October 2015

artus® Mycobac. diff. LC PCR Kit Handbook

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Σ 96

Version 1

Quantitative in vitro diagnostics

For use with the LightCycler® 1.1/1.2/1.5 and LightCycler 2.0 instruments

IVD

CE

REF 4556063 (24 reactions)
4556065 (96 reactions)

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Sample to Insight

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Contents

Summary and Explanation 4

Principle of the Procedure 4

Materials Provided 6

- Kit contents 6

Materials Required but Not Provided 7

Warnings and Precautions 8

- Warnings 8

Reagent Storage and Handling 8

Procedure 9

- Important points before starting 9
- DNA isolation 10
- Internal Control 12
- Quantitation 12
- Preparing the PCR 13
- Programming of the LightCycler instruments 16
  - Programming the LightCycler 1.1/1.2/1.5 instrument 16
  - Programming the LightCycler 2.0 instrument 19

Interpretation of Results 22

- Data analysis of the PCR data on the LightCycler 1.1/1.2/1.5 instrument 22
- Data analysis of the PCR data on the LightCycler 2.0 instrument 25
- Troubleshooting 29

Quality Control 32

Limitations 32

Performance Characteristics 33

- Analytical sensitivity 33
- Specificity 34
- Precision 37
- Robustness 39
- Reproducibility 39

References 40
Symbols 40

Summary and Explanation

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 TB. The incidence of TB worldwide is about eight million and about three million people die each year. Even though developing countries are particularly affected, TB is a reemerging disease in industrialized nations, mainly due to the immigration of infected people and the development of drug-resistant TB. Minorities, such as the homeless, drug users and immunocompromised persons are affected disproportionately.

TB is a chronic, cyclic disease, mainly affecting the lung and the associated lymph nodes. However, depending on the immune status of the patient, the *M. tuberculosis* bacteria can also colonize other organs. TB is primarily transmitted from person to person via aerosols. Only people with active disease are contagious. Especially in immunosuppressed people, *M. tuberculosis* bacteria can be reactivated (recrudescent) even years after the initial infection.

Mycobacterium avium and *Mycobacterium intracellulare* are environmental bacteria which can be found in water and in soil. In contrast to *M. tuberculosis* infections, humans can be infected by *M. avium* and *M. intracellulare* via contaminated drinking water or food. Transmission from person to person is very unlikely. In immune competent individuals, infections with *M. avium* and *M. intracellulare* are usually asymptomatic, whereas immunocompromised persons, especially in HIV-infected patients, may be challenged with a massive dissemination of the bacteria over the entire body, which is usually fatal.

Principle of the Procedure

Pathogen diagnosis 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 reopen the reaction tubes after the PCR run (1).

The *artus* Mycobac. diff. 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. pinnipedii*) as well as members of the *M. avium* complex (*M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominissuis* and *M. intracellulare*) using polymerase chain reaction (PCR) in the

LightCycler instrument. The Mycobac. diff. LC Master contains reagents and enzymes for the specific amplification of a 163 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 instruments. In addition, the *artus* Mycobac. diff. LC PCR Kit contains a second heterologous amplification system to identify possible PCR inhibition.

	Selection of the fluorescence channels	
PCR product	**LightCycler 1.1/1.2/1.5 Instrument**	**LightCycler 2.0 Instrument**
M. tuberculosis/M. avium complex	**F2/Back-F1**	**640/Back 530**
Mycobac. diff 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/M. avium* complex PCR (see "Analytical sensitivity," page 33).

To distinguish the *M. tuberculosis* complex from the different *M. avium* subspecies and from *M. intracellulare*, the system utilizes the specific melting temperatures of the probes. During the melting curve step, a signal is detected in fluorescence channel **F2** or **640** for the members of the *M. tuberculosis* complex at 60°C, for all *M. avium* subspecies at 63.5°C and for *M. intracellulare* at 55°C. Variations between LightCycler instruments may cause deviations of melting points by 1–2°C. However, this deviation will be the same for all 3 melting points. Various extraction conditions and buffers can result in melting points slightly different from those of the controls supplied. The PCR should be repeated if the deviation between the melting point of the analyzed sample and the control is more than 1°C.

External positive controls (M. avium LC Control, M. intracellulare LC Control) are supplied for the detection of all *M. avium* subspecies and *M. intracellulare*. Additionally, the kit contains M. tuberculosis quantitation standards (M. tuberculosis LC QS 1–4) which allow the determination of the *M. tuberculosis* complex pathogen load. For further information, refer to "Quantitation," page 12.

Materials Provided

Kit contents

***artus* Mycobac. diff. LC PCR Kit**				
Catalog number			**4556063**	**4556065**
Number of reactions			**24**	**96**
Cap color	**Reagent name**	**Symbol**	**Amount**	**Amount**
Blue	Mycobac. diff. LC Master		2 x 12 reactions	8 x 12 reactions
Yellow	Mycobac. diff. LC Mg-Sol*	**Mg-Sol**	1 x 400 µl	1 x 400 µl
Red	M. tuberculosis LC QS† 1 (5×10^4 copy/µl)	**QS**	1 x 200 µl	1 x 200 µl
Red	M. tuberculosis LC QS 2 (5×10^3 copy/µl)	**QS**	1 x 200 µl	1 x 200 µl
Red	M. tuberculosis LC QS 3 (5×10^2 copy/µl)	**QS**	1 x 200 µl	1 x 200 µl
Red	M. tuberculosis LC QS 4 (5×10^1 copy/µl)	**QS**	1 x 200 µl	1 x 200 µl
Red	M. avium LC Control		1 x 200 µl	1 x 200 µl
Red	M. intracellulare LC Control		1 x 200 µl	1 x 200 µl
Green	Mycobac. diff LC IC‡	**IC**	1 x 1000 µl	2 x 1000 µl
White	Water (PCR grade)		1 x 1000 µl	1 x 1000 µl

* Mg-Sol: Magnesium solution.

† QS: Quantitation Standard

‡: IC: Internal Control

Materials Required but Not Provided

Important: Make sure that the instruments used in these procedures have been checked and calibrated according to the manufacturer's recommendations.

- Disposable powder-free gloves
- DNA isolation kit (see "DNA isolation," page 10)
- Lysozyme mix (see "DNA isolation," page 10)
- Pipets (adjustable)
- Sterile pipet tips with filters
- Vortex mixer
- Heating block capable of heating from 37°C to 95°C
- Desktop centrifuge with rotor for 2 ml reaction tubes
- Color Compensation Set (Roche Life Science, cat. no. 12 158 850 001) 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 (Roche Life Science, cat. no. 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

Warnings and Precautions

The user should always pay attention to the following:

- Use sterile pipet 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.

Warnings

For safety information of the *artus* Mycobac. diff. LC PCR Kit, please consult the appropriate safety data sheets (SDSs). The SDSs are available online in PDF format at **www.qiagen.com/safety**.

Reagent Storage and Handling

Store components of the *artus* Mycobac. diff. LC PCR Kit at –15 to –30°C. The components are stable until the expiration date stated on the label. Do not thaw and freeze the components more than twice as this may reduce assay performance. If the reagents are to be used only intermittently, aliquot the reagents and store. Do not have components at 4°C for more than 5 hours at a time.

Procedure

Important points before starting

- 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, 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).
- 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 (> 2x) of a carrier RNA aliquot.
- Use 1 µg carrier RNA per 100 µl lysis buffer. For instance, if the extraction protocol suggests 200 µl lysis buffer, add 2 µl carrier RNA (1 µg/µl) directly into the lysis buffer. Before beginning each extraction, a mixture of lysis buffer, carrier RNA and internal control (see "Internal Control," page 12) should be prepared freshly according to the following pipetting scheme:

	Number of samples	
Reagent	**1**	**12**
Lysis buffer	e.g., 200 µl	e.g., 2400 µl
Carrier RNA (1µg/µl)	2 µl	24 µl
Mycobac. diff. LC IC	10 µl	120 µl
Total volume	**212 µl**	**2544 µl**
Volume per extraction	**200 µl**	**each 200 µl**

- 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, carry out an additional centrifugation step (3 minutes, 13,000 rpm) before the elution step to remove any remaining ethanol. This prevents possible inhibition of PCR.
- The *artus* Mycobac. diff. LC PCR Kit should not be used with phenol-based isolation methods.
- **Important**: The internal control of the *artus* Mycobac. diff. LC PCR Kit is used directly in the isolation procedure (see "Internal Control," page 12).

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. Carry out the DNA isolation according to the manufacturer's instructions. QIAGEN recommends the following isolation kit:

Sample material	Nucleic acid isolation kit	Catalog number	Manufacturer	Carrier RNA
Sputum, BAL, bronchial secretion, CSF, stomach fluid, peritoneal punction	QIAamp® DNA Mini Kit (50)	51304	QIAGEN	Not included

Important: 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, we highly recommend the following amendments of the QIAamp DNA Mini Kit supplementary protocol.

Always pay attention to the following to prevent cross-contamination during 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 lid.
- Do not touch the inside of the tube lid. If you do touch it, change the potentially contaminated glove immediately.
- The use of a water bath is 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.

Always pay attention to the following to prevent cross-contamination during the DNA isolation procedure:

- 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 pipet 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 use.

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.

1. Transfer between 250 µl and 500 µl of the NALC-NaOH–decontaminated sample into a 1.5 ml screw cap tube.
2. Centrifuge for 10 minutes at 17,000 x *g* (13,000 rpm) in a desktop centrifuge.
3. Carefully discard the supernatant by pipetting.
4. 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.
5. Incubate for at least 1 hour at 37°C in a heating block.
6. Centrifuge briefly to remove drops from the inside of the lid.
7. 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 10 µl internal control (see “Internal Control, “ page 12).
8. Mix well by vortexing.
9. Incubate for 30 minutes at 56°C in a heating block.
10. Centrifuge briefly to remove drops from the inside of the lid.
11. Incubate 15 minutes at 95°C.
    Important: Incubation time should not be exceeded as this may cause DNA degradation.
12. **Note**: After completion of the incubation at 95°C the samples are no longer infectious. Cool the sample to room temperature.
13. 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* (Third Edition, June 2012) starting with the addition of ethanol at step 6, and perform the final DNA elution using 100 µl Buffer AE.

Internal Control

An internal control (Mycobac. diff. LC IC) is supplied. This allows the user both to control the DNA isolation procedure and to check for possible PCR inhibition (see Figure 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 volume of the internal control is dependent on the elution volume. The use of 10 µl is only valid for an elution volume of 100 µl (0.1 µl per 1 µl elution volume). The internal control and carrier RNA (see "DNA isolation," page 10) 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, 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 at 4°C for only a few hours may lead to internal control failure and reduced extraction efficiency. Do not add the internal control and the carrier RNA to the sample material directly.

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 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

  To define the standards, 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 that the function **Enable Controls** is not activated. Otherwise the selection of analysis options for the data analysis is restricted (see "Data analysis of the PCR data on the LightCycler 2.0 instrument," page 25).

The standard curve generated as above can also be used for subsequent runs, provided that at least 1 standard of 1 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.

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:

$$\text{Results (copies/ml)} = \frac{\text{Result (copies/µl)} \times \text{Elution volume (µl)}}{\text{Sample volume (ml)}}$$

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 instruments is provided at the following:

Technical Note: Quantitation of pathogen copy numbers using LightCycler 1.1/1.2/1.5 instrument.

www.qiagen.com/gb/resources/resourceLC1

Quantitation of pathogen copy numbers using CE-IVD-marked artus *LC PCR Kits and the LightCycler 2.0 Instrument.*

www.qiagen.com/gb/resources/resourceLC2

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. Make sure that at least one quantitation standard and/or positive control (M. avium LC Control, M. intracellulare LC Control) as well as one negative control (Water, PCR grade) is 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.

To monitor the DNA isolation procedure and to check for possible PCR inhibition, the internal control has already been added to the isolation (see "Internal Control," page 12).

Use the following pipetting scheme (for a schematic overview, see Figure 1):

		Number of samples	
		1	**12**
Preparation of Master Mix	Mycobac diff. LC Master	13 µl	156 µl
	Mycobac. diff. LC Mg-Sol	2 µl	24 µl
	Total volume	**15 µl**	**180 µl**
Preparation of PCR assay	Master Mix	15 µl	15 µl each
	Sample	5 µl	5 µl each
	Total volume	**20 µl**	**20 µl each**

1. Pipet 15 µl of the Master Mix into the plastic reservoir of each capillary.
2. 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) or positive controls (M. avium LC Control, M. intracellulare LC Control) must be used as a positive control and 5 µl of water (Water, PCR grade) as a negative control.
3. Close the capillaries.
4. To transfer the mixture from the plastic reservoir into the capillary, centrifuge the adapters containing the capillaries in a desktop centrifuge for 10 seconds at a maximum of 400 x *g* (2,000 rpm).

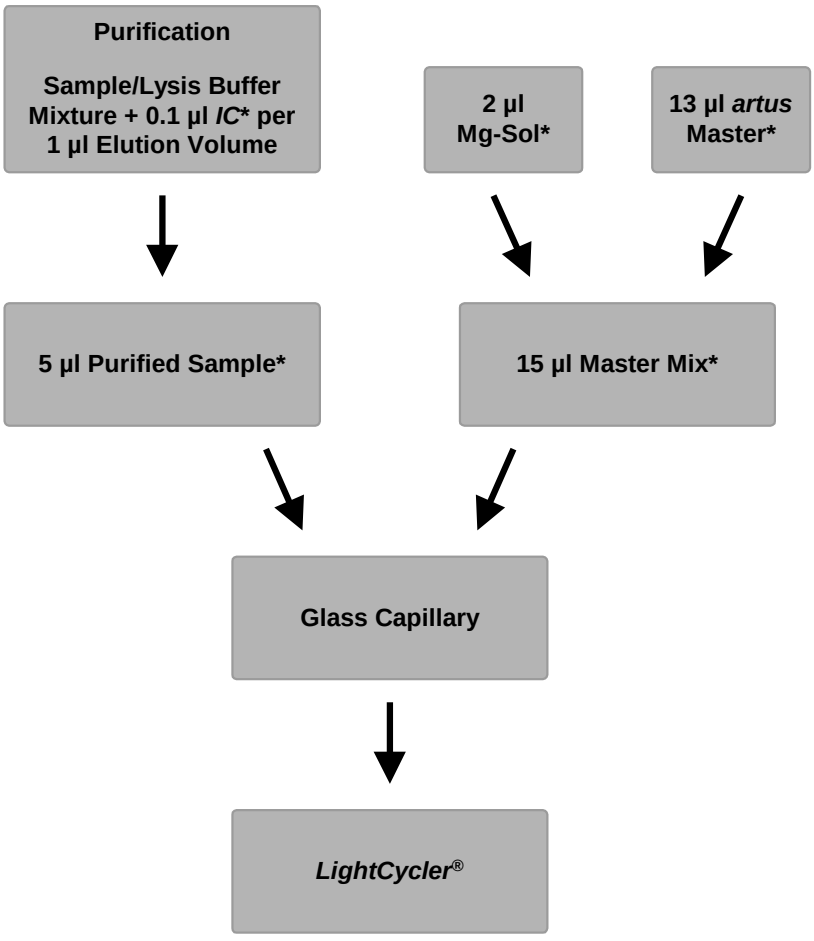

Figure 1. Schematic workflow for the control of both the purification procedure and PCR inhibition.

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

Programming of the LightCycler instruments

Programming the LightCycler 1.1/1.2/1.5 instrument

For the detection of DNA of the members of the *M. tuberculosis* complex and the *M. avium* complex, create a temperature profile on your LightCycler 1.1/1.2/1.5 instrument according to the following 5 steps (see Figures 2–6).

Initial activation of the Hot Start enzyme	**Figure 2**
Touch down step	**Figure 3**
Amplification of the DNA	**Figure 4**
Melting curve	**Figure 5**
Cooling	**Figure 6**

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. Find further information on programming the LightCycler 1.1/1.2/1.5 instrument in the *LightCycler Operator's Manual*.

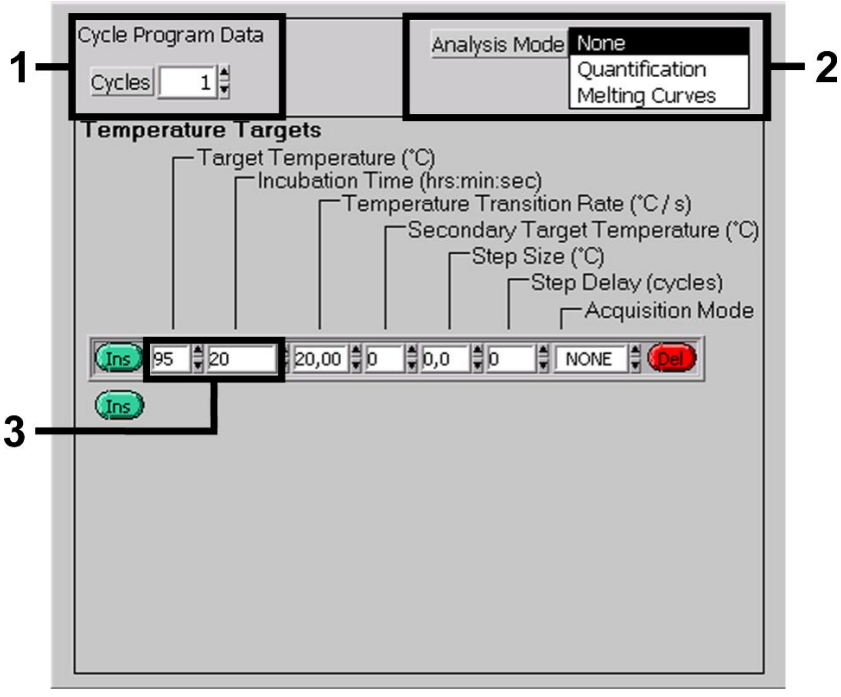

Figure 2. Initial activation of the Hot Start enzyme.

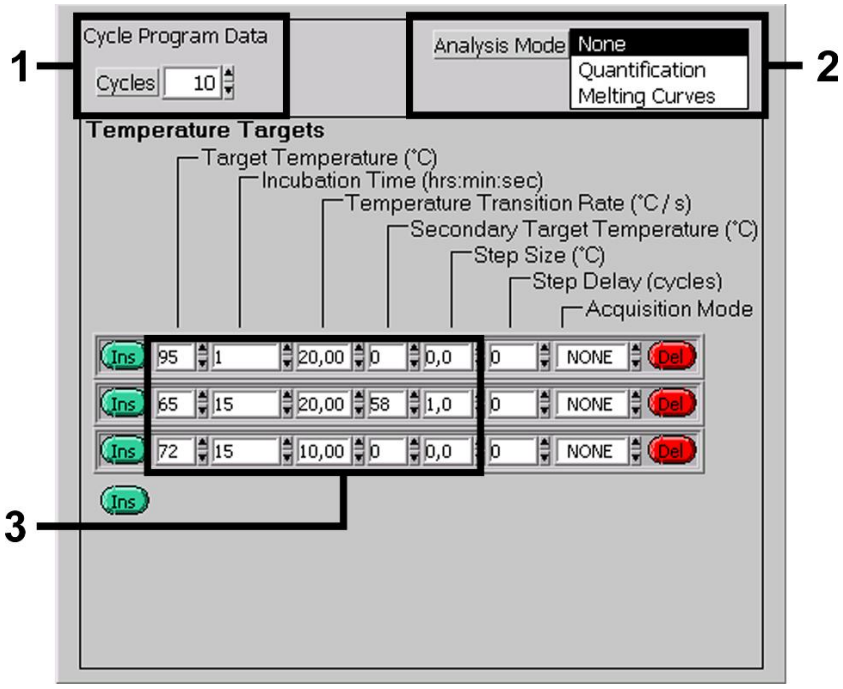

Figure 3. Touch down step.

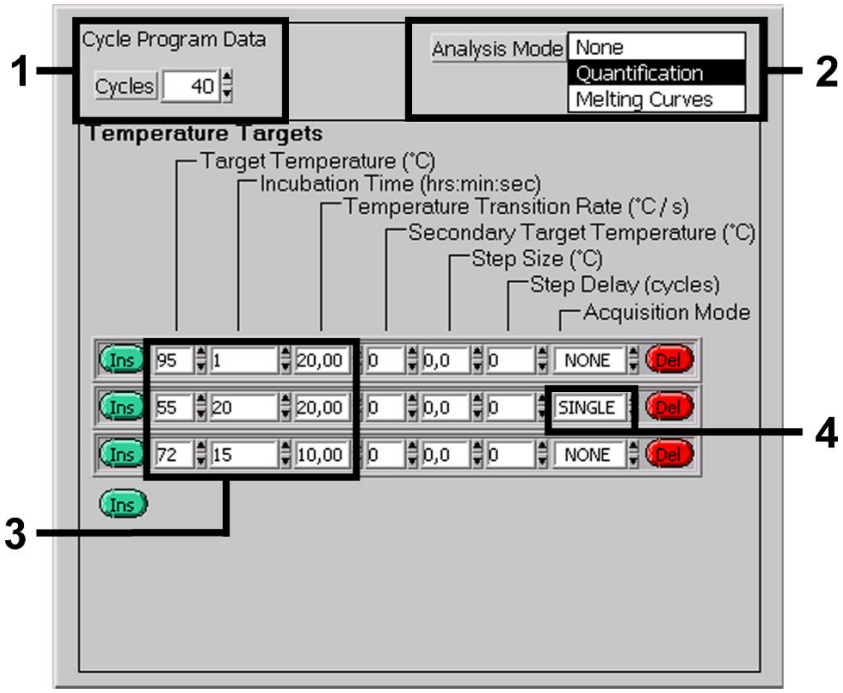

Figure 4. Amplification of the DNA.

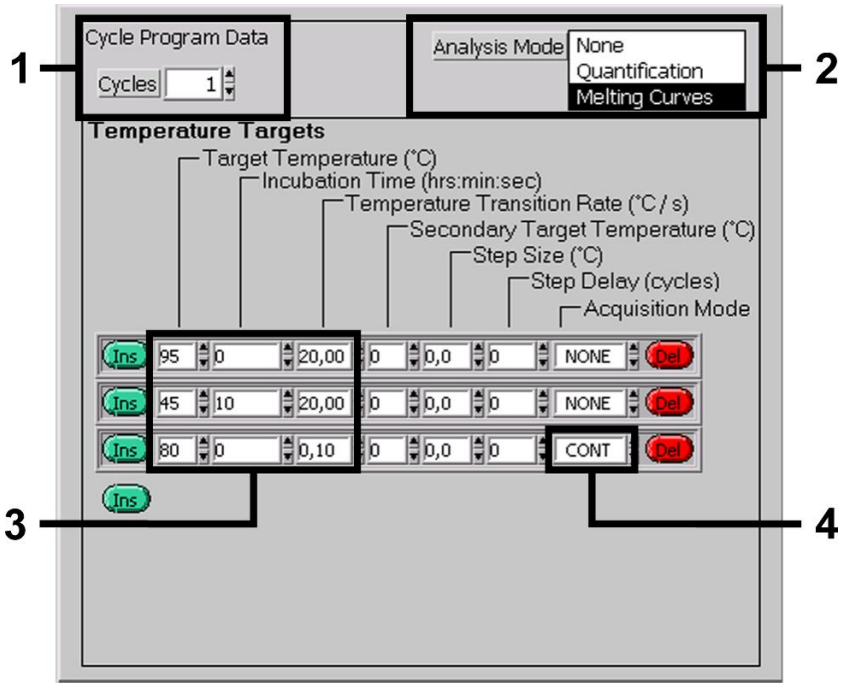

Figure 5. Melting curve

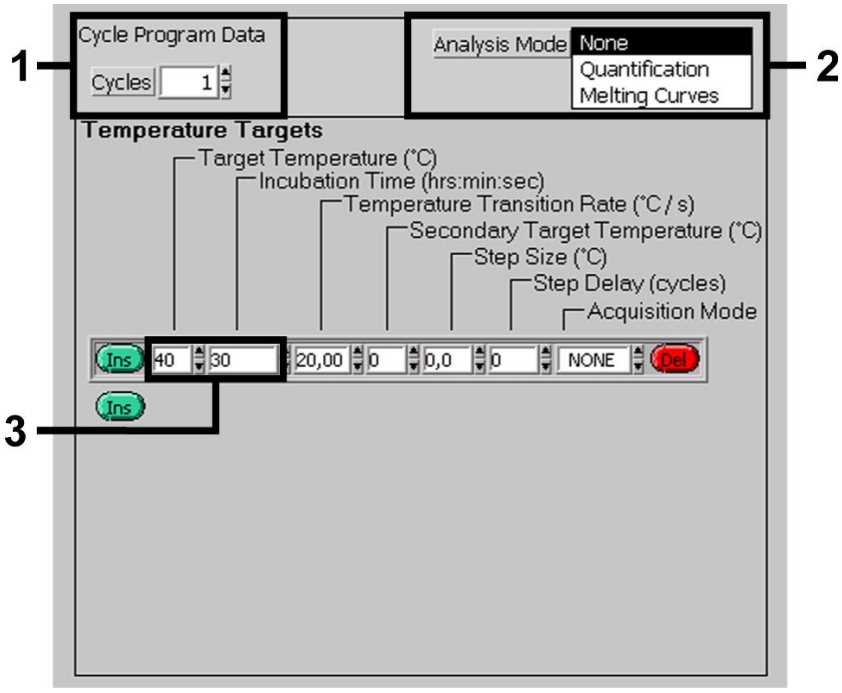

Figure 6. Cooling.

Programming the LightCycler 2.0 instrument

1. To program a PCR run with LightCycler 2.0 instrument, activate the option **New...** in the main menu and select **LightCycler Experiment**.
2. **Important**: First enter the number of capillaries prepared for this PCR run (**Max. Seek Pos.**, see [1] in Figure 7).
3. For the detection of DNA of members of the *M. tuberculosis* complex and *M. avium* complex, create a temperature profile on your LightCycler 2.0 instrument according to the 5 steps shown in the following table.

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
Touch down	95	00:00:01	20	0	0	0	None	10	None
	65	00:00:15	20	58	1	0	None		
	72	00:00:15	10	0	0	0	None		
Amplification of the DNA	95	00:00:01	20	0	0		None	40	Quantification
	55	00:00:20	20	0	0		Single		
	72	00:00:15	10	0	0		None		
Melting curve	95	00:00:00	20	0	0		None	1	Melting Curves
	45	00:00:10	20	0	0		None		
	80	00:00:00	0.1	0	0		Cont.		
Cooling	40	00:00:30	20	0	0	0	None	1	None

4. To enter the sample specifications, activate the button **Samples**.
5. In the window **Capillary View**, first enter the total number of planned PCR preparations for the PCR run (**Sample Count**).
6. Assign names to the samples under **Sample Name**.

7. Under **Selected Channels,** select the fluorescence channel **640** for the detection of the analytical *M. tuberculosis/M. avium* complex PCR**.** .
8. Under **Selected Channels,** select the fluorescence channel **705** for the detection of the internal control PCR.
9. To define the standards and to assign the corresponding concentrations, select the option **Absolute Quantification** under **Analysis Type** (see "Quantitation," page 12).

   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 "Data analysis of the PCR data on the LightCycler 2.0 instrument," page 24).
10. Under **Target Name**, assign the target sequences to be detected (*M. tuberculosis/M. avium* complex) in the selected fluorescence channel **640**.
11. Under **Target Name**, assign the target sequence to be detected (internal control) in the selected fluorescence channel **705**.
12. 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.
13. To generate a standard curve for data analysis, define the quantitation standards with their corresponding concentrations. Select **Standard** under **Sample Type** and enter the corresponding concentration for each standard under **Concentration**.
14. 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**. A new window appears.
15. Select, under **Templates** and **Macros**, the submenu **Run Templates** and save the data under an appropriate name.

16. To start the PCR run, change to the field **Run** and activate the function **Start Run** (see [2] in Figure 7).

    The PCR program will start after entry of the location where the data should be saved.

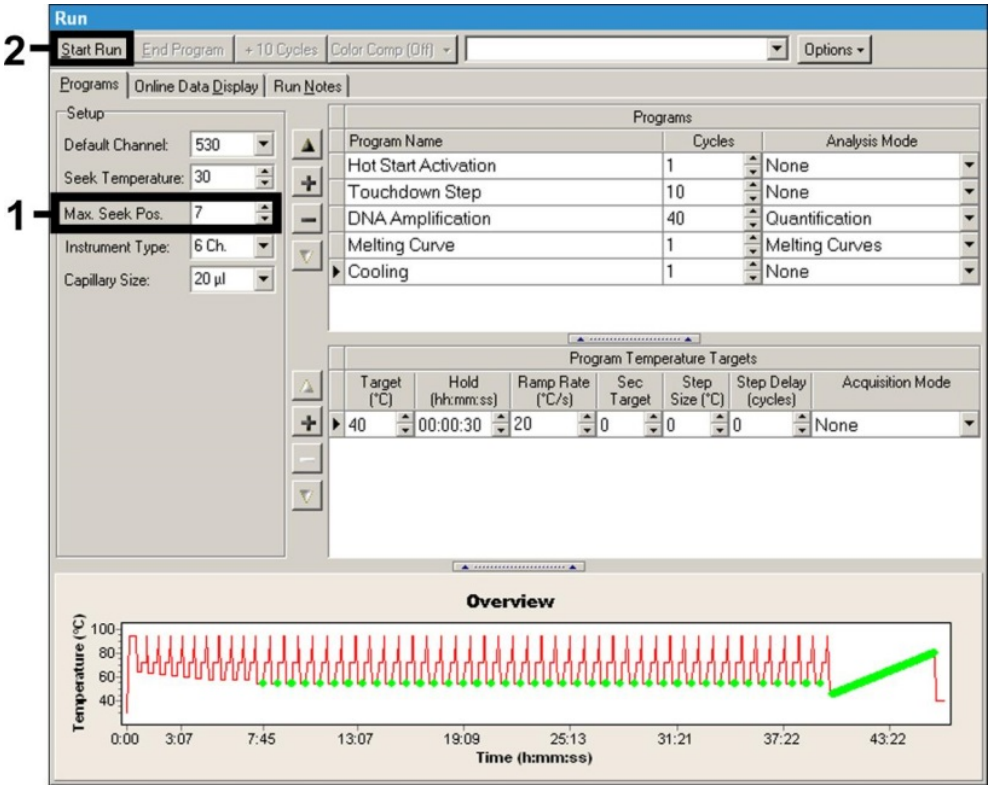

Figure 7. Start of the PCR run.

Interpretation of Results

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 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.

1. Open the **Color Compensation File** before, during or after the PCR run by activating the **Choose CCC File** or the **Select CC Data** button respectively.
2. 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**.
3. For analysis of PCR results with the *artus* Mycobac. diff. LC PCR Kit, select fluorescence display options **F2/Back-F1** for the analytical *M. tuberculosis*/*M. avium* complex PCR and **F3/Back-F1** for the internal control PCR, respectively.

   For the analysis of quantitative runs, follow the instructions given in "Quantitation," page 12.

The following results are possible:

- A signal is detected in fluorimeter channel **F2/Back-F1**.

  The result of the analysis is positive. The sample contains DNA of one or more members of the *M. tuberculosis* complex and/or the *M. avium* 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 **F2/Back-F1** channel) can lead to a reduced or absent fluorescence signal of the internal control in the **F3/Back-F1** channel (competition).

  Differentiation can be made between the *M. tuberculosis* complex, the *M. avium* subspecies and *M. intracellulare* on the basis of melting points (channel **F2/Back-F1**, program **Melting Curve**). The melting point for the members of the *M. tuberculosis* complex is expected to be 60°C, for the *M. avium* subspecies 63.5°C and for *M. intracellulare* 55°C. Differentiation between the *M. tuberculosis* complex and the *M. avium* complex is shown in Figure 8.

  Variations between LightCycler instruments may cause deviations of melting points by 1–2°C. However, this deviation will be the same for all 3 melting points. Various extraction conditions

and buffers can result in melting points slightly different from those of the controls supplied. The PCR should be repeated if the deviation between the melting point of the analyzed sample and the control is more than 1°C. For some mycobacteria species, melting points which deviate from those given above may be observed (see "Troubleshooting," page **Error! Bookmark not defined.**).

- No signal is detected in fluorimeter channel **F2/Back-F1**. 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 or the *M. avium* complex is detectable. It can be considered negative.

  In the case of a negative *M. tuberculosis/M. avium* complex PCR, the detected signal of the internal control rules out the possibility of PCR inhibition.

- No signal is detected in the **F2/Back-F1** or in the **F3/Back-F1** channel.

  No diagnosis can be concluded.

  Examples of positive and negative PCR reactions are given in Figure 9 and Figure 10. Information regarding error sources and their solution can be found in "Troubleshooting," page **Error! Bookmark not defined.**.

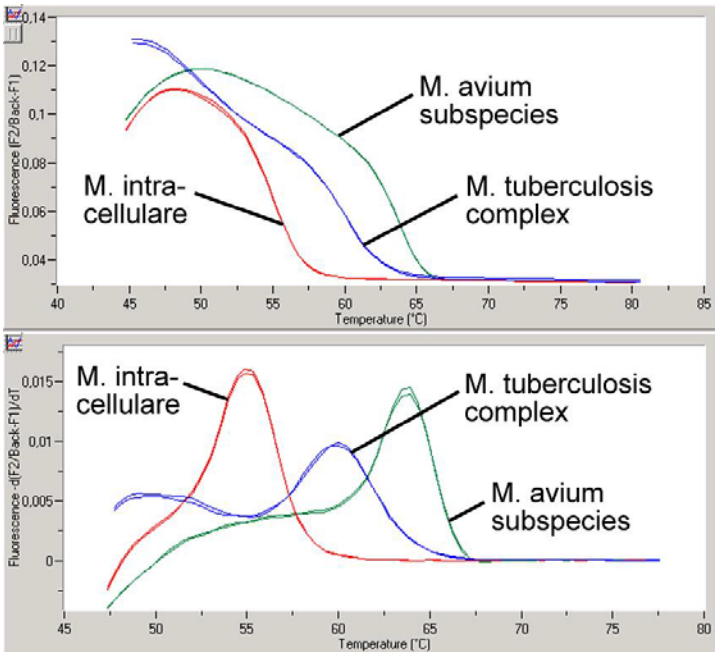

Figure 8. Differentiation between the *M. tuberculosis* complex and the *M. avium* complex in fluorimeter channel F2/Back-F1 of the LightCycler 1.1/1.2/1.5 instrument (Program: Melting Curve).

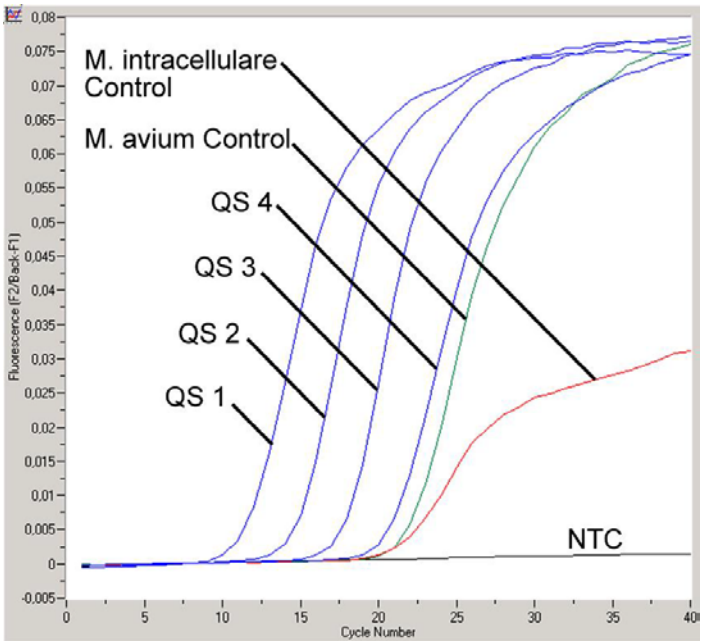

Figure 9. Detection of the quantitation standards (M. tuberculosis LC QS 1 – 4) and positive controls (M. avium LC Control, M. intracellulare LC Control) in fluorimeter channel F2/Back F1 of the LightCycler 1.1/1.2/1.5 instrument. NTC: non-template control (negative control).

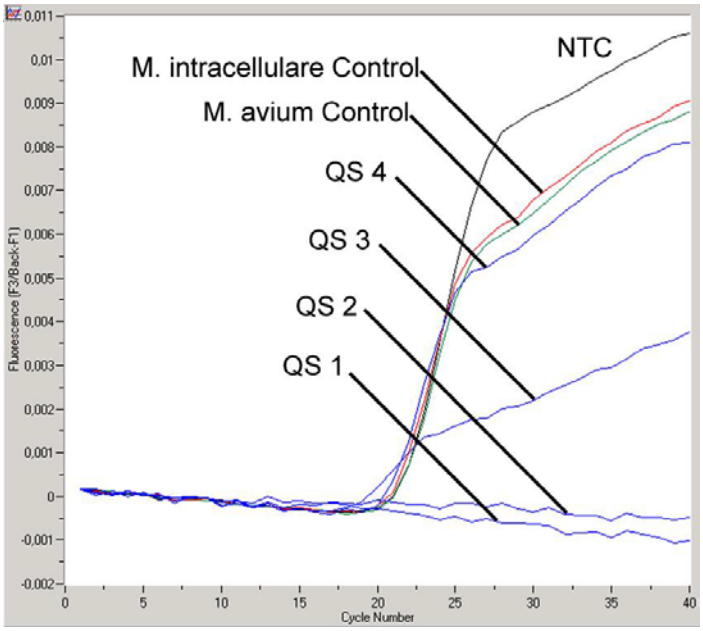

Figure 10. Detection of the internal control in fluorimeter channel F3/Back F1 of the LightCycler 1.1/1.2/1.5 Instrument with simultaneous amplification of quantitation standards (M. tuberculosis LC QS 1–4) and positive controls (M. avium LC Control, M. intracellulare LC Control). NTC: non-template control (negative control).

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, use the LightCycler Software Version 4.0. Consider the instructions given in the *LightCycler 2.0 Instrument Operator's Manual*, Version 4.0.

For the analysis of PCR data proceed as follows (see Figure 11):

1. 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* Mycobac. diff. LC PCR Kit should be analyzed with this function.

2. The LightCycler Software Version 4.0 contains a file termed **Color Compensation File** which compensates multicolor analyses for interferences between fluorescence channels. Open this file during or after the PCR run by activating **Color Comp (On/Off)** and then the **Select Color Compensation** button (see Figure 11).

3. 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.

4. For analysis of the PCR results gained with the *artus* Mycobac. diff. LC PCR Kit, select fluorescence display options **640/Back 530** for the analytical M. *tuberculosis*/M. *avium* complex PCR and **705/Back 530** for the Internal Control PCR, respectively.

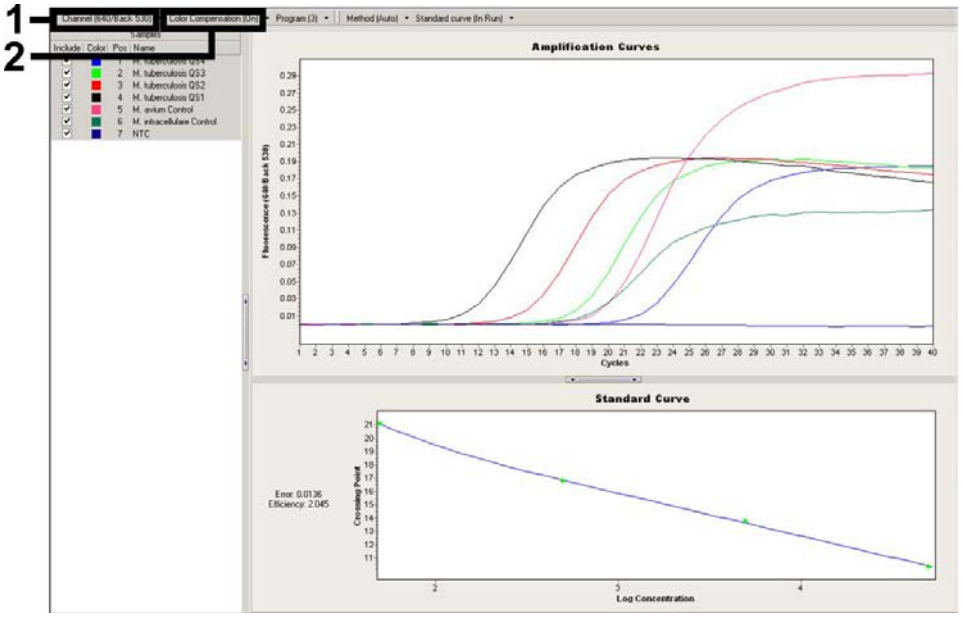

Figure 11. Activation of the Color Compensation File and selection of the fluorescence channel.

For the analysis of quantitative runs, follow the instructions given in "Quantitation," page 12.

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

- A signal is detected in fluorescence channel **640/Back 530**.

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

  In this case, the detection of a signal in the **705/Back 530** channel is dispensable, since high initial concentrations of *M. tuberculosis* complex DNA (positive signal in the **640/Back 530** channel) can lead to a reduced or absent fluorescence signal of the internal control in the **705/Back 530** channel (competition).

  Differentiation can be made between the *M. tuberculosis* complex, the *M. avium* subspecies and *M. intracellulare* on the basis of melting points (channel **640/Back 530**, program: **Melting Curve**). The melting point for the members of the *M. tuberculosis* complex is expected to be 60°C, for the *M. avium* subspecies 63.5°C and for *M. intracellulare* 55°C. Differentiation between the *M. tuberculosis* complex and the *M. avium* complex in fluorescence channel **640/Back 530** of the LightCycler 2.0 instrument is shown in Figure 12.

  Variations between LightCycler instruments may cause deviations of melting points by 1–2°C. However, this deviation will be the same for all 3 melting points. Various extraction conditions and buffers can result in melting points slightly different from those of the controls supplied. The PCR should be repeated if the deviation between the melting point of the analyzed sample and the control is more than 1°C. For some mycobacteria species, melting points which deviate from those given above may be observed (see "Troubleshooting," page Error! Bookmark not defined.).

- No signal is detected in fluorescence channel **640/Back 530**. At the same time, a signal from the internal control appears in the **705/Back 530** channel.

  No DNA of members of the *M. tuberculosis* complex or *M. avium* complex is detectable in the sample. It can be considered negative.

  In the case of a negative *M. tuberculosis*/*M. avium* complex PCR, the detected signal of the internal control rules out the possibility of PCR inhibition.

- No signal is detected in the **640/Back 530** or in **705/Back 530** channels.

  No diagnosis can be concluded.

  Examples of positive and negative PCR reactions are given in Figure 13 and Figure 14. Information regarding error sources and their solution can be found in "Troubleshooting," page Error! Bookmark not defined..

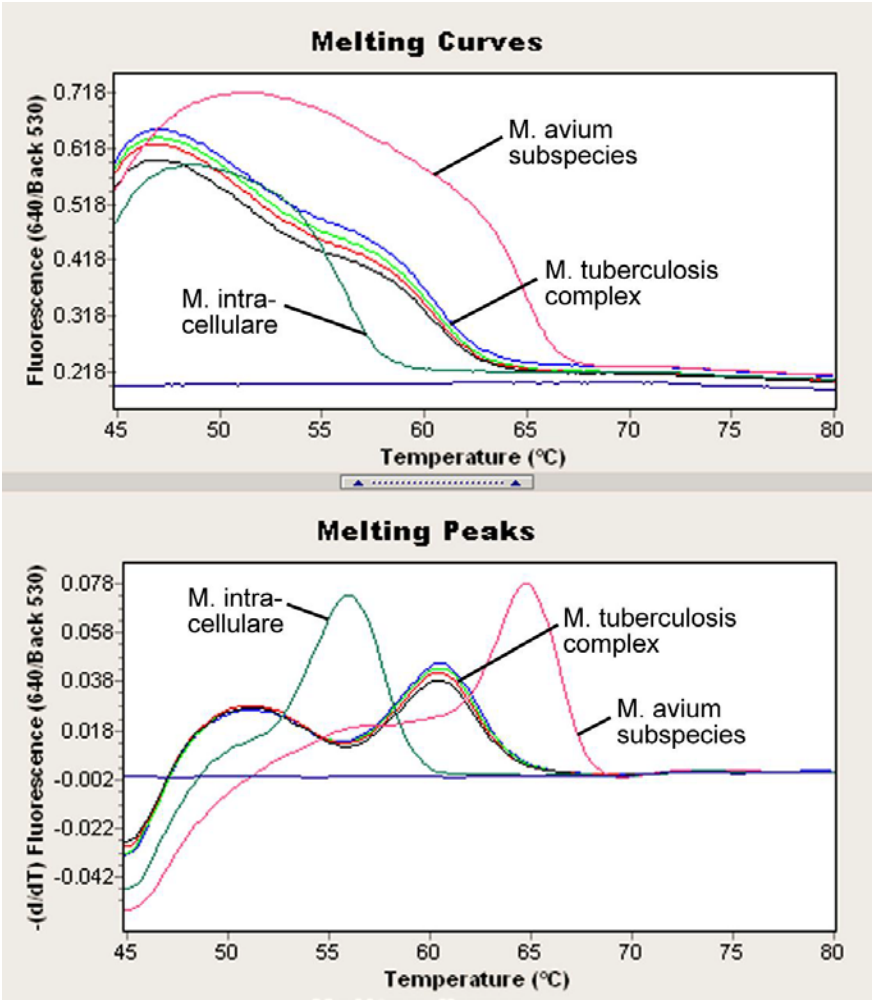

Figure 12. Differentiation between the *M. tuberculosis* complex and the *M. avium* complex in fluorescence channel 640/Back 530 of the LightCycler 2.0 instrument (Program: Melting Curve).

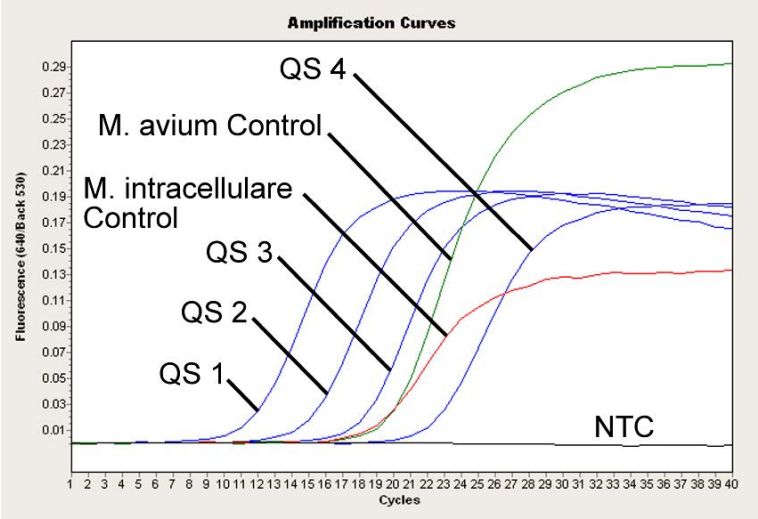

Figure 13. Detection of the quantitation standards (M. tuberculosis LC QS 1–4) and positive controls (M. avium LC Control, M. intracellulare LC Control) in fluorescence channel 640/Back 530 of the LightCycler 2.0 instrument. NTC: non-template control (negative control).

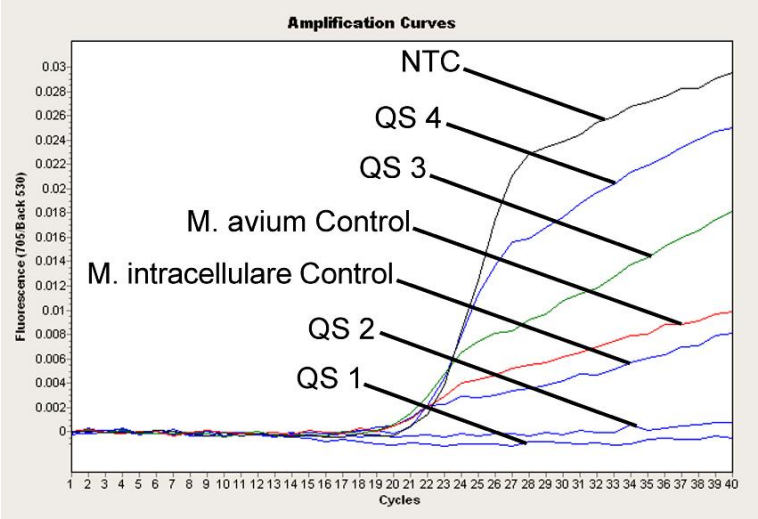

Figure 14. Detection of the internal control in fluorescence channel 705/Back 530 of the LightCycler 2.0 instrument with simultaneous amplification of quantitation standards (M. tuberculosis LC QS 1–4). NTC: non-template control (negative control).

		Comments and suggestions
No signal with quantitation standards (M. tuberculosis LC QS 1–4) and positive controls (M. avium LC Control, M. intracellulare LC Control) in fluorescence channel F2/Back-F1 or 640/Back 530		
a)	The selected fluorescence channel for PCR data analysis does not comply with the protocol	For data analysis, select the fluorescence channel **F2/Back-F1** or **640/Back 530** for the analytical *M. tuberculosis/M. avium* complex PCR and the fluorescence channel **F3/Back-F1** or **705/Back 530** for the internal control PCR.
b)	Incorrect programming of the temperature profile of the LightCycler 1.1/1.2/1.5 or LightCycler 2.0 instruments	Compare the temperature profile with the protocol (see "Programming of the LightCycler instruments," page 16).
c)	Incorrect configuration of the PCR reaction	Check your work steps by means of the pipetting scheme (see "Preparing the PCR," page 13) and repeat the PCR, if necessary.
d)	The storage conditions for one or more kit components did not comply with the instructions or the *artus* Mycobac. diff. LC PCR Kit has expired	Check the storage conditions (see "Reagent Storage and Handling," page 8) 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 F2/Back F1 or 640/Back 530 for the specific *M. tuberculosis/M. avium* complex PCR		
a)	The PCR conditions do not comply with the protocol	Check the PCR conditions (see "No signal with quantitation standards and positive controls," above) and repeat the PCR with corrected settings, if necessary.

		Comments and suggestions
b)	The PCR was inhibited	Make sure that you use a recommended isolation method (see "DNA isolation," page 10) and closely follow 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 "DNA isolation," page 10).
c)	DNA was lost during 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 "DNA isolation," page 10) and closely follow the manufacturer's instructions.
d)	The storage conditions for one or more kit components did not comply with the instructions or the *artus* Mycobac. diff. LC PCR Kit had expired	Check the storage conditions (see "Reagent Storage and Handling," page 8) 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 F2/Back-F1 or 640/Back 530 of the analytical PCR

a)	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. Always pipet the positive controls last. Make sure that work space and instruments are decontaminated at regular intervals.

		Comments and suggestions
b)	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.

The melting curve analysis in fluorescence channel F2/Back-F1 or 640/Back 530 does not allow a clear assignment to one of the 3 detectable mycobacterial groups

a)	The sample contains DNA of the *M. intracellulare* serovar 7 or 18	The melting point is approximately 52°C.
b)	The sample contains DNA of *M. marinum* and/or *M. ulcerans*	The melting point is approximately 53°C.
c)	The sample contains DNA of *M. haemophilum*	The melting point is approximately 62°C.
d)	The sample contains DNA of a mycobacterial species which is different from the members of the *M. avium* and *M. tuberculosis* complex and also from *M. marinum* and *M. ulcerans*	An assignment to a defined mycobacterial species is not possible.

If you have any further questions or if you encounter problems, contact QIAGEN Technical Services.

Quality Control

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

Limitations

- The product is to be used by personnel specially instructed and trained in the in vitro diagnostics procedures only.
- 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.
- Although rare, mutations within the highly conserved regions of the bacterial genome covered by the kit's primers and/or probe may result in underquantitation or failure to detect the presence of the bacteria in these cases. Validity and performance of the assay design are evaluated at regular intervals.

Performance Characteristics

Analytical sensitivity

In order to determine the analytical sensitivity of the *artus* Mycobac. diff. LC PCR Kit, a standard dilution series was set up from 50 to nominal 0.05 *M. tuberculosis* copy* equivalents/µl and from 50 to nominal 0.39 *M. avium* as well as *M. intracellulare* copy equivalents/µl and analyzed on the LightCycler 1.1/1.2/1.5 instrument in combination with the *artus* Mycobac. diff. LC PCR Kit. Testing was carried out on 3 different days on 8 replicates. The results shown in Table 1 were determined by probit analyses. Graphical illustrations are shown in Figures 15–17.

Table 1. Detection limits of targets with the *artus* Mycobac. diff. LC PCR Kit

Target	Detection limit (p = 0.05)
M. tuberculosis complex	1.5 copies/µl
M. avium subspecies	3.8 copies/µl
M. intracellulare	2.8 copies/µl

This means that there is a 95% probability that 1.5 copies/µl (*M. tuberculosis* complex), 3.8 copies/µl (*M. avium* subspecies) and 2.8 copies/µl (*M. intracellulare*) will be detected.

Probit analysis: *Mycobacterium tuberculosis* (LightCycler1.1/1.2/1.5)

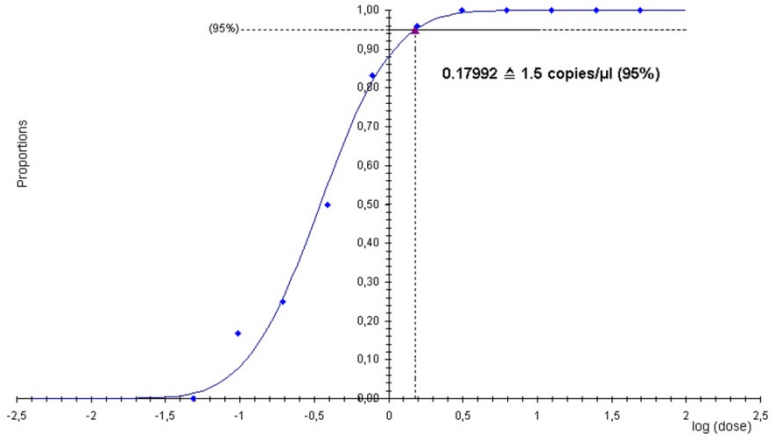

Figure 15. Analytical sensitivity of the *artus* Mycobac. diff. LC PCR Kit (*M. tuberculosis*) on the LightCycler 1.1/1.2/1.5 instrument.

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

Probit analysis: *Mycobacterium avium* (LightCycler1.1/1.2/1.5)

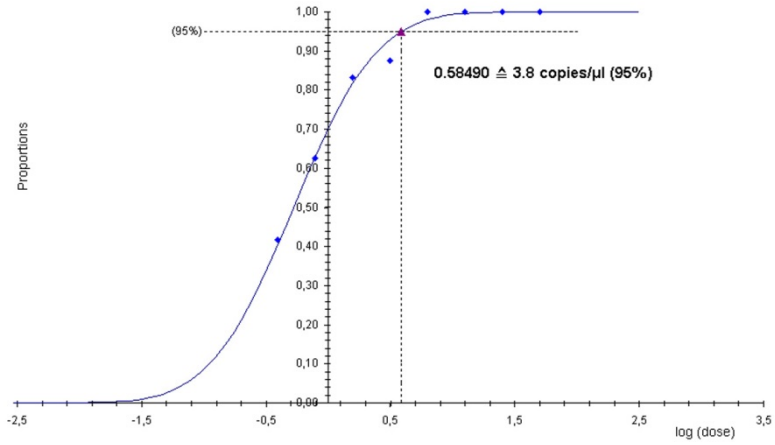

Figure 16. Analytical sensitivity of the *artus* Mycobac. diff. LC PCR Kit (*M. avium*) on the LightCycler 1.1/1.2/1.5 instrument.

Probit analysis: *Mycobacterium intracellulare* (LightCycler1.1/1.2/1.5)

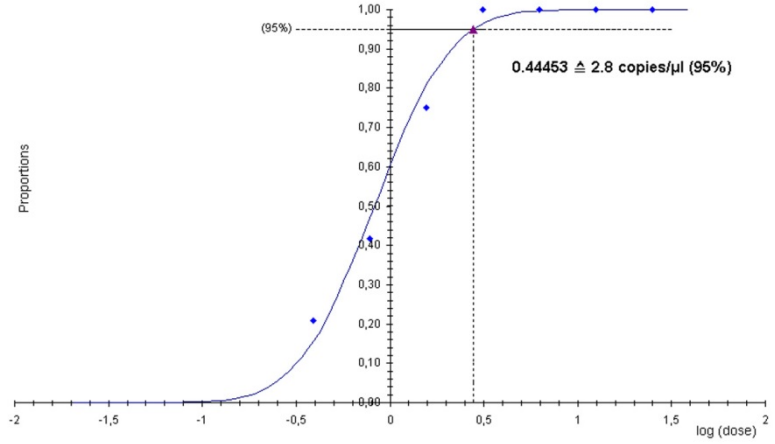

Figure 17. Analytical sensitivity of the *artus* Mycobac. diff. LC PCR Kit (*M. intracellulare*) on the LightCycler 1.1/1.2/1.5 instrument.

Specificity

The specificity of the *artus* Mycobac. diff. 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 and *M. avium* complex has thus been ensured.

Moreover, the specificity was validated with 90 different *M. tuberculosis* and *M. avium* complex negative samples (30 sputum, 30 BAL and 30 bronchial secretion samples). These did not generate any signals with the *M. tuberculosis*/*M. avium* complex specific primers and probes, which are included in the Mycobac. diff. LC Master. To determine the specificity of the *artus* Mycobac. diff. LC PCR Kit, the control group listed in Table 2 was tested for cross-reactivity. None of the tested pathogens was reactive.

Table 2. Testing the specificity of the kit with potentially cross-reactive pathogens

Control group	***M. tuberculosis*/ *M. avium* complex (F2/Back-F1 or 640/Back 530)**	**Internal control (F3/Back-F1 or 705/Back 530)**
Actinomyces israelii	–	+
Aeromonas hydrophila	–	+
Bordetella pertussis	–	+
Candida albicans	–	+
Chlamydia trachomatis	–	+
Chlamydia pneumoniae	–	+
Citrobacter freundii	–	+
Corynebacterium diphtheriae	–	+
Corynebacterium jeikeium	–	+
Cryptococcus neoformans	–	+
Eikenella corrodens	–	+
Enterobacter aerogenes	–	+
Enterobacter cloacae	–	+
Enterococcus faecalis	–	+
Enterococcus faecium	–	+
Escherichia coli	–	+
Fusobacterium nucleatum ssp. *polymorphum*	–	+
Haemophilus influenzae	–	+
Haemophilus parainfluenzae	–	+

Control group	*M. tuberculosis*/ *M. avium* complex (F2/Back-F1 or 640/Back 530)	Internal control (F3/Back-F1 or 705/Back 530)
Klebsiella pneumoniae	–	+
Lactobacillus acidophilus	–	+
Neisseria gonorrhoeae	–	+
Neisseria meningitidis	–	+
Nocardia asteroides	–	+
Nocardia brasiliensis	–	+
Nocardia farcinia	–	+
Nocardia otitidiscaviarum	–	+
Peptostreptococcus productus	–	+
Porphyromonas gingivalis	–	+
Prevotella denticola	–	+
Propionibacterium acnes	–	+
Pseudomonas aeruginosa	–	+
Salmonella enteritidis	–	+
Salmonella typhi	–	+
Staphylococcus aureus	–	+
Staphylococcus epidermidis	–	+
Streptococcus agalactiae	–	+
Streptococcus pyogenes	–	+
Streptococcus mutans	–	+
Streptococcus pneumoniae	–	+
Streptomyces venezuelae	–	+
Veillonella parvula	–	+
Xanthomonas maltophilia	–	+

High amounts of DNA of mycobacterial species not belonging to the *M. tuberculosis/M. avium* complex might lead to a failure of the internal control (see Table 3). In addition, the generation of an amplification curve in fluorimeter channel **F2/Back-F1** and/or of a melting curve is possible. But for all mycobacterial species tested, the melting curve analysis allowed a clear differentiation from the *M. tuberculosis/M. avium* complex (see Table 3).

Table 3: Testing the specificity of the kit with potentially cross-reactive mycobacteria.

Species	***M. tuberculosis/ M. avium* complex (F2/Back-F1 or 640/Back 530)**	**Internal control (F3/Back-F1 or 705/Back 530)**	**Melting point (F2/Back-F1 or 640/Back 530)**
Mycobacterium celatum	+	–	49.5°C
Mycobacterium chelonae	–	–	–
Mycobacterium fortuitum	–	–	–
Mycobacterium gordonae	–	+	–
Mycobacterium haemophilum	+	–	62.0°C
Mycobacterium kansasii	–	–	49.0°C
Mycobacterium lentiflavum	+	–	50.0°C
Mycobacterium malmoense	–	–	50.0°C
Mycobacterium marinum	+	–	53.5°C
Mycobacterium scrofulaceum	–	–	–
Mycobacterium szulgai	–	–	50.5°C
Mycobacterium ulcerans	+	–	53.5°C
Mycobacterium xenopi	–	+	–

Precision

The precision data of the *artus* Mycobac. diff. 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* Mycobac. diff. LC PCR Kit have been collected using the quantitation standard of the lowest concentration (QS 4; 50 copies/µl). Testing was performed with 8 replicates. The precision data were calculated on basis of the C_T values of the amplification curves (C_T: threshold cycle, see Table 4). In addition, precision data for quantitative results in copies/µl were determined using the corresponding C_T values (see Table 4). Based on these results, the overall statistical spread of any given sample with the mentioned concentration is 1.42% (C_T) or 12.17% (copies/µl), and for the detection of the internal control 1.36 % (C_T). These values are based on the totality of all single values of the determined variabilities.

Table 4. Precision data on basis of the C_T values

	Standard deviation	Variance	Coefficient of variation (%)
Intra-assay variability: M. tuberculosis LC QS 4	0.15	0.02	0.73
Intra-assay variability: Internal Control	0.16	0.02	0.78
Inter-assay variability: M. tuberculosis LC QS 4	0.23	0.06	1.42
Inter-assay variability: Internal Control	0.33	0.11	1.65
Inter-batch variability: M. tuberculosis LC QS 4	0.25	0.06	1.23
Inter-batch variability: Internal Control	0.23	0.06	1.17
Total variance: M. tuberculosis LC QS 4	0.29	0.08	1.42
Total variance: Internal Control	0.27	0.07	1.36

Table 5. Precision data on basis of the quantitative results (in copies/µl)

	Standard deviation	Variance	Coefficient of variation (%)
Intra-assay variability: M. tuberculosis LC QS 4	5.35	28.57	10.64
Inter-assay variability: M. tuberculosis LC QS 4	7.44	55.40	14.73
Inter-batch variability: M. tuberculosis LC QS 4	5.07	25.67	10.08
Total variance: M. tuberculosis LC QS 4	6.13	37.56	12.17

Robustness

The verification of the robustness allows the determination of the total failure rate of the *artus* Mycobac. diff. LC PCR Kit. A total of 30 *M. tuberculosis/M. avium* complex-negative samples of each sputum, BAL and bronchial secretion were spiked with 12.5 copies/µl elution volume of *M. avium* control DNA (approximately 3-fold concentration of the analytical sensitivity limit). After extraction using the QIAamp DNA Mini Kit (see "DNA isolation," page 10), these samples were analyzed with the *artus* Mycobac. diff. LC PCR Kit. For all *M. avium* samples, the failure rate was 0%. In addition, the robustness of the internal control was assessed by purification and analysis of *M. tuberculosis/M. avium* complex-negative sputum, BAL and bronchial secretion samples (30 each). The total failure rate was 0%. Inhibitions were not observed. Thus, the robustness of the *artus* Mycobac. diff. LC PCR Kit is ≥ 99%.

Reproducibility

Reproducibility data permit a regular performance assessment of the *artus* Mycobac. diff. LC PCR Kit as well as an efficiency comparison with other products. These data are obtained by the participation in established proficiency programs.

References

1. Mackay I.M. (2004) Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. **10**, 190.

Symbols

Symbol	Symbol definition
	Use by
	Batch code
	Manufacturer
	Catalog number
	Material number
	CE mark for European conformity
	In vitro diagnostic medical device
	Contains reagents sufficient for <N> tests

Symbol	Symbol definition
COMP	Components
CONT	Contains
NUM	Number
GTIN	Global Trade Item Number
	Temperature limitation
QS	Quantitation Standard
IC	Internal Control
Mg-Sol	Magnesium solution

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