (yellow, A2) in the dark. Prepare the necessary amount of LightCycler®-capillaries in a pre-cooled LightCycler®-Cooling Block and consider additional 2 capillaries for controls (red A3, green A4). Keep DNA samples ready and mix well before use.

**Enzyme mix preparation (ready to use)**
Centrifuge shortly both blue vials (A1a and A1b) to collect the solutions at the bottom of the vials. Transfer now the content of solution A1b (enzyme buffer) with sterile filter tip quantitative into vial A1a (enzyme) and mix well by pipetting (ca. 15x, do not vortex!). This ready to use enzyme mix is stable for about 3 month at -20°C; after freezing, this solution can be thawed twice at 8°C provided that it was not stored longer than one hour (cooled) during the working steps.

**Master mix preparation**
Following table shows the composition for one reaction (final volume: 20 µl). For analysis of several samples in parallel, a master mix should be prepared in a sterile vial multiplying each single volume by the number N of samples (incl. 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, A2)</td>
<td>16 µl</td>
<td>16 µl x (N + 10%)</td>
</tr>
<tr>
<td>Enzyme Mix ready to use (blue, A1)</td>
<td>2 µl</td>
<td>2 µl x (N + 10%)</td>
</tr>
</tbody>
</table>

Mix prepared master mix well by gently pipetting (about 15 – 20 x) and aliquot 18 µl into each LightCycler®-capillary.

**Samples**
Add 2 µl of each sample DNA in the corresponding LightCycler®-capillaries; use first two capillaries for the both controls (1. negative control, 2 µl and 2. positive control, 2 µl).

Close the filled LightCycler®-capillaries with their tips (if there is no capillary-tool from Roche use sterile tweezers to avoid contamination).

Transfer capillaries into the LightCycler® Carousel and keep position of capillaries (respectively samples).

Spin down samples in the LightCycler® Carousel-centrifuge (if a table centrifuge is used, insert the LightCycler®-Cooling Block with the capillaries inside and centrifuge at 3.000 rpm for 15 sec.).
Protocol

Insert the LightCycler®-Carousel with all sample-loaded capillaries into the LightCycler®-instrument. Activate following **PCR-protocol** and perform subsequently the LightCycler®-**real time PCR**:

### Experimental Protocol

<table>
<thead>
<tr>
<th>Program:</th>
<th>Denaturation</th>
<th>Type:</th>
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<th>Cycles</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segment Number</td>
<td>Temperature Target (°C)</td>
<td>Hold Time</td>
<td>Stepé (°C/sec)</td>
<td>2° Target Temp (°C)</td>
<td>Stepsize (°C)</td>
</tr>
<tr>
<td>1</td>
<td>95</td>
<td>600</td>
<td>20</td>
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<table>
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<td>Stepé (°C/sec)</td>
<td>2° Target Temp (°C)</td>
<td>Stepsize (°C)</td>
</tr>
<tr>
<td>1</td>
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<td>10</td>
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<td>0</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
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<td>20</td>
<td>0</td>
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</tr>
<tr>
<td>3</td>
<td>72</td>
<td>10</td>
<td>20</td>
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<table>
<thead>
<tr>
<th>Program:</th>
<th>Melting Curve</th>
<th>Type:</th>
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<tbody>
<tr>
<td>Segment Number</td>
<td>Temperature Target (°C)</td>
<td>Hold Time</td>
<td>Stepé (°C/sec)</td>
<td>2° Target Temp (°C)</td>
<td>Stepsize (°C)</td>
</tr>
<tr>
<td>1</td>
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<td>20</td>
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<td>3</td>
<td>75</td>
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<table>
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<th>Program:</th>
<th>Cooling</th>
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<tbody>
<tr>
<td>Segment Number</td>
<td>Temperature Target (°C)</td>
<td>Hold Time</td>
<td>Stepé (°C/sec)</td>
<td>2° Target Temp (°C)</td>
<td>Stepsize (°C)</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>30</td>
<td>20</td>
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</table>

9. ANALYSIS OF GENOTYPES AND INTERPRETATION OF RESULTS

For interpretation of melting curves it is necessary to load (and save) an appropriate **Color Compensation File** under „select CC Data“.

Results of melting curve analysis for the T/C (-13910) polymorphism are shown in channel F2 at 640 nm (choose F2/F1).

The melting curve analysis should be performed with following settings:

- Calculation mode: polynomial
- Digital filter: enabled
- Degrees to average: 9

The **positive control (A3)** contains template **heterozygous** for T/C (-13910) polymorphism (one allel carries T-allel, the another allel carries the C-allel).

Following figures shows typical examples for **homozygous** as well as **heterozygous** samples - indicated temperatures should be found again within +/- 1°C:

Date: 2009/01/28
10. TROUBLESHOOTING

No fluorescence peak with positive control or samples at 640 nm (F2):
- Proof PCR-program of the LightCycler®:
  ⇒ repeat analysis with corrected protocol.
- MutaREAL® 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 MutaREAL® Laktase reagents (LightCycler® PCR Kit).
- low quality of DNA-template:
  ⇒ exactly follow the manufacturer’s manual for DNA extraction.

Low fluorescence peak at 640 nm (F2):
- mix single components carefully 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) LightCycler®-Cooling Block is recommended.