

artus[®] M. tuberculosis LC PCR Kit

Handbook



24 (catalog no. 4555003)



96 (catalog no. 4555005)

For research use only. Not for use in diagnostic procedures.

For use with the

LightCycler[®] 1.1/1.2/1.5 and LightCycler 2.0 Instrument

July 2009 – Version 1



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artus M. tuberculosis LC PCR Kit

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artus[®] M. tuberculosis LC PCR Kit

For use with the *LightCycler 1.1/1.2/1.5* or *LightCycler 2.0* Instrument.

For research use only. Not for use in diagnostic procedures.

1. Contents

	Labelling and contents	Art. No. 4555003 24 reactions	Art. No. 4555005 96 reactions
Blue	<i>M. tuberculosis</i> LC Master	2 x 12 rxns	8 x 12 rxns
Yellow	<i>M. tuberculosis</i> LC Mg-Sol [¶]	1 x 400 µl	1 x 400 µl
Red	<i>M. tuberculosis</i> LC QS 1 5 x 10 ⁴ cop/µl	1 x 200 µl	1 x 200 µl
Red	<i>M. tuberculosis</i> LC QS 2 [¶] 5 x 10 ³ cop/µl	1 x 200 µl	1 x 200 µl
Red	<i>M. tuberculosis</i> LC QS 3 [¶] 5 x 10 ² cop/µl	1 x 200 µl	1 x 200 µl
Red	<i>M. tuberculosis</i> LC QS 4 [¶] 5 x 10 ¹ cop/µl	1 x 200 µl	1 x 200 µl
Green	<i>M. tuberculosis</i> LC IC [¶]	1 x 1,000 µl	2 x 1,000 µl
White	Water (PCR grade)	1 x 1,000 µl	1 x 1,000 µl

- ¶ QS = Quantitation Standard
IC = Internal Control
Mg-Sol = Magnesium Solution

2. Storage

The components of the *artus* M. tuberculosis LC PCR Kit should be stored at -20°C and are stable until the expiry date stated on the label. Repeated thawing and freezing (> 2 x) should be avoided, as this may reduce the sensitivity. If the reagents are to be used only intermittently, they should be frozen in aliquots. Storage at +4°C should not exceed a period of five hours.

3. Additionally Required Materials and Devices

- Disposable powder-free gloves
- DNA isolation kit (see **8.1 DNA Isolation**)
- Lysozyme mix (see **8.1 DNA Isolation**)
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- *Color Compensation Set* (Roche Diagnostics, Cat. No. 2 158 850) for the installation of a *Crosstalk Color Compensation* file for the *LightCycler 1.1/1.2/1.5* or *LightCycler 2.0* Instrument
- *LightCycler Multicolor Demo Set* (Cat. Nr. 03 624 854 001) for the *LightCycler 2.0* Instrument
- *LightCycler* Capillaries (20 µl)
- *LightCycler* Cooling Block
- *LightCycler 1.1/1.2/1.5* (Software Version 3.5) or *LightCycler 2.0* (Software Version 4.0) Instrument
- *LightCycler* Capping Tool

4. General Precautions

The user should always pay attention to the following:

- Use sterile pipette tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice or in the *LightCycler* Cooling Block.

5. Pathogen Information

Tuberculosis (TB) is still one of the most important infectious diseases worldwide. Some two billion people, one-third of the world's population, are infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The incidence of tuberculosis worldwide is about eight million and about three million people die each year. Even though the Third World countries are particularly affected, tuberculosis is a reemerging disease in industrialized nations, mainly due to the immigration of infected people and the development of drug resistant TB.

Tuberculosis is a chronic, cyclic disease, mainly affecting the lung and the associated lymph nodes. However, the *M. tuberculosis* bacteria can also colonize other organs. TB is primarily transmitted from person to person via aerosols.

6. Principle of Real-Time PCR

Pathogen detection by the polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR the amplified product is detected via fluorescent dyes. These are usually linked to oligonucleotide probes which bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e. in real-time) allows the detection and quantitation of the accumulating product without having to re-open the reaction tubes after the PCR run (Mackay, 2004).

7. Product Description

The *artus* *M. tuberculosis* LC PCR Kit constitutes a ready-to-use system for the detection of DNA of all members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. microti*, *M. pinnipedi*) using PCR in the *LightCycler* Instrument. The *M. tuberculosis* LC Master contains reagents and enzymes for the specific amplification of a 159 bp region of the mycobacterial genome, and for the direct detection of the specific amplicon with the *LightCycler* 1.1/1.2/1.5 or *LightCycler* 2.0 Instrument. In addition, the *artus* *M. tuberculosis* LC PCR Kit contains a second heterologous amplification system to identify possible PCR inhibition.

PCR product	Selection of the fluorescence channels	
	<i>LightCycler</i> 1.1/1.2/1.5 Instrument	<i>LightCycler</i> 2.0 Instrument
<i>M. tuberculosis</i> complex	F1	530
<i>M. tuberculosis</i> LC IC	F3/Back-F1	705/Back 530

The amplification and detection of this *Internal Control* (IC) do not reduce the detection limit of the analytical *M. tuberculosis* complex PCR (see **11.1 Analytical Sensitivity**). External positive controls (*M. tuberculosis* LC QS 1 - 4) are supplied which allow the determination of the amount of bacterial DNA. For further information, please refer to section **8.3 Quantitation**.

8. Protocol

8.1 DNA Isolation

Before the DNA isolation, large sample volumes or strongly acidic samples must first be concentrated or neutralized, respectively. For the analysis of sputum, we recommend a NALC-NaOH decontamination; stomach fluid should be neutralized with phosphate buffer. After a final centrifugation, the bacteria pellet can be used for the following DNA isolation.

Various manufacturers offer DNA isolation kits. Sample amounts for the DNA isolation procedure depend on the protocol used. Please carry out the DNA isolation according to the manufacturer's instructions. The following isolation kit is recommended:

Sample Material	Nucleic Acid Isolation Kit	Catalogue Number	Manufacturer	Carrier RNA
Sputum, BAL, bronchial secretion, CSF, stomach fluid, peritoneal puncture	QIAamp® DNA Mini Kit (50)	51 304	QIAGEN	not included

Attention: It is important to follow the instructions in **Appendix D** (Protocols for bacteria) described in the *QIAamp DNA Mini and Blood Mini Handbook*. To ensure an effective and contamination-free lysis of the mycobacteria the following amendments (highlighted bold) of QIAamp DNA Mini Kit supplement Protocol D are highly recommended:

Important: All pipetting steps before the incubation at 95°C have to be performed in a class II safety cabinet, since the samples are potentially infectious.

- **Transfer between 250 µl and 500 µl of the NALC-NaOH-decontaminated sample into a 1.5 ml screw cap tube.**
- Centrifuge 10 minutes at **17,000 x g (13,000 rpm)** in a desktop centrifuge.
- **Carefully discard the supernatant by pipetting.**
- Add 180 µl lysozyme mix (20 mg/ml lysozyme; 20 mM Tris-HCl (pH 8.0); 2 mM EDTA; 1.2 % Triton®) and resuspend the pellet by pipetting up and down.

- Incubate for **at least one hour** at 37°C in a thermoblock.
- **Centrifuge briefly to remove drops from the inside of the lid.**
- Add 20 µl Proteinase K and 200 µl AL buffer **supplemented with carrier RNA** (2 µg RNA Homopolymer Poly[rA], not included in the QIAamp DNA Mini Kit, per 200 µl AL buffer) **and optionally 10 µl *Internal Control*** (see **8.2 Internal Control**).
- Mix well by vortexing.
- Incubate for 30 minutes at 56°C in a thermoblock.
- **Centrifuge briefly to remove drops from the inside of the lid.**
- Incubate 15 minutes at 95°C (**incubation time should not be exceeded as this may cause DNA degradation**).

Important: Please note that only after completion of the incubation at 95°C the samples are no longer infectious.

- **Cool the sample down to room temperature.**
- Centrifuge briefly to remove drops from the inside of the lid.
- Follow the “Protocol: DNA Purification from Tissues” in the *QIAamp DNA Mini and Blood Mini Handbook* (Second Edition, November 2007) starting with the addition of ethanol at step 6, and **perform the final DNA elution using 100 µl Buffer AE.**

Important remarks for the prevention of cross-contaminations:

a) Regarding the lysis of the bacteria:

- The use of screw cap tubes is absolutely essential.
- The screw cap tubes must always be locked tightly.
- After each incubation step centrifuge the tube briefly to remove drops from the inside of the lid.
- Do not touch the inside of the tube lid. If so, please change the potentially contaminated glove immediately.
- The use of a waterbath is generally not recommended.
- Make sure the samples cool down to room temperature after the 95°C heating step, since otherwise the risk of aerosol-mediated contamination after opening the tube is extremely high.

b) Regarding the DNA isolation

- Make sure that you do not wet the rim of a QIAamp spin column.
- Do not touch the lid of a QIAamp spin column on the inside. If so, change the potentially contaminated glove immediately.
- Do not use the same pipette tip for different samples, not even to apply the washing buffers AW1 and AW2 or the elution buffer AE. This avoids cross-contamination between samples and the contamination of a buffer.
- Use each 2 ml collection tube only once. If you run out of collection tubes you may also use 2 ml microcentrifuge tubes, the lids of which have to be removed before.

General remarks:

- The use of **carrier RNA** is critical for the extraction efficiency and, consequently, for DNA/RNA yield. If the selected isolation kit does not contain carrier RNA, please note that the addition of carrier (RNA Homopolymer Poly[rA]) is strongly recommended for the extraction of nucleic acids from cell free body fluids and material low in DNA/RNA content (e.g. CSF). Please proceed as follows in these cases:
 - a) Resuspend the lyophilised carrier RNA using the elution buffer (do not use lysis buffer) of the extraction kit (e.g., Buffer AE of the QIAamp DNA Mini Kit) and prepare a dilution with a concentration of 1 µg/µl. Divide this carrier RNA solution in a number of aliquots adequate to your needs and store them at -20°C. Avoid repeated thawing (> 2 x) of a carrier RNA aliquot.
 - b) Use 1 µg carrier RNA per 100 µl lysis buffer. For instance, if the extraction protocol suggests 200 µl lysis buffer, please add 2 µl carrier RNA (1 µg/µl) directly into the lysis buffer. Before beginning of each extraction, a mixture of lysis buffer and carrier RNA (and *Internal Control*, where applicable, see **8.2 Internal Control**) should be prepared freshly according to the following pipetting scheme:

Number of samples	1	12
Lysis buffer	e.g. 200 µl	e.g. 2,400 µl
Carrier RNA (1 µg/µl)	2 µl	24 µl
Total Volume	202 µl	2,424 µl
Volume per extraction	200 µl	each 200 µl

- c) Please use the freshly prepared mixture of lysis buffer and carrier RNA instantly for extraction. Storage of the mixture is not possible.
- When using isolation protocols with **ethanol**-containing washing buffers, please carry out an additional centrifugation step (three minutes, 13,000 rpm) before the elution to remove any remaining ethanol. This prevents possible inhibition of PCR.
 - The *artus* M. tuberculosis LC PCR Kit should not be used with **phenol**-based isolation methods.

Important: The *Internal Control* of the *artus* M. tuberculosis LC PCR Kit can be used directly in the isolation procedure (see **8.2 Internal Control**).

8.2 Internal Control

An *Internal Control* (*M. tuberculosis* LC IC) is supplied. This allows the user **both to control the DNA isolation procedure and to check for possible PCR inhibition** (see Fig. 1). For this application, add the *Internal Control* to the isolation at a ratio of 0.1 µl per 1 µl elution volume. For example, using the QIAamp DNA Mini Kit, the DNA is eluted in 100 µl Buffer AE. Hence, 10 µl of the *Internal Control* should be added initially. The quantity of *Internal Control* used depends **only** on the elution volume. The *Internal Control* and carrier RNA (see **8.1 DNA Isolation**) should be added only

- to the mixture of lysis buffer and sample material or
- directly to the lysis buffer.

The *Internal Control* must not be added to the sample material directly. If added to the lysis buffer please note that the mixture of *Internal Control* and lysis buffer/carrier RNA has to be prepared freshly and used instantly (storage

of the mixture at room temperature or in the fridge for only a few hours may lead to *Internal Control* failure and a reduced extraction efficiency). Please do **not** add the *Internal Control* and the carrier RNA to the sample material directly.

The *Internal Control* can optionally be used **exclusively to check for possible PCR inhibition** (see Fig. 2). For this application, add 0.5 µl of the *Internal Control* and 2 µl *M. tuberculosis LC Mg-Sol* per reaction directly to 13 µl *M. tuberculosis LC Master*. For each PCR reaction use 15 µl of the Master Mix produced as described above* and add 5 µl of the purified sample. If you are preparing a PCR run for several samples please increase the volume of the *M. tuberculosis LC Master*, the *M. tuberculosis LC Mg-Sol* and the *Internal Control* according to the number of samples (see **8.4 Preparing the PCR**).

8.3 Quantitation

The enclosed *Quantitation Standards* (*M. tuberculosis LC QS 1 - 4*) are treated as previously purified samples and the same volume is used (5 µl). To generate a standard curve on the *LightCycler* Instrument, all four *Quantitation Standards* should be used as follows:

LightCycler 1.1/1.2/1.5 Instrument

Define the *M. tuberculosis LC QS 1 - 4* and defined in the *Sample Loading Screen* as standards with the specified concentrations (see *LightCycler Operator's Manual*, Version 3.5, Chapter B, 2.4. Sample Data Entry).

LightCycler 2.0 Instrument

In order to define the standards, please activate the function *Analysis Type* in the menu of the window *Samples* and select *Absolute Quantification*. You can now define the *M. tuberculosis LC QS 1 - 4* as standards and enter the corresponding concentrations for each standard (see *LightCycler Operator's Manual*, Version 4.0, Chapter 2.2 Entering Sample Information). Make sure

* The volume increase caused by adding the *Internal Control* is neglected when preparing the PCR assay. The sensitivity of the detection system is not impaired.

that the function *Enable Controls* is **not** activated. Otherwise the selection of analysis options for the data analysis is restricted (see **9.2 Data Analysis of the PCR Data on the *LightCycler 2.0* Instrument**).

The standard curve generated as above can also be used for subsequent runs, provided that at least one standard of **one** given concentration is used in the current run. For this purpose, the previously generated standard curve needs to be imported (see *LightCycler Operator's Manual*, Version 3.5, Chapter B, 4.2.5. Quantitation with an External Standard Curve or Version 4.0, Chapter 4.2.2 Saving a Standard Curve). However, this quantitation method may lead to deviations in the results due to variability between different PCR runs.

Attention: The *Quantitation Standards* are defined as copies/μl. The following equation has to be applied to convert the values determined using the standard curve into copies/ml of sample material:

$\text{Result (copies/ml)} = \frac{\text{Result (copies/}\mu\text{l)} \times \text{Elution Volume (}\mu\text{l)}}{\text{Sample Volume (ml)}}$

Please note that as a matter of principle the initial sample volume should be entered in the equation above. This has to be considered when the sample volume has been changed prior to the nucleic acid extraction (e.g. narrowing the volume by centrifugation or increase of volume by replenishment to the volume required for the isolation).

Important: A guideline for the quantitative analysis of *artus* systems on the *LightCycler 1.1/1.2/1.5* or *LightCycler 2.0* Instrument is provided at www.qiagen.com/Products/ByLabFocus/MDX (Technical Note for quantitation on the *LightCycler 1.1/1.2/1.5* or *LightCycler 2.0* Instrument).

8.4 Preparing the PCR

Make sure that the Cooling Block as well as the capillary adapters (accessories of the *LightCycler* Instrument) are pre-cooled to +4°C. Place the desired number of *LightCycler* capillaries into the adapters of the Cooling Block. Please make sure that at least one *Quantitation Standard* as well as one negative control (*Water, PCR grade*) are included per PCR run. To generate a standard curve, use all supplied *Quantitation Standards* (*M. tuberculosis LC QS 1 - 4*) for each PCR run. Before each use, all reagents need to be thawed completely, mixed (by repeated up and down pipetting or by quick vortexing) and centrifuged briefly.

If you want to use the *Internal Control to monitor the DNA isolation procedure and to check for possible PCR inhibition*, it has already been added to the isolation (see **8.2 Internal Control**). In this case, please use the following pipetting scheme (for a schematic overview see Fig. 1):

	Number of samples	1	12
1. Preparation of Master Mix	<i>M. tuberculosis LC Master</i>	13 µl	156 µl
	<i>M. tuberculosis LC Mg-Sol</i>	2 µl	24 µl
	<i>M. tuberculosis LC IC</i>	0 µl	0 µl
	Total Volume	15 µl	180 µl
2. Preparation of PCR assay	Master Mix	15 µl	15 µl each
	Sample	5 µl	5 µl each
	Total Volume	20 µl	20 µl each

If you want to use the *Internal Control exclusively to check for PCR inhibition*, it must be added directly to the *M. tuberculosis LC Master*. In this case, please use the following pipetting scheme (for a schematic overview see Fig. 2):

	Number of samples	1	12
1. Preparation of Master Mix	<i>M. tuberculosis</i> LC Master	13 µl	156 µl
	<i>M. tuberculosis</i> LC Mg-Sol	2 µl	24 µl
	<i>M. tuberculosis</i> LC IC	0.5 µl	6 µl
	Total Volume	15.5 µl*	186 µl*
2. Preparation of PCR assay	Master Mix	15 µl*	15 µl each*
	Sample	5 µl	5 µl each
	Total Volume	20 µl	20 µl each

Pipette 15 µl of the Master Mix into the plastic reservoir of each capillary. Then add 5 µl of the eluted sample DNA. Correspondingly, 5 µl of at least one of the *Quantitation Standards (M. tuberculosis* LC QS 1 - 4) must be used as a positive control and 5 µl of water (*Water, PCR grade*) as a negative control. Close the capillaries. To transfer the mixture from the plastic reservoir into the capillary, centrifuge the adapters containing the capillaries in a desktop centrifuge for ten seconds at a maximum of 400 x g (2,000 rpm).

* The volume increase caused by adding the *Internal Control* is neglected when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Addition of the *Internal Control* to the Purification Procedure

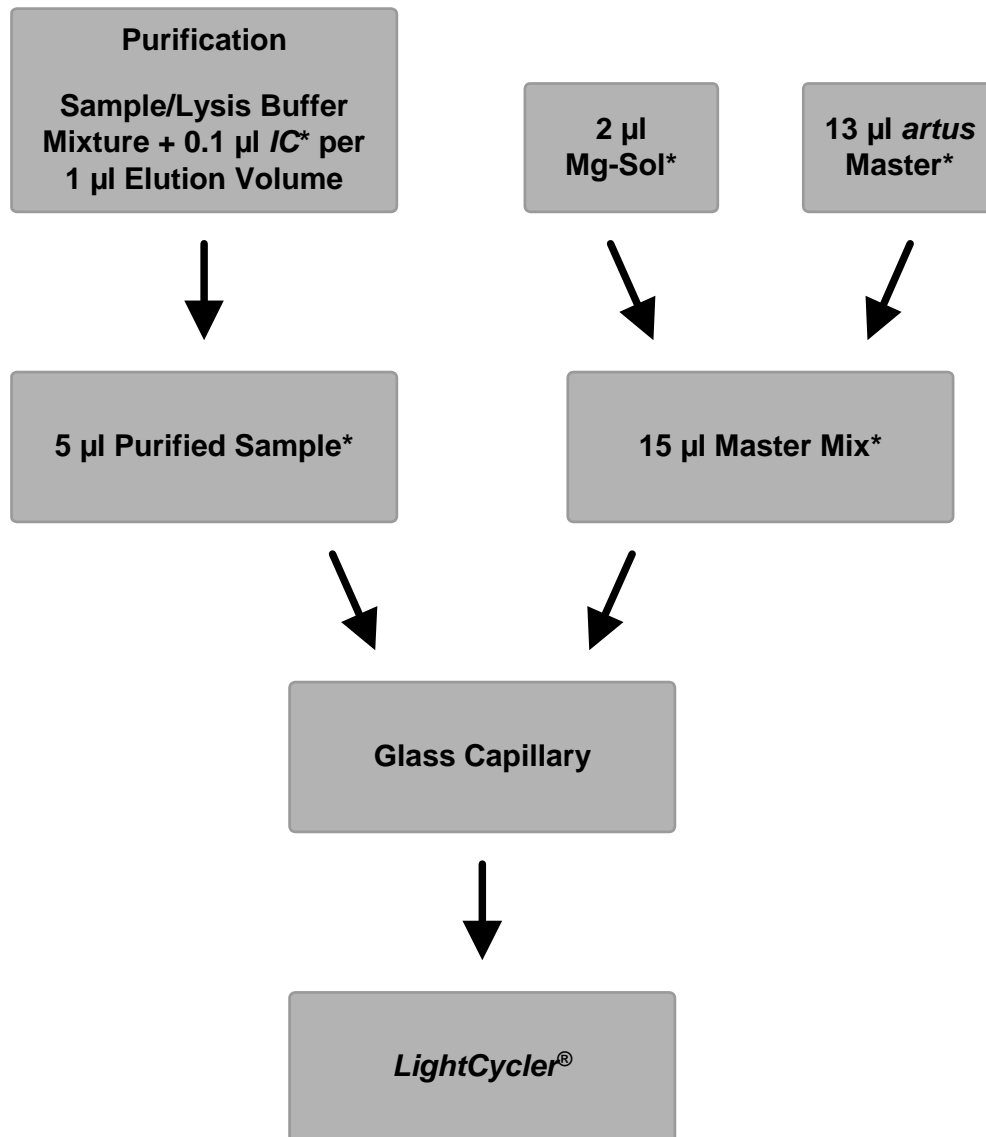


Fig. 1: Schematic workflow for the control of both the purification procedure and PCR inhibition.

* Please make sure that the solutions are thawed completely, mixed well and centrifuged briefly.

Addition of the *Internal Control* into the *artus* Master

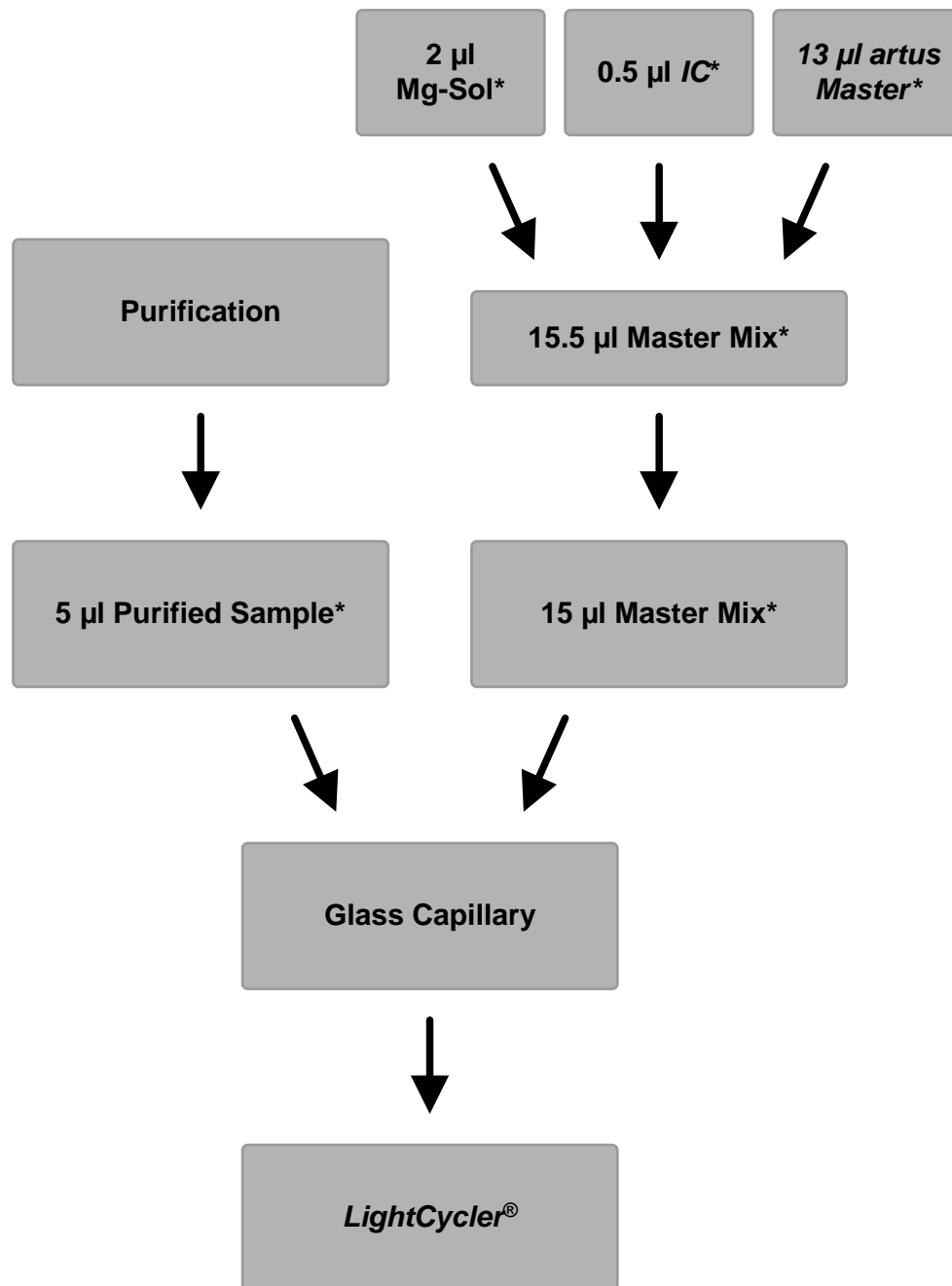


Fig. 2: Schematic workflow for the control of PCR inhibition.

* Please make sure that the solutions are thawed completely, mixed well and centrifuged briefly.

8.5 Programming of the *LightCycler* Instruments

8.5.1 Programming the *LightCycler* 1.1/1.2/1.5 Instrument

For the detection of *M. tuberculosis* complex DNA, create a temperature profile on your *LightCycler* 1.1/1.2/1.5 Instrument according to the following three steps (see Fig. 3 - 5).

- A. Initial Activation of the Hot Start Enzyme Fig. 3
- B. Amplification of the DNA Fig. 4
- C. Cooling Fig. 5

Pay particular attention to the settings for *Analysis Mode*, *Cycle Program Data* and *Temperature Targets*. In the illustrations these settings are framed in bold black. Please find further information on programming the *LightCycler* 1.1/1.2/1.5 Instrument in the *LightCycler Operator's Manual*.

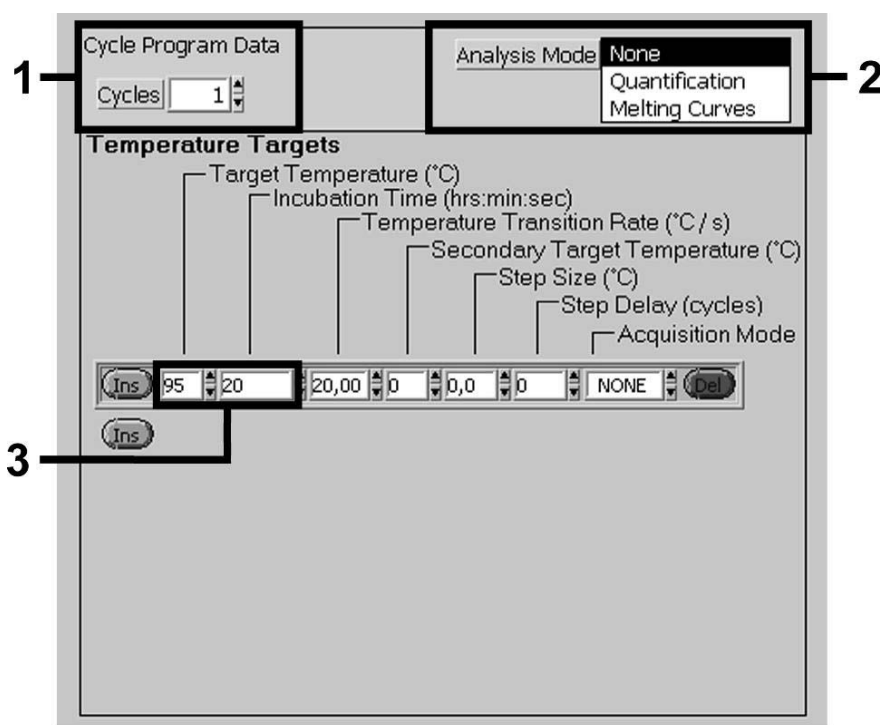


Fig. 3: Initial Activation of the Hot Start Enzyme.

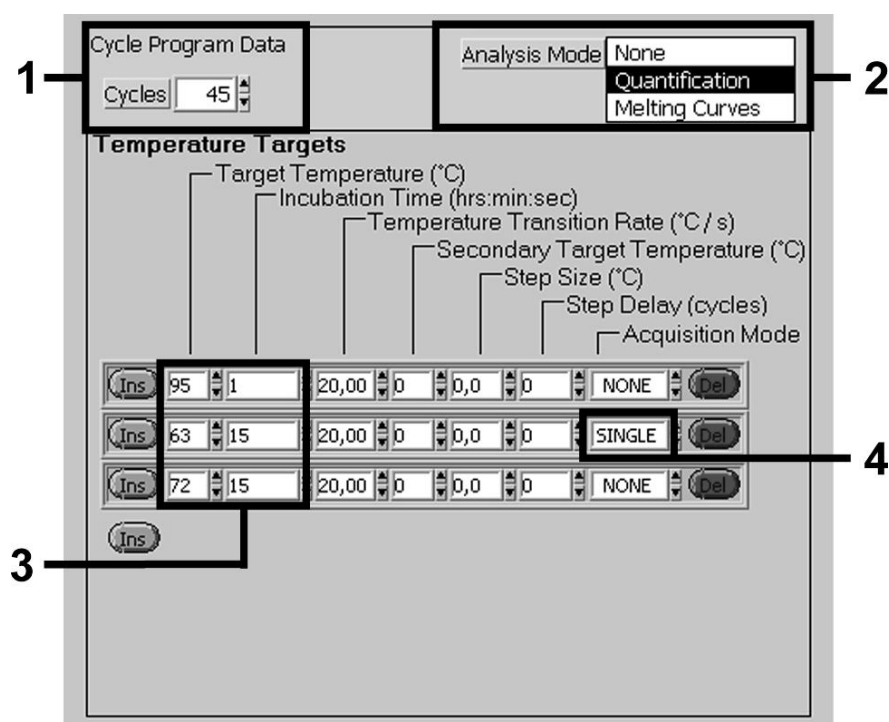


Fig. 4: Amplification of the DNA.

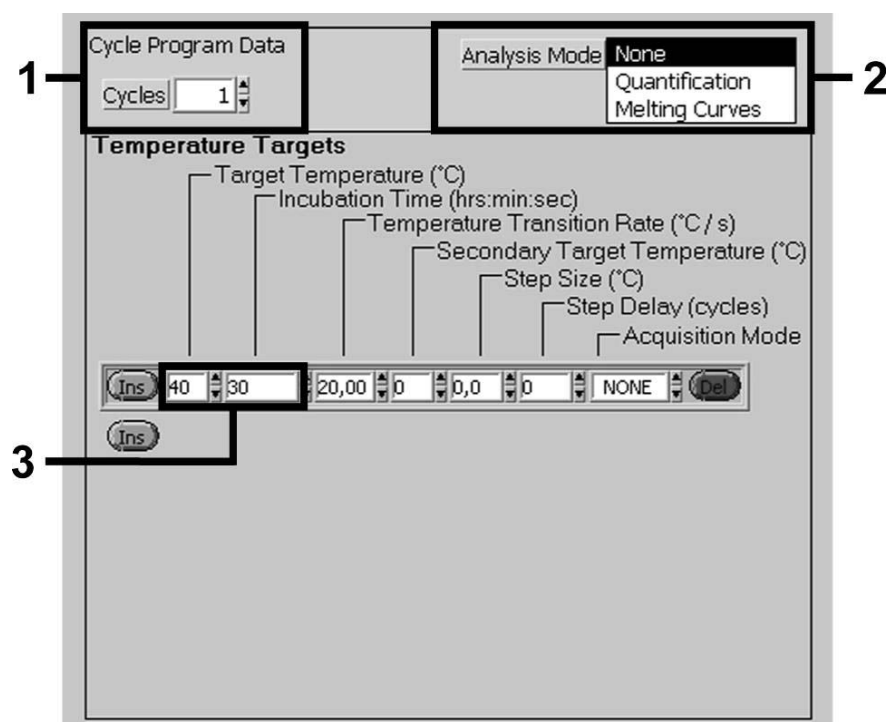


Fig. 5: Cooling.

8.5.2 Programming the *LightCycler 2.0* Instrument

To programme a PCR run with *LightCycler 2.0* Instrument please activate the option *New* in the main menu and select *LightCycler Experiment*.

Subsequently, for the detection of *M. tuberculosis* complex DNA, create a temperature profile on your *LightCycler 2.0* Instrument according to the following three steps (see Table 1).

- A. Initial Activation of the Hot Start Enzyme
- B. Amplification of the DNA
- C. Cooling

Make sure that you first enter the number of capillaries prepared for this PCR run (*Max. Seek Pos.*, see Fig. 6).

Table 1: Creating the temperature profile.

Program	Target [°C]	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Sec Target	Step Size [°C]	Step Delay [cycles]	Acq. Mode	Cycles	Analysis Mode
Activation	95	00:00:20	20	0	0	0	None	1	None
Amplification of the DNA	95	00:00:01	20	0	0	0	None	45	Quantification
	63	00:00:15	20	0	0	0	Single		
	72	00:00:15	20	0	0	0	None		
Cooling	40	00:00:30	20	0	0	0	None	1	None

To enter the sample specifications, please activate the button *Samples*.

- In the window *Capillary View* first enter the total number of planned PCR preparations for the PCR run (*Sample Count*).
- Then, you can assign names to the samples under *Sample Name*.
- Also select under *Selected Channels* the fluorescence channels 530 for the detection of the analytical *M. tuberculosis* complex PCR and 705 for the detection of the *Internal Control* PCR.
- To define the standards and to assign the corresponding concentrations, please select the option *Absolute Quantification* under *Analysis Type* (see **8.3 Quantitation**).
- Make sure that the function *Enable Controls* is **not** activated. Otherwise the selection of analysis options for the data analysis is restricted (the

mode *Fit Points* is not available, see **9.2 Data Analysis of the PCR Data on the *LightCycler 2.0* Instrument**). Under *Target Name* you can assign the target sequences to be detected (*M. tuberculosis* complex or *Internal Control*) in the selected fluorescence channels 530 and 705. The completion of the column *Target Name* can be facilitated with the function *Auto Copy...* To define the *Target Name* helps to get a better overview, but it is not strictly required for data analysis.

- To generate a standard curve for data analysis, the *Quantitation Standards* should be defined with their corresponding concentrations. Therefore, please select *Standard* under *Sample Type* and enter the corresponding concentration for each standard under *Concentration*.
- The programmed temperature profile can be stored on the computer's hard drive, to make use of it again for further runs. For this purpose, activate the function *Save As* under the menu *File*, upon which a new window appears. Please select under *Templates and Macros* the submenu *Run Templates* and save the data under an appropriate name.
- In order to start the PCR run, change to the field *Run* and activate the function *Start Run* (see Fig. 6). The PCR programme will start after entering the location, where the data should be saved.

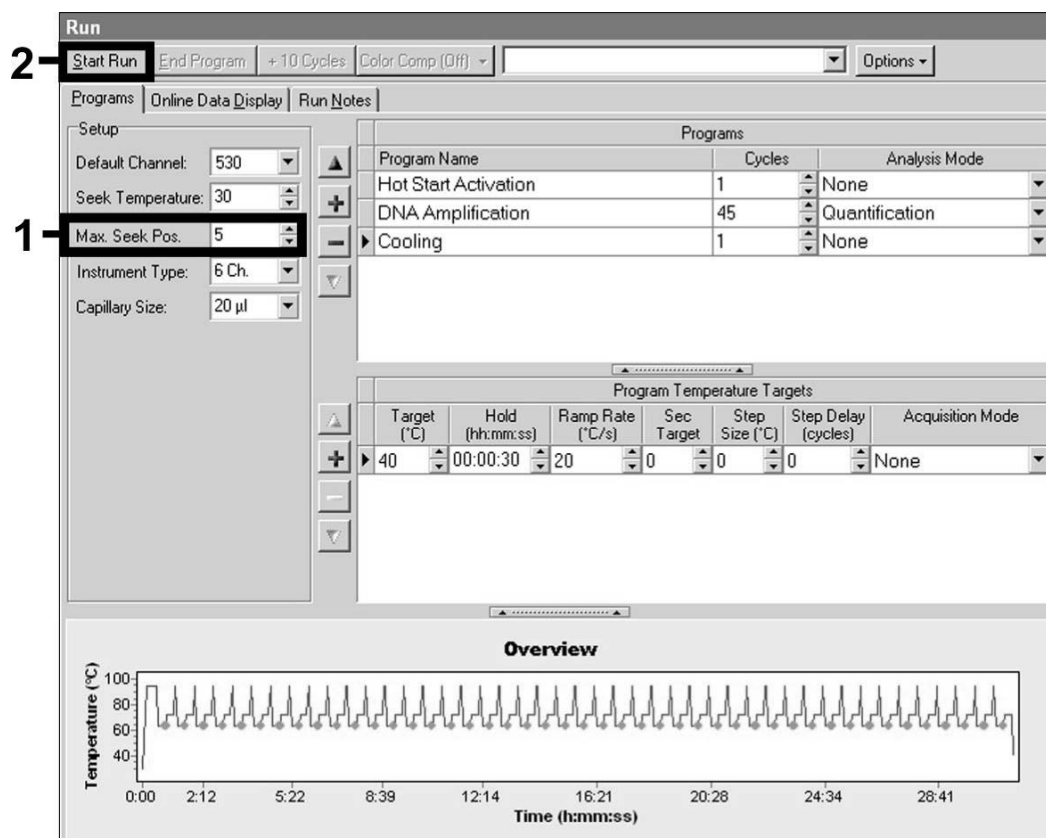


Fig. 6: Start of the PCR run.

9. Data Analysis

9.1 Data Analysis of the PCR Data on the *LightCycler*

1.1/1.2/1.5 Instrument

For the analysis of the PCR data collected with the *LightCycler* 1.1/1.2/1.5 Instrument, we recommend the use of the *LightCycler* Software Version 3.5.

In multicolor analyses interferences occur between fluorimeter channels. The *LightCycler* 1.1/1.2/1.5 Instrument's software contains a file termed *Color Compensation File*, which compensates for these interferences. Open this file before, during or after the PCR run by activating the *Choose CCC File* or the *Select CC Data* button respectively. If no *Color Compensation File* is installed, generate the file according to the instructions in the *LightCycler Operator's Manual*. After the *Color Compensation File* has been activated, separate signals appear in fluorimeter channels F1, F2 and F3. For analysis of the PCR results gained with the *artus M. tuberculosis LC PCR Kit* please select fluorescence display options F1 for the analytical *M. tuberculosis* complex PCR and F3/Back-F1 for the *Internal Control* PCR, respectively. For the analysis of quantitative runs, please follow the instructions given in **8.3 Quantitation** and in the **Technical Note for quantitation on the *LightCycler* 1.1/1.2/1.5 or *LightCycler* 2.0 Instrument** at www.qiagen.com/Products/ByLabFocus/MDX.

The following results are possible:

1. A signal is detected in fluorimeter channel F1.

The result of the analysis is positive: The sample contains DNA of one or more members of the *M. tuberculosis* complex.

In this case, the detection of a signal in the F3/Back-F1 channel is dispensable, since high initial concentrations of *M. tuberculosis* complex DNA (positive signal in the F1 channel) can lead to a reduced or absent fluorescence signal of the *Internal Control* in the F3/Back-F1 channel (competition).

2. In fluorimeter channel F1 no signal is detected. At the same time, a signal from the *Internal Control* appears in the F3/Back-F1 channel.

In the sample no DNA of members of the *M. tuberculosis* complex is detectable. It can be considered negative.

In the case of a negative *M. tuberculosis* complex PCR the detected signal of the *Internal Control* rules out the possibility of PCR inhibition.

3. No signal is detected in the F1 or in the F3/Back-F1 channel.

No result can be concluded.

Information regarding error sources and their solution can be found in **10. Troubleshooting.**

Examples of positive and negative PCR reactions are given in Fig. 7 and Fig. 8.

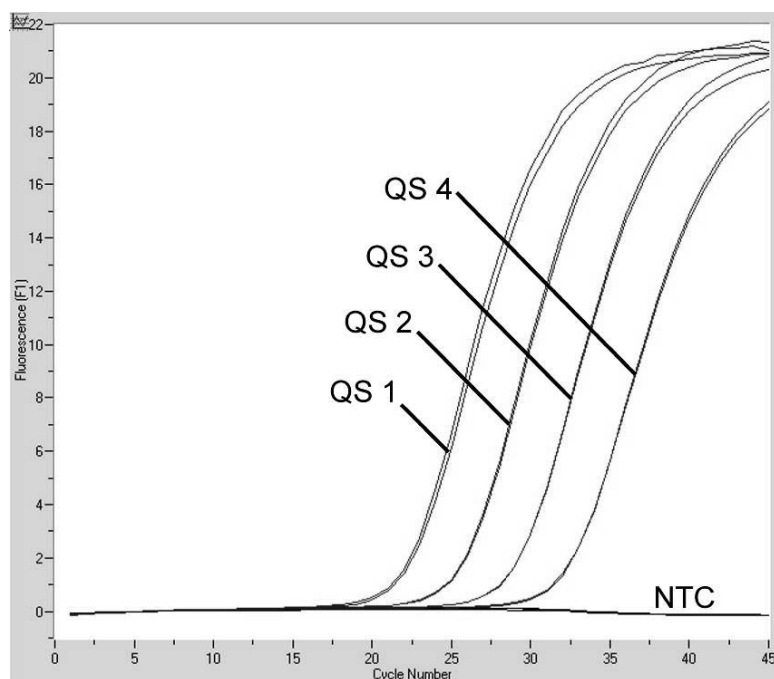


Fig. 7: Detection of the *Quantitation Standards* (*M. tuberculosis* LC QS 1 - 4) in fluorimeter channel F1 of the *LightCycler 1.1/1.2/1.5* Instrument. NTC: non-template control (negative control).

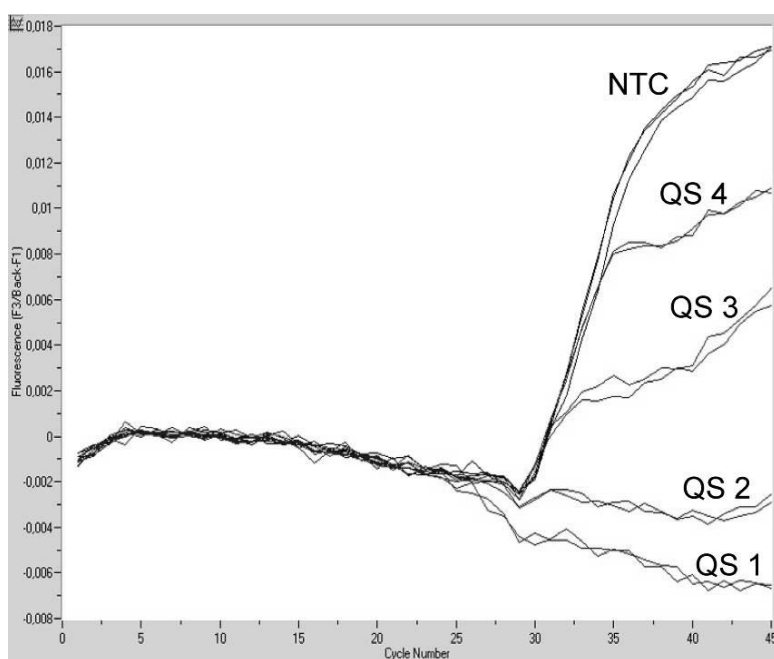


Fig. 8: Detection of the *Internal Control* (IC) in fluorimeter channel F3/Back-F1 of the *LightCycler 1.1/1.2/1.5* Instrument with simultaneous amplification of the *Quantitation Standards* (*M. tuberculosis* LC QS 1 - 4). NTC: non-template control (negative control).

9.2 Data Analysis of the PCR Data on the *LightCycler 2.0* Instrument

For the analysis of the PCR data collected with the *LightCycler 2.0* Instrument please use the *LightCycler* Software Version 4.0. Please consider the instructions given in the *LightCycler 2.0 Instrument Operator's Manual Version 4.0*.

For the analysis of PCR data please proceed as follows (see Fig. 9):

- Activate the function *Analysis* in the menu strip and select the option *Absolute Quantification*. As a matter of principle, all amplification data generated with the *artus* LC PCR Kit should be analyzed with this function.
- The *LightCycler* Software Version 4.0 contains a file termed *Color Compensation File*, which compensates multicolor analyses interferences between fluorescence channels. Open this file during or after the PCR run by activating the *Color Comp (On/Off)* and then the *Select Color Compensation* button (see Fig. 9). If no *Color Compensation File* is installed, generate the file according to the instructions given in the *LightCycler Operator's Manual*.
- After the *Color Compensation File* has been activated, separate signals appear in the fluorescence channels. For analysis of the PCR results gained with the *artus M. tuberculosis* LC PCR Kit please select fluorescence display options 530 for the analytical *M. tuberculosis* complex PCR and 705/Back 530 for the *Internal Control* PCR, respectively.

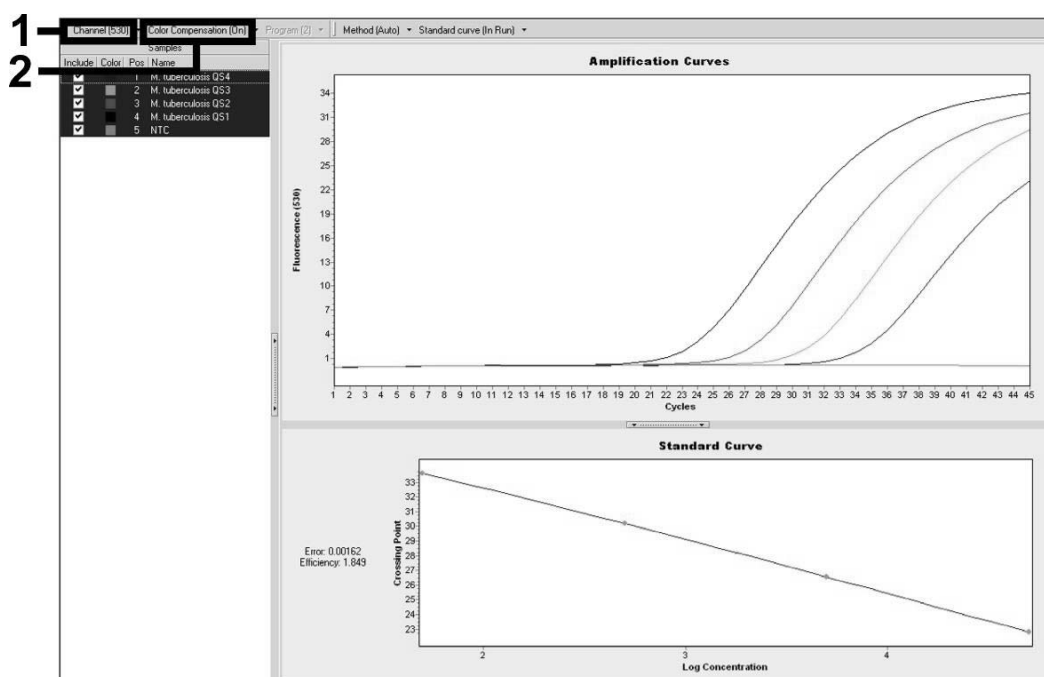


Fig. 9: Activation of the *Color Compensation File* and selection of the fluorescence channel.

For the analysis of quantitative runs, please follow the instructions given in **8.3 Quantitation** and in the **Technical Note for quantitation on the LightCycler 1.1/1.2/1.5 or LightCycler 2.0 Instrument** at www.qiagen.com/Products/ByLabFocus/MDX.

Once the setting of analysis options is completed, following results are possible:

1. A signal is detected in fluorescence channel 530.

The result of the analysis is positive: The sample contains DNA of one or more members of the *M. tuberculosis* complex.

In this case, the detection of a signal in the 705/Back 530 channel is dispensable, since high initial concentrations of *M. tuberculosis* DNA (positive signal in the 530 channel) can lead to a reduced or absent fluorescence signal of the *Internal Control* in the 705/Back 530 channel (competition).

2. In fluorescence channel 530 no signal is detected. At the same time, a signal from the *Internal Control* appears in the 705/Back 530 channel.

In the sample no DNA of members of the *M. tuberculosis* complex is detectable. It can be considered negative.

In the case of a negative *M. tuberculosis* complex PCR the detected signal of the *Internal Control* rules out the possibility of PCR inhibition.

3. No signal is detected in the 530 or in 705/Back 530 channel.

No result can be concluded.

Information regarding error sources and their solution can be found in **10. Troubleshooting.**

Examples of positive and negative PCR reactions are given in Fig. 10 and Fig. 11.

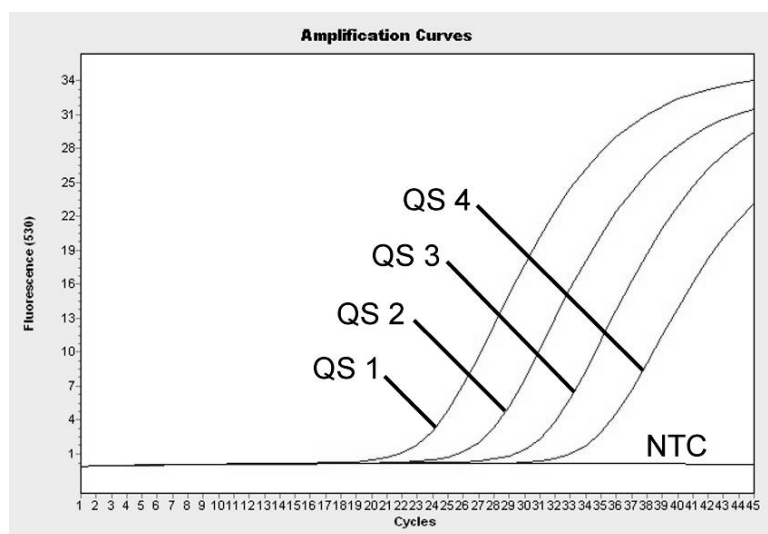


Fig. 10: Detection of the *Quantitation Standards* (*M. tuberculosis* LC QS 1 - 4) in fluorescence channel 530 of the *LightCycler 2.0* Instrument. NTC: non-template control (negative control).

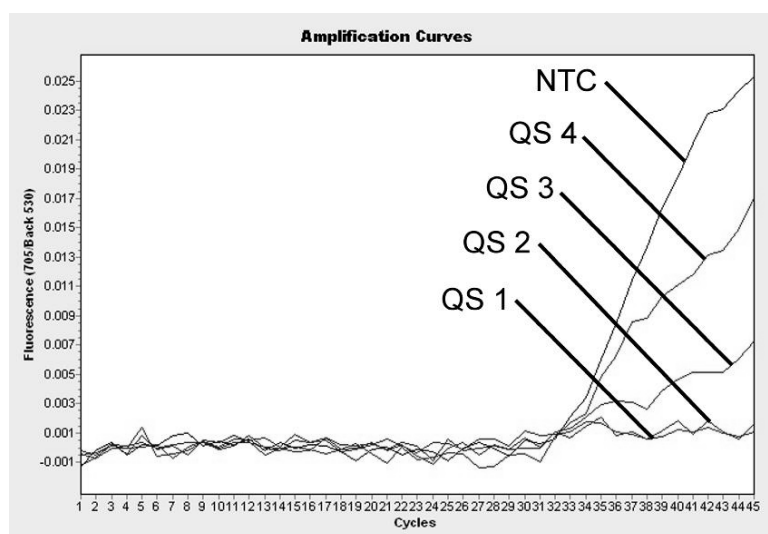


Fig. 11: Detection of the *Internal Control* (IC) in fluorescence channel 705/Back 530 of the *LightCycler 2.0* Instrument with simultaneous amplification of *Quantitation Standards* (*M. tuberculosis* LC QS 1 - 4). NTC: non-template control (negative control).

10. Troubleshooting

No signal with positive controls (*M. tuberculosis* LC QS 1 - 4) in fluorescence channel F1 or 530:

- The selected fluorescence channel for PCR data analysis does not comply with the protocol.
 - For data analysis select the fluorescence channel F1 or 530 for the analytical *M. tuberculosis* complex PCR and the fluorescence channel F3/Back-F1 or 705/Back 530 for the *Internal Control* PCR.
- Incorrect programming of the temperature profile of the *LightCycler* 1.1/1.2/1.5 or *LightCycler* 2.0 Instrument.
 - Compare the temperature profile with the protocol (see **8.5 Programming of the *LightCycler* Instruments**).
- Incorrect configuration of the PCR reaction.
 - Check your work steps by means of the pipetting scheme (see **8.4 Preparing the PCR**) and repeat the PCR, if necessary.
- The storage conditions for one or more kit components did not comply with the instructions given in **0**.
- Storage of the *artus* *M. tuberculosis* LC PCR Kit had expired.
 - Please check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.

Weak or no signal of the *Internal Control* in fluorescence channel F3/Back-F1 or 705/Back 530 and simultaneous absence of a signal in channel F1 or 530:

- The PCR conditions do not comply with the protocol.
 - Check the PCR conditions (see above) and repeat the PCR with corrected settings, if necessary.
- The PCR was inhibited.
 - Make sure that you use a recommended isolation method (see **8.1 DNA Isolation**) and stick closely to the manufacturer's instructions.

- Make sure that during the DNA isolation the recommended additional centrifugation step has been carried out before the elution in order to remove any residual ethanol (see **8.1 DNA Isolation**).
- DNA was lost during extraction.
 - If the *Internal Control* had been added to the extraction, an absent signal of the *Internal Control* can indicate the loss of DNA during the extraction. Make sure that you use a recommended isolation method (see **8.1 DNA Isolation**) and stick closely to the manufacturer's instructions.
- The storage conditions for one or more kit components did not comply with the instructions given in **0**.
- Storage of the *artus* M. tuberculosis LC PCR Kit had expired.
 - Please check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.

Signals with the negative controls in fluorescence channel F1 or 530 of the analytical PCR.

- A contamination occurred during preparation of the PCR.
 - Repeat the PCR with new reagents in replicates.
 - If possible, close the PCR tubes directly after addition of the sample to be tested.
 - Strictly pipette the positive controls at last.
 - Make sure that work space and instruments are decontaminated at regular intervals.
- A contamination occurred during extraction.
 - Repeat the extraction and PCR of the sample to be tested using new reagents.
 - Make sure that work space and instruments are decontaminated at regular intervals.

If you have any further questions or if you encounter problems, please contact our Technical Service.

11. Specifications

11.1 Analytical Sensitivity

In order to determine the analytical sensitivity of the *artus* M. tuberculosis LC PCR Kit, a standard dilution series has been set up from 4.3 to nominal 0.43 *M. tuberculosis* complex copy equivalents*/ μ l and analyzed on the *LightCycler* 1.1/1.2/1.5 Instrument in combination with the *artus* M. tuberculosis LC PCR Kit. Testing was carried out on three different days on eight replicates. The result was determined by a probit analysis. A graphical illustration of the probit analysis is shown in Fig. 12. The analytical detection limit of the *artus* M. tuberculosis LC PCR Kit in combination with the *LightCycler* 1.1/1.2/1.5 Instrument is consistently 1.6 copies/ μ l ($p = 0.05$). This means that there is a 95 % probability that 1.6 copies/ μ l will be detected.

Probit analysis: *Mycobacterium tuberculosis* (LightCycler 1.1/1.2/1.5)

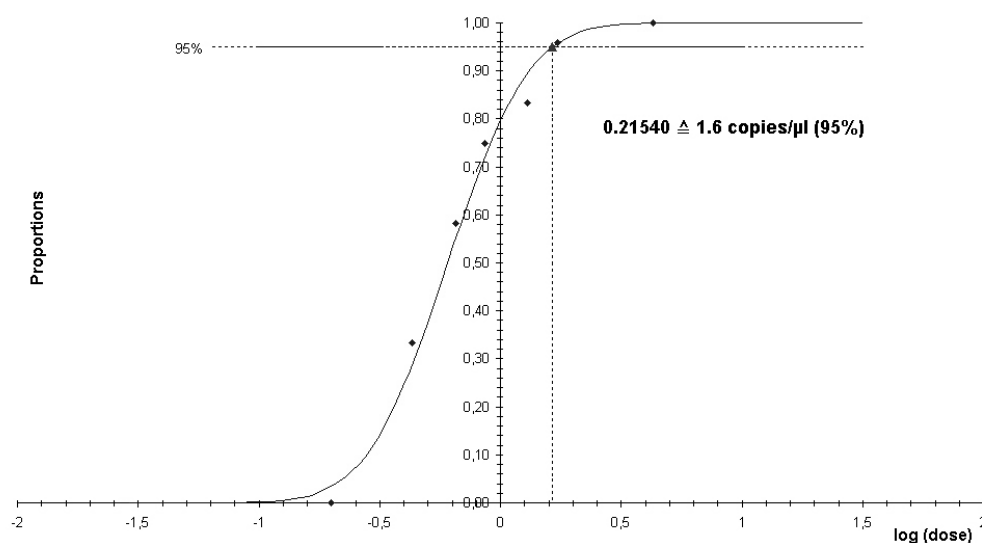


Fig. 12: Analytical sensitivity of the *artus* M. tuberculosis LC PCR Kit on the *LightCycler* 1.1/1.2/1.5 Instrument.

* The standard is a cloned PCR product, the concentration of which has been determined by absorption and fluorescence spectroscopy.

11.2 Specificity

The specificity of the *artus* M. tuberculosis LC PCR Kit is first and foremost ensured by the selection of the primers and probes, as well as the selection of stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The detectability of all members of the *M. tuberculosis* complex has thus been ensured.

Moreover, the specificity was verified with 90 different *M. tuberculosis* complex negative samples (30 sputum, 30 BAL and 30 bronchial secretion samples). These did not generate any signals with the *M. tuberculosis* complex specific primers and probes, which are included in the *M. tuberculosis* LC Master.

To determine the specificity of the *artus* M. tuberculosis LC PCR Kit the control group listed in the following table (see Table 2) has been tested for cross-reactivity. None of the tested pathogens has been reactive.

Table 2 (part I): Testing the specificity of the kit with potentially cross-reactive pathogens.

Control Group	<i>M. tuberculosis</i> (F1 or 530)	Internal Control (F3/Back-F1 or 705/Back 530)
<i>Actinomyces israelii</i>	-	+
<i>Aeromonas hydrophila</i>	-	+
<i>Bordetella pertussis</i>	-	+
<i>Candida albicans</i>	-	+
<i>Chlamydia trachomatis</i>	-	+
<i>Chlamydia pneumoniae</i>	-	+
<i>Citrobacter freundii</i>	-	+
<i>Corynebacterium diphtheriae</i>	-	+
<i>Corynebacterium jeikeium</i>	-	+
<i>Cryptococcus neoformans</i>	-	+
<i>Eikenella corrodens</i>	-	+
<i>Enterobacter aerogenes</i>	-	+
<i>Enterobacter cloacae</i>	-	+
<i>Enterococcus faecalis</i>	-	+
<i>Enterococcus faecium</i>	-	+
<i>Escherichia coli</i>	-	+
<i>Fusobacterium nucleatum ssp. polymorphum</i>	-	+
<i>Haemophilus influenzae</i>	-	+
<i>Haemophilus parainfluenzae</i>	-	+
<i>Klebsiella pneumoniae</i>	-	+
<i>Lactobacillus acidophilus</i>	-	+
<i>Mycobacterium avium ssp. avium</i>	-	+
<i>Mycobacterium celatum</i>	-	+
<i>Mycobacterium chelonae</i>	-	+
<i>Mycobacterium fortuitum</i>	-	+
<i>Mycobacterium gordonae</i>	-	+
<i>Mycobacterium intracellulare</i>	-	+
<i>Mycobacterium kansasii</i>	-	+
<i>Mycobacterium lentiflavum</i>	-	+
<i>Mycobacterium malmoeense</i>	-	+
<i>Mycobacterium marinum</i>	-	+
<i>Mycobacterium scrofulaceum</i>	-	+
<i>Mycobacterium szulgai</i>	-	+

Table 2 (part II): Testing the specificity of the kit with potentially cross-reactive pathogens.

Control group	<i>M. tuberculosis</i> (F1 or 530)	Internal Control (F3/Back-F1 or 705/Back 530)
<i>Mycobacterium ulcerans</i>	-	+
<i>Mycobacterium xenopi</i>	-	+
<i>Neisseria gonorrhoeae</i>	-	+
<i>Neisseria meningitidis</i>	-	+
<i>Nocardia asteroides</i>	-	+
<i>Nocardia brasiliensis</i>	-	+
<i>Nocardia farcinia</i>	-	+
<i>Nocardia otitidiscaviarum</i>	-	+
<i>Peptostreptococcus productus</i>	-	+
<i>Porphyromonas gingivalis</i>	-	+
<i>Prevotella denticola</i>	-	+
<i>Propionibacterium acnes</i>	-	+
<i>Pseudomonas aeruginosa</i>	-	+
<i>Salmonella enteritidis</i>	-	+
<i>Salmonella typhi</i>	-	+
<i>Staphylococcus aureus</i>	-	+
<i>Staphylococcus epidermidis</i>	-	+
<i>Streptococcus agalactiae</i>	-	+
<i>Streptococcus pyogenes</i>	-	+
<i>Streptococcus mutans</i>	-	+
<i>Streptococcus pneumoniae</i>	-	+
<i>Streptomyces venezuelae</i>	-	+
<i>Veillonella parvula</i>	-	+
<i>Xanthomonas maltophilia</i>	-	+

11.3 Precision

The precision data of the *artus* M. tuberculosis LC PCR Kit have been collected by means of the *LightCycler 1.1/1.2/1.5* Instrument and allow the determination of the total variance of the assay. The total variance consists of the **intra-assay variability** (variability of multiple results of samples of the same concentration within one experiment), the **inter-assay variability** (variability of multiple results of the assay generated on different instruments of the same type by different operators within one laboratory) and the **inter-batch variability** (variability of multiple results of the assay using various batches). The data obtained were used to determine the standard deviation, the variance and the coefficient of variation for the pathogen specific and the *Internal Control* PCR.

Precision data of the *artus* M. tuberculosis LC PCR Kit have been collected using the *Quantitation Standard* of the lowest concentration (QS 4; 50 copies/µl). Testing was performed with eight replicates. The precision data were calculated on basis of the Ct values of the amplification curves (Ct: threshold cycle, see Table 3). In addition, precision data for quantitative results in copies/µl were determined using the corresponding Ct values (see Table 4). Based on these results, the overall statistical spread of any given sample with the mentioned concentration is 1.58 % (Ct) or 12.58 % (conc.), for the detection of the *Internal Control* 1.47 % (Ct). These values are based on the totality of all single values of the determined variabilities.

Table 3: Precision data on basis of the Ct values.

	Standard Deviation	Variance	Coefficient of Variation [%]
Intra-assay variability: <i>M. tuberculosis</i> LC QS 4	0.18	0.03	0.56
Intra-assay variability: <i>Internal Control</i>	0.17	0.03	0.58
Inter-assay variability: <i>M. tuberculosis</i> LC QS 4	0.45	0.20	1.39
Inter-assay variability: <i>Internal Control</i>	0.36	0.13	1.23
Inter-batch variability: <i>M. tuberculosis</i> LC QS 4	0.45	0.20	1.37
Inter-batch variability: <i>Internal Control</i>	0.49	0.24	1.68
Total variance: <i>M. tuberculosis</i> LC QS 4	0.51	0.26	1.58
Total variance: <i>Internal Control</i>	0.43	0.18	1.47

Table 4: Precision data on basis of the quantitative results (in copies/µl).

	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay variability: <i>M. tuberculosis</i> LC QS 4	6.00	36.06	11.93
Inter-assay variability: <i>M. tuberculosis</i> LC QS 4	6.77	45.82	13.42
Inter-batch variability: <i>M. tuberculosis</i> LC QS 4	6.26	39.20	12.43
Total variance: <i>M. tuberculosis</i> LC QS 4	6.34	40.14	12.58

11.4 Robustness

The verification of the robustness allows the determination of the total failure rate of the *artus* M. tuberculosis LC PCR Kit. 30 *M. tuberculosis* complex negative samples of each sputum, BAL and bronchial secretion were spiked with 3 copies/μl elution volume of *M. tuberculosis* control DNA (approx. threefold concentration of the analytical sensitivity limit). After extraction using the QIAamp DNA Mini Kit (see **8.1 DNA Isolation**) these samples were analyzed with the *artus* M. tuberculosis LC PCR Kit. For all *M. tuberculosis* samples the failure rate was 0 %. In addition, the robustness of the *Internal Control* was assessed by purification and analysis of *M. tuberculosis* complex negative sputum, BAL and bronchial secretion samples (30 each). The total failure rate was 0 %. Inhibitions were not observed.

12. Product Use Limitations

- The *artus* M. tuberculosis LC PCR Kit is for research use only. Not for use in diagnostic procedures.
- No claim or representation is intended for their use for a specific clinical use (diagnostic, prognostic, or therapeutic).
- Strict compliance with the user manual is required for optimal PCR results.
- Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

13. Safety Information

For safety information of the *artus* M. tuberculosis LC PCR Kit, please consult the appropriate material safety data sheet (MSDS). The MSDS are available online in convenient and compact PDF format at www.qiagen.com/support/msds.aspx.

14. Quality Control

In accordance with QIAGEN's ISO 9001 and ISO 13485-certified Total Quality Management System, each lot of *artus* M. tuberculosis LC PCR Kit is tested against predetermined specifications to ensure consistent product quality.

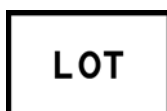
15. References

Mackay IM. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. 2004; 10 (3): 190 - 212.

16. Explanation of Symbols



Use by



Batch code



Manufacturer



Catalogue number



Material number



Handbook



Components



Contains



Number



<N>

Contains sufficient for <N> tests



Temperature limitation



Refer to information given in the handbook

QS

Quantitation Standard

IC

Internal Control

Mg-Sol *Magnesium Solution*

www.qiagen.com

Australia ■ Orders 03-9840-9800 ■ Fax 03-9840-9888 ■ Technical 1-800-243-066

Austria ■ Orders 0800/28-10-10 ■ Fax 0800/28-10-19 ■ Technical 0800/28-10-11

Belgium ■ Orders 0800-79612 ■ Fax 0800-79611 ■ Technical 0800-79556

Brazil ■ Orders 0800-557779 ■ Fax 55-11-5079-4001 ■ Technical 0800-557779

Canada ■ Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

China ■ Orders 021-3865-3865 ■ Fax 021-3865-3965 ■ Technical 800-988-0325

Denmark ■ Orders 80-885945 ■ Fax 80-885944 ■ Technical 80-885942

Finland ■ Orders 0800-914416 ■ Fax 0800-914415 ■ Technical 0800-914413

France ■ Orders 01-60-920-926 ■ Fax 01-60-920-925 ■ Technical 01-60-920-930 ■ Offers 01-60-920-928

Germany ■ Orders 02103-29-12000 ■ Fax 02103-29-22000 ■ Technical 02103-29-12400

Hong Kong ■ Orders 800 933 965 ■ Fax 800 930 439 ■ Technical 800 930 425

Ireland ■ Orders 1800-555-049 ■ Fax 1800-555-048 ■ Technical 1800-555-061

Italy ■ Orders 02-33430411 ■ Fax 02-33430426 ■ Technical 800-787980

Japan ■ Telephone 03-5547-0811 ■ Fax 03-5547-0818 ■ Technical 03-5547-0811

Korea (South) ■ Orders 1544 7145 ■ Fax 1544 7146 ■ Technical 1544 7145

Luxembourg ■ Orders 8002-2076 ■ Fax 8002-2073 ■ Technical 8002-2067

Mexico ■ Orders 01-800-7742-639 ■ Fax 01-800-1122-330 ■ Technical 01-800-7742-639

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