BactoReal®
Listeria monocytogenes

Manual

For use with the

- **ABI PRISM® 7500 (Fast)**
- **Mx3005P®**
- **LightCycler® 480**
- **LightCycler® 1.2/1.5/2.0**

For research use only

REF **RTGM200**

Σ **50**

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1. Product description

BactoReal® *Listeria monocytogenes* is a real-time PCR assay for detection of DNA of *L. monocytogenes.* This test was developed for the ABI PRISM® 7500 (Fast) instrument (Applied Biosystems), for the LightCycler® 1.2/1.5/2.0/480 instruments (Roche) and for Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments.

BactoReal® *Listeria monocytogenes* contains an assay for the amplification and detection of the hlyA gene of *L. monocytogenes* and an external positive control for *L. monocytogenes.* The amplification mix is not included. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *L. monocytogenes* specific DNA (see 8. Interpretation of PCR-data). This test allows the rapid and sensitive detection of DNA of *L. monocytogenes* from samples purified either from liquor, blood or other samples (e.g. with the QIAamp DNA Mini Kit).

Internal positive control (IPC, optional):
BactoReal® *Listeria monocytogenes* can be performed in a multiplex PCR (depending on the PCR-platform) with ControlReal or Internal Positive Control Assays. Order numbers see 6. Additionally required materials and devices.
When using PCR-platforms not validated by ingenetix, an evaluation of the multiplex-PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing of a multiplex-PCR.

- Tests for exclusion of false-negative interpretation of results due to PCR inhibition: ControlReal assays containing primers, a probe and an internal control PCR target included in the assay mix.
- Tests for control of DNA extraction and real-time PCR: Internal Positive Control Assays containing primers, a probe and an internal control PCR target in an extra tube which can be extracted with the sample.

For use with the ABI PRISM® 7500 (Fast) instrument or the Mx3005P® instrument:
Detection in VIC/HEX channel (554 nm): ControlReal 1 or Internal Positive Control Assay 1
Detection in Cy5 channel (667 nm): ControlReal 3 or Internal Positive Control Assay 3

For use with the LightCycler® 480 instrument:
Detection in Cy5 channel (667 nm): ControlReal 3 or Internal Positive Control Assay 3

For use with the LightCycler® 1.2/1.5/2.0 instrument:
Detection in channel F2 (610 nm): ControlReal 2 or Internal Positive Control Assay 2

Ingenetix ViroReal®, BactoReal® and ParoReal assays detecting viral, bacterial and parasitic DNA are optimized to run under the same thermal cycling conditions and with the same amplification mix.

2. Pathogen information

Listeriosis is an infection caused by eating food (milk, cheeses, ice cream, vegetables, sausages, meats and fish) contaminated with the bacterium called *Listeria monocytogenes*. Although there are other types of *Listeria (L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seegligeri* and *L. grayi),* most cases of listeriosis are caused by *L. monocytogenes*. *Listeria* are ubiquitously found in soil and water. Gastrointestinal symptoms may precede more serious forms of listeriosis or may be the only symptoms expressed. The two main severe clinical manifestations of listeriosis are sepsis and meningitis. People at highest risk include the elderly, the fetuses of pregnant women, and the immunosuppressed. *Listeria monocytogenes* is a gram positive, facultative anaerobic, and facultative intracellular bacterium. Its incubation time is at least seven days. Its ability to grow at temperatures as low as 0°C permits multiplication in refrigerated foods. *Listeria monocytogenes* is killed by pasteurization and cooking. It expresses a Beta hemolysin (hlyA gene) which causes destruction of red blood cells and has the ability to target other cells.

References:
Nogva, H.K. et. al. 2000. Application of 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. Appl. Environ. Microbiol. 66: 4266-4271.

3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplificates.

4. General Precautions

The user should always pay attention to the following:

- Always include a negative control per PCR-run (water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during DNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with *L. monocytogenes* DNA during extraction.
- Be careful when handling the positive control.
- 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 workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.

5. Contents

Assay

Labelling	Content	Amount
Listeria monocytogenes Assay Mix (green cap)	Primer and probe for *L. monocytogenes*-detection	2 x 25 µl

Positive Control

Labelling	Content	Amount
Listeria monocytogenes Positive Control (red cap)*	Control-DNA (approx. 1,000 target copies/µl)	1 x 25 µl

*Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 100 target copies/µl).

The components of BactoReal® *Listeria monocytogenes* should be stored at -20°C and are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- PCR-grade water
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes

6.1. For ABI PRISM® 7500 Fast instrument (Applied Biosystems)

- *Either:* MicroAmp Fast Reaction Tubes (125 strips; 8 tubes/strip) (order no. 4358293) + MicroAmp Optical 8-Cap Strip (300 strips; 8 Tubes/Strip) (order no. 4323032)
- *Or:* MicroAmp Fast Optical 96-well reaction plate with barcode (0.1 ml) (20 plates) (order no. 4346906) + MicroAmp Optical Adhesive Film (100 pieces) (order no. 4311971)
- 7500 Fast Precision Plate Holder for MicroAmp Tube Strips
- TaqMan® Gene Expression Master Mix (order no. 4369016; 1 x 5 ml for 500 reactions based on 20 µl reaction volume).
- Optional: ControlReal 1 (ingenetix order no. RTGMCR-1; 50 reactions), ControlReal 3 (ingenetix order no. RTGMCR-3; 50 reactions), Internal Positive Control Assay 1 (ingenetix order no. RTGMIPC-1; 100 reactions) or Internal Positive Control Assay 3 (ingenetix order no. RTGMIPC-3; 100 reactions).

6.2. For ABI PRISM® 7500 instrument (Applied Biosystems)

- *Either:* ABI PRISM™ Optical Tubes (8 Tubes/ Strip) (125 strips; 8 tubes/strip) (order no. 4316567) + MicroAmp Optical 8-Cap Strip (300 strips; 8 Tubes/Strip) (order no. 4323032)
- *Or:* 96-Well Optical Reaction Plate with barcode (20 plates) (order no. 4306737) + Optical Adhesive Cover Starter Kit (20 pieces) (order no. 4313663)
- TaqMan® Gene Expression Master Mix (order no. 4369016; 1 x 5 ml for 500 reactions based on 20 µl reaction volume).
- Optional: ControlReal 1 (ingenetix order no. RTGMCR-1; 50 reactions), ControlReal 3 (ingenetix order no. RTGMCR-3; 50 reactions), Internal Positive Control Assay 1 (ingenetix order no. RTGMIPC-1; 100 reactions) or Internal Positive Control Assay 3 (ingenetix order no. RTGMIPC-3; 100 reactions).

6.3. For Mx3005P® QPCR System (Agilent)

- 96-well PCR plates, 0.2 ml, non-skirted (order no. 401333)
  Or: 8 x strip tubes, 0.2 ml (order nr. 401428) and 8 x optical strip caps (order no. 401425)
- TaqMan® Gene Expression Master Mix (order no. 4369016; 1 x 5 ml for 500 reactions based on 20 µl reaction volume).
- Optional: ControlReal 1 (ingenetix order no. RTGMCR-1; 50 reactions), ControlReal 3 (ingenetix order no. RTGMCR-3; 50 reactions), Internal Positive Control Assay 1 (ingenetix order no. RTGMIPC-1; 100 reactions) or Internal Positive Control Assay 3 (ingenetix order no. RTGMIPC-3; 100 reactions).

6.4. For LightCycler® 480 (Roche)

- LightCycler® 480 Multiwell Plate 96, white with sealing foils (order no. 04729692001)
- TaqMan® Gene Expression Master Mix (order no. 4369016; 1 x 5 ml for 500 reactions based on 20 µl reaction volume).
- Optional: ControlReal 3 (ingenetix order no. RTGMCR-3; 50 reactions) or Internal Positive Control Assay 3 (ingenetix order no. RTGMIPC-3; 100 reactions).

6.5. For LightCycler® 1.2/1.5/2.0 (Roche)

- LC™-FastStart DNA Master Kit Hybridisation Probes (order no. 12239272001: Kit for 480 reactions of 20 µl final reaction volume, *or* order no. 03003248001: Kit for 96 reactions of 20 µl final reaction volume)
- LightCycler® Capillaries (20 µl) (order no. 04929292001: 1 pack containing 5 boxes, each with 96 capillaries and stoppers)
- LightCycler® Cooling Block
- LightCycler® Capping Tool
- LightCycler® 1.1/1.2/1.5 or 2.0 Instrument
- LightCycler® Carousel Centrifuge
- Optional: ControlReal 2 (ingenetix order no. RTGMCR-2; 50 reactions) or Internal Positive Control Assay 2 (ingenetix order no. RTGMIPC-2; 100 reactions).

7. Preparation of real-time PCR

Please make sure that at least one negative control (water), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates which increases the probability of detection of the pathogen and facilitates interpretation of results.

7.1. Pipetting scheme

Sample: 1-5 µl of the sample can be used. When using < 5 µl of the sample, the amount of H_2O has to be changed accordingly.

Positive Control: As positive control please use 1 µl of the *Listeria monocytogenes* Positive Control + 4 µl H_2O.

Use of Internal Positive Control (optional):

- ControlReal Assays contain the CR Assay Mix (primers, probe and an internal control PCR target included in the assay mix). They exclude false-negative interpretation of results caused by inhibition of real-time PCR.
- Internal Positive Control Assays contain the IPC Assay Mix (primers and probe) and the IPC Target. They exclude false-negative interpretation of results caused by inhibition of real-time PCR and serve as an extraction control.

  → When IPC Target used directly as target for PCR (for control of PCR inhibition):
  Dilute IPC Target freshly 1:100 with water and add to the master mix (use 1 µl/reaction).

  → When IPC Target added during extraction (for control of extraction and PCR inhibition):
  Spike 1 µl of the undiluted IPC Target into the sample material after the lysis buffer was added. Do not add the IPC Target directly to the sample material.

  Caution: The IPC Target is stored in DNA stabilizer which contains guanidinium thiocyanate/Triton X-100.

7.1.1. For use with the ABI PRISM® 7500 (Fast) instrument, Mx3005P® and LightCycler® 480

Without internal positive control assay

		Per sample
Preparation of master mix (mix well)	H_2O	4.0 µl
	TaqMan® Gene Expression Master Mix (2x)	10.0 µl
	Listeria monocytogenes Assay Mix	1.0 µl
	Total volume	**15.0 µl**
Preparation of PCR assay	Master mix	15.0 µl
	Sample	5.0 µl
	Total volume	**20.0 µl**

With internal positive control assay (IPC, optional)

		Per sample
Preparation of master mix (mix well)	H_2O	3.0 µl
	TaqMan® Gene Expression Master Mix (2x)	10.0 µl
	Listeria monocytogenes Assay Mix	1.0 µl
	CR or IPC Assay Mix (ControlReal or Internal Positive Control Assay)	1.0 µl
	Total volume	**15.0 µl**
Preparation of PCR assay	Master mix	15.0 µl
	Sample	5.0 µl
	Total volume	**20.0 µl**

7.1.2. For use with the LightCycler® 1.2/1.5/2.0 instrument

Without internal positive control assay

		Per sample
Preparation of master mix (mix well)	H_2O	9.6 µl
	$MgCl_2$ stock solution [25 mM]	2.4 µl
	LC-HYBR**	2.0 µl
	Listeria monocytogenes Assay Mix	1.0 µl
	Total volume	**15.0 µl**
Preparation of PCR assay	Master mix	15.0 µl
	Sample	5.0 µl
	Total volume	**20.0 µl**

With internal positive control assay (IPC, optional)

		Per sample
Preparation of master mix (mix well)	H_2O	8.6 µl
	$MgCl_2$ stock solution [25 mM]	2.4 µl
	LC-HYBR*	2.0 µl
	Listeria monocytogenes Assay Mix	1.0 µl
	CR-2 or IPC-2 Assay Mix (ControlReal 2 or Internal Positive Control Assay 2)	1.0 µl
	Total volume	**15.0 µl**
Preparation of PCR assay	Master mix	15.0 µl
	Sample	5.0 µl
	Total volume	**20.0 µl**

*LC-HYBR (LC™-FastStart DNA Master Kit Hybridisation Probes). Pipet 60 µl from vial 1b in vial 1a. See instructions of the manufacturer.

7.2. Programming of the real-time PCR instrument

Please find further information on programming the instrument in the respective operator's manual.

7.2.1. ABI PRISM® 7500 (Fast) instrument

Instrument parameter for *Absolute Quantification*:

- **Thermal Cycler Conditions:** without "fast cycling" parameter
- **Detectors:** For detection of *Listeria monocytogenes:* FAM-TAMRA
  Optional for detection of IPC: Cy5-NONE (ControlReal 3, Internal Positive Control Assay 3) **or** VIC-TAMRA (ControlReal 1, Internal Positive Control Assay 1)
- **Passive Reference:** ROX
- **Sample Volume:** 20 µl
- **Temperature Profile:** see below

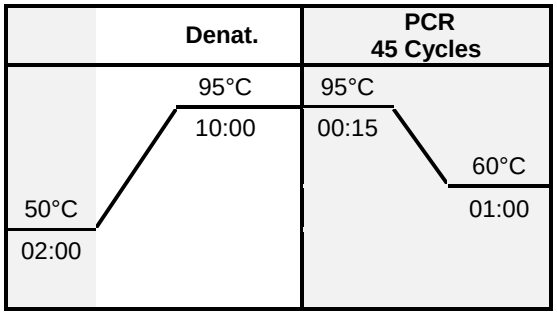
7.2.2. Mx3005P® instrument

Instrument parameters for *Quantitative PCR*:

- **Collect fluorescence data**
- **Select dyes:** For detection of *Listeria monocytogenes:* FAM
  Optional for detection of IPC: Cy5 (ControlReal 3, Internal Positive Control Assay 3) **or** HEX (ControlReal 1, Internal Positive Control Assay 1)
- **Select passive reference dye**: ROX
- **Filter set gain settings:** ROX x2, Cy5 or HEX x4, FAM x8
- **Temperature Profile:** see below

Temperature Profile for:

ABI PRISM® 7500 and Mx3005P®



Attention: Cycling conditions are valid for the TaqMan® Gene Expression Master Mix. When using other reaction mixes the thermal profile has to be changed according to the manufacturer's instruction manual.

7.2.3. LightCycler® 480 instrument

	Program name	Acquisition mode	Cycle	Analysis mode
1: Program	50°C, 2 min	none	1 cycle	None
2: Program	95°C, 10 min	none	1 cycle	None
3: Program	95°C, 15 sec 60°C, 1 min	none single	45 cycles	Quantification
4: Program	40°C, 10 sec	none	1 cycle	None

Detection format:
Name: One Color Hydrolysis Probe (without IPC) **or** two Color Hydrolysis Probe (with IPC)
Integration time mode: Dynamic
Filter combinations:

Active	Name	Melt factor	Quant factor	Max integration time
Yes	FAM (465-510)	1	10	2 second(s)
*Yes	Cy 5 / Cy 5.5 (618-660)	1	10	2 second(s)

*Optional for detection of IPC

Attention: Cycling conditions are valid for the TaqMan® Gene Expression Master Mix. When using other reaction mixes the thermal profile has to be changed according to the manufacturer's instruction manual.

7.2.4. LightCycler® 1.2/1.5/2.0 instrument

Temperature profile:

Program Name: Activation **Cycles:** 1 Cycle **Analysis Mode:** None

Target Temp.	Incubation time	Temp. Trans. Rate	Sec. Target Temp.	Step Size	Step Delay	Acquisition Mode
95	00:10:00	20.00	0	0.0	0	NONE

Program Name: Amplification **Cycles:** 45 Cycles **Analysis Mode:** Quantification

Target Temp.	Incubation time	Temp. Trans. Rate	Sec. Target Temp.	Step Size	Step Delay	Acquisition Mode
95	00:00:10	20.00	0	0.0	0	NONE
62	00:00:40	20.00	0	0.0	0	SINGLE

Program Name: Cool **Cycles:** 1 Cycle **Analysis Mode:** None

Target Temp.	Incubation time	Temp. Trans. Rate	Sec. Target Temp.	Step Size	Step Delay	Acquisition Mode
40	00:00:10	20.00	0	0.0	0	NONE

Attention: Cycling conditions are valid for the LC™-FastStart DNA Master Kit Hybridisation Probes. When using other reaction mixes the thermal profile has to be changed according to the manufacturer's instruction manual.

8. Interpretation of PCR-data

Examples for interpretation of positive reactions are shown in the amplification plots below.

For a valid interpretation, the following criteria must be fulfilled:

	Ct/Cp *L. monocytogenes* target	Ct/Cp IPC target (optional)	Interpretation
Negative control	Negative	36.0 ± 2	Valid
Positive control (undiluted, 1 µl/PCR)	30.0-33.0	36.0 ± 2	Valid
Or: positive control (1:10 diluted, 1 µl/PCR)	33.0-36.0	36.0 ± 2	Valid
Extraction negative control (recommended)	Negative	36.0 ± 2	Valid
Negative sample	Negative	36.0 ± 2	Valid
Positive sample	Positive	Positive / Negative	Valid

8.1. For use with the ABI PRISM® 7500 (Fast) instrument, Mx3005P® and LightCycler® 480

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with BactoReal® *Listeria monocytogenes* please select FAM channel for the *L. monocytogenes* target and VIC/HEX channel (ControlReal 1, Internal Positive Control Assay 1) or Cy5 channel (ControlReal 3, Internal Positive Control Assay 3) for the internal positive control target (optional). Samples with a positive Ct/Cp-value are considered positive. Please also check the presence of amplification-curves manually.

Once the analysis is completed, the following results are possible:

1. Signal in FAM channel:
→ DNA of *L. monocytogenes* was amplified. The sample has to be interpreted as positive.
L. monocytogenes DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

2. No signal in FAM channel:
→ No *L. monocytogenes* DNA is detectable in the sample. The sample has to be interpreted as negative.
An inhibition of PCR cannot be excluded.

2a. No signal in FAM channel but signal of the internal positive control (optional):
→ No *L. monocytogenes* DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the internal positive control assay excludes a putative PCR inhibition.

2b. No signals in FAM channel and no signal with internal positive control (optional):
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

8.2. For use with the LightCycler® 1.2/1.5/2.0 instrument

For analysis of PCR data please proceed as follows:

- Important: Make sure that the colour compensation is deactivated. A signal at 530 nm (F1 channel) and at 610 nm (F2 channel) can be observed with the *L. monocytogenes* target, while the IPC target is exclusively detected at 610 nm (F2 channel). Therefore, amplification of the IPC target cannot lead to false-positive signals at 530 nm.
- Activate the function *Analysis* in the menu strip and select the option *Absolute Quantification*.
- For analysis of PCR results gained with BactoReal® *Listeria monocytogenes* please select fluorescence display options 530 nm (F1 channel) for the *L. monocytogenes* target and 610 nm (F2 channel) for the IPC target (optional). Samples with a positive Cp-value are considered positive. Please also check the presence of amplification-curves manually. For this, click with left mouse button on the respective sample positions.

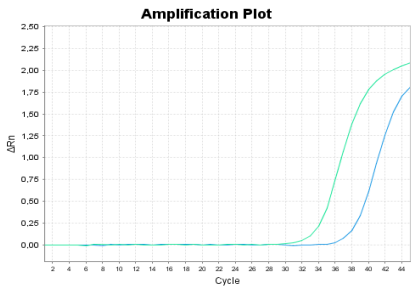
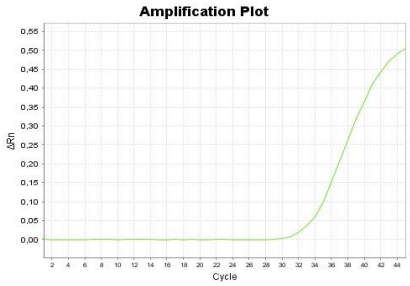
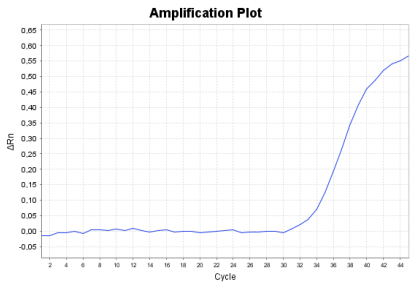
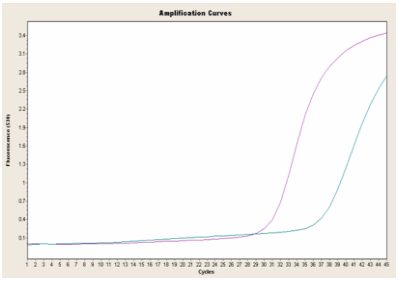
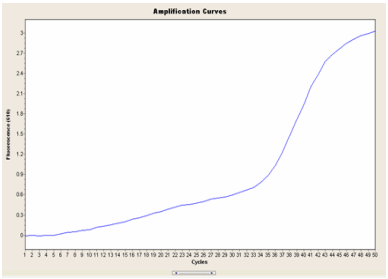
Once analysis is completed, the following results can be observed with the samples:

1. Signal at 530 nm (channel F1):
→ DNA of *L. monocytogenes* was amplified. The sample has to be interpreted as positive.
In this case fluorescence is also detected at 610 nm (channel F2), since no colour compensation is used.

2. No signal at 530 nm (channel F1):
→ No *L. monocytogenes* DNA is detectable in the sample. The sample has to be interpreted as negative.
An inhibition of the PCR cannot be excluded.

2a. No signal at 530 nm (channel F1) but signal at 610 nm (channel F2) (signal of the IPC, optional):
→ No *L. monocytogenes* DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the internal positive control assay excludes a putative PCR inhibition.

2b. No signals at 530 nm (channel F1) and at 610 nm (optional) (channel F2):
→ No interpretation can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

Detection of ***Listeria monocytogenes***	Detection of **internal positive control (optional)**	
		
ABI Prism® 7500: FAM channel 1:10 serial dilution of *L. monocytogenes* DNA	**ABI Prism® 7500:** Cy5 channel Internal positive control (ControlReal 3)	**ABI Prism® 7500:** VIC channel Internal positive control (ControlReal 1)
		
LightCycler® 2.0: channel F1, 530 nm 1:10 serial dilution of *L. monocytogenes* DNA	**LightCycler® 2.0:** channel F2, 610 nm Internal positive control (ControlReal 2)	

9. Troubleshooting

1. No *L. monocytogenes* specific signal with positive control:

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.

2. When using an internal positive control assay (optional): no signal with the internal positive control and no *L. monocytogenes* specific signal with the sample:

- The PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extraction can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate amount of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.
  For use with the LightCycler® 1.2/1.5 or 2.0 instrument: make sure no colour compensation is activated.

3. *L. monocytogenes* specific signal with the negative control:

- A contamination occurred during preparation of the PCR.
  → Repeat the PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

4. *L. monocytogenes* specific signal with the negative control of DNA-extraction (optional):

- A contamination occurred during extraction.
  → Repeat the extraction and PCR using new reagents.
  → Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications

10.1. Analytical sensitivity

The analytical sensitivity is 5-10 fg *Listeria monocytogenes* DNA/PCR.

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. The primers and probes were checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known *L. monocytogenes* strains.
BactoReal® *Listeria monocytogenes* was tested on different bacterial isolates (*B. parapertussis, B. pertussis, C. pneumoniae, E. coli, H. influenzae, L. pneumophila, L. innocua, M. pneumoniae, N. meningitides, P. multocida, S. agalactiae* and *S. pneumoniae*). No cross reactions were observed.

11. Annex – symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufactured by
Σ	Contains sufficient for <n> tests		Store at