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- Trockeneiszuschlag
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Lactose Intolerance LCT

REF: RT-37 or RT-37R

Determination of the C13910T and G22018A polymorphisms in Real Time *PCR*

INTRODUCTION AND PURPOSE OF USE

The lactose intolerance LCT Kit is a qualitative test that allows the allelic discrimination, by means of *Real Time PCR*, of C13910T and G22018A polymorphisms associated to lactose intolerance. The polymorphisms are localized in MCM6 gene located upstream of the LCT gene.
The procedure allows the amplification of Wild-type alleles and mutated alleles of LCT for both polymorphisms, using amplification mix contained in the blue cap tube and in the green cap tube.
Allelic discrimination is performed making a scatter plot of mutated allele's fluorescence versus wild-type allele's fluorescence; discriminating in this way the three possible genotypes: Homozygote Wild-Type, Homozygote Mutated and Heterozygote Mutated for both polymorphisms.
The analysis of the results is made by an instrument of Real Time PCR, composed by a thermal cycler with a system of fluorescence detection.

CONTENT

The kit contains reagents enough to perform 48 amplification tests:

	Quantity	Description
R1	3 x 440 µl	**Amplification mMix** dNTPs, Tris-HCl, KCl, $MgCl_2$, Taq Polymerase, Nuclease-free water, ROX
R2	3 x 130 µl	**LCT C13910Tprobe mix** LCT C/T upstream primer, LCT C13910Tdownstream primer, LCT C/T WT Probe (FAM), LCT C/T MUT Probe (VIC), water.
R3	3 x 130 µl	**LCT G22018A probe mix** LCT G/A upstream primer, LCT G/A downstream primer, LCT G/A WT Probe (FAM), LCT G/A MUT Probe (VIC), water.
R4	3 x 35 µl	**Positive control Wild-Type C13910T (C/T)** Cloned DNA corresponding to Wild-Type MCM6 gene.
R5	3 x 35 µl	**Positive Control Mutated C13910T (C/T)** Cloned DNA corresponding to Mutated MCM6 gene.
R6	3 x 35 µl	**Positive Control Wild-Type G22018A (G22018A)** Cloned DNA corresponding to Wild-Type MCM6 gene.
R7	3 x 35 µl	**Positive Control Mutated G22018A (G22018A)** cloned DNA corresponding to Mutated MCM6 gene
R8	1 x 30 µl	**Negative Control**

Instruction for use: **ST.RT37-ENG.4**

MATERIALS AND STRUMENTATION REQUIRED BUT NOT SUPPLIED

Disposable latex powder-free gloves or similar material;
Bench microcentrifuge (12,000 - 14,000 rpm);
Micropipettes and Sterile tips with aerosol filter;
Vortex;
Plastic materials (microplate and optical adhesive cover);
Dry block shacker for 1.5ml conical tubes
Magnetic rack for 1.5ml conical tubes
EZ1 ADV XL DSP DNA Blood Card (ref. 9018702)

Reagents

The **Lactose Intolerance LCT** kit was developed and validated to be used with the following extraction method:

Manual Extraction

Ref. 51304/51306
QIAmp DNA mini kit. The kit allows the manual DNA extraction from Human samples. The kit contains reagents enough to perform the DNA extraction for 50/250 samples. (QIAGEN)

Automatic extraction

Ref. 62124
EZ1 DSP DNA Blood kit. The kit allows the automatic DNA extraction from Human samples. The kit contains reagents enough to perform the DNA extraction for 48 samples. (QIAGEN)

Manual/Automatic extraction (Siemens)

10629800 - VERSANT® Sample Preparation 1.2 Reagents kit box 1. The kit allows the manual DNA extraction from Human samples. The kit contains reagents enough to perform the DNA extraction for 96 samples.

10629801 - VERSANT® Sample Preparation 1.2 Reagents kit box 2. The kit allows the manual DNA extraction from Human samples. The kit contains reagents enough to perform the DNA extraction for 96 samples.

Strumentation

Automatic Extraction

Ref. 9001492.
EZ1 Advanced XL. Robotic Workstation. (QIAGEN)

The kit **Lactose Intolerance LCT** was developed and validated to be used with the following real time PCR instruments:

Real Time PCR

- 7500 Fast from Lifetechnologies
- StepOne plus from Lifetechnologies
- VERSANT kPCR AD from Siemens or Stratagene MX3005P
- Rotor Gene-Q MDxMDx from QIAGEN
- CFX96 Real Time PCR System from Bio-Rad

Please ensure that the instruments have been installed, calibrated, checked and maintained according to the manufacturer's instruction and recommendations.

SAMPLES AND STORAGE

The **lactose intolerance LCT** system must be used with extracted DNA from the following biological samples: **whole Blood EDTA**. Collected samples must be shipped and stored at +2 - +8°C and used within 3 days from the collected data.
Store the sample at -20°C if it is used after 3 days.

PRECAUTIONS USE

This kit is for in *vitro* diagnostic (IVD), for professional use only and not for in vivo use.
After reconstitution, the amplification master mix must be used in one time (16 reactions). Repeat thawing and freezing of reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently.
At all times follow Good Laboratory Practice (GLP) guidelines.
Wear protective clothing such as laboratory coats and disposable gloves while assaying samples.
Avoid any contact between hands and eyes or nose during specimens collection and testing.
Handle and dispose all used materials into appropriate bio-hazard waste containers. It should be discarded according to local law.
Keep separated the extraction and the reagents preparation.
Never pipette solutions by mouth.
Avoid the air bubbles during the master mix dispensing. Eliminate them before starting amplification.
Wash hands carefully after handling samples and reagents.
Do not mix reagents from different lots.
It is not infectious and hazardous for the health (see Material Safety data Sheet – MSDS).
Do not eat, drink or smoke in the area where specimens and kit reagents are handled.
Read carefully the instructions notice before using this test.
Do not use beyond the expiration date which appears on the package label.
Do not use a test from a damaged protective wrapper.

LIMIT OF THE METHOD

The extreme sensitivity of gene amplification may cause false positives due to cross-contamination between samples and controls. Therefore, you should:

- physically separate all the products and reagents used for amplification reactions from those used for other reactions, as well as from post-amplification products;
- use tips with filters to prevent cross-contamination between samples;
- use disposable gloves and change them frequently;
- carefully open test tubes to prevent aerosol formation;
- close every test tube before opening another one.

The proper functioning of the amplification mix depends on the correct collection, correct transportation, correct storage and correct preparation of a biological sample.
As with any diagnostic device, the results obtained with this product must be interpreted taking in consideration all the clinical data and other laboratory tests done on the patient.
As with any diagnostic device, with this product there is a residual risk of obtaining invalid, false positives or false negatives results.

STORAGE AND STABILITY

Store the product **Lactose Intolerance LCT** at **–20°C**.
The **Lactose Intolerance LCT** kit is shipped on dry ice. The kit components should be frozen. An intact and well stored product has a stability of 12 months from the date of production. Do not use beyond the expiration date which appears on the package label.
Repeat thawing and freezing of reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently.

ANALYTICAL PROCEDURE

Human DNA Extraction

Manual Extraction

Ref. 51304/51306 - QIAmp DNA mini kit (QIAGEN).
Follow the instructions inside the kit *QIAmp DNA Mini Kit*. Elute the sample in 50 µl of buffer AE.

Automatic extraction

Ref. 62124 - EZ1 DSP DNA Blood kit on EZ1 Advanced XL instrument.
Follow the instructions inside the kit *EZ1 DSP DNA Blood kit*. Start from 200 µl of sample and elute it in 50 µl of buffer AE.

Manual extraction (SIEMENS)

Ref. 10629800 - VERSANT® Sample Preparation 1.2 Reagents kit box 1.
Ref. 10629801 - VERSANT® Sample Preparation 1.2 Reagents kit box 2.
Follow the instructions supplied by Siemens and elute it in 70 µl of Elution buffer. Transfer 55 µl of eluted sample to an appropriately size tube.
Sample can be stored at -20°C.

SOFTWARE SETTING:

Lifetechnologies 7500 fast/StepOne plus

Turn the instrument and the computer on and open the control software. Click on "**Advance Setup**": by default the software will shows the page "**experiment properties**". Write in the "**experiment name**" the file name, choose the type of instrument (**7500 or 7500fast**), the type of reaction (**Genotyping**), the type of used reagent (**Taqman·Reagents**) and the reaction time of analysis (**Standard ~ 2 hours to complete a run**).

Open the page named "**page setup**".
In the window "**Assign SNP assay to the selected wells**" open "**Create new SNP Assay**" and set:

SNP Assay Name: LCT C/T

	Reporte	Quencer
Allele 1 Name: LCT C13910T WT	FAM	None
Allele 2 Name: LCT C13910T MUT	VIC	None

SNP Assay Name: LCT G22018A

	Reporte	Quencer
Allele 1 Name: LCT G22018A WT	FAM	None
Allele 2 Name: LCT G22018A MUT	VIC	None

In the page "**plate setup**", move on the area "**Assign Sample to the selected Wells**": set the name of the analyzing samples, of positive controls and negative controls.

Choose an area of the plate where positive controls will be placed: select in the blank "**Assign SNP assay to the selected well**" and assign the SNP Assay LCT C13910T. After set these tasks:
- "**task Positive control Allele1/Allele1**" for LCT C13910T homozygous wild-type;
- "**task Positive control Allele2/Allele2**" for LCT C13910T homozygous mutated;

Choose an area of the plate where positive controls will be placed: select in the blank "**Assign SNP assay to the selected well**" and assign the SNP Assay LCT G22018A. After set these tasks:
- "**task Positive control Allele1/Allele1**" for LCT G22018A Wild Type homozygote;
- "**task Positive control Allele2/Allele2**" for LCT G22018A mutated homozygote;

Choose an area of the plate where negative control will be placed: select "**Assign SNP assay to the selected well**" the "**task Negative control**" for SNP Assay LCT C13910T.

Choose an area of the plate where negative control will be placed: select "**Assign SNP assay to the selected well**" the "**task Negative control**" for SNP Assay LCT G22018A.

Select an area of the plate where samples will be placed: select the wells and set SNP Assay LCT C13910T. Link every well to a sample, through the window "**Assign samples to selected wells**".
For each sample, select in the blank "**Assign SNP to selected wells**" the "**task UnKnown (U)**" for the SNP Assay LCT C13910T.

Select an area of the plate where samples will be placed: select the wells and set SNP Assay LCT G22018A. Link every well to a sample, through the window "**Assign samples to selected wells**".
For each sample, select in the blank "**Assign SNP to selected wells**" the "**task UnKnown (U)**" for the SNP Assay LCT G22018A.

Wt- LCT C/T		C2- LCT C/T		C2- LCT G/A
Mut- LCT C/T		C3- LCT C/T		C3- LCT G/A
Neg- LCT C/T		C4- LCT C/T		C4- LCT G/A
		C5- LCT C/T		C5- LCT G/A
		C6- LCT C/T		C6- LCT G/A
Wt- LCT G/A		C7- LCT C/T		C7- LCT G/A
Mut- LCT G/A		C8- LCT C/T		C8- LCT G/A
Neg- LCT G/A		C9- LCT C/T		C9- LCT G/A

Mix **LCT C13910T**   Mix **LCT G22018A**

Set ROX as passive reference, using as normalizer of detecting fluorescence.

Open "**Run Method**" (sheet **Graphic View**) and set the right thermal cycling:

cycles	Pre PCR Read	denaturation	annealing/extension	Post PCR Read
1	60°C 1min			
1		95°C 10min		
35		95°C 15sec	53°C 1min	
1				60°C1min

In the window "**Reaction volume plate per well**" set a volume of 25 µl.
After making the plate, and correctly inserting it in the instrument, press the button "**Start Run**".

Rotor Gene-Q MDxMDx

New runs can be set up using the Quick Start wizard or the Advanced wizard, which appear when the software is started up. Select Advanced wizard. As a first step, select the desired template for the run by double-clicking on the template from the list in the "New Run" window. Select **Two Step** Reaction.

In the next window, select the **rotor type** from the list. Check the "**Locking Ring Attached**" checkbox and then click "**Next**".

Insert the operator name and reaction volume of 25µl and click "**Next**".

In the next window click on "**edit profile**". Set the following thermal cycle:

cycles	denaturation	annealing/extension
1	50° C 2 min	
1	95°C 10min	
35	95°C 15sec	53°C 1min

Select the annealing / extension from the thermal profile and click on "**Acquiring A to cycling**."

In the next window, select yellow from the available channels and add it to acquiring channel along with the green channel and click "**OK**". In the next window click on "**OK**" and then click "**Next**".

click on "**Edit Gain**" button and set the following values for each channel:

Reporter	Gain
Green	5
Yellow	4

To begin the course, click on the button "**Start Run**". You can save the model before you begin your run by clicking on "**Save Template**".
After clicking on the button "**Start Run**" window appears "**Save As**". The stroke can be saved in the desired position by the user.

Once the run started, the window "**Edit Samples**" allows you to set the name of samples and controls in the positions in which they were loaded on the instrument.

Select the locations where they were positioned the Wild Type and Mutant controls designate them as **LCT C13910T positive CTR and LCT C13910T Mutant Positive CTR**. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**Positive Controls** ".

Select the locations where they were positioned the Wild Type and Mutant controls designate them as **LCT G22018A positive CTR and LCT G22018A Mutant Positive CTR**. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**Positive Controls** ".

Select the location where you placed the Negative Control for each polymorphism and name it as **Negative Control**. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**Negative Controls**"

Select the location of each sample (2 wells for each sample to allow the discrimination of both the polymorphism) and enter the name or code of the patient. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**UnKnown**"

At the end of the operation click "**OK**" in the "**edit samples**" and wait until the end of the race for the analysis (see "**Interpretation of Results**").

Versant kPCR AD or Stratagene MX3005P

Turn on the instrument, the computer and start the control software. Turn on the light at least 20 minutes before starting a new experiment. You can click on the lamp image for turning on the light from toolbar or you can select "**Lamp On**" from menu "**Instruments**".
Verify the correct setting of fluorescent reporters gains: In the setting menu choose and then **"Filter set gain setting"**
.

Reporter	Gain
FAM	8
JOE/HEX	2
ROX	1

From main screen of the software a window will open "**New option –Select experiment/Project type**": select "**Allelic Discrimination/SNPs Real Time**". The software will automatically open the windows **plate set up**.

Choose a zone of the plate where it will be placed **LCT C13910T Wild Type control** and **LCT G22018A Wild Type control** and select in the toolbar on the right of the software the menu:

Well type:	Collect Fluorescent Data:	Reference Dye:	Replicate Symbol:
Pos. Control FAM	FAM/HEX/ROX	ROX	None

Clicking on every well it will appear the dialogue window "**well information**" here you can set the name of the calibrator: **LCT C13910T Wild Type** or **LCT G22018A Wild Type**

Choose a zone of the plate where it will be placed **LCT C13910T Mutated control** and **LCT G22018A Mutated control** and select in the toolbar on the right of the software the menu:

Well type:	Collect Fluorescent Data:	Reference Dye:	Replicate Symbol:
Pos. Control HEX	FAM/HEX/ROX	ROX	None

Clicking on every well it will appear the dialogue window "**well information**" here you can set the name of the calibrator: **LCT C13910T Mutated and LCT G22018A Mutated**

Choose a plate zone where you can put the unknown samples and select in the bar on the right from the menu.

Well type:	Collect Fluorescent Data:	Reference Dye:	Replicate Symbol:
UnKnown	FAM/HEX/ROX	ROX	None

Clicking on every well it will appear the dialogue window "**well information**" here you can set the name of the sample:
Every well "**Unknown**" should be singularly named clicking on single name it will open the window "**well information**" in this window it is possible to insert the sample name in analysis.

Choose a plate zone where you can put the negative control and select in the right bar on the right from the menu.

Well type:	Collect Fluorescent Data:	Reference Dye:	Replicate Symbol:
NTC	FAM/HEX/ROX	ROX	None

Clicking on every single well will appear the window "**well information**" where it can be possible setting the name of the target.

Select every well corresponding to occupied places (make multiple selections with the mouse, using the **Ctrl** button) from **LCT C13910T** mix; click right button of the mouse and select the voice "**well information**". In correspondence to fluorescent reporter **FAM** set in the specific space (**Assay**): **LCT C13910T WT**. In correspondence to fluorescent reporter **HEX** set in the specific place (**Assay**): **LCT C13910T MUT**.

Select every well corresponding to occupied places (make multiple selections with the mouse, using the **Ctrl** button) from **LCT G22018A** mix; click right button of the mouse and select the voice "**well information**". In correspondence to fluorescent reporter **FAM** set in the specific space (**Assay**): **LCT G22018A WT**. In correspondence to fluorescent reporter **HEX** set in the specific place (**Assay**): **LCT G22018A MUT**.

Open the page "**Thermal Profile Setup**" and set the correct thermal cycle:

cycles	denaturation	annealing/extension
1	50°C 2 min	
1	95°C 10min	
40	95°C 15sec	53°C 1min

After preparing the plate and correctly insert it in the instrument, push the button "**Start Run**". the software will ask the file name for the saving data.

CFX 96 Real Time PCR

Turn the instrument and the computer on and start the control software. In the principal screen will appear the window "**Startup wizard**": select "**CFX96"** and press **"ok".** In the next window push "**create new**" and set the thermal protocol and the reaction volume (25µl):

cycles	denaturation	annealing/extension
1	50°C 2 min	
1	95°C 10min	
35	95°C 15sec	53°C 1min

Save the protocol and click the next button. The software will open in default the sheet "**plate**". Click "**create new**", select **"Fluorophores button"** to choose fluorophores (FAM and VIC). Select the locations where they were positioned the controls of known concentration and choose the **"Sample Type" Standards.** Click "**Load**" check boxes to load fluorophores and Type or select Target Name. Select the location where you placed the Negative Control. Choose the **"Sample Type" NTC.** Click "**Load**" check boxes to load fluorophores and Type or select Target Name
Select the location of each sample and enter the name or code of the patient. Choose the **"Sample Type" Unknown.** Click "**Load**" check boxes to load fluorophores and Type or select Target Name
Save the plate clicking the next button and start the experiment

PREPARATION OF THE REACTIONS:

Defrost a tube of **Amplification mMix**;
Defrost a tube of **LCT C13910T probe mix** and a tube of **LCT G22018A probe mix**
Mix carefully through vortex **210µ**l of **Amplification mMix** with **126 µl** of **LCT C13910Tprobe mix**
Mix carefully through vortex **210µl** of **Amplification mMix** with **126µl** of **LCT G22018A probe mix**
The mix is enough for 16 amplification reactions: **2 positive controls**, **1 negative control** and **13 samples**.
Distribute, in the amplification plate, **20 µ**l of just reconstituted **C13910T (C/T) mix** in chosen positions and already setted on the instrument software.
Distribute, in the amplification plate, **20 µl** of just reconstituted **G22018A (G/A) mix** in chosen positions and already setted on the instrument software.
Distribute, in the negative control position, **5 µl** of solution taken by **the negative control** vial.
Distribute, in chosen position for each sample, **5 µ**l of corresponding **sample.**
Distribute, in chosen positions for the positive controls, **5 µ**l of **Positive Control Wild-Type** and **5 µl of Positive Control Mutated**.
Seal up accurately the plate using an optical adhesive film and verify that there aren't air bubbles in the mix, to avoid the amplification interferences.
Transfer the plate in the instrument and push the button "**Start Run**".

QUALITATIVE ANALYSIS

Lifetechnologies 7500 Fast/StepOne Plus.

Scatter Plot Analysis C13910T (C/T) and G22018A (G/A)

At the end of the amplification reaction, the software automatically shows the obtained results in the "**Allelic Discrimination Plot**".
The Lifetechnologies 7500Fast/StepOne Plus instrument automatically perform the genotyping of unknown samples by comparing controls versus homozygous wild-type and homozygous mutated contained in the kit.

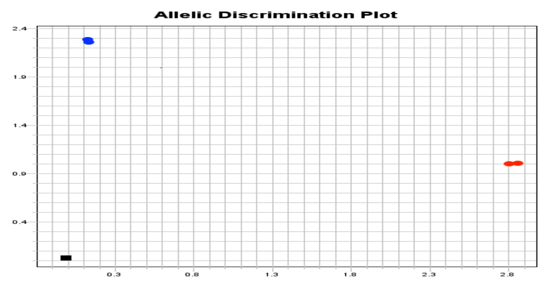
A proper results' analysis needs a correct settings of the instrumentation. For this aim, set:

Baseline fluorescence level from cycle 6;

	LCT CT-WT - **FAM** Threshold	LCT GA-MUT - **VIC** Threshold
7500 Fast	0.05	0.05
StepOne Plus	0.05	0.05

	LCT GA-WT - **FAM** Threshold	LCT GA-MUT - **VIC** Threshold
7500 Fast	0.05	0.05
StepOne Plus	0.05	0.05

It is recommended to verify correct placement of each individual sample on the scatter plot. For viewing the report containing all data obtained during the analysis, click the sheet "**view table well**".



The position of the negative control in other plot region could be a contamination of the reaction mix.
Be sure that in the negative control not be increasing of specific fluorescence by examinating targets (FAM and VIC).

C13910T (C/T) ΔCt Analysis

Further analysis can be performed with the ΔCt study of the results. For this purpose it is necessary a correct setting of the software

Set the level of background fluorescence (Baseline) from cycle 6; Set the following threshold:

	LCT CT-WT - **FAM** Threshold	LCT CT-MUT - **VIC** Threshold
7500 Fast	0.05	0.05
StepOne Plus	0.05	0.05

Export data to Excel and set the formula for each sample and control:

Allele2 Ct (LCT CT-MUT) – Allele1 Ct (LCT CT-WT)

See paragraph "**INTERPRETATION OF RESULTS**"

G22018A (G/A) ΔCt Analysis

Further analysis can be performed with the ΔCt study of the results. For this purpose it is necessary a correct setting of the software

Set the level of background fluorescence (Baseline) from cycle 6;
Set the following threshold:

	LCT GA-WT - **FAM** Threshold	LCT GA-MUT - **VIC** Threshold
7500 Fast	0.1	0.1
StepOne Plus	0.1	0.1

Export data to Excel and set the formula for each sample and control:
Allele2 Ct (LCT GA-MUT) – Allele1 Ct (LCT GA-WT)

See paragraph "**INTERPRETATION OF RESULTS**"

Rotor Gene-Q MDxMDx
C13910T (C/T) Scatter Plot Analysis
Select **C13910T (LCT C/T) Wild type, Mutant, negative controls** and **samples.**
Click on **Analysis**. In the **Analysis window** select **Allelic Discrimination** sheet, click on **Cycling A green-Cycling A yellow** and click **"show"**. The amplification plot will appear.
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Click **genotypes** button and set:

	Reacting channel	Reacting channel
Wild type	Cycling A green	
Heterozygote	Cycling A green	Cycling A Yellow
Mutant		Cycling A Yellow

In the **Discrimination threshold** set as Threshold.

	LCT C/T-WT **Green (FAM)** Threshold	LCT C/T-MUT **Yellow (VIC)** Threshold
Rotor Gene-Q MDx	0.1	0.1

In the **Analysis window** select **scatter** sheet, click on **Cycling A green-Cycling A yellow** and click **"show"**.
The scatter plot will appear:
Wild Type (high left), Heterozygous (in the middle) and Mutant (bottom right).

C13910T (C/T) ΔCt Analysis
Further analysis can be performed with the ΔCt study of the results. For this purpose you need a different setting of analysis and a correct setting of the software:

At the end of the PCR run open the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A (green)**".
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Set the correct setting of the threshold in the space provided "**CT calculation – Threshold**".

	LCT CT-WT – **Green (FAM)** Threshold
Rotor Gene-Q MDx	0.02

Open the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A (yellow)**".
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Set the correct setting of the threshold in the space provided "**CT calculation – Threshold**".

	LCT CT-MUT – **Yellow (VIC)** Threshold
Rotor Gene-Q MDx	0.02

Export data to Excel, save the file as "Excel Analysis Sheet" and enter the following formula for each sample and control:

Yellow Ct (LCT CT-MUT) – Green Ct (LCT CT-WT)

See paragraph "**INTERPRETATION OF RESULTS**"

G22018A (G/A) Scatter Plot Analysis
Select **G22018A (LCT G/A) Wild Type, Mutant, Negative Controls and Samples.**
Click on **Analysis**. In the **Analysis window** select **Allelic Discrimination** sheet, click on **Cycling A green-Cycling A yellow** and click **"show"**. The amplification plot will appear.
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Click **genotypes** button and set:

	Reacting channel	Reacting channel
Wild type	Cycling A green	
Heterozygote	Cycling A green	Cycling A Yellow
Mutant		Cycling A Yellow

In the **Discrimination threshold** set as Threshold.

	LCT G/A-WT **Green (FAM)** Threshold	LCT G/A -MUT **Yellow (VIC)** Threshold
Rotor Gene-Q MDx	0.3	0.3

In the **Analysis window** select **scatter plot** sheet, click on **Cycling A green-Cycling A yellow** and click **"show"**.
The scatter plot will appear:
Wild Type (high left), Heterozygous (in the middle) and Mutant (bottom right).

G22018A (G/A) ΔCt Analysis
Further analysis can be performed with the ΔCt study of the results. For this purpose you need a different setting of analysis and a correct setting of the software:

At the end of the PCR run open the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A (green)**".
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Set the correct setting of the threshold in the space provided "**CT calculation – Threshold**".

	LCT GA-WT – **Green (FAM)** Threshold
Rotor Gene-Q MDx	0.2

Open the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A (yellow)**".
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Set the correct setting of the threshold in the space provided "**CT calculation – Threshold**".

	LCT GA-MUT – Yellow **VIC** Threshold
Rotor Gene-Q MDx	0.2

Export data to Excel, save the file as "Excel Analysis Sheet" and enter the following formula for each sample and control:

Yellow Ct (LCT GA-MUT) – Green Ct (LCT GA-WT)

See paragraph "**INTERPRETATION OF RESULTS**"

Versant kPCR AD or Stratagene MX3005P
C13910T (C/T) Scatter Plot Analysis
Click on button "**Analysis**" in the toolbar. The software will open the sheet "**Analysis Term Setting**". Activate the button FAM and HEX in the low part of the screen and select samples and controls for the LCT C13910T mix.
From window "**Analysis Term Setting**" open the sheet "**Results**". Select in the right of the screen the area "**Area to Analysis**" and the voice "**Amplification Plot**".
Set in the area "**Threshold Fluorescence**" the values:

	LCT C/T-WT **FAM** Threshold	LCT C/T-MUT **HEX** Threshold
Versant kPCR AD	0.3	0.3

Select in the right of the screen in the area "**Area to Analysis**" the voice "**Dual Colour Scatter Plot**".
Select in the area "Display value for" the button "**Fluorescence**" and choice from the menu the voice "**Rlas/Rfirst**".

Select in the area "**Allele Association**":
Allele A: **LCT C/T Wild Type**
Allele B: **LCT C/T Mutated**

At the end it will be possible obtain the detailed account, clicking "**Text Report**" in the area "**Area to Analysis**".
Only for Versant kPCR AD from Siemens or Stratagene MX3005P/MX3000PMX3000P
If the software can't discriminate the wild type and mutated positive controls click "**Show all genotypes**". It will be now possible to modify the genotyping windows, select the samples placed near the positive wild type and identify them as wild type. Select the samples placed near the positive mutated and identify them as mutated. Select the samples placed in an intermediate position between wild type positive control and mutated positive control and identify them as Heterozygote for the Leiden Mutation.

C13910T (C/T) ΔCt Analysis
Further analysis can be performed with the ΔCt study of the results. For this purpose you need a different setting of analysis and a correct setting of the software:

Click "Analysis" in the toolbar.
Click the sheet "Results"; and choose the analysis "Amplification plot". Check the correct setting of the threshold in the window "Threshold fluorescence" and set the following values:

	LCT CT-WT - **FAM** Threshold	LCT CT-MUT - **HEX** Threshold
Versant kPCR AD	0.1	0.1

From the Text Report window you can export the results by clicking on the main menu: file, export
Export data to Excel and set the formula for each sample and control:
HEX Ct (LCT CT-MUT) – FAM Ct (LCT CT-WT)

See paragraph "**INTERPRETATION OF RESULTS**"

G22018A (G/A) Scatter Plot Analysis
Click on button "**Analysis**" in the toolbar. The software will open the sheet "**Analysis Term Setting**". Activate the button FAM and HEX in the low part of the screen and select samples and controls for the LCT G22018A mix.
From window "**Analysis Term Setting**" open the sheet "**Results**". Select in the right of the screen the area "**Area to Analysis**" and the voice "**Amplification Plot**".
Set in the area "**Threshold Fluorescence**" the values:

	LCT G/A-WT **FAM** Threshold	LCT G/A -MUT **HEX** Threshold
Versant kPCR AD	0.5	0.5

Select in the right of the screen in the area "**Area to Analysis**" the voice "**Dual Colour Scatter Plot**".
Select in the area "Display value for" the button "**Fluorescence**" and choice from the menu the voice "**Rlas/Rfirst**".

Select in the area "**Allele Association**":
Allele A: **LCT G/A Wild Type**
Allele B: **LCT G/A Mutated**

At the end, it will be possible, clicking on area "**Area to Analysis**" the voice "**Text Report**", obtain the report of the results.
Only for Versant kPCR AD from Siemens or Stratagene MX3005P/MX3000PMX3000P
If the software can't discriminate the wild type and mutated positive controls click "**Show all genotypes**". It will be now possible to modify the genotyping windows, select the samples placed near the positive wild type and identify them as wild type. Select the samples placed near the positive mutated and identify them as mutated. Select the samples placed in an intermediate position between wild type positive control and mutated positive control and identify them as Heterozygote for the Leiden Mutation.

G22018A (G/A) ΔCt Analysis
Further analysis can be performed with the ΔCt study of the results. For this purpose you need a different setting of analysis and a correct setting of the software:

Click "Analysis" in the toolbar.
Click the sheet "Results"; and choose the analysis "Amplification plot". Check the correct setting of the threshold in the window "Threshold fluorescence" and set the following values:

	LCT GA-WT - **FAM** Threshold	LCT GA-MUT - **HEX** Threshold
Versant kPCR AD	0.5	0.5

From the Text Report window you can export the results by clicking on the main menu: file, export
Export data to Excel and set the formula for each sample and control:
HEX Ct (LCT GA-MUT) – FAM Ct (LCT GA-WT)

See paragraph "**INTERPRETATION OF RESULTS**"

CFX96 Real Time PCR System
C13910T (C/T)and G22018A (G/A) Scatter Plot Analysis
At the end of the PCR, select the "**Allelic Discrimination**" sheet. On the bottom of the screen, set "**Selected fluorophores**": X = FAM and Y = VIC. Choose **RFU** from "**Display Mode**" and "**Normalize data**". Select "**settings**" from the menu and choose "**Baseline Threshold…**"

You can export the report pushing the paper block figure on the top of the screen

INTERPRETATION OF RESULTS
The use of positive and negative control in each amplification session allows to verify the correct functioning of the amplifcation mix and the absence of any contamination.

The instrument software is able to analyze the fluorescences that are emitted by the specific probe for LCT CT and LCT GA Wild-Type (FAM) and by the specific probe for the LCT CT and LCT GA mutated (VIC/HEX).

C13910T (C/T) and G22018A (G/A) Scatter Plot Analysis
A regular functioning of the amplification mix can be verified analyzing the correct position of positive controls and negative controls on the scatter plot.

- Positive control Homozygote Wild-Type: horizontal position on X axis (down on the right) Ct< 30
- positive Control homozygote Mutated: vertical position on Y axis (up on the left) Ct< 30
- negative Control: placed at the origin of cartesian plane (down on the left)

Genotyping tests (allelic discrimination) are endpoint experiments: fluorescence data are collected at the end of the reaction (Post PCR Read) and subtracted to initial read fluorescence (Pre PCR Read).
The software makes a scatter plot with obtained results: Y axis is the normalized fluorescence of Mutated Allele, while X axis shows the normalized fluorescence of Wild-Type Allele. The diagnosis obtained with the comparison between unknown samples and Homozygote Wild-Type and Homozygote Mutate, given by the kit. Selecting the controls, we obtain their disposition on the scatter plot, depending of their relation between fluorescence emitted by two probes FAM (Wild-Type) and VIC/HEX (Mutated).

The genotyping of polymorphisms C13910T and G22018A is a genetic susceptibility test that evaluates the major or minor predisposition of an individual to develop the disease.

	C13910T	**G22018A**	**Predisposition to the disease**
Genotype	**TT** Wild type	**AA** Wild type	Not predisposed
Genotype	**CT** Heterozygous	**GA** Heterozygous	Not predisposed
Genotype	**CC** Mutant	**GG** Mutant	Predisposed

Studies about genomic association demonstrate that CC genotype in C13910T polymorphism and GG genotype in G22018A polymorphism are predisposed to lactose malabsorption.

On the contrary, TT and CT genotype in C13910T polymorphism and AA and GA genotype in G22018A polymorphism don't predisposed lactose intolerance.

C13910T (C/T) and G22018A (G/A) ΔCt Analysis
By analyzing ΔCt you can identify the correct genotype of the sample being analyzed by performing subtraction of the assigned Mutated allele Ct to assigned wild-type allele Ct.
The genotype is determined by following the table below:

Genotype	ΔCt (Ct Mutated - Ct Wild Type)
Wild Type	ΔCt > 2
Mutated	ΔCt < - 2
Heterozygous	-2 > ΔCt < 2

In the amplification of each positive control (wild-type and Mutated), the Ct values of the allele-specific probe are used to validate the assay.
Make sure that the fluorescence emitted by the amplification allele has a correct Ct <30.
If the result of the allele-specific amplification of each control has a Ct > 30 or undetermined the session can not be considered valid and must be repeated.

In the amplification of each sample, the Ct values of the allele-specific probe are used to validate the assay from the extraction process up to the stage of detection.
Make sure that the fluorescence emitted by the allele-specific amplification of the sample, identified after analysis, has not a Ct > 30.

Sample	Wild Type Allele (FAM)	Mutated Allele (VIC)	Assay and Genotype
Wild Type	Ct < 30	Not relevant	Valid
Mutated	Not relevant	Ct < 30	Valid
Heterozygous	Ct < 30	Ct < 30	Valid

If a sample has a Ct > 27 means that there are problems in the extraction phase or in the amplification and therefore could be assigned to a sample genotype wrong. Repeat sample.

Sample	Wild Type Allele (FAM)	Mutated Allele (VIC)	Assay and Genotype
Wild Type	Ct > 30	Not relevant	Not Valid
Mutated	Not relevant	Ct > 30	Not Valid
Heterozygous	Ct > 30	Ct > 30	Not Valid

PERFORMANCES
Clinical Sensitivity:
For the purposes of this evaluation is considered as clinic sensitivity the skill of the method of determining real positive samples in the whole screened samples. The analysis is performed on samples with different genotypes and the test is made following the informations present in the methodology.

Obtained results show a clinical sensitivity of 100%.

Diagnostic Specificity:
For the purposes of this evaluation is considered as diagnostic specificity the skill of the method of determining real negative samples. The diagnostic specificity of the system is valued analyzing human genomic samples tested and confirmed with favourable genotype for C13910T and G22018A polymorphisms, by sequencing.

Diagnostic specificity is 100% for material extracted from EDTA blood.

Analytical Specificity:
Test's specificity is guaranteed by the use of specific primers for determining MCM6 gene and of probes intentionally designed on **C/T mutation for C13910T polymorphism and G/A mutation for G22018A polymorphism**.
The alignment of the choose regions for specific primers' hybridization with available sequences of present in database, demonstrated: their conservation and the complete specificity for the analyzed targets. Samples that are defined as positive for a determined genotype as much must be recognized by the amplification system.

INTERFERENCES:
Verify that in DNA extracted from the sample there aren't nucleoproteins and haemoglobin, in way of exclude possible inhibition of PCR reactions. The interference due to contaminants can be highlighted through the spectrophotometric analysis and obtained data report at 260 nm (maximum absorbtion of Nucleic Acids) and 280 nm (maximum absorbtion of Proteins). A pure DNA might have a rate of approximately 1.8.

QUALITY CONTROL
It is therefore recommended to insert as quality control of every extraction session, amplification and detection of a negative sample and of a positive sample which have already tested before or referential material with known concentration.

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TECHNICAL ASSISTANCE
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IVD	*In vitro* diagnostic device
	Read the instruction's manual
	Range of temperature
	Use within (dd/mm/yyyy: year-month)
LOT	Lot (xxxx)
REF	Code
	Manufacturer
	Contains sufficient for <n> tests

EDMA: 16010490
CND: W01060104

The kit ***Lactose Intolerance LCT*** is CE marked diagnostic kit according to the European *in vitro* diagnostic directive 98/79/CE.

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IVD
for *in vitro* diagnostic use