eurofins | GeneScan

BAC*Gene* *Salmonella* spp.

Test kit for qualitative real-time PCR detection of *Salmonella* spp.

Cat. No. 5123221801:

96 lysis and real-time PCR reactions

Cat. No. 5123221810 (10x high throughput (HTP) kit):

10 x 96 lysis and real-time PCR reactions

Cat. No. 5123221811 (10x content of cat. no. -01):

10 x 96 lysis and real-time PCR reactions

BY AFNOR CERTIFICATION NF VALIDATION EN ISO 16140

EGS 38/01-03/15
ALTERNATIVE ANALYTICAL
METHODS FOR AGRIBUSINESS
www.afnor-validation.com

PERFORMANCE TESTED AOAC RESEARCH INSTITUTE LICENSE NUMBER 121501

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1 INTRODUCTION

The BAC*Gene Salmonella* spp. detection kit provides materials for rapid detection of *Salmonella* spp. DNA from food, feed (including pet food) and environmental samples.

The BAC*Gene Salmonella* spp. detection kit may also be useful for other purposes in food product research and analysis field, e.g. microbial monitoring of production processes.

It is intended that this kit is used by trained laboratory personnel.

The BAC*Gene Salmonella* spp. kit is validated for use with the Agilent AriaMx™, Bio-Rad CFX96 Touch™ and CFX96 Touch™ Deep Well PCR platforms.

1.1 Certifications

1.1.1 AFNOR Certification

The BAC*Gene Salmonella* spp. kit is certified by AFNOR Certification as an alternative method for *Salmonella* spp. detection in all human food products, feed and environmental samples according to EN ISO 16140:

- 25 g samples of all human food products including:
  - Meat and meat products
  - Milk and dairy products
  - Produces (vegetables, fruits and related products)
  - Eggs and egg products
  - Fish and seafood products
- 25 g of feed (including pet food)
- up to 375 g of pet food
- up to 375 g of milk powders & infant formula (with and without probiotics)
- Environmental samples

BY AFNOR CERTIFICATION NF VALIDATION EN ISO 16140

EGS 38/01-03/15
ALTERNATIVE ANALYTICAL METHODS FOR AGRIBUSINESS
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The end of the validity of the NF VALIDATION certification is indicated on the certificate EGS 38/01-03/15.

1.1.2 AOAC Performance Testing

The BAC*Gene Salmonella* spp. kit is certified by AOAC-Research Institute under the Performance Tested Methods[SM] Program for detection of *Salmonella* spp. in:

- Fresh raw ground beef (15 % fat, 25 g)
- Frozen spinach (25 g)
- Pasteurized whole liquid eggs (25 mL)
- Frozen cod fillet (25 g)
- Raw whole milk (25 mL)
- Dog pâté (composed of beef meat and animal by-products, cereals, carrots and vegetable by-products, up to 375 g)
- Dry dog pellets (25 g)
- Infant formula milk powder supplemented with probiotics (*Bifidus lactis*) (up to 375 g)
- Process water from scalding tank (25 mL)
- Stainless steel environmental surface (1" x 1" area)
- Cocoa powder, cocoa liquor, cocoa butter, cocoa crumb and milk chocolate (up to 375 g each)
- Ground beef (up to 375 g)
- Ground pork (up to 250 g)
- Marinated chicken with brine (up to 250 g)
- Pork carcass cloth

The end of the validity of the AOAC-RI certification is indicated on the certificate no. 121501.

1.2 Important Notes

Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

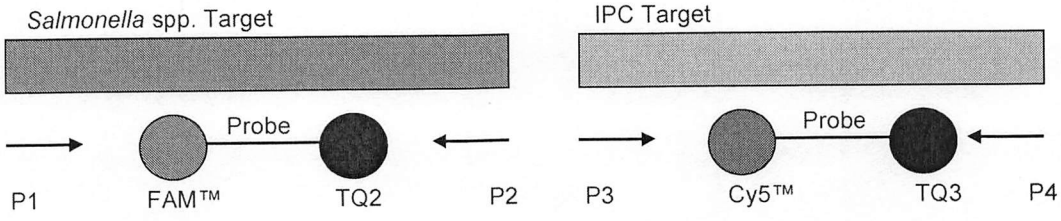
1.3 Test Principle

DNA amplification and detection methods take advantage of the nucleotide sequence conservation found in bacterial genomes that ensures the potential for high specificity and sensitivity in detection of food-borne, pathogenic bacteria.

After enrichment, the microbial DNA is extracted by a simple thermal lysis step and rapidly analysed by real-time PCR. In this way *Salmonella* species are detected from enrichment cultures with extraordinary high sensitivity.

By means of specific primers (P) a nucleotide sequence of the species *Salmonella* is amplified during PCR. Primers do not react with DNA derived from closely related species from the *Enterobacteriaceae* family.

The amplified fragments are detected with a FAM™ fluorescence-labelled hybridization probe quenched by non-fluorescent Tide Quencher™ 2 (TQ2). An internal positive control (IPC) is included in the MasterMix. IPC DNA is amplified in parallel and detected using a Cy5™ fluorescence-labelled hybridisation probe quenched by non-fluorescent Tide Quencher™ 3 (TQ3). IPC detection indicates the proper functioning of the PCR.



1.4 Components of the Kit

Important Notes:

- Store all reagents as indicated below, through to the expiration date printed on the label. Never store components of the kit together with samples or post-PCR products. Shelf life is indicated on the labels of the components.

For Lysis: Cat. No. 5123221801 / 5123221811

1x/10x	**Lysis plate for sample preparation,** empty, rippable (high profile)
1x/10x	**Domed caps*,** for use with Lysis plate, set of 12 strips
2x/20x	**Lysis buffer I,** 4 mL, store at -20 °C ± 2°C or 4°C ± 2°C; after addition of proteinase K store at 4°C ± 2°C for maximum 2 weeks
2x/20x	**Proteinase K,** vials with blue cap, each with 500 µL, store at -20°C ± 2°C

For *Lysis*: Cat. No. 5123221810 (10x HTP)

10x	**Lysis plate for sample preparation,** empty, rippable (high profile)
10x	**Domed caps*,** for use with lysis plate, set of 12 strips
2x	**Lysis buffer I,** 40 mL, store at -20 °C ± 2°C or 4°C ± 2°C; after addition of proteinase K store at 4°C ± 2°C for maximum 2 weeks
2x	**Proteinase K,** 5 mL, store at -20°C ± 2°C

*Note: additional domed caps for lysis in a package of 250 x 8 cap strips with Cat no. 5613900301 can be purchased at kits@eurofins.com.

For PCR: Cat. No. 5123221801 / 5123221810 (1x/10x)

1x/10x/10x	**BAC*Gene* *Salmonella* spp. PCR plate,** with pre-dispensed MasterMix and PCR support grid. Store light protected at -20°C ± 2°C.
1x/10x/10x	**Optical caps,** for use with PCR plate. Mat of 12 strips, rippable.
2x/4x/20x	***Salmonella* positive control plasmid DNA,** vial with yellow cap, 50 µL, store at -20°C ± 2°C. Do not freeze/thaw more than 6 times.

1.5 Additional Equipment, Consumables and Reagents Required

1.5.1 Sampling / Enrichment

- Swabs / sponges / wipes / cloths (for **environmental samples**)*
- Dey-Engley neutralizing buffer (for **environmental samples**)
- Letheen broth (for **environmental samples**)
- MRB medium (for **carcass sampling**)
- Diluents according to EN ISO 6887-1 (for **carcass sampling**)
- Stomacher® or paddle blender, capable of running between 15.000 and 20.000 rev/min
- Enrichment bags with side filter
- Buffered Peptone Water (BPW), prepared according to EN ISO 6579 and EN ISO 11133 standards
- UHT milk (for **cocoa products** primary enrichment)
- Brilliant Green (for **cocoa products** primary enrichment)
- BHI medium (for **cocoa products** secondary enrichment)
- Tween-80® (**for products containing >20 % fat, including cocoa containing products**, unless the products already contain sufficient emulsifier).
  - Add sufficient amount of polysorbate 80 [polyoxyethylene (20) sorbitan monooleate] to improve emulsification during suspension (diluent between 1 g/L and 10 g/L, according to estimated fat content).
  - Alternative surfactants and emulsifiers are available under various trade names, but the proportions to use should be determined by the laboratory (according to EN ISO 6884-4:2017).

***Note:** Products pre-moistened with neutralizing buffers containing aryl sulfonate (sodium dodecylbenzenesulfonate) should not be used in conjunction with this kit. Recommended is the use of Dey-Engley neutralizing buffer or Letheen broth.

1.5.2 Lysis

Equipment

- 1x 96 well cooling block (e.g. cat. no. 5613900501)
- 2x Heating block 96 well, 0.2 mL
  (e.g. cat. no. 5613900701 for 37°C, cat. no. 5613900801 for 95°C)
- 2x Insert for heating block 0.2 mL wells
  (e.g. cat. no. 5613900901)
- Blue work rack for lysis
  (e.g. cat. no. 5613901101)
- Centrifuge for microtiter-plates/or -strips - depending on throughput:
  - Capacity of 2x 8-well strips: (e.g. Carl Roth GmbH, Rotilabo® centrifuge with butterfly rotor cat. no. T465.1)
  - Capacity of 4x 8-well strips: (e.g. Sigma Aldrich Co LLC, MyFuge™ 12 mini centrifuge cat. no. Z681733-1EA)

  Recommended:
  - Capacity of two times 12x 8-well strips: (e.g. Benchmark Scientific, PlateFuge™ microplate microcentrifuge, cat. no. 5613901701)
- Capping/Uncapping tool for 8-well strips (e.g. MicroAmp® Cap Installing Tool, cat. no. 5613900401)
- Stepper pipette (1 mL) (e.g. HandyStep® S (Brand®), cat. no. 5617703401)
- Single channel pipettes (1 mL, 100 µL)
  (e.g. Transferpette® S 100 - 1000 µL (Brand®), ca. no. 5617703301)
- Pipette filler for serological pipettes

Consumables

- DNA-/Nuclease-free pipette tips
- 1 mL stepper tips
- Sterile container for enrichment storage
- Serological pipettes (e.g. cat. no. 5617702405 (1 mL) or cat. no. 5617702505 (10 mL))
- Powder free gloves

1.5.3 PCR Setup

Equipment

- 1x 96-well cooling block (e.g. cat. no. 5613900601)
- Pink work rack (e.g. cat. no. 5613901201)
- Capping/Uncapping tool for 8-well strips
  (e.g. MicroAmp® Cap Installing Tool, cat. no. 5613900401)
- Multichannel pipette (5 µL)
  (e.g. Transferpette® S-8 Kanal / 0.5 - 10 µL (Brand®), cat. no. 5617703501)
- Single channel pipettes (5 µL)
  (e.g. Transferpette® S, 0.5 - 10 µL (Brand®), ca. no. 5617703101)
- Disposable transfer rack (e.g. an empty tip box) as mount for the yellow support grid and the PCR plate
- Real-time PCR Thermocycler:
  Agilent AriaMx™ (cat no.: G8830A) (Software v. 1.0) with min. FAM™ and Cy5™ filter set
  or Bio-Rad CFX96 Touch™ (CFX Manager™ Software v. 3.1)
  or Bio-Rad CFX96 Touch™ Deep Well (CFX Manager™ Software v. 3.1)
- Windows 7 PC with Microsoft Excel 2010 32 bit
  (Excel Version 14.7140.xxxx or later)
- BAC*Gene* Evaluation Sheet

Consumables

- Roti® Nucleic Acid-free (Carl Roth GmbH, cat. no. HP69) or 1% HCl (for DNA decontamination)
- DNA-/Nuclease-free pipette tips
- Nuclease free water (molecular biology grade)
- Powder free gloves

2 HOW TO USE THIS PRODUCT

2.1 Safety Precautions

- All samples should be handled with caution as they are potentially infectious.
- Do not eat, drink or apply cosmetics in the work area where the test is performed.
- Do not pipette by mouth.
- Avoid contact of kit components with injured skin.
- *Salmonella* spp. should not be handled by pregnant women, children, the elderly and immuno-compromised individuals due to the high infection risk and fatal health consequences for this group, in particular for the unborn child in case of pregnant women.
- The BAC*Gene Salmonella* spp. kit contains proteinase K which may cause allergic reactions (EUH 208).

The BAC*Gene Salmonella* spp kit contains no hazardous components.
For more information, please refer to the BAC*Gene Salmonella* spp kits safety data sheet.

2.2 Working guidelines

- Comply with Good Laboratory Practice (refer to EN ISO 7218 standard)
- Refer to EN ISO 22174:2005 for the general requirements for the in vitro amplification of nucleic acid sequences
- Perform cleaning protocol (outlined in next section)
- Clearly separate the area for taking samples from the enrichment, from the area where lysis and particularly PCR setup are performed
- Use different equipment (e.g. pipettes) for sampling the enrichment, performing lysis and PCR
- Use DNA, nuclease-free and sterile labware
- Wear gloves
- Change gloves after removing samples from the enrichment, before starting the lysis protocol

2.2.1 Cleaning protocol

Before commencing work and after completion of work, ensure workspaces to be clean:

Workspace	**Cleaning protocol**
Enrichment preparation area	Disinfect surfaces with 80% ethanol.
Lysis area	Disinfect surfaces with 80% ethanol. Decontaminate surface with Roti® Nucleic Acid-free or 1% HCl. Pipettes used during lysis should also be decontaminated with Roti® Nucleic Acid-free.
PCR area	Decontaminate surfaces with Roti® Nucleic Acid-free or 1% HCl.

2.3 Waste Disposal

Dispose any waste which is potentially contaminated with pathogenic bacteria according to your internal and local regulations.

2.4 Enrichment

For preparation of test samples and initial suspensions follow instructions of EN ISO 6579, EN ISO 6887, EN ISO 17604, EN ISO 7218 and EN ISO 18593 standards. Especially for higher sample volumes it may be applicable to homogenize samples by hand massage, otherwise use a stomacher.

It is strongly recommended to prepare samples in stomacher bags containing a side filter. It is also strongly recommended to prepare enrichment controls (positive and negative) in parallel with the samples. Growth in the negative enrichment control indicates contamination of the medium used and therefore enrichment should be repeated.

After the enrichment protocol, samples can be stored at 2-8°C for up to 72 h before testing.

<table>
<tr><th colspan="6">Certified categories by AFNOR (EGS 38/01-03/15)</th></tr>
<tr><th rowspan="2">Sample Category</th><th colspan="3">Enrichment</th><th colspan="2">Volume for Analysis</th></tr>
<tr><th>Medium</th><th>Temp</th><th>Time</th><th>Enrichment</th><th>Lysate</th></tr>
<tr><td>All human food products (except Milk & Dairy)
25 g</td><td rowspan="3">1:10 BPW</td><td rowspan="10">37 ± 1°C</td><td>16 - 24 h</td><td rowspan="11">10 µL</td><td rowspan="11">5 µL</td></tr>
<tr><td>Feed products (incl. pet food)
25 g</td><td rowspan="10">18 - 24 h</td></tr>
<tr><td>Dusts, process water
25 g / mL</td></tr>
<tr><td>Swab
1</td><td>10 mL BPW</td></tr>
<tr><td>Sponge, wipe, cloth
1</td><td>225 mL BPW</td></tr>
<tr><td>Milk powders & infant formula without probiotics
up to 375 g</td><td>1:10 BPW (pre-warmed to 37 ± 1°C)</td></tr>
<tr><td>Milk powders & infant formula containing probiotics
up to 375 g</td><td>1:10 double strength BPW (pre-warmed to 37 ± 1°C)</td></tr>
<tr><td>Pet food
up to 375 g</td><td>1:10 BPW (pre-warmed to 37 ± 1°C)</td></tr>
<tr><td>Milk & Dairy
25 g / mL</td><td>1:10 BPW</td><td>41.5 ± 1°C</td></tr>
</table>

<table>
<tr><th colspan="6">Certified matrices by AOAC (License No: 121501)</th></tr>
<tr><th rowspan="2">Matrix</th><th colspan="3">Enrichment</th><th colspan="2">Volume for Analysis</th></tr>
<tr><th>Medium</th><th>Temp</th><th>Time</th><th>Enrichment</th><th>Lysate</th></tr>
<tr><td>Fresh raw ground beef (15 % fat)
Frozen Spinach
Pasteurized whole liquid eggs
Frozen cod fillet
25 g</td><td rowspan="3">1:10 BPW</td><td rowspan="6">37 ± 1°C</td><td>16 - 24 h</td><td rowspan="7">10 µL</td><td rowspan="7">5 µL</td></tr>
<tr><td>Dry dog pellets
25 g</td><td rowspan="6">18 - 24 h</td></tr>
<tr><td>Process water from scalding tank
25 mL</td></tr>
<tr><td>Stainless steel environmental surface
1" x 1" area</td><td>10 mL BPW</td></tr>
<tr><td>Infant formula milk powder supplemented with probiotics (Bifidus lactis)
up to 375 g</td><td>1:10 double strength BPW (pre-warmed to 37 ± 1°C)</td></tr>
<tr><td>Dog pâté (composed of beef meat and animal by-products, cereals, carrots and vegetable by products
up to 375 g</td><td>1:10 BPW (pre-warmed to 37 ± 1°C)</td></tr>
<tr><td>Raw whole milk
25 mL</td><td>1:10 BPW</td><td>41.5 ± 1°C</td></tr>
</table>

Certified matrices by AOAC (License No: 121501): Rapid protocols of selected meat matrices & carcass cloths					
Matrix	Enrichment			Volume for Analysis	
	Medium	Temp	Time	Enrichment	Lysate
Ground beef **up to 375 g**	1:10 BPW (pre-warmed to 41.5 ± 1°C)	41.5 ± 1°C	**10** – 18 h	50 µL	5 µL
Ground pork **up to 250 g**					
Pork carcass cloths Pre-moistened with MRB **1 cloth**	90 mL BPW (pre-warmed to 41.5 ± 1°C)				
Marinated chicken with brine **up to 250 g**	1:10 BPW (pre-warmed to 37 ± 1°C)	37 ± 1°C			
Pork carcass cloths Pre-moistened with diluents (according to 6887-1) **1 cloth**	90 mL BPW (pre-warmed to 37 ± 1°C)				

Certified matrices by AOAC (License No: 121501): For cocoa-containing products up to 375 g**								
Matrix	Primary Enrichment			Secondary Enrichment			Volume for Analysis	
	Medium	Temp	Time	Medium	Temp	Time	Enrich-ment	Lysate
Cocoa powder Cocoa liquor Milk chocolate Cocoa butter Cocoa crumb **up to 375 g**	1:10 skimmed/non-fat milk (0.1 – 0.3% fat) (pre-warmed to 37 ± 1°C) + Brilliant Green (0.018 g/L)	37 ± 1°C	18 - 24 h	10 µL primary + 500 µL BHI (RT*)	41.5 ± 1°C	3 - 5 h	10 µL (of 2nd enrich-ment)	5 µL

*RT = Room Temperature (20-25 °C)

**Note:
For nut chocolate pastes (e.g. hazelnut cocoa spreads, praline fillings and similar, excluding chocolates with whole nuts or nut pieces) it is recommended to follow a modification of the cocoa protocol.
It has been demonstrated that replacing skimmed / non-fat milk (0.1 – 0.3% fat) by Buffered Peptone Water results in a significant increase in *Salmonella* growth for these matrix items.
Incubation times (18 - 24h) and temperature (37 ± 1°C) as well as the BHI step
(at 41.5 ± 1°C for 3 – 5 h) shall be performed as in the above mentioned cocoa protocol.

2.5 Before you begin

1. Place cooling block for lysis at 4°C ± 2°C overnight
2. Place cooling block for PCR at -20°C ± 2°C overnight
3. Turn on heating block to 37°C ± 2°C and 95°C ± 2°C
4. Thaw proteinase K just before adding it to Lysis buffer I
5. Prepare final lysis buffer

   Cat no. 5123221801 (1x):
   Add 500 µL proteinase K (vial with blue cap) to 4 mL lysis buffer I (transparent container).

   Cat no. 5123221810 (10x):
   Add 5 mL proteinase K (small transparent container) to 40 mL lysis buffer I.
6. Mix gently by inverting 5 times.
7. Dispense the final lysis buffer by placing the lysis plate into the blue working rack and aliquoting 90 µL (**Exceptions:** 50 µL for ground beef up to 375 g, ground pork up to 250 g, marinated chicken with brine up to 250g and pork carcass cloths) of the complete lysis buffer into each well.
8. Seal wells with domed caps.
9. Mark date of preparation and name on the label provided with kit and place onto the storage rack for lysis buffer plate/strips.
10. The final lysis buffer can be stored for 2 weeks at 4°C ± 2°C.

The volume of lysis buffer I of the kits with cat nos. 5123221801 and 5123221811 (4.5 mL) is sufficient for 48 reactions.

Lysis buffer I volume of the kit with cat no. 5123221810 (45 mL) is sufficient for 480 reactions or five 96 well plates.

2.6 Sample lysis

1. Label lysis buffer plate/strips to ensure correct orientation when pipetting samples.
2. Using a serological pipette, remove an aliquot of enrichment culture and dispense into a sterile container. This step is highly recommended to avoid cross-contamination, as opposed to inserting a micropipette directly into the enrichment bag.
3. Use an Un-/Capping Tool (with blue lysis label, dedicated to lysis area) to open the first lysis strip.

Note: Open each strip individually, add samples and close. Only then proceed to the next strip. The correct method to use this tool is to place the "teeth" of the tool under the connection between the caps and lever the caps open.

4. Transfer **10-50* µL** of enrichment into appropriate wells.

Exceptions:

For up to 375 g **meat samples** and **pork carcass cloths** transfer **50 µL** of enriched sample to a lysis well containing **50 µL** of final lysis buffer.

Note: Do not add enrichment samples to wells **A1 and B1** to ensure the same layout in the following PCR (A1: positive control, B1: negative control).

5. Seal the strip using the Un-/Capping Tool.
6. Proceed to the next lysis strip until all samples have been included.
7. Place lysis plate/strips with blue frame onto the **37°C** (± 2°C) heating block for **20 min**.
8. Transfer lysis plate/strips with blue frame onto the **95°C** (± 2°C) heating block for **10 min**.
9. Finally transfer lysis plate/strips with blue frame onto the blue 96 well cooling block (stored at 4°C ± 2°C) for **5 min**.
10. Ensure that **no condensation** remains in the lids by transferring the lysis plate/strips with the blue frame to a 96-well plate centrifuge or a centrifuge with adapter for 0.2 mL in 8-strip format. Spin down samples for 30 sec within the range of 400 x g to 2000 x g. If condensate remains in the lids, please repeat this step.
11. If **particles** are visible in the lysis vessel, centrifuge the plate/strip following the procedure in the step above.
12. Transfer lysis plate and blue frame into the working rack with transparent inlay.

2.7 PCR

2.7.1 Special precautions during PCR analysis

PCR is an exponential reaction. The detection of single DNA targets is possible. The extreme sensitivity requires special precautions for handling and equipment. After a successful amplification, several billion amplicons are present in the reaction tube. Each of them might lead to a false positive result when contaminating sample material, i.e. by spreading in aerosols.

The most important rules to avoid PCR contamination:

- Separate the different procedures spatially.
  Ideally use separate rooms for sample preparation and PCR setup, or at least dedicated different areas, equipment and consumables for each procedure.
- Use DNA/Nuclease-free filter tips.
- Wear disposable powder-free gloves.
- Store BAC*Gene Salmonella* spp. kit components for PCR and lysis only in dedicated areas, and apart from enrichment preparation areas and sample storage.
- Always use PCR controls and preferably also enrichment controls.
- Consider the PCR/post-PCR area/room where the PCR cycler is located as potentially contaminated with DNA-, decontaminate regularly and make sure to avoid carry over to other areas by using separate equipment, lab coats, shoes.
- Dispose of used PCR plates/strips very carefully, make sure that caps do not open, move waste only in tightly closed bags.
- Use separate equipment and materials for cleaning of different areas, in particular for the PCR cycler room/area and for PCR setup. Instruct cleaning personnel accordingly.
- Control all areas for DNA/amplicon contamination on a regular basis (swabs/PCR analysis).

2.7.2 General Information

PCR is performed in a volume of 25 µL in 0.1 mL reaction tubes/plates according to the real-time PCR cycler instructions.

Test samples:
20 µL MasterMix + 5 µL lysate per reaction

Positive control (C+):
20 µL MasterMix + 5 µL *Salmonella* positive control plasmid DNA

Negative control (C-):
20 µL MasterMix + 5 µL heat-inactivated lysis buffer (prepared alongside samples in well B1, 37°C for 20 min and 95°C for 10 min)

2.7.3 Control Reactions

Important:

For every PCR, it is necessary to prepare a positive and a negative control reaction. These are required for the evaluation of the PCR results! The layout should start with the positive control in A1, the negative control in B1, samples in the following wells.

Required:

- 1 Positive control (C+):
  5 µL *Salmonella* positive control plasmid DNA
- 1 Negative control (C-):
  5 µL heat-inactivated lysis buffer added to MasterMix

Recommended:

- 1 Negative enrichment control (E-):
  Salmonella free enrichment medium control
- 1 Positive enrichment control (E+):
  Lysed enrichment medium inoculated with *Salmonella*

2.7.4 PCR

Plate Setup

The following plate document shows the recommended distribution of reactions:

	1	2	3	4	5	6	7	8	9	10	11	12
A	C+	7	15	23	31	39	47	55	63	71	79	87
B	C-	8	16	24	32	40	48	56	64	72	80	88
C	1	9	17	25	33	41	49	57	65	73	81	89
D	2	10	18	26	34	42	50	58	66	74	82	90
E	3	11	19	27	35	43	51	59	67	75	83	91
F	4	12	20	28	36	44	52	60	68	76	84	92
G	5	13	21	29	37	45	53	61	69	77	85	E-
H	6	14	22	30	38	46	54	62	70	78	86	E+

Plate layout for 92 samples;
C+ = positive control
C- = negative control
E- = negative enrichment control
E+ = positive enrichment control

PCR Setup

Before starting the practical working steps, make sure you have switched on the computer, the PCR instrument and ensure the sample layout for the PCR plate is suitably documented and programmed.

Only remove the amount of PCR strips containing pre-dispensed MasterMix necessary for testing the required amount of samples and store the remainder at -20°C ± 2°C. Frequent freezing and thawing might cause inactivation of the reagents. Do not freeze-thaw any more than 3 times.

1. **Important**: When taking out the PCR plate/strips from the packaging, please ensure that the MasterMix is frozen at the bottom of the wells by visually checking the level of the mix. If frozen MasterMix is i.e. in the lids of the PCR strips, allow it to thaw at room temperature, spin down immediately after thawing and refreeze the mix.
2. Place PCR strips together with the yellow PCR support grid into the 96-well cooling block which has been cooled at -20°C ± 2°C. Avoid exposing the PCR plate to light for long periods of time.
3. The lysis and PCR plate should be arranged in the same orientation in order to facilitate handling with the multichannel pipette and in a way that the pipette with the lysate is moved across the PCR plate as little as possible in order to avoid cross contamination of wells (place the lysis plate i.e. behind the PCR plate).
4. Open the first strip of the lysis plate with the Un-/Capping Tool (dedicated to the PCR area).
5. Open the corresponding strip of the PCR plate (make sure that the mix is still frozen to avoid spilling while opening the PCR strips).
   Discard the caps of the PCR plate/strips.
6. Add 5 µL positive control DNA (vial with yellow cap) to well A1.
   Note: Do not freeze/thaw positive control more than 6 times.
7. Transfer 5 µL of each lysate by using an 8-well multichannel pipette to the corresponding PCR strip.
   Note: Make sure that the lysate is taken from the upper half of the lysis volume, avoid touching a potential pellet of debris. Ensure the lysate is pipetted directly on top of the frozen MasterMix.
8. Using a fresh strip of optical caps (provided in the kit) seal the PCR strip by hand (do not use the Un-/ Capping Tool). Re-close lysis strip with domed caps using the Un-/Capping Tool.
9. Repeat steps 5, 6, 8 and 9 with the remaining strips.
10. Ensure that all samples were pipetted on top of the MasterMix. In case some samples are not on top of the MasterMix, quickly spin down the strips in a centrifuge.
11. Transfer the PCR plate to the real-time instrument (use e.g. empty pipet tip box as mount) and start the run.
12. Store lysis plate at -20°C ± 2°C in case of PCR repetition.

- For specific instruction for PCR set-up and result export for the **Agilent AriaMx™**, proceed to **section 3**.
- For specific instruction for PCR set-up and result export for the **Bio-Rad CFX96 Touch™ (Deep Well)**, proceed **to section 4**.

Other PCR platforms should not be used with this kit.

3 AGILENT ARIAMX™: INSTRUCTIONS FOR USE WITH BAC*GENE* *SALMONELLA* SPP. KIT

The BAC*Gene Salmonella* spp. kit is validated for use on the Agilent AriaMx with software version 1.0.

The PCR platform should be prepared prior to commencing the practical work. For more detailed description of the instrument programming please refer to the user manual of the Agilent AriaMx.

3.1 PCR Setup

The following instructions outline how to setup a PCR run on the Agilent AriaMx™ using the touchscreen on the instrument.

3.1.1 Import template to platform

1. Turn on Agilent AriaMx™
2. Insert USB containing the "BACGene_Salmo_AriaMx_Template.amxt" file into slot at the front of the platform
3. Select "New experiment"
4. Select "Open Template"
5. Choose "Import". Select "BACGene_Salmo_AriaMx_Template.amxt" from USBDisk. The template is now imported onto the platform.

Note: This procedure only needs to be carried out the first time of usage.

3.1.2 Running PCR using template (for Agilent AriaMx™)

1. Turn on Agilent AriaMx™
2. Select "New experiment"
3. Select "Open Template"
4. Open "BACGene_Salmo_AriaMx_Template.amxt"
5. Select "Sync Plate"
   Please check that following parameters are included in the template:

   Plate Setup Tab:

   - Fluorophores:

     FAM™ and Cy5™ are selected for all 96 wells

   • Target Names: If more than one system is analysed on one plate, please use the following target names for respective wells of the BAC*Gene Salmonella* kit:

BAC*Gene Salmonella*	Target Name	Dye Name
Salmonella spp.	2014a	FAM™
IPC *Salmonella*	2014i	Cy5™

   - Sample type:
     - A1 should be defined as 'Calibrator'
     - B1 should be defined as 'Buffer'
     - All other wells should be defined as 'Unknown'

   Thermal Profile:

1 HOLD	42 CYCLES	
enzyme activation	denaturation	annealing & extension
15 min at 95°C	15 sec at 95°C	60 sec at 60°C
no data collection	no data collection	data collection

Note: The run should always be started using the provided template!

6. In "Thermal Profile" tab select "Run Experiment" to start the PCR run. Save the experiment under desired name.

3.2 Evaluation (for Agilent AriaMx™)

After run completion, data must be evaluated using the BAC*Gene* Evaluation Spreadsheet. The data exports from the Agilent AriaMx™ are described below.

3.2.1 Export of data (for Agilent AriaMx™)

1. When running the Agilent AriaMx™ without a connected PC, follow these instructions:
   a. Insert USB into slot on the front of the platform
   b. Go to 'Saved files' and select the run file
   c. Copy and paste this to the USB drive (listed on left side)
   d. Transfer file to desired location
2. Open Agilent AriaMx™ software ver. 1.0 on your PC for data evaluation and open the run file.

To name samples:

   a. Create a text (.txt) file with the following format: WELL NAME, SAMPLE ID e.g.
      A1, C+
      B1, C-
      C1, 12345
      ...
   b. In the run file, right click on any well and select "Import Well Name..."
   c. Select the .txt file to import and click "Open"
3. Ensure all wells are selected in the "Plate Setup" screen.
4. As a final check before export go to "Graphical Displays" tab and ensure that the "Background Based Threshold" is set from Cycle Range **5** thru **9** (this option can be found under "Amplification Plots").
5. To prepare a default configuration for result export, follow these instructions (**Note:** this step only needs to be carried out the first time you use the software):
   a. Go to "Export Data" tab
   b. To set up a default export:
      i From the "Definition" drop down menu select "Add new"
      ii Name this as "BACGene Salmo export"
      iii In "File Type" select "Text"
      iv In Items list, select "Amplification plots" and "Tabular Results"
   c. Press Save icon beside the "Definition" drop down menu to save this export configuration

6. To export results, follow these instructions (for Agilent AriaMx™):
   a. Go to "Export Data" tab
   b. Select "BAC*Gene* Salmo export" from the Definition drop-down menu
   c. Edit Tabular results by clicking on the blue pen so that the following columns are selected:
      - Well
      - Well Type
      - Sample Name*
      - Well Name*
      - Dye
      - Target
      - ΔR Last*
      - Threshold (ΔR)*
      - Cq (ΔR)*

      *In the current software version, these columns will need to be selected EVERY time you export the results
   d. Select "Export Data". Save to chosen location (Note: it is necessary to save both export files in the same folder)
   e. Proceed to 'BAC*Gene* Evaluation Sheet for data interpretation.

4 BIO-RAD CFX96 TOUCH™(DEEP WELL) INSTRUCTIONS FOR USE WITH BAC*GENE SALMONELLA* SPP. KIT

The BAC*Gene Salmonella* spp. kit is validated for use on the Bio-Rad CFX96 Touch™ and Bio-Rad CFX96 Touch™ Deep Well with software version 3.1.

The PCR platform should be prepared prior to commencing the practical work. For more detailed description of the instrument programming please refer to the user manual of the Bio-Rad CFX96 Touch™ or CFX96 Touch™ Deep Well.

4.1 PCR Setup

4.1.1 Running PCR using template

1. Turn on Bio-Rad CFX96 Touch™ (Deep Well)
2. Open the Bio-Rad CFX Manager™ 3.1
3. Choose 'Run setup'
4. Select instrument CFX96
5. Select run type 'User-defined'
6. In the protocol tab choose 'Select existing…'
7. Navigate to and select the protocol template 'BACGene_Salmo_CFX_Protocol' for the Bio-Rad CFX96 Touch™ and 'BACGene_Salmo_CFX_DW_Protocol' for Bio-Rad CFX96 Touch™ Deep Well
8. Select 'Next'
9. In the plate tab choose 'Select existing…'
   - Navigate to and select the plate template 'BACGene_Salmo_CFX_Plate'

   Optional for naming the samples:
   - Select 'Edit Selected…'
   - Select 'Spreadsheet View/Importer' from the panel or from 'Editing tools' menu
   - Include the sample names into the list
   - Select 'OK'
   - Select 'OK' again to leave the plate view
   - Select 'Next' to get to the 'Start run' tab

The run should always be started using the provided protocol and plate templates!

Please check that following parameters are included in the protocol (for Bio-Rad CFX96 Touch™(Deep Well)):

- Sample volume: 25 µL
- Thermal cycler times and temperatures

1 HOLD	**41 REPETITIONS**	
enzyme activation	denaturation	annealing & extension
15 min at 95°C	15 sec at 95°C	60 sec at 60°C
no data collection	no data collection	data collection

Please check that following parameters are included in the plate:

- Plate Type: BR White or Clear
- Scan Mode: All Channels
- Fluorophores: FAM™ and Cy5™ are selected for all 96 wells
- Target Names: If more than one system is analysed on one plate, please use the following target names for respective wells of the BAC*Gene Salmonella* kit:

BAC*Gene Salmonella*	**Target Name**	**Dye Name**
Salmonella spp.	2014a	FAM™
IPC *Salmonella*	2014i	Cy5™

- Sample type:
  - A1 should be defined as 'Positive control'
  - B1 should be defined as 'Negative control'
  - All other wells should be defined as 'Unknown'

4.2 Evaluation (for Bio-Rad CFX96 Touch™(Deep Well))

After run completion, data should be evaluated using the 'BAC*Gene* Evaluation Sheet'. The data exports from the Bio-Rad CFX96 Touch™ or Bio-Rad CFX96 Touch™ Deep Well are described below.

4.2.1 Export of data

1. When running the Bio-Rad CFX96 Touch™ (Deep Well) without a connected PC, follow these instructions:
   a. Insert USB into slot on the front of the platform
   b. Go to 'Saved files' folder and choose 'Real Time Data"
   c. Select desired file and choose 'File Options' to export to the USB
   d. Transfer file to desired location
2. Open Bio-Rad CFX Manager™ software version 3.1 and select the saved run. Save the resulting Optical file at desired folder.
3. Once the optical file is opened, go to 'Settings' tab and ensure the following options are selected:
   a. Cq Determination Mode = Single Threshold
   b. Baseline Setting = Baseline Subtracted Curve Fit
   c. Ensure to select 'Apply Fluorescence Drift Correction' under 'Baseline Settings'
   d. If you are running a mixed plate (e.g. BAC*Gene Salmonella* spp. and *Listeria* Multiplex, *Listeria monocytogenes* or *Listeria* spp. in the same run), make sure that the Plate type "BR White" is selected
4. To export results, follow these instructions:
   a. Go to 'Export' tab
   b. Select 'All Data Sheets' and export as text files (*.txt). Choose location for files to be saved.
   c. Select 'Custom Export' in text format with 'Tab' separator (*.txt) with at least the following columns and save in chosen location
   (Note: save all export files in the same folder):
      - Well
      - Fluorophore
      - Target Name
      - Content
      - Cq
      - Sample Name
      - End RFU
5. Proceed to 'BAC*Gene* Evaluation Sheet for data interpretation.

5 DATA INTERPRETATION

Data is interpreted using the 'BAC*Gene* Evaluation Sheet' provided. Data should be uploaded as described in 'BAC*Gene* Evaluation Sheet' file.

Interpretation of final sample results is summarized in the following table:

Salmonella	**Internal Positive Control**	**Final results**
Reaction positive	Not relevant	Positive for *Salmonella* spp.
Reaction negative	Valid	Negative for *Salmonella* spp.
Reaction negative	Invalid	Questionable*

* Refer to troubleshooting section (see 7.)

6 CONFIRMATION OF PRESUMPTIVE POSITIVE RESULTS

In the context of NF VALIDATION and AOAC-PTM certification, all samples identified as positive by the BAC*Gene Salmonella* spp. kit must be confirmed by (one of) the following test(s):

Option 1:

Using the conventional tests described in the methods standardized by CEN or ISO from colonies (including the purification step). The confirmation step must start from the enrichment broth.

Option 2:

By subculture of 100 µL from the enrichment broth in 9.9 mL RVS (incubation 21-27 h at 41.5°C ± 1°C) followed by streaking onto XLD* and another chromogenic agar followed by latex test (i.e. Oxoid) and biochemical galleries directly on isolated typical colonies without a purification step.

* Choose a second selective plating medium which is complementary to XLD agar and is based on different diagnostic characteristics to those of XLD agar to facilitate detection of, for instance, lactose positive or H2S-negative *Salmonella*. For examples of isolation media, see ISO 6579-1:2017, Annex E.

Option 3:

Using any other method certified NF VALIDATION or AOAC, respectively, the principle of which must be different from the BAC*Gene Salmonella* spp. kit. The protocol of detection of the second validated method used for the confirmation shall be followed entirely. All steps which are before the step from which the confirmation is started shall be common to both methods. The BAC*Gene Salmonella* spp. kit and the second validated method must have common first steps, for instance the same enrichment broth.

Independent of the confirmation method used, in the event of discordant results (presumptive positive with the alternative method, non-confirmed by one of the means described above, in particular by the latex test) the laboratory must follow the necessary steps to ensure the validity of the result obtained.

7 TROUBLESHOOTING

7.1 Troubleshooting problems upon delivery

Observation:
MasterMix plate is not frozen upon arrival

Possible Cause:
Plates defrosted during shipping or were not stored at -20°C upon arrival

Solution:
Contact Eurofins GeneScan Technologies Technical Service
(kits@eurofins.com or +49–(0)761–5038–100)
or your local distributor to arrange a new shipment and to return the defrosted plates.

7.2 Troubleshooting data export and import to BACGene Evaluation Sheet

Observation:
No results displayed in BACGene Evaluation Sheet after successful import of results

Possible Cause:
The content has not been enabled and Macros are not running properly

Solution:
Ensure the content is enabled by following instructions in links provided in the BACGene Evaluation Sheet or at the official Microsoft Office website (http://office.microsoft.com/). Then repeat the evaluation.

Possible Cause:
No C+ or C- has been defined

Solution:
Ensure that both C+ and C- have been defined. This can be done by defining "Task" in the drop-down menu for the specific wells in the "Results-tab view" worksheet.

Possible Cause:
The correct columns for Tabular results (Agilent AriaMx) or Custom Export (Bio-Rad CFX96 Touch™ (Deep Well)) were not selected when exporting results from the PCR run file.

Solution:
Ensure the correct columns are selected for the export and repeat the export and evaluation of results.

Observation:
Data import into the BACGene Evaluation Sheet was not successful

Agilent AriaMx™:

Possible Cause:
FAM™ or Cy5™ dye was deselected during examination of the run file

Solution:
Ensure both FAM™ and Cy5™ channels are selected and repeat result export. Also ensure all wells are selected.

Possible Cause:
Tabular results and/or amplification plots were not exported correctly

Solution:
Ensure result export was in the correct format as described in 'Instruction' tab of the BACGene Evaluation Sheet and in section 3.2.1 of the manual above. Then repeat the evaluation.

Bio-Rad CFX96 Touch™ (Deep Well):

Possible Cause:
The correct columns in Custom Export were not selected.

Solution:
Ensure result export was in the correct format as described in 'Instruction' tab of the BACGene Evaluation Sheet and in section 4.2.1 of the manual above. Then repeat the evaluation.

Possible Cause:
Custom Export text file was exported with 'Comma' separator instead of 'Tab' separator

Solution:
Ensure that the Custom Export is carried out correctly as described in 'Instruction' tab of the BACGene Evaluation Sheet and in section 4.2.1 above. Then repeat the evaluation.

Observation:
Second export file was not automatically detected during import into evaluation sheet

Possible Cause:
All required export files were not exported

Solution:
Ensure all required export files are successfully exported.

Possible Cause:
The names of the export files were changed

Solution:
When prompted, manually choose files or repeat export.

Possible Cause:
Both files were not saved in the same folder

Solution:
When prompted, manually choose appropriate file or ensure all export files are saved in the same folder.

Observation:
Fluorescent drift observed in curves from a Bio-Rad CFX96 Touch™ (Deep Well) PCR run

Possible Cause:
The "Apply Fluorescent Drift Correction" option was not selected during the run evaluation

Solution:
Ensure this option is selected and repeat the export of results. Then continue with the evaluation.

7.3 Troubleshooting results post evaluation

Observation:
C- negative control is positive (in the Salmonella system)

Possible Cause:
Cross contamination occurred

Solution:
Follow decontamination procedure.
Repeat lysis from enrichment and repeat PCR using fresh aliquots of all reagents.
If C- still continues to show a positive signal, contact Eurofins GeneScan Technologies.

Observation:
C- not in range (in the IPC system) – evaluation is not possible

Evaluation of the IPC is dependent on the C- control reaction. If this reaction is not in range, the IPC cannot be evaluated in the sample reactions.

Possible Cause:
Incorrect threshold was used

Solution:
The threshold is automatically set by the software (Agilent AriaMx™ software or Bio-Rad CFX96 Touch™) and should not be manually manipulated. Please refer to section 3.2.1 (AriaMx) or 4.2.1 (Bio-Rad CFX96 Touch™ (Deep Well)) to ensure correct export settings are used.

Possible Cause:
MasterMix plate was freeze-thawed more than 3 times

Solution:
The MasterMix plate should not be freeze-thawed more than 3 times as it may cause inactivation of the reagents.

Possible Cause:
95°C Lysis step was not performed correctly

Solution:
Ensure the heating block is reaching the correct temperature (95°C ± 2°C) by measurement with a thermometer. Ensure this step is performed for the correct amount of time (10 min).

Observation:
Cq value of the C+ is late or negative (in the Salmonella system) – evaluation is not possible

Possible Cause:
Pipetting error where no positive control or too little positive control material was added

Solution:
Repeat PCR ensuring 5 µL of the positive control material is pipetted into appropriate well (A1).

Possible Cause:
The positive control was not stored correctly

Solution:
If there is suspicion of incorrect storage, use a fresh tube of positive control material. When starting a new kit, always use the positive control material from that kit.

Possible Cause:
The positive control material has undergone too many freeze-thaw cycles

Solution:
Use a fresh tube of positive control material. Do not freeze/thaw more than 6 times. If C+ still continues to show a negative signal, contact Eurofins GeneScan Technologies.

Agilent AriaMx™:

Possible Cause:
In the automatic analysis mode, the AriaMx™ software sometimes sets the "Background Based Threshold" (Cycle Range 5 thru 9 and Sigma Multiplier 10) not in the early exponential amplification phase.

Solution:
Check amplification plots and thresholds for Cy5™ and FAM™. Set the threshold for the *Salmonella* system (FAM™ channel) manually in the early exponential phase of the amplification plots and ensure that the C+ amplification plot is included.

Observation:
A "Q" result is observed for sample in the BACGene Evaluation sheet

Possible Cause:
Missing PCR tubes

Solution:
Check layout of tubes in PCR platform to ensure it matches the correct layout

Possible Cause:
The frozen MasterMix was not in the bottom of the PCR tube, but in the lid

Solution:
Always visually check the level of the MasterMix before use. If the MasterMix is not at the bottom of the tubes, allow to thaw, spin down and re-freeze before use.

Possible Cause:
95°C Lysis step was not performed correctly

Solution:
Ensure the heating block is reaching the correct temperature (95°C ± 2°C) by measurement with a thermometer. Repeat lysis from enrichment and repeat the PCR.

Possible Cause:
Sample lysate was not taken from upper half of tube when transferring to PCR

Solution:
Repeat lysis from enrichment and repeat PCR. Ensure to take sample from upper half of tube.

Possible Cause:
Inhibition of PCR

Solution:
Repeat lysis from enrichment and repeat PCR.
If PCR remains inhibited, dilute the lysate sample with DNA-/Nuclease free water (one part sample plus four parts water and one part sample plus nine parts water).
If inhibition persists, review instructions of EN ISO 6579 and EN ISO 6887 for preparation of initial suspensions. Check if the matrix type has been validated for use with the BAC*Gene Salmonella* spp. kit. Only validated matrices should be used with this kit.

8 PRODUCT WARRANTIES, SATISFACTION GUARANTEE

Eurofins GeneScan Technologies GmbH ("GeneScan") warrants the products manufactured by it will be free of defects in materials and workmanship when used in accordance with the applicable instructions for a period equal to or shorter of one year from the date of shipment of the product(s) or the expiration date marked on the product packaging under the storage conditions, recommended in the instructions and/or on the package. GeneScan makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose. GeneScan's sole obligation with the respect to the foregoing warranties shall be, at its option, to either replace or to refund the purchase price of the product(s) or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies GeneScan promptly of any such defect. GeneScan shall not be liable for any direct, indirect or consequential damages resulting from economic loss or property damages sustained by buyer or any customer from the use of the product(s). A copy of Eurofins GeneScan GmbH terms and conditions can be obtained on request, and is also provided in our price lists.

9 TECHNICAL SUPPORT SERVICE

For technical assistance and more information please contact the Eurofins GeneScan Technical Service or your local distributor.

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