



PCRFast[®]
Salmonellen / Salmonella

Realtime (Sonde)
Realtime (probe)

IF/MR1001

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PCRFast[®] Salmonella Realtime (probe)

Brief information

Simple, molecular biological test (PCR) for detecting salmonella in realtime (probe with Fam reporter and non fluorescence quencher) in foodstuffs and animal feed (detection of the invA gene). The test kit has 96 reaction vials, each reaction vial contains a specific primer pair the probe and an internal amplification control (probe with a HEX reporter and non fluorescence quencher) for investigating possible inhibiting effects (ITC).

8 reaction vials (red marking) contain additionally salmonella DNA for PCR positive controls (PTC).

Time required:	enrichment.....	approx. 24 h
(10 samples)	extraction.....	25 min
	PCR setup.....	15 min
	PCR.....	2 h

PCRFast[®]
ist ein eingetragenes Warenzeichen der ifp Institut für Produktqualität GmbH.
ifp führt auch Auftragsanalytik durch.

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is a registered trademark of ifp Institut für Produktqualität GmbH.
ifp also offers contract analyses.

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1. Principle of the test

PCRFast® Salmonella is a simple, molecular biological test (PCR) for detecting salmonella in foodstuff and animal feed (detection of the *invA* gene). The analytical procedure described here complies with the international standards (ISO) for PCR analysis and the § 64 of the German Foodstuffs and Animal Feed Code (LFGB).

All the reaction vials contain the for the PCR reaction necessary specific primers and probe in an optimum amount and an **internal amplification control ITC** for investigating inhibitory effects.

The **colourless reaction vials** are used for the detection of the **specific DNA** extracted from the sample, for the **negative controls NTC** (checking the MasterMix for contamination) and for the **extraction control ETC** (check on extraction for contamination).

In addition to the primers, **the red – coloured reaction vials** contain **salmonella DNA**. These reaction vials are used for the **positive control PTC** (check on functionality of MasterMix).

12.5 µl of double concentrated MasterMix and, subsequently, **12.5 µl of the extracted DNA sample** is added into the reaction vial. The MasterMix contains an optimum concentration of polymerase, nucleotides and magnesium chloride. The target sequence is then amplified in a PCR thermocycler. Is the target present the probe hybridises on the built up DNA fragments. Thereby the reporter (Fam/HEX) and the quencher (non fluorescence) of the probe will be separated and the reporter radiates a fluorescence signal (Fam: 520 nm, HEX: 553 nm). The intensity of the signal increases with the amount of synthesised DNA which could be measured and displayed. If the target sequence is not present no increase in the fluorescence is detectable.

2. Package contents

The test kit contains:

- 11 x **strips (colourless)** with eight 0.2 ml reaction vials each (88 reaction vials), coated with specific primers, probe (for sample determinations and for the negative controls NTC) and an internal amplification control.
- 1 x **strips (red)** with eight 0.2 ml reaction vials each (8 reaction vials), coated with specific primers, probe and additionally salmonella DNA (for the positive controls PTC) and an internal amplification control.

3. Additionally required instruments and reagents

Lab material and instruments

- homogenizer (Stomacher)
- stomacher bag, sterile
- measuring cylinder, sterile
- erlenmeyer flask, sterile
- culture tube, sterile
- 37 °C (98.6 °F) and 42 °C (107.6 °F) incubator
- water bath or heating block 95 °C (203 °F)
- vortexer
- biocentrifuge, min. 14,000 x g (for 1.5 ml and 2.0 ml reaction vials)
- PCR thermocycler:**
 - e.g. Applied Biosystems 7500, STRATAGENE Mx 3005 P, Mx 3000 P
- microliter pipettes 2 - 20 µl, 20 - 200 µl, 100 - 1,000 µl, e.g. Gilson Pipetman P with filter tips
- 1.5 ml or 2.0 ml reaction vials

Reagents

Enrichment

- pre - enrichment: peptone water, buffered
- selective enrichment: Rappaport - Vassiliadis (RVS)

DNA extraction

- ethanol >95 % (denatured)
- 0.1 x TE - buffer (TRIS 1 mmol / l, EDTA 0.1 mmol / l)
- water deionised, DNA free
- DNA-extraction kit, e.g. PCRFast[®] Microbe Extraction

PCR

Realtime

double concentrated MasterMix (Hotstart recommended),
e.g. AmpliTaq Gold® PCR MasterMix, Applied Biosystems No. 4318739;
Brilliant® II QPCR MasterMix, Stratagene No. 600804; QuantiTect Probe
PCR Kit, Qiagen No. 204343

4. Precautions

- all tasks should be performed taking all precautions common in labs, and all PCR tasks should be performed in accordance with the CEN / ISO recommendations
- to avoid carryovers, perform all work wearing protective gloves and use filter tips
- perform sample preparation, PCR setup and detection in separate rooms

5. Storage instructions

Store the test kit and the reaction vials at 2 - 8 °C (35.6 - 46.4 °F).

6. Sample enrichment and DNA isolation

6.1. Enrichment

Foodstuff samples are enriched according to § 64 of the German Foodstuffs and Animal Feed Code (LFGB). With each analyzing series one sample without matrix should be done as an extraction control. A positive extraction control indicates a contamination in the reagents.

6.1.1. Pre - enrichment

25 g (ml) of a foodstuff sample is weighed in a sterile stomacher bag, diluted at a ratio of 1 : 10 (w / v) with peptone water (buffered) and incubated at 37 °C (98.6 °F) for 18 h (e.g. 25 g sample + 225 ml peptone water, buffered).

6.1.2. Selective enrichment

0.1 ml of the pre - enrichment from 6.1.1 is transferred to a culture tube with 10 ml RVS broth and incubated at 42 °C (107.6 °F) for 5 - 6 h.

6.2. DNA isolation

Thermal Extraction procedure

- 1) for DNA - extraction take 1 ml of the enrichment culture (6.1.2.) and centrifuge at 14,000 x g for 10 min, discard supernatant
- 2) resuspend the pellet in 200 µl 0,1 x TE – buffer, centrifuge at 14,000 x g for 10 min, discard supernatant
- 3) resuspend the pellet again in 200 µl 0,1 x TE – buffer
- 4) thermal lysis: heat the pellet for 15 min. at 95 °C (203 °F)
- 5) cool down the solution (e.g. 1 - 2 min at - 20 °C (- 4 °F))
- 6) mix the solution and centrifuge at 14,000 x g for 5 min

After the extraction the supernatant has to be diluted with a ratio of 1 : 10 with 0.1 x TE – buffer and can be used for the PCRFast[®] reaction (e.g. 10 µl supernatant + 90 µl 0,1 x TE – buffer).

Also conventional extraction systems can be used for the DNA – extraction (e.g. PCRFast[®] Microbe Extraction).

6.3. Confirming suspect colonies

To confirm suspect colonies on solid culture medium, it suffices to slurry one loop of this colony in 0.2 ml of deionized water and heat it at 95 °C (203 °F) for 10 min. The sample is then cooled and centrifuged at 14,000 x g for 5 min. The supernatant can be used directly for PCRFast[®].

7. PCR setup

7.1. Preparations

- remove strip from the plastic bag and separate required number of reaction vials
- place the remaining strips / reaction vials and the desiccant back into the bag, seal bag and store at 2 - 8 °C (35.6 - 46.4 °F)
- have the required amount of MasterMix at hand (12.5 µl per reaction)

7.2. Procedure

–pipette the following volumes into the reaction vials:

per sample	reaction vial	MasterMix	DNA from sample
	colourless	12.5 µl	12.5 µl
inhibition control ITC	red	12.5 µl	12.5 µl
per analysis run	reaction vial	Master-Mix	0.1 x TE buffer
negative control NTC	colourless	12,5 µl	12.5 µl
positive control PTC	red	12.5 µl	12.5 µl

Tab. 1: PCRFast[®] Salmonella pipetting steps

–close the reaction vials (centrifuging recommended) and place them into the PCR thermocycler.

7.4. Instrument settings

Reporter: Fam and HEX
Quencher: non fluorescence
Reference dye: ROX (depending on used master mix)

Perform amplification according to the following temperature / cycler profile:

Cycler profile

10 min	95 °C (203 °F)	
30 sec	95 °C (203 °F)	
45 sec	60 °C (140 °F)	35 cycles
30 sec	72 °C (161.6 °F)	

Note:

Validation was performed using TaqMan[®] Universal PCR Master Mix by Applied Biosystems and Brilliant[®] II QPCR MasterMix by Stratagene and the

thermocyclers specified in section 3. The specified cycler profile may need to be adjusted to the respective instrument and the MasterMix.

7.5. Detection

- assessment of the amplification in realtime is done via the amplification plot
- in principle the evaluation should be done using the software of the realtime thermocycler. Are the results not satisfactory using the instrument presettings, the threshold and the baseline settings could be changed

8. Evaluation

The following table summarizes the evaluation. With a positive sample the final point of the fluorescence curve lies clearly above the threshold.

8.1. Evaluation matrix

Sample	Inhibition control (ITC)	Negative control NTC	Positive control PTC	Result
+	+			sample positive
no amplification	+			sample negative
no amplification	no amplification			inhibition*
		no amplification		MasterMix not contaminateded
			+	MasterMix functional

* dilute extracted sample DNA once more and amplify again

Tab. 3: Evaluation of PCRFast® Salmonella

8.2.2. Examples of amplification plot evaluation

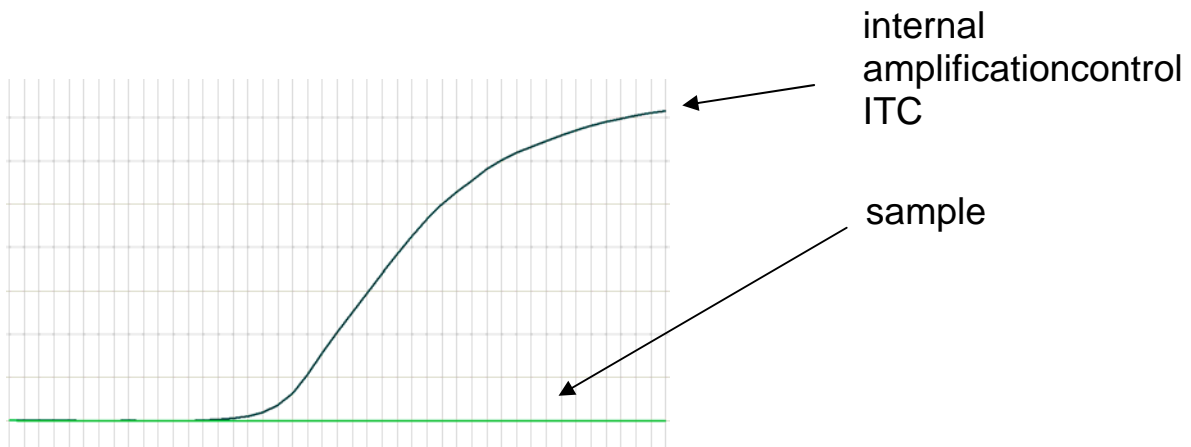


Fig. 1: sample negative; sample without amplification plot

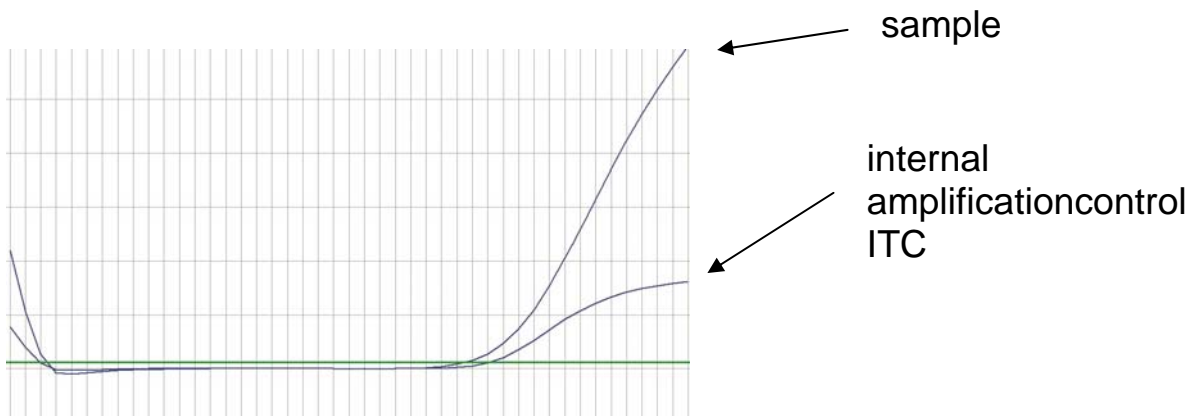


Fig. 2: sample positive

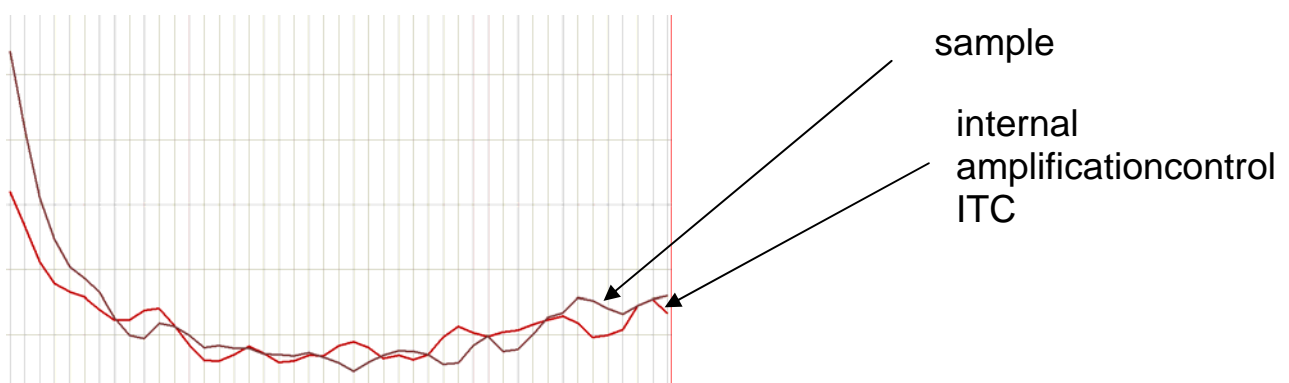


Fig. 3: reaction inhibition, further diluting the isolated DNA

9. Sensitivity

Limit of detection: 1 salmonella in 25 g sample.

10. Specificity

PCRFast[®] Salmonella is 100% specific. The following species have each been tested for cross-reactivity with at least 2500 copies:

Species		Species		Species	
Salmonella	+	Listeria monocytogenes	-	Campylobacter jejuni	-
Escherichia coli	-	Enterobacter sakazakii	-	Legionella pneumophilia	-
Staphylococcus aureus	-	Bacillus cereus	-	Yersinia enterocolitica	-
Clostridium perfringens	-	Legionella erythra	-	Shigella flexneri	-
VTEC stx1	-	VTEC stx2	-		

Tab. 4: Specificity of PCRFast[®] Salmonella

+ amplification

+ no amplification

Should you have any questions on how to conduct this test, on sample preparations or on PCR analysis in general, please contact the Competence Centre for DNA Analysis of ifp, Institut für Produktqualität.

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You will also find more information on conducting PCRFast[®] at www.produktqualitaet.com.

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11. References

ISO 6579

Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.

ISO 22174

Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of food - borne pathogens - General requirements and definitions

ISO 20837

Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of food - borne pathogens - Requirements for sample preparation and qualitative detection

ISO 20838

Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of food - borne pathogens - Requirements for amplification and detection for qualitative methods

Official collection of analysis methods after §64 LFGB

L 00.00 - 98 „Method for qualitative detection of *Salmonella* in food by Real-time PCR“

Stark, R., Mäde, D.

„Nachweis von *Salmonellen* in Lebensmitteln“

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