BactoReal® Kit *Salmonella enterica*

Instructions for Use

IFU Version 2.0
Kit Version 2.0

For veterinary use only

REF	DVEB02313	Σ	100
REF	DVEB02353	Σ	50

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Explanation of symbols

LOT	Batch code		Use by date
REF	Catalogue number		Manufacturer
Σ	contains sufficient for <n> tests		Temperature limit (store at)
	Consult instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents

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1. Product description

BactoReal® Kit *Salmonella enterica* is a non-automated real-time PCR test for the qualitative detection of DNA of the invasion protein A (invA) gene of *Salmonella enterica* (*S. enterica*).

A probe-specific amplification-curve in the FAM channel (530 nm) indicates the amplification of *S. enterica* specific DNA. The internal DNA positive control (IPC) serves as a control for DNA extraction and possible real-time PCR inhibition and is detected in the Cy5 channel. The target for the DNA IPC (artificial target DNA) is added during sample extraction.

This test has been validated with the ABI® 7500 Fast Real-Time PCR System (fast cycle parameters are not supported, Thermo Fisher Scientific) and was also tested with LightCycler® 480 II (Roche Diagnostics) and Mx3005P® (Agilent). It is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM and Cy5 channel (eg. QuantStudio™ 5/6/7 Real-Time PCR System (Thermo Fisher Scientific), qTOWER^{3}G (Analytik Jena), Mic instrument (bio molecular systems), cobas z 480 Analyzer (Roche)).

When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Keep in mind that some PCR platforms first must be calibrated with the corresponding dye before performing multiplex-PCR.

Ingenetix ViroReal®, BactoReal®, MycoReal®, ParoReal® and PanReal products are optimized to run under the same thermal cycling conditions. RNA and DNA can be analysed in one run.

2. Pathogen information

Salmonella is a genus of Gram-negative bacteria of the *Enterobacteriaceae* family. This genus consists of only two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella bongori* mostly infects cold blooded animals. *Salmonella enterica* has six subspecies and more than 2500 serovars. Subspecies are *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *enterica*, *S. enterica* subsp. *houtenae*, *S. enterica* subsp. *indica* and *S. enterica* subsp. *salamae*. *Salmonella* infections in warm-blooded animals are generally caused by strains from *S. enterica* subspecies *enterica*. Typhoidal serovars include *Salmonella* Typhi and *Salmonella* Paratyphi, which only infect humans. Nontyphoidal serovars are more common, and usually cause self-limiting gastrointestinal disease. They can infect a range of animals, and are zoonotic. Although many other *Salmonella* serovars may cause enteric disease, the more common ones in cattle are *Salmonella Typhimurium, Salmonella Dublin* and *Salmonella Newport*. In sheep and goat the most common are *Salmonella Typhimurium, Salmonella Dublin, Salmonella Abortusovis, Salmonella Anatum* and *Salmonella Montevideo*. In pigs the most common are *Salmonella Typhimurium* and *Salmonella Choleraesuis*, and in poultry *Salmonella Enteritidis*, *Salmonella Typhimurium, Salmonella Gallinarum* and *Salmonella Pullorum*.

3. Principle of real-time PCR

The test is based on multiplex real-time PCR by 5'-nuclease-assay technology. Specific DNA sequences are amplified and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes. This allows the sequence-specific detection of PCR amplificates.

During PCR, primers are extended by Taq polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of Taq polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fuorescence wavelengths.

The Cq value (Cq = Quantification cycle, Ct = Cycle threshold, Cp = Crossing point) describes the cycle at which the fluorescence rises significantly above the background fluorescence.

4. Contents of the kit, stability and storage

Table 1. BactoReal® Kit *Salmonella enterica* order no. DVEB02313 or DVEB02353

Labelling	Content	Amount		Storage
		DVEB02313	DVEB02353	
Salmonella enterica + IPC3 Assay Mix (green cap)	Primer and probe for the detection of - *S. enterica* (**FAM**) - DNA IPC (**Cy5**)	2 x 50 µl	1 x 50 µl	-25 to -15 °C
IPC-Target (DNA) (orange cap)	Target for DNA IPC (internal DNA positive control system)	1 x 200 µl	1 x 200 µl	-25 to -15 °C
Salmonella enterica Positive Control (red cap)	DNA positive control (approx. 1000 target copies/µl)	1 x 300 µl	1 x 300 µl	-25 to -15 °C
DNA Reaction Mix (white cap)	PCR reaction mix for DNA amplification	2 x 500 µl	1 x 500 µl	**-25 to -15 °C after opening at 2 to 8 °C**
Nuclease-Free Water (blue cap)	Nuclease-free water	1 x 1000 µl	1 x 1000 µl	-25 to -15 °C

DNA Reaction Mix: The Reaction Mix provided with the kit has been designed for reliable, high-sensitivity real-time PCR. The Master Mix contains a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs with dUTP and Uracil-N-glycosylase (UNG) to eliminate amplicon carryover, ROX™ dye (passive reference) and buffer components – additives optimized to handle real-time PCR inhibitors.

Delivery and Storage: Shipment is with dry ice or with coolpacks. When stored properly, the kit components are stable until the specified expiration date. This also applies after opening. Store kit protected from light.

Quality Control Release Testing: In accordance with the ISO 13485 certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality. Quality control is performed with a linearised plasmid containing parts of the pathogen DNA.

5. Additionally required materials and devices

- Reagents and devices for DNA-extraction which are appropriate for the listed sample material (see Section 8)
- Nuclease-free water
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Vortex-Mixer
- Benchtop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument detecting and differentiating fluorescence in FAM and Cy5 channel (see Section 1)
- Appropriate 96-well optical reaction plates or reaction tubes with corresponding (optical) closing material
- Optional: Laminar flow box
- Optional: PCR workstation

6. Precautions and safety information

6.1. General Information

- The use of this kit is limited to qualified personnel instructed in real-time PCR.
- Transportation of specimens must comply with local regulations for the transport of biological materials.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Discard specimens, reagents, and waste according to your local safety regulations.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- The real-time PCR instrument should be calibrated, serviced, and cleaned regularly.
- Protect kit components from light.
- Avoid mixing reagents of different kits and lots and check expiry date of the kit.

6.2. Specific information

A workflow must be followed to avoid false-positive results due to detection of contaminating DNA.

Recommended measures to avoid DNA contamination

- Use separated workspaces for specimen preparation, set-up of real-time PCR and amplification. Supplies and equipment must be dedicated to each of these separate workspaces to ensure workflow in the laboratory from pre- to post-PCR.
- Laboratory benches and devices must be cleaned regularly.
- Sample preparation should be performed in a laminar flow box. Clean laminar flow box regularly in all areas.
- If possible, leave consumables and pipettes in the laminar flow box and PCR workstation.
- Preparation of real-time PCR should be done in a PCR workstation.
- The use of sterile aerosol-resistant pipette tips is required.
- Use only DNAse-free consumables.
- Wear lab coat.
- Work only with powder-free disposable gloves, do not touch the outer surface of the palm and fingers of the gloves when putting them on. Change gloves frequently. To avoid skin contact, wear gloves over the sleeves of the lab coat. Use disposable sleeve protectors if possible.
- Do not touch the rim or threads of open vials.
- Caution is advised when handling specimens and positive control to avoid cross-contamination.
- Store positive and potentially positive material separately from all other reagents.
- For a valid interpretation of results, a negative control shall be included during DNA extraction (e.g., extraction of water instead of sample material), to exclude false-positive results due to contamination with pathogen DNA during extraction.
- Optional: Include a negative control of PCR per PCR run (nuclease-free water instead of sample, NTC).

7. Limitations

- Reliable results with this test are only achieved by appropriate specimen collection, transport and storage, as well as an appropriate DNA extraction procedure.
- A negative test result does not exclude the possibility of infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or quantities below the assay sensitivity.
- For this kit highly specific primers and probes have been selected. However, false-negative results or results with low sensitivity (high Cq values) might be obtained due to sequence heterogeneity within the target region of yet unkown subtypes.
- Results should be interpreted in context of other laboratory findings.

8. Preparation of samples

BactoReal® Kit *Salmonella enterica* is suitable for analysis of DNA extracts of samples from swabs or feces.

Sample preparation should be performed with the recommended measures to avoid DNA contamination (see Section 6). Always include an extraction negative control during DNA extraction (e.g., extraction of water instead of sample material).

Purified DNA should be frozen at -25 to -15 °C for long-term storage.

8.1. Sample collection and storage

For short-term storage samples can be kept at 2 °C to 8 °C until further processing. If native samples need to be frozen prior to extraction add lysis buffer immediately upon thawing.

8.2. Recommended extraction methods

Ensure that the extraction system used is not contaminated with pathogen DNA detected with BactoReal® Kit *Salmonella enterica*. Extract samples with an DNA extraction system compatible with real-time PCR technology and appropriate for the sample material. Extract DNA from 100-200 µl sample (depending on the extraction method) and elute in approx. 50-100 µl.

For manual extraction recommended:

- NucleoSpin® VET, MACHEREY-NAGEL
- QIAamp® DNA Mini Kit, QIAGEN

For automated extraction recommended:

- NucleoMag® VET, MACHEREY-NAGEL
- Maxwell® RSC Pathogen Total Nucleic Acid Kit, Promega

When using extraction methods not recommended by ingenetix, an evaluation of the extraction method should be performed.

8.3. Quality control for DNA extraction and real-time PCR inhibition with IPC

The DNA IPC system (internal DNA positive control) is used as a control for DNA extraction, identifies possible real-time PCR inhibition and confirms the integrity of kit reagents. For this, an artificial DNA sequence (IPC-Target (DNA), approx. 10^5 copies/µl) is added during extraction.

NOTE

The Cq values of the IPC depend on the extraction method and on the type of sample material. Negative samples should show Cq values of the IPC of 28 ± 2. The extraction method used must be validated accordingly with sample material. Use the IPC-Target (DNA) freshly diluted (1:10 with nuclease-free water) in the extraction, if Cq values < 26 are determined during validation with samples.

8.4. Application of IPC as quality control of DNA extraction and real-time PCR

The IPC-Target (DNA) must be added during extraction.

→ Per sample spike 1 µl IPC-Target (DNA) (orange cap) directly into the appropriate volume of lysis buffer (or spike into the sample after the lysis buffer has been added), then continue the extraction procedure.

CAUTION
The IPC-Target (DNA) shall not be added to sample material in the absence of lysis buffer, as degradation may occur. It must be added to the lysis buffer.

8.5. Application of IPC as quality control for real-time PCR

If the IPC-Target (DNA) has not been added during extraction, it can be added at a later stage to the PCR master mix.

→ Freshly dilute the IPC-Target (DNA) 1:100 with nuclease-free water and add 1 µl of the dilution/PCR reaction.

CAUTION
The IPC-Target (DNA) shall not be added undiluted to the master mix.

9. Preparation of real-time PCR

- Include one Positive Control (red cap), one extraction negative control and optionally one negative control of PCR (NTC, e.g., nuclease-free water) per PCR run.
- It is generally recommended to analyse samples in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.
- Best use DNA shortly after extraction and always keep on ice. Alternatively, use DNA stored at -25 °C to -15 °C. Thaw DNA on ice, avoid prolonged exposure to room temperature and refreeze after use.
- Thaw kit components completely at room temperature. When thawed, mix the DNA Reaction Mix gently by inverting 2 to 3 times to ensure a homogenous solution. Mix the other kit components carefully and centrifuge briefly with low speed.
- **Positive Control**
  Use 9 µl of the Positive Control (red cap). Always, pipette Positive Control at last. It should be stored at -25 °C to -15 °C. Before use, gently mix the Positive Control, do not vortex. To avoid frequent freeze/thaw cycles, it can also be temporarily stored at 2 °C to 8 °C if used several times on the same day.

9.1. Pipetting scheme

		per reaction
Preparation of Master Mix (mix well)	DNA Reaction Mix	10.0 µl
	Salmonella enterica + IPC3 Assay Mix	1.0 µl
	Total volume Master Mix	**11.0 µl**
Preparation of PCR	Master Mix	11.0 µl
	DNA-sample*	9.0 µl
	Total volume	**20.0 µl**
*9 µl sample can be used. When using a volume other than 9 µl, the final volume has to be adjusted accordingly with nuclease free water.		

If IPC-Target (DNA) was not added during extraction: Freshly dilute the IPC-Target (DNA) (orange cap) 1:100 with nuclease-free water and add 1 µl per reaction directly to the master mix. In this case, the IPC-Target (DNA) is used as a quality control for the real-time PCR reaction (see Section 8.5).

- Prepare the Master Mix according to the number of PCR reactions, calculating an additional volume of approx. 10% for pipetting loss.
- Pipette 11 µl of the prepared Master Mix per reaction into the well of the optical reaction plate.
- Then add 9 µl of the extracted sample or controls. Pipette the 9 µl of Positive Control at last.
- Seal the plate with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

9.2. Programming of temperature profile

Further information on programming of the real-time PCR instrument can be found in the respective operator's manual. Keep in mind that some PCR-platforms must be calibrated with the corresponding dye before performing a multiplex-PCR.

Reaction volume: 20 µl

Ramp speed: "Standard" without "fast cycling" parameter for ABI® 7500 Fast Instrument, QuantStudio™ (Thermo Fisher Scientific)

Program 1 Cycles: 1 Analysis: None	Program 2 Cycles: 1 Analysis: None	Program 3 Cycles: 45 Analysis: Quantification Acquisition at 60°C
50 °C 2 min	95 °C 2 min	95 °C 5 sec 60 °C 60 sec

Add. program 1: If viral RNA should be also detected in the same PCR run, program 1 must be prolonged to 15 min at 50 °C. This temperature profile can be used for all ingenetix ViroReal®, BactoReal®, MycoReal®, ParoReal® and PanReal kits for the detection of DNA or RNA.

Add. program 2: the previous temperature profile with 20 sec in program 2 can still be used.

Select detection channels

FAM channel: Detection of *S. enterica* **Cy5 channel**: Detection of DNA IPC
For ABI® 7500 instrument, QuantStudio™ 5/6/7 (ThermoFisher Scientific), Mx3005P® (Agilent) FAM-TAMRA Cy5-NONE Passive reference dye: ROX
For CFX Opus Real-time PCR System (Bio-Rad), CFX96 Real-time PCR System (Bio-Rad), T-COR 8 (Tetracore) FAM Cy5
For MIC instrument (bio molecular systems) FAM: Green Cy5: Red

For cobas z 480 Analyzer / LightCycler® 480 II (Roche)
Detection format: 2 Color Hydrolysis Probe
FAM: Excitation at 465 nm, Emission at 510 nm
Cy5: Excitation at 610 / 618 nm, Emission at 670 / 660 nm

10. Interpretation of data

For information on the fluorescence display options for the pathogen and the IPC see Section 9.2.

IMPORTANT
Samples should be checked in both logarithmic (Roche instrument: Abs Quant/Fit Points) and linear view and compared to the negative control. In addition to the Cq values, also check the amplification curves and adjust the threshold (noise band) if necessary. After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Assessment of sample test results should be performed after data of the positive and negative control and of the IPC have been examined and determined to be valid. If results of controls are not valid, no interpretation of results with samples is possible.

Negative samples: Samples without amplification curves (no Cq values, undetermined) are regarded as negative. No DNA was detected in these samples because there is no infection with the corresponding pathogen or the pathogen DNA concentration is below the detection limit of the test.

Positive samples: Samples with Cq values < 45 in the fluorescence channel for the pathogen target are regarded as positive. Table 2 shows the criteria for valid postive and negative controls. Table 3 shows interpretation of data with samples.

10.1. Controls

Table 2. Criteria for valid controls, IPC-Target (DNA) was added during extraction

	Cq pathogen *S. enterica* FAM channel	Cq IPC Cy5[1]	Interpretation	Action
Positive Control	< 30	Negative	Valid	-
Positive Control	Negative	Negative	Invalid	see Section 11.1
Extraction negative control	Negative	28 ± 2[2]	Valid	-
Extraction negative control	Negative	Negative	Invalid	see Section 11.1
Extraction negative control	Positive	28 ± 2[2]	Invalid	see Section 11.3
PCR negative control (NTC, optional)	Negative	Negative	Valid	-
PCR negative control (NTC, optional)	Positive	Negative	Invalid	see Section 11.2

[1] If the IPC-Target (DNA) was added directly to the master mix, all samples in the Cy5 channel must be positive.
[2] Cq values of the IPC are dependent on the extraction method and should show Cq values of 28 ± 2 (see Section 8).

10.2. Samples

Samples with positive Cq-values are considered positive, see Table 3.

Table 3. Interpretation of data with samples

	Cq pathogen *S. enterica* FAM channel	Cq IPC Cy5[1]	Interpretation	Action
Sample	Negative	28 ± 2	Negative	-
Sample	Positive	28 ± 2[2]	Positive	-
Sample	Negative	Negative	Invalid	see Section 11.4

[1] A positive signal of the IPC excludes potential PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- values can indicate a partial inhibition of PCR. Cq values of the IPC are dependent on the extraction method and should show Cq values of 28 ± 2 (see Section 8).
[2] High pathogen load in the sample can lead to a reduced or absent fluorescence signal of the IPC.

In case of invalid data, analysis must be repeated with the remaining or newly extracted DNA sample (see Section 11).

11. Troubleshooting

11.1. No signal with Positive Control and IPC

- Incorrect programming of the temperature profile or incorrect setting of detection channels on the real-time PCR instrument.
  - Compare temperature profile and setting of detection channels with details specified in the protocol.
- Incorrect configuration of the PCR reaction.
  - Check your pipetting steps with the pipetting scheme and repeat PCR, if necessary.
- For control of DNA extraction and PCR inhibition, the undiluted IPC-Target (DNA) must be added during extraction to the lysis buffer. If no IPC-Target (DNA) was added to lysis buffer during extraction:
  - Repeat DNA extraction.
- For control of the real-time PCR only, 1 µl of freshly 1:100 diluted IPC-Target (DNA) has to be added to the mastermix. If no IPC-Target (DNA) was added to the Master Mix:
  - Freshly dilute IPC-Target (DNA) and repeat PCR.
- The IPC-Target (DNA) was added undiluted directly to the Master Mix and not freshly diluted 1:100.
  - Freshly dilute IPC-Target (DNA) 1:100 and repeat PCR.
- No Positive Control was added.
  - Repeat PCR in case all samples are negative.
- The DNA may have been degraded. Thawing frozen samples releases DNAses, causing degradation.
  - Repeat DNA extraction. Start lysis right away and let the samples thaw upon lysis incubation.

11.2. Pathogen specific signal with negative control of PCR

- A contamination occured during preparation of the real-time PCR reaction.
  - Repeat the real-time PCR with new reagents in replicates.
  - Strictly pipette Positive Control at last.
  - Make sure that workspace and instruments are cleaned at regular intervals.

11.3. Pathogen specific signal with negative control of extraction

- A contamination occurred during extraction.
  - Repeat DNA extraction and real-time PCR using new reagents.
  - Make sure that workspace and instruments are cleaned at regular intervals.

11.4. No signal with IPC

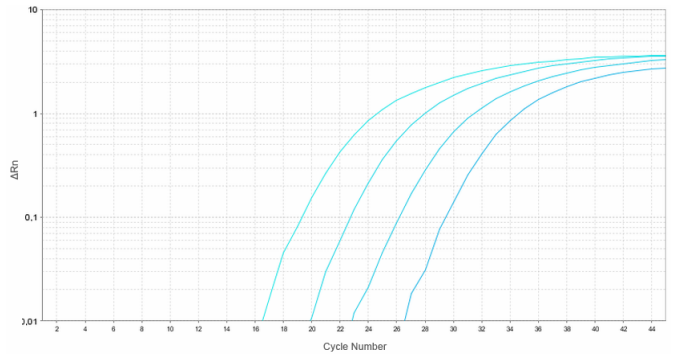
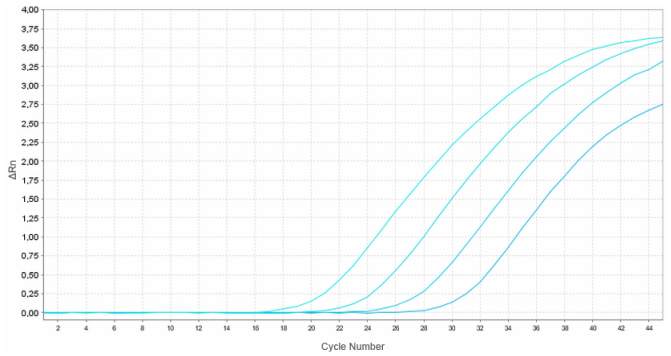
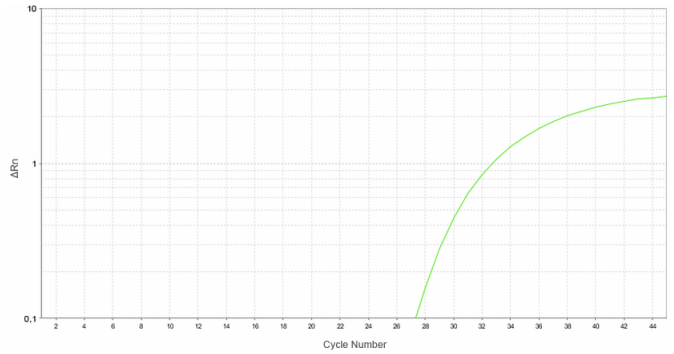
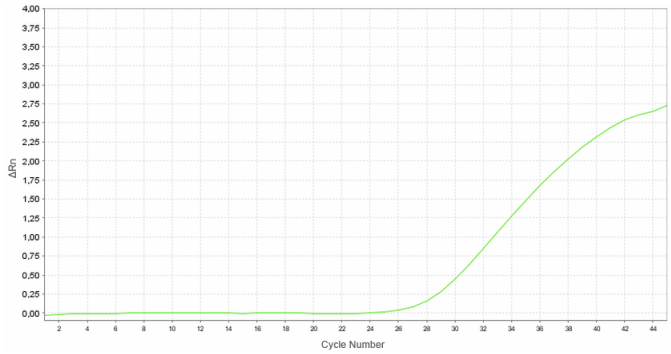
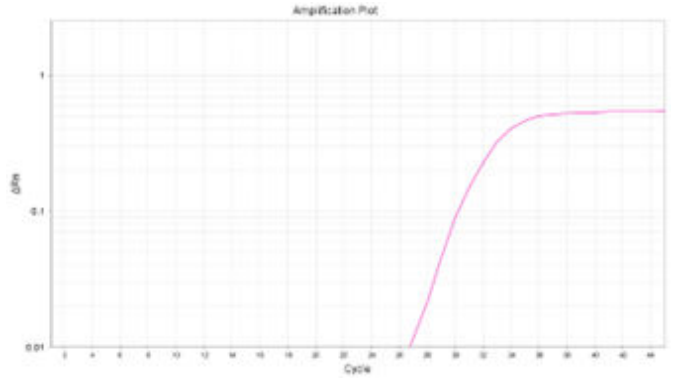
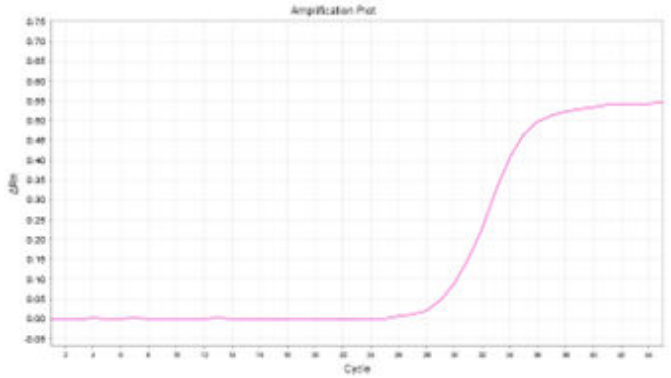
- Incorrect assignment of detection channels in sample.
  - Please verify the correct assignment of detection channels.
- Incorrect real-time PCR conditions.
  - Check the real-time PCR conditions and repeat the real-time PCR, if necessary.
- The DNA might be degraded.
  - Repeat DNA extraction.
- If IPC-Target (DNA) was added during extraction:
  - Inhibition of real-time PCR may have occurred.
  - DNA extraction was unsuccessful.
  - The IPC-Target (DNA) was not added to the lysis buffer of the sample.
  - The extracted sample was not added to the real-time PCR reaction.
  - No statement is possible. Verify you use a recommended method for DNA isolation and re-examine the single steps of the DNA extraction.
- If no operating mistakes during DNA extractions can be retraced, it is recommended to repeat the real-time PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of nuclease-free water).

12. Specifications and performance evaluation

12.1. Test performance on different real-time PCR instruments

Performance of BactoReal® Kit *Salmonella enterica* with an ABI® 7500 Real-time PCR System (Thermo Fisher Scientific) is shown in Table 4.

Table 4. Performance data

Logarithmic view	Linear view
	
ABI® 7500: FAM channel 1:10 serial dilution of an *S. enterica* DNA target	
	
ABI® 7500: FAM channel *S. enterica* Positive Control	
	
ABI® 7500: Cy5 channel DNA IPC System	

This test has been validated with the ABI® 7500 Fast Real-Time PCR System (fast cycle parameters are not supported, Thermo Fisher Scientific) and tested with LightCycler® 480 II (Roche Diagnostics) and Mx3005P® (Agilent), but is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, and Cy5 channel.

12.2. Analytical test performance

Analytical sensitivity: The analytical sensitivity is 7 target copies/PCR reaction. The limit of detection (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) is 19 target copies/reaction and was determined by several replicates around the detection limit.

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 *Salmonella enterica* strains. The kit is specific for *Salmonella enterica*. *Salmonella bongori* is detected with less sensitivity.

13. References

Wilkins, W et al. “Comparison of bacterial culture and real-time PCR for the detection of Salmonella in grow-finish pigs in Western Canada using a Bayesian approach.” Zoonoses and public health vol. 57 Suppl 1 (2010): 115-20. doi:10.1111/j.1863-2378.2010.01365.x

Mainali, C et al. “Validation of a real-time polymerase chain reaction assay for the detection of Salmonella in crops of broiler chickens.” Poultry science vol. 90,3 (2011): 660-4. doi:10.3382/ps.2010-00634

Mainar-Jaime, R C et al. “Estimation of the diagnostic accuracy of the invA-gene-based PCR technique and a bacteriological culture for the detection of Salmonella spp. in caecal content from slaughtered pigs using Bayesian analysis.” Zoonoses and public health vol. 55,2 (2008): 112-8. doi:10.1111/j.1863-2378.2007.01096.x

14. Revision History

Version	Date	Info
2.0	2024-03-31	General Update