iQ-Check® *S.* Enteritidis Kit

Catalog #: 357-8142

User Guide

Test for the real-time PCR detection of *Salmonella* Enteritidis in food and environmental samples

BIO-RAD

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I. INTRODUCTION

Even though number of cases is decreasing in some areas, millions of human salmonellosis are still reported each year in the world. *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*Salmonella* Enteritidis or SE) is the most often implicated, sometimes representing more than 50 % of the cases. Even though sources such as raw milk, pork, beef, sprouts and almonds have been identified, eggs and poultry have been the most common food source linked to these SE infections. SE has an unusual ability to colonize the ovarian tissue of hens and be present in intact shell eggs, in very low number.

In the USA, to reduce the health impact of this pathogen, USDA (through the NPIP) and FDA have put regulations in place for poultry and egg production fields. Environmental testing of poultry houses is required; if SE is detected, then eggs must be tested. In these samples, the detection is hampered by very low level of contamination or by high level of interfering flora. In the EU, regulations have been implemented to monitor *Salmonella* spp. and specific serotypes including *S.* Enteritidis in poultry, from the primary production stage to the food products.

The iQ-Check *S.* Enteritidis kit allows for the rapid and specific detection of *Salmonella* Enteritidis in food and environmental samples (including environment of primary production) within a few hours after the end of the microbiological enrichment. Up to 94 samples can be processed with a minimized risk of contamination and an easy-to-use procedure.

II. THE iQ-Check *S.* ENTERITIDIS TECHNOLOGY

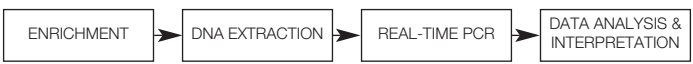
The iQ-Check *S.* Enteritidis kit is a test based on gene amplification and detection by real-time PCR. Ready-to-use PCR reagents contain oligonucleotides (primers and probes) specific for *Salmonella* Enteritidis, as well as DNA polymerase and nucleotides. Detection and data analysis are optimized for use with a Bio-Rad real-time PCR instrument, such as the MiniOpticon™, the CFX96™ or the CFX96 Deep Well™ systems.

PCR is a powerful technique used to generate many copies of target DNA. During the PCR reaction, several cycles of heating and cooling allow DNA denaturation, by heat, followed by primers binding to the target region. The DNA polymerase then uses these primers and deoxynucleotide triphosphates (dNTPs) to extend the DNA, creating copies of the target DNA. These copies are called amplicons.

In real-time PCR, specific probes are used to detect the DNA during the amplification, by hybridizing to the amplicons. These probes are linked to a fluorophore which fluoresces only when hybridized to the target sequence; FAM is the fluorophore linked to the probe hybridizing to the *Salmonella* Enteritidis specific DNA sequence. In the absence of target DNA, no fluorescence will be detected. As the amount of amplicons increases with each round of amplification, fluorescence intensity also increases. During each PCR cycle, at the annealing step, the optical module or detector measures this fluorescence, whereas the associated software plots the fluorescence intensity versus number of cycles. This method allows a simple determination of the presence, or absence, of *Salmonella* Enteritidis in a sample.

A synthetic DNA "internal control" is included in the reaction mix. This control is amplified with a specific probe at the same time as the *Salmonella* Enteritidis target DNA sequence, and detected by a second fluorophore. It allows for the validation of any negative result.

This test allows the detection of *Salmonella* Enteritidis in food products and environmental samples (including environment of primary production) previously enriched by culture. It includes the following four main steps:



III. KIT COMPONENTS

The iQ-Check *S.* Enteritidis kit contains sufficient reagents for 96 tests.

Reference ID	Reagent	Quantity Provided
A	Lysis reagent	1 bottle (20 mL)
B	Fluorescent probes	1 tube (0.55 mL)
C	Amplification mix	1 tubes (4.4 mL)
D	PCR negative control	1 tube (0.5 mL)
E	PCR positive control	1 tube (0.25 mL)

IV. SHELF LIFE AND STORAGE

Once received, the kit must be stored between +2°C and +8°C. Reagents stored at this temperature can be used until the expiration date indicated on the reagent tube.

V. MATERIAL REQUIRED BUT NOT SUPPLIED

Equipment

- Stomacher®, masticator or equivalent for homogenizing test samples.
- Incubator for sample microbiological enrichment.
- Specific for extraction in 1.5 ml tube
  - Bench top centrifuge (max. 10,000-12,000 g), optional for the Easy protocol
  - Dry heat block (100°C ± 5°C).
- Specific for extraction in deepwell plate
  - Dry heat block (100°C ± 5°C).
  - or agitator-incubator for deepwell plates, such as a "Thermomixer" (Eppendorf)
- Vortex apparatus.
- Magnetic stir plate.
- 20 µL, 200 µL and 1,000 µL micropipettes.
- Combitip pipettes or equivalent repeat pipettors.
- Bio-Rad real-time PCR system*, e.g. MiniOpticon, CFX96 or CFX96 Deep Well systems.

Note: We recommend using a universal power source (UPS) with the thermal cycler.

* Contact Bio-Rad for detailed information on instruments recommended by our technical department.

Supplies

- Enrichment medium: buffered peptone water, (E.g. Bio-Rad cat.#: 356-4684, 500 g; 355-4179, 225 mL x 6 bottles; 355-5789, 2.3 L x 5 bags; 355-5790, 5 L x 2 bags).
- Specific for environmental primary production samples: supplement for enrichment: RAPID' *Salmonella* Capsule, Bio-Rad cat.#: 356-4710, x100, Quantity for 250 mL; 356-4709, x100, Quantity for 2.5 L, 356-4712, Quantity for 100 tests.
- Stomacher bag with incorporated filter.
- Environmental sponges.
- Environmental swabs.
- Specific for extraction in tube
  - 1.5 mL conical screwcap sterile tubes (E.g. Bio-Rad cat. #: 224-0110).
- Specific for extraction in deepwell plate
  - 1 mL deepwell plate, Bio-Rad cat.#: 359-0132.

- Plastic sealing film, Bio-Rad cat.#: 359-0139.
- Pre-pierced sealing film, such as "X-Pierce™ Sealing Films", Bio-Rad cat #: 360-0040, for North America only; cat #: 359-3977, x100.

- RAPID' *Salmonella* Agar, Bio-Rad cat #: 356-3961, 90 mm x 20 dishes; 356-3962, 90 mm x 100 dishes; 356-4705, 500 g.
- Immune-sera: Bio-Rad offers a full range of anti-sera for the serotying of *Salmonella*. Contact Bio-Rad for detailed information.
- PCR plates, tubes, sealing tape and caps, see real-time PCR system user guide for iQ-Check kits.
- 1 mL and 10 mL pipettes.
- Sterile filter tips, adaptable to 20 µL, 200 µL and 1,000 µL micropipettes.
- Tips for Combitip pipettes or equivalent repeat pipettors, sterile, individual package.
- 2 mL and 5 mL sterile test tubes.
- Powder-free gloves.
- Distilled sterile water.
- Bleach 5 %.
- Cleaning agent such as DNA AWAY® or RNase AWAY®.

VI. PRECAUTIONS AND RECOMMENDATIONS FOR BEST RESULTS

- This test must be performed by adequately trained personnel.
- Samples and enrichment cultures must be handled as potentially infectious material and eliminated according to local rules and regulations.
- All potentially infectious material should be autoclaved before disposal.
- The quality of results depends on strict compliance with the following Good Laboratory Practice (for example the EN ISO 7218 standard), especially concerning PCR:
  - The laboratory equipment (pipettes, tubes, etc.) must not circulate from one work station to another.
  - It is essential to use a positive control and a negative control for each series of amplification reactions.
  - Do not use reagents after their expiration date.
  - Vortex reagents from the kit before using them to ensure homogeneity.
  - Periodically, verify the accuracy and precision of pipettes, as well as correct functioning of the instruments.
  - Change gloves often, especially if you suspect they are contaminated.
  - Clean work spaces periodically with at least 5% bleach and other decontaminating agent such as DNA AWAY®.
  - Use powder-free gloves and avoid fingerprints and writing on caps of tubes. Both cases will interfere with data acquisition.

- It is strongly advised to follow the general requirements described in the standard EN ISO 22174:2005 “Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food pathogens – General requirements and definitions”.

VII. PROTOCOL

It is strongly recommended to read the entire protocol before starting the test.

Enrichment conditions are detailed hereafter. DNA extraction can be performed according to different protocols; the Easy I protocol requires a 100 µL sample volume, the Standard I protocol requires a 1 mL sample volume.

Any DNA that has been extracted according to protocols indicated in the iQ-Check *Salmonella* spp. II user guide can be tested with iQ-Check *S.* Enteritidis. The following table outlines the main protocols that can be used depending on the application.

Scope (matrices)	Enrichment	DNA extraction	
		method	format
Food products (including eggs), animal feed, & environmental[1] samples	BPW 18 h ± 2 h 37°C ± 1°C	Standard I	Tube
	BPW 21 h ± 1 h 37°C ± 1°C	Easy I	Tube Deepwell
Pool of eggs	BPW (5X) 20 h ± 2 h 37°C ± 1°C	Easy I	Tube Deepwell
Environmental primary production samples (boot swabs, hygiene swabs, dust, litters, feces, drinker waters…)	Suppl. BPW 19 h ± 1 h 41.5°C± 1°C + BPW 5 h ± 1 h 37°C ±1°C	Easy I	Tube Deepwell

[1] *Except environmental primary production samples.*

A. Sample Enrichment

Enrichment media must be warmed at room temperature before use.

Homogenize n g of sample in 9 x n mL (for example 25 g in 225 mL) of buffered peptone water, in a stomacher bag with incorporated filter.

Incubate, without shaking, for times and at temperatures indicated in the table above.

Further information:

1. Pooled eggs samples

- Prepare a pool of 20 shell eggs as indicated in the FDA Bacteriological Analytical Manual, Chapter 5: *Salmonella* - eggs chapter (December 2007 Edition), available at www.fda.gov.
  - Disinfect eggs by immersion for at least 10 seconds, with a solution consisting of 3 parts of 70% alcohol (ethyl or isopropyl) to 1 part iodine/potassium iodide solution.
  - Remove eggs and allow to air dry.
  - Crack 20 eggs aseptically into a Whirl-Pak bag.
  - Mix thoroughly by hand until yolks are completely mixed with the albumen.
- Enrich the egg mixture
  - Add 200 mL of 5X autoclaved BPW to the pooled eggs.

  Note: see preparation in Appendix B.
  - Well homogenise.
  - Incubate 20 h (± 2 h) at 37°C.
- Prepare the sample for DNA extraction
  - After the enrichment, distribute 990 µL of BPW (1x) in tubes or in wells of a deepwell plate.
  - Add 10 µL of the enriched sample.
  - Follow the Easy I protocol for the DNA extraction.

2. Environmental primary production samples

- Incubate the sample in buffered peptone water supplemented with the RAPID'*Salmonella* Capsule (refer to the table for incubation conditions).
- After this first enrichment, for each sample, dispense 900 µL of buffered peptone water in tube or in well of a deepwell plate.
- Transfer 100 µL of pre-enriched sample.
- Seal the deepwell plate and refer to the table for incubation conditions.
- Follow the Easy I protocol for DNA extraction.

Note: the Primary Enrichment can be stored between +2°C and +8°C for 16 hours following the end of the incubation at 41.5°C, before to proceed to the Secondary Enrichment.

B. DNA Extraction

General recommendations:

- Before starting the test, turn on the heat block to preheat it and set it to 95°C - 100°C.
- In general, avoid shaking the enrichment bag and collecting large fragments of food debris. For food samples with a fatty supernatant,

collect the sample just below this layer.
- Open tubes and wells carefully to avoid any possible cross contaminations.
- Cool the deepwell plate before pipetting directly through pre-pierced sealing film.
- Pipette the lysis reagent while it is stirring at medium speed with the magnetic bar contained in the bottle, in order to keep it in suspension.

Standard I protocol

1 - Collect 1 mL of decanted enriched sample into a tube.
2 - Centrifuge at 10,000-12,000 g. for 5 minutes.
3 - Discard all the supernatant.
4 - Add 200 µL of the lysis reagent (reagent A) to the pellet.
5 - Resuspend pellet by pipetting the reagent up and down in the tube.
6 - Vortex at high speed.
7 - Place the tube in the heat block at 95°C - 100°C for 10 to 15 minutes.
8 - Vortex at high speed.
9 - Centrifuge at 10,000-12,000 g for 5 minutes.

If you choose to temporarily stop the procedure, this is the recommended stopping point.
The supernatant can be stored for up to 1 year at -20°C. Before reusing it, always allow it to thaw, homogenize, and then centrifuge at 10,000-12,000 g for 5 minutes.

Easy I protocol

1 - Aliquot 100 µL of homogenized lysis reagent A to tubes or wells of a deepwell plate.
2 - Add 100 µL of the decanted enriched sample.
Mix by pipetting up and down and close the tube with caps or seal the deepwell plate with pre-pierced sealing film.
3 - Incubate in the appropriate heat block at 95°C - 100°C for 10 to 15 minutes or in the plate incubator-agitator for 15 to 20 minutes at 1,300 rpm.
4 - Vortex tubes at high speed.
5 - If using a deepwell plate, allow to cool down to room temperature.
6 - As an optional step to eliminate debris, centrifuge tubes at 10,000-12,000 g at least 2 minutes.
Centrifugation is not needed for deepwell plate.

If you choose to temporarily stop the procedure, this is the recommended stopping point.

The supernatant can be stored for up to 1 year at -20°C. Before reusing it, always allow it to thaw, homogenize, and then centrifuge tubes at 10,000-12,000 g for 5 minutes.

C. Real-time PCR

1. Instrument and software setup

For instrument and software setup, follow instructions in the real-time PCR system user guide for iQ-Check kits.

2. PCR mix preparation

2.1 Prepare a PCR mix containing the amplification solution (reagent **C**) and the fluorescent probes (reagent **B**) depending on the number of samples and controls to analyze (at least one positive and one negative control must be included in each PCR run). Use the pipetting table in Appendix to find the correct volumes to use for each reagent.

2.2 After preparation, the PCR mix (reagent B + C) must be used immediately or is stable for **1 hour maximum at 2°C - 8°C**.

2.3 Pipette **45 µL** of this PCR mix in each well according to your plate setup.

2.4 Add **5 µL** of sample or reagent **D** (negative control) or reagent **E** (positive control). Do not vortex the sample before pipetting. Seal hermetically the wells of the plate or strips. It is important to avoid bubbles at the bottom of the wells by pipetting carefully. As an optional step, to eliminate any bubbles, centrifuge the sealed PCR plate or the PCR strips (quick spin).

2.5 Place the plate or strips in the thermal cycler. Be sure to place the plate correctly: A1 well at the upper left corner. Close the reaction module.

3. PCR Start

To start the PCR run, follow instructions in the real-time PCR system user guide for iQ-Check kits.

D. Data Analysis

Data can be analyzed directly at the end of the PCR run or at a later time by opening the stored data file. Follow instructions in the corresponding

real-time PCR system user guide for iQ-Check kits for opening data files and setting the data analysis parameters.

1. Interpreting Results

Once the data analysis parameters have been set, results are interpreted by analyzing the Cq values of each sample (the cycle at which the amplification curve crosses the threshold).

The CFX Manager™ IDE allows a complete automated analysis for the CFX96/CFX96 Deep Well and the MiniOpticon, #: 93893b.

1.1 Controls

Before interpreting sample results, it is necessary to verify the positive and negative controls.

For the experiment to be valid, the controls must have the following results, as summarized in the table below, otherwise the PCR reaction needs to be repeated.

	***S.* Enteritidis detection (FAM)**	**Internal control detection (HEX Channel)**
Negative control	Cq = N/A*	28 ≤ Cq ≤ 40
Positive control	26 ≤ Cq ≤ 36	Not significant

* The software indicates a Cq value of N/A (not applicable) when the fluorescence of a sample does not rise significantly above the background noise, and hence does not cross the threshold.

If results of negative and positive controls differ from those in the table above, it is necessary to repeat the PCR.

1.2 Samples

A **positive** *Salmonella* Enteritidis sample must have a Cq value ≥ 10 for the FAM fluorophore.

If the Cq value is below 10, verify that as raw data the curve is a regular amplification curve (with a flat base line, followed by a rapid increase of fluorescence and then a flattening out). If the curve seems correct, it may be considered a positive *Salmonella* Enteritidis sample.

If there is no Cq value (Cq=N/A) for FAM, or the curve is not a typical amplification curve, the internal control for that sample must then be analyzed:

- This sample is considered as a **negative** *Salmonella* Enteritidis sample if there is no Cq value in FAM, and the internal control has a Ct ≥ 28.

- Should the internal control also not have a Cq value (Cq = N/A), this probably indicates an inhibition of the PCR reaction. The sample needs to be diluted (perform a 1/10 dilution in distilled sterile water, using 10 µL of DNA extract, then use 5 µL of the dilution for amplification), and the PCR repeated.

- Should the Cq value for the internal control be < 28 it is not possible to interpret the result. Verify that the threshold was correctly placed, or that the curve as raw data is a regular amplification curve. If the curve does not have a characteristic shape, it will be necessary to repeat the PCR test.

Interpretation of sample results is summarized in the following table:

***S.* Enteritidis detection (FAM)**	**Internal control detection (HEX Channel)**	**Interpretation**
Cq ≥ 10	Not significant	Positive
Cq = N/A	Cq ≥ 28	Negative
Cq = N/A	Cq = N/A	Inhibition**

** When both *Salmonella* and internal control detection give a Cq value = N/A, the sample must be tested again but diluted (1/10).

VIII. CONFIRMATION OF POSITIVE RESULTS

Positive iQ-Check results need to be confirmed (cross-reactions have been observed with a few *Salmonella* strains; contact Bio-Rad for more details):

A. All samples but primary production samples

1 - Using standard tests described in the standardized methods (ISO, USDA MLG or FDA BAM). For the confirmation test, it is necessary to start from the buffered peptone water enrichment broth after the full 18 h ± 2 h enrichment at 37°C.

2 - Using any other method based on a principle different from that used in the iQ-Check *S.* Enteritidis PCR test, for example the chromogenic medium RAPID' *Salmonella* with a double enrichment (includes a selective enrichment step in RVS medium; refer to the product technical sheet for instructions for use). Complete the method with serological determination. The protocol of this second method must be followed entirely; the confirmation is carried out from the buffered peptone water enrichment broth, if this step is common to both methods.

B. Primary production sample
For environmental primary production samples, the confirmation shall be processed starting from the Primary Enrichment, then follow standardized methods (ISO 6579/A1, annex D, USDA MLG or FDA BAM). For the Appendix D of the ISO 6579/A1 standard, follow the MSRV method then the RAPID' *Salmonella* medium for the isolation. Complete the method with serological determination.

Results that are not in agreement between iQ-Check *S.* Enteritidis and the confirmation method described above are possibly due to the presence of non motile *Salmonellae*. In that case, we recommend to follow the RAPID' *Salmonella* method, double enrichment protocol (refer to the product technical sheet for instructions for use). This protocol includes a selective enrichment step in RVS medium.

Primary enrichment bags can be stored until 24 hours between +2°C and +8°C before to proceed to the confirmation.

IX. CONFIRMATION OF SINGLE COLONIES USING iQ-Check

iQ-Check *S.* Enteritidis may also be used for confirming single isolated *Salmonella* Enteritidis colonies on agar plates.

1. Pick an isolated colony, from an agar plate, selective or non-selective, with a tooth-pick or sterile loop, or other adapted consumable (e.g. pipette tip).
2. Resuspend the colony in 100 µL tryptone salt or distilled sterile water in a microfuge tube. Homogenize using a vortex.
3. Use 5 µL of the suspension with 45 µL of PCR mix (see section VII.C Real-time PCR) and follow the rest of the iQ-Check *Salmonella* Enteritidis protocol for the data and result interpretation.

XI. REFERENCES

1. Guidance for Industry: Prevention of *Salmonella* Enteritidis in Shell Eggs During Production, Storage, and Transportation, 74 FR 33030.
2. Kaufmann-White scheme, Antigenic Formulae of the *Salmonella* Serovars, 2007, 9th edition, WHO Collaborating Centre for Reference and Research on *Salmonella*.
3. National Poultry Improvement Plan and Auxiliary Provisions, Code of Federal Regulations Title 9, Animals and Animal Products Parts 145–147 and Part 56.
4. Production, Storage, and Transportation of shell eggs, Code of Federal Regulations, 21 CFR part 118.
5. Standard NF EN ISO 6579, Annex D. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. December 2002.
6. United States Department of Agriculture, Food Safety and Inspection Service. Microbiology Laboratory Guidebook – Chapter 4.03. Isolation and Identification of *Salmonella* from Meat, Poultry and Egg Products. October 1, 2004. On line at http://www.fsis.usda.gov/PDF/MLG_4_03.pdf
7. United States Food and Drug Administration, Center for Food Safety and Applied Nutrition. Bacteriological Analytical Manual - Chapter 5. *Salmonella*. June 2006. On line at: http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/UCM070149).

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APPENDIX A - PCR Mix Calculation Guide

To find the correct volumes to use when preparing the PCR mix, add the total number of samples and controls to be analyzed, and find the corresponding volumes of reagent B and reagent C in the table.

Total number of samples & controls	Probes Reagent B (µL)	Amplification mix Reagent C (µL)
1	5	40
2	11	86
3	16	130
4	22	173
5	27	216
6	32	259
7	38	302
8	43	346
9	49	389
10	54	432
11	59	475
12	65	518
13	70	562
14	76	605
15	81	648
16	86	691
17	92	734
18	97	778
19	103	821
20	108	864
21	113	907
22	119	950
23	124	994
24	130	1000
25	135	1100
26	140	1100
27	146	1200
28	151	1200
29	157	1300
30	162	1300
31	167	1300
32	173	1400
33	178	1400
34	184	1500
35	189	1500
36	194	1600
37	200	1600
38	205	1600
39	211	1700
40	216	1700
41	221	1800
42	227	1800
43	232	1900
44	238	1900
45	243	1900
46	248	2000
47	254	2000
48	259	2100

Total number of samples & controls	Probes Reagent B (µL)	Amplification mix Reagent C (µL)
49	265	2100
50	270	2200
51	275	2200
52	281	2200
53	286	2300
54	292	2300
55	297	2400
56	302	2400
57	308	2500
58	313	2500
59	319	2500
60	324	2600
61	329	2600
62	335	2700
63	340	2700
64	346	2800
65	351	2800
66	356	2900
67	362	2900
68	367	2900
69	373	3000
70	378	3000
71	383	3100
72	389	3100
73	394	3200
74	400	3200
75	405	3200
76	410	3300
77	416	3300
78	421	3400
79	427	3400
80	432	3500
81	437	3500
82	443	3500
83	448	3600
84	454	3600
85	459	3700
86	464	3700
87	470	3800
88	475	3800
89	481	3800
90	486	3900
91	491	3900
92	497	4000
93	502	4000
94	508	4100
95	513	4100
96	518	4100

APPENDIX B - Preparation of 5X buffered peptone water

- Weight 100 g of dehydrated buffered peptone water (Bio-Rad cat. # 356-4684)
- Add 1 L of distilled water
- Homogenize
- Autoclave 15 min at 121°C

In the United States, for technical assistance, please call (800) 4BIORAD. Select option 2 for technical support and option 2 again for the Food Science Division. To place an order, please call (800) 4BIORAD and press option 1 for customer service. Orders can also be faxed to (800) 879-2289.

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