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Research Article

MICROBIOLOGICAL METHODS

Validation of the CompactDry "Nissui" BC for Enumeration of *Bacillus cereus* in a Variety of Foods: AOAC *Performance Tested Method*^{SM} 092201

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Abstract

Background: The CompactDry "Nissui" BC is a ready-to-use dry media sheet using a chromogenic medium with selective agents for the detection and enumeration of *Bacillus cereus* in products after incubation at 30 ± 1°C for 24 ± 2 h.
Objective: The CompactDry "Nissui" BC method was validated to achieve AOAC *Performance Tested Methods*^{SM} certification.
Method: The performance of the CompactDry "Nissui" BC was compared to that of ISO 7932:2004 for 10 matrixes, including panna cotta, double cream, dried baby food, dried vegetable soup mix, seafood sticks, salmon pâté, sliced ham, pork liver pâté, ham and cheese sandwich, and Caesar pasta salad with chicken and bacon. Performance indicators included repeatability, difference of means (DOM), and inclusivity/exclusivity.
Results: After $\log_{10}$ transformation of the data, the relative standard deviation of repeatability (RSD_r) was ≤9.2% for 28 of the 30 materials (10 matrixes each at three contamination levels) analyzed by the CompactDry "Nissui" BC method and ≤13% for 27 of the 30 matrix/level combinations analyzed by the reference method. Method equivalence was demonstrated in 28 of the 30 matrix/level combinations based on the 90% confidence interval of the DOM being within (-0.5, 0.5). For inclusivity, 47 of 50 strains tested showed typical colonies and confirmed positive. For exclusivity, 28 of 33 strains tested resulted in no growth or were negative, and five were positive. Inclusivity and exclusivity results were similar on the reference method agar. The method was shown to be robust to changes in sample volume, incubation temperature, and incubation time, and data are presented supporting product consistency and 18-month shelf life.
Conclusions: The CompactDry "Nissui" BC method is validated for the determination of *Bacillus cereus* in a variety of matrixes.
Highlights: The CompactDry "Nissui" BC method is equivalent to the ISO 7932:2004 reference method and is suitable for *Performance Tested Methods*^{SM} certification for the matrixes tested.

General Information

Bacillus cereus is widespread in the environment and often is isolated from soil and vegetation. It is found in a wide variety of foods and causes two types of food poisoning: diarrheal type and vomiting type. Foods including meats, milk, vegetables, and fish have been associated with the diarrheal-type food poisoning. The vomiting-type outbreaks generally have been associated with rice products; however, other starchy foods, such as potato, pasta, and cheese products, also have been implicated. Food mixtures, such as sauces, puddings, soups, casseroles, pastries, and salads, have frequently been linked with food-poisoning outbreaks (1). Foods susceptible to the presence of *B. cereus* need a robust test method to demonstrate whether this organism is present at levels likely to cause food poisoning

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and therefore be a concern to health. This method is designed to allow the enumeration of presumptive *Bacillus cereus*. Similar to the ISO reference method, it may also enumerate other members of the wider *B. cereus* group such as *Bacillus thuringiensis*, *Bacillus mycoides*, and *Bacillus weihenstephanensis*.

To save operator time and to allow a trained laboratory scientist to perform microbial count tests without difficulty, Nissui developed the CompactDry series of products based on a unique concept and technology applicable to the food industry. CompactDry "Nissui" BC requires the addition of 1 mL of specimen on the device, and results are available in 24 ± 2 h.

Principle of the Method

CompactDry "Nissui" BC are ready-to-use dry media sheets comprising culture medium and a cold-soluble gelling agent. The film is rehydrated by inoculating 1 mL of the sample into the center of the self-diffusible medium. The CompactDry "Nissui" BC culture medium contains nutrients, mannitol, selective agents, chromogenic enzyme substrate, and gelling agent for the detection and enumeration of *Bacillus cereus* after incubation at 30 ± 1°C for 24 ± 2 h. Colonies appear blue/pale blue and must be confirmed according to ISO 7932:2004 (2).

Scope of Method

(a) *Analyte(s)*.—The target analyte is presumptive *Bacillus cereus*. The method may also detect other members of the *Bacillus cereus* group such as *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, and *Bacilllus mycoides*.

(b) *Matrixes*.—Matrixes included panna cotta (with raspberries), double cream (50% fat), dried baby food (cereal-based with strawberry and raspberry flakes), dried vegetable soup mix, surimi seafood sticks, salmon pâté, sliced ham, pork liver pâté, sandwiches (ham and cheese on malted brown bread), and pasta salad (with chicken, bacon, and Caesar dressing).

(c) *Summary of validated performance claims*.—Performance is equivalent to that of ISO 7932:2004, *Microbiology of Food and Animal Feeding Stuffs—Horizontal Method for the Enumeration of Presumptive Bacillus cereus—Colony count technique at 30°C* (2) for the matrixes tested.

Definitions

(a) *Repeatability (s_r)*.—Standard deviation of replicates for each analyte at each concentration of each matrix for each method.

(b) *Difference of means (DOM)*.—Mean difference between candidate and reference method transformed results with 95% confidence interval for each analyte at each concentration of each matrix.

Materials and Methods

Test Kit Information

(a) *Kit name*.—CompactDry "Nissui" BC.

(b) *Catalog number*.—06533 (40 plates), 06534 (240 plates).

(c) *Ordering information*.—Customer Support Section, Nissui Pharmaceutical Co., Ltd., 3-24-6, Ueno, Taito-ku, Tokyo 110-0005 Japan, Telephone: +81-3-5846-5707/Fax: +81-3-5846-5629, e-mail: contact@nissui-pharm.jp.

Test Kit Component

(a) *CompactDry "Nissui" BC plates*.

Additional supplies and reagents

(a) *Filtered sample bags*.—80–400 mL capacity.

(b) *Maximum Recovery Diluent (MRD)*.—Also known as Peptone Salt Diluent. Prepare according to ISO 6887-1:2017 (3) or source commercially (e.g., Oxoid CM0733 or equivalent).

(c) *Pipets*.—Capable of delivering 1.0 mL.

(d) *Pipet tips*.—Capable of delivering 1.0 mL.

(e) *Colony counter*.

Apparatus

(a) *Laboratory paddle blender*.—Stomacher® 400 (Seward, West Sussex, UK) or equivalent.

(b) *Incubator*.—Capable of maintaining 30 ± 1°C.

Safety Precautions

(a) Wash immediately with water if medium or reagent comes into contact with eyes or mouth. Consult a physician.

(b) Manipulations with microorganisms involve certain risks of laboratory-acquired infections. Carry out manipulations under supervision of trained laboratory personnel with biohazard protection measures.

(c) Treat laboratory equipment or medium that comes in contact with the specimen as infectious and sterilize appropriately.

(d) Sterilize any medium, reagent, or materials by autoclaving or boiling after use, and then dispose as industrial waste according to local laws and regulations for disposal of such material.

General Precautions

(a) Read and precisely follow the warnings and directions for use described in the package insert and/or label.

(b) Do not use for human or animal diagnosis.

(c) Do not use product after its expiration date. The quality of the product is not guaranteed after its shelf life.

(d) Do not use product that contains any foreign materials, is discolored, is dehydrated, or has a damaged container.

(e) Use plates as soon as possible after opening. Any unused plates should be returned to the aluminum bag and sealed with tape to avoid light and moisture.

(f) During inoculation, do not touch the surface of medium and/or tip of pipet, and be careful to avoid any contamination by falling microorganism.

(g) During incubation, keep the lid of CompactDry tight to avoid any possible dehydration.

(h) It is recommended to use a stomacher bag with a filter to eliminate risks of carry-over of tiny pieces of food into the surface of the medium.

(i) The specimen should be diluted by buffer solution to the level of concentration of <150 CFU/plate. If more than 10^4 CFU are inoculated in a plate, no independent colonies will form and the entire plate may become colored.

(j) If the nature of the specimen does affect the result, the specimen should be inoculated only after the cause is eliminated by means such as dilution and others—for example, specimens having high viscosity, reactivity with chromogens, deep color, or too high or too low pH.

Sample Preparation

(a) Weigh a 10 g test portion into a filtered sample bag.
(b) Add 90 mL MRD and pummel for 1 min ± 10 s in paddle blender.
(c) Make 10-fold serial dilutions in MRD.

Analysis

(a) Open aluminum pouch and remove the set of four plates.
(b) Detach the number of plates needed by bending up and down while pressing the lid. Alternatively, use the set of four connected plates for serial dilutions of one sample. Label the plates with sample and dilution information.
(c) For each plate, remove the lid and add 1 mL of diluted sample in the middle of the dry sheet. Replace the lid. The sample diffuses automatically and evenly over the sheet and rehydrates the gel.
(d) Invert the plates and incubate at 30 ± 1°C for 24 ± 2 h.
(e) Count blue/pale blue colonies. White paper placed under the plate can be useful for counting.
(f) The enumeration range is 1–150 CFU/plate. If >150 colonies are present, dilute the sample further to achieve a concentration in the countable range.

Interpretation

The size of the plate is 20 cm^2, and the back of the container has a carved grid of 1 cm × 1 cm to make colony counting easier. When it is difficult to count the colonies due to a large number of colonies grown in the medium, the total viable count can be obtained by multiplying the average number of colonies from representative grids (1 cm × 1 cm) by 20.

Validation Study

This validation study was conducted under the MicroVal program following ISO 16140–2:2016 *Microbiology of the food chain—Method validation—Part 2: Protocol for the validation of alternative proprietary methods against a reference method* (4) by the MicroVal Expert Laboratory, Campden BRI (Campden, UK), which conducted the inclusivity/exclusivity study and the matrix study. The method developer, Nissui Pharmaceuticals, conducted the *Performance Tested Methods* (PTM)-specific studies, including robustness and product consistency and stability.

Independent Laboratory Studies

Inclusivity/Exclusivity Study

The inclusivity of the CompactDry "Nissui" BC method was determined using 50 strains of the *Bacillus cereus* group, including *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. weihenstephanensis*. *B. anthracis*, although part of the *B. cereus* group, was not included in the study due to being a biothreat agent. Each strain was grown overnight in a nonselective broth and diluted to a level of approximately 100 colony-forming units (CFU)/mL (100 × LOQ of the method). Exclusivity was established with 33 nontarget microorganisms. Each organism was grown for at least 24 h in a suitable nonselective broth under optimal conditions of growth. Exclusivity organisms were diluted to a level of approximately 10^5 CFU/mL, a level approximating the maximum level of contamination expected to occur in any of the claimed matrixes. All cultures were tested on CompactDry "Nissui" BC and the ISO 7932:2004 reference method (2), which consisted of plating on mannitol egg yolk polymyxin agar (MYP) and confirmation on Sheep Blood Agar (SBA).

The inclusivity results are presented in Table 1. Of the 50 inclusivity strains tested, 47 showed typical growth on the CompactDry "Nissui" BC medium and confirmed positive on SBA. *B. cytotoxicus* (German Collection of Microorganisms and Cell Cultures (DSM) 22905), *B. mycoides* (Campden BRI Culture Collection (CRA) 16597), and *B. pseudomycoides* (CRA 16382) did not grow on CompactDry "Nissui" BC. MYP showed typical colonies that confirmed positive on SBA for 48 of the 50 inclusivity strains. For MYP, *B. cytotoxicus* (DSM 22905) showed typical growth but did not confirm on SBA, and *B. mycoides* (CRA 16597) did not grow.

Table 2 shows the exclusivity results. Initially, species 1–30 were tested. Of these 30, 26 were negative on the CompactDry "Nissui" BC medium and MYP. Three of the 26, *B. amyloliquefaciens*, *B. subtilis*, and *Lysinibacillus sphaericus*, showed growth on MYP but confirmed negative on SBA, so the result was negative. Four species, including *B. coagulans*, *B. laterosporus*, *Brevibacillus brevis*, and *Paenibacillus polymyxa*, resulted in growth on CompactDry "Nissui" BC medium and MYP that confirmed positive on SBA. To check these results, an additional strain each of *B. coagulans*, *B. laterosporus*, and *Paenibacillus polymyxa* were tested. The additional testing showed one of the additional three strains, *B. laterosporus* CRA 1515, was also confirmed positive on both CompactDry BC and MYP.

The results indicate that the CompactDry "Nissui" BC has similar selectivity for *B. cereus* and the wider *B. cereus* group as MYP. The use of a confirmation procedure on SBA must be done according to ISO 7932 (2).

Matrix Study

Ten food matrixes from five food categories were included in the method comparison study. The food products used in the matrix study are given in Table 3, along with the specific strain of inoculum used artificial contamination. All cultures were maintained on storage beads (Protect, Technical Service Consultants, Ltd.) at -75°C in the Campden BRI Culture Collection. Prior to use, each strain was subcultured onto a pre-poured plate of Plate Count Agar (PCA, Oxoid, CM0325) and incubated for 24 to 48 h at 30 ± 1°C. An isolated colony from each strain was then subcultured onto PCA slant and incubated for 24 to 48 h at 30 ± 1°C. The culture slant was then kept at 2–8°C until required for use. Slants were kept for a period of up to 4 weeks. Each strain used for the matrix study was cultured separately by transferring growth from the chilled OCA slope into 10 mL Nutrient Broth (NB, Oxoid, CM0001) incubated for up to 48 h at 30 ± 1°C.

The number of cells in the culture was enumerated microscopically, and the culture was then serially diluted in Maximum Recovery Diluent (MRD, Oxoid, CM0733) to the desired level for the subsequent contamination of the samples. There were three levels (low, medium, and high) for each matrix, and five replicate test portions from each level were tested. For each level of panna cotta, double cream, seafood sticks, salmon pâté, sliced ham, pork liver pâté, sandwiches, and pasta salad, a 100 g sample was inoculated with 1 mL of appropriate culture using a sterile 1 mL pipet and homogenized thoroughly

Table 1. Inclusivity testing on CompactDry "Nissui" BC and MYP Agar

No.	Species	Source[a]	Origin	CD BC	MYP
1	*Bacillus cereus*	CRA 84	Meatloaf	+[b]	+
2	*Bacillus cereus*	CRA 193	Environmental	+	+
3	*Bacillus cereus*	CRA 1549	Dried milk	+	+
4	*Bacillus cereus*	CRA 1731	Chocolate ice cream	+	+
5	*Bacillus cereus*	CRA 1740	Cream cake	+	+
6	*Bacillus cereus*	CRA 1741	Flour	+	+
7	*Bacillus cereus*	CRA 1749	Cream cake	+	+
8	*Bacillus cereus*	CRA 1964	Milk/cream	+	+
9	*Bacillus cereus*	CRA 4110	Contaminated flask	+	+
10	*Bacillus cereus*	CRA 6295	Flavoring	+	+
11	*Bacillus cereus*	CRA6452	Flour	+	+
12	*Bacillus cereus*	CRA7616	Dairy	+	+
13	*Bacillus cereus*	CRA 8711	Infant formula	+	+
14	*Bacillus cereus*	CRA 16100	Flavor	+	+
15	*Bacillus cereus*	CRA 16101	Flavor	+	+
16	*Bacillus cereus*	CRA 16381	Environmental	+	+
17	*Bacillus cereus*	CRA 16438	Environmental	+	+
18	*Bacillus cereus*	CRA 16563	Unknown	+	+
19	*Bacillus cereus*	CRA 16564	Food poisoning	+	+
20	*Bacillus cereus*	CRA 16565	Pharmaceutical	+	+
21	*Bacillus cereus*	CRA 16566	Unknown	+	+
22	*Bacillus cereus*	CRA 16569	Meatloaf	+	+
23	*Bacillus cereus*	CRA 16570	Food poisoning	+	+
24	*Bacillus cereus*	CRA 16571	Unknown	+	+
25	*Bacillus cereus*	CRA 16579	Industrial	+	+
26	*Bacillus cereus*	CRA 16580	Industrial	+	+
27	*Bacillus cereus*	CRA 16582	Environmental	+	+
28	*Bacillus cereus*	CRA 16583	Industrial	+	+
29	*Bacillus cereus*	CRA 16662	Dried potato	+	+
30	*Bacillus cereus*	CRA 17010	Mangoes	+	+
31	*Bacillus cereus*	CRA17011	Water	+	+
32	*Bacillus cereus*	CRA 17012	Milk	+	+
33	*Bacillus cereus*	CRA 17013	Soil	+	+
34	*Bacillus cytotoxicus*	DSM 22905	Vegetable puree	–[c]	–[d]
35	*Bacillus mycoides*	CRA 16597	UHT custard	–	–
36	*Bacillus mycoides*	CRA 1522	Dried milk	+	+
37	*Bacillus mycoides*	CRA 16646	Soft drink factory	+	+
38	*Bacillus mycoides*	CRA 1510	Dried milk	+	+
39	*Bacillus mycoides*	CRA 8504	Food environment	+	+
40	*Bacillus pseudomycoides*	CRA 16382	Soil	–	+
41	*Bacillus thuringiensis kurstaki*	CRA 17032	Insecticide	+	+
42	*Bacillus thuringiensis aizawai*	CRA 17033	Insecticide	+	+
43	*Bacillus thuringiensis israelensis*	CRA 17034	Insecticide	+	+
44	*Bacillus thuringiensis*	CRA 16616	Broccoli	+	+
45	*Bacillus thuringiensis*	CRA 16314	Flour moth	+	+
46	*Bacillus thuringiensis*	CRA 1744	Flour	+	+
47	*Bacillus thuringiensis*	CRA 16619	Broccoli	+	+
48	*Bacillus weihenstephanensis*	CRA 16578	Pasteurized milk	+	+
49	*Bacillus weihenstephanensis*	DSM 104135	Soil	+	+
50	*Bacillus weihenstephanensis*	DSM 104109	Soil	+	+

[a] CRA = Campden Culture Collection (Campden BRI, Chipping Campden, UK); DSM = DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

[b] "+" indicates growth occurred with typical morphology and confirmation on SBA.

[c] Unless otherwise noted, "-" indicates growth did not occur.

[d] This strain showed typical colonies on MYP, but did not confirm on SBA, so was deemed confirmed negative.

in a laboratory paddle blender. The samples were stored for 72 h at 2–8°C prior to analysis. For dried baby food and dried vegetable soup mix, lyophilized spores were used, and samples were stored at room temperature for 14 days. Briefly, washed spore suspensions were diluted in sterile 10% nonfat dried milk powder and lyophilized by freeze-drying. The lyophilized suspensions were ground into a homogenous powder in a sterile container, added to the dried food in a large sterile bag, and shaken to evenly distribute. Samples of this initial preparation were diluted (e.g., 10 g amount in a further 90 g of product) to achieve the correct levels. Levels of spores were determined by heating a 1:10 suspension of product in MRD in a water bath for

Table 2. Exclusivity testing on CompactDry "Nissui" BC and MYP agar with confirmation on SBA

No.	Species	Source[a]	Origin	CD BC[b]	MYP[b]
1	*Allicyclobacillus acidoterrestris*	CRA 5331	Apple juice	–	–
2	*Alicyclobacillus cycloheptanicus*	CRA 16823	Soil	–	–
3	*Alicyclobacillus fastidiosus*	CRA 16831	Apple juice	–	–
4	*Alicyclobacillus pomorum*	CRA 16830	Fruit juice	–	–
5	*Aneurinibacillus aneurinolyticus*	CRA 7751	Flavor	–	–
6	*Anoxybacillus flavithermus*	CRA 17047	Food isolate	–	–
7	*Bacillus amyloliquefaciens*	CRA 6317	Crumpets	–	–[c]
8	*Bacillus circulans*	CRA 16584	Cream	–	–
9	*Bacillus coagulans*	CRA 10205	Evaporated milk	+	+
10	*Bacillus fusiformis*	CRA 16652	Soft drinks	–	–
11	*Bacillus laterosporus*	CRA 1523	Dried milk	+	+
12	*Bacillus licheniformis*	CRA 6335	Pesto	–	–
13	*Bacillus megaterium*	CRA 16512	Soil	–	–
14	*Bacillus oceanisediminis*	CRA 17220	Food isolate	–	–
15	*Bacillus pumilus*	CRA 16594	Industrial isolate	–	–
16	*Bacillus psychrodurans*	CRA 16694	Soil	–	–
17	*Bacillus smithii*	CRA 7240	Pineapple	–	–
18	*Bacillus sonorensis*	CRA 17231	Food isolate	–	–
19	*Bacillus sphaericus*	CRA 7950	Flavoring	–	–
20	*Bacillus subtilis*	CRA 14161	Milk shake	–	–[c]
21	*Brevibacillus brevis*	CRA 7748	Flavor	+	+
22	*Brevibacillus parabrevis*	CRA 7757	Flavor	–	–
23	*Leuconostoc mesenteroides*	CRA 16022	Soft ham	–	–
24	*Listeria ivanovii*	CRA 1123	Soft cheese	–	–
25	*Lysinibacillus sphaericus*	CRA 7746	Unknown	–	–[c]
26	*Paenibacillus amylolyticus*	CRA 16606	Barley	–	–
27	*Paenibacillus macerans*	CRA 16488/DSM 357	Unknown	–	–
28	*Paenibacillus pabuli*	CRA 16605	Barley	–	–
29	*Paenibacillus polymyxa*	CRA 7747	Food isolate	+	+
30	*Staphylococcus aureus*	CRA 1224	Margarine	–	–
31	*Bacillus coagulans*	CRA 17185	Industrial isolate	–	–
32	*Bacillus laterosporus*	CRA 1515	Dried milk	+	+
33	*Paenibacillus polymyxa*	CRA 16386/ATCC 43865	Unknown	–	–

[a] CRA = Campden Culture Collection (Campden BRI, Chipping Campden, UK); DSM = DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany); ATCC = American Type Culture Collection (Manassas, VA, USA).
[b] Unless otherwise noted, "-" indicates growth did not occur; "+" indicates growth occurred with typical morphology and confirmation on SBA.
[c] Strain showed typical growth on MYP, but the colonies did not confirm on SBA, so they were deemed confirmed negative.

10 min at 80°C followed by enumeration on PCA. From each inoculated sample, five replicate 10 g test portions were weighed into filtered sample bags, diluted with 90 mL MRD, and homogenized for 1 min ± 10 s. Serial dilutions were made in MRD, and appropriate dilutions were plated out on the candidate and reference method plates.

For CompactDry "Nissui" BC, the method was followed as written above without confirmations. The colony count was multiplied by the dilution factor, divided by the test portion size (10 g), and recorded as the number of CFU per gram.

For ISO 7932:2004, 1.0 mL aliquots of appropriate dilutions were spread onto MYP and incubated at 30 ± 1°C for 18–24 h. If colonies were not clearly visible, the plates were reincubated for a further 24 h. Up to five typical and five atypical colonies (pink without halos) were confirmed by stabbing onto SBA and examining for zones of clearance after incubation at 30 ± 1°C for 24 ± 2 h. As per ISO 7218:2007 (5) with Amendment 1:2013 (6), single plates of successive dilutions were prepared as a minimum. To increase reliability, duplicate plates were carried out when considered necessary based on the expected contamination level and dilution plated. If only one dilution was plated, then duplicate plates were used.

Prior to data analysis, a logarithmic transformation of the data was carried out as follows:

Log_{10} (CFU/g + 0.1), where 0.1 is an offset to allow for inclusion of data for "0" CFU.

Graphs were generated plotting the log transformed candidate method results verses the log transformed reference method results for each matrix (*see* Figure 1) to look for any apparent discrepancies. None were observed; however, the dried baby food and dried vegetable soup mix, which were inoculated with spores, did not have as wide a range of concentrations as the other eight matrixes. Statistical analysis was performed using the AOAC workbook for quantitative microbiology statistics (7).

A summary of the method comparison data and statistical analyses is presented in Table 4. The standard deviation of repeatability (s_r) of the five replicate test portions for each product at each inoculum level was generally similar between the CompactDry and ISO methods. A few notable exceptions include the high level of panna cotta, the low level of dried soup mix, the low level of sandwiches, and the low level of pasta salad. For three of these four exceptions, however, the CompactDry method shows better repeatability than the ISO method.

Table 3. Categories, types, strains, food item, and target levels for the matrix study

Category/Type	Strain[a]	Food item	Target levels	Test portions
Dairy products	*B. weihenstephanensis* CRA 16578	Panna cotta	Low: 10^3 CFU/g	5
			Medium: 10^4 CFU/g	5
			High: 5×10^5 CFU/g	5
		Double cream	Low: 10^3 CFU/g	5
			Medium: 10^4 CFU/g	5
			High: 5×10^5 CFU/g	5
Dried cereals, fruits, nuts, seeds, and vegetables	*B. cereus* CRA 8711	Dried baby food	Low: 10^4 CFU/g	5
			Medium: 10^5 CFU/g	5
			High: 10^6 CFU/g	5
		Dried vegetable soup mix	Low: 10^4 CFU/g	5
			Medium: 10^5 CFU/g	5
			High: 10^7 CFU/g	5
Ready-to-eat fish products	*B. cereus* CRA 6295	Seafood sticks	Low: 10^2 CFU/g	5
			Medium: 5×10^3 CFU/g	5
			High: 5×10^5 CFU/g	5
		Salmon pâté	Low: 10^2 CFU/g	5
			Medium: 5×10^3 CFU/g	5
			High: 5×10^5 CFU/g	5
Ready-to-eat meat and poultry products	*B. cereus* CRA 16569	Sliced ham	Low: 10^2 CFU/g	5
			Medium: 5×10^3 CFU/g	5
			High: 10^5 CFU/g	5
		Pork liver pâté	Low: 10^2 CFU/g	5
			Medium: 5×10^3 CFU/g	5
			High: 10^5 CFU/g	5
Multicomponent foods	*B. thuringiensis* CRA 1744	Sandwiches	Low: 10^2 CFU/g	5
			Medium: 5×10^3 CFU/g	5
			High: 5×10^5 CFU/g	5
		Pasta salad	Low: 10^2 CFU/g	5
			Medium: 5×10^3 CFU/g	5
			High: 5×10^5 CFU/g	5

[a] CRA = Campden Culture Collection (Campden BRI, Chipping Campden, UK).

The difference of mean (DOM) log10 CFU/g values with confidence intervals between the candidate and reference methods are also shown in Table 4. DOM values were between -0.43 (90% CI -0.57, -0.3) log10 CFU/g for pork liver pâté at the medium level and 0.29 (90% CI -0.06, 0.63) log10 CFU/g for sandwiches at the low level. The bias appears random (both positive and negative values) rather than systematic (all positive or all negative values). In 2020, AOAC proposed draft standard method performance requirements (SMPRs®) for quantitative microbiological methods (8). According to the SMPR, to demonstrate method equivalence, the 90% confidence interval on the DOM must be within (-0.5, 0.5). Applying this criterion, the two materials at the extremes of DOM just mentioned are the only two materials not meeting this requirement. One of those materials is the low level of sandwiches with DOM 0.29 (90% CI -0.06, 0.63) for which the CompactDry method had very good repeatability and the reference method had poor repeatability. The other material is the medium level of pork liver pâté with DOM -0.43 (90% CI -0.57, -0.30), for which the 90% CI was just barely outside the (-0.5, 0.5) 90% confidence interval range. The remaining 28 materials demonstrate statistical equivalence of the CompactDry "Nissui" BC and ISO 7932:2004 methods.

Method Developer Studies

Robustness Study

This study evaluates the ability of the method to remain unaffected by small variations in method parameters that might be expected to occur when the method is performed by an end user. The parameters varied included sample volume (0.95–1.05 mL), incubation temperature (28–32°C), and incubation time (22–26 h). The study followed a factorial design, and each treatment combination was evaluated with five replicates each of *Bacillus cereus* at ~10^6 CFU/mL as a high level and at ~10^2 CFU/mL as a low level and *Bacillus circulans* at ~10^6 CFU/mL as a negative control.

B. cereus (ATCC 14579) and *B. circulans* (ATCC 4513) were cultured in 10 mL SCD (Soybean Casein Digest) broth and incubated 24 h at 30°C. Each culture was diluted with sterilized Peptone salt solution to the above concentrations.

Robustness results are summarized in Table 5. The *B. circulans* negative control replicates showed no growth under any conditions. The *B. cereus* high level mean values ranged from 7.08 $\log_{10}$ to 7.20 $\log_{10}$ in treatment combinations 1–8 compared to 7.11 $\log_{10}$ for treatment combination 9 using nominal conditions. At the low level, the mean values from treatment combinations 1–8 varied from 1.68 to 1.78 $\log_{10}$ compared to 1.73 $\log_{10}$ for the nominal mean result. Thus, none of the mean values for treatment combinations 1–8 varied more than one standard deviation from the nominal result at either concentration of *B. cereus*; therefore, none of the method parameter variations caused a significant effect.

Product Consistency and Stability Study

Three retained lots of CompactDry "Nissui" BC product stored under recommended conditions (1–30°C) that were 1, 12, and

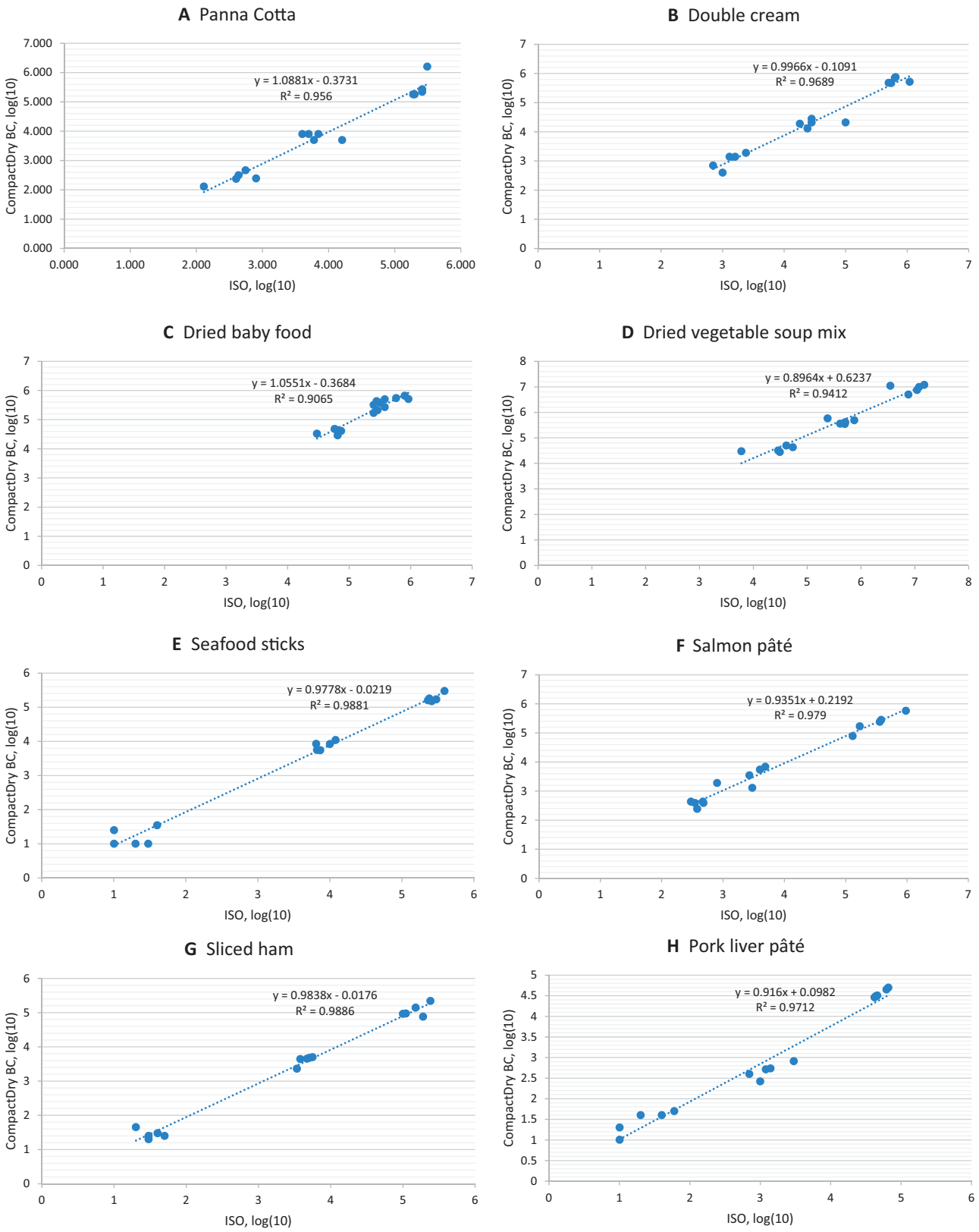

Figure 1. Plots of candidate method results verses reference method results for each food matrix.

18 months past the manufacturing date were compared. Cultures were prepared as for the robustness study, and five replicates of each culture were tested on each lot of product.

Results are shown in Table 6. The *B. circulans* (ATCC 4513) negative control replicates showed no growth for any of the lots. The *B. cereus* (ATCC 14579) replicates yielded similar mean

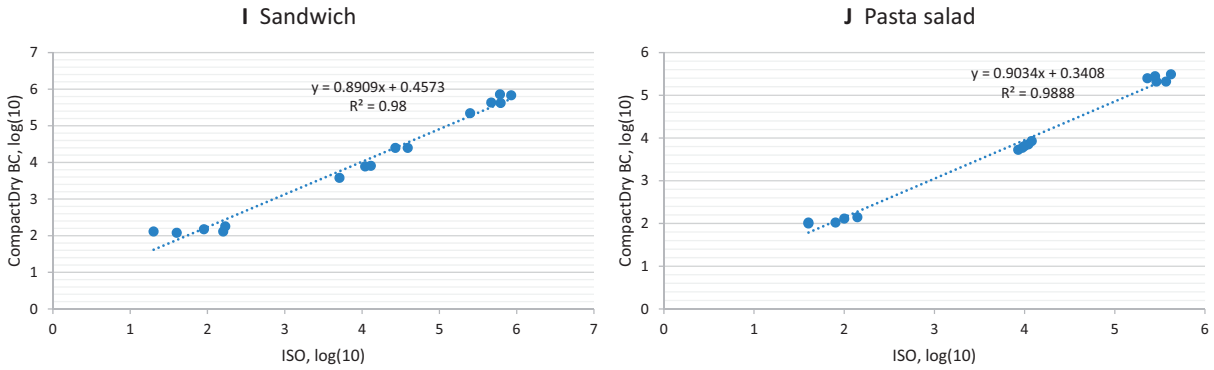

Figure 1. (Continued).

Table 4. Method comparison data summary and statistics

			CompactDry "Nissui" BC		ISO 7932:2004			95% CI[e]		90% CI	
Matrix	Contamination level	n[a]	Mean $\log_{10}$ CFU/g[b]	s_r[c]	Mean $\log_{10}$ CFU/g	s_r	DOM[d]	LCL[f]	UCL[g]	LCL	UCL
Panna cotta	Low	5	2.41	0.202	2.61	0.296	−0.19	−0.44	0.06	−0.38	0.00
	Medium	5	3.82	0.112	3.83	0.230	0.00	−0.40	0.39	−0.31	0.30
	High	5	5.50	0.402	5.38	0.089	0.12	−0.30	0.53	−0.20	0.44
Double cream	Low	5	3.00	0.275	3.11	0.203	−0.11	−0.32	0.11	−0.27	0.06
	Medium	5	4.30	0.120	4.51	0.287	−0.21	−0.57	0.15	−0.48	0.06
	High	5	5.76	0.097	5.82	0.133	−0.06	−0.26	0.14	−0.21	0.09
Dried baby food	Low	5	4.58	0.091	4.74	0.154	−0.16	−0.35	0.03	−0.31	−0.02
	Medium	5	5.68	0.147	5.76	0.178	−0.08	−0.25	0.10	−0.21	0.06
	High	5	5.45	0.172	5.44	0.046	0.01	−0.18	0.21	−0.14	0.16
Dried vegetable soup mix	Low	5	4.55	0.108	4.42	0.372	0.14	−0.26	0.54	−0.17	0.44
	Medium	5	5.63	0.093	5.66	0.181	−0.02	−0.31	−0.27	−0.24	0.20
	High	5	6.94	0.154	6.94	0.248	−0.01	−0.36	0.35	−0.28	0.27
Seafood sticks	Low	5	1.19	0.262	1.28	0.272	−0.09	−0.50	0.32	−0.40	0.23
	Medium	5	3.88	0.130	3.92	0.119	−0.04	−0.16	0.08	−0.13	0.05
	High	5	5.27	0.120	5.45	0.093	−0.18	−0.25	−0.10	−0.24	−0.12
Salmon pâté	Low	5	2.57	0.104	2.59	0.087	−0.02	−0.19	0.15	−0.15	0.11
	Medium	5	3.50	0.305	3.42	0.307	0.08	−0.25	0.42	−0.18	0.34
	High	5	5.34	0.318	5.49	0.340	−0.15	−0.26	−0.04	−0.24	−0.06
Sliced ham	Low	5	1.45	0.131	1.51	0.150	−0.07	−0.37	0.24	−0.30	0.17
	Medium	5	3.61	0.139	3.65	0.090	−0.04	−0.15	0.06	−0.12	0.04
	High	5	5.06	0.184	5.18	0.159	−0.11	−0.31	0.08	−0.27	0.04
Pork liver pâté	Low	5	1.44	0.287	1.34	0.349	0.10	−0.12	0.33	−0.07	0.28
	Medium	5	2.68	0.181	3.11	0.234	−0.43	−0.61	−0.26	−0.57	−0.30
	High	5	4.56	0.106	4.71	0.091	−0.15	−0.17	−0.13	−0.16	−0.13
Sandwiches	Low	5	2.15	0.070	1.86	0.400	0.29	−0.16	0.74	−0.06	0.63
	Medium	5	4.04	0.356	4.18	0.346	−0.14	−0.23	−0.06	−0.21	−0.08
	High	5	5.66	0.207	5.72	0.200	−0.06	−0.17	0.05	−0.14	0.03
Pasta salad	Low	5	2.06	0.065	1.85	0.243	0.21	−0.02	0.44	0.03	0.39
	Medium	5	3.82	0.078	4.01	0.058	−0.19	−0.22	−0.16	−0.21	−0.17
	High	5	5.40	0.075	5.49	0.104	−0.10	−0.24	0.05	−0.21	0.01

[a] n = Number of replicate test portions.
[b] CFU = Colony-forming units.
[c] s_r = Standard deviation of repeatability.
[d] DOM = Difference of means.
[e] CI = Confidence interval for DOM.
[f] LCL = Lower confidence limit for DOM.
[g] UCL = Upper confidence limit for DOM.

values for each of the three lots, and ANOVA analysis demonstrated no statistical difference between lots at the 5% level ($P > 0.05$). Thus, the combined study demonstrates both lot-to-lot repeatability and product stability over the 18-month shelf life.

Discussion

The results of these studies indicate that the CompactDry "Nissui" BC method can be used for rapid and accurate enumeration of *Bacillus cereus* in a variety of food commodities,

Table 5. Robustness testing of CompactDry "Nissui" BC

Treatment combination	Sample volume	Incubation temperature	Incubation time	*B. cereus* High level[a]		*B. cereus* Low level[a]		*B. circulans* High level[a]	
				Mean $\log_{10}$	s_r[b]	Mean $\log_{10}$	s_r	Mean $\log_{10}$	s_r
1	0.95 mL	28°C	22 h	7.08	0.10	1.72	0.07	NG[c]	NG
2	0.95 mL	28°C	26 h	7.10	0.08	1.72	0.04	NG	NG
3	0.95 mL	32°C	22 h	7.18	0.08	1.71	0.03	NG	NG
4	0.95 mL	32°C	26 h	7.15	0.10	1.73	0.10	NG	NG
5	1.05 mL	28°C	22 h	7.20	0.05	1.78	0.05	NG	NG
6	1.05 mL	28°C	26 h	7.20	0.10	1.73	0.07	NG	NG
7	1.05 mL	32°C	22 h	7.18	0.13	1.76	0.03	NG	NG
8	1.05 mL	32°C	26 h	7.16	0.06	1.68	0.04	NG	NG
9 (nominal)	1.00 mL	30°C	24 h	7.11	0.13	1.73	0.07	NG	NG

[a] Five replicates tested.
[b] s_r = Standard deviation.
[c] NG = No growth.

Table 6. Product consistency and stability for the CompactDry "Nissui" BC

Lot	Time post-manufacturing (months)	*B. cereus* High level[a]		*B. cereus* Low level[a]		*B. circulans* High level[a]	
		Mean $\log_{10}$	s_r[b]	Mean $\log_{10}$	s_r	Mean $\log_{10}$	s_r
A	1	6.62	0.02	1.75	0.04	NG[c]	NG
B	12	6.60	0.02	1.79	0.03	NG	NG
C	18	6.60	0.03	1.80	0.06	NG	NG
ANOVA *P*-value		0.133		0.287		NA[d]	

[a] Five replicates tested.
[b] s_r = Standard deviation.
[c] NG = No growth.
[d] NA = Not applicable.

including panna cotta, double cream, dried baby food, dried vegetable soup mix, seafood sticks, salmon pâté, sliced ham, pork liver pâté, sandwich, and pasta salad. The CompactDry "Nissui" BC method shows similar repeatability to the ISO 7932:2004 reference method and equivalent mean results.

The inclusivity and exclusivity results showed selectivity of the CompactDry "Nissui" BC with 47 of 50 inclusivity strains positive and 28 of 33 exclusivity strains negative. These results were similar on the reference method agar.

The CompactDry method was shown to be robust to changes in sample volume, incubation temperature, and incubation time. While the data demonstrated no significant difference when incubation temperature varied between 28 and 32°C and incubation time varied between 22 and 26 h, for best results it is recommended to incubate at 30 ± 1°C for 24 ± 2 h. The product consistency and stability study demonstrated no significant lot-to-lot variation or loss of performance over 18-month shelf life.

The CompactDry "Nissui" BC method eliminates unstable and variable steps over the reference method. There is a reduction in the amount of technical labor required in preparation of agar, and there is no need for confirmation procedures. There are additional advantages in reduction of storage space, waste disposal, and required incubator space.

Conclusions

The CompactDry "Nissui" BC method for enumeration of *B. cereus* was shown to be equivalent to the ISO 7932:2004 reference method for the 10 claimed matrixes and therefore should be certified as an AOAC *Performance Tested Method*SM.

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Conflict of Interest

None declared.

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