Kit Reference: LC-LacIDuplex-LP **Last revision date:** 16/05/2018

Lamp Human Lactose Intolerance Duplex KIT

LC-LacIDuplex-LP

24 reactions: LC-LacIDuplex-LP-24

96 reactions: LC-LacIDuplex-LP-96

Detection of the polymorphisms -13910 C/T, -13915 T/G, -14010 G/C, -14009 T/G and surrounding mutations in the MCM6 gene linked to lactase persistence in adults.

Performance Testing

INSTRUCTIONS FOR USE

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Revision notes compared to previous version:

Version	Description of the modification
01	Creation

1. INTRODUCTION

The **LAMP Human Lactose Intolerance Duplex KIT (LC-LacIDuplex-LP)** is intended as an aid to the diagnosis of lactase persistence or primary lactose intolerance.

The **LAMP Human Lactose Intolerance Duplex KIT (LC-LacIDuplex-LP)** is an in vitro diagnostic assay allowing the qualitative detection of the MCM6 -13910 C/T polymorphism (rs4988235), the MCM6 -13915 T/G polymorphism (rs41380347), the MCM6 -14010 G/C polymorphism (rs145946881), the MCM6 -14009 T/G (rs869051967) and other surrounding mutations by Loop-mediated isothermal amplification (LAMP), in blood samples from patients potentially tolerant to lactose. Next to the -13910C/T and -13915T/G polymorphisms also polymorphisms on positions -13907, -13908, -13913 and -13914 can be detected.

LAMP is a relatively new DNA amplification technique, using an isothermal nucleic acid amplification, which eradicates the need for expensive thermal cyclers used in conventional PCR or real-time PCR and could provide major advantages due to its simplicity, ruggedness, and low cost. In LAMP, the target sequence is amplified at a constant temperature around 65 °C using together three sets of primers and a polymerase with high strand displacement activity in addition to a replication activity. Typically, 6 different primers are used to identify 8 distinct regions on the target gene, which increases specificity. Due to the specific nature of the action of these primers, the amount of DNA produced in LAMP is considerably higher than amplification based on PCR. Detection of homozygous wild-type, heterozygous and homozygous mutant genotype is performed by melting curve analysis after amplification.

2. GLOSSARY

DNA	Deoxyribonucleic acid
LAMP	Loop-mediated isothermal amplification
PCR	Polymerase chain reaction
FIP	Forward Inner Primer
BIP	Backward Inner Primer
SNP	Single Nucleotide Polymorphism
Tm	Melting temperature
MCM6	Mini-chromosome maintenance complex component 6
C	Cytosine
T	Thymine
G	Guanine
A	Adenine

3. GENERAL INFORMATION

Intended Use

The **Lamp Human Lactose Intolerance Duplex KIT (LC-LacIDuplex-LP)** is an in vitro diagnostic test intended for the qualitative detection of the -13910 C/T polymorphism, the -13915 T/G polymorphism, the -14010 G/C polymorphism, the -14009 T/G polymorphism and surrounding mutations by Loop-mediated isothermal amplification (LAMP) in patients with suspected lactose intolerance.

This assay is dedicated to professional use in diagnostic laboratory. The device is not for self-testing.

Disease Information

Lactose intolerance is an impaired ability to digest lactose. Lactose is normally broken down by lactase, an enzyme which is produced by cells in the lining of the small intestine.

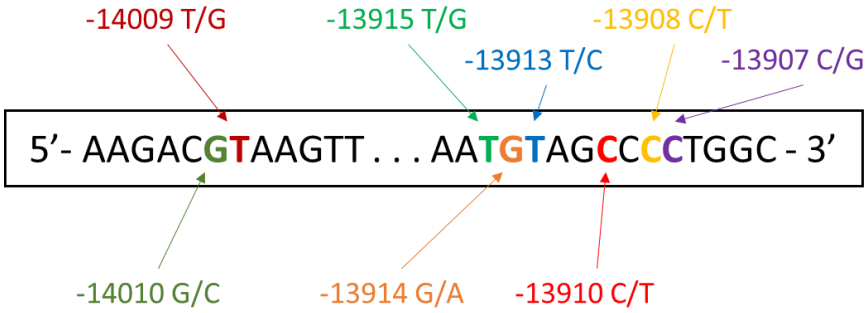
Lactose intolerance in adults is different from congenital lactase deficiency, which is a disorder in which infants are unable to break down lactose. Lactose intolerance in adulthood is caused by reduced production of lactase after infancy, called lactase non-persistence or adult type hypolactasia and is the most common type of lactose intolerance. The expression of the enzyme lactase, encoded by the LCT gene, is regulated by a DNA sequence located nearby, MCM6. Changes in this element have led to sustained lactase production in the small intestine and the ability to digest lactose throughout life. People without these changes have a reduced ability to digest lactose as they get older, resulting in the signs and symptoms of lactose intolerance.

Most people with lactase non-persistence retain some lactase activity, resulting in different capabilities of digesting lactose. Often there is a difference seen between digesting fresh milk and other dairy products, as these have been processed and a large part of the lactose has been already broken down.

Real-time PCR detection methods have been proposed for the detection and typing of the -13910C/T mutation, which was shown to be a useful marker for lactase persistence. -13910 CC is associated with lactose intolerance. -13910 C/T and -13910 T/T is associated with lactase persistence.

Recently other SNPs have been described within intron 13 of MCM6, some in close proximity to the -13910 location, of which at least one, the -13915G has been associated with lactase persistence.

Overview of the mutations described in the -13910 and -14010 regions of the MCM6 gene:



-13910 C/T: rs4988235, associated with lactase persistence in Caucasian population.

-13915 T/G: rs41380347, associated with lactase persistence in Saudi Arabia and is frequent in African and Arabian populations.

-14010 G/C: rs145946881, associated with persistence in east Africa.

-14009 T/G: rs869051967, associated with persistence in east Africa.

-13907 C/G: associated with persistence in east Africa.

-13908 C/T: variant described with unknown significance.

-13913 T/C: present in Jordan/Saudi Arabia/Africa. Significance not clear.

-13914G/A: shown to have lactase activity above the cut-off level for adult-type hypolactasia.

The four above mutations can be detected by the LC-LacIDuplex-LP assay and can be discriminated from the -13910 C/T polymorphism, even though the kit will not be validated for those four additional mutations.

Operating Principle

The assay with the **"Lamp Human Lactose Intolerance Duplex KIT"** is performed on whole blood samples either freshly collected or stored at -20°C and allows the amplification of the MCM6 target sequence and the detection of the -13910 C/T, -13915 T/G, -14010 G/C and -14009 T/G polymorphisms and surrounding mutations in a very short time.

The **"Lamp Human Lactose Intolerance Duplex KIT" contains** 6 specific primers allowing the loop-mediated amplification of a specific region surrounding the -13910 C/T polymorphism. Each amplified target sequence is detected by a mutation specific probe through the detection of fluorescence quenching. After amplification, the temperature is decreased to 35°C and the probe hybridizes the amplified fragment, bringing the fluorophore and the quencher in close proximity, resulting in quenching of the fluorescence. During the melting curve analysis, the temperature is gradually

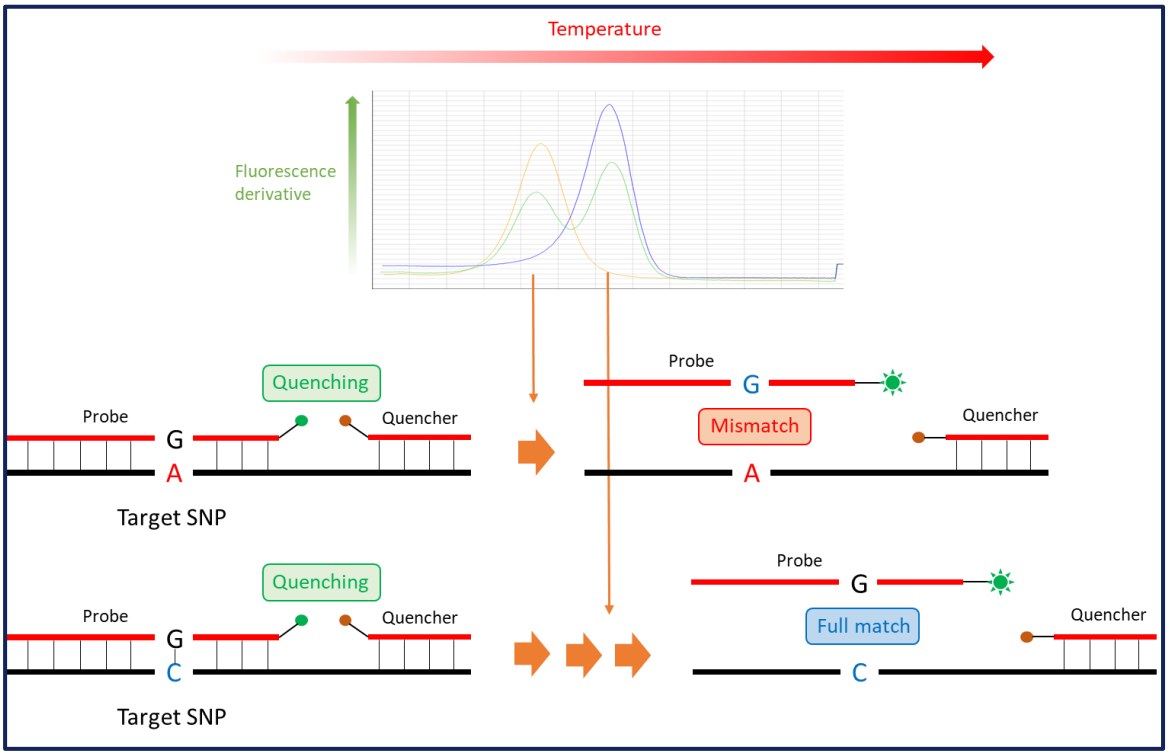
increased to 80°C while the change in fluorescence emission is measured. Because the amplified wild type DNA fragment does not match perfectly with the mutation-specific probe, the probe will be released at a lower temperature from wild type DNA fragment than for the amplified mutated DNA fragment. Fragments with additional mutations will show at least two mismatches with the -13910 T specific probe and therefore the probe will be released at an even lower temperature from these amplified fragments (the same thing is observed with the -14009 probe). The change in fluorescence at a different temperature allows to make the difference between the different nucleotide variants in the -13910 region.

Principle of LAMP:

6 types of primers are designed to hybridize distinct regions surrounding the polymorphism: the FIP and F3 primers at the 3' side and the BIP and B3primers at the 5' side. In addition, there are two Loop primer: Loop Primer F and Loop Primer B.

FIP	Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end.
F3 Primer	Forward Outer Primer consists of the F3 region that is complementary to the F3c region.
BIP	Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end.
B3 Primer	Backward Outer Primer consists of the B3 region that is complementary to the B3c region.
Loop Primer F	Sequence complementary to the single stranded loop region between the F1 and F2 regions
Loop primer B	Sequence complementary to the single stranded loop region between the B1 and B2 regions

Principle of melting curve analysis:



In the LAMP Human Lactose Intolerance Duplex KIT the probes have been designed to hybridize the amplified -13910T DNA fragment and the amplified -14009G fragment.

Contents of the LC-LacIDuplex-LP-24 kit

The LC-LacIDuplex-LP-24 kit is composed of one carton box containing 5 vials:

Name	Description	Number of tubes	Volume	Label color
1.1 LC-LacIDuplex-LB	Lysis Buffer	1	12000 µL	Yellow (FFFF00)
1.2 LC-LacIDuplex-LB	Lysis Buffer	1	12000 µL	Yellow (FFFF00)
2. LC-LacIDuplex-RB	Reaction Buffer	1	480 µL	Skyblue (87CEBB)
3. LC-LacIDuplex-Ctrl+	Positive Control	1	30 µL	Red (FF0000)
4. LC-LacIDuplex-Ctrl-	Negative Control	1	30 µl	GreenYellow (ADFF2F)

There is enough quantity of reaction buffer, lysis buffer and controls to perform 24 reactions of 25µl.

Contents of the LC-LacI-LP-96 kit

The LC-LacIDuplex-LP-96 kit is composed of one carton box containing 6 vials:

Name	Description	Number of tubes	Volume	Label color
1.1 LC-LacIDuplex-LB	Lysis Buffer	1	12000 µL	Yellow (FFFF00)
1.2 LC-LacIDuplex-LB	Lysis Buffer	1	12000 µL	Yellow (FFFF00)
2.1 LC-LacIDuplex-RB	Reaction Buffer	1	960 µL	Skyblue (87CEBB)
2.2 LC-LacIDuplex-RB	Reaction Buffer	1	960 µL	Skyblue (87CEBB)
3. LC-LacIDuplex-Ctrl+	Positive Control	1	30 µL	Red (FF0000)
4. LC-LacIDuplex-Ctrl-	Negative Control	1	30 µl	GreenYellow (ADFF2F)

There is enough quantity of reaction buffer for 96 LAMP reactions with a final volume of 25 µl with a high throughput protocol.

Quality control

Quality control procedures are intended to monitor reagent and assay performance.

Quality control requirements must be performed in conformance with local state and/or federal regulations or accreditation requirements and the laboratory standard quality control procedures.

There is a positive and negative control included in the kit. For each run, the negative and positive control should be included to monitor test performance.

The positive control is essential for evaluating the efficiency of the procedure. It allows the verification of the reagents' quality (eg. probes and primers integrity, enzyme activity). The positive control consists of plasmid DNA containing the mutated sequence (-13910T and -14009G polymorphisms) of the MCM6 gene surrounding the polymorphisms and plasmid DNA containing the wild type sequence of the MCM6 gene surrounding the polymorphisms, in a 1:1 ratio.

The negative control is essential for detecting contaminated reagents or environmental contamination by nucleic acids. The negative control is PCR grade water and should not generate any amplification or peak in the melting curve.

4. REAGENTS STORAGE, HANDLING AND STABILITY

- Our kits are shipped frozen, should arrive frozen and should be stored frozen at ≤ – 20°C.
- At -20°C kits can be stored until the expiration date stated on the label.
- Do not subject the reagents to more than 8 freeze-thaw cycles.
- Once open, our kits can be stored either at -20 until the expiration date or between 2 and 8°C for up to 12 weeks.
- Do not refreeze reagents that have been stored at 4°C.
- Minimize reagents exposure to light.

5. WARNINGS AND PRECAUTIONS

If you receive a damaged parcel or thawed kits, please contact your local distributor.

- Read the instructions for use before performing the experiment.
- Do not use reagents if the protective box is open or torn upon arrival.
- Do not use reagents if the tubes are open or damaged.
- Do not use expired reagents and/or materials.
- Do not mix different batches of products.
- Do not substitute reagents from the kits with different batch numbers or from other manufacturers.
- Use of this product should be limited to scientists/laboratory technicians who have been trained in the LAMP technology.
- Good Laboratory Practices must be adhered to, in particular:
    - Perform quality control as mentioned
    - Wear protective clothing and disposable gloves while handling kit reagents.
    - Keep all vials closed when not in use.
    - Do not mix caps between tubes or re-use caps as contamination may occur and compromise test results.
    - Do not pipette by mouth.
    - Do not smoke, drink or eat in the laboratory areas.
- The experiments should not be carried out by pregnant woman.
- Discard any kit suspected of being contaminated.
- After use, material- reagents –waste must be properly discarded in a specific waste bin for biological substances in accordance with country, federal, provincial, state and local regulations.
- Do not ingest any component from the kit.
- **Important contamination precautions:** this product generates amplified DNA targets. When performing the test, caution must be taken to prevent amplicon contamination of work areas. Always use filter pipette tips, perform amplification in an isolated area with dedicated pipettes, separated from lysis and mix preparation. Use tips and tubes that are DNA free. Clean the work area on regular intervals, with appropriate products (DNA away)

  Do not re-open test tubes after amplification.

6. SAMPLES COLLECTION, STORAGE AND TRANSPORT

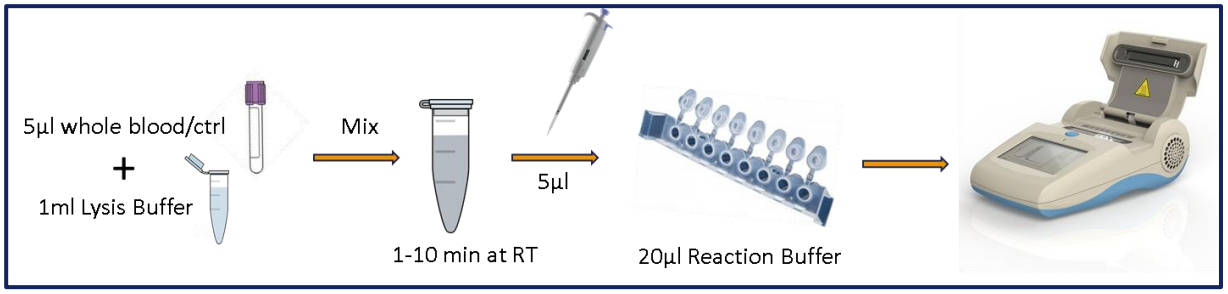
Specimen	Whole blood sample
Collection	EDTA tubes
Storage	At 2-8°C for ≤ 30 days At -20°C for ≤ 12 months
Transport	At 2-8°C

Specimen	DNA samples
Collection	Extraction from whole blood sample
Storage	At 2-8°C for ≤ 7 days At -20°C for ≤ 12 months
Transport	At -20°C

- Avoid multiple freeze-thaw cycles for all sample types.
- Ensure that the transport of human specimens meets all local and national regulations for the transport of etiologic agents.

7. PROTOCOL FOR LC-GENIE III

Overview of the procedure



Laboratory equipment and disposables

- LC-Genie III v3.17
- Micropipettes
- Timer
- Microcentrifuge tubes 1.5ml (DNA free)
- Vortex mixer
- Minicentrifuge
- Genie strips 0.2ml (LC-GenieStrip)
- Strip holder, for example the Genie strip holder (LC-GenieStripHolder)
- Pipette tips with filter barrier (DNA free)
- Optional: Dymo LabelWriter

Recommendation before starting:

- Manipulate the reaction buffer in an area far or at least separated from the amplification room.
- Thaw the reagents and samples at room temperature.
- Before preparing the reaction, vortex shortly and spin down all kit solutions briefly when thawed.
- Vortex shortly and spin down all specimen samples briefly when thawed.
- Perform all the steps on the same day.
- Keeping the specimen lysed at room temperature for a long period of time can lead to the degradation of nucleic acids and consequently decrease the LAMP efficacy.

Protocol:

Prepare the whole blood samples as follows:

Mix the lysis buffer (LC-LacIDuplex-LB) to homogenize.

Dispense 1000µl of lysis buffer (LC-LacIDuplex-LB) in the 1.5ml microcentrifuge tubes, Add 5µl of whole blood sample to the lysis buffer. Vortex in order to homogenize and incubate 1-10 minutes at room temperature. Use this solution and "specimen lysed".

Prepare the DNA samples as follows:

Mix the lysis buffer (LC-LacIDuplex-LB) to homogenize.

Dispense 10µl of lysis buffer (LC-LacIDuplex-LB) in the 1.5ml microcentrifuge tubes, Add 2µl of DNA sample with a concentration between 1ng/µl and 20ng/µl to the lysis buffer. Pipet up and down in order to homogenize and incubate 1-10 minutes at room temperature. Use this solution and "specimen lysed".

Prepare the positive and negative controls as follows:

Dispense 1000µl of lysis buffer (LC-LacIDuplex-LB) in two 1.5 ml microcentrifuge tubes. Add 5µl of positive (LC-LacIDuplex-Ctrl+) and negative (LC-LacIDuplex-Ctrl-) control to the lysis buffer. Vortex in order to homogenize and incubate 1-10 minutes at room temperature. Use these two solutions as "controls for reaction".

Lysed samples can be immediately used after incubation or can be stored for maximum 24h in the fridge.

Alternative volumes can be used if the ratio of sample/lysis buffer is respected.

Prepare the Lamp reaction as follows:

Add 5 µl of "specimen lysed or controls for reaction" to the 20 µl of Reaction Buffer (LC-LacIDuplex-RB) previously dispensed in the 0.2 ml strips set over the strip holder. Close the tubes correctly with the caps. Spin the strip and load the tubes onto the LC-Genie III machine and close the cover.

In order to validate a run, the positive and the negative controls must be included in each run.

System set-up:

Read and follow the LC-Genie III Manual on supplied with the LC-Genie III machine before setting-up the machine.

Switch on the LC-Genie III instrument by using the power switch at the back of the instrument.

Inserting tubes

Press the button on the front of the unit and the lid should open upwards. Close the lid after deposing the strip, by lowering and pressing down firmly.

Use only the recommended LC-Genie Strips, other tubes and strips will not fit.

IMPORTANT! The shape of the tubes is such that they will only fit in one way. The locating pins on the block have corresponding holes in the strips.

LC-Genie III welcome screen

The LC-Genie III uses a touchscreen for viewing and inputting data.

Touch the screen gently and press the appropriate keys when required. The touch screen can be operated while wearing protective gloves or by using the stylus included with the instrument.

IMPORTANT! Do not use a pen or any other sharp implements to touch the screen.

When switching on, the LED above the screen will be amber in color. Wait for the light to change to green, then touch the screen to access the main menu.

Login

All names can be used for login; no password is necessary, except for supervisor access (see LC-GENIE III manual for more information on supervisor access).

Run

Start a run by touching in the main menu the predefined profile “LacIDuplex Melting” on the instrument.

Press ‘Next’

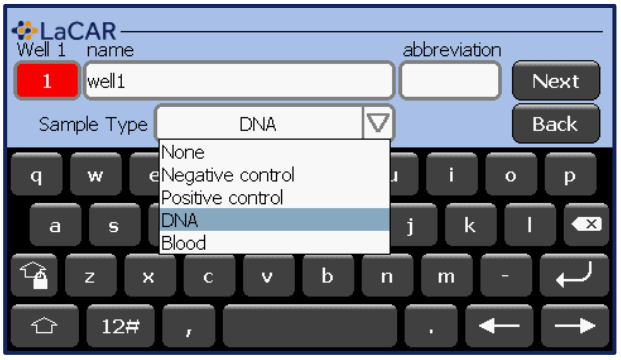
Add the name of the experiment. The name of the chosen profile followed by date and time is automatically proposed.

Click ‘Next’

Scan the barcode of the kit. A warning will be given when the kit has passed the expiration date.

IMPORTANT! When the kit is out of date, the run can be started by clicking “Next”, however it is not recommended to perform a test with a kit that has expired.

Add the names to the well blocks and add the appropriate sample type. ‘Next’ switches to the next name and saves the current well name.



IMPORTANT! Different criteria are used for interpretation of the different sample types, therefor it is important to add the correct sample type.

Once a run is started, the software will go to the ‘Temperature’ screen. The other windows can be accessed via the touchscreen.

All LC-Genie III runs are automatically saved in the ‘LOG’ folder on the machine and classified corresponding to the year/month/day of the run.

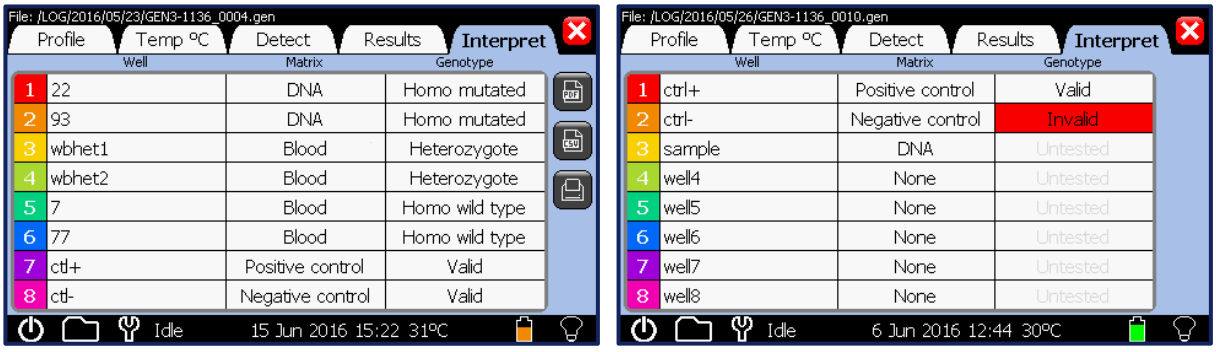
Interpretation of results:

Interpretation of the sample results on the LC-Genie III is not yet completely automated. The interpretation for polymorphism other than -13910 C/T and -14009T/G should be performed manually based on the melting temperature. A software update will be performed for automatic interpretation.

When the run is finished a graphical presentation of the results can be seen in the tab “Detect”. The melting temperatures corresponding to the peaks are presented in the tab “Results”.

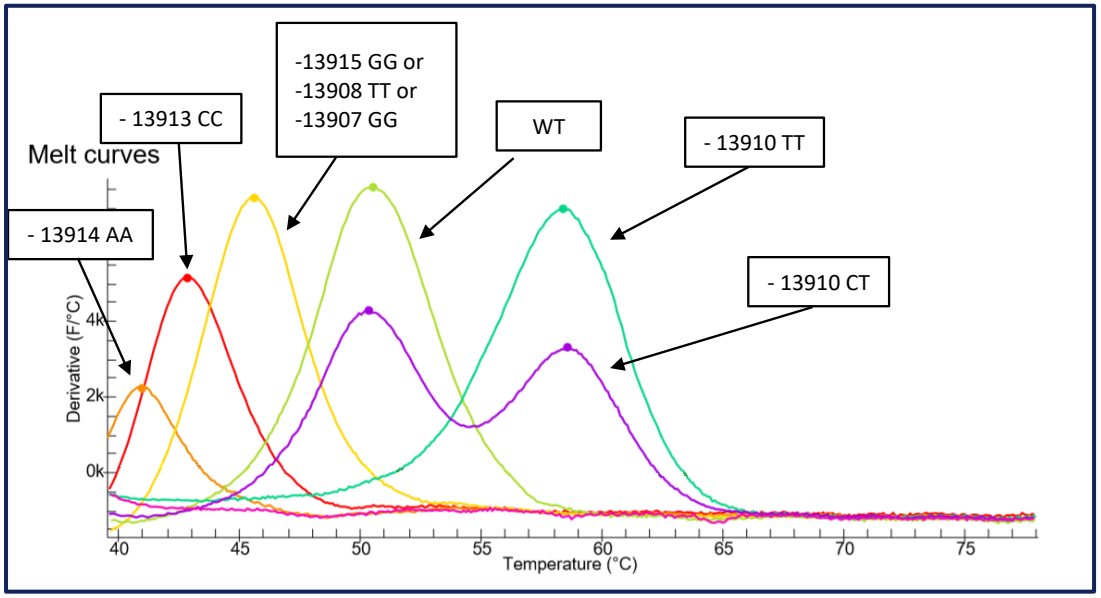
In the tab “Interpret” the results of the controls are interpreted automatically as valid/invalid. When the result is not valid for one of the controls the software will block the results of the samples and indicate in red the invalid control.

IMPORTANT! No genotype should be accepted without a visual control of the melting curve.

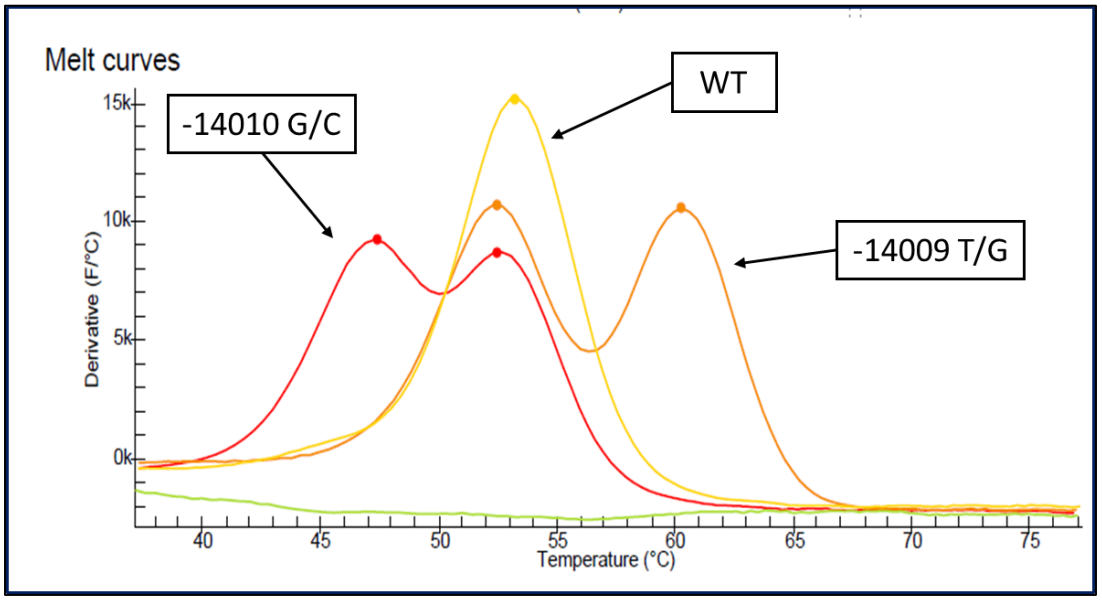


The final genotype of the invalid samples should be manually interpreted at this moment.

Blue: 13910/13915



Yellow: 14010/14009



Results are interpreted based on following melting temperatures and thresholds:

Interpretation criteria for Controls:

Blue : 13910/13915

Name	Peaks – T (°C)	Fluorescence level
Positive control (LC-LacIDuplex-Ctrl+)	50.60-53.60	>700
	58.70-61.70	>700
Negative control (LC-LacIDuplex-Ctrl-)	No peak	No peak

Yellow: 14010/14009

Name	Peaks – T (°C)	Fluorescence level
Positive control (LC-LacIDuplex-Ctrl+)	55.10-58.10	>700
	63.80-66.80	>700
Negative control (LC-LacIDuplex-Ctrl-)	No peak	No peak

Interpretation criteria for blood samples:

Blue: 13910/13915

50.60-53.60 Fluo > 700	58.70-61.70 Fluo > 700	Interpretation by software	Suggested Genotype
Present	Absent	Homozygous Wild Type	Wild type -13910 CC
Absent	Present	Homozygous mutant	Homozygote mutant -13910 TT
Present	Present	Heterozygous	Heterozygote -13910 CT

Peak between 45.00°C and 48.00°C: mutation at position -13915 (or -13907/-13908) present.

Peak between 42.00°C and 44.00°C: mutation at position -13913 present

Peak between 40.00°C and 42.00°C: mutation at position -13914 present

Yellow : 14010/14009

55.10-58.10 Fluo > 700	63.80-66.80 Fluo > 700	Interpretation by software	Suggested Genotype
Present	Absent	Homozygous Wild Type	Wild type -14009 TT
Absent	Present	Homozygous mutant	Homozygote mutant -14009 GG
Present	Present	Heterozygous	Heterozygote -14009 TG

Interpretation criteria for DNA samples:

Blue: 13910/13915

51.20-54.20 Fluo >700	57.00-60.00 TBD* Fluo >700	Interpretation by software	Suggested Genotype
Present	Absent	Homozygous Wild Type	Wild type -13910 CC
Absent	Present	Homozygous mutant	Homozygote mutant -13910 TT
Present	Present	Heterozygous	Heterozygote -13910 CT

Peak between 45.00°C and 48.00°C: mutation at position -13915 (or -13907/-13908) present.

Peak between 42.00°C and 44.00°C: mutation at position -13913 present

Peak between 40.00°C and 42.00°C: mutation at position -13914 present

Yellow: 14010/14009

55.40-58.40 Fluo >700	57.00-60.00 TBD* Fluo >700	Interpretation by software	Suggested Genotype
Present	Absent	Homozygous Wild Type	Wild type -14009TT
Absent	Present	Homozygous mutant	Homozygote mutant -14009 GG
Present	Present	Heterozygous	Heterozygote -14009 TG

Invalid results

Melting temperature of one of the peaks is out of specification.

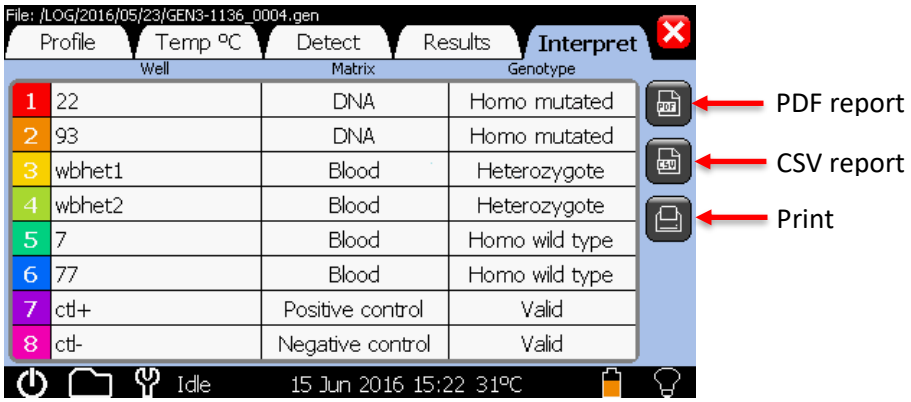
Melting curve shows one or more peaks below threshold level or shows not well separated peaks.

Melting curve shows more than two peaks.

Report

A pdf report containing all run information and results can be created by clicking the pdf button in the "interpret" window. The report is saved in the folder "REPORT" or on a USB key inserted in the machine.

The melting temperatures of the peaks can be exported as .CSV by clicking the csv button in the "interpret" window. The file is saved in the folder "REPORT". This file can be used for import in excel for further analysis of the melting temperatures when needed.

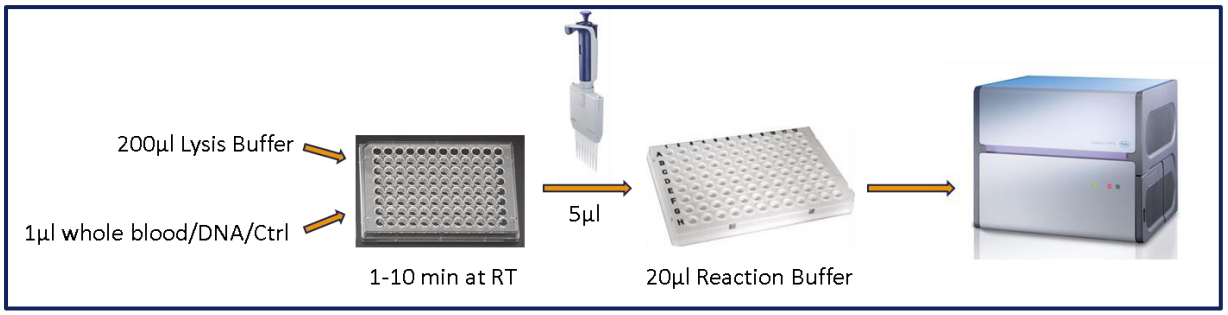


When the LC-GENIE III is attached to the computer, the generated files in the "REPORT" folder can be copied to the computer.

The result table can be immediately printed from the machine using the Dymo LabelWriter™, by clicking on the print button.

8. USE ON LIGHTCYCLER®480 (I & II) / COBAS® Z 480 (ROCHE)

Overview of the procedure



Laboratory equipment and disposables

- LightCycler® 480 (I & II) / Cobas® z 480 (Roche)
- Micropipettes, optional: multichannel pipette
- Timer
- Vortex mixer
- Centrifuge
- 96-well microplate (DNA free)
- LightCycler 480 Multiwell plate 96 (White) and LC480 sealing foil
- Pipette tips with filter barrier (DNA free)
- LC-Films (Adhesive pre-pierced films)

LaCAR optional control kit

- LC-LacIDuplexCTRL-LP: Control Kit including 4 additional controls: one -13910 plasmid DNA Homozygous Wild-Type, one -13910 and -14009 plasmid DNA Homozygous Mutant, one -13915 and -14010 plasmid DNA Homozygous Mutant & one -13915 and -14010 plasmid DNA Heterozygous.

Recommendation before starting:

- Manipulate the reaction buffer in an area far or at least separated from the amplification room.
- Thaw the reagents and samples at room temperature.
- Before preparing the reaction, vortex shortly and spin down all kit solutions briefly when thawed.
- Vortex shortly and spin down all specimen samples briefly when thawed.
- Perform all the steps on the same day.

- Keeping the specimen lysed at room temperature for a long period of time can lead to the degradation of nucleic acids and consequently decrease the LAMP efficacy.

Protocol:

If you use LaCAR's Control Kit please refer to its own Instructions for Use.

If not, please proceed as described here below.

Prepare the whole blood samples as follows:

Pipette up and down to homogenize the lysis buffer (LC-LacIDuplex-LB).

Add 1µl of whole blood to the ELISA plate. Afterwards seal the plate with a pre-pierced sealing foil and add carefully 200µl of lysis buffer (LC-LacIDuplex-LB) to the plate through the film. Perform some up-and-down to homogenize the "lysed specimen".

Prepare the DNA samples as follows:

Pipette up and down to homogenize the lysis buffer (LC-LacIduplex-LB).

Add 1µl of DNA (with a concentration between 10ng/µl and 50ng/µl) to the ELISA plate. Afterwards seal the plate with a pre-pierced sealing foil and add carefully 200µl of lysis buffer (LC-LacIDuplex-LB) to the plate through the film. Perform some up-and-down to homogenize the "lysed specimen".

Prepare the positive and negative controls as follows:

Pipette up and down to homogenize the lysis buffer (LC-LacIDuplex-LB).

Add 1µl of each control (one well for each) to the ELISA plate. Afterwards seal the plate with a pre-pierced sealing foil and add carefully 200µl of lysis buffer (LC-LacIDuplex-LB) to the plate through the film. Perform some up-and-down to homogenize the "lysed specimen".

Alternative volumes can be used if the ratio of sample/lysis buffer is respected.

Incubation:

Incubate the samples during **1 to 10 minutes** at room temperature. Use this solution as "lysed specimen".

Prepare the LC480 96-well plate as follows:

Dispense 20µl of Reaction Buffer (LC-LacIDuplex-RB) in the white LC480 96-well plate, one well for each of the samples and controls included.

Pipette carefully up and down to homogenize the lysed solution. Add 5 µl of "lysed specimen" to the 20 µl of Reaction Buffer (LC-LacIDuplex-RB). Pipette carefully up and down to mix the sample with the reaction buffer. Seal the plate correctly with the appropriate seal. Spin the plate to eliminate air bubbles before placing it in the machine.

In order to validate a run, the positive and the negative controls must be included in each run.

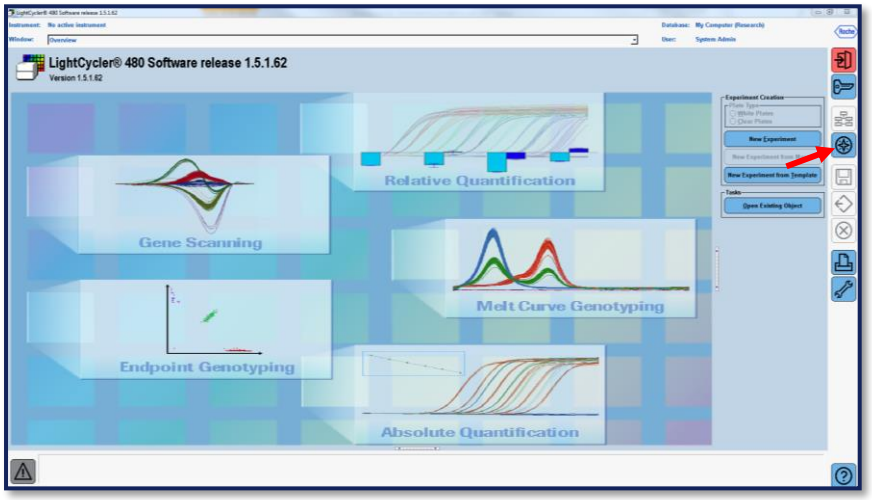
System set-up:

Read and follow the LC480 manual before setting-up the machine.

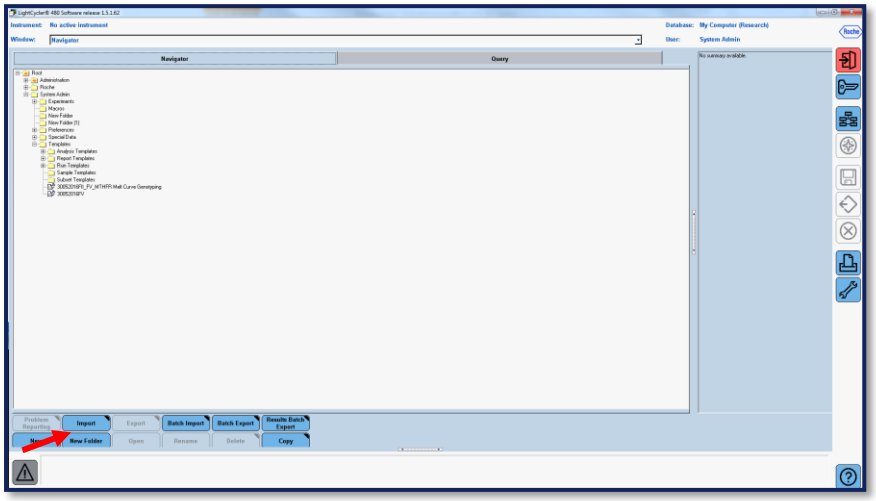
Installation using the available template file

Open the LightCycler® Software

Click on the navigator icon.

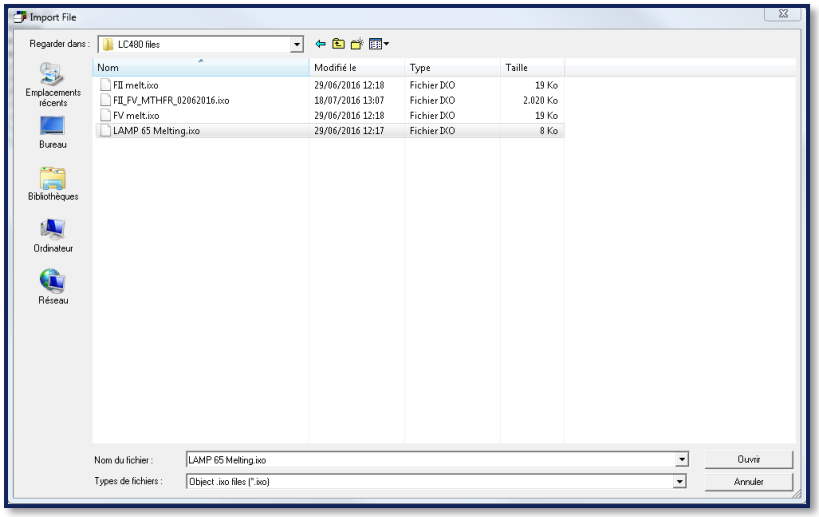


The following window opens:

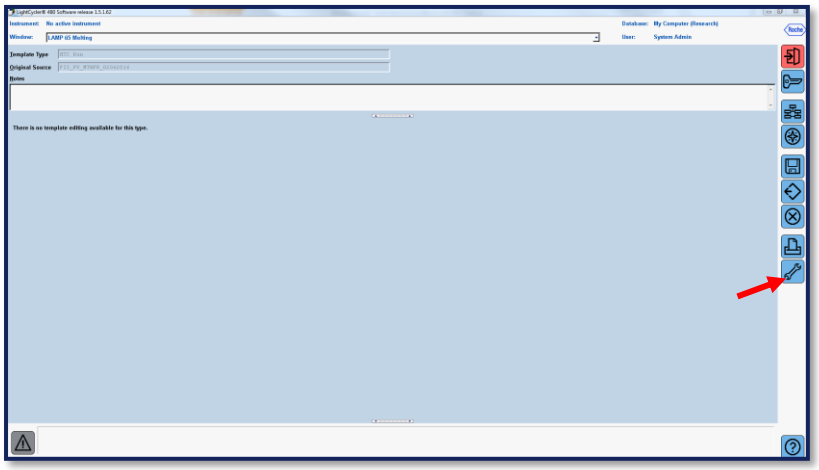


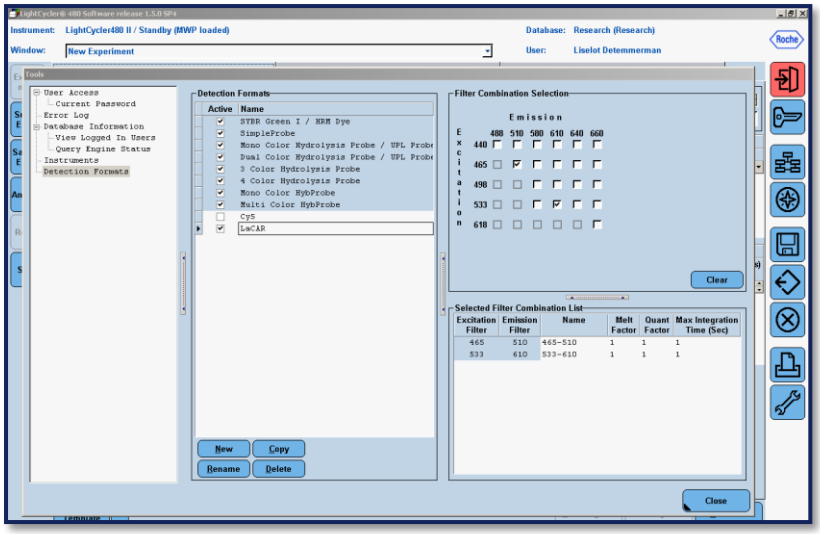
Click “Import”

Select the provided LightCycler® 480 “.ixo” file called ”LAMP LacIDuplex.ixo” and click “Open”



The following window opens:





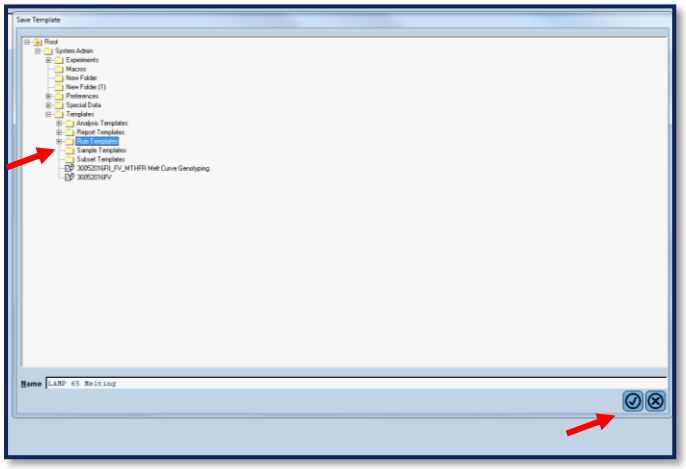
Go to “Detection Formats” and create a new detection format by selecting the correct filter combination as indicated.

LC480 I (Roche) please select following filter combinations:
Excitation filter: 483- Emission filter: 533
Excitation filter: 558-Emission filter: 610

LC480 II (Roche) please select following filter combinations:
Excitation filter: 465- Emission filter: 510
Excitation filter: 533-Emission filter: 610

Cobas® z 480 (Roche) please select following filter combinations:
Excitation filter: 465- Emission filter: 510
Excitation filter: 540-Emission filter: 610

Click “Close”, then click “Save”.

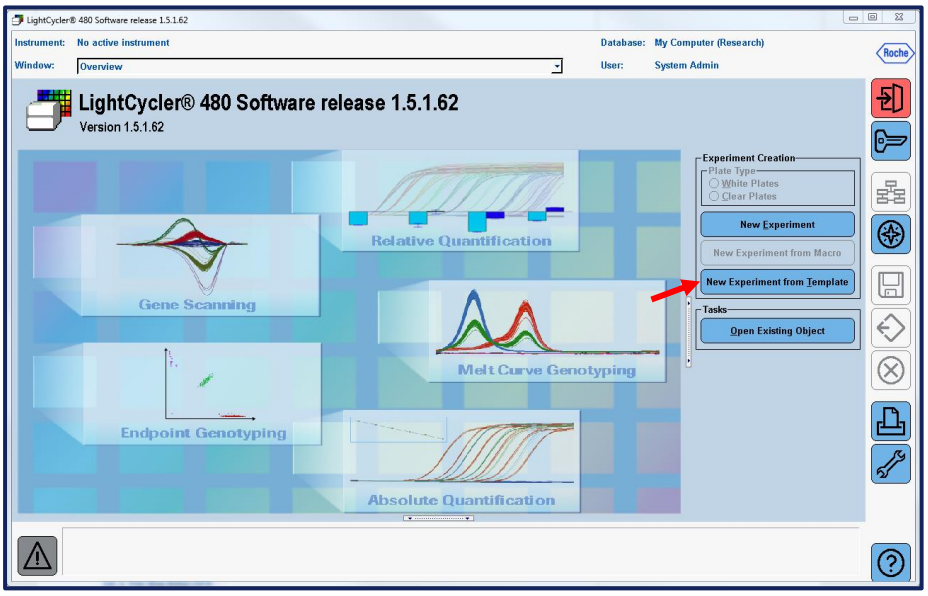


Store the file as Run Template as “LAMP LacIDuplex”

The program is now available as a run template and can be selected by clicking “New experiment from Template” after opening the LightCycler® software.

Starting a run

Switch on the LC480 instrument by using the power switch at the side of the instrument.



Click the "New experiment from template" button to start a run. Select the "LAMP LacIDuplex" program. Add the correct subsets and sample names.

LAMP 65 melting program:

Cycles: 70 **Analysis mode:** Quantification

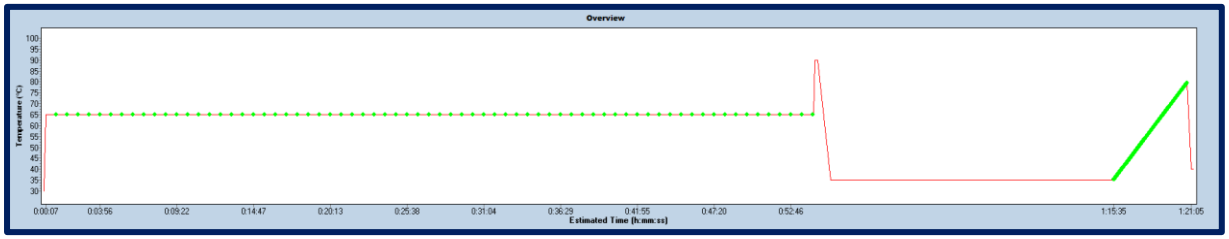
Target (°C)	Acquisition Mode	Hold (hh :mm :ss)	Ramp rate (°C/s)	Acquisitions (per °C)
65°C	None	00:00:01	4.40	
65°C	Single	00:00:22	4.40	

Melting:

Cycles: 1 **Analysis mode:** Melting curves

Target (°C)	Acquisition Mode	Hold (hh :mm :ss)	Ramp rate (°C/s)	Acquisitions (per °C)
90	None	00 :00 :02	4.4	
35	None	00:20:00	1.0	
80	Continuous		0.14	2
40	None	00:00:01	2.2	

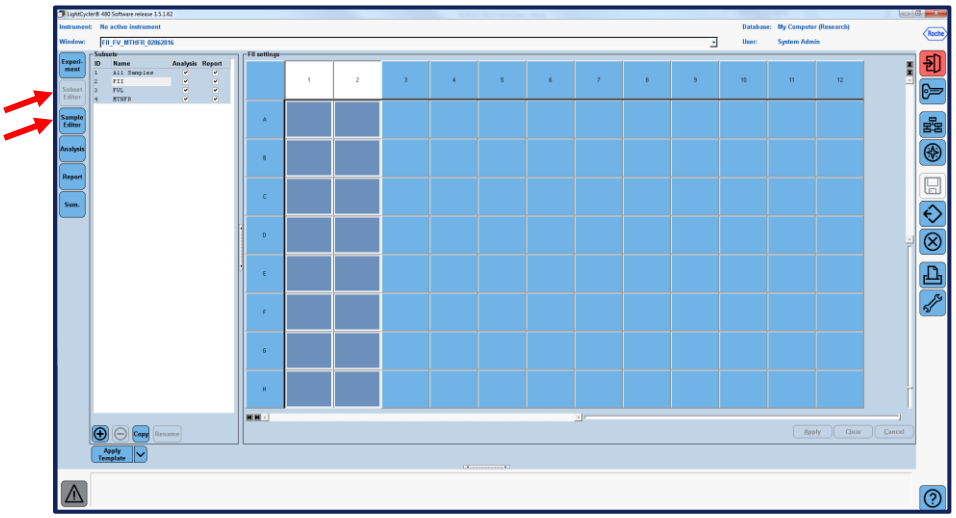
Overview:



Click the “start run” button to start the run, the software will automatically ask you to save the run before starting.

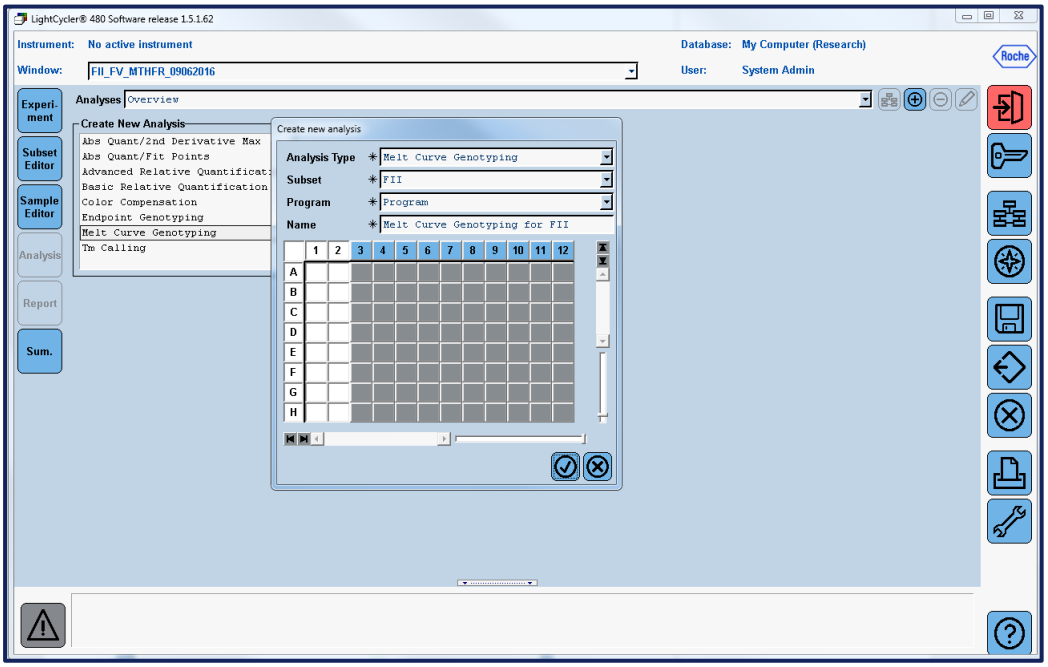
Add in the “Sample Editor” window the correct samples names.

For easier interpretation, subsets can be added in the “Subset Editor” window. This option should be used when combining different assays on the same plate.



Interpretation of results

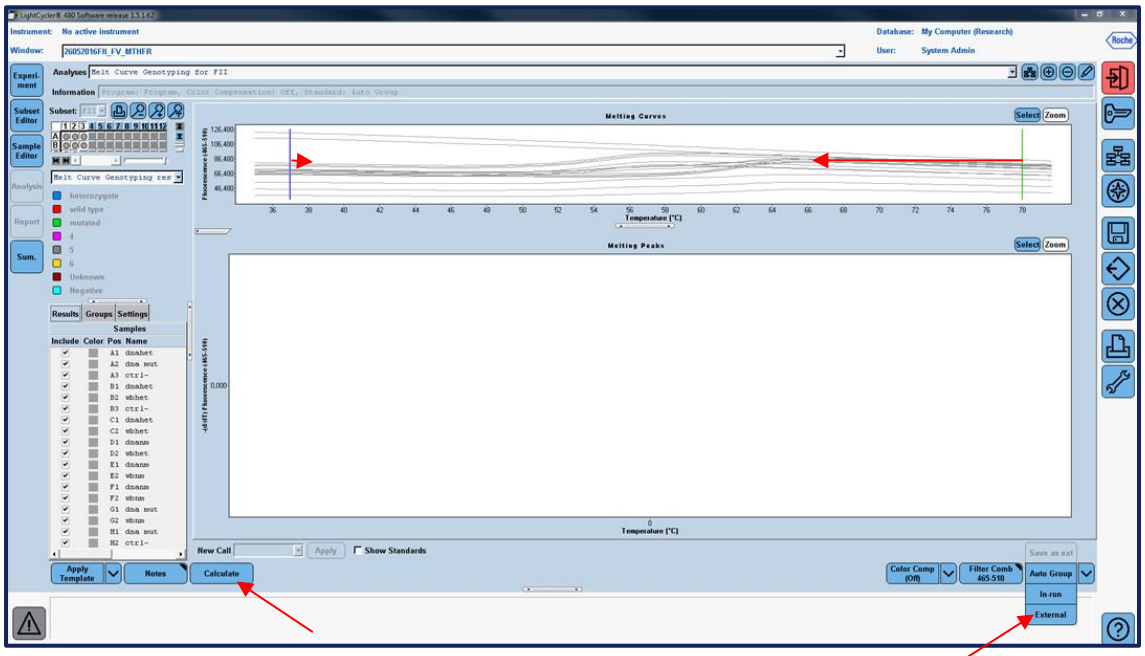
For the interpretation of the result, go to the analysis button and select “Melt curve genotyping”. If subsets were created, the correct subset should be chosen.



Analyse the results using the “melt curve genotyping” analysis in the LightCycler480 software 1.5 using an external melt standard.

Choose the correct melting standards file from the available standards (LacIDuplex -139** Melt (39-66) and LacIDuplex -140** Melt (42-71)). Melting standards are based on results of whole blood samples. Place the left calculation range at 39°C and the right one at 66°C and at 42°C and 71°C.

Click “Calculate”

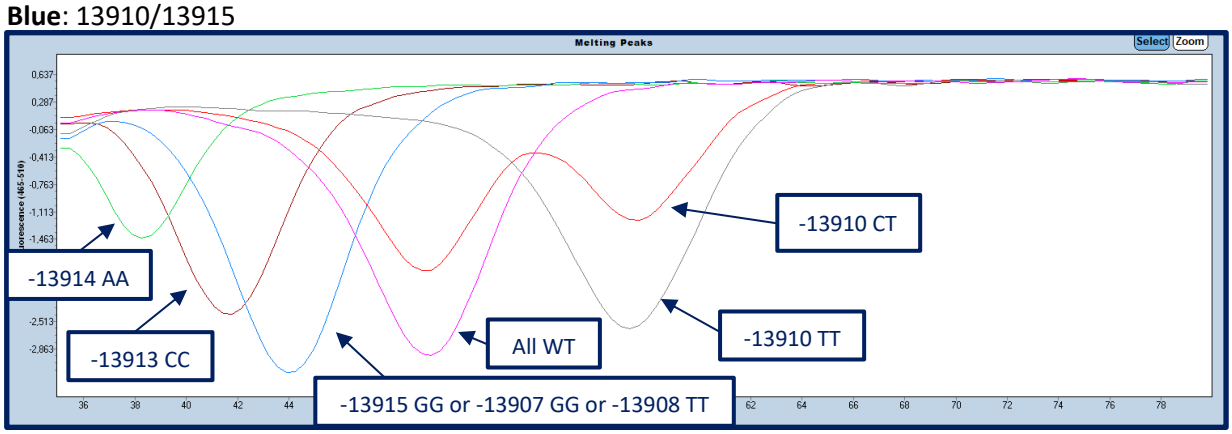


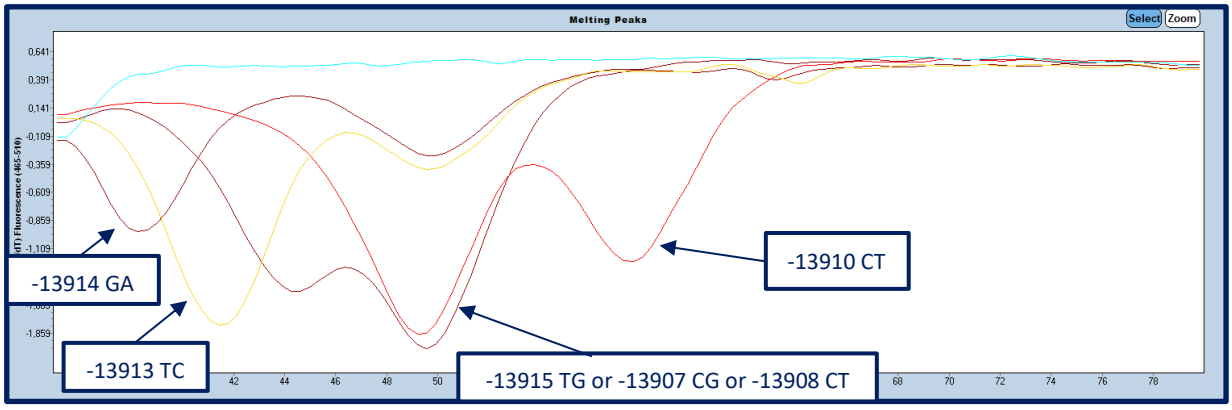
Interpretation:

To consider a run as valid, all controls must be reported in the correct group and all negative controls must be reported as a negative.

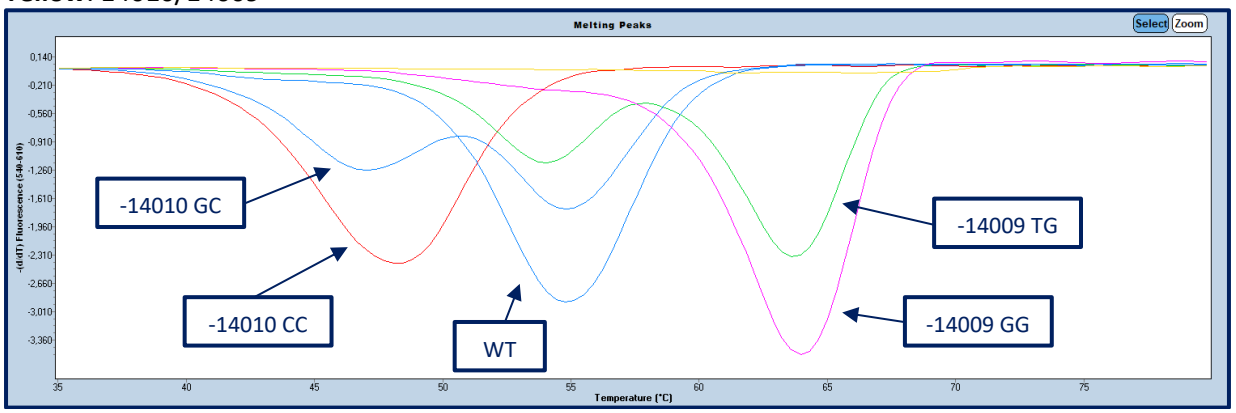
The color of the sample indicates the suggested genotype. Samples with the same color have the same identification.

Blue: 13910/13915





Yellow: 14010/14009



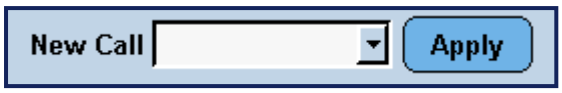
Visual interpretation:

Samples that do not show sufficient similarity to one of the reference standards will be shown in brown and grouped as unknown. These samples can be interpreted by comparing the melting peak to the external reference curves ("show standards").

The -139** defined limits are around 45°C (between 43°C and 47°C) for the first peak, around 51°C (between 49°C and 53°C) for the second peak, and around 59°C (between 57°C and 61°C) for the third peak (here above the red peak).

The -140** defined limits are around 49°C (between 47°C and 51°C) for the first peak, around 56°C (between 54°C and 58°C) for the second peak, and around 65°C (between 63°C and 67°C) for the third peak (here above the red peak).

The identification of the unknown sample can be changed by clicking on the corresponding samples and by adding the genotype in the New Call window and clicking "Apply".



Samples showing more than two peaks or a peak outside the defined limits are considered as invalid and should be repeated.

Save the result by clicking the "Save" button on the right sight of the screen.

Report

Click the "Report" button to create a report.

Select the appropriate information (Results, Group, Melting curves, and Melting peaks) to include in the report.

The suggested genotype is mentioned in the table, in the column "Group".

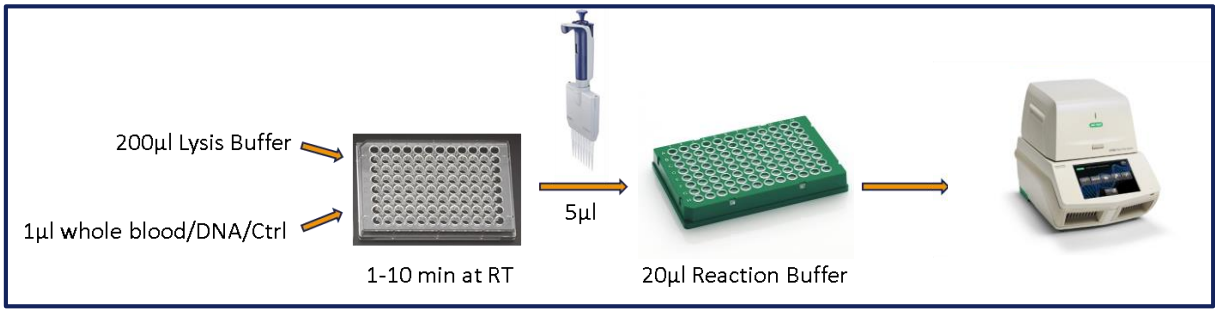
Results

Inc	Pos.	Sample Name	Sample Type	Group	Score	Resolution	Status
☑	A2	0022	Unknown	Heterozygote	0,97	0,83	
☑	B1	0078	Unknown	wild type	0,97	0,97	
☑	B2	0078	Unknown	wild type	0,97	0,97	
☑	C1	0065	Unknown	wild type	0,98	0,98	
☑	C2	0065	Unknown	wild type	0,98	0,98	
☑	D1	0007	Unknown	wild type	0,98	0,98	
☑	D2	0007	Unknown	wild type	0,93	0,93	
☑	E1	dna 22 1ng	Unknown	Heterozygote	1,00	0,87	
☑	E2	AA	Melting Standar	mutant	1,00	0,87	
☑	F1	dna 22 5ng	Unknown	Heterozygote	0,77	0,34	
☑	F2	GG	Melting Standar	wild type	0,89	0,89	
☑	G1	dna 5010 1ng	Unknown	wild type	1,00	0,99	
☑	G2	ctrl+	Melting Standar	Heterozygote	0,88	0,66	
☑	H1	dna 5010 5ng	Unknown	wild type	0,95	0,95	
☑	H2	ctrl-	Negative Contro	Negative	1,00	1,00	

If the sample is considered as invalid or designated by the software as unknown, the sample should be tested again starting back from the whole blood sample.

9. USE ON BIO-RAD CFX96™

Overview of the procedure



Laboratory equipment and disposables

- Bio-Rad CFX96 Touch
- Micropipettes, optional: multichannel pipette
- Timer
- Vortex mixer
- Centrifuge
- 96-well microplate (DNA free)
- Hard-shell white well 96-well skirted PCR plates (HSP-9655) and sealing foil
- Pipette tips with filter barrier (DNA free)
- LC-Films (Adhesive pre-pierced films)

Recommendation before starting

- Manipulate the reaction buffer in an area far or at least separated from the amplification room.
- Thaw the reagents and samples at room temperature.
- Before preparing the reaction, vortex shortly and spin down all kit solutions briefly when thawed.
- Vortex shortly and spin down all specimen samples briefly when thawed.
- Perform all the steps on the same day.
- Keeping the specimen lysed at room temperature for a long period of time can lead to the degradation of nucleic acids and consequently decrease the LAMP efficacy.

Protocol

Prepare the whole blood samples as follows

Add 1µl of whole blood to the ELISA plate. Afterwards seal the plate with a pre-pierced sealing foil and add carefully 200µl of lysis buffer to the plate through the film. Perform some up-and-down to homogenize the “lysed specimen”.

Prepare the DNA samples as follows

Add 1µl of DNA (at a concentration between 5ng/µl and 30ng/µl) to the ELISA plate. Afterwards seal the plate with a pre-pierced sealing foil and add carefully 200µl of lysis buffer to the plate through the film. Perform some up-and-down to homogenize the “lysed specimen”.

Prepare the positive and negative controls as follows

Add 1µl of control to the ELISA plate. Afterwards seal the plate with a pre-pierced sealing foil and add carefully 200µl of lysis buffer to the plate through the film. Perform some up-and-down to homogenize the “lysed specimen”.
Alternative volumes can be used if the ratio of sample/lysis buffer is respected.

Incubation

Incubate the samples during 1 to 10 minutes at room temperature. Use this solution as “lysed specimen”.

Prepare the 96-well plate as follows

Dispense 20µl of Reaction Buffer (LC-LacIDuplex-RB) in the white 96-well plate, one well for each of the samples and controls included.
Pipette carefully up and down to homogenize the lysed solution. Add 5 µl of “lysed specimen” to the 20µl of Reaction Buffer. **Do not pipet completely at the bottom nor at the top of the lysed sample**.
Pipette carefully up and down to mix the sample with the reaction buffer. Seal the plate correctly with the appropriate seal. Spin the plate to eliminate air bubbles before placing it in the machine.
In order to validate a run, the positive and the negative controls must be included in each run.

System set-up

Read and follow the CFX96 manual before setting-up the machine.

Installation

Start the CFX Manager software by clicking twice on the Bio-Rad CFX Manager icon.

The Startup Wizard opens. Go to the Run setup window to perform a run. Click User-defined.

The LAMP LacIDuplex protocol should be programmed by clicking 'Create New' in the protocol window and adding the correct program as shown underneath.

Target (°C)	Acquisition Mode	Hold (hh :mm :ss)	Increment
65°C	None	00:30:00	
90°C	None	00 :00 :02	
35C	None	00 :10 :00	
30°C	None	00:00:05	
30 to 80°C	Plate Read		0.5°C for 0 :05

Store the file as Protocol Template as "LAMP LacIDuplex"

The program is now available as a template and can be selected by clicking "select existing"

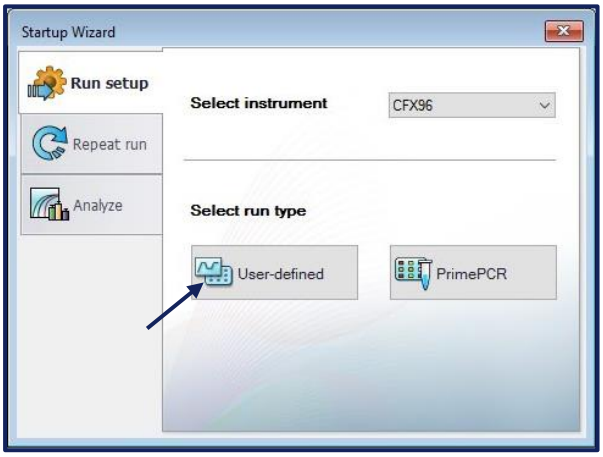
Starting a run

Switch on the machine and open the Bio-Rad CFX Manager software.

Go to the Run setup window to perform a run. Click user defined.

The run setup window included three tabs:

Protocol: Click the protocol tab to select the "**LAMP LacIDuplex**" protocol to run.



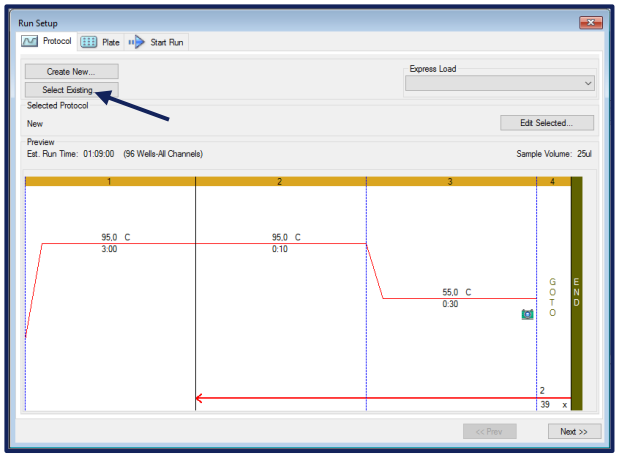


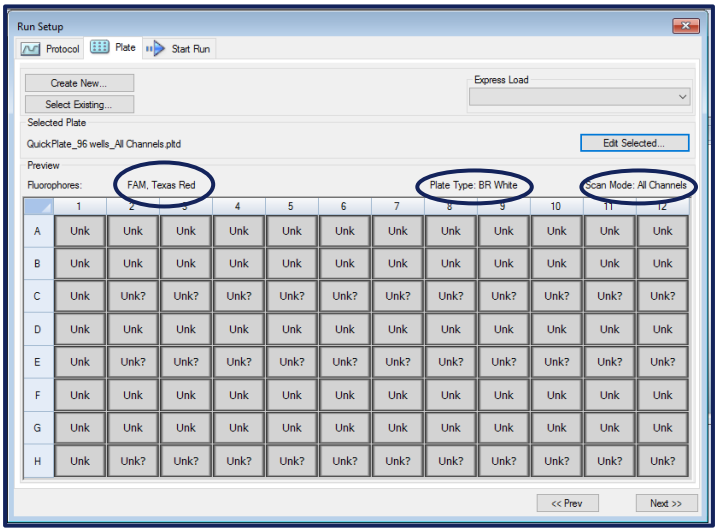
Plate: Click the plate tab to select an existing plate to run or edit, or to create a new plate in the plate editor window.

Fluorophores: FAM / TR

Plate type: BR White

Scan Mode: All Channels

Plate: Click the plate tab to select an existing plate to run or edit, or to create a new plate in the plate editor window.



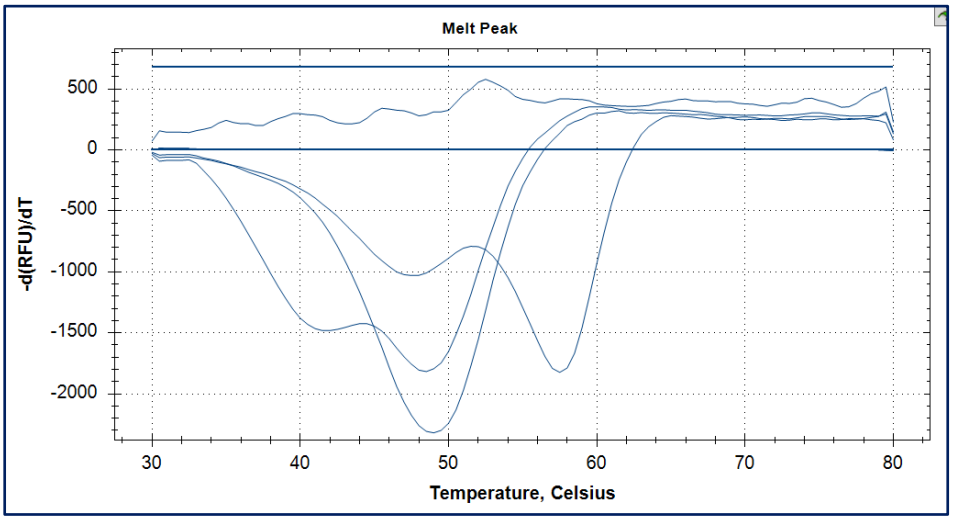
Interpretation of results

For the interpretation of the results, go to the Data analysis window.

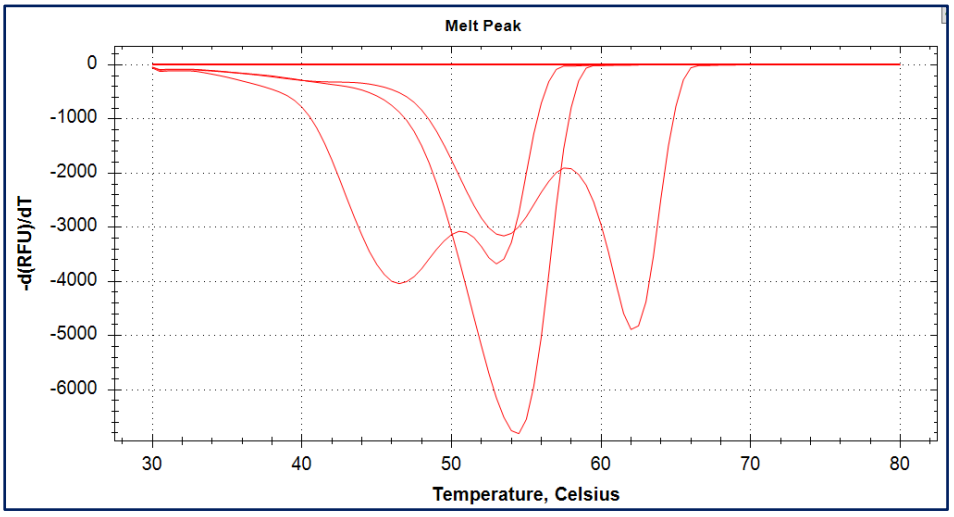
Go to the Melt curve tab.

The software plots the RFU data collected during a melt curve as a function of temperature. To analyze melt peak data, the software assigns a beginning and ending temperature to each peak by moving the threshold bar.

Blue: 13910/13915



Yellow: 14010/14009



Open the **Melt Curve tab** to determine the Tm of the melting peaks. This tab shows the melt curve data in these four views:

- Melt Curve. View the real-time data for each fluorophore as RFUs per temperature for each well
- Melt Peak. View the negative regression of the RFU data per temperature for each well
- Well selector. Select wells to show or hide the data

- Peak spreadsheet. View a spreadsheet of the data collected in the selected well

Adjust the Melt Curve data:

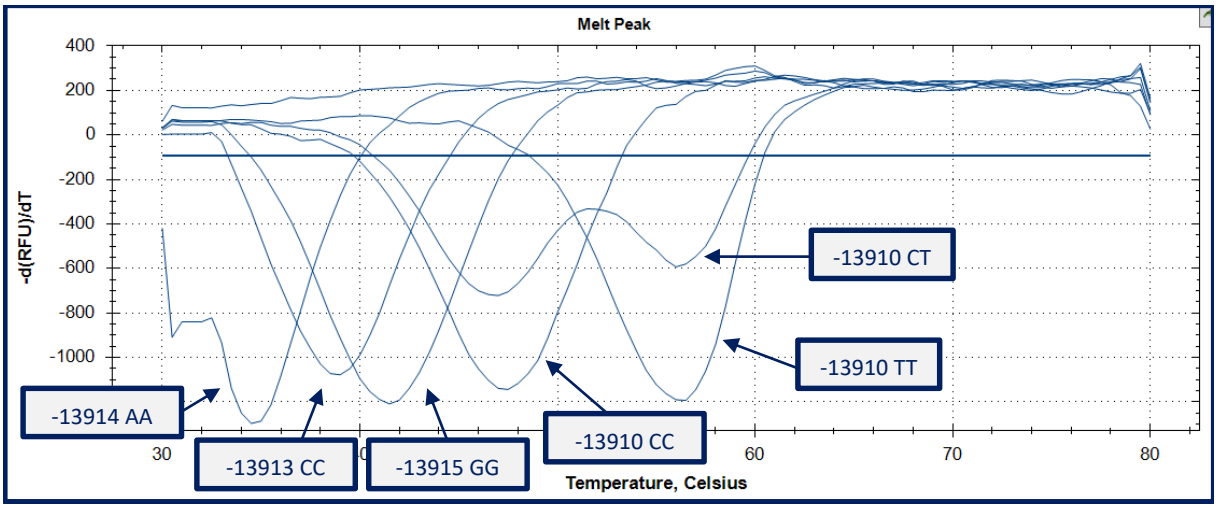
- Select Negative to view the spreadsheet data for the peaks below the Melt Threshold line. Adjust the threshold line to -100.
- Select a well group to view and analyze a subset of the wells in the plate. Select each well group by name in the Well Group pull-down menu in the toolbar.

Melt Curve Data Tab:

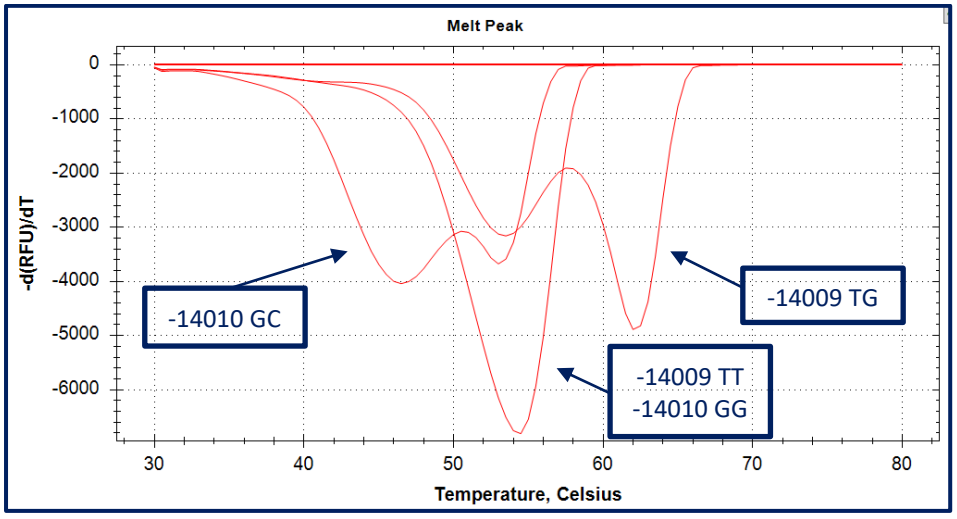
The Melt Curve Data tab shows the data from the Melt Curve tab in multiple spreadsheets that include all the melt peaks for each trace.

Interpretation

Blue: 13910/13915



Yellow: 14010/14009



Results are interpreted based on melting temperatures and fluorescence values.

To consider a run as valid, all controls must be within the set limits and all negative controls must be reported as a negative.

Interpretation criteria for Controls:

Blue: 13910/13915

	47.00°C- 51.00°C	55.50°C – 59.50°C
Positive control	Present	Present
Negative control	Absent	Absent

Yellow: 14010/14009

	51.50°C- 55.50°C	60.00°C – 64.00°C
Positive control	Present	Present
Negative control	Absent	Absent

Interpretation of unknown samples is based on the criteria underneath:

Blue: 13910/13915

39.50°C-43.50°C	47.00°C- 51.00°C	55.50°C – 59.50°C	Suggested Genotype
Absent	Present	Present	Heterozygous -13910 CT
Absent	Present	Absent	Homozygote wild type
Absent	Absent	Present	Homozygous Mutated -13910 TT
Present	Absent	Absent	Homozygous mutated -13915 GG
Present	Present	Absent	Heterozygous -13915 T/G

Yellow: 14010/14009

39.50°C-43.50°C	47.00°C- 51.00°C	55.50°C – 59.50°C	Suggested Genotype
Absent	Present	Present	Heterozygous -14009 TG
Absent	Present	Absent	Homozygote wild type
Absent	Absent	Present	Homozygous Mutated -14009 GG
Present	Absent	Absent	Homozygous mutated -14010 CC
Present	Present	Absent	Heterozygous -14010 G/C

10. TROUBLESHOOTING GUIDE

	Signal	Putative explanation	Solutions
C+ C- Samples	Valid Valid Invalid/Negative	Inappropriate lysis	Repeat the test from the lysis process
C+ C- Samples	Valid/Invalid Valid/Invalid Invalid	Inappropriate storage conditions, error in reaction preparation	Check the kit's Storage conditions Check the reaction preparation
C+ C- Samples	Valid Invalid (positive) Valid	Reagent contamination and/or environmental contamination	Ensure Good Laboratory Practices is adhered to Repeat the test with a new kit starting from extraction step
C+ C- Samples	Valid/Invalid Valid Invalid/Negative	Inappropriate storage conditions	Check the kit's Storage conditions
C+ C- Samples	Invalid (Negative) Invalid (Positive) Valid	Inversion of positive and negative control	Repeat the test
C+ C- Samples	Multiple low peaks present in one or more curves	Bubbles might create small fluorescent changes resulting in small peaks	Repeat the test, avoid bubble formation while pipetting
C+ C- Samples	Invalid (Tm shift) Valid/Invalid Invalid (Tm shift)	Melting temperature shift in one or multiple samples/controls	Pipetting errors can cause a shift in melting temperature

11. TEST LIMITATIONS

- A strict compliance with the instruction for use and the manuals is required for optimal results.
- Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.
- There is a risk of false negative results due to a loss of nucleic acid in sample. The positive control does not indicate whether the quality of the sample is valid.
- There is a risk of false positive values resulting from cross-contamination by amplification product or by the positive control.
- It is recommended that the laboratory assess the possibility of any additional rare mutations that may generate false genotyping results and report this as a limitation, if applicable.