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Research Article

MICROBIOLOGICAL METHODS

Validation of the 3M™ Petrifilm™ Coliform Count Plate for Enumeration of Coliforms in Bottled Water: AOAC *Performance Tested Method*℠ 082101

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Abstract

Background: The 3M™ Petrifilm™ Coliform Count (CC) Plate is a sample-ready-culture medium system which contains modified Violet Red Bile nutrients, a cold-water-soluble gelling agent, and a tetrazolium indicator that facilitates colony enumeration.
Objective: To validate the 3M Petrifilm CC Plate method for bottled water through the AOAC® *Performance Tested Method(s)*℠ program.
Methods: The performance of the 3M Petrifilm CC Plate was compared to the U.S. Food and Drug Administration *Bacteriological Analytical Manual* Chapter 4, Section III.D for the enumeration of coliforms in bottled water. Matrix data were normalized by $\log_{10}$ transformation and performance indicators included repeatability and difference of means with 90 and 95% confidence intervals. Inclusivity, exclusivity, robustness, and product consistency and stability were evaluated.
Results: This study demonstrated that the 3M Petrifilm CC Plate method detects and enumerates coliforms from bottled water. The average $\log_{10}$ counts of the 3M Petrifilm CC Plate method were equivalent to or better than the average $\log_{10}$ counts of the reference method. Results from inclusivity and exclusivity studies demonstrated that the 3M Petrifilm CC Plate method differentiated coliforms from non-coliform strains. In product consistency, stability, and robustness testing, different lots of 3M Petrifilm CC Plates and small deviations in incubation time and temperature did not affect test results.
Conclusion: The 3M Petrifilm CC Plate method is an accurate and robust method for the enumeration of coliforms in bottled water.
Highlights: The 3M Petrifilm CC Plate allows for detection of confirmed coliforms within 24 h. Up to 20 sample-ready plates can be stacked during incubation, providing flexibility and saving space.

General Information

Bottled water consumption is increasing worldwide. In the United States, 14.4 billion gallons of bottled water were consumed in 2019 (1). Potable water is regulated by the US Environmental Protection Agency; however bottled water is legally classified as food in the United States and regulated by the US Food and Drug Administration (FDA; 21 CFR 165.110 [b]). "Coliform organisms are

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not necessarily pathogens and are rarely found in bottled water; however, they serve as an indicator of insanitation or possible contamination. Surveys have shown that coliforms are useful indicators of bottled water quality, but some countries also monitor additional microbial populations as indicators of bottle water quality" (2). The 3M™ Petrifilm™ Coliform Count (CC) Plate method was developed to provide rapid and quantitative coliform results from food, beverages, and bottled water samples. Incorporating the use of a mixed cellulose ester (MCE) filter for bottled water samples, the 3M Petrifilm CC Plate method is reproducible, tests large volumes (100 mL), and is faster than the most probable number and agar methods, providing confirmed coliform results in 24 h. 3M Petrifilm CC Plates have previously received AOAC *Official Methods of Analysis*℠ final action status for milk (method **986.33**; 3), dairy products (method **989.10**; 4), and foods (method **991.14**; 5).

Principle of the Method

The 3M Petrifilm CC Plate is a sample-ready-culture medium system which contains modified Violet Red Bile nutrients, a cold-water-soluble gelling agent, and a tetrazolium indicator that facilitates colony enumeration. 3M Petrifilm CC Plates are used for the enumeration of coliforms in the food and beverage and bottled water industries.

Scope of Method

(a) *Analytes*.—Coliforms.
(b) *Matrixes*.—Milk, dairy products, foods, and bottled water: purified water, treated spring water, non-carbonated natural spring water, carbonated natural spring water.
(c) *Summary of validated performance claims*.—No statistical difference detected compared to the FDA *Bacteriological Analytical Manual* (BAM) Chapter 4, Section III for the enumeration of coliform count in bottled water (2) or FDA BAM Chapter 4, Section I (2).

Definitions

(a) *Repeatability (s_r)*.—Precision where independent test results are obtained with the same method on equivalent test items in the same laboratory by the same operator using the same equipment within a short interval of time.
(b) *Difference of means (DOM)*.—Difference in $\log_{10}$ of the average results between the candidate and reference method for one level of contamination.
(c) *Confidence interval (CI)*.—A CI displays the probability that a parameter will fall between a pair of values around the mean. CIs are calculated at the 90 and 95% levels.
(d) *Statistical equivalence*.—The acceptance criterion for statistical equivalence is that the 90% CI on the bias between the methods falls within −0.5, 0.5.
(e) *Standard deviation of* s_r.—$s_r = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$
(f) *Relative standard deviation of* s_r *(RSD_r)*.—$RSD_r = [s_r/mean_{cand}] \times 100$.

Materials and Methods

Test Kit Information

(a) *Kit name*.—3M Petrifilm Coliform Count Plate.
(b) *Catalogue numbers*.—6410, 6411, 6416, 6443.
(c) *Ordering information*.—https://www.3m.com/foodsafety.

Test Kit Components

(a) *3M Petrifilm CC Plate*.
(b) *3M Petrifilm Spreader*.

Additional Supplies and Reagents

(a) *47 mm, 0.45 μ pore size MCE filter*.
(b) *Diluents*.—Butterfield's phosphate buffer, sterile irrigation-water (VWR catalogue # 76353-870 or equivalent), sterile distilled water, sterile deionized (DI) water, and sterile reverse osmosis (RO) water.

Apparatus

(a) *Pipets*.—Capable of delivering 1.0 mL.
(b) *Incubator*.—Capable of maintaining at 35 ± 1°C.
(c) *Vacuum filtration manifold or equivalent filtration equipment*.

Reference Materials

(a) *Lauryl tryptose broth (LST) with Durham tube*.
(b) *Brilliant green bile lactose broth (BGLB) with Durham tube*.

Safety Precautions

The user should read, understand, and follow all safety information in the instructions for the 3M Petrifilm CC Plate. Retain the safety instructions for future reference.

(a) To reduce the risks associated with exposure to biohazards and environmental contamination, follow current industry standards and local regulations for disposal of biohazardous waste.
(b) To reduce the risks associated with release of contaminated product, follow all product storage instruction contained in the instructions for use. Do not use beyond the expiration date.
(c) To reduce the risks associated with bacterial infection and workplace contamination, perform 3M Petrifilm CC Plate testing in a properly equipped laboratory under the control of a skilled microbiologist. Users must be trained in current proper testing techniques: for example, Good Laboratory Practices (6), ISO 7218, or ISO 17025.

Other Precautions

(a) To reduce the risks associated with misinterpretation of results, 3M has not documented 3M Petrifilm CC Plates for use in industries other than food and beverage including bottled water. For example, 3M has not documented 3M Petrifilm CC Plates for testing pharmaceuticals or cosmetics. 3M has not documented 3M Petrifilm CC Plates for testing surface and municipal waters, or waters used in the pharmaceutical or cosmetic industries. The use of 3M

Petrifilm CC Plates to test water samples in compliance with local water testing regulations is at the sole discretion and responsibility of the end user. 3M Petrifilm CC Plates have not been tested with all possible bottled water samples, testing protocols, or with all possible strains of microorganisms.

(b) Do not use the 3M Petrifilm CC Plates in the diagnosis of conditions in humans or animals.

(c) 3M Petrifilm CC Plates do not differentiate any one coliform strain from another.

(d) Foods with high sugar content may increase the potential for gas production from non-coliform *Enterobacteriaceae*.

(e) Consult the Safety Data Sheet for additional information.

General Preparation

Use proper aseptic techniques. Use proper precautions for biosafety level 2 microorganisms.

Store unopened packages at ≤8°C (≤46°F). Use before expiration date on package. In areas of high humidity where condensate may be an issue, it is best to allow packages to reach room temperature before opening.

Sample Preparation

3M Petrifilm CC Plate assay—bottled water samples.—

(a) Place the 3M Petrifilm CC Plate on a flat, level surface.

(b) Lift the top film and dispense 1 mL of an appropriate sterile hydration diluent onto the center of bottom film. Appropriate sterile hydration diluents include distilled water, DI water, and RO water.

(c) Roll the top film down onto the sample to prevent trapping air bubbles.

(d) Place the 3M Petrifilm Spreader with the flat side down on the center of the plate. Press gently on the center of the spreader to distribute the diluent evenly. Spread the diluent over the entire 3M Petrifilm Plate growth area before the gel is formed. Do not slide the spreader across the film.

(e) Remove the spreader and allow the plates to remain closed for a minimum of 1 h before use.

(f) Store hydrated 3M Petrifilm CC Plates in a sealed pouch or plastic bag. Protect plates from light and refrigerate at 2–8°C (36–46°F) for up to 7 days.

(g) Following standard procedures for water analysis, membrane filter water sample using a 47 mm, 0.45 μ pore size MCE filter.

(h) Carefully lift the top film of the 3M Petrifilm CC Plate. Avoid touching the circular growth area. Place the filter in the center of the hydrated area. Minimize trapping bubbles under the filter.

(i) Slowly roll top film onto the filter. Minimize trapping air bubbles and creating gaps between the filter and the 3M Petrifilm CC Plate.

(j) Lightly apply pressure by using the 3M Petrifilm Plate spreader or sliding a finger lightly across the entire disk area (including edges) to ensure uniform contact of the filter with the gel and to eliminate any air bubbles.

(k) Incubate 3M Petrifilm CC Plates at 35 ± 1°C for 24 ± 2 h or 36 ± 1°C for 24 ± 2 h in a horizontal position with the clear side up in stacks of no more than 20.

Interpretation of the 3M Petrifilm CC Plate.—

(a) 3M Petrifilm CC Plates can be counted using a standard colony counter or other illuminated magnifier. Do not count colonies on the foam dam since they are removed from the selective influence of the medium. Do not count artifact bubbles that may be present.

(b) Red colonies associated with gas bubbles are counted as coliforms. Gas bubbles may form a circular or star-shaped pattern around the colony. Gas produced by coliforms may disrupt the colony so that the colony "outlines" the bubble. This should be counted as a single coliform. Red colonies without closely associated gas bubbles may be coliforms and should be picked and tested with appropriate confirmation methods (2, 7). When colonies are present in large numbers, 3M Petrifilm CC Plates will have a deepening of the gel color associated with many small, indistinct colonies or gas bubbles. When this occurs, record results as too numerous to count (TNTC). *Note: Delayed counting of 3M Petrifilm CC Plates with filters is not recommended.*

(c) Colonies may be isolated for further identification. Lift the top film and pick the colony from the gel or the filter surface. When lifting the top film, the filter may adhere to either the top film or the bottom film. If the filter adheres to the top film, separate the filter from the top film and pick colonies. Test using standard procedures.

(d) For further information, refer to the appropriate 3M Petrifilm CC Plate Interpretation Guide. If you have questions about specific applications or procedures, please visit our website at www.3M.com/foodsafety or contact your local 3M representative or distributor.

Confirmation.—

(a) No confirmation is required for red colonies associated with gas bubbles on 3M Petrifilm CC Plates.

(b) To confirm red colonies without closely associated gas bubbles, lift top film of 3M Petrifilm CC Plate and pick at least 10 representative coliform colonies and transfer each to a tube of LST and incubate tubes at 35 ± 0.5°C for 48 h. Subculture any gas positive LST tubes to BGLB and incubate at 35 ± 0.5°C for 48 h. Gas production in BGLB within 48 h is a confirmed coliform test (2). Report results as number of coliform colonies per 100 mL.

Validation Study

This validation study was conducted under the AOAC Research Institute *Performance Tested Method(s)* program and the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* (8). Method developer studies were conducted in the laboratories of 3M Company and included a subset of the inclusivity study and matrix study for one of the claimed matrixes. The independent laboratory study was conducted by SGS Vanguard Sciences and included a matrix study for three of the claimed matrixes, inclusivity/exclusivity study, product consistency and stability studies, and robustness testing.

Inclusivity/Exclusivity Study

Methodology.—Inclusivity organisms (Table 1) and exclusivity organisms (Table 2) were cultured in non-selective broth at 35 ± 1°C for 24 ± 2 h or incubation temperature and time appropriate for organism growth. The organisms were then decimally

Table 1. Inclusivity results for 3M Petrifilm CC Plates—bottled water method

Number	Organism	Source	Origin	Results[a]	Confirmation[b]
1	*Citrobacter braakii*	ATCC 51113	Snake	G, G–	+
2	*Citrobacter braakii*	ATCC 43162	Clinical	G, G–	+
3	*Citrobacter freundii*	ATCC 8090	Unknown	G, G–	+
4	*Citrobacter freundii*	ATCC 43864	Unknown	G, G+	NA
5	*Cronobacter muytjensii*	ATCC 51329	Unknown	G, G+	NA
6	*Cronobacter sakazakii*	ATCC 29544	Clinical, child's throat	G, G+	NA
7	*Enterobacter aerogenes*[c]	U37[d]	Unknown	G, G+	NA
8	*Enterobacter aerogenes*	ATCC 13048	Clinical, sputum	G, G+	NA
9	*Enterobacter aerogenes*	ATCC51697	Unknown	G, G+	NA
10	*Enterobacter amnigenus*[c]	ATCC 51816	Milk	G, G+	NA
11	*Enterobacter amnigenus*	ATCC 51818	Milk	G, G+	NA
12	*Enterobacter cancerogenus*	ATCC 49817	Poplar	G, G+	NA
13	*Enterobacter cloacae*	ATCC 23355	Unknown	G, G+	NA
14	*Enterobacter cloacae*	ATCC 35030	Unknown	G, G+	NA
15	*Enterobacter cloacae*	ATCC 13047	Clinical, spinal fluid	G, G+	NA
16	*Enterobacter cloacae*	NCTC 13464[e]	Unknown	G, G+	NA
17	*Enterobacter hormaechei*	ATCC 700323	Unknown	G, G+	NA
18	*Enterobacter gergoviae*	ATCC 33028	Clinical, urine	G, G+	NA
19	*Escherichia coli*	ATCC 51813	Food, MN	G, G+	NA
20	*Escherichia coli*	ATCC 11229	Unknown	G, G+	NA
21	*Escherichia coli*	NCTC 13216	Unknown	G, G+	NA
22	*Escherichia coli*	3M PWW4[d]	Wastewater	G, G+	NA
23	*Escherichia coli*	3M FR1[d]	Food	G, G+	NA
24	*Escherichia coli*	AFLCC[g]	Food	G, G+	NA
25	*Escherichia coli*	ATCC 35218	Clinical, canine	G, G+	NA
26	*Escherichia coli*	ATCC 11229	Unknown	G, G+	NA
27	*Escherichia coli*	ATCC 25922	Clinical	G, G+	NA
28	*Escherichia coli*	ATCC 8739	Clinical, feces	G, G+	NA
29	*Escherichia coli*[c]	ATCC 10536	Unknown	G, G+	NA
30	*Escherichia coli*[c]	ATCC 14948	Unknown	G, G+	NA
31	*Escherichia coli*[c]	FSD723[d]	Unknown	G, G+	NA
32	*Escherichia coli*[c]	FR2[d]	Unknown	G, G+	NA
33	*Escherichia coli*[c]	ATCC 35218	Canine	G, G+	NA
34	*Escherichia vulneris*	ATCC 33821	Clinical, wound	G, G+	NA
35	*Hafnia alvei*	ATCC 51815	Milk	G, G+	NA
36	*Klebsiella aerogenes*	ATCC 35029	Unknown	G, G+	NA
38	*Klebsiella aerogenes*	NCIMB[f] 10102	Clinical, sputum	G, G+	NA
39	*Klebsiella oxytoca*	ATCC 43165	Clinical	G, G+	NA
40	*Klebsiella oxytoca*[c]	ATCC 43086	Unknown	G, G+	NA
41	*Klebsiella oxytoca*	ATCC 13182	Clinical, tonsil	G, G+	NA
42	*Klebsiella oxytoca*[c]	ATCC 700324	Unknown	G, G+	NA
43	*Klebsiella oxytoca*[c]	U33[d]	Unknown	G, G+	NA
44	*Klebsiella oxytoca*[c]	ATCC 8724	Unknown	G, G+	NA
45	*Klebsiella oxytoca*[c]	ATCC 51817	Milk	G, G+	NA
46	*Klebsiella pneumoniae*	ATCC 4352	Cow's milk	G, G+	NA
47	*Klebsiella pneumoniae*[c]	ATCC 23357	Cow's milk	G, G+	NA
48	*Klebsiella pneumoniae*[c]	U29[d]	Unknown	G, G+	NA
49	*Klebsiella pneumoniae*[c]	U28[d]	Unknown	G, G+	NA
50	*Kluyvera georgiana*	ATCC 51603	Clinical, human sputum	G, G+	NA

[a] Results on 3M Petrifilm CC Plates for growth with gas is indicated by G, G+ and growth with no gas is indicated by G, G–.
[b] The confirmation method was completed and the strain was confirmed as a coliform (+) or was not confirmed as a coliform (–). No confirmation steps were completed (NA) because the coliform result was confirmed by the presence of gas on the 3M Petrifilm CC Plate.
[c] Strain tested by 3M Company Laboratory.
[d] 3M Company Laboratory Culture Collection.
[e] NCTC = National Collection of Type Cultures.
[f] NCIMB = National Collection of Industrial Food and Marine Bacteria.
[g] SGS Vanguard Sciences, Inc Culture Collection.

diluted in sterile irrigation water to 100 colony-forming units (CFU)/100 mL for the inclusivity organisms and 1000 CFU/100 mL for the exclusivity organisms.

All organisms were randomized and blind-coded prior to testing. The standard procedures for water analysis were followed with the membrane filtration of the 100 mL samples

Table 2. Exclusivity results for 3M Petrifilm CC Plates—bottled water method

Number	Organism	Source	Origin	Results[a]	Confirmation[b]
1	*Acinetobacter baumanii*	ATCC 19606	Urine	–	NA
2	*Aeromonas hydrophila*	ATCC 35654	Unknown	G, G–	–
3	*Alcaligenes faecalis*	ATCC 8750	Unknown	G, G–	–
4	*Bacillus cereus*	ATCC 11778	Unknown	–	NA
5	*Bacillus subtilis*	ATCC 27370	Unknown	G, G–	–
6	*Bordetella parapertussis*	ATCC 15311	Clinical	NG	NA
7	*Carnobacterium maltaromaticum*	ATCC 27865	Raw milk	NG	NA
8	*Edwardsiella tarda*	ATCC 15947	Clinical, feces	G, G–	–
9	*Enterococcus faecalis*	ATCC 14506	Unknown	G, G–	–
10	*Enterococcus faecium*	NRRL B2354[c]	Dairy utensils	G, G–	–
11	*Haemophilus influenzae*	ATCC 35056	Clinical	G, G–	–
12	*Lactococcus garvieae*	ATCC 43921	Mastitis	G, G–	–
13	*Leuconostoc mesenteroides*	ATCC 8293	Olives	G, G–	–
14	*Listeria innocua*	ATCC 33090	Cow brain	G, G–	–
15	*Listeria ivanovii*	ATCC 19119	Sheep	G, G–	–
16	*Listeria monocytogenes*	ATCC 19115	Clinical	G, G–	–
17	*Micrococcus luteus*	NCIMB 8166[d]	Air	G, G–	–
18	*Microbacterium testaceum*	ATCC 15829	Paddy	G, G–	–
19	*Proteus mirabilis*	ATCC 25933	Clinical	–	NA
20	*Proteus vulgaris*	ATCC 6380	Unknown	–	NA
21	*Pseudomonas aeruginosa*	ATCC 15442	Animal room water bottle	G, G–	–
22	*Salmonella* Anatum	ATCC 9270	Pork liver	–	NA
23	*Salmonella* Typhimurium	ATCC 13311	Clinical, feces	G, G–	–
24	*Shigella flexneri*	ATCC 9199	Unknown	–	NA
25	*Shigella sonnei*	ATCC 25931	Clinical, feces	–	NA
26	*Shigella boydii*	ATCC 9207	Unknown	–	NA
27	*Staphylococcus aureus*	ATCC 6538	Clinical	G, G–	–
28	*Streptococcus pyogenes*	ATCC 12384	Unknown	–	NA
29	*Vibrio parahaemolyticus*	NCTC 10885[e]	Oyster	G, G–	–
30	*Yersinia enterocolitica*	ATCC 23715	Clinical, eye	–	NA

[a] Results on 3M Petrifilm CC Plates with no growth (–) and growth with no gas (G, G–).
[b] The confirmation method was completed and the strain was not confirmed as a coliform (–). No confirmation steps were completed (NA) because there was no growth on the 3M Petrifilm CC Plate.
[c] NRRL = Agricultural Research Service Culture Collection.
[d] NCIMB = National Collection of Industrial Food and Marine Bacteria.
[e] NCTC = National Collection of Type Cultures.

using a 47 mm, 0.45 μ pore size MCE filter. The filter was placed on the 3M Petrifilm CC Plate as indicated in the instructions for use. The plates were incubated at 35 ± 1°C for 24 ± 2 h. After incubation, the plates were observed for red colonies associated with gas bubbles. Red colonies not associated with a gas bubble were subcultured and tested with the appropriate confirmation method.

Results.—Results from the inclusivity testing and exclusivity testing conducted at SGS Vanguard Sciences, Inc. and 3M Company were combined and are presented in Table 1 (inclusivity) and Table 2 (exclusivity). Of the 50 coliform inclusivity isolates tested, 47 out of 50 isolates had typical coliform growth (red colonies associated with gas) on 3M Petrifilm CC Plates. Three *Citrobacter* strains [*C. braakii* American Type Culture Collection® (ATCC) 51113, *C. braakii* ATCC 43162, and *C. freundii* ATCC 8090) had atypical coliform growth (red colonies without associated gas) on 3M Petrifilm CC Plates. Per the product instructions, red colonies without closely associated gas bubbles from bottled water samples may be coliforms and should be confirmed per FDA BAM chapter 4, Section III.D. A subset of these colonies was subcultured and confirmed to be coliforms. All 30 exclusivity strains had no growth or atypical growth (red colonies without associated gas) that did not confirm as coliforms per the confirmation method (18 strains had no growth and 12 strains had atypical growth).

Matrix Study

Methodology.—For the method comparison analysis four bottled water samples were analyzed: purified water, treated spring water, non-carbonated natural spring water, and carbonated natural spring water. All bottled water samples utilized for testing were tested for coliforms prior to inoculation and had results of <1 CFU/100 mL. The bottled water samples were then inoculated at low, medium, and high levels with the organisms listed in Table 3. Purified water samples were prepared and tested by 3M Company. Individual colonies of *Enterobacter cloacae* (ATCC 13047) were grown in tryptic soy broth (TSB) overnight at 35 ± 1°C. Serial dilutions were prepared in tubes of sterile irrigation water diluent to achieve an inoculation level of 1–50, 51–100, or 101–150 CFU/100 mL. There were five paired samples inoculated for each inoculation level. The inoculated samples were equilibrated for 48–72 h at 2–8°C. There was an additional set of bottle water samples that remained uninoculated and were tested for coliform count.

Bottled water samples of treated spring water, non-carbonated natural spring water, and carbonated natural spring

Table 3. Matrix preparation

Matrix	Inoculation organism	Inoculum condition	Storage conditions	Contamination level, CFU/100 mL	Replicates per method	Test portions per replicate per method, mL
Carbonated natural spring water	*Citrobacter freundii* (ATCC 8090)	Unstressed culture	48–72 h at 2–8°C	0	5	100
				1–50	5	
				51–100	5	
				101–150	5	
Non-carbonated natural spring water	*Klebsiella pneumonia* (ATCC 4352)	Unstressed culture	48–72 h at 2–8°C	0	5	100
				1–50	5	
				51–100	5	
				101–150	5	
Treated spring water	*E. coli* (ATCC 8739)	Unstressed culture	48–72 h at 2–8°C	0	5	100
				1–50	5	
				51–100	5	
				101–150	5	
Purified water	*Enterobacter cloacae* (ATCC 13047)	Unstressed culture	48–72 h at 2–8°C	0	5	100
				1–50	5	
				51–100	5	
				101–150	5	

water samples were prepared by SGS Vanguard Sciences, Inc. Individual colonies of *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 4352), and *C. freundii* (ATCC 8090) were grown in brain heart infusion broth (BHI) overnight at 35–37°C. The cultures were stored at 2–8°C for 24 h while the inoculum level was determined. Serial dilutions were prepared in tubes of sterile distilled water diluent to achieve an inoculation level of 1–50, 51–100, or 101–150 CFU/100 mL. There were 10 samples inoculated for each inoculation level. The inoculated samples were equilibrated for 48–72 h at 2–8°C. There was an additional set of bottle water samples that remained uninoculated and were tested for aerobic plate count and coliform count.

Candidate Method

All analyses were performed using paired test portions. Five samples from each inoculation level were randomized and blind-coded prior to testing. The standard procedures for water analysis were followed with the membrane filtration of the 100 mL samples using a 47 mm, 0.45 µ pore size MCE filter. The filter was placed on the 3M Petrifilm CC Plate as indicated in the instructions for use. The plates were incubated at 35 ± 1°C for 24 ± 2 h. After incubation, the plates were observed for red colonies associated with gas bubbles. Any red colonies not associated with a gas bubble were picked and tested according to FDA BAM Chapter 4, Section III.D.

Reference Method

Five samples from each inoculation level were randomized and blind-coded prior to testing per FDA BAM Chapter 4, Section III.D (2). Each 100 mL test portion was filtered using 47 mm, 0.45 µ pore size filter for the enumeration of bacteria. The filter was transferred to LES Endo Agar and incubated at 35 ± 0.5°C for 22–24 h. The number of colonies that were pink to dark red with a green metallic surface sheen were counted. To confirm, if there were 5–10 green sheen colonies on the filter, all colonies were confirmed by inoculating growth from each green sheen colony into tubes of LST and incubated at 35 ± 0.5°C for 48 h. If the number of sheen colonies exceeded 10, 10 representative suspect colonies were randomly selected and confirmed. Any gas positive LST tubes were subcultured to BGLB and incubated at 35 ± 0.5°C for 48 h. Gas production in BGLB within 48 h were considered a confirmed coliform test. If typical colonies were not present, atypical colonies were picked to screen for the presence of atypical reacting coliforms.

Results.—Statistical analysis was conducted for each contamination level comparing the 3M Petrifilm CC Plate method result to the FDA BAM Chapter 4, Section III.D reference method result (LES Endo Agar). For each test portion, results were logarithmically ($\log_{10}$) transformed using the equation CFU/100 mL + 0.1, according to the AOAC statistical workbook (9). After logarithmic ($\log_{10}$) transformations of the counts, DOM with 90 and 95% upper and lower confidence limits, s_r, and RSD_r were determined for each contamination level using the Least Cost Formulations Workbook for Paired Method Analysis for Micro Testing, version 1.2 (9). A mean difference between methods of <0.5 $\log_{10}$ with a 90% CI containing values between −0.5 and 0.5 was used as guidance to determine statistically significant differences between two methods being compared (10). The matrix study data are presented in Table 4.

Carbonated Natural Spring Water

The low inoculation load for carbonated natural spring water had a mean level of coliform bacteria of 1.48 $\log_{10}$ CFU/100 mL with the 3M Petrifilm CC Plate method and 1.72 $\log_{10}$ CFU/100 mL with the FDA BAM method. The medium inoculation load had a mean level of coliform bacteria of 1.82 $\log_{10}$ CFU/100 mL on the 3M Petrifilm CC Plate method and 2.09 $\log_{10}$ CFU/100 mL on the BAM method. The high inoculation load had a mean level of coliform bacteria of 1.91 $\log_{10}$ CFU/100 mL with the 3M Petrifilm CC Plate method and 2.28 $\log_{10}$ CFU/100 mL with the BAM method (Table 4). The colonies were tiny red colonies with no gas associated with the colonies on the 3M Petrifilm CC Plate method and were typical, green metallic colonies on the reference plates. A portion of the colonies were confirmed as coliforms. The methods were statistically equivalent for carbonated natural spring water as the 90 and 95% CIs for the mean difference fell between −0.5 and 0.5 (Table 4).

Table 4. Matrix study: 3M Petrifilm CC Plate—bottled water method versus reference method results

		3M Petrifilm CC Plate			Reference method[c]				90% CI[e]		95% CI[f]	
Matrix	Cont. level[a]	Mean[b]	s_r	RSD_r, %	Mean	s_r	RSD_r, %	DOM[d]	LCL[g]	UCL[h]	LCL	UCL
Carbonated natural	Un[j]	0.000	NA	NA	0.000	NA	NA	NA	NA	NA	NA	NA
spring water	Low	1.48	0.08	5.41	1.72	0.03	1.74	−0.24	−0.31	−0.17	−0.33	−0.15
(*C. freundii* ATCC 8090)	Med	1.82	0.04	2.25	2.09	0.01	0.72	−0.27	−0.30	−0.23	−0.32	−0.22
	High	1.91	0.12	6.28	2.28	0.05	2.02	−0.37	−0.49	−0.26	−0.53	−0.22
Non-carbonated natural	Un[j]	0.000	NA	NA	0.000	NA	NA	NA	NA	NA	NA	NA
spring water	Low	1.17	0.13	11.47	−1.00	0.00	0.00	2.17	2.04	2.30	2.00	2.34
(*K. pneumonia* ATCC	Med	1.79	0.04	2.29	−1.00	0.00	0.00	2.79	2.75	2.89	2.74	2.84
4352)	High	1.99	0.04	1.86	−1.00	0.00	0.00	2.99	2.95	3.02	2.94	3.03
Treated spring water	Un[j]	0.000	NA	NA	0.000	NA	NA	NA	NA	NA	NA	NA
(*E. coli* ATCC 8739)	Low	1.50	0.14	9.1	1.49	0.06	3.95	0.01	−0.14	0.15	−0.18	0.20
	Med	1.78	0.03	1.6	1.99	0.04	1.91	−0.21	−0.26	−0.17	−0.27	−0.15
	High	1.98	0.05	2.3	2.20	0.03	1.32	−0.23	−0.29	−0.16	−0.32	−0.14
Purified water (*E. cloacae*	Un[j]	0.000	NA	NA	0.000	NA	NA	NA	NA	NA	NA	NA
ATCC 13047)[i]	Low	1.11	0.22	0.90	0.99	0.42	42.10	0.13	−0.13	0.39	−0.21	0.47
	Med	1.72	0.49	28.36	1.70	0.33	19.51	0.02	−0.13	0.17	−0.18	0.22
	High	2.23	0.05	2.38	2.06	0.04	1.75	0.17	0.12	0.23	0.10	0.25

[a] All matrixes are artificially contaminated when an uncontaminated (Un) level is reported.
[b] Mean of five replicate portions after logarithmic transformation: $\log_{10}$[CFU/100 mL + (0.1)f].
[c] BAM Ch. 4, Section III.D.
[d] Mean difference between the candidate and reference methods.
[e] 90% CI based on paired statistical analysis. If the confidence internal of the methods does not fall between −0.5 and 0.5, then the methods would not be considered equivalent.
[f] 95% CI based on paired statistical analysis.
[g] Lower confidence limit for DOM.
[h] Upper confidence limit for DOM.
[i] Purified water samples were tested by 3M company.
[j] NA = Not applicable.

Non-Carbonated Natural Spring Water

The non-carbonated natural spring water had a mean level of coliform bacteria of 1.17, 1.79, and 1.99 $\log_{10}$ CFU/100 mL for the low, medium, and high inoculation levels, respectively, with the 3M Petrifilm CC Plate method. The reference method did not have growth at any of the inoculation levels (Table 4). The colonies on the 3M Petrifilm CC Plate were large, red colonies with gas associated with the colonies. A portion of the colonies were confirmed as coliforms. As there was no growth on the reference media plates, the methods were not statistically equivalent for non-carbonated natural spring water as the 90 and 95% CI for the mean difference did not fall between −0.5 and 0.5 (Table 4).

Treated Spring Water

The low inoculation load for treated spring water had a mean level of coliform bacteria of 1.50 $\log_{10}$ CFU/100 mL with the 3M Petrifilm CC Plate method and 1.49 $\log_{10}$ CFU/100 mL with the FDA BAM method. The medium inoculation load had a mean level of coliform bacteria of 1.78 $\log_{10}$ CFU/100 mL on the 3M Petrifilm CC Plate method and 1.99 $\log_{10}$ CFU/100 mL on the BAM method. The high inoculation load had a mean level of coliform bacteria of 1.98 $\log_{10}$ CFU/100 mL with the 3M Petrifilm CC Plate method and 2.20 $\log_{10}$ CFU/100 mL with the BAM method (Table 4). The colonies were large red colonies associated with gas on the 3M Petrifilm CC Plate method for the low inoculation level and smaller red colonies associated with gas on the higher contamination level plates. The colonies were typical, green metallic on the reference plates. A portion of the colonies were confirmed as coliforms. The methods were statistically equivalent for treated spring water as the 90 and 95% CI for the mean difference fell between −0.5 and 0.5 (Table 4).

Purified Water

The low inoculation load for purified water had a mean level of coliform bacteria of 1.11 $\log_{10}$ CFU/100 mL with the 3M Petrifilm CC Plate method and 0.99 $\log_{10}$ CFU/100 mL with the FDA BAM method (Table 4). The medium inoculation load had a mean level of coliform bacteria of 1.72 $\log_{10}$ CFU/100 mL on 3M Petrifilm CC Plates and 1.70 $\log_{10}$ CFU/100 mL on the BAM method. The high inoculation load had a mean level of coliform bacteria of 2.23 $\log_{10}$ CFU/100 mL with the 3M Petrifilm CC Plate method and 2.06 $\log_{10}$ CFU/100 mL with the BAM method. The colonies were red with gas associated with the colonies on the 3M Petrifilm CC Plate method and were typical, green metallic on the reference plates. A portion of the colonies were confirmed as coliforms. The methods were statistically equivalent for purified water as the 90 and 95% CI for the mean difference fell between −0.5 and 0.5 (Table 4).

Product Consistency and Stability

Methodology.—Three lots of 3M Petrifilm CC Plates at the beginning (418320073A; 4 months), middle (418320024C; 7 months), and end (3346CY; 16 months) of the product shelf-life were tested for consistency and stability. The 3M Petrifilm CC Plate has a shelf life of 18 months when stored at less than or equal to 8°C (46°F). One *E. coli* (ATCC 25922) and one S. Typhimurium (ATCC 14028 [non-coliform]) isolate were cultured in BHI broth overnight at 35–37°C. The cultures were stored at 2–8°C for 24 h

Table 5. Consistency and stability of 3M Petrifilm CC Plates—bottled water method

3M Petrifilm CC Plate	Cont. level	Lot 3346CY Exp 9-8-2020 16 months			Lot 418320024C Exp 7-24-2021 7 months			Lot 418320073A Exp 09-11-2021 4 months		
		DOM[a]	90% CI[b]	95% CI	DOM	90% CI	95% CI	DOM	90% CI	95% CI
Lot 3346CY	Non[c]	0.00	NA[d]	NA	0.00	NA	NA	0.00	NA	NA
	Low	NA	NA	NA	0.03	−0.08, 0.13	−0.11, 0.17	0.06	−0.04, 0.17	−0.07, 0.20
	High	NA	NA	NA	0.01	−0.02, 0.05	−0.03, 0.06	0.03	−0.01, 0.08	−0.02, 0.09
Lot 418320024C	Non	0.00	NA	NA	0.00	NA	NA	0.00	NA	NA
	Low	NA	NA	NA	NA	NA	NA	0.04	−0.08, 0.15	−0.11, 0.18
	High	NA	NA	NA	NA	NA	NA	0.02	−0.04, 0.08	−0.05, 0.10

[a] Mean difference between the sample lots.
[b] CI based on paired statistical analysis. If the confidence internal of the methods falls between −0.5 and 0.5, then the methods would be considered equivalent.
[c] Non = Non-coliform inoculated sample. No counts were observed.
[d] NA = Not applicable.

Table 6. Robustness of 3M Petrifilm CC Plates—bottled water method

Incubation parameters	Cont. level	3M Petrifilm CC Plate			Reference condition (35°C, 24 h)			DOM[b]	90% CI[c]		95% CI[d]	
		Mean[a]	s_r	RSD_r, %	Mean	s_r	RSD_r, %		LCL[e]	UCL[f]	LCL	UCL
33°C, 22 h	Un[g]	0.00	NA[h]	NA	0.00	NA	NA	0.00	NA	NA	NA	NA
	Low	0.996	0.097	9.73	0.902	0.135	14.97	−0.094	−0.171	−0.018	−0.194	0.005
	High	1.459	0.088	6.03	1.428	0.076	5.32	−0.032	−0.140	0.076	−0.173	0.109
33°C, 26 h	Un	0.00	NA	NA	0.00	NA	NA	0.00	NA	NA	NA	NA
	Low	0.996	0.097	9.73	0.902	0.135	14.97	−0.094	−0.171	−0.018	−0.194	0.005
	High	1.459	0.088	6.03	1.428	0.076	5.32	−0.032	−0.140	0.076	−0.173	0.109
37°C, 22 h	Un	0.00	NA	NA	0.00	NA	NA	0.00	NA	NA	NA	NA
	Low	0.886	0.184	20.76	0.902	0.135	14.97	−0.110	−0.302	0.083	−0.360	0.141
	High	1.448	0.068	4.70	1.428	0.076	5.32	−0.012	−0.093	0.069	−0.093	0.069
37°C, 26 h	Un	0.00	NA	NA	0.00	NA	NA	0.00	NA	NA	NA	NA
	Low	0.886	0.184	20.76	0.902	0.135	14.97	−0.110	−0.302	0.083	−0.360	0.141
	High	1.451	0.066	4.55	1.428	0.076	5.32	−0.009	−0.091	0.074	−0.116	0.099

[a] Mean of five replicate portions after logarithmic transformation: $\log_{10}$[CFU/100 mL + (0.1)f].
[b] Mean difference between the 3M Petrifilm Plate at different incubation conditions and 3M Petrifilm Plates at nominal incubation conditions.
[c] 90% CI based on paired statistical analysis. If the confidence internal of the methods does not fall between −0.5 and 0.5, then the methods would not be considered equivalent.
[d] 95% CI based on paired statistical analysis.
[e] Lower confidence limit for DOM.
[f] Upper confidence limit for DOM.
[g] Un = Non-coliform inoculated sample. No counts were observed.
[h] NA = Not applicable.

while the inoculum level was determined. Serial dilutions were prepared in tubes of sterile distilled water diluent to achieve an inoculation level of 1–50 or 101–150 CFU/100 mL for the target culture and 101–150 CFU/100 mL for the non-target culture. Samples of purified water were inoculated with *S.* Typhimurium (ATCC 14028) as a non-target organism and *E. coli* (ATCC 25922) as a target organism. Each lot of 3M Petrifilm CC Plates had five replicates of the target culture at the high level, five replicates of the target culture at the low level, and five replicates of non-target culture in a randomized blind coded fashion plated. After incubation, the plates were observed for red colonies associated with gas bubbles. Any red colonies not associated with a gas bubble were picked and tested according to BAM Chapter 4, Section III.D (2).

Results.—The non-target organism was not present on any of the lots of 3M Petrifilm CC Plates (Table 5). The mean of the low inoculated samples was 1.42–1.49 and 1.82–1.86 $\log_{10}$ CFU/100 mL for the high inoculated samples. When the mean difference between the lots of 3M Petrifilm CC Plates was determined, the results for each plate lot were statistically equivalent as the 90 and 95% CIs for the mean difference fell between −0.5 and 0.5 (Table 5).

Robustness

Methodology.—This study evaluated variations in method parameters that might be expected to occur when the 3M Petrifilm CC Plate method is performed by an end user. The effects of perturbations in two method parameters were investigated; incubation temperature and incubation time. Four treatment combinations were evaluated and compared to the nominal test condition: (1) 33°C; 22 h, (2) 33°C; 26 h, (3) 37°C; 22 h, and (4) 37°C; 26 h. The nominal test condition was 34–36°C; 23–25 h. One *E. coli* (ATCC 25922) and one *S.* Typhimurium ATCC 14028 (non-coliform) isolate was cultured in BHI broth overnight at 35–37°C. The cultures were stored at 2–8°C for 24 h while the inoculum level was determined. Serial dilutions were prepared in tubes of sterile distilled water diluent to achieve an inoculation level of 1–50 CFU/100 mL or 101–150 CFU/100 mL for the target culture and 101–150 CFU/100 mL for the non-target culture. Samples of purified water were inoculated with

S. Typhimurium (ATCC 14028) as a non-target organism and *E. coli* (ATCC 25922) as a target organism. Each condition had five replicates of the target culture at the high level, five replicates of the target culture at the low level, and five replicates of non-target in a randomized blind coded fashion plated. After incubation, the plates were observed for red colonies associated with gas bubbles. Any red colonies not associated with a gas bubble were subcultured and tested according to BAM Chapter 4, Section III.D (2).

Results.—The samples that were inoculated with a low level of organisms had mean level of organisms of 0.88–0.99 $\log_{10}$ CFU/100 mL and the high-level samples had a mean level of organisms of 1.43–1.46 $\log_{10}$ CFU/100 mL water (Table 6). When the mean difference between the incubation time and temperatures were determined, the results for each plate lot were statistically equivalent as the 90 and 95% CI for the mean difference fell between −0.5 and 0.5 (Table 6).

Discussion

In the matrix studies conducted in this evaluation, the 3M Petrifilm Coliform Count (CC) Plate method was compared to the FDA/BAM Chapter 4, Section III.D for the enumeration of coliform count in bottled water reference method at 24 h. Four bottled water matrixes—purified water, treated spring water, non-carbonated natural spring water, and carbonated natural spring water—were inoculated with coliforms at low, medium, and high levels. The $\log_{10}$ counts from the 3M Petrifilm CC Plate method were compared with $\log_{10}$ counts from the reference method.

The mean differences of $\log_{10}$ transformed results between the 3M Petrifilm CC Plate and the reference method was less than 0.5 $\log_{10}$ and had a 90% CI containing values between −0.5 and 0.5 for all contamination levels for purified water, treated spring water, and carbonated natural spring water. The CIs were calculated on the mean differences for each comparison and were between −0.5 and 0.5. In fact, the differences were 0.37 $\log_{10}$ lower and also had a 95% CI between −0.5 and 0.5, indicating that the 3M Petrifilm CC Plate is statistically equivalent to the reference method for purified water, treated spring water, and carbonated natural spring water. For the non-carbonated natural spring water, the mean differences of $\log_{10}$ transformed results between the 3M Petrifilm CC Plate and the reference method did not meet the equivalency requirements of less than 0.5 $\log_{10}$ and a 90% CI containing values between −0.5 and 0.5, however, this was due to the fact that *K. pneumonia* (ATCC 4352) was not recovered on the reference media at any of the inoculation levels and the difference in recovery is in favor of the 3M Petrifilm CC Plate method. The root cause of the poor recovery of *K. pneumonia* (ATCC 4352) on the reference method was not determined.

In strain studies, of the 50 coliform inclusivity isolates tested, 47 out of 50 strains had typical coliform growth (red colonies associated with gas) on 3M Petrifilm CC Plates. Three strains—*C. braakii* ATCC 51113, *C. braakii* ATCC 43162, and *C. freundii* ATCC 8090—had atypical coliform growth (red colonies without associated gas) that were confirmed as coliforms per the product instructions. All 30 exclusivity strains had no growth or atypical growth (red colonies without associated gas) that did not confirm as coliforms per the product instructions.

The 3M Petrifilm CC Plate has a shelf life of 18 months when stored at less than or equal to 8°C. A combined lot-to-lot and stability study was conducted on three unique lots of 3M Petrifilm CC Plates at the beginning, middle, and end of the product shelf-life. No difference in recovery or appearance was observed between the 3M Petrifilm CC Plate lots and all lots were statistically equivalent (Table 5).

Robustness testing proved that the performance of the 3M Petrifilm CC Plate was not adversely affected by small variations in key parameters (incubation time and incubation temperature), such that may occur during routine laboratory use (Table 6).

Conclusions

These studies have demonstrated that the 3M Petrifilm CC Plate method is an accurate, specific, sensitive, and rugged method that detects and enumerates coliforms at 24 h from bottled water matrixes, yielding equivalent values to the FDA BAM Chapter 4, Section III.D reference method for the enumeration of coliform count in bottled water.

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Conflict of Interest

None declared.

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