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MICROBIOLOGICAL METHODS

# Evaluation of the Thermo Scientific SureTect Salmonella Species PCR Assay in a Broad Range of Foods and Select Environmental Surfaces: Pre-Collaborative and Collaborative Study: First Action 2021.02

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## Abstract

**Background:** The Thermo Scientific™ SureTect™ Salmonella species PCR Assay utilizes Solaris™ reagents for performing PCR for the rapid and specific detection of *Salmonella* species in a broad range of foods and select environmental surfaces.
**Objective:** The aims were to demonstrate the reproducibility of the Thermo Scientific SureTect Salmonella species PCR Assay in a collaborative study using a challenging matrix, cocoa powder, and to extend the scope of the method.
**Method:** In the collaborative study, the candidate method was compared to the US Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) Chapter 5 *Salmonella* reference method. The candidate method used two PCR thermocyclers, the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR instrument (QS5) and the Applied Biosystems 7500 Fast Real-Time PCR instrument (7500 Fast). Fourteen participants from nine laboratories located within the United States and Europe were solicited for the collaborative study, with 12 participants submitting valid data. Three levels of contamination were evaluated for each matrix. Statistical analysis was conducted according to the probability of detection statistical model. In addition, 11 matrix studies were performed comparing the candidate method to the FDA/BAM Chapter 5 or US Department of Agriculture, Food Safety and Inspection Service, *Microbiology Laboratory Guidebook* 4.10 Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg, and Siluriformes (Fish) Products and Carcass and Environmental Sponges reference method. Nine of these matrices were also compared to the EN ISO 6579–1:2017/Amd.1:2020(E) Microbiology of the food chain—Horizontal method for the detection, enumeration and serotyping of Salmonella—Part 1: Detection of Salmonella spp.—AMENDMENT 1: Broader range of incubation temperatures, amendment to the status of Annex D, and correction of the composition of MSRV and SC reference method.

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**Results:** In the collaborative study, the difference in laboratory results indicates equivalence between the candidate method and reference method for the matrix evaluated, and the method demonstrated acceptable interlaboratory reproducibility as determined in the collaborative evaluation. False-positive and false-negative rates were determined for the matrix and produced values of <2%. The two PCR thermocyclers (QS5, 7500 Fast) performed equivalently. There were no result differences between candidate method confirmations and reference method confirmations. In the pre-collaborative matrix extension, the results from the matrix studies showed a comparable performance between the candidate method and the tested reference methods.
**Conclusions:** Based on the data generated, the method demonstrated acceptable interlaboratory reproducibility data and statistical analysis.
**Highlights:** Due to the COVID-19 pandemic, some participants had to be trained remotely. Additionally, 375 g cocoa powder is known to be a challenging matrix for PCR methods. No unusual cross-contamination or false-positive/negative was reported, highlighting the ease of use, reproducibility, and robustness of the method.

Low-moisture foods typically do not support the growth of *Salmonella*; however, studies have indicated that the organism can remain viable for extended periods of time. In fact, *Salmonella* does not need to propagate to cause illness; infections can occur with products contaminated with as low as 1 CFU/g (1). While rare, *Salmonella* outbreaks associated with low-moisture products often impact large numbers of people (1). Over the last few decades, several low-moisture product outbreaks have occurred involving chocolate, raw almonds, dry seasonings, pet food, and peanut butter (1).

Typically, Salmonellae are sensitive to heat processing; however, these organisms can become resistant as the water activity of a product becomes lower (2). Manufacturers must ensure that their heat-processed, low-moisture products do not become contaminated after processing through the addition of additives if the food comes in contact with contaminated material (3).

The Thermo Scientific™ SureTect™ Salmonella species PCR Assay is based upon the use of Solaris™ reagents for performing PCR. Dye-labeled probes target unique DNA sequences specific to *Salmonella* species, and an internal positive control (IPC). Target DNA, if present, is detected by real-time PCR. Analysis software provides interpretation of results. The IPC template, primers, and probe provide an internal control with each reaction to show that the PCR process has occurred. It is unnecessary to incorporate positive control organisms with routine testing of samples.

Prior to the collaborative study, the SureTect Salmonella species PCR Assay was validated according to the current AOAC INTERNATIONAL guidelines (4) in an AOAC *Performance Tested Method*℠ study in a broad range of foods and select environmental surfaces. The SureTect Salmonella species PCR Assay was awarded *Performance Tested Method* certificate 051303 in May 2013 for nine food matrices and one environmental surface. Since the original study, matrix extensions were conducted in August 2015 and August 2021. In order to extend the scope of the candidate method, 11 matrix studies were performed against the appropriate US Food and Drug Administration (FDA) *Bacteriological Analytical Manual (BAM)* Chapter 5 *Salmonella* (5) and US Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS) *Microbiology Laboratory Guidebook (MLG)* 4.10 Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg, and Siluriformes (Fish) Products and Carcass and Environmental Sponges (6) reference methods, and nine of them were also compared to the EN ISO 6579–1:2017/Amd.1:2020(E) Microbiology of the food chain—Horizontal method for the detection, enumeration and serotyping of Salmonella—Part 1: Detection of Salmonella spp.—AMENDMENT 1: Broader range of incubation temperatures, amendment to the status of Annex D, and correction of the composition of MSRV and SC (7) reference method. Shortened incubation times, large test portions, challenging matrixes with polyphenols, high background or technological microflora, and high fat content were tested. The following matrix studies were performed by Q Laboratories in Cincinnati, OH, USA: fresh bagged spinach (375 g), raw beef trim (375 g), cocoa powder (375 g), cocoa butter (375 g), cocoa liquor (375 g), and dark chocolate (>70% cocoa solids; 375 g). Cut cabbage (25 g), cut mango (25 g), grated Cheddar cheese (25 g), single cream (8% fat; 25 g), and feta cheese (25 g) were tested by the Thermo Fisher Scientific laboratory in Basingstoke, UK. *See* Table 1 for an overview of the previously and newly validated matrixes for *Performance Tested Method* certification.

## Experimental

### *Collaborative Study*

The purpose of this collaborative study was to compare the reproducibility of the SureTect Salmonella species PCR Assay to the FDA/BAM Chapter 5 (5) reference method for cocoa powder (375 g).

### *Study Design*

In this collaborative study cocoa powder was evaluated. The matrix was obtained from a local retailer and screened for the presence of *Salmonella* by the FDA/BAM Chapter 5 reference method and the SureTect Salmonella species PCR Assay. The cocoa powder was artificially contaminated with a lyophilized culture of *Salmonella enterica* serovar Kentucky American Type Culture Collection (ATCC; Manassas, VA, USA) 9263 (antigenic properties: C2 (8) 20; i; z6 and origin: unknown). The matrix was inoculated at two levels of contamination: a high inoculation level of approximately 2–5 CFU/test portion and a low inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions (0 CFU/test portion) was also included. The inoculated test portions were held for 2 weeks at ambient temperature (20–25°C), prior to initiating testing.

Twelve replicate samples from each of the three inoculation levels were analyzed by each participant. Due to different test portion sizes, enrichment media, and enrichment conditions, an unpaired study design was followed. A total of 72 samples, 36 for the SureTect Salmonella species PCR Assay (375 g test portion) and 36 for the reference method (25 g test portion) were sent to each participating technician. Collaborators were also sent an uninoculated test portion for determining the total

**Table 1.** Performance tested method validation study summary for the Thermo Scientific SureTect Salmonella species PCR Assay

| Thermo Scientific SureTect Salmonella species PCR Assay— *Performance Tested Method* **051303** | | | | | | |
|---|---|---|---|---|---|---|
| Original *Performance Tested Method* certificate issued | Matrixes | Sample size | Enrichment media/dilution | Enrichment time, h | Enrichment temperature, °C | Reference method |
| May, 2013 | Raw ground beef (80% lean) | 25 g | BPW[a]/1-in-10 | 8–24 | 41.5 ± 1 | ISO[b] |
| | Raw ground beef | 25 g | BPW/1-in-10 | 18–24 | 34–38 | ISO |
| | Pork frankfurters | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Raw chicken breast | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Bagged lettuce | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Non-fat dried milk powder | 25 g | BPW/1-in-10 | 18–26 | 34–38 | ISO |
| | Cooked shrimp (heads off) | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Chilled ready-to-eat meal (containing beef) | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Pasteurized liquid whole egg | 25 g | BPW/1-in-10 | 18–26 | 34–38 | ISO |
| | Stainless steel (slab, 304 series, brushed finish) | 1 inch × 1 inch, swab | BPW/10 mL | 18–26 | 34–38 | ISO |
| | Stainless steel (slab, 304 series, brushed finish) | 4 inch × 4 inch, sponge | BPW/100 mL | 18–26 | 34–38 | ISO |
| Method Modification[c] | Matrixes | Sample size | Enrichment dilution | Enrichment time, h | Enrichment temperature, °C | Reference method |
| August, 2015 | Wet cat food | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Raw ground pork | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Dry dog food | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Pasteurized 2% milk | 25 mL | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Mung bean sprouts | 25 g | BPW + 20 mg/L novobiocin/1-in-10 | 20–28 | 41.5 ± 1 | ISO |
| | Cut cantaloupe | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Chilled pizza dough | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Whole black peppercorns | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Peanut butter | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Ice cream (vanilla) | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO |
| | Dark chocolate (85% cocoa solids) | 25 g | CSR[d]/1-in-10 | 20–28 | 34–38 | ISO |
| | Raw ground beef (80% lean) | 375 g | mTSB[e]/1-in-5 | 9–24 | 41.5 ± 1 | ISO |
| | Plastic surface (polystyrene Petri dish) | 1 in. × 1 in., swab | BPW/10 mL | 18–26 | 34–38 | ISO |
| | Plastic surface (large polystyrene Petri dish) | 4 in. × 4 in., sponge | BPW/100 mL | 18–26 | 34–38 | ISO |
| Method modification[f] | Matrixes | Sample size | Enrichment dilution | Enrichment Time, h | Enrichment temperature, °C | Reference method |
| January, 2021 | Cocoa powder | 375 g | BPW or NFDM[g]/1-in-10[h] | 22–30 | 34–38 | ISO & FDA/BAM[i] |
| | Cocoa liquor | 375 g | BPW or NFDM/1-in-10 | 22–30 | 34–38 | ISO & FDA/BAM |
| | Cocoa butter | 375 g | BPW or NFDM/1-in-10 | 22–30 | 34–38 | ISO & FDA/BAM |
| | Dark chocolate (>70% cocoa solids) | 375 g | BPW or NFDM/1-in-10 | 22–30 | 34–38 | ISO & FDA/BAM |
| Method modification[j] | Matrixes | Sample size | Enrichment dilution | Enrichment time, h | Enrichment temperature, °C | Reference method |
| April, 2021 | Cut cabbage | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO & FDA/BAM |
| | Cut mango | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO & FDA/BAM |
| | Grated Cheddar cheese | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO & FDA/BAM |
| | Single cream (8% fat) | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO & FDA/BAM |
| | Feta cheese | 25 g | BPW/1-in-10 | 20–28 | 34–38 | ISO & FDA/BAM |
| | Raw beef trim | 375 g | mTSB/1-in-5 | 8–24 | 41.5 ± 1 | MLG[k] |
| | Fresh bagged spinach | 375 g | BPW/1-in-10 | 8–24 | 41.5 ± 1 | FDA/BAM |

[a]BPW = Buffered peptone water.
[b]ISO = EN ISO 6579: Microbiology of the food chain—Horizontal method for the detection, enumeration and serotyping of Salmonella—Part 1: Detection of Salmonella spp.
[c]AOAC *Performance Tested Method* Certificate No. 51303.
[d]CSR = Cocoa sample recovery broth.
[e]mTSB = Modified tryptone soya broth.
[f]Matrices approved in January 2021 for *Performance Tested Method* certification (the data are also included in this report).
[g]NFDM = Non-fat dried milk. NFDM was supplemented with Brilliant Green Due (0.018 g/L).
[h]Cocoa and chocolates matrices were enriched with BPW and NFDM and tested against both the ISO and FDA/BAM reference methods.
[i]US Food and Drug Administration *Bacteriological Analytical Manual* (BAM), Chapter 5—Salmonella.
[j]Matrices approved with First Action *Official Methods*℠.
[k]US Department of Agriculture Food Safety and Inspection Service Microbiology Laboratory Guidebook (USDA FSIS MLG) 4.10 Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg, and Siluriformes (Fish) Products and Carcass and Environmental Sponges.

aerobic plate count (APC) following the US FDA (2001) *Bacteriological Analytical Manual* Chapter 3: Aerobic Plate Count reference method (8) on the day samples were received.

A detailed collaborative study packet outlining all necessary information related to the study including media preparation, test portion preparation, and documentation of results was sent to each collaborating laboratory prior to the initiation of the study.

### *Preparation of Inoculum and Test Portions*

The *Salmonella* strain used in this evaluation was propagated onto tryptone soy agar (TSA) with 5% sheep blood (SBA) from a Q Laboratories frozen stock culture stored at –70°C. The organism was incubated for 24 ± 2 h at 35 ± 1°C. Isolated colonies were picked to 10 mL brain heart infusion (BHI) broth and incubated for 18–24 h at 35 ± 1°C.

A lyophilized culture was used to artificially contaminate the cocoa powder. The culture was prepared by diluting the overnight BHI broth culture in a sterile cryoprotectant, reconstituted non-fat dry milk (NFDM), and placing it onto a freeze-dry system for 48–72 h. Afterward, a bulk lot of the instant NFDM was inoculated, in a drop-wise manner, with the lyophilized culture and mixed thoroughly with sterile sampling devices to ensure even distribution. For the preparation of the candidate method test portions, 25 g inoculated test product was mixed with 350 g uninoculated test product to prepare 375 g test portions, which were packaged in sterile Whirl-Pak® bags. For the reference method test portions, 25 g samples from the bulk lots were directly sampled and packaged in sterile Whirl-Pak® bags. After inoculation, the test matrix was held for 2 weeks at room temperature (24 ± 2°C) prior to analysis.

To determine the level of *Salmonella* in the test matrix, a five-tube most probable number (MPN) method was conducted on the first day of analysis following the FDA/BAM Chapter 5 reference method. The MPN was determined by analyzing 5 × 50 g test portions, the reference method test portions from the collaborating laboratories, and 5 × 10 g test portions. The 25 g test portions from the collaborative study were used as the middle level. To the 50 g test portions, 450 mL reconstituted NFDM (supplemented with 1% Brilliant Green Dye solution) was added, and for the 10 g test portions, 90 mL reconstituted NFDM (supplemented with 1% Brilliant Green Dye solution) was added and incubated following the FDA/BAM Chapter 5 reference method. The total number of positive results was documented, and the MPN and 95% confidence intervals were calculated using the Least Cost Formulation (LCF) MPN Calculator, Version 1.6, provided by AOAC Research Institute (RI) (9).

### *Test Portion Distribution*

All samples were labeled with a randomized, blind-coded three-digit number affixed to the sample container. All international test portions were shipped on a Monday while all domestic test portions were shipped on a Wednesday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transport Association. Upon receipt, samples were held by the collaborating laboratory at ambient temperature (20–25°C) until the following Monday when analysis was initiated after a total equilibration time of 2 weeks. A temperature recorder was included in each shipment to track the temperature of the package during transit. Participants were instructed to obtain the temperature of their package upon receipt and document the results on the Sample Receipt Confirmation form provided, and fax or e-mail it back to the study director.

### *Test Portion Analysis*

Collaborators were instructed to follow the appropriate preparation and analysis as outlined in the study protocol. Two separate sets of 36 test portions (12 high, 12 low, and 12 uninoculated controls for each method) were analyzed due to the unpaired study design. The SureTect Salmonella species PCR Assay test portions (375 g) were enriched with 3375 mL prewarmed (34–38°C) buffered peptone water (BPW) ISO formulation, homogenized by hand for at least 30 s and incubated for 20–28 h at 34–38°C. After enrichment the samples were diluted 1-in-5 in BPW before proceeding to the lysis step. The lysis step was performed using the Applied Biosystems™ SimpliAmp™ Thermal Cycler. The SureTect Salmonella species PCR Assay analysis was then conducted using the same procedure on two thermocyclers: the Applied Biosystems QuantStudio™ 5 Real-Time PCR instrument and the Applied Biosystems 7500 Fast Real-Time PCR instrument. Both instruments have been included in the *Performance Tested Method* evaluations of the SureTect Salmonella species PCR Assay. Out of the 12 collaborators that successfully conducted the testing, six participants conducted analysis using the QuantStudio 5 and six participants conducted analysis using the 7500 Fast instrument. Table 2 presents a summary of the collaborator participation along with instrument utilized.

Regardless of the presumptive result, all test portions were confirmed following the FDA/BAM Chapter 5 reference method beginning with a transfer to secondary enrichments (tetrathionate [TT] broth and Rappaport–Vassiliadis [RV] broth). The RV tubes were incubated at 42 ± 0.2°C for 24 ± 2 h. Because the cocoa powder APC results were $<10^4$ CFU/g, the TT tubes were incubated at 35 ± 2°C for 24 ± 2 h. After incubation, secondary enrichments were streaked to selective agars xylose lysine deoxycholate (XLD), Hektoen enteric (HE), and bismuth sulfite (BS) and incubated at 35 ± 2°C for 24 ± 2 h. If no visible colonies were present after 24 h of incubation on the BS plates, they were reincubated for an additional 24 ± 2 h at 35 ± 2°C. Typical isolated colonies from the selective agars were transferred to triple sugar

**Table 2.** Participation of each collaborating laboratory in the collaborative study for cocoa powder

| Collaborator ID No. | Participating laboratory | Analyst | Instrument | Participated[a] |
|---|---|---|---|---|
| 1[b] | 1 | 1 | QS5[c] | N |
| 2 | 2 | 1 | 7500 Fast[d] | Y |
| 3 | 3 | 1 | QS5 | Y |
| 4[e] | 4 | 1 | QS5 | N |
| 5 | 5 | 2 | 7500 Fast | Y |
| 6 | 6 | 1 | QS5 | Y |
| 7 | 3 | 2 | QS5 | Y |
| 8 | 5 | 2 | 7500 Fast | Y |
| 9 | 7 | 1 | 7500 Fast | Y |
| 10 | 7 | 2 | 7500 Fast | Y |
| 11 | 8 | 1 | QS5 | Y |
| 12 | 9 | 1 | QS5 | Y |
| 13 | 9 | 2 | QS5 | Y |
| 14 | 9 | 3 | 7500 Fast | Y |

[a]Y = Collaborator analyzed the food type. N = Collaborator did not analyze the food type.
[b]Collaborator voluntarily withdrew prior to testing.
[c]Applied Biosystems™ QuantStudio™ 5 Real-Time PCR instrument.
[d]Applied Biosystems™ 7500 Fast Real-Time PCR instrument.
[e]Collaborator dropped due to extreme cross-contamination, resulting in nearly all positive results.

iron (TSI) agar and lysine iron agar (LIA) slants and incubated for 24 ± 2 h at 35 ± 2°C. Typical isolates were confirmed positive by serological confirmation (polyvalent O and H) and biochemical confirmation by the API 20 E (AOAC *Official Method*SM **978.24**; 10), VITEK™ 2 GN biochemical identification test (AOAC *Official Method*SM **2011.17**; 11) or Bruker MALDI Biotyper™ system (AOAC *Official Method*SM **2017.09**; 12). In addition to following the confirmation as outlined in the reference method, all SureTect Salmonella species PCR Assay test portions were also confirmed following an alternative confirmation procedure. Regardless of presumptive results, all SureTect Salmonella species PCR Assay primary enrichments were directly streaked to Oxoid™ *Brilliance*™ Salmonella agar (BSA). From the BSA plates, typical colonies were subjected to a *Salmonella* agglutination using an Oxoid™ Salmonella Test Kit. All colonies that produced typical agglutination reactions were confirmed using Thermo Scientific Oxoid™ Microbact™ GNB 24E.

For the reference method test portions, 25 g samples were enriched in reconstituted NFDM (supplemented with 1% Brilliant Green Dye solution) and analyzed according to the procedures in the FDA/BAM Chapter 5 reference method as described in the preceding paragraph.

### *Statistical Analysis*

Each collaborating laboratory reported the test results on the data sheets provided. The data sheets were submitted to the study director at the end of testing for statistical analysis. Data for each contamination level were analyzed using the probability of detection (POD) statistical model (13) and conducted using the AOAC Micro Stats Workbook software V2.3 (14). The POD was calculated as the number of positive outcomes divided by the total number of trials. Laboratory POD (LPOD) values were calculated as the total POD values for all collaborators. LPOD values were calculated for the candidate presumptive results, $LPOD_{CP}$, the candidate confirmatory results (including false-negative results), $LPOD_{CC}$, the difference in the candidate presumptive and confirmatory results, $dLPOD_{CP}$, presumptive candidate results that confirmed positive (excluding false-negative results), $LPOD_C$, the reference method, $LPOD_R$, and the difference between the confirmed candidate and reference methods, $dLPOD_C$. A $dLPOD_C$ confidence interval not containing the point zero would indicate a statistically significant difference between the candidate method and the reference method at the 95% confidence level. In addition to POD values, the repeatability standard deviation ($s_r$), the among-laboratory repeatability standard deviation ($s_L$), the reproducibility standard deviation ($s_R$), and the interlaboratory correlation coefficient (ICC) were calculated.

## AOAC *Official Method*SM 2021.02

### *Salmonella* Species in a Broad Range of Foods and Selected Environmental Surfaces: PCR Assay

### First Action 2021

[Applicable to the detection of *Salmonella* in raw ground beef (~80% lean, 25 g and 375 g), pork frankfurters (25 g), raw chicken breast (25 g), bagged lettuce (25 g), NFDM powder (25 g), raw ground pork (25 g), cooked shrimp (heads off; 25 g), chilled ready-to-eat meal (containing beef; 25 g), pasteurized liquid whole egg (25 g), wet cat food (25 g), dry dog food (25 g), pasteurized liquid milk (2% fat; 25 g), mung bean sprouts (25 g), cut cantaloupe (25 g), chilled pizza dough (25 g), whole black peppercorns (25 g), peanut butter (25 g), ice cream (vanilla; 25 g), dark chocolate (85% cocoa solids; 25 g), and the environmental surfaces stainless steel (slab, 304 series, brushed finish; 4 in. × 4 in., sponge) and plastic (large polystyrene petri dish; 4 in. × 4 in., sponge)]. Matrices were compared to EN ISO 6579:2002/COR 1:2004 *Microbiology of food and animal feeding stuffs—Horizontal method for the detection of Salmonella spp. – Technical Corrigendum 1* (15).

The newly tested matrices are included as part of the precollaborative study: cut cabbage (25 g), cut mango (25 g), grated Cheddar cheese (25 g), single cream (8% fat; 25 g), feta cheese (25 g), fresh bagged spinach (375 g), raw beef trim (375 g), dark chocolate (>70% cocoa solids; 375 g), cocoa liquor (375 g), cocoa butter (375 g), and cocoa powder (375 g).

For the cut cabbage, cut mango, grated cheese, single cream, feta cheese, dark chocolate, cocoa liquor, cocoa butter, and cocoa butter, the candidate method is compared to FDA/BAM Chapter 5 (5) and to ISO 6579–1:2017 (7).

For the beef trim the candidate method is compared to the USDA/FSIS *MLG* 4.10 (6).]

*See* Table **2021.02A** for a summary of results of the interlaboratory study.

*See* Table **2021.02B** for detailed results of the interlaboratory study.

*Caution*:

*General Safety*

(a) Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

(b) Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.

*Chemical Safety*

(a) Read and understand the safety data sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.

(b) Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).

(c) Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, a fume hood).

(d) Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.

(e) Handle chemical wastes in a fume hood.

(f) Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)

(g) After emptying a waste container, seal it with the cap provided.

(h) Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.

(i) Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

*Biological Hazard Safety*

**Table 2021.02A.** Collaborative study: Summary of results for the detection of *Salmonella* in 375 g cocoa powder test portions by the Thermo Scientific SureTect *Salmonella* species PCR Assay vs FDA/BAM Chapter 5[a]

| Method | Thermo Scientific SureTect Salmonella species PCR Assay | | |
|---|---|---|---|
| Inoculation level | Un-inoculated | Low | High |
| Candidate presumptive positive/total number of samples analyzed | 0/144 | 60/144 | 144/144 |
| Candidate presumptive $LPOD_{CP}$ | 0.00 (0.00, 0.03) | 0.42 (0.32, 0.52) | 1.00 (0.97, 1.00) |
| $s_r$[c] | 0.00 | 0.49 | 0.00 |
| $s_L$[d] | 0.00 | 0.06 | 0.00 |
| $s_R$[e] | 0.00 | 0.55 | 0.00 |
| ICC[f] | N/A | 0.02 (–0.07, 0.10) | N/A |
| Candidate confirmed positive/total number of samples analyzed | 0/144 | 61/144 | 144/144 |
| Candidate confirmed $LPOD_{CC}$ | 0.00 (0.00, 0.03) | 0.42 (0.32, 0.53) | 1.00 (0.97, 1.00) |
| $s_r$[c] | 0.00 | 0.49 | 0.00 |
| $s_L$[d] | 0.00 | 0.08 | 0.00 |
| $s_R$[e] | 0.00 | 0.57 | 0.00 |
| ICC[f] | N/A | 0.02 (–0.07, 0.11) | N/A |
| Candidate method positive/total number of samples analyzed | 0/144 | 60/144 | 144/144 |
| Candidate presumptive positive that confirmed $LPOD_C$ | 0.00 (0.00, 0.03) | 0.42 (0.32, 0.52) | 1.00 (0.97, 1.00) |
| $s_r$[c] | 0.00 | 0.49 | 0.00 |
| $s_L$[d] | 0.00 | 0.06 | 0.00 |
| $s_R$[e] | 0.00 | 0.55 | 0.00 |
| ICC[f] | N/A | 0.02 (–0.07, 0.10) | N/A |
| Reference positive/total number of samples analyzed | 0/144 | 55/144 | 144/144 |
| Reference $LPOD_R$ | 0.00 (0.00, 0.03) | 0.38 (0.31, 0.46) | 1.00 (0.97, 1.00) |
| $s_r$[c] | 0.00 | 0.49 | 0.00 |
| $s_L$[d] | 0.00 | 0.00 | 0.00 |
| $s_R$[e] | 0.00 | 0.49 | 0.00 |
| ICC[f] | N/A | 0.00 (–0.07, 0.07) | N/A |
| dLPOD (candidate vs reference)[b] | 0.00 (–0.03, 0.03) | 0.04 (–0.09, 0.16) | 0.00 (–0.03, 0.03) |
| dLPOD (candidate presumptive vs candidate confirmed)[b,g] | 0.00 (–0.02, 0.02) | –0.01 (–0.03, 0.02) | 0.00 (–0.02, 0.02) |

[a] Results include 95% confidence intervals in parentheses.
[b] A confidence interval for dLPOD that does not contain the value 0 indicates a statistically significant difference between the two methods.
[c] Repeatability standard deviation.
[d] Among-laboratory standard deviation.
[e] Reproducibility standard deviation.
[f] Both the alternative confirmation procedure and the confirmation procedure following the FDA BAM Chapter 5 produced identical results.
[g] Interlaboratory correlation coefficient.

**(a)** Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

**(b)** *Salmonella* are a major foodborne pathogen that are responsible for salmonellosis in humans and animals. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations (16, 17).

**(c)** Dispose of all inoculated culture media as hazardous microbiological waste, even if shown to be negative for the target organism, according to local guidelines.

### A. *Principle*

The SureTect Salmonella species PCR Assay is based upon the use of Solaris reagents for performing PCR, for the rapid and specific detection of *Salmonella* species in a broad range of food types and select environmental surfaces. The method utilizes dye-labeled probes to target unique DNA sequences specific to *Salmonella* species, and an IPC. Target DNA, if present, is detected by real-time PCR. The IPC template, primers, and probe provide an internal control with each reaction to show that the PCR process has occurred. Analysis software for the QuantStudio 5 and the 7500 Fast provides interpretation of results.

### B. *Apparatus*

Items available from Thermo Fisher Scientific (www.thermofisher.com):

**(a)** *Homogenizer, laboratory blender or dilutor*.—One of the following or equivalent, DB5000A, DB4100A, DB4150A.

**(b)** *Homogenizer bags appropriate for the sample size*.—DB4100A or DB4150A; DB4011A, DB4012A, DB4013A, DB4014A, or equivalent.

**(c)** *Incubators fitted with racks for homogenizer bags*.—Set to 34–38°C or 41.5±1°C.

**(d)** *Thermal cycler*.—Applied Biosystems SimpliAmp A24811; or equivalent.

**Table 2021.02B.** Comparative results for the detection of Salmonella in 375 g cocoa powder test portions by the Thermo Scientific SureTect Salmonella species PCR Assay vs FDA/BAM Chapter 5 in a collaborative study

| | | | Candidate presumptive(CP) | | | Candidate confirmed (CC) [a] | | | Candidate result (C) | | | Reference method (R) | | | C vs R | CP vs CC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Statistic | Matrix/MPN | Collaborator | n[b] | x[c] | $POD_{CP}$ | n | x | $POD_{CC}$ | n | x | $POD_C$ | n | x | $POD_R$ | $dLPOD_{C,R}$ | $dLPOD_{CP,CC}$ |
| | | | | | | Uninoculated control | | | | | | | | | | |
| | Cocoa powder | 1 | NA[d] | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 2 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 1 | 0.08 | –0.08 | 0.00 |
| | | 3 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 1 | 0.08 | –0.08 | 0.00 |
| | | 4 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 5 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 6 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 7 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 8 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 9 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 10 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 11 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 12 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 13 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | 14 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 12 | 0 | 0.00 | 0.00 | 0.00 |
| | | | | | | MPN/test portion | | | | | | | | | | |
| Estimate | NA | All | 144 | 0 | 0.00 | 144 | 0 | 0.00 | 144 | 0 | 0.00 | 144 | 0 | 0.00 | 0.00 | 0.00 |
| LCL*e* | NA | | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.03 | –0.02 |
| UCL*f* | NA | | | | 0.03 | | | 0.03 | | | 0.03 | | | 0.03 | 0.03 | 0.02 |
| $s_r$[c] | | | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | | |
| $s_L$[d] | | | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | | |
| $s_R$[e] | | | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | | |
| ICC[f] | | | | | NA | | | NA | | | NA | | | NA | | |
| LCL | | | | | NA | | | NA | | | NA | | | NA | | |
| UCL | | | | | NA | | | NA | | | NA | | | NA | | |
| | | | | | | Low inoculum level | | | | | | | | | | |
| | Cocoa powder | 1 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 2 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 0.00 | 0.00 |
| | | 3 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 12 | 3 | 0.25 | 0.08 | 0.00 |
| | | 4 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 5 | 12 | 6 | 0.50 | 12 | 7 | 0.58 | 12 | 6 | 0.50 | 12 | 4 | 0.33 | 0.17 | 0.00 |
| | | 6 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 12 | 6 | 0.50 | –0.17 | 0.00 |
| | | 7 | 12 | 3 | 0.25 | 12 | 3 | 0.25 | 12 | 3 | 0.25 | 12 | 3 | 0.25 | 0.00 | 0.00 |
| | | 8 | 12 | 5 | 0.42 | 12 | 5 | 0.42 | 12 | 5 | 0.42 | 12 | 4 | 0.33 | 0.07 | 0.00 |
| | | 9 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 12 | 4 | 0.33 | 0.00 | 0.00 |
| | | 10 | 12 | 8 | 0.67 | 12 | 8 | 0.67 | 12 | 8 | 0.67 | 12 | 6 | 0.50 | 0.17 | 0.00 |
| | | 11 | 12 | 2 | 0.17 | 12 | 2 | 0.17 | 12 | 2 | 0.17 | 12 | 6 | 0.50 | –0.33 | 0.00 |
| | | 12 | 12 | 8 | 0.67 | 12 | 8 | 0.67 | 12 | 8 | 0.67 | 12 | 7 | 0.58 | 0.09 | 0.00 |
| | | 13 | 12 | 6 | 0.50 | 12 | 6 | 0.50 | 12 | 6 | 0.50 | 12 | 3 | 0.25 | 0.25 | 0.00 |
| | | 14 | 12 | 6 | 0.50 | 12 | 6 | 0.50 | 12 | 6 | 0.50 | 12 | 5 | 0.42 | 0.08 | 0.00 |
| | | | | | | MPN/test portion | | | | | | | | | | |
| Estimate | 0.48 | All | 144 | 60 | 0.42 | 144 | 61 | 0.42 | 144 | 61 | 0.42 | 144 | 55 | 0.38 | 0.04 | –0.01 |
| LCL | 0.36 | | | | 0.32 | | | 0.32 | | | 0.32 | | | 0.31 | –0.09 | –0.03 |
| UCL | 0.62 | | | | 0.52 | | | 0.53 | | | 0.52 | | | 0.46 | 0.16 | 0.02 |
| $s_r$[c] | | | | | 0.49 | | | 0.49 | | | 0.49 | | | 0.49 | | |
| $s_L$[d] | | | | | 0.06 | | | 0.08 | | | 0.06 | | | 0.00 | | |
| $s_R$[e] | | | | | 0.55 | | | 0.57 | | | 0.55 | | | 0.49 | | |
| ICC[f] | | | | | 0.02 | | | 0.02 | | | 0.02 | | | 0.00 | | |
| LCL | | | | | –0.07 | | | –0.07 | | | –0.07 | | | –0.07 | | |
| UCL | | | | | 0.10 | | | 0.11 | | | 0.10 | | | 0.07 | | |
| | | | | | | High inoculum level | | | | | | | | | | |
| | Cocoa powder | 1 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 2 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 3 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |

*(continued)*

**Table 2021.02B.** *(continued)*

| | | | Candidate presumptive(CP) | | | Candidate confirmed (CC) [a] | | | Candidate result (C) | | | Reference method (R) | | | C vs R | CP vs CC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Statistic | Matrix/MPN | Collaborator | n[b] | x[c] | $POD_{CP}$ | n | x | $POD_{CC}$ | n | x | $POD_C$ | n | x | $POD_R$ | $dLPOD_{C,\ R}$ | $dLPOD_{CP,\ CC}$ |
| | | 4 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 5 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 6 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 7 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 8 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 9 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 10 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 11 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 12 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 13 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | 14 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 12 | 12 | 1.00 | 0.00 | 0.00 |
| | | | | | | | | MPN/test portion | | | | | | | | |
| Estimate | 5.42 | All | 144 | 144 | 1.00 | 144 | 144 | 1.00 | 144 | 144 | 1.00 | 144 | 144 | 1.00 | 0.00 | 0.00 |
| LCL | 3.53 | | | | 0.97 | | | 0.97 | | | 0.97 | | | 0.97 | –0.03 | –0.02 |
| UCL | 8.30 | | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.03 | 0.02 |
| $s_r^g$ | | | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | | |
| $s_L^h$ | | | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | | |
| $s_R^i$ | | | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | | |
| ICC[j] | | | | | NA | | | NA | | | NA | | | NA | | |
| LCL | | | | | NA | | | NA | | | NA | | | NA | | |
| UCL | | | | | NA | | | NA | | | NA | | | NA | | |

[a] The alternative confirmation and the FDA/BAM Chapter 5 confirmation produced identical results.
[b] n = Number of test portions.
[c] x = Number of positive test portions.
[d] NA = Collaborator did not complete testing or was dropped due to extremely contamination.
[e] LCL = Lower confidence limit.
[f] UCL = Upper confidence limit.
[g] $s_r$ = Repeatability standard deviation.
[h] $s_L$ = Among-laboratory standard deviation.
[i] $s_R$ = Reproducibility standard deviation.
[j] ICC = Interlaboratory correlation coefficient.

**(e)** *MicroAmp 96-well tray/retainer set for Veriti Systems*.—4381850.

**(f)** *Real-time PCR instrument*.—Applied Biosystems QuantStudio 5. 0.1 mL block, with Thermo Scientific RapidFinder Analysis Software v1.1 or later for use with SureTect Salmonella species PCR Assay and Pathogen Assay File: SalmonellaSpp_SureTect_QS5 version 2.1 or later [A36320 (desktop), A36328 (laptop)].

**(g)** *Fast Real-Time PCR instrument*.—Applied Biosystems 7500 with Applied Biosystems RapidFinder Express Software v2.0 or later for use with SureTect Salmonella species PCR Assay and Pathogen Assay File: Salmonella species SureTect 2.0 or later [A30304 (desktop), A30299 (laptop)].

**(h)** *MicroAmp 96-well tray for VeriFlex block*.—4379983.

**(i)** *Precision plate holder for SureTect assays*.—PT0690.

**(j)** *7500 Fast precision plate holder*.—0.1 mL tube strips (A29252).

**(k)** *PCR carry plate for SureTect assays*.—PT0695.

**(l)** *VersiPlate PCR strip tube plate*.—96-well, low profile (AB1800).

**(m)** *Ultra clear qPCR caps*.—Strips of 8 (AB0866).

**(n)** If using 7500 Fast Precision Plate Holder for 0.1 mL tube strips (Cat. No. A29252).

*(1)* *MicroAmp Fast 8-Tube strip*.—0.1 mL (4358293).

*(2)* *MicroAmp Optical 8-cap strips*.—4323032.

### *C. Reagents*

**(a)** *SureTect Salmonella species PCR assay*.—96 tests (PT0100A).

*(1)* *Lysis reagent 1*.— Tube (clear, pale blue liquid containing fine white particles), 12 strips of eight tubes.

*(2)* *Lysis tube caps*.—Domed, 12 strips of eight tubes.

*(3)* *Proteinase K*.—Clear colorless liquid, one tube.

*(4)* *SureTect Salmonella species PCR tubes*.—Twelve strips of eight tubes one pellet each.

*(5)* *PCR caps*.—Twelve strips of eight caps.

**(b)** *Oxoid BPW (ISO)*.—CM1049B, CM1211B or equivalent.

**(c)** *Oxoid Modified Tryptone Soya Broth*.—CM0989B (or equivalent).

**(d)** *Oxoid novobiocin selective supplement*.—SR0181E, or equivalent.

**(e)** *Cocoa sample recovery (CSR) broth*.—CM1155B.

**(f)** *Dey–Engley broth or other neutralizing broth*.

**(g)** *Peptone water*.

**(h)** *Oxoid Rappaport–Vassiliadis soya (RVS) peptone broth*.—CM0866B (or equivalent).

**(i)** *Oxoid BSA*.—CM1092B (base), SR0194E (supplement). Outside North America: R110374 (monoplate).

**(j)** *Oxoid Salmonella test kit (latex test)*.—DR1108A.

(k) *Thermo Scientific Microbact GNB 24E Kit, 40 tests*.—MB1131A.
(l) *Thermo Scientific Microbact GNB Reagent Set D*.—MB1082A.

Additional items required:

(m) *Disposable gloves*.
(n) *Variable volume single-channel pipette*.—1–10 mL.
(o) *96-well rack*.
(p) *Filtered pipette tips*.—1–10 mL.
(q) *Sample tubes*.—1.5 mL.
(r) *Sterile sampling swabs or sponges*.—Remel bio-spo Sponge or equivalent.
(s) *Single-channel pipette or electronic adjustable spacing multichannel pipette*.—10–100 μL
(t) *Single-channel stepper pipette*.—10–100 μL.
(u) *Filtered pipette tips*.—10–100 μL.
(v) *Compact PCR tube rack*.—Mixed colors.
(w) *Tool for capping and decapping*.—Optional.
(x) *Timer*.
(y) *Vortex mixer*.
(z) *8-channel pipette*.—5–50 μL.
(aa) *Filtered pipette tips*.—10–100 μL.

### D. General Instructions

(a) *Guidelines for sample enrichment*.
  (1) For preparation of master suspensions, follow the instructions of BS EN ISO 6887: Microbiology of food and animal feed. Preparation of test samples, initial suspension, and decimal dilutions for microbiological examination (Parts 1–6) (18) and EN ISO 6579 (7) standards. Comply with good laboratory practices (refer to ISO 7218:2007 Microbiology of food and animal feeding stuffs—General requirements and guidance for microbiological examinations [19]).
  (2) Follow the manufacturer's instructions for the preparation of culture media.
  (3) When following the short enrichment protocol, ensure that the enrichment broth is prewarmed for 18–24 h before adding it to the sample.
  (4) Use filtered homogenizer bags to help with fat and particle separation.
  (5) For consistent PCR results, use a ventilated incubator.
  (6) Follow the specified temperature allowances.
(b) *Guidelines for sample lysis*.
  (1) Follow the specified temperature allowances.
  (2) For downstream PCR on the 7500 Fast instrument or the QuantStudio 5 instrument—Prepare a mock-purified sample using sterile enrichment media as a negative extraction control. (The negative extraction control is required for RapidFinder Express Software; it is optional but recommended for RapidFinder™ Analysis Software.) Add the enriched sample or negative extraction control to the bottom of the lysis tube.
  (3) To prevent crushing of the tubes, use the MicroAmp 96-Well Tray/Retainer Set provided with the SimpliAmp Thermal Cycler. See the SimpliAmp Thermal Cycler User Guide (Pub. No. MAN0009889). Alternatively, use at least four complete tube strips in the heat block. We recommend spacing the strips evenly across the heat block. If needed, add empty SureTect tubes to make four complete strips.
(c) *Guidelines for PCR*.
  (1) *Important*: After the lysate has been added to the pellets, ensure that the pellet rehydrates immediately by tapping the tubes on the laboratory bench. Start the PCR run within 30 min.
  (2) Tube and cap strips can be cut when less than a full strip is required. Do not cut the strips of caps or tubes too close to the wall of the tube or the cap lid, otherwise the lid might not seal adequately during PCR.
  (3) After the PCR tubes have been opened, add the lysate within 10 min.
  (4) Particulate matter from the lysate can inhibit the PCR. To ensure that no particles are transferred from the Lysis Reagent 1 Tube to the PCR tube, remove lysate from the top half of the liquid, taking care not to disrupt the particles at the bottom of the tube. If the particles become disturbed, allow the particles to resettle for 1–2 min before lysate removal.
  (5) Ensure that the pellet is fully dissolved. The solution changes from blue to green when the pellet is dissolved.
  (6) For ease of use, a multi-channel pipettor can be used to transfer multiple lysates to the PCR tubes.
  (7) Follow "Good laboratory practices for PCR." For more information search online for the PCR Learning Center | Thermo Fisher Scientific—US.

### E. Sample Enrichment

(a) *Specific enrichments*.
  (1) *Raw ground beef (25 g, short protocol)*.—Add 225 mL prewarmed (41.5±1°C) BPW (ISO). Homogenize for 30 s to 1 min using a homogenizer. Incubate at 41.5±1°C for 8–24 h.
  (2) *Raw ground beef (375 g, short protocol)*.—Add 1.5 L prewarmed (41.5±1°C) Oxoid Modified Tryptone Soya Broth. Homogenize for 30 s to 1 min using a homogenizer. Incubate at 41.5±1°C for 9–24 h.
  (3) *NFDM powder, raw ground beef, pasteurized liquid whole egg (25 g)*.—Add 225 mL BPW (ISO). Homogenize for 30 s to 1 min using a homogenizer. Incubate at 34–38°C for 18–24 h.
  (4) *Cooked shrimp, pork frankfurters, bagged lettuce, chilled ready-to-eat meal (containing beef), raw ground pork, raw chicken breast, wet cat food, dry dog food, pasteurized liquid 2% milk, cut cantaloupe, chilled pizza dough, black peppercorns, ice cream (vanilla), peanut butter (25 g)*.—Add 225 mL BPW (ISO). Homogenize for 30 s to 1 min using a homogenizer. For samples containing hard particles, such as bone—squeeze the bag by hand until the sample is mixed thoroughly with the media. Incubate at 34–38°C for 20–28 h.
  (5) *Mung bean sprouts (25 g)*.—Add 225 mL prewarmed (41.5±1°C) BPW (ISO) with 20 mg/L novobiocin. Homogenize for 30 s to 1 min using a homogenizer. Incubate at 41.5±1°C for 20–28 h.
  (6) *Dark chocolate (85% cocoa solids; 25 g)*.—Add 225 mL CSR broth. Homogenize for 30 s to 1 min using a homogenizer. Incubate at 34–38°C for 20–28 h.
  (7) *Cocoa powder, cocoa liquor, cocoa butter, dark chocolate (>70% cocoa solids; 375 g)*.—Add 3375 mL BPW (ISO). Homogenize for 30 s to 1 min using a homogenizer. Incubate at 34–38°C for 22–30 h.
  (8) *Cocoa powder, cocoa liquor, cocoa butter, dark chocolate (>70% cocoa solids; 375 g)*.—Add 3375 mL reconstituted

NFDM supplemented with Brilliant Green Dye (0.018 g/L) for products with high background microflora. Note that NFDM can be replaced with UHT milk. Homogenize for 30 s to 1 min using a homogenizer. Incubate at 34–38°C for 22–30 h.

(9) *Cut cabbage, cut mango, grated Cheddar cheese (25g).*—Add 225 mL BPW (ISO). Homogenize for 30 s to 1 min using a homogenizer. Incubate at 34–38°C for 20–28 h.

(10) *Raw beef trim (375g, short protocol).*—Add 1.5 L prewarmed (41.5±1°C) Oxoid Modified Tryptone Soya Broth. Homogenize for 30 s to 1 min using a homogenizer. Incubate at 41.5±1°C for 8–24 h.

(11) *Fresh bagged spinach (375g, short protocol).*—Add 3375 mL prewarmed (41.5±1°C) BPW (ISO). Homogenize for 30 s to 1 min using a homogenizer. Incubate at 41.5±1°C for 8–24 h.

(12) *Environmental surface swabs and sponges.*—Pre-moisten a sterile sampling swab or sponge. For sampling areas that have been cleaned or treated with disinfectants and other cleaning agents, use a neutralizing broth, such as Dey–Engley broth. For other areas, use sterile peptone water or other suitable diluents. Rub the swab or sponge in both a horizontal and a vertical direction across the entire sampling area. Place the sample in the original packaging or other material that is suitable for transport. Samples may be held for up to 2 h at room temperature (23±5°C) or 8 h in the refrigerator (2–8°C) prior to adding the samples to media. Add swabs to 10 mL BPW (ISO). Add sponges to 100 mL BPW (ISO). Homogenize thoroughly. Incubate at 34–38°C for 18–26 h.

**(b)** For all samples, remove the enriched sample from the incubator, briefly mix the liquid in the homogenizer bag/tube by hand, transfer an aliquot of sample from the filtered side of the bag to a new tube, and then close the tube and briefly mix.

**(c)** Retain enough sample for confirmation or repeat testing.

### *F. Test Portion Analysis*

**(a)** *Lysis using a SimpliAmp Thermal Cycler*

(1) Equilibrate the lysis reagent 1 tubes to room temperature (23±5°C).

(2) Check that there is no liquid around the plastic seal and the reagents are collected at the bottom of the tube.

(3) Allow the tubes to remain at room temperature (23±5°C) for approximately 10 min before opening.

(4) Remove the plastic seal from each lysis reagent 1 tube, then add 10 µL proteinase K to the tube. These tubes are referred to as lysis tubes in the rest of the procedure.

(5) *Important*: Avoid contamination of the proteinase K stock tube. Use a new filtered pipette tip each time proteinase K is withdrawn from the stock tube. Use a 10–100 µL repeat pipettor to reduce the number of tips required.

(6) For cocoa and chocolate samples only, dilute the enrichment 1-in-5 (1 part enriched sample and 4 parts sterile BPW).

(7) Transfer 10 µL of the enriched sample (or diluted cocoa/chocolate sample) to a lysis tube. For the negative extraction controls, transfer 10 µL sterile enrichment media to a lysis tube. Ensure that the pipette tip reaches the bottom of the lysis tube, to facilitate complete mixing of the sample with lysis reagent 1.

(8) Seal the tubes with the domed lysis tube caps, ad then incubate the samples in the SimpliAmp Thermal Cycler using the program outlined in Table 2021.02C.

(9) *Important:* To prevent crushing the tubes in the SimpliAmp Thermal Cycler, use the MicroAmp 96-well tray/retainer set or include at least four complete SureTect lysis tube strips.

(10) Ensure that the lid heater is on and set to 105°C, and the volume is set to maximum.

(11) Proceed directly to PCR. (Optional: Store the samples at 2–8°C for up to 24 h, including any time stored at 4°C in the thermal cycler).

**(b)** *Lysis using heat blocks.*

(1) Ensure that two heating blocks are set to 37±2°C and 95±2°C.

(2) Equilibrate the lysis reagent 1 tubes to room temperature (23±5°C).

(3) Place the required number of lysis reagent 1 tubes in a suitable rack.

(4) Check that there is no liquid around the plastic seal and the reagents are collected at the bottom of each tube.

(5) Allow the tubes to remain at room temperature (23±5°C) for approximately 10 min before opening.

(6) Remove the plastic seal from each lysis reagent 1 tube, and then add 10 µL of proteinase K to the tube. These tubes are referred to as lysis tubes in the rest of the procedure.

(7) *Important*: Avoid contamination of the proteinase K stock tube. Use a new filtered pipette tip each time proteinase K is withdrawn from the stock tube. Use a 10–100 µL repeat pipettor to reduce the number of tips required.

(8) For cocoa and choc olate samples only, dilute the enrichment 1-in-5 (1 part enriched sample and 4 parts sterile BPW (ISO)).

(9) Transfer 10 µL the enriched sample (or diluted chocolate sample) to a lysis tube. For the negative extraction controls, transfer 10 µL sterile enrichment media to a lysis tube. Ensure that the pipette tip reaches the bottom of the lysis tube, to facilitate complete mixing of the sample with lysis reagent 1.

(10) Seal the tubes with domed lysis tube caps, and then incubate the samples in the appropriate heating blocks:

(i) 37±2°C for 10 min.

(ii) 95±2°C for 5 min.

**Table 2021.02C.** SimpliAmp Thermal Cycler program

| Step | Temperature (°C) | Time (min) |
|---|---|---|
| 1 | 37 | 10 |
| 2 | 95 | 5 |
| 3 | 10 | 2 |
| 4 | 4 | Hold[a] |

[a] For convenience, samples can be held at 4 °C until proceeding to PCR or transfer to storage at 2–8°C.

(iii) Ambient temperature for 2 min. For convenience, samples can be transferred to storage at 2–8°C for up to 24 h.

(11) Proceed directly to PCR. (Optional: Store the samples at 2–8°C for up to 24 h.)

### G. Analysis

**(a)** *PCR with the QuantStudio 5 Instrument and RapidFinder Analysis Software v1.1 or later*.

(1) The plate layout is determined by the user. See the "Help" function in the software for detailed instructions. On the home screen of the RapidFinder Analysis Software, click "Create Experiment", and then enter or edit the well parameters. Select "SalmonellaSpp_SureTect_QS5" version 2.1 or later for the assay. Before starting this procedure, ensure that you are familiar with *Guidelines for PCR*.

(2) Following the plate layout previously set up in the software, place the required number of SureTect Salmonella species PCR Tubes (PCR tubes) in the MicroAmp 96-Well Tray for VeriFlex Block. Place the block on the MicroAmp Splash-Free 96-Well Base. Press the PCR tubes to the tray to ensure they sit firmly, then tap the tubes on the bench to ensure that the pellets are located at the bottom of the tubes.

(3) Allow the PCR tubes to remain on the bench for approximately 5 mins, to bring to room temperature (23±5°C), and then open one strip of PCR tubes by removing the seal.

*Important:*

(i) If all sample lysates can be applied to the PCR tubes in 10 min, open all strips of the PCR tubes.

(ii) If all sample lysates cannot be applied to the PCR tubes in 10 min, open only one strip of the PCR tubes, and then proceed to the next step.

(iii) PCR pellets are pale yellow. If the pellet is collapsed or not pale yellow, do not use.

(iv) If the pellet is not positioned at the bottom of a tube, gently move the pellet to the bottom of the tube with a sterile, empty, pipette tip. Do not use a tip containing lysate.

(4) Uncap the lysis tubes.

(5) Transfer 20 µL lysate or mock-purified sample (negative extraction control reaction) to the appropriate PCR tube to rehydrate the pellet. Tap the rack to ensure that the lysate is at the bottom of the tube and touching the pellet.

*Important:* Remove lysate from the top half of the liquid to ensure that no lysis particles are transferred from the lysis tube to the PCR tube. Do not touch the pellet when adding the lysate.

(6) Seal the PCR tubes with the flat optical PCR Caps provided with the kit. Ensure that the tubes are properly sealed by pressing down firmly over each opening. Do not use the capping tool to seal the PCR tubes.

(7) If only one strip of PCR tubes was opened, repeat steps G(a), (2–5) for the remaining strips of PCR tubes.

(8) Mix all the PCR tubes thoroughly for 10–15 s to ensure that the pellet is fully rehydrated. This can be performed using a vortex mixer. Ensure that the liquid is at the bottom of the tube before placing it in the PCR instrument. If needed, hold the tubes upright, and flick sharply downward or spin down using a centrifuge.

*Important:* Start the PCR run within 30 min of the addition of the sample lysates to the PCR tubes.

(9) Eject the instrument drawer. Use the MicroAmp 96-Well Tray for VeriFlex Block to transfer the tubes to the instrument in the same configuration as the plate layout determined in the software, then close the instrument drawer.

(10) In the Run tab of the experiment file in RapidFinder Analysis Software, select the instrument's serial number from the instrument drop-down list.

(11) Click Start Run, and then follow the software prompts. Data analysis is automated by the software. For detailed instructions, and options for reporting, export, and storage of results, see the Help function in the software.

**(b)** *PCR with the 7500 Fast Instrument and RapidFinder Express Software v2.0 or later*.

(1) RapidFinder Express Software determines the Run Layout (plate layout) for your samples based on the information entered and creates a run file. Refer to the Help function in the software for more details. On the main page of RapidFinder Express Software, select Create/Edit a Run File, and then enter or edit the Run File information at the prompts. If desired, you can manually customize the plate layout in the software. Select Salmonella species SureTect 2.0 or later for the assay. Before starting this procedure, ensure that you are familiar with *Guidelines for PCR*.

(2) Following the plate layout previously set up in the software, place the required number of SureTect Salmonella species PCR tubes in a suitable rack with a PCR carry plate, and then tap the rack of tubes on the bench to ensure that the pellets are located at the bottom of the tubes. If required by the plate layout, place empty SureTect PCR tubes in the rack to balance the tray when the tubes are placed in the instrument.

(3) Allow the PCR tubes to remain on the bench for approximately 5 min, to bring them to room temperature (23±5°C), and then open one strip of PCR tubes by removing the seal.

*Important:*

(i) If all sample lysates can be applied to the PCR tubes in 10 min, open all strips of the PCR tubes.

(ii) If all sample lysates cannot be applied to the PCR tubes in 10 min, open only one strip of the PCR tubes, and then proceed to the next step.

(iii) PCR pellets are pale yellow. If the pellet is collapsed or not pale yellow, do not use.

(iv) If the pellet is not positioned at the bottom of a tube, gently move the pellet to the bottom of the tube with a sterile, empty, pipette tip. Do not use a tip containing lysate.

(4) Uncap the lysis tubes.

(5) Transfer 20 µL lysate or mock-purified sample (negative extraction control reaction) to the appropriate PCR tube to rehydrate the pellet. Tap the rack to ensure

that the lysate is at the bottom of the tube and touching the pellet.

*Important*: Remove lysate from the top half of the liquid to ensure that no lysis particles are transferred from the lysis tube to the PCR tube. Do not touch the pellet when adding the lysate.

(6) Seal the PCR tubes with the flat optical PCR caps provided with the kit. Ensure that the tubes are properly sealed by pressing down firmly over each opening. Do not use the capping tool to seal the PCR tubes.
(7) If only one strip of PCR tubes was opened, repeat steps **G(b)**, (2–5) for the remaining strips of PCR tubes.
(8) Mix all the PCR tubes thoroughly for 10–15 s to ensue that the pellet is fully rehydrated. This can be performed using a vortex mixer. Ensure that the liquid is at the bottom of the tube before placing it in the PCR instrument. If needed, hold the tubes upright, and flick sharply downward or spin down using a centrifuge.

*Important:* Start the PCR run within 30 min of the addition of the sample lysates to the PCR tubes.

(9) In the RapidFinder Express Software, select Start Instrument Run on the main page, select the appropriate run file, and follow the software prompts.
(10) Use the PCR carry plate to transfer the tubes to the instrument in the same configuration as the run layout. Use the Precision Plate Holder for SureTect assays. Be sure to load empty SureTect PCR tube strips as directed by the software.
(11) Close the tray to the instrument and follow the RapidFinder Express Software prompts to start the run. Data analysis is automated by the software.

### *H. Test Result Report and Interpretation of Results*

**(a)** On the home screen of the RapidFinder Analysis Software (*see* Figure 1), click Results, and then click the sub-tab for the desired view of the data.
(1) *Summary*.—Plate format.
(2) *Results*.—Table format.
(3) *Details*.—Amplification plot.

**(b)** In the RapidFinder Express Software (Figure 2), select View Results on the main page, select the appropriate run file, and follow the prompts to view the results. To display a list of results in table format, click Table View. Select a sample, and then click View Details to see replicate information about samples.

### *I. Candidate Confirmation*

**(a)** *Thermo Scientific SureTect Salmonella species PCR Assay Test Result Confirmation*.
(1) Streak 10 µL primary enrichment onto Oxoid™ BSA and incubate for 22–26 h at 34–38°C.
Troubleshooting: For cocoa and chocolate products BSA can be replaced with XLD. For products with high background microflora, it is recommended to perform a subculture of the primary enrichment broth in RVS broth followed by incubation for 21–27 h at 41.5 ± 1°C before streaking onto BSA or XLD and incubating for 22–26 h at 34–38°C.

(2) *Confirm characteristic and well-isolated Salmonella* colonies using:
(*a*) Oxoid Salmonella latex kit or
(*b*) Microbact GNB 24E kit.
(3) Characteristic colonies can also be confirmed using the methods described in reference methods depending on the legislation territory or matrix:
(*a*) FDA/BAM Chapter 5.
(*b*) USDA/FSIS MLG 4.10.
(*c*) EN ISO 6579-1.
(4) Any other appropriate national reference method or using an appropriate AOAC *Official Method of Analysis*℠ validated confirmation method; an EN ISO 16140-6:2019 (20) validated confirmation method.

| Result icon | Result |
|---|---|
| | Positive result |
| | Negative result |
| | Result warning |

**Figure 1.** RapidFinder™ Analysis Software results icons.

| Result icon[a] | Result |
|---|---|
| | Positive result |
| | Negative result |
| | Result warning |

[a] RapidFinder™ Express displays results pictorially.

**Figure 2.** RapidFinder™ Express Software results icons.

In the event of discordant results (presumptive positive with the alternative method, not confirmed by one of the means described above), the laboratory must employ adequate means to ensure the validity of the result obtained.

## Results

### *Collaborative Study*

The collaborative study involved a method comparison evaluation of the SureTect Salmonella species PCR Assay to the FDA/BAM Chapter 5 reference method for cocoa powder. A total of 14 participants throughout the continental United States and Europe participated in this study. In two of the laboratories, two separate analysts participated and in one of the laboratories, three separate analysts participated. The remaining seven laboratories each had one participating analyst. Twelve out of the 14 participants submitted valid data. One laboratory voluntarily withdrew from participation prior to initiating testing and one laboratory had unacceptable data due to cross-contamination for all test portions that were analyzed. The root cause of the contamination was not determined but was most likely due to the nature of working with a powdered matrix. For the laboratories that did participate, each participant analyzed 36 unpaired test portions for the SureTect Salmonella species PCR Assay and the FDA/BAM Chapter 5 reference method: 12 inoculated with a high level of *Salmonella*, 12 inoculated with a low level of *Salmonella*, and 12 uninoculated controls. In addition to the test portions, all participants set up an APC to determine the total microbial load of the test matrix and the appropriate incubation conditions

for the TT broth. The average APC result obtained by the collaborators was $2.0 \times 10^2$ CFU/g. The highest count documented out of all of the participants was $6.0 \times 10^2$ CFU/g and the lowest was $1.0 \times 10^1$ CFU/g.

A background screen of the matrix, following the FDA/BAM Chapter 5 reference method and using the SureTect Salmonella species PCR Assay, indicated an absence of indigenous *Salmonella* species. Ten replicate 375 g test portions (randomly sampled from 50% of the total packages used in the analysis) were screened for the presence of *Salmonella*. All test portions produced negative results for the target analyte.

Table **2021.02A** summarizes the interlaboratory results. As per criteria outlined in Appendix J of the *Official Methods of Analysis* manual (4), fractional positive results were obtained. Detailed results for each laboratory are presented in Table **2021.02B**. The level of *Salmonella* was determined by MPN on the day of initiation of analysis by the coordinating laboratory. The MPN levels obtained, with a 95% confidence interval, were 0.48 MPN/test portion (0.36, 0.62) for the low inoculum level and 5.42 MPN/test portion (3.53, 8.30) for the high inoculum level. MPN results are presented in the second column of Table **2021.02B**.

### *Cocoa Powder*

Detailed results of the LPOD statistical analysis are presented in Table **2021.02B** and Figures 3–6.

For the low inoculation level, 60 out of 144 test portions ($LPOD_{CP}$ of 0.42) were reported as presumptive positive by the SureTect Salmonella species PCR Assay with 61 out of 144 test portions ($LPOD_{CC}$ of 0.42) confirming positive (following both the alternative confirmation and the FDA/BAM Chapter 5 reference method). For samples that produced presumptive positive results by the SureTect Salmonella species PCR Assay, 60 out of 144 samples confirmed positive ($LPOD_C$ of 0.42; value includes only presumptive positive results that confirmed positive). For the reference method, 55 out of 144 test portions were reported as positive ($LPOD_R$ of 0.38). A $dLPOD_C$ value of 0.04 with 95% confidence interval of (–0.09, 0.16) was obtained between the candidate and reference methods, indicating no statistically significant difference between the two methods. A $dLPOD_{CP}$ value of –0.01 with 95% confidence intervals of (–0.03, 0.02) was obtained between the presumptive and confirmed results, indicating no statistically significant difference between the presumptive and confirmed results (following both the alternative confirmation and the FDA/BAM Chapter 5 reference method).

For the high inoculation level, 144 out of 144 test portions ($LPOD_{CP}$ of 1.00) were reported as presumptive positive by the SureTect Salmonella species PCR Assay. There were 144 out of 144 reported test portions ($LPOD_{CC}$ of 1.00) that confirmed positive (following both the alternative confirmation and the FDA/BAM Chapter 5 reference method). For samples that produced presumptive positive results by the SureTect Salmonella species PCR Assay, 144 out of 144 samples confirmed positive ($LPOD_C$ of 1.00). For the reference method, 144 out of 144 test portions were reported as positive ($LPOD_R$ of 1.00). A $dLPOD_C$ value of 0.00 with 95% confidence interval of (–0.03, 0.03) was obtained between the candidate and reference methods, indicating no statistically significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (–0.02, 0.02) was obtained between the presumptive and confirmed results, indicating no statistically significant difference between the presumptive and confirmed results (following both the alternative confirmation and the FDA/BAM Chapter 5 reference method).

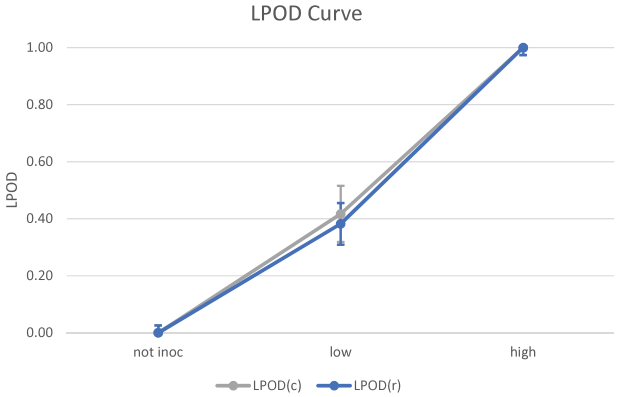


**Figure 3.** LPOD values of SureTect Salmonella species PCR Assay and FDA/BAM Chapter 5 for the collaborative study of cocoa powder.

For the uninoculated controls, 0 out of 144 samples ($LPOD_{CP}$ of 0.00) produced a presumptive positive result by the SureTect Salmonella species PCR Assay with 0 out of 144 test portions ($LPOD_{CC}$ of 0.00) confirming positive (following both the alternative confirmation and the FDA/BAM Chapter 5 reference method). There were 0 out of 144 samples that produced a presumptive positive result by the SureTect Salmonella species PCR Assay that confirmed positive ($LPOD_C$ of 0.00). For the reference method, 0 out of 144 test portions were reported as positive ($LPOD_R$ of 0.00). A $dLPOD_C$ value of 0.00 with 95% confidence interval of (–0.03, 0.03) was obtained between the candidate and reference methods, indicating no statistically significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (–0.02, 0.02) was obtained between the presumptive and confirmed results, indicating no statistically significant difference between the presumptive and confirmed results (following both the alternative confirmation and the FDA/BAM Chapter 5 reference method).

Results of the APC for the collaborating laboratories are listed in Table 3. Details of the shipment temperatures for the collaborating laboratories are listed in Table 4.

## Pre-Collaborative Study

### *Study Overview—Additional Matrices (January 2021)*

The following matrix studies were performed by Q Laboratories, >70% cocoa solids dark chocolate (375 g), cocoa powder (375 g), cocoa liquor (375 g), and cocoa butter (375 g).

Dark chocolate (>70% cocoa solids), cocoa powder, cocoa liquor, and cocoa butter were all purchased from a local supplier and prescreened for natural contamination of *Salmonella* species following FDA/BAM Chapter 5 and ISO 6579–1:2017 reference methods for both NFDM and BPW enriched samples. Total APC was determined following the FDA/BAM Chapter 3 reference method (8). Natural contamination was not detected in the screening of any of the four matrixes; therefore, the matrixes were artificially contaminated. The matrix study consisted of evaluating 30 paired and 30 unpaired samples of >70% cocoa solids dark chocolate (375 g), cocoa powder (375 g), cocoa liquor (375 g), and cocoa butter (375 g). Within the 30 paired and 30 unpaired samples for each matrix, there were 5 uninoculated samples (0 CFU/test portion), 20 low-level inoculated samples (0.2-2

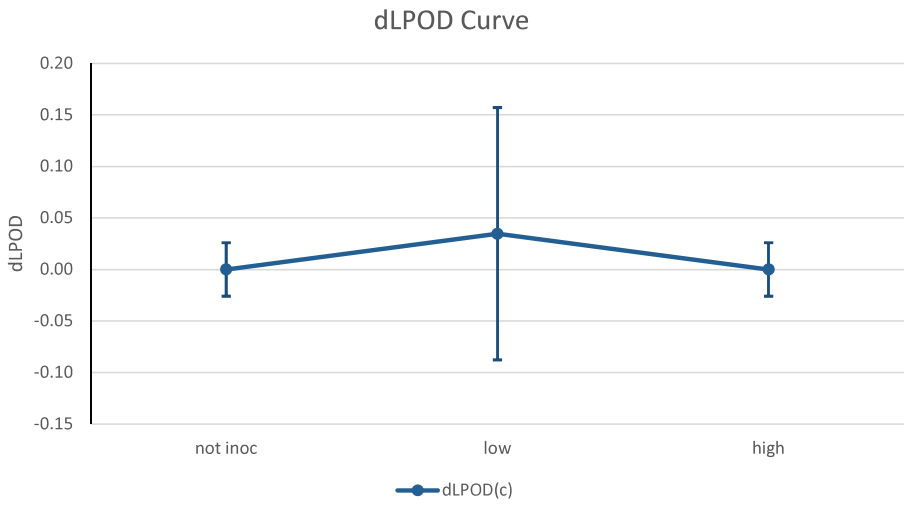


**Figure 4.** $dLPOD_C$ values of SureTect Salmonella species PCR Assay and FDA/BAM Chapter 5 for the collaborative study of cocoa powder.

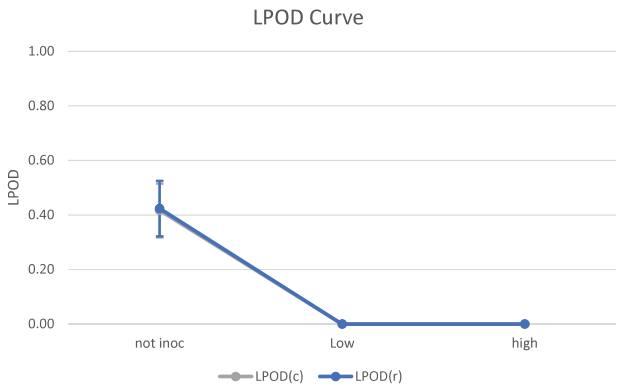


**Figure 5.** LPOD values of presumptive Thermo Scientific SureTect Salmonella species PCR Assay and confirmed results for the collaborative study of cocoa powder.

CFU/test portion), and 5 high-level inoculated samples (2–10 CFU/test portion). All samples were evaluated at 20–28 h for NFDM (supplemented with Brilliance Green Dye) enriched samples and at 22–30 h for BPW (ISO) enriched samples. All samples were analyzed using the QuantStudio 5 and 7500 Fast instruments. Regardless of the presumptive results, all samples were culture-confirmed following their respective reference method. Final confirmation was achieved using a Bruker MALDI Biotyper following AOAC *Official Method*$^{SM}$ **2017.09**. An alternative confirmation was also performed by streaking 10 μL of each sample on XLD and BSA following incubation. XLD and BSA plates were incubated at 34–38°C for 24 ± 2 h. Presumptive positive *Salmonella* colonies were confirmed using latex agglutination and a Microbact$^{TM}$ GNB 24E kit.

### *Organism Preparation and Inoculation*

For the cocoa powder matrix, inoculation of a lyophilized culture of *Salmonella enterica* serovar Infantis QL 052016.18S was used. The lyophilized culture was prepared as follows. The *Salmonella* culture was propagated on SBA from a stock culture stored at –70°C. SBA was incubated for 24 ± 4 h at 37 ± 1°C. A single colony was then transferred to BHI broth and incubated for 24 ± 4 h at 37 ± 1°C. The culture was then diluted in a sterile cryoprotectant, reconstituted NFDM, and freeze-dried for 48–72 h. A bulk lot of the matrix was inoculated with a lyophilized culture that was diluted in powdered NFDM to a low level expected to yield fractional positive results (5–15 positive results), and a high level expected to yield all positive results. After inoculation and mixing by inverting in a sterile bag, samples were held for 2 weeks at room temperature (20–25°C). For 25 g samples, individual 25 g samples were aseptically weighed into sterile stomacher bags for the reference method. For 375 g test portions, 25 g inoculated product was added to 350 g uninoculated product.

For the >70% cocoa solids dark chocolate, cocoa liquor, and cocoa butter matrixes, a liquid heat-stressed culture was used. For the >70% cocoa solids dark chocolate *Salmonella* Typhimurium ATCC 14028 was used, for cocoa liquor *Salmonella* Enteritidis ATCC 13076 was used, and for cocoa butter *Salmonella* Senftenberg ATCC 43845 was used. The liquid heat-stressed culture was prepared as follows. The *Salmonella* culture was propagated on SBA from a stock culture stored at –70°C. SBA was incubated for 24 ± 4 h at 34–38°C. The pure culture was transferred to BHI broth and incubated for 24 ± 4 h at 37 ± 1°C. Following incubation, the culture was heat stressed by heating the culture at 55 ± 0.1°C for 10–20 min. The heat-stressed culture was plated on the non-selective agar TSA and the selective XLD agar and incubated for 24 ± 4 h at 35 ± 1°C. Following incubation, the percentage injury was determined using the following formula, and the inoculating culture had to have an injury of 50–80%:

$$\left(1 - \frac{n_{\text{select}}}{n_{\text{nonselect}}}\right) \times 100$$

where $n_{\text{select}}$ = number of colonies on selective agar; and $n_{\text{nonselect}}$ = number of colonies on nonselective agar.

The *Salmonella* levels were confirmed by performing 10-fold serial dilutions using phosphate-buffered saline to obtain a proper inoculation level. The >70% cocoa solids dark chocolate, cocoa liquor, and cocoa butter were melted using a double-boiler system at 50 ± 2°C. Each matrix was heated until melted and then tempered to 37 ± 2°C in a water bath. A bulk sample of the >70% cocoa solids dark chocolate, cocoa liquor, and cocoa butter was inoculated in a spot-wise manner with

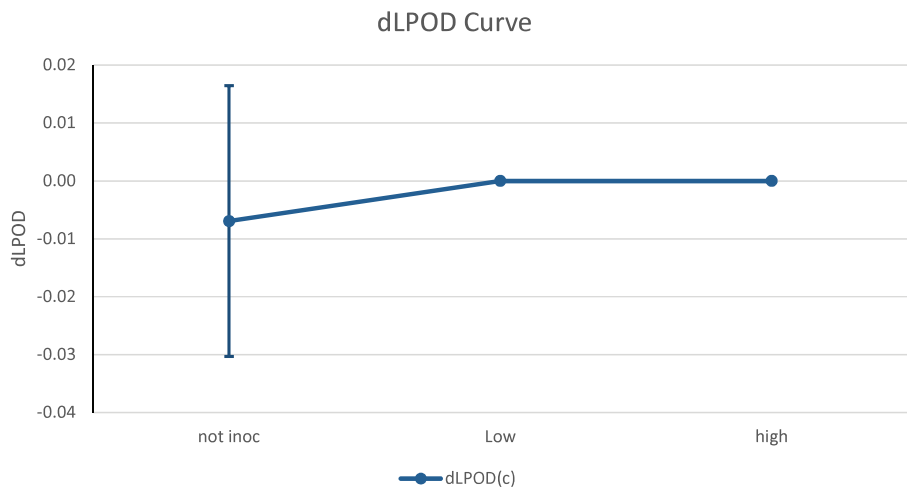


**Figure 6.** $dLPOD_{(CP)}$ values of presumptive SureTect Salmonella species PCR Assay and confirmed results for the collaborative study of cocoa powder.

**Table 3.** Results of aerobic plate count for the collaborative study of cocoa powder

| Collaborator | Cocoa powder (CFU/g[a]) |
|---|---|
| 1 | N/A[b] |
| 2 | $1.0 \times 10^2$ |
| 3 | $3.0 \times 10^2$ |
| 4 | N/A |
| 5 | $2.0 \times 10^2$ |
| 6 | $1.0 \times 10^2$ |
| 7 | $3.0 \times 10^2$ |
| 8 | $6.0 \times 10^2$ |
| 9 | $1.0 \times 10^2$ |
| 10 | $1.0 \times 10^2$ |
| 11 | $1.0 \times 10^2$ |
| 12 | $2.0 \times 10^2$ |
| 13 | $2.0 \times 10^2$ |
| 14 | $2.0 \times 10^2$ |

[a] Samples (25 g) analyzed by the FDA/BAM Chapter 3 Reference Method.
[b] N/A = Not applicable.

an appropriate volume of the organism, which was small enough not to adversely affect the water activity of the sample, and at a dilution that considered the initial die-off and achieved each of the desired contamination levels: a low level expected to yield fractional positive results (5–15 positive results), and a high level expected to yield all positive results. The >70% cocoa solids dark chocolate, cocoa liquor, and cocoa butter were mixed thoroughly by swirling in a sterile sampling device to ensure homogenous distribution and 25 g individual test portions were placed into sterile laboratory blender bags for both reference methods. For 375 g test portions, 25 g inoculated product was added to 350 g uninoculated product and mixed by swirling in a sterile sampling device. Samples were stored for 2 weeks at ambient temperature (20–25°C) before being tested.

**Table 4.** Shipment temperatures for the collaborative study of cocoa powder

| Collaborator | Temperature measured by recorder, °C |
|---|---|
| 1 | N/A[a] |
| 2 | 8.2 |
| 3 | 6.7 |
| 4 | N/A |
| 5 | 6.5 |
| 6 | 9.0 |
| 7 | 9.3 |
| 8 | 10.1 |
| 9 | 10.6 |
| 10 | 11.1 |
| 11 | 5.2 |
| 12 | 19.2 |
| 13 | 20.1 |
| 14 | 20.9 |

[a] N/A = Not applicable.

### *MPN Analysis*

The level of *Salmonella* species in the low-level inoculum for all 25 g test portions was determined by the MPN method on the day of analysis by evaluating $5 \times 50$ g and $20 \times 25$ g (reference method test portions), and $5 \times 10$ g inoculated test samples. The level of *Salmonella* species in the high-level inoculum for all 25 g test portions was determined by MPN analysis by evaluating $5 \times 25$ g (reference method test portions), and $5 \times 10$ g and $5 \times 5$ g inoculated test samples. To the 25 g portions, 225 mL reference method enrichment broth was added, to the 10 g portions 90 mL reference method enrichment broth was added, and to the 5 g portions 45 mL enrichment broth was added. All 25 g portions were utilized from the unpaired reference method test potions analyzed following the FDA/BAM Chapter 5 reference method. The number of positive results from the three test levels was used to calculate the MPN using the LCF MPN calculator (version 1.6) provided by AOAC-RI.

### *FDA/BAM Chapter 5 Salmonella*

Test portions of 25 g each were combined with $225 \pm 5$ mL NFDM (supplemented with Brilliant Green Dye). All test portions were homogenized by hand-massaging and were stood at room temperature (20–25°C) for $60 \pm 5$ min. The pH of the enrichments needed to be adjusted to $6.8 \pm 0.2$ if not in that range. Subsequently, all enrichments were incubated at $35 \pm 2$°C for $24 \pm 2$ h.

Following incubation, 0.1 mL primary enrichment was transferred into 10 mL RV and 1.0 mL was transferred into 10 mL TT broth. The RV tubes were incubated at 42 ± 0.2 °C for 24 ± 2 h. The TT tubes were incubated at 35 ± 2°C for 24 ± 2 h. Following incubation, a loopful of the secondary enrichments was streaked onto BS agar, HE agar, and XLD and incubated at 35 ± 2°C for 24 ± 2 h. If no visible colonies were present after 24 h of incubation on the BS plates, they were reincubated for an additional 24 ± 2 h at 35 ± 2°C. A minimum of two suspect colonies from each selective agar was transferred to TSI and LIA slants and incubated at 35 ± 2°C for 24 ± 2 h. Following incubation, the TSI and LIA slants were examined for typical reactions. Slants producing typical reactions were streaked onto TSA and incubated for 35 ± 2°C for 18–24 h. Following incubation, isolates were serologically tested for both somatic O and flagellar H agglutination. Additionally, purified TSA isolates were identified using the Bruker MALDI Biotyper, AOAC *Official Method*SM **2017.09**.

### *ISO 6579–1:2017/Traditional Confirmation*

For all test portions, a 375 g sample was enriched with 3375 mL NFDM and incubated at 34–38°C for 18 ± 2 h. Following incubation, 0.1 mL primary enrichment was transferred into 10 mL RVS while 1.0 mL of the primary enrichment was transferred into 10 mL of Muller–Kauffmann TT–novobiocin broth (MKTTn). The RVS tubes were incubated at 41.5 ± 1°C for 24 ± 3 h and the MKTTn tubes were incubated at 37 ± 1°C for 24 ± 3 h. Following incubation, a loopful of the secondary enrichments was streaked to two different selective agars, XLD and HE. All plates were inverted and incubated at 34–38°C for 24 ± 3 h. One suspected colony from either selective agar plate was transferred to TSA plates and incubated at 34–38°C for 24 ± 3 h. After incubation, typical isolates were examined for purity on TSA, underwent poly O and H serology, and were identified using the Bruker MALDI system (MicroVal 2017LR72).

### *Alternative Confirmation Procedure*

Following incubation, 10 µL of each sample was streacked on to XLD and BSA and incubated at 34–38°C for 24 ± 2 h. Presumptive positive *Salmonella* colonies were confirmed using latex agglutination and a Microbact™ GNB 24E kit.

### *Thermo Scientific™ SureTect™ Salmonella Species PCR Assay*

All test portions were prepared and incubated according to the protocol described previously in *Sample Enrichment*. All samples were analyzed by the SureTect Salmonella species PCR Assay at 20–28 h for NFDM enriched samples and 22–30 h for BPW (ISO) enriched samples. All samples, regardless of presumptive results, were confirmed following their respective reference method with final confirmation by Bruker MALDI Biotyper AOAC *Official Method of Analysis*SM **2017.09** (MicroVal 2017LR72).

### *Results*

Prior to inoculation, an APC result of $6.7 \times 10^4$ CFU/g was obtained from cocoa powder, <10 CFU/g for >70% cocoa solids dark chocolate, $2.6 \times 10^2$ CFU/g for cocoa liquor, and $3.4 \times 10^2$ CFU/g for cocoa butter. The injury for the *Salmonella* Typhimurium ATCC 14028 used to inoculate the >70% cocoa solids dark chocolate was 77.22%, with 77.06% for the *Salmonella* Enteritidis ATCC 13076 used to inoculate the cocoa liquor, and 73.85% for the *Salmonella* Senftenberg ATCC 43845 used to inoculate the cocoa butter. As per criteria outlined in Appendi x J of the *Official Methods of Analysis* manual, fractional positive results were obtained in all test portions. The POD was calculated as the number of positive outcomes divided by the total number of trials (10). The POD was calculated for the candidate presumptive results, $POD_{CP,}$ the candidate confirmatory results, $POD_{CC}$, the difference in the candidate presumptive and confirmatory results, $dPOD_{CP,}$ presumptive candidate results that confirmed positive, $POD_{C,}$ the reference method, $POD_R$, and the difference in the confirmed candidate and reference methods, $dPOD_C$. The POD analysis between the Thermo Scientific SureTect Salmonella species PCR Assay and the reference methods indicated that there was no significant difference at the 5% level between the number of positive results by the methods. The POD analysis between the SureTect Salmonella species PCR Assay presumptive and confirmed results indicated that there was no significant difference at the 5% level for all methods following both the traditional confirmation procedure and the alternative confirmation procedure. A summary of POD analyses is presented in Tables 5–7.

### *Study Overview—Additional Matrices (April 2021)*

The following matrix studies were performed by Q Laboratories: fresh bagged spinach (375 g) and raw beef trim (375 g); and by the Thermo Fisher Scientific laboratory in Basingstoke, UK: cut cabbage (25 g), cut mango (25 g), grated Cheddar cheese (25 g), single cream (8% fat; 25 g), and feta cheese (25 g).

All food matrices were obtained from local supermarkets or food wholesale companies. For the reference method, the USDA FSIS MLG 4.10 was used when analyzing the raw beef trim matrix, and the FDA/BAM Chapter 5 was used for fresh bagged spinach, cut cabbage, cut mango, and grated Cheddar cheese matrices. In addition, the EN ISO 6579-1:2017 reference method was carried out for the cut cabbage, cut mango, grated Cheddar cheese, single cream (8% fat), and feta cheese matrices.

To prescreen the 25 g samples, relevant portions of the samples were enriched in BPW (ISO). The bags were then incubated at 34–38°C for 20 h. After incubation, samples were tested using the SureTect Salmonella species PCR Assay. Presumptive positives were streaked onto a suitable selective plate and confirmed using the Oxoid Salmonella Latex kit. Samples were also prescreened using the FDA/BAM Chapter 5 reference method.

To prescreen the 375 g samples, the USDA FSIS MLG 4.10 reference method was carried out for the beef trim matrix and FDA/BAM Chapter 5 reference was carried out for the fresh bagged spinach matrix.

The results of the prescreen showed that the cut mango was naturally contaminated at low level with *Salmonella* species. The rest of the matrices were not found to be naturally contaminated.

For the comparison to the USDA FSIS MLG 4.10 reference method, the matrix study consisted of evaluating a total of 30 unpaired 375 g portions and 30 unpaired 25 g portions for fresh raw beef trim.

For the comparison to the FDA/BAM Chapter 5 reference method, the matrix study consisted of evaluating a total of 30 unpaired 375 g portions and 30 unpaired 25 g portions for fresh bagged spinach. A total of 30 paired 25 g portions were evaluated for the cut mango, 60 unpaired 25 g portions for the cut cabbage, 60 unpaired 25 g portions for the grated Cheddar cheese, 60 unpaired 25 g portions for the single cream, and 60 unpaired 25 g portions for the feta cheese.

**Table 5.** Pre-collaborative: Thermo Scientific SureTect Salmonella species PCR Assay—cocoa and chocolate matrices—NFDM enrichment results, presumptive versus confirmed and candidate versus FDA/BAM Chapter 5 reference method—POD results[a]

| | | | Candidate presumptive(CP) | | | Candidate confirmed (CC) | | | Candidate result (C)[b] | | | Reference method (R) | | | C versus R dPOD | CP versus CC dPOD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Statistic | Matrix/organism | MPN[c] | n[d] | x[e] | $POD_{CP}$[f] | n | x | $POD_{CC}$[g] | n | x | $POD_C$[h] | n | x | $POD_R$[i] | (C, R)[j] | (CP, CC)[k] |
| | | N/A[o] | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| LCL[l] | Cocoa powder (375 g) | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL[m] | | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.57 | 20 | 13 | 0.65 | 20 | 13 | 0.65 | 20 | 13 | 0.65 | 20 | 10 | 0.50 | 0.15 | 0.00 |
| LCL | | 0.36 | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.30 | –0.15 | –0.13 |
| UCL | *Salmonella* Infantis QL 052016.18 | 1.02 | | | 0.82 | | | 0.82 | | | 0.82 | | | 0.70 | 0.41 | 0.13 |
| | | 2.06 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 0.98 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 4.17 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Dark chocolate (>70% dark solids: 375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.83 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 12 | 0.60 | –0.10 | 0.00 |
| LCL | | 0.49 | | | 0.30 | | | 0.30 | | | 0.30 | | | 0.39 | –0.37 | –0.13 |
| UCL | | 1.40 | | | 0.70 | | | 0.70 | | | 0.70 | | | 0.78 | 0.19 | 0.13 |
| | *Salmonella* Typhimurium ATCC 14028 | 3.70 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 1.05 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 9.02 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Cocoa liquor (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.70 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 11 | 0.55 | –0.05 | 0.00 |
| LCL | | 0.42 | | | 0.30 | | | 0.30 | | | 0.30 | | | 0.34 | –0.33 | –0.13 |
| UCL | *Salmonella* Enteritidis ATCC 13076 | 1.09 | | | 0.70 | | | 0.70 | | | 0.70 | | | 0.74 | 0.24 | 0.13 |
| | | 2.58 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 1.15 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 5.80 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Cocoa butter (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.59 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 13 | 0.65 | –0.15 | 0.00 |
| LCL | | 0.42 | | | 0.30 | | | 0.30 | | | 0.30 | | | 0.43 | –0.41 | –0.13 |
| UCL | *Salmonella* Senftenberg ATCC 43845 | 1.14 | | | 0.70 | | | 0.70 | | | 0.70 | | | 0.82 | 0.15 | 0.13 |
| | | 2.12 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 1.17 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 5.27 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |

[a] All results were identical for all time points for both PCR instruments evaluated.
[b] Candidate test portions and ISO 6579 reference method test portions were paired.
[c] MPN = Most probable number is calculated using the LCF MPN calculator version 1.6 provided by AOAC-RI, with 95% confidence interval.
[d] n = Number of test portions.
[e] x = Number of positive test portions.
[f] $POD_{CP}$ = Candidate method presumptive positive outcomes divided by the total number of trials.
[g] $POD_{CC}$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[h] $POD_C$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[i] $POD_R$ = Reference method confirmed positive outcomes divided by the total number of trials.
[j] $dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[k] $dPOD_C$ = Difference between the confirmed candidate method result and reference method confirmed result POD values.
[l] LCL = Lower confidence limit.
[m] UCL = Upper confidence limit.
[o] N/A = Not applicable.

For the comparison to the EN ISO 6579 reference method, the matrix study consisted of evaluating a total of 30 paired 25 g portions for the cut mango, 30 paired 25 g portions for the cut cabbage, 30 paired portions for the 25 g grated Cheddar cheese, 30 paired portions for the 25 g single cream, and 30 paired portions for the 25 g feta cheese.

Within each sample set, there were 5 uninoculated samples (0 CFU/test portion), 20 low-level inoculated samples (0.2–2 CFU/test portion), and 5 high-level inoculated samples (2–10 CFU/test portion).

**Table 6.** Pre-collaborative: Thermo Scientific SureTect Salmonella species PCR Assay—cocoa and chocolate matrices—BPW enrichment results, presumptive versus confirmed and candidate versus FDA/BAM Chapter 5 reference method—POD results[a]

| | | | Candidate presumptive(CP) | | | Candidate confirmed (CC) | | | Candidate result (C) | | | Reference method (R) | | | C versus R dPOD | CP versus CC dPOD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Statistic | Matrix/organism | MPN[b] | n[c] | x[d] | $POD_{CP}$[e] | n | x | $POD_{CC}$[f] | n | x | $POD_{C}$[g] | n | x | $POD_{R}$[h] | (C, R)[i] | (CP, CC)[j] |
| | | N/A[o] | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| LCL[k] | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL[l] | Cocoa powder (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.57 | 20 | 15 | 0.75 | 20 | 15 | 0.75 | 20 | 15 | 0.75 | 20 | 10 | 0.50 | 0.25 | 0.00 |
| LCL | | 0.36 | | | 0.53 | | | 0.53 | | | 0.53 | | | 0.30 | –0.05 | –0.13 |
| UCL | *Salmonella* Infantis QL 052016.18 | 1.02 | | | 0.89 | | | 0.89 | | | 0.89 | | | 0.70 | 0.49 | 0.13 |
| | | 2.06 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 0.98 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 4.17 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | Dark chocolate | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | (>70% dark solids: | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | 375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.83 | 20 | 12 | 0.60 | 20 | 12 | 0.60 | 20 | 12 | 0.60 | 20 | 12 | 0.60 | 0.00 | 0.00 |
| LCL | | 0.49 | | | 0.39 | | | 0.39 | | | 0.39 | | | 0.39 | –0.28 | –0.13 |
| UCL | | 1.40 | | | 0.78 | | | 0.78 | | | 0.78 | | | 0.78 | 0.28 | 0.13 |
| | *Salmonella* Typhimurium ATCC 14028 | 3.70 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 1.05 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 9.02 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Cocoa liquor (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.70 | 20 | 7 | 0.35 | 20 | 7 | 0.35 | 20 | 7 | 0.35 | 20 | 11 | 0.55 | –0.20 | 0.00 |
| LCL | *Salmonella* Enteritidis ATCC 13076 | 0.42 | | | 0.18 | | | 0.18 | | | 0.18 | | | 0.34 | –0.46 | –0.13 |
| UCL | | 1.09 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.74 | 0.10 | 0.13 |
| | | 2.58 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 1.15 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 5.80 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Cocoa butter (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.59 | 20 | 13 | 0.65 | 20 | 13 | 0.65 | 20 | 13 | 0.65 | 20 | 13 | 0.65 | 0.00 | 0.00 |
| LCL | *Salmonella* Senftenberg ATCC 43845 | 0.42 | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | –0.28 | –0.13 |
| UCL | | 1.14 | | | 0.82 | | | 0.82 | | | 0.82 | | | 0.82 | 0.28 | 0.13 |
| | | 2.12 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 1.17 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 5.27 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |

[a] All results were identical for all time points for both PCR instruments evaluated.
[b] MPN = Most probable number is calculated using the LCF MPN calculator version 1.6 provided by AOAC-RI, with 95% confidence interval.
[c] n = Number of test portions.
[d] x = Number of positive test portions.
[e] $POD_{CP}$ = Candidate method presumptive positive outcomes divided by the total number of trials.
[f] $POD_{CC}$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[g] $POD_{C}$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[h] $POD_{R}$ = Reference method confirmed positive outcomes divided by the total number of trials.
[i] $dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[j] $dPOD_{C}$ = Difference between the confirmed candidate method result and reference method confirmed result POD values.
[k] LCL = Lower confidence limit.
[l] UCL = Upper confidence limit.
[o] N/A = Not applicable.

### *Organism Preparation and Inoculation*

Despite the cut mango being naturally contaminated, this matrix was artificially contaminated. All inoculated matrixes tested were spiked with a liquid, unstressed culture. The cut cabbage matrix was spiked with Trials Culture Collection (TCC; Basingstoke, UK) 2717 *Salmonella* Montevideo, the grated cheese matrix was spiked with TCC 2100 *Salmonella* Urbana, the cut mango matrix was spiked with Research and Development Culture Collection (RDCC; Basingstoke, UK) 2725 *Salmonella* Agona, the single cream (8% fat) matrix was spiked with *Salmonella* Muenchen RDCC 2130, the feta cheese matrix was

**Table 7.** Pre-collaborative: Thermo Scientific SureTect Salmonella species PCR Assay—cocoa and chocolate matrices—BPW enrichment results, presumptive versus confirmed and candidate versus ISO 6579:2017 reference Method—POD results[a]

| | | | Candidate presumptive (CP) | | | Candidate confirmed (CC) | | | Candidate result (C) | | | Reference method (R) | | | C versus R dPOD | CP versus CC dPOD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Statistic | Matrix/organism | MPN[b] | n[c] | x[d] | $POD_{CP}$[e] | n | n | $POD_{CC}$[f] | n | x | $POD_C$[g] | n | x | $POD_R$[h] | (C, R)[i] | (CP, CC)[j] |
| | | N/A[o] | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| LCL[k] | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL[l] | Cocoa powder (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.57 | 20 | 15 | 0.75 | 20 | 15 | 0.75 | 20 | 15 | 0.75 | 20 | 13 | 0.65 | 0.10 | 0.00 |
| LCL | | 0.36 | | | 0.53 | | | 0.53 | | | 0.53 | | | 0.43 | –0.18 | –0.13 |
| UCL | *Salmonella* Infantis QL 052016.18 | 1.02 | | | 0.89 | | | 0.89 | | | 0.89 | | | 0.82 | 0.36 | 0.13 |
| | | 2.06 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 0.98 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 4.17 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | Dark chocolate | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | (>70% dark solids: | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | 375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.83 | 20 | 12 | 0.60 | 20 | 12 | 0.60 | 20 | 12 | 0.60 | 20 | 10 | 0.50 | 0.10 | 0.00 |
| LCL | | 0.49 | | | 0.39 | | | 0.39 | | | 0.39 | | | 0.30 | –0.19 | –0.13 |
| UCL | | 1.40 | | | 0.78 | | | 0.78 | | | 0.78 | | | 0.70 | 0.37 | 0.13 |
| | *Salmonella* | 3.70 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | Typhimurium | 1.05 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | ATCC 14028 | 9.02 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Cocoa liquor (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.70 | 20 | 7 | 0.35 | 20 | 7 | 0.35 | 20 | 7 | 0.35 | 20 | 10 | 0.50 | –0.15 | 0.00 |
| LCL | *Salmonella* | 0.42 | | | 0.18 | | | 0.18 | | | 0.18 | | | 0.30 | –0.41 | –0.13 |
| UCL | Enteritidis ATCC | 1.09 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.70 | 0.15 | 0.13 |
| | 13076 | 2.58 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 1.15 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 5.80 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Cocoa butter (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.59 | 20 | 13 | 0.65 | 20 | 13 | 0.65 | 20 | 13 | 0.65 | 20 | 10 | 0.65 | 0.15 | 0.00 |
| LCL | *Salmonella* | 0.42 | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | –0.15 | –0.13 |
| UCL | Senftenberg ATCC | 1.14 | | | 0.82 | | | 0.82 | | | 0.82 | | | 0.82 | 0.41 | 0.13 |
| | 43845 | 2.12 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 1.17 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 5.27 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |

[a] All results were identical for all time points for both PCR instruments evaluated.
[b] MPN = Most probable number is calculated using the LCF MPN calculator version 1.6 provided by AOAC-RI, with 95% confidence interval.
[c] n = Number of test portions.
[d] x = Number of positive test portions.
[e] $POD_{CP}$ = Candidate method presumptive positive outcomes divided by the total number of trials.
[f] $POD_{CC}$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[g] $POD_C$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[h] $POD_R$ = Reference method confirmed positive outcomes divided by the total number of trials.
[i] $dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[j] $dPOD_C$ = Difference between the confirmed candidate method result and reference method confirmed result POD values.
[k] LCL = Lower confidence limit.
[l] UCL = Upper confidence limit.
[o] N/A = Not applicable.

spiked with *Salmonella* Mbandaka RDCC 2099, the raw beef trim matrix was spiked with ATCC 51957 *Salmonella* Agona, and the fresh bagged spinach matrix was spiked with ATCC 9270 *Salmonella* Anatum.

For the cut cabbage, mango, grated Cheddar cheese, single cream (8% fat), and feta cheese matrices, spiking cultures were prepared by removing the required strains from the –80°C culture collection freezer, subculturing to TSA, and incubating plates at 34–38°C for 24 ± 1 h. After incubation, the test trains were subcultured into 10 mL Tryptone Soya Broth and incubated at 34–38°C for 24 ± 1 h. After this incubation, strains were diluted to the equivalent of a 0.5 McFarland standard using sterile saline and then further diluted to $10^{-6}$ in maximum recovery diluent. The liquid culture was spiked into 3000 g bulk material for all matrixes and then stored for 48–72 h at 2–8°C. Additionally, 50 μL of the $10^{-4}$ and $10^{-5}$ dilutions were subcultured onto TSA

**Table 8.** Pre-collaborative: Thermo Scientific SureTect Salmonella species PCR Assay, presumptive versus confirmed and candidate versus FDA/BAM reference or USDA/FSIS MLG 4.10– POD results[a]

| | | | Candidate presumptive (CP) | | | Candidate confirmed (CC) | | | Candidate result (C) | | | Reference method (R)[b] | | | C versus R dPOD | CP versus CC dPOD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Statistic | Matrix/organism | MPN[c] | n[d] | x[e] | $POD_{CP}$[f] | n | x | $POD_{CC}$[g] | n | x | $POD_{C}$[h] | n | x | $POD_{R}$[i] | (C, R)[j] | (CP, CC)[k] |
| | | N/A[p] | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| LCL[l] | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL[m] | Fresh bagged spinach (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.45 | 20 | 8 | 0.40 | 20 | 8 | 0.40 | 20 | 8 | 0.40 | 20 | 7 | 0.35 | 0.05 | 0.00 |
| LCL | | 0.22 | | | 0.22 | | | 0.22 | | | 0.22 | | | 0.18 | –0.23 | –0.13 |
| UCL | *Salmonella* Anatum ATCC 9270 | 0.78 | | | 0.61 | | | 0.61 | | | 0.61 | | | 0.57 | 0.32 | 0.13 |
| | | 1.97 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 0.91 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 4.27 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Raw beef trim (375 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.45 | 20 | 8 | 0.40 | 20 | 8 | 0.40 | 20 | 8 | 0.40 | 20 | 6 | 0.30 | 0.10 | 0.00 |
| LCL | *Salmonella* Agona ATCC 51957 | 0.22 | | | 0.22 | | | 0.22 | | | 0.22 | | | 0.15 | –0.18 | –0.13 |
| UCL | | 0.78 | | | 0.61 | | | 0.61 | | | 0.61 | | | 0.52 | 0.36 | 0.13 |
| | | 1.97 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 0.91 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 4.27 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 2[o] | 0.40 | 5 | 2 | 0.40 | 5 | 2 | 0.40 | 5 | 2 | 0.40 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.12 | | | 0.12 | | | 0.12 | | | 0.12 | –0.47 | –0.47 |
| UCL | Cut mango (25 g)[n] | N/A | | | 0.77 | | | 0.77 | | | 0.77 | | | 0.77 | 0.47 | 0.47 |
| | | 0.98 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 0.00 | 0.00 |
| LCL | *Salmonella* Agona RDCC 2725 | 0.53 | | | 0.30 | | | 0.30 | | | 0.30 | | | 0.30 | –0.13 | –0.13 |
| UCL | | 1.46 | | | 0.70 | | | 0.70 | | | 0.70 | | | 0.70 | 0.13 | 0.13 |
| | | 4.38 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 2.29 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.47 | –0.47 |
| UCL | | 9.25 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.47 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Grated Cheddar cheese (25 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.45 | 20 | 7 | 0.35 | 20 | 7 | 0.35 | 20 | 7 | 0.35 | 20 | 8 | 0.40 | –0.05 | 0.00 |
| LCL | | 0.22 | | | 0.18 | | | 0.18 | | | 0.18 | | | 0.22 | –0.93 | –0.13 |
| UCL | *Salmonella* Urbana TCC 2100 | 0.79 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.61 | 0.23 | 0.13 |
| | | 2.29 | 5 | 4 | 0.80 | 5 | 4 | 0.80 | 5 | 4 | 0.80 | 5 | 5 | 1.00 | –0.20 | 0.00 |
| LCL | | 1.05 | | | 0.38 | | | 0.38 | | | 0.38 | | | 0.57 | –0.62 | –0.47 |
| UCL | | 5.02 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.28 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Cut cabbage (25 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.26 | 20 | 11 | 0.55 | 20 | 11 | 0.55 | 20 | 11 | 0.55 | 20 | 5 | 0.25 | 0.30 | 0.00 |
| LCL | *Salmonella* Montevideo TCC 2717 | 0.10 | | | 0.34 | | | 0.34 | | | 0.34 | | | 0.11 | –0.002 | –0.13 |
| UCL | | 0.48 | | | 0.75 | | | 0.75 | | | 0.74 | | | 0.46 | 0.54 | 0.13 |
| | | 1.00 | 5 | 4 | 0.80 | 5 | 4 | 0.80 | 5 | 4 | 0.80 | 5 | 4 | 0.80 | 0.00 | 0.00 |
| LCL | | 0.48 | | | 0.38 | | | 0.38 | | | 0.38 | | | 0.38 | –0.43 | –0.47 |
| UCL | | 1.98 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | Single Cream (8%) (25 g) | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.60 | 20 | 9 | 0.45 | 20 | 9 | 0.45 | 20 | 9 | 0.45 | 20 | 8 | 0.40 | 0.05 | 0.00 |
| LCL | | 0.37 | | | 0.26 | | | 0.26 | | | 0.26 | | | 0.22 | –0.24 | –0.13 |
| UCL | *Salmonella* Muenchen RDCC 2130 | 0.91 | | | 0.67 | | | 0.67 | | | 0.67 | | | 0.61 | 0.33 | 0.13 |
| | | 0.50 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 0.24 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 1.06 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 | 0.00 |

*(continued)*

**Table 8.** *(continued)*

| Statistic | Matrix/organism | MPN[c] | Candidate presumptive (CP) | | | Candidate confirmed (CC) | | | Candidate result (C) | | | Reference method (R)[b] | | | C versus R dPOD | CP versus CC dPOD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | n[d] | x[e] | $POD_{CP}$[f] | n | x | $POD_{CC}$[g] | n | x | $POD_C$[h] | n | x | $POD_R$[i] | (C, R)[j] | (CP, CC)[k] |
| LCL | Feta cheese (25 g) | N/A | | | 0.00 | | | 0.00 | | | 0.00 | | | 0.00 | –0.43 | –0.47 |
| UCL | | N/A | | | 0.43 | | | 0.43 | | | 0.43 | | | 0.43 | 0.43 | 0.47 |
| | | 0.10 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 0.00 | 0.00 |
| LCL | *Salmonella* Mbandaka RDCC 2099 | 0.03 | | | 0.30 | | | 0.30 | | | 0.30 | | | 0.30 | –0.28 | –0.13 |
| UCL | | 0.30 | | | 0.70 | | | 0.70 | | | 0.70 | | | 0.70 | 0.28 | 0.13 |
| | | 1.65 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 | 0.00 |
| LCL | | 0.80 | | | 0.57 | | | 0.57 | | | 0.57 | | | 0.57 | –0.43 | –0.47 |
| UCL | | 3.40 | | | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | 0.43 | 0.47 |

[a] All results were identical for all time points for both PCR instruments evaluated.
[b] Results obtained following the alternative confirmation were identical to results obtain from confirmation by FDA/BAM Chapter 5 and USDA/FISIS-MLG 4.10 reference method. Only the raw beef trim was tested against the USDA/FSIS-MLG 4.10 reference method. All other matrices were tested against FDA/BAM chapter 5.
[c] MPN = Most probable number is calculated using the LCF MPN calculator version 1.6 provided by AOAC-RI, with 95% confidence interval.
[d] n = Number of test portions.
[e] x = Number of positive test portions.
[f] $POD_{CP}$ = Candidate method presumptive positive outcomes divided by the total number of trials.
[g] $POD_{CC}$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[h] $POD_C$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[i] $POD_R$ = Reference method confirmed positive outcomes divided by the total number of trials.
[j] $dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[k] $dPOD_C$ = Difference between the confirmed candidate method result and reference method confirmed result POD values.
[l] LCL = Lower confidence limit.
[m] UCL = Upper confidence limit.
[n] Paired enrichment.
[o] Natural contamination that has been confirmed using both the candidate and reference method.
[p] N/A = Not applicable.

in triplicate and then incubated at 34–38°C for 24 ± 1 h. The plate results were used to enumerate the CFU in the bulk material and this was then used to calculate the amount of spiked material to combine with the non-inoculated material to create the 25 g test portions.

For the cut cabbage and cut mango bulk material, the matrix was cut into small pieces measuring ≤2 cm. The pre-prepared spiking culture was spot-inoculated with a pipette onto the surface of the food and the food was then mixed by sterile gloved hand inside a sterile bag to evenly distribute the inoculum. The bag opening was folded over and sealed with a rubber band with as much air excluded as possible, with the complete bulk stored at 4 °C until the day of use.

For the grated Cheddar cheese, single cream (8% fat), and feta cheese bulk material, the pre-prepared spiking culture was spot-inoculated with a pipette onto the surface of the food and the food was then mixed by hand inside a sterile bag to evenly distribute the inoculum. The bag opening was folded over and sealed with a rubber band with as much air excluded as possible, with the complete bulk stored at 4 °C until the day of use.

Samples were inoculated so that on the day of testing the level of inoculum was such that fractionally positive results, 5–15 positives per 20 replicates for low spike, and 5/5 positives per five replicates for high spike, were targeted.

Five samples for each matrix were not inoculated.

For the fresh bagged spinach and raw beef trim matrices, spiking cultures were prepared by propagating from a stock culture stored at –70°C to SBA and incubating for 24 ± 2 h at 35 ± 1°C. A single colony was then transferred into BHI broth and incubated at 35 ± 1°C for 24 ± 2 h. Following incubation, the culture was diluted to a target level using BHI as the diluent and added to the matrix in a drop-wise manner at an appropriate amount where the low-level (0.2–2 CFU/test portion) inoculated samples would yield expected fractional positive results (5–15 positive results) and the high-level (2–10 CFU/test portion) inoculated samples would yield expected all positive results. Inoculated matrix was mixed with a sterile spatula in a sterile container to ensure homogeneous distribution of the organisms within the matrix and was held for 48–72 h at 2–8° C to allow for equilibration of the organism as per AOAC guidelines. For the 375 g test portions, 25 g from each contamination level was mixed with a sterile spatula in a sterile container with 350 g uninoculated matrix on the day of analysis.

For all matrices, enrichment was carried out as detailed above in the *General Instructions* section. After enrichment the lysis step was performed using the Applied Biosystems™ SimpliAmp™ Thermal Cycler. Samples were then analyzed using the SureTect Salmonella species PCR assay on the 7500 Fast and QuantStudio 5 instruments and results were interpreted with RapidFinder Express (v2.0 or later) and RapidFinder Analysis (v1.1 or later) software, respectively. Following the final incubation time point, all samples regardless of presumptive results were confirmed using the candidate method and reference method confirmation procedures previously described in the *Candidate Confirmation* section.

### *MPN Analysis*

For all matrices, the level of the respective spiking organism in the low-level inoculum for all 25 g test portions was determined using the MPN method on the day of analysis by evaluating 5 × 50 g and 20 × 25 g (reference method test portions), and

**Table 9.** Pre-collaborative: Thermo Scientific SureTect Salmonella species PCR Assay, candidate versus ISO 6579–1:2017 reference method—POD results[a]

| | | | Candidate result (C)[b] | | | Reference method (R) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Statistic | Matrix/organism | MPN[c] | n[d] | x[e] | $POD_C$[f] | n | x | $POD_R$[g] | C versus R $dPOD_{C,R}$[h] |
| | | N/A[m] | 5 | 2[l] | 0.40 | 5 | 2 | 0.40 | 0.00 |
| LCL[i] | | N/A | | | 0.12 | | | 0.12 | –0.47 |
| UCL[j] | Cut mango (25 g)[k] | N/A | | | 0.77 | | | 0.77 | 0.47 |
| | | 0.98 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 0.00 |
| LCL | *Salmonella* Agona RDCC 2725 | 0.53 | | | 0.30 | | | 0.30 | –0.13 |
| UCL | | 1.46 | | | 0.70 | | | 0.70 | 0.13 |
| | | 4.38 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 |
| LCL | | 2.29 | | | 0.57 | | | 0.57 | –0.47 |
| UCL | | 9.25 | | | 1.00 | | | 1.00 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | –0.47 |
| UCL | Grated Cheddar (25 g) | N/A | | | 0.43 | | | 0.43 | 0.47 |
| | | 0.45 | 20 | 7 | 0.35 | 20 | 7 | 0.35 | 0.00 |
| LCL | | 0.22 | | | 0.18 | | | 0.18 | –0.13 |
| UCL | | 0.79 | | | 0.57 | | | 0.57 | 0.13 |
| | *Salmonella* Urbana TCC 2100 | 2.29 | 5 | 4 | 0.80 | 5 | 5 | 1.00 | 0.00 |
| LCL | | 1.05 | | | 0.38 | | | 0.57 | –0.47 |
| UCL | | 5.02 | | | 1.00 | | | 1.00 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | –0.47 |
| UCL | Cut cabbage (25 g) | N/A | | | 0.43 | | | 0.43 | 0.47 |
| | | 0.26 | 20 | 11 | 0.55 | 20 | 11 | 0.55 | 0.00 |
| LCL | *Salmonella* Montevideo TCC 2717 | 0.10 | | | 0.34 | | | 0.34 | –0.13 |
| UCL | | 0.48 | | | 0.74 | | | 0.74 | 0.13 |
| | | 1.00 | 5 | 4 | 0.80 | 5 | 4 | 0.80 | 0.00 |
| LCL | | 0.48 | | | 0.38 | | | 0.38 | –0.47 |
| UCL | | 1.98 | | | 1.00 | | | 1.00 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | –0.47 |
| UCL | Single cream (8%) (25 g) | N/A | | | 0.43 | | | 0.43 | 0.47 |
| | | 0.60 | 20 | 9 | 0.45 | 20 | 9 | 0.45 | 0.00 |
| LCL | | 0.37 | | | 0.26 | | | 0.26 | –0.13 |
| UCL | *Salmonella* Muenchen RDCC 2130 | 0.91 | | | 0.67 | | | 0.67 | 0.13 |
| | | 0.50 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 |
| LCL | | 0.24 | | | 0.57 | | | 0.57 | –0.47 |
| UCL | | 1.06 | | | 1.00 | | | 1.00 | 0.47 |
| | | N/A | 5 | 0 | 0.00 | 5 | 0 | 0.00 | 0.00 |
| LCL | | N/A | | | 0.00 | | | 0.00 | –0.47 |
| UCL | Feta cheese (25 g) | N/A | | | 0.43 | | | 0.43 | 0.47 |
| | | 0.10 | 20 | 10 | 0.50 | 20 | 10 | 0.50 | 0.00 |
| LCL | *Salmonella* Mbandaka RDCC 2099 | 0.03 | | | 0.30 | | | 0.30 | –0.13 |
| UCL | | 0.30 | | | 0.70 | | | 0.70 | 0.13 |
| | | 1.65 | 5 | 5 | 1.00 | 5 | 5 | 1.00 | 0.00 |
| LCL | | 0.80 | | | 0.57 | | | 0.57 | –0.47 |
| UCL | | 3.40 | | | 1.00 | | | 1.00 | 0.47 |

[a] All results were identical for all time points for both PCR instruments evaluated.
[b] Candidate test portions and ISO 6579 reference method test portions were paired.
[c] MPN = Most probable number is calculated using the LCF MPN calculator version 1.6 provided by AOAC-RI, with 95% confidence interval.
[d] n = Number of test portions.
[e] x = Number of positive test portions.
[f] $POD_C$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[g] $POD_R$ = Reference method confirmed positive outcomes divided by the total number of trials.
[h] $dPOD_C$ = Difference between the confirmed candidate method result and reference method confirmed result POD values (paired).
[i] LCL = Lower confidence limit.
[j] UCL = Upper confidence limit.
[k] Paired enrichment.
[l] Natural contamination that has been confirmed using both the candidate and reference method.
[m] N/A = Not applicable.

5 × 10 g inoculated samples. The level of the respective spiking organism in the high-level inoculum for all 25 g test portions was determined by MPN analysis on the day of analysis by evaluating 5 × 25 g (reference method test portions), and 5 × 10 g and 5 × 5 g inoculated test samples. To the 50 g portions 450 mL reference method enrichment broth was added, to the 10 g portions 90 mL reference method enrichment broth was added, and to the 5 g portions 45 mL reference method enrichment broth was added.

As per the criteria outlined in Appendix J of the *Official Methods of Analysis* manual, fractional positive results were obtained for fresh bagged spinach (375 g) and raw beef trim (375 g) at 8 and 24 h time points, and at 20 h for cut cabbage (25 g), cut mango (25 g), grated Cheddar cheese (25 g), single cream (8% fat; 25 g), and feta cheese (25 g) for the SureTect Salmonella species PCR Assay.

### *Results*

Prior to inoculation, an APC result of $3.20 \times 10^4$ CFU/g was obtained for the fresh bagged spinach, $4.20 \times 10^5$ CFU/g was obtained for the raw beef trim, $4.00 \times 10^3$ CFU/g was obtained for the cut cabbage, $1.60 \times 10^3$ CFU/g was obtained for the cut mango, $1.44 \times 10^7$ CFU/g was obtained for the grated Cheddar cheese, $5.28 \times 10^8$ CFU/g was obtained for the single cream (8% fat), and $3.90 \times 10^7$ CFU/g was obtained for the feta cheese.

The POD was calculated as the number of positive outcomes divided by the total number of trials. The POD was calculated for the candidate presumptive results, $POD_{CP}$, the candidate confirmatory results, $POD_{CC}$, the difference in the candidate presumptive and confirmatory results, $dPOD_{CP}$, presumptive candidate results that confirmed positive, $POD_{C}$, the reference method, $POD_{R}$, and the difference in the confirmed candidate and reference methods, $dPOD_{C}$. The POD analysis between the SureTect Salmonella species PCR Assay and the reference method indicated that there was no significant difference at the 5% level between the number of positive results by the methods at all time points evaluated, regardless of the reference method. The POD analysis between the SureTect Salmonella species PCR Assay presumptive and confirmed results indicated that there was no significant difference at the 5% level for all methods at all time points following both the traditional confirmation procedure and the alternative confirmation procedure. A summary of POD analyses is presented in Tables 8 and 9.

## Discussion

### *Collaborative Study*

No negative feedback was provided regarding the performance of the SureTect Salmonella species PCR Assay. One false-negative result was observed from the analysis of the SureTect Salmonella species PCR Assay. The false-negative rate was 0.01%. During the collaborative study, both the QuantStudio 5 and the 7500 Fast PCR instruments were evaluated. The data suggest the instruments are considered equivalent. There were no differences in result between the candidate method confirmations and reference method confirmations.

Overall, the data generated during this evaluation demonstrate the reproducibility of this method. No statistically significant detectable differences were observed between the presumptive method and the confirmed results.

### *Pre-Collaborative Study*

The data from these studies supports the product claims of the SureTect Salmonella species PCR Assay as a reliable detection method for *Salmonella* species in a broad range of foods and environmental samples. The POD analysis results for the matrix studies demonstrated that, based on inadequate sample size, equivalency was not proven, except for mango and feta cheese. For the non-cocoa matrices, the performance of the candidate method was better than the performance of the reference method (except for grated Cheddar cheese), characterized by positive dPOD values.

## Recommendations

It is recommended that the Thermo Scientific SureTect Salmonella species PCR Assay adopts First Action *Official Methods*$^{SM}$ for the detection of *Salmonella* species from a broad range of foods and categories: raw meat, heat-processed milk and dairy products, fresh produce and fruits, ready-to-eat and ready-to-reheat meals, low-moisture foods, cocoa and chocolate products, egg products, and pet food and environmental samples.

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## Conflict of Interest

None declared.

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