



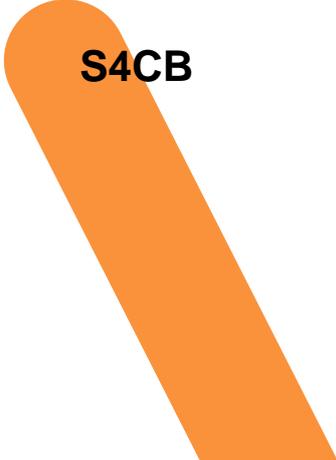
DNA  
DIAGNOSTIC

# User Manual

## Salmonella 4 Cows

DNA extraction – qPCR

Cat. No.:



**S4CB**

**USER MANUAL**  
Cat No. S4CB  
DNA Diagnostic A/S  
[www.dna-diagnostic.com](http://www.dna-diagnostic.com)  
Revision 2019.09.12

## TABLE OF CONTENTS

1. PURPOSE OF THE TEST.....	3
2. KIT COMPONENTS AND STORAGE.....	4
3. REQUIRED EQUIPMENT AND MATERIALS NOT INCLUDED IN THE KIT.....	5
4. PROTOCOL.....	6

**Trademarks:**

Black Hole Quencher® (BHQ®) and CAL Fluor® dye technologies incorporated in this product are used under licensing agreement with Biosearch Technologies, Inc. and protected by U.S. and world-wide patents issued, or in application.

## 1. PURPOSE OF THE TEST

*Salmonella* species can infect both cows and calves. *Salmonella* Dublin is the most common serotype of *Salmonella* in cattle. The infection often causes clinical diseases and production loss. Animals infected by salmonella can suffer from diarrhea and/or abortion. The source of infection is usually through fecal shedding of bacteria from infected cows, which can show disease symptoms or no symptoms.

The Salmonella 4 Cows kit is a fast, low-cost and easy-to-use method for the detection *Salmonella* species and *Salmonella* Dublin directly from feces samples, swabs of feces/rectum, or swabs of the environment. The Salmonella 4 Cows kit contains material for Fecal/Swab DNA extraction using a simple 96 deepwell-based extraction protocol (single tubes can be used) and specific qPCR mix pre-aliquoted in 8-strip tubes. The entire protocol from DNA extraction to result can be carried out in as little as 3 hours for 96 samples.

The complete kit has a limit of detection (LOD) of  $1 \times 10^3$  CFU per gram of fecal matter, or  $1 \times 10^2$  CFU per sample input (one swab or 0,1g feces).

## 2. KIT COMPONENTS AND STORAGE

The **Salmonella 4 Cows kit** contains material for DNA extraction and qPCR of **4x96 samples**. The Salmonella 4 Cows kit contains **one box** for storage at room temperature (RT) and **another box** for storage at **-20°C**:

<b>Box</b>	<b>COMPONENTS</b>	<b>AMOUNT</b>	<b>STORAGE</b>
I	96 deepwell plates (2mL) with beads solution and glass beads, sealed	4 x 96 well plates	Room Temperature
I	Adhesive seals	12 sheets	Room Temperature
I	Wash Buffer	4 bottles of 110 mL	Room Temperature
I	Optical lids for 0.2/0.1 mL plates	8 x 96 caps for (q)PCR	Room Temperature
I	96 well plates (0.2 mL), clear, empty	4 x 96 well plates	Room Temperature
I	Buffer P	2 bottles of 30 mL	Room Temperature
II	Lysis Buffer-I	2 bottles of 12 mL	-20°C
II	Mix I Additive for Lysis Buffer-I	4 vials of 1.334 mL	-20°C
II	<b>Salmonella 4 Cows qPCR Master Mix</b> in sealed 96 well plates (0.1 mL or 0.2 mL white tubes)	4 x 96 well plates	-20°C

NOTE: Buffer F is acquired separately – See 3. REQUIRED EQUIPMENT AND MATERIALS NOT INCLUDED IN THE KIT

### 3. REQUIRED EQUIPMENT AND MATERIALS NOT INCLUDED IN THE KIT

- Centrifuge for 96 well plates. Centrifuge **must be capable of running at 5000 x g**.  
*Example: Heraeus Multifuge X3R Centrifuge with HIGHPlate 6000 rotor.*
- Vacuum system capable of aspirating supernatant from 2mL Deepwell plates.  
*Example: Vacuum system connected to an 8-channel adaptor, together with the 'Pipetting Box for 2mL Deepwells' from DNA Diagnostic, and 'BRAND 2-200 µL pipette tips'. Contact DNA Diagnostic for information*  
*Example: 2mL deepwell platewasher. Contact DNA Diagnostic for further information.*  
*Example: Vacuum system with pipette tips (35mm from tip to rim stop).*  
*Note: Do not use manual pipetting*
- qPCR instrument with filters for FAM (492nm-516nm), HEX (535nm-555nm), ROX (585nm-610nm), CY5 (635nm-665nm).  
*Examples: ABI 7500/7500 Fast, ABI QuantStudios, ABI ViiA 7, Bio-Rad CFX96, BMS Mic qPCR, Agilent MX3005p/3000p, Agilent AriaMX, Roche LightCycler 480, etc*
- Vortexer for 96 deepwell plates and for single tubes  
*Example: IKA MS 3 basic/digital with Standard attachment (single tubes) and Microtiter attachment (96 wells)*
- Ice bucket or 0-4 °C cooling block.
- Standard lab pipettes and sterile filter tips.
- 0,2 mL 96-well PCR strip rack (plate support)

#### ***For sample collection and DNA extraction using Sarstedt Feces Tube***

- 6 bottles of 520mL Buffer F (DNA Diagnostic Cat. no. BF520)
- Sarstedt Feces Tube: Sarstedt 'Faeces Tube 76mm x 20 mm'.

#### ***For sample collection using eNAT swabs***

- eNAT swabs: COPAN eNAT™ kit (cat. 608CS01R): '1ml eNAT™ transport and preservation medium in 12x80mm screw cap tube and 1 regular FLOQSwab™'
- One bottle of 520mL Buffer F (DNA Diagnostic Cat. no. BF520)

#### ***For sample collection using boot swabs***

- Boot swabs (boot socks, bootswabs, sock swabs), including plastic bag. Adaptations of fabric swabs to be worn over the feet. Different fabrics and manufactures are accepted. See ISO 13307 for further information.
- One bottle of 520mL Buffer F (DNA Diagnostic Cat. no. BF520)

#### ***For Direct Feces extraction in single tubes***

- One bottle of 520mL Buffer F (DNA Diagnostic Cat. no. BF520)

## 4. PROTOCOL

Samples can be processed from Sarstedt tubes, eNAT swabs, feces samples directly, or boot swabs.

### Sample collection using eNAT swabs:

1. Open peel pouch and take out swab. Insert swab tip in rectum, swab feces directly, or swab surface. Be sure that the swab contains a layer of feces (for maximum sensitivity), but not an excessive layer (see figure).

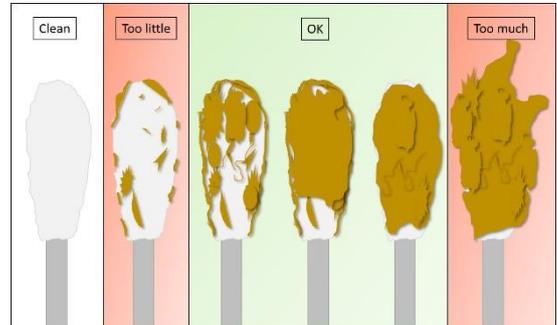
*Note: If swabbing a dry surface, wet the swab tip in the eNAT solution, or sterile/DNA-free H<sub>2</sub>O, before sampling.*

2. Open the eNAT tube, insert the swab tip into the tube with eNAT solution and break off the tip at the red break-point. Close the tube with the swab inside. Tighten the lid to avoid leakage.

3. Write sample name/number on tube.

4. Send the tube to analysis lab. The sample can stay at ambient temperatures for 3 days, at 0-5 °C for 14 days, or for 1 year at -20 °C

*Note: For most target bacteria, store the tube at 0-5 °C until extraction (maximum 7 days).*



### Sample collection using Sarstedt Tubes:

5. Open tube. With the scoop attached to the lid, collect approximately 1 gram of feces (0,5g to 1,5 g), but not an excessive layer (see figure).

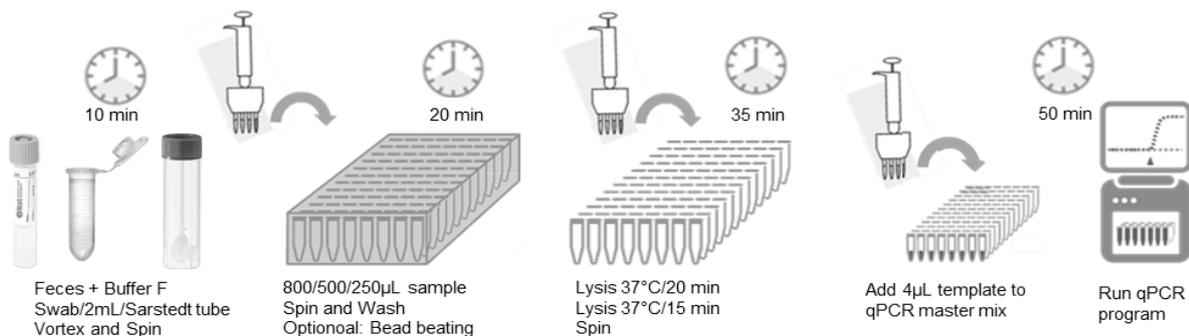
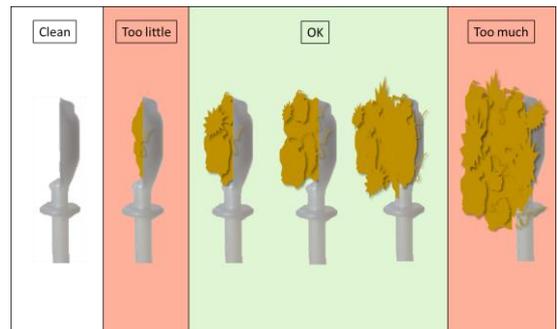
*Note: If feces are liquid or watery, collect 5 scoops of sample*

6. Insert the scoop into the tube and close the tube. Tighten the lid to avoid leakage.

7. Write sample name/number on tube.

8. Send the tube to analysis lab. Store the sample tube cold (4 °C ± 3 °C) until extraction, for maximum 7 days.

*Note: Storage times affect different bacterial species differently.*



Overview of protocol

### **eNAT swab extraction:**

1. Open eNAT tube and add **1 mL Buffer F**. Do not remove swab from lid. Close tube (including swab).
2. **Vortex** vigorously for **15 seconds** (1<sup>st</sup> vortex).
3. Open tube, remove and discard swab, by pulling it off the cap. Close the tube with the cap.  
*Note: Be careful not to cross-contaminate samples. E.g. by using the same two gloved fingers for all samples, and discarding the gloves after.*
4. **Vortex** tube vigorously for **15 seconds** (2<sup>nd</sup> vortex). Be sure sample is completely homogenized; otherwise prolong mixing time/intensity.
5. Spin tube **1000 x g** for **1 minute**. Spin can be performed in the eNAT tube directly, or by transferring of all sample to a 2mL tube or 2mL deepwell.
6. Use **800 µL** supernatant for further extraction; see **DNA extraction** protocol.

### **Sarstedt tube extraction:**

1. Open tube and add **7 mL Buffer F**. Close and tighten lid.
2. **Vortex** the tube vigorously for **15 seconds**. Be sure sample is completely homogenized with the buffer; otherwise prolong mixing time/intensity.  
*Note: Due to foam formation, do not stop the vortexing during the 15 seconds, as efficient homogenization can otherwise be hard to obtain.*
3. Spin tube **1000 x g** for **1 minute**. Spin can be performed in the Sarstedt tube directly, or by transferring 1,5mL liquid to a 2mL tube or 2mL deepwell plate.
4. Carefully open the tube/deepwell without disturbing the pellet.  
Use **250 µL** supernatant for further extraction (avoid pellet); see **DNA extraction** protocol.

### **Direct Feces extraction in single tubes:**

1. Homogenize the feces sample and transfer **0,1g ± 0,03g feces** to a 2mL tube.  
*Note: If feces are liquid or watery, transfer 100 µL of sample to a tube.*
2. Add **1,2 mL Buffer F** and close lid.
3. Vortex tube vigorously for **20 seconds**, or continue until samples are homogenized.  
Alternatively, secure the tubes on a mixer/vortexer and mix vigorously for **1 to 10 minutes**, until samples are homogenized; otherwise prolong mixing time/intensity.
4. Spin tube at **1000 x g** for **1 minute**.
5. Use **500 µL** supernatant for further extraction; see **DNA extraction** protocol.

### **Boot swab extraction:**

1. Add **100 mL** sterile saline or PBS buffer to the boot swab in a plastic bag or container.  
Mix samples **thoroughly and vigorously**, either manually or by automatic shaking.
2. Transfer **500 µL** of the liquid to a well in a 2mL deepwell plate
3. Add **800 µL Buffer F** and mix by pipetting up and down. Seal wells.
4. Spin deepwell at **1000 x g** for **1 minute**.
5. Use **800 µL** supernatant for further extraction; see **DNA extraction** protocol.

## DNA extraction

1. Carefully open deepwell and discard seal. Transfer **800 µL** (eNAT swab or Boot swab extraction), **250 µL** (Sarstedt tubes) **or 500µL** (Direct Feces extraction) of supernatant to the wells in a 2 mL Beads solution deepwell plate (or 2 mL tubes) carrying 650 µL Beads solution and glass beads.
2. Spin **5000 x g for 5 minutes**
3. Remove and discard the seal and place the deepwell plate in a 96 well deepwell plate washer. Remove supernatant with the plate washer.

Note:

- *It is important to remove all supernatant from the pellet without touching the pellet. Therefore, correct adjustment of the plate washer syringes is required. Using an empty deepwell plate and colored water, the program should leave approximately 20 µL liquid in all wells. Contact DNA Diagnostic for program example*
- *Vacuum system with pipette tips (35mm from tip to rim stop). Aspire along the sides of the wells, on the opposite side of the pellet, until the pipette tip rim stop hits the top of the wells. Check that supernatant is removed but pellet intact.*
- *Using the 'Pipetting Box for 2mL Deepwells' from DNA Diagnostic, BRAND 2-200 µL pipette tips, and a Vacuum system. Contact DNA Diagnostic for information*

4. Wash: Add **1 mL Wash Buffer** and seal the plate with **adhesive seals**.
5. Spin **5000 x g for 5 minutes**
6. Remove and discard the seal and place the deepwell plate in a 96 well deepwell plate washer. Remove supernatant with the plate washer
7. Prepare per reaction **6 µL Mix I additive** to **54 µL Lysis buffer-I** and **60 µL Buffer P**. Mix.

Lysis-I Mix	1 reaction	9 reaction	100 reactions
Lysis Buffer-I	54 µL	486 µL	5400 µL
Mix I additive	6 µL	54 µL	600 µL
Buffer P	60 µL	540 µL	6000 µL
TOTAL	120 µL	1080 µL	12000 µL

8. Add **120 µL Lysis-I Mix**, to each pellet and cover with **adhesive seal**.
9. Mix briefly and spin down shortly to bring the liquid to the bottom (e.g. 10 seconds at 1500 x g).
10. Remove caps from clear 0.2 mL tubes (96 well plate in 8-well strips).
11. Use an 8-channel pipette with filter tips to transfer **60 µL** (up to 120 µL) from each well of the deepwell plate into the corresponding tubes in the clear 0.2 mL tubes. Close tubes with the caps.
12. Incubate the tubes at: **37°C for 20 min.** → **95°C for 15 min.** → **4°C for 5 min.**  
A thermocycler/(q)PCR instrument can be used.
13. Centrifuge the 96 well plate at **5000 x g for 5 min.**

*Note it is important to use a 96 tube support for the 96 well plate during centrifugation.*

*Note: The extracted DNA can be stored at -20°C.*

**qPCR:**

1. Thaw Salmonella 4 Cows qPCR Mix (can be divided in 8-tube strips) on ice or a cooling block. Spin down briefly (e.g. 1500 x g for 10 seconds). When removing the seal, be sure that the tubes are stable to avoid spillage of content.  
Keep qPCR Mix on ice/cooling block until it is loaded into the qPCR instrument
2. Carefully remove the caps from the 96 well plate with extracted DNA. Use an 8-channel pipette with filter tips to transfer **4 µL** of each supernatant (avoid pellet) directly to the corresponding tubes of the 96 well plate with qPCR Mix.
3. Close the qPCR tubes with the optical lids. Spin down briefly (e.g. 1500 x g for 10 seconds)
4. Load the qPCR tubes into the qPCR (real time PCR) instrument. Run the following program:

Denaturation	95°C	1 min	1x
Denaturation	95°C	5 sec	40 x
Annealing + Elongation	60°C	25 sec	

Use **FAM, ROX and HEX** filters for detection of the Internal Amplification Control (IAC), Salmonella species and Salmonella Dublin, respectively. Read fluorescence at end of each annealing/elongation step. Salmonella 4 Cows is compatible with fast thermocyclers and the qPCR master mix is supplied in 0.1 mL white low profile (WLP) or 0.2 mL white regular profile (WRP).

**Important notes for Agilent MX3005p and MX3000 users**

- Targets: Define the three targets: ROX, HEX and FAM.
- Read fluorescence at end of each annealing/elongation step.
- Set “gain settings” to FAM=2, ROX=1 and HEX=1

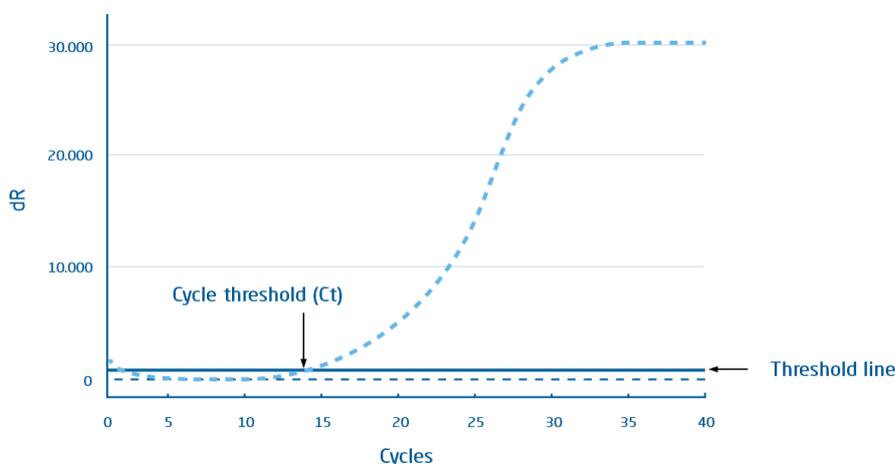
**Important notes for ABI 7500 users**

- Targets: While defining the three targets: ROX, HEX and FAM, choose for all targets “None” as Quencher.
- Passive reference dye: Select “None” instead of “ROX or HEX” as passive reference dye.

**Important notes for CFX 96 touch users**

- Targets: Define the three targets: ROX, HEX and FAM.
- Read fluorescence at end of each annealing/elongation step.

## Interpretation of analysis:



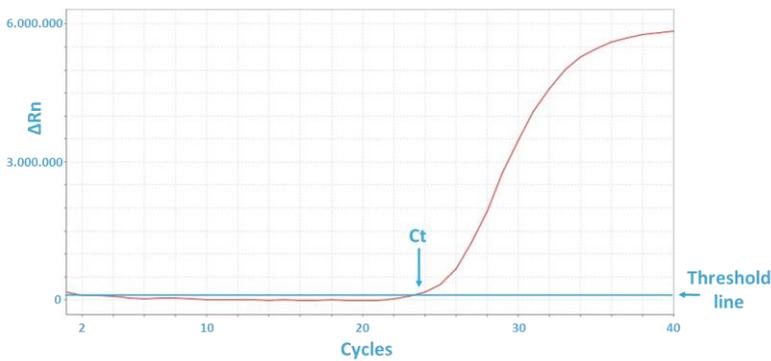
Amplification plot with the number of PCR cycles is shown on the x-axis, and the background subtracted fluorescence from the amplification reaction is shown on the y-axis. The threshold line is used to find the Ct value, where it intersects the amplification curve when the fluorescence increases due to PCR amplification. The threshold line is calculated automatically by the instrument giving in this example a Ct value of 14.

1. Check amplification plot for **FAM (IAC)** signals and corresponding Ct-values. Manually check assigned thresholds and baselines, and adjust if necessary, according to normal qPCR analysis standards (see 'Extended amplification plot analysis').  
The Ct must be in the interval **27-34 to confirm the qPCR reaction is functional**.  
*Reactions with no FAM signal, signal absent or outside the interval 27-34 **must be retested**.*  
*Retest inhibited samples by diluting the lysate 1:5 in Buffer P, and running qPCR again.*  
*Note: the FAM Ct can be > 34 or missing if a Ct for ROX, HEX is low. Retesting not needed.*
2. Check amplification plot for **ROX** and **HEX** signals and corresponding Ct-values. Manually check assigned thresholds and baselines, and adjust if necessary, according to normal qPCR analysis standards (see 'Extended amplification plot analysis').

Target	Filter	Positive	Negative	Retest
		<b>Ct</b>	<b>Ct</b>	<b>Ct</b>
Salmonella species	ROX	≤38	absent	38-40*
Salmonella Dublin	HEX	≤38	absent	38-40*
IACB	FAM	≤34		>34 or absent

\*Inconclusive. Repeat the qPCR with the same DNA extraction template to confirm or reject the result. If the second test is negative, the sample is considered negative. If the second test is positive (Ct < 40), the sample is considered positive.

### Extended Amplification plot analysis (if needed):



**Figure:** Amplification plot from ABI 7500 Fast with X-axis showing cycle number and Y-axis showing background subtracted fluorescence. The threshold line is used to find the Ct value. Ct is the intersection between the amplification curve and the threshold line. The threshold line is set just above the base line in this example resulting in a Ct value of 23,5.

Ct values are calculated automatically by the instrument. The algorithms used to calculate Ct values differ between qPCR instrument software packages. The threshold line in the amplification plot should be set manually to allow accurate Ct determination. The threshold line should be set just above the background fluorescence, so that the threshold line intersects the amplification curves when the fluorescence increases due to PCR amplification. The threshold level should however be set sufficiently high to allow for fluctuations in background fluorescence and background fluorescence drift (a linear increase in fluorescence). Sometimes the baseline and the amplification curves can be better separated by looking at the curves in the 'Log Scale', instead of the 'Linear Scale'.

### The threshold level can be set manually using the following the steps:

**ABI; 7500, 7500 Fast, ViiA 7:** In the Analysis window, choose "Plot Type: deltaRn vs Cycle", and "Graph Type: Linear". Unmark "Auto Threshold" and "Auto Baseline". Then click "Show Threshold" and with the mouse move the threshold line above the baseline.

**Bio-Rad CFX96:** Click the "Settings" tab, choose "Baseline Setting"; "Baseline Subtracted Curve Fit" and "Apply Fluorescence Drift Correction". Baseline Correction: Click the "Settings" tab, choose "Baseline Threshold"; "Baseline Cycles" choose "User defined (10-15)" and "Single Threshold" choose "User defined (750)" (see 'Extended Amplification plot analysis' above if needed).

**Qiagen Rotor-Gene Q:** Click "Analysis", choose the fluorescence to analyze, click "Linear Scale", "Slope Correct" and "Auto-Scale". In the right side panel, under "CT Calculation", click the button next to "Threshold" and mark the threshold level on the amplification plot.

**Roche LightCycler 480:** Choose "Absolut Quantification" and "Fit Points". Then click the tab "Noise Band" and click the "Noiseband" drop down button and choose "Noiseband (Fluoresc)". Use the mouse to set the Noise Band.

**Agilent MxPro:** The automatic threshold level can be used. Choose "Adaptive Baseline Correction" and "Background-based Threshold" using cycle 7-11 and a sigma multiplier of 10.

# DNA DIAGNOSTIC

## **For more information, contact**

DNA Diagnostic A/S  
Voldbjergvej 14  
8240 Risskov  
Denmark  
Tel.: +45 8732 3050  
E-mail: [info@dna-diagnostic.com](mailto:info@dna-diagnostic.com)  
[www.dna-diagnostic.com](http://www.dna-diagnostic.com)

*DNA Diagnostic A/S was established in 1992. DNA Diagnostic A/S is an ISO 13485 certified developer, manufacturer, and worldwide supplier of PCR based in vitro diagnostic kits.*

**Cat. No.:**

**S4CB**

**USER MANUAL**