

## **COPYRIGHT NOTICE & PERMISSION TO USE**

3M Food Safety has purchased the right to make AOAC® *Official Method of Analysis* **996.02** <http://www.eoma.aoac.org/> available to you in electronic form on Condition that you accept the following terms of use. Please read and respect these terms of use.

### **Copyright**

AOAC INTERNATIONAL, Rockville, MD, USA owns the copyright of this Official Method publication. All rights are reserved.

### **Permission to Use**

You may download an electronic file of this method publication for the purposes of viewing and/or printing one copy of the method. Neither the electronic file nor a hard copy print may be reproduced. You may not remix, transform, build upon or modify this material in any way for redistribution.

### 17.3.03A

**AOAC Official Method 996.02**  
**Coliform Count**  
**in Dairy Products**  
**High-Sensitivity Dry Rehydratable Film Method**  
**First Action 1996**  
**Revised First Action 1999**  
**Final Action 2002**

(Applicable to determination of low levels of coliform bacteria in dairy products.)

See Table 996.02A for the results of the interlaboratory study supporting acceptance of the method. See Table 996.02B for comparison of the modified Petrifilm HSCC plate method to the original Petrifilm HSCC plate method and the VRBA method for enumeration of coliforms at low, medium, and high levels of inoculation. See Table 996.02C for repeatability standard deviations of the modified Petrifilm HSCC plate and the original Petrifilm HSCC plate methods.

#### A. Principle

Undiluted or diluted test portions are added to plates of dry medium and cold H<sub>2</sub>O-soluble gel at a rate of 5.0 mL/plate. Pressure, when applied to plastic spreader placed on overlay film, spreads test suspensions over ca 60 cm<sup>2</sup> growth area. Gelling agent is allowed to solidify, plates are incubated, and coliforms are counted.

#### B. Apparatus and Reagent

(a) *High-Sensitivity Coliform Count (HSCC) Plates*.—Plates contain modified violet red bile, cold H<sub>2</sub>O-soluble gelling agent, and 2,3,5-triphenyltetrazolium chloride indicator. Petrifilm High-Sensitivity Coliform Count Plates (3M Microbiological Products, 3M Center Bldg, 275-5W-05, St. Paul, MN 55144, USA, No. 6405) or equivalent product producing similar method performance statistics meet these specifications.

(b) *Plastic spreader*.—Provided with Petrifilm plates; designed to spread test portion evenly over plate growth area.

(c) *Pipets*.—Calibrated for bacteriological use. Automatic pipet to deliver 5.0 mL may be used.

(d) *Colony counter*.—Standard apparatus, Quebec Model preferred (Fisher Laboratory Products, 200 Park Ln, Pittsburgh, PA 15275, USA, No. 07-908-7), or one providing equivalent magnification (1.5 $\times$ ) and visibility.

(e) *Dilution water*.—Prepare stock solution as follows: Dissolve 34 g KH<sub>2</sub>PO<sub>4</sub> in 500 mL H<sub>2</sub>O, adjust to pH 7.2 with 1M NaOH (40 g/L; ca 175 mL), and dilute to 1 L with H<sub>2</sub>O. Prepare buffered H<sub>2</sub>O for dilutions by diluting 1.25 mL stock solution to 1 L with boiled and cooled H<sub>2</sub>O. Autoclave 15 min at 121°C.

(f) *Stomacher laboratory blender*.

#### C. Preparation of Test Suspensions

Specified dilutions are for maximum sensitivity. Higher dilutions may be plated as needed. Do not use diluents containing citrate or

thiosulfate. Mix all dilutions by shaking 25 times through 30 cm arc in 7 s. Stomach or blend solids 2 min to homogenize.

(a) *Whole milk, 2% milk, 1% milk, skim milk, and raw milk*.—Plate 5 mL on dry-film coliform count plate. Incubate. Multiply colony count by 0.2 to obtain count/g.

(b) *Ice cream and mixes, sherbert, cream, half-and-half, condensed milk, and chocolate milk*.—Make 1 + 5 dilution (24.75 g/99 mL dilution water). Plate 5 mL on dry-film coliform count plate. Incubated colony count on plate is count/g.

(c) *Butter and margarine*.—Proceed as in (b) with diluent prewarmed to 40–45°C. Do not use citrate buffer to homogenize test portion.

(d) *Sour cream, dips, buttermilk, yogurt, and frozen yogurt*.—Make a 1 + 10 dilution (11 g/99 mL dilution water). After dilution, adjust pH to 6.5–7.5 with 1M NaOH (ca 0.1 mL/g product). Plate 5 mL on dry-film coliform count plate. Multiply colony count by 2 to obtain count/g.

(e) *Cheese, cottage cheese, and related products*.—Proceed as in (d) with diluent prewarmed to 40–45°C. Do not use citrate buffer to homogenize test portion.

#### D. Analysis

Place dry-film coliform count plate on a flat surface. Lift top film and inoculate 5 mL test suspension, C, onto center of film base. Carefully place top film down on inoculum. Distribute inoculum over prescribed growth area with downward pressure in center of plastic spreader. Leave plate undisturbed 2–5 min to allow gel to solidify. Place plates in incubator in horizontal position, clear side up, in stacks not exceeding 10 plates. Incubate plates 24 h at 32°C.

Count plates promptly after incubation period. Magnifier-illuminator may be used to facilitate counting. Coliforms appear as red colonies with one or more gas bubbles associated with them (within one colony diameter). Count all colonies in countable range (15–150 colonies). Red colonies without gas bubbles are not counted as coliform organisms.

To calculate coliform count, multiply total number of coliform colonies/plate (or average number of colonies/plate, if counting duplicate plates of same dilution) by appropriate dilution factor. When counting colonies on duplicate plates of consecutive dilutions, calculate mean number of colonies for each dilution before determining average bacterial count.

Estimated counts can be made on plates with >150 colonies and reported as estimated counts. In making such counts, circular growth area can be considered to contain ca 60 cm<sup>2</sup>. To isolate colonies for further identification, lift top film and pick colony from gel.

Reference: [J. AOAC Int. 80, 505\(1997\)](#).

**Table 996.02A. Interlaboratory study results for determination of coliform in dairy products by high-sensitivity dry rehydratable film method**

Product	Level	Mean log, CFU/g	$s_r$	$s_R$	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %	$r^a$	$R^b$
2% milk	Low	0.681	0.058	0.101	8.4	14.9	0.162	0.283
	Medium	1.576	0.067	0.082	4.3	5.2	0.188	0.230
	High	2.437	0.071	0.109	2.9	4.5	0.199	0.305
Chocolate milk	Low	0.612	0.241	0.241	39.4	39.4	0.675	0.675
	Medium	1.637	0.088	0.090	5.4	5.5	0.246	0.252
	High	2.607	0.114	0.116	4.4	4.5	0.319	0.325
Cream	Low	0.638	0.255	0.255	39.9	39.9	0.714	0.714
	Medium	1.661	0.046	0.071	2.8	4.3	0.129	0.199
	High	2.679	0.081	0.095	3.0	3.5	0.227	0.266
Vanilla ice cream	Low	0.147	0.203	0.208	138.1	141.2	0.568	0.582
	Medium	1.098	0.146	0.178	13.3	16.2	0.409	0.498
	High	1.899	0.087	0.170	4.6	9.0	0.244	0.476
Cottage cheese	Low	1.420	0.139	0.185	9.8	13.0	0.389	0.518
	Medium	2.410	0.112	0.131	4.7	5.4	0.314	0.367
	High	3.361	0.115	0.125	3.4	3.7	0.322	0.35
Cheddar cheese	Low	0.311	0.055	0.055	17.7	17.7	0.154	0.154
	Medium	1.633	0.105	0.180	6.4	11.0	0.294	0.504
	High	3.122	0.071	0.226	2.3	7.2	0.199	0.633

<sup>a</sup>  $r = 2.8$   $s_r$   
<sup>b</sup>  $R = 2.8$   $s_R$

**Table 996.02B. Comparison of the modified Petrifilm HSCC plate method to the original Petrifilm HSCC plate method and the VRBA method for enumeration of coliforms at low, medium, and high levels of inoculation**

Food	log mHSCC – log HSCC <sup>a</sup>				log mHSCC – log VRBA <sup>b</sup>			
	N	Mean log difference	T-value	p-value	N	Mean log difference	T-value	p-value
Low level of inoculation								
Butter	8	0.109	0.832	0.433	8	-0.228	-1.082	0.315
Buttermilk	9	-0.039	-0.277	0.789	9	-0.146	-1.275	0.238
Cheese	10	-0.026	-0.356	0.730	10	0.124	1.521	0.163
Chocolate milk	7	0.014	0.306	0.770	8	-0.111	-0.787	0.457
Cream	10	0.094	1.183	0.267	10	0.136	3.092	0.013 <sup>c</sup>
Frozen yogurt	10	0.170	2.603	0.029 <sup>c</sup>	9	0.102	0.880	0.405
Ice cream	10	0.052	0.906	0.388	9	0.136	1.836	0.104
Sour cream	8	-0.055	-0.368	0.724	8	-0.379	-2.155	0.068
Sweetened condensed milk	7	-0.106	-1.032	0.342	7	-0.301	-2.537	0.044 <sup>c</sup>
Whole milk	10	0.030	0.789	0.450	10	0.428	4.570	0.001 <sup>c</sup>
All foods	89	0.029	1.013	0.314	88	-0.001	-0.029	0.977
Medium level of inoculation								
Butter	10	0.048	0.516	0.617	10	-0.317	-2.580	0.030 <sup>c</sup>
Buttermilk	10	0.048	0.638	0.539	10	-0.150	2.330	0.045 <sup>c</sup>
Cheese	10	-0.035	-0.658	0.527	10	0.131	2.072	0.068
Chocolate milk	8	-0.097	-1.098	0.308	8	-0.136	-3.042	0.019 <sup>c</sup>
Cream	10	0.024	0.699	0.502	10	0.048	0.649	0.532
Frozen yogurt	10	0.136	1.826	0.101	10	0.146	2.627	0.028 <sup>c</sup>
Ice cream	10	0.025	1.405	0.194	10	0.065	1.595	0.145
Sour cream	10	0.192	3.579	0.006 <sup>c</sup>	10	0.308	3.559	0.006 <sup>c</sup>
Sweetened condensed milk	10	0.034	2.144	0.061	10	-0.073	-1.317	0.220
Whole milk	10	-0.003	-0.104	0.919	10	0.341	6.646	<0.0001 <sup>c</sup>
All foods	98	0.040	2.083	0.040 <sup>c</sup>	98	0.070	2.460	0.016 <sup>c</sup>
High level of inoculation								
Butter	9	-0.064	-4.143	0.003 <sup>c</sup>	9	0.012	0.092	0.929
Buttermilk	10	0.102	2.484	0.035 <sup>c</sup>	10	0.141	2.429	0.038 <sup>c</sup>
Cheese	10	0.028	3.687	0.005 <sup>c</sup>	10	0.007	-0.390	0.706
Chocolate milk	8	0.013	0.260	0.802	8	-0.122	-3.003	0.020 <sup>c</sup>
Cream	10	0.083	4.434	0.002 <sup>c</sup>	10	0.103	4.091	0.003 <sup>c</sup>
Frozen yogurt	10	0.041	2.528	0.032 <sup>c</sup>	10	-0.024	-1.195	0.263
Ice cream	10	0.020	1.242	0.246	10	0.041	2.376	0.042 <sup>c</sup>
Sour cream	10	0.148	3.770	0.004 <sup>c</sup>	10	0.192	2.555	0.031 <sup>c</sup>
Sweetened condensed milk	10	0.021	1.039	0.326	10	-0.024	-0.556	0.592
Whole milk	10	0.001	0.033	0.974	10	-0.011	-0.352	0.733
All foods	97	0.041	4.179	<0.0001 <sup>c</sup>	97	0.033	1.776	0.079

<sup>a</sup> Comparison of the modified Petrifilm HSCC plate and the original Petrifilm HSCC plate methods.

<sup>b</sup> Comparison of the modified Petrifilm HSCC plate and the VRBA method.

<sup>c</sup> Significantly different ( $p < 0.05$ ).

**Table 996.02C. Repeatability standard deviations of the modified Petrifilm HSCC plate and the original Petrifilm HSCC plate methods**

Food	Modified Petrifilm HSCC plate $s_r$	Original Petrifilm HSCC plate $s_r$
Butter	0.1936	0.1912
Buttermilk	0.1288	0.1411
Cheese	0.1073	0.1000
Chocolate milk	0.1264	0.1009
Cream	0.1034	0.1489
Frozen yogurt	0.1148	0.1338
Ice cream	0.1404	0.1276
Sour cream	0.1516	0.1443
Sweetened condensed milk	0.1170	0.1231
Whole milk	0.0883	0.1050