Salmofast®

Real-time PCR-based assay for the detection of *Salmonella* spp.

Products for human consumption and animal feeding
Environmental samples in food production and food handling

INTRODUCTION

Salmonella is a bacterium that can cause food poisoning in humans and animals. It is commonly found in raw or undercooked meats, eggs, and contaminated water or soil. Symptoms can include fever, diarrhea, abdominal cramps, and vomiting, which can be prevented through proper food handling and hygiene practices.

Salmonellosis is a major global health problem affecting millions yearly. *Salmonella* can be found in contaminated food and water sources, and the disease is most prevalent in low- and middle-income countries. In severe cases, salmonellosis can be fatal. Proper food handling and hygiene practices can help prevent the spread of this disease.

Salmofast® has been specifically designed to provide a simple and reliable method for detecting the presence of *Salmonella* in various samples by real-time PCR. The kit contains all the necessary PCR amplification and detection components: master mix including primers and probes, and positive and negative controls for quality assurance. This user-friendly kit allows for accurate and sensitive detection of *Salmonella* in various sample types, including food, water, and environmental swabs. Please read the instructions carefully before proceeding with the assay to ensure accurate and reliable results.

PRINCIPLE

Salmofast® is based on detecting a specific sequence in a target gene in all *Salmonella* species using real-time PCR. A positive amplification confirms the presence of these microorganisms in the sample analyzed.

Salmofast® can also be used to confirm colonies that grow on agar plates.

COMPONENTS

Presentation

Salmofast® is presented in vials containing enough reagents to perform 48 or 96 reactions and positive and negative controls for quality assurance.

Supplied Reagents

Reaction Mix

The Reaction Mix contains the appropriate amounts of buffer, dNTPs, Hot-start DNA polymerase, oligonucleotides, sterile bidistilled DNA-free water, and $MgCl_2$ to perform the number of reactions indicated in the box. The Reaction Mix includes an internal amplification control (IAC), whose detection shows the absence of PCR inhibitors. Salmonella and IAC detection probes are labeled with CY5 and HEX/VIC fluorophores, respectively. The Reaction Mix does not contain ROX.

DNAready

Protein K-containing Lysis buffer.

Controls

Positive control with target DNA from *Salmonella* and non-template control (DNA-free water) are included.

Conservation

Store at -20ºC until the product's expiry date on the package. Distributing the master mix by pipetting 15 µl into PCR tubes upon reception to avoid repeated freeze and thaw cycles is highly recommended. Consider to aliquot C+, as well. Work in a DNA-free space.

Materials required but not supplied

· Incubators
. Real-time Thermocyclers

Manufacturer	Equipment	Catalog. Num.
Applied Biosystems	QuantStudio 5	A36328
Biorad	CFX96 Touch Real-Time PCR Detection System	185-5095 184-5096
	CFX Opus DeepWell	17007992

. Salmonella Latex Agglutination. Oxoid. Ref. DR1108A
. Oxoid™ Brilliance™ Salmonella agar Ref. PO5098
. Piston or positive displacement pipettes and filter tips.
. Microcentrifuge and PCR tubes/strips.
. Disposable powder-free examination gloves

PCR PROTOCOL

Thaw the required number of reaction mix PCR tubes and controls included in the kit.

Load 5 µl of the samples DNA extraction and positive and non-template controls (C+ and NTC). Avoid exposing reagents to direct light.

PCR setup and analysis

Use the following software and version for each thermocycler

- **QuantStudio 5**: QuantStudio™ Design & Analysis v. 1.5.2
- **Biorad CFX96**: CFX Manager™ Software v. 3.1
- **Biorad CFX Opus DeepWell** CFX Manager™ Software v. 3.1

See Annex 1 for detailed setup and PCR analysis instructions.

Place the PCR tubes into the real-time thermocycler. Set the fluorescence reading at the channels corresponding to the fluorophores CY5 and HEX (Biorad CFX96, CFX Opus DeepWell) or VIC (QS 5). Use the following program to perform the amplification:

PCR program

Step	Event	Temperature	Time
1	DNA polymerase activation and initial nucleic acids denaturing	95 ºC	10 minutes
2	Denaturing	95 ºC	15 seconds
(40 cycles)	Hybridization/extension	60 ºC	1 minute*

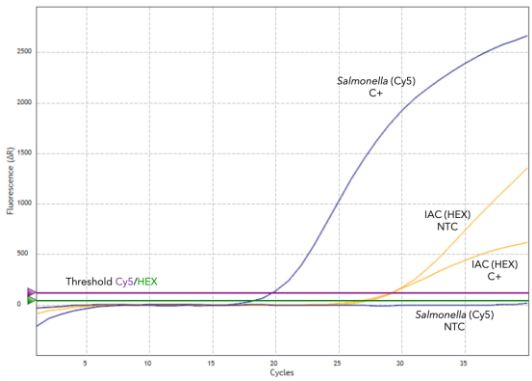
*Fluorescence reading (channels Cy5 and HEX/VIC at the end of each Hybridization / Extension step at 60ºC.

The name of the optical channels for the fluorochromes used may vary depending on the thermocycler equipment used. Excitation and emission values for the included fluorophores are:

	IAC-HEX	Salmonella – Cy5
Excitation	530 nm	646 nm
Emission	556 nm	669 nm

Results and interpretation

Select the appropriate fluorescent channel to check DNA amplification from both *Salmonella* (Cy5) and IAC (HEX or VIC).



The result of *Salmonella* detection by PCR is positive whenever the amplification curve of the Cy5 channel crosses the threshold established by the software. The lack of IAC amplification is only relevant for interpreting negative results.

For a proper interpretation of negative *Salmonella* PCR results, inhibition must be discarded by checking that IAC amplifies correctly. IAC amplification in negative samples must display a Ct value not exceeding 3 cycles that of the NTC reaction.

When inhibition is detected, the PCR must be repeated using a 1/10 dilution of the original DNA extract. Dilute with Molecular Biology grade water.

QUALITY ASSURANCE

For results quality assurance, follow ISO 22174 "Microbiology of food and animal feeding stuff - Polymerase chain reaction (PCR) for detecting foodborne pathogens - General requirements and definitions."

Good Laboratory Practice must be observed to obtain reliable results with this technique. The high sensitivity of this test requires extreme care to maintain the purity of all reagents. Avoid contaminants from the environment (both DNA and nucleases) and discard all suspicious reagents. The use of this product is limited to qualified personnel.

Do not use Salmofast® after expiry or best before date. Store this product at the indicated temperature and conditions. Do not interchange components from different lots.

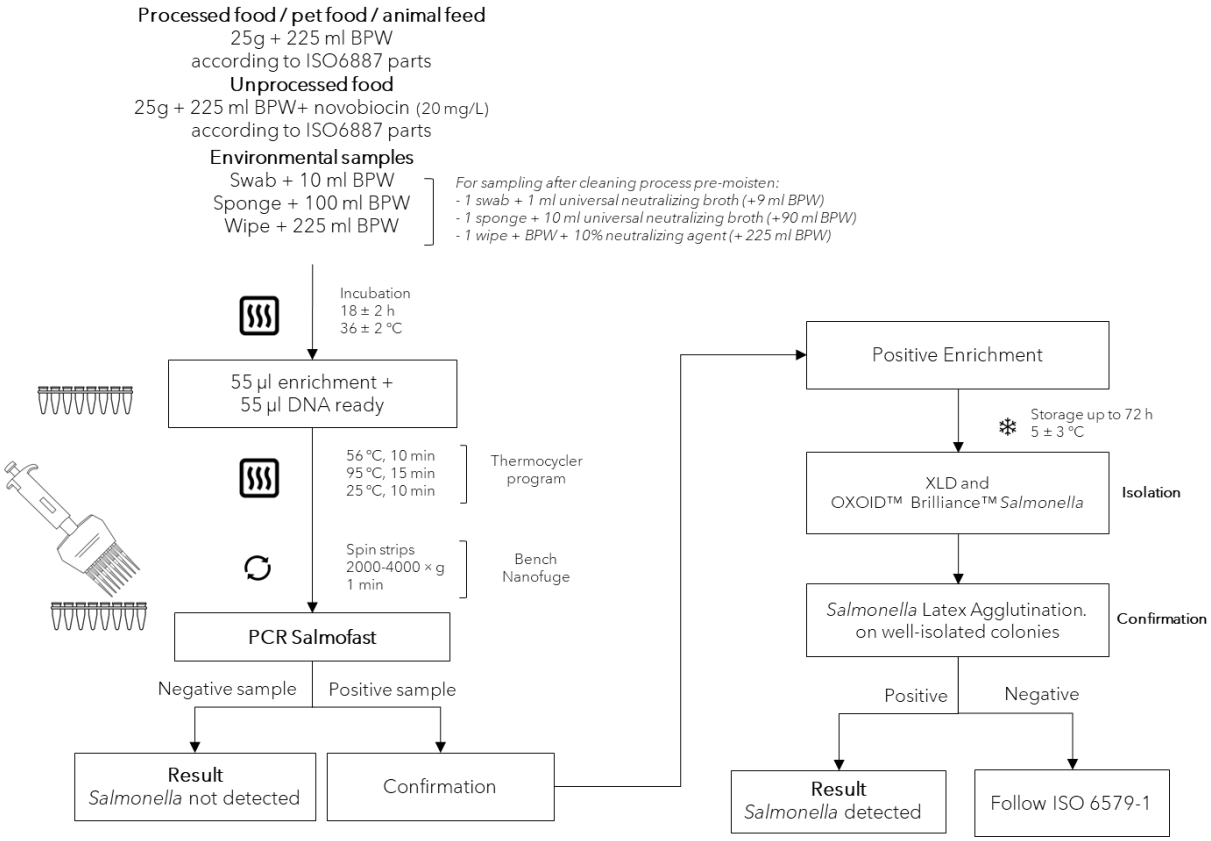
INCLUSIVITY AND EXCLUSIVITY

The inclusivity of Salmofast® primers and probes has been satisfactorily assayed with 628 reference strains of *Salmonella*, mostly foodborne and environmental. Exclusivity has been checked with 146 non-target strains.

DISCLAIMER

This test has been designed to investigate the presence of *Salmonella* in environmental and food samples or for other purposes related to R&D. Do not use Salmofast® as a diagnostic tool in clinical samples.

PROTOCOL for the detection of *Salmonella* in food, animal feed, and environmental samples from food production and handling



TROUBLESHOOTING

Problem	Cause	Solution
Neither *Salmonella* nor IAC-specific signals are detected.	PCR inhibition.	Dilute the DNA extract 1/10 and repeat the analysis.
	Inadequate storage of the Reaction Mix.	Store the Reaction Mix at the recommended temperature and avoid direct contact with light. Check the expiry date.
Salmonella-specific amplification is detected in non-template control (NTC).	Contamination of materials or reagents.	Repeat the analysis with fresh reagents and cleaned pipettes. Wash surfaces with freshly diluted bleach (10%) or a similar reagent. If contamination persists, contact our Technical Department.
No amplification of *Salmonella* is obtained in positive control tubes.	If no IAC signal is observed: bad storage of the Reaction Mix.	Store the Reaction Mix at the recommended temperature and avoid contact with light. Repeat the analysis with fresh reagents.
	If an IAC signal is detected: pipetting error or positive control degradation.	Repeat the analysis and ensure an appropriate positive control is added to the corresponding tube.
Amplification curves are not sigmoid-shaped. "Aberrant" curves	The reaction tube is not completely closed.	Repeat the reaction and make sure the tube is hermetically closed.
	Non-optimal baseline defined by the software	Change the *begin* and *end* cycles for the Baseline calculation, as required.

Annex 1

Instructions:
Operation of thermocyclers Applied Biosystems QuantStudio 5, Biorad CFX96 Touch Real-Time PCR Detection System and Biorad CFX Opus DeepWell PCR System for the analysis of *Salmonella* using Salmofast

Contenido

1. Applied Biosystems QuantStudio 5

1.1. Plate setup

Software version:

- Setup: QuantStudio™ Design&Analysys v.1.5.2
- Post PCR data analysis: z™ Design&Analysys v.2.6.0

First time setting up a plate for the analysis of Salmonella
Make sure the computer is connected to your QuantStudio™ 5
Click on "Create New Experiment."

"Properties"

Select the following options

- *Instrument type*: QuantStudio™5 System
- *Block type*: 96-Well 0.1 ml
- *Experiment type*: Presence/Absence
- *Chemistry*: Taqman® Reagents
- *Run mode*: Standard

"Method"

Set up the thermal profile
Check the Reaction volume (20.0 ul) and the Heated Cover Temperature 105.0 °C
Set two stages

1. Hold Stage: 10 min at 95 °C
2. PCR Stage: (40 cycles) 15 seconds at 95 °C and 1 min at 60 °C (data collection)
3. Post-Read Stage: leave it as is

"Plate"

"Plate Attributes" section: set "Passive reference" to "None"
Click on the "Advanced Setup" tab
On the "Targets" box, add a new target to make two. Click each target to edit with the following parameters

- Target 1:
  - Name: Salmonella
  - Reporter: CY5
  - Quencher: NFQ-MGB
- Target 2:
  - Name: IAC
  - Reporter: VIC
  - Quencher: NFQ-MGB

On the plate representation, select the sample-containing wells. Assign both targets by clicking on the respective checkboxes. On the "**task** "column, select "Unknown **U**" and internal control: **I** for Salmonella and IAC targets, respectively. Select the Salmonella target task as **N** for the NTC well (no template control). The names of both targets (Salmonella and IAC) should appear inside the cell.

To assign cell names, select the cell and click **Add** to generate individual sample names or import from the **Action** drop-down menu following the software instructions.

"Run"

Click "START RUN" and follow the prompted options to save the file.

1.2. Results Analysis and Interpretation

Software: QuantStudio™ Design&Analysys v.2.6.0

Open the file generated after the QuantStudio™ run (".eds" extension) (**Open file).**
Click on the "Analyze" button. A green tick should appear in the top-right corner of the button.

Click on the "**Run Summary**" Tab to ensure no issues have occurred during the run.

Result Interpretation
Click on the "**Presence Absence**" Menu tab.
Both the table with the call and the amplification plots are visible.
Filter for Salmonella channel (Cy5).
Check that NTC does not produce amplification for this channel.
The presence/absence of Salmonella is shown in the corresponding column with the Cq value (threshold crossing) in case of positive amplification.

If no Cy5 amplification is produced for the sample to be considered negative (absence of Salmonella), check the corresponding result for IAC (VIC channel) and make sure that amplification has been produced with a Cq value that is lower or equal to that of NTC + 3 Cq units.

For general criteria on data interpretation, see the decision chart at the end of this document (section 3).

2. Biorad CFX96 and Biorad CFX Opus Deepwell

Software: CFX 3.1

2.1 Plate setup

Create a new plate in the IDE Plate Manager.
Select" Type: Qualitative"
Select the wells to be used and assign the corresponding categories on the right panel: **Positive control**, **Unknown,** or **Negative control.**

Set up the parameters for **Salmofast** for the first time
On the main menu, click "RUN" and then "User-defined run."

A new window opens with three options: **Protocol**, **Plate,** and **Start Run**
Click on "Create new."
Adjust the reaction volume to 20 µl

On the "**Protocol Screen,**" set up the thermal cycling:

- Step 1: 10 min at 95 °C
- Step 2: 15 sec at 95 °C
- Step 3: 1 min at 60 °C + Plate read
- Step 4: GOTO (Step) 2, 39 more times
- END

Save the protocol to a file for further use.
Click "OK". Now, the Protocol window displays the selected conditions.
For the subsequent runs, click on "Select Existing" and take the one saved before

Click on the "**Plate**" tab.
A scheme of the 96-well plate shows up.
Click on "Create new."
A new window with the configurable plate opens. Now it's time to

A. Select the reaction-containing wells. Click or click&drag the wells where the samples are placed
B. Assign type of experiment (Positive control, Unknown, or Negative Control). With **Salmofast,** we need to create
   - One Positive Control,
   - The desired number of samples for that specific run and
   - One non-template control (NTC)
C. Select the fluorescence channels to read: on the right panel, click "Select fluorophores." Click boxes for channels 2 (HEX) and 4 (Cy5)

Edit the target names (IAC and Salmonella for HEX and Cy5, respectively) by clicking the box beside the channel name. The channel name should now appear inside the well's cell. Save the configuration for later analyses. Click "OK."

Click on the "**Run**" tab to launch the PCR. Follow all indications about lid temperatures. When everything is ready, click "Start Run."

2.2. Results Analysis and Interpretation

Open the file generated after the PCR run (extension ".pcrd").
On the amplification curves panel, uncheck the HEX box to see only the Cy5 curves. Select the well containing the positive control and check its End RFU (Relative fluorescence units) value.

Select "Settings>Baseline Threshold" from the menu. At the single threshold dialog box, select "User defined" and manually enter a value of 10% of Positive Control's End RFU whenever the auto-calculated threshold is lower than this value.

Check that the PCR control results (Positive Control and NTC) are as expected: positive control shows a curve crossing the threshold. NTC does not produce any amplification curve. When hovering the mouse over the amplification curves, sample ID and Cq value appear highlighted in the table on the right.

Samples: the result is positive for Salmonella when an amplification curve is produced in the Cy5 channel, and a Cq value appears on the table.

When no amplification curve is produced in the Cy5 channel, the result is negative (absence of Salmonella) when amplification in the HEX channel (IAC) is produced, and its Cq value does not exceed 3 Cq units from that of NTC.

For general criteria on data interpretation, see the decision chart at the end of this document (section 3).

3. General decision chart for Salmofast analysis on any platform.

- Does the amplification curve of CY5/Salmonella cross the threshold line and produce a positive Cq value?
  - YES
    - Does the Cy5 channel of NTC show an amplification curve crossing the threshold
      - YES: Mastermix contaminated. Repeat with fresh reagents
      - NO: **Positive** (Presence of Salmonella)
  - NO: go to the next question

- Does Cq (HEX/VIC) show amplification?
  - YES
    - Is Cq_{SAMPLE} value ≤ Cq_{NTC} + 3?
      - YES: Sample result is **Negative** (Absence of Salmonella)
      - NO: PCR inhibition (see next point)
  - NO: PCR inhibition. Repeat analysis by diluting the DNA sample 1/10 using Molecular Biology grade water

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