Readycult® Coliforms 100 Presence/Absence Test for Detection and Identification of Coliform Bacteria and *Escherichia coli* in Finished Waters

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Version 1.0

MERCK KGaA
Frankfurter Strasse 250
64271 DARMSTADT
GERMANY

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1.0 Scope and Application

1.1 This method is for use in the Environmental Protection Agency's (EPA's) data gathering and monitoring programs under the Safe Drinking Water Act.

1.2 Readycult® Coliforms is a selective and differential medium for determining the presence or absence of total coliforms and *E. coli* in finished waters. It is a one-step, ready-to-use, dehydrated, and granulated culture medium supplied in a gamma-irradiated snap pack. Each unit is added to a 100 ml water sample. The composition of Readycult® is identical to the composition of Merck´s dehydrated culture medium named Fluorocult® LMX Broth (Manafi and Ossmer, Merck Cat. No. 1.10620).

1.3 This method tests for coliform bacteria and/or *E. coli* in 24 ± 1 h. Confirmation or verification steps are not required. Optional confirmation or verification with the use of KOVAC´s indole reagent (for the confirmation of *E. coli*) is approved but not required.

1.4 The detection limit of Readycult® Coliforms is 1 colony forming unit (CFU) of coliform bacteria or *E. coli* per 100 ml of medium.

2.0 Summary of Method

2.1 This medium determines the presence or absence of coliform bacteria and *E. coli* in finished water. The content of one blister pack is added to a 100 ml sample of water followed by incubation at 36 ± 1°C or 35 ± 0.5°C for 24 ± 1 h. If coliform bacteria are present, the medium changes color from slightly yellow to blue-green. In addition, if *E. coli* is present, the medium will emit a bright blue fluorescence when subjected to a long wave (366 nm) ultraviolet (UV) light.

2.2 Readycult® Coliforms is based on the detection of two enzymes (ß-galactosidase and ß-glucuronidase) that are characteristic of the total coliform group and *E. coli,* respectively (1, 2). Identification of these enzymes is accomplished by resuscitation of the target organisms with a combination of tryptose, salts, phosphate buffers, and carbohydrates, followed by hydrolysis of the chromogenic and/or fluorogenic enzyme substrates. At the same time, lauryl sulfate sodium salt selectively inhibits the growth of accompanying bacterial flora.

2.3 Readycult® Coliforms contains the chromogenic enzyme substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-GAL) for the detection of ß-galactosidase (an enzyme indicative of the coliform group). Upon hydrolysis by ß-D-galactosidase, X-GAL releases a chromogenic compound (indigo-blue) that turns the medium from slightly yellow to a blue-green color. The endpoint of this reaction in the medium is distinct and not subject to interference in water samples discolored by humic acids, turbidity or other materials.

2.4 Readycult® Coliforms contains the fluorogenic enzyme substrate 4-methyl-umbelliferyl-ß-D-glucuronide (MUG) for the detection of ß-glucuronidase (an enzyme

specific to *E. coli*). Upon hydrolysis by ß-glucuronidase, MUG releases 4-methylumbelliferone that fluoresces when exposed to ultraviolet light. The fluorescence differentiates the presence of *E. coli* from the rest of the coliform group as required by the Total Coliform Rule (3) in the analysis of drinking water.

2.5 Readycult® Coliforms contains the enzyme substrate tryptophane for the detection of tryptophanase (another enzyme specific to *E. coli*). Upon cleavage by tryptophanase, tryptophane releases indole that immediately forms a red ring when KOVAC´s indole reagent is added directly to the broth. This reaction may be used as an additional confirmation for the presence of *E. coli* in vessels with positive fluorescence.

3.0 Definitions

3.1 Defined Substrate - An enzyme substrate that has a specific known chemical structure.

3.2 Chromogenic Enzyme Substrate - A substrate that releases a chromogenic compound upon hydrolysis by an enzyme.

3.3 Fluorogenic Enzyme Substrate - A substrate that releases a fluorogenic compound upon hydrolysis by an enzyme.

3.4 Specific Enzymes - Enzymes which react with only one particular substrate or very closely related compounds, e.g., ß-D-galactosidase is a specific enzyme that reacts with lactose or lactose analogs such as X-GAL. In addition, specific enzymes can also refer to those associated with a certain species or group of microorganisms.

3.5 Hydrolysis - Enzyme catalyzed decomposition of a substrate by addition of a water molecule (H_20).

3.6 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-GAL). A defined substrate that changes color from colorless to blue-green after hydrolysis by ß-D-galactosidase.

3.7 4-Methylumbelliferyl-ß-D-glucuronide (MUG) - A defined enzyme substrate that fluoresces under long wave UV light after hydrolysis by ß-D-glucuronidase.

3.8 Tryptophane - A defined enzyme substrate that releases indole upon cleavage by tryptophanase. A red ring forms in the presence of indole when KOVAC´s indole reagent is added directly to the broth.

3.9 Coliform Bacteria (Total Coliforms) - Bacteria that possess the enzyme ß-D-galactosidase. In Readycult® Coliforms, this enzyme is capable of producing a blue-green color.

3.10 *Escherichia coli* - Bacteria that possess the enzymes ß-D-galactosidase, ß-glucuronidase, and tryptophanase. In Readycult® Coliforms, these enzymes are capable of producing a blue-green color, fluorescence under UV light, and a positive indole reaction (red ring) upon addition of KOVAC's indole reagent (if desired).

3.11 Positive Control - Addition of a known organism to medium that produces a positive reaction.

3.12 Negative Control - Addition of a known organism to medium that produces a negative (no) reaction.

4.0 Interferences

4.1 Chemical / Physical - There are no known interferences contained in drinking or potential source water that interfere with the distinct color change or development of fluorescence in Readycult® Coliforms

5.0 Safety

5.1 The analyst must know and practice normal safety procedures for working in a microbiology laboratory where handling potentially biohazardous cultures is possible.

5.2 Solid and liquid waste containing viable microorganisms should be decontaminated by autoclaving or using an appropriate disinfectant before discarding.

5.3 Readycult® Coliforms is not a listed or suspected carcinogen. Contact with the skin or eyes may cause irritation or redness.

6.0 Equipment and Supplies

6.1 Equipment

6.1.1 Incubator or Waterbath – 36 ± 1°C or 35 ± 0.5°C.

6.1.2 Long wave UV Light - 366 nm, 6W minimum

6.2 Glassware and Supplies - All glassware and plastic vessels used for determining the presence of coliform bacteria or *E. coli* should be handled according to recommended guidelines(1). All glassware and containers should be washed with a suitable laboratory detergent, rinsed thoroughly with tap water followed by rinsing with reagent water(4) and sterilized by autoclaving at 121°C for 15 minutes prior to use.

6.2.1 Sterile Graduated Glass or Plastic Sample Collection Vessels – minimum 120 ml capacity for 100 ml water sample

6.2.2 Biohazardous Waste Container

6.3 Sterile Inoculating Loop or Needle

7.0 Reagents and Standards

7.1 Readycult® Coliforms 100 is provided as ready-to-use, pre-measured, dehydrated, granulated culture medium in a snap pack for 100 ml water sample format. If

properly stored at room temperature (+15 to + 25 °C), the product has a maximum shelf life of 36 months from date of manufacture.

7.2 Composition (gram/snap pack) of Readycult® Coliforms 100

Tryptose	0.5
Sodium chloride	0.5
Sorbitol	0.1
Tryptophan	0.1
Di-potassium hydrogen phosphate	0.27
Potassium dihydrogen phosphate	0.2
Laurylsulfate sodium salt	0.01
X-GAL	0.008
MUG	0.005
IPTG	0.01

7.3 Readycult® Coliforms is provided in a ready-to-use, pre-measured, sterile snap pack that requires no preparation. This media should be purchased from a commercially available source and should not be prepared from basic ingredients.

7.4 Sodium thiosulfate ($Na_2S_20_3$) - 3% solution (w/v) prepared with ACS grade $Na_2S_20_3$ (or equivalent) and reagent water.

7.5 KOVAC´s indole reagent is provided as a ready to use solution in 100 ml glass bottle or in a 30ml convenient dropper bottle (Bactident® Indole).

8.0 Sample Collection, Dechlorination, Preservation, Shipment and Storage (1)

8.1 Water Sample Collection. Detailed guidance of current aseptic sample collection techniques may be found in: 1) Chapter V, 6.1-6.5 *Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures Quality Assurance* – 5th Edition. U.S. EPA. Office of Ground Water and Drinking Water, EPA 815-R-05-004. and/or 2) Section 9060 A-B. *Standard Methods for the Examination of Water and Wastewater* – 21st Edition – 2005. American Public Health Association, American Water Works Association, and Water Environment Federation.

8.1.1 Collection Bottle or Bag – Sample containers should be clean, wide-mouth plastic or borosilicate glass bottles with non-leaking stoppers or caps that can withstand repeated sterilization. Presterilized plastic bottles or bags may also be used. The capacity of sample containers should be at least 120 ml. Containers should be non-fluorescing under long wave UV light.

8.1.2 Sampling Procedure – Remove faucet attachments such as screen or splash guard. Open tap fully and let water run to waste for 2-3 minutes. Reduce water flow to permit filling bottle without splashing.

8.2 Dechlorination - Chlorinated drinking water should be collected into a sterile collection vessel containing sufficient sodium thiosulfate ($Na_2S_20_3$) to neutralize the chlorine present. For a 100 ml water sample, 0.1 ml 3% $Na_2S_20_3$ should be used. Alternately, an equivalent amount of $Na_2S_20_3$ in tablet form may be substituted provided the tablet contains no binding agent that inhibits growth.

8.3 Preservation - Samples should be tested as soon as possible after collection. Water samples should be shipped or held at <10°C prior to testing and processed within 30 hours of collection. See current regulations in Chapter V, 6.3-6.4 *Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures Quality Assurance* – 5th Edition. U.S. EPA. Office of Ground Water and Drinking Water, EPA 815-R-05-004.

9.0 Quality Control

9.1 Each new lot of Readycult® Coliforms should be tested with positive and negative controls according to Chapter V, 5.1.6.4 *Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures Quality Assurance* – 5th Edition. U.S. EPA. Office of Ground Water and Drinking Water, EPA 815-R-05-004.

9.2 For each quality control sample, aseptically transfer the content of one snap pack to 100 ml sterile reagent water. Shake gently to completely dissolve.

9.3 Inoculate the individual bottles of rehydrated Readycult® Coliforms with a loopful of growth from a 21 ± 3 h broth culture of the following: *Citrobacter freundii* (ATCC 8090 or a characterized ß-D-galactosidase positive environmental strain) or *Klebsiella pneumoniae* (ATCC 31488) as a positive control for total coliform, *E. coli* (ATCC 11775, ATCC 25922 or a characteristic MUG positive environmental strain) as a positive control for *E. coli*, and *Enterococcus faecalis* (ATTC 19433) or *Pseudomonas Aeruginosa* (ATCC 10145) as a negative control for both target organisms. Include an uninoculated bottle of rehydrated media as an additional negative control.

9.4 Incubate at 36 ± 1°C or 35 ± 0.5°C for 24 ± 1 h and observe for the following results. Ensure that the specified incubation period is followed in air-type incubators according to Chapter V, 5.6.8 *Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures Quality Assurance* – 5th Edition. U.S. EPA. Office of Ground Water and Drinking Water, EPA 815-R-05-004.

Readycult ® Coliforms Control Organism Reactions

Organism	Color	Fluorescence	Indole
Klebsiella pneumoniae	Blue-Green	Negative	Negative
Citrobacter freundii	Blue-Green	Negative	Negative
E. coli	Blue-Green	Positive	Positive
Pseudomonas aeruginosa	Yellowish	Negative	Negative
Enterococcus faecalis	Yellowish	Negative	Negative
None	Yellowish	Negative	Negative

9.5 For additional quality control testing, positive Readycult® Coliforms may be inoculated into brilliant green lactose bile, EC plus MUG or other media used for confirmation.

9.6 Routine laboratory QA/QC procedures for applicable glassware, incubators, and other equipment should conform to : 1) Chapter V, Section 3, *Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures Quality Assurance* – 5th Edition. U.S. EPA. Office of Ground Water and Drinking Water, EPA 815-R-05-004. and/or 2) Section 9020. *Standard Methods for the Examination of Water and Wastewater* – 21st Edition – 2005. American Public Health Association, American Water Works Association, and Water Environment Federation.

10.0 Calibration and Standardization

10.1 Readycult® Coliforms - there is no required calibration or standardization of the method. Acceptable performance of the Readycult® Coliforms by the user is determined by routine quality control using known cultures and is described in Section 9.0.

10.2 Equipment: All equipment described in Section 6.0 should be maintained and calibrated according to the manufacturers' recommendations and the general QC guidelines in . 1) Chapter V, Section 3, *Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures Quality Assurance* – 5th Edition. U.S. EPA. Office of Ground Water and Drinking Water, EPA 815-R-05-004. and/or 2) Section 9020. *Standard Methods for the Examination of Water and Wastewater* – 21st Edition – 2005. American Public Health Association, American Water Works Association, and Water Environment Federation.

11.0 Procedure

11.1 Test Procedure

11.1.1 Add 100 ml water sample into a sterile, transparent vessel with at least a 120 ml capacity. (refer to section 8.0)

11.1.2 Take out one Readycult® Coliforms snap pack, tap the pack to ensure the granulated media does not stick to the bottom, and bend the upper part of the pack until it breaks open. Do not touch the opening.

11.1.3 Add the contents to the water sample. Seal the vessel and shake to completely dissolve the granules.

11.1.4 Incubation: 24 ± 1 h at 36 ± 1°C or 35 ± 0.5°C. Ensure that the specified incubation period is followed in air-type incubators according to Chapter V, 5.3 *Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures Quality Assurance* – 5th Edition. U.S. EPA. Office of Ground Water and Drinking Water, EPA 815-R-05-004

11.2 Sample Interpretation

11.2.1 Visually check each vessel for a blue-green color. If the sample is blue-green, coliform bacteria are present in the test sample.

11.2.2 If the solution is blue-green, examine for fluorescence using a long wave UV lamp. If fluorescence is present, the solution will glow a uniform, bright, light-blue throughout. The presence of fluorescence indicates that *E. coli* is present in the sample.

11.2.3 Optional: If a secondary confirmation of *E. coli* is desired in the vessel with positive fluorescence, add approximately 2.5 ml KOVAC´s indole reagent directly to the broth. A red ring will immediately form (indole reaction) to confirm the presence of *E. coli*.

11.2.4 If no blue-green color is observed, the sample is negative for coliform bacteria.

12.0 Data Analysis, Calculation, Interpretation and Reporting Results

12.1 Total Coliforms

12.1.1 A change of color from slight yellow to blue-green indicates the presence of at least 1 CFU of coliform bacteria. Report as a positive Presence/Absence Test result. An absence of a change of color should be reported as a negative Presence/ Absence Test result. No further data analysis or calculation is required.

12.2 *E. coli*

12.2.1 A fluorescence observed under long wave UV light indicates a positive result and the presence of at least 1 CFU of *E. coli*. Report as a positive Presence/Absence Test result.

13.0 Method Performance Characteristics

13.1 Specificity - the specificity of Readycult® Coliforms was assessed for recovery of total coliforms and *E. coli*. Both positive and negative results were evaluated according to the EPA's ATP(5) format for the presence or absence of total coliforms and *E. coli* as compared to the reference methods. Discrepancies between Readycult® Coliforms and reference method results in this study were resolved with an identification to species by a clinically approved commercial kit (#4345000 BBL Crystal Enteric/Nonfermenter). Both unadjusted (identification-to-species data not considered) and adjusted (identification-to-species data considered) results of that study appear below:

13.1.1 Total Coliforms (unadjusted) - False positive error was 7.00%. False negative error was 5.10%.

13.1.2 *E. coli* (unadjusted) - False positive error was 5.00%. False negative error was 6.86%.

13.1.3 Total Coliforms (adjusted) - False positive error was 3.0%. False negative error was 3.96%.

13.1.4 *E. coli* (adjusted) - False positive error was 0.00%. False negative error was 6.86%.

13.2 Comparability - the comparability of Readycult® Coliforms was assessed for recovery of total coliforms and *E. coli*. Both positive and negative results were evaluated according to the EPA's ATP(5) format for the presence or absence of total coliforms and *E. coli* as compared to the reference methods. Chi-square, Cochran-Mantel-Haenszel, and Breslow-Day statistics were calculated on 16 sets of data for total coliforms and 15 sets of data for *E. coli*. The results are too extensive to summarize here. See report from Montana Environmental Laboratory.

13.3 Additional note: The use of 120ml test vessel has been validated according to the EPA Microbiological Alternate Test Procedure (ATP) protocol for Drinking Water, Ambient Water, and Waste Water Methods. Based on a review of the study data, the EPA's Office of Ground Water and Drinking Water (OGWDW) and Statistics and Analytical Support Branch (SASB) concluded that there is no significant difference between the performance of the method with 150ml bottles at 36°C versus when run in 120ml bottles at 35°C. These findings were ratified by the USEPA with a formal letter of approval dated 6th May 2005.

13.4 Precision and Bias - This method is a qualitative test, and as such, precision and bias statements cannot be provided.

14.0 Pollution Prevention

14.1 Wherever possible, it is recommended that laboratory personnel use pollution control techniques to minimize waste generation. When waste cannot be reduced at the source, recycling is recommended.

15.0 Waste Management

15.1 It is the responsibility of each laboratory to comply with all federal, state and local regulations governing waste management particularly to hazardous waste identification rules and land disposal restrictions and to protect the air, water, and land by minimizing and controlling all release from fume hoods and bench operations. Compliance is also required with any sewage discharge permits and regulations (6). For further information, Federal, State or local agencies should be contacted.

16.0 References

1. American Public Health Association, American Water Works Association, Water Environment Federation. Microbiological Examination, Part 9000. *IN:* Standard Methods for the Examination of Water and Wastewater, 18th ed. Greenberg, A.E., L.S. Clesceri, and A.D. Eaton, eds. Washington, D.C., American Public Health Association. 1993, pp. 9-1 9-69.

2. Krieg., N.R**.** and J.G. Holt., eds. Bergey's Manual of Systematic Bacteriology, vol 1. Baltimore, Williams and Wilkins, 1989.

3. U.S. Environmental Protection Agency. National Primary Drinking Water Regulations, Total Coliforms (Including Fecal coliforms and *E. coli*; Final Rule. Federal Register 54(124): 27547-27568. Washington, D.C., Office of Federal Register. June 29, 1989.

4. American Society of Testing Materials. Specifications for Reagent Water, (Type III Grade), D1193-91. *IN:* Annual Book of ASTM Standards, vol. 11.01. Philadelphia, American Society for Testing Materials. 1991, p. 45.

5. U.S. Environmental Protection Agency. Protocol for Alternative Test Procedures for Coliform Bacteria in Compliance with Drinking Water Regulations – Presence / Absence Liquid Culture Methods for Finished Waters – Version 1.2 December , 1995

6. U.S. Environmental Protection Agency. National Primary and Secondary Drinking water Regulations: Analytical Methods for Regulated Drinking Water Contaminants; Proposed Rule, F6. Federal Register 58(129): 65626. Washington, D. C, Office of the Federal Register. December 15, 1993

7. Manafi, M. and Rosmann, H., Evaluation of Readycult Presence-Absence Test for Detection of Total Coliforms and *E. coli* in Water. 98th. American Society for Microbiology, Atlanta, 17-21 May 1998

8. Lee, J.V., Lightfoot, N.F. and Tillett, H.E. An evaluation of presence / absence tests for coliform organisms and *Escherichia coli*. International Conference on Coliforms and *E. coli* : Problem or solution? . 24-27 September 1995 , University of Leeds , UK

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

Jennifer Singh
EMD Chemicals
Market Segment Manager
Environmental & Molecular Microbiology
480 S. Democrat Rd.
Gibbstown, NJ 08027

1/5/2007

Dear Ms. Singh

EPA's Office of Ground Water and Drinking Water has reviewed EMD's method "Readycult® Coliforms 100 Presence/Absence Test for Detection and Identification of Coliform Bacteria and *Escherichia coli* in Finished Waters, Version 1.1, January 2007" and has determined that it is acceptable for monitoring total coliforms and *E. coli* under the Total Coliform Rule (40 CFR 141.21). This current version incorporates changes that were approved in May 2005 (ATP Case No. BD03-001).

We appreciate EMD Chemicals' continued interest in the development of environmental compliance monitoring methods. If you have any questions regarding our determination, please contact Steven C. Wendelken at 513-569-7491.

Sincerely,

Steven C. Wendelken, Ph.D.
ATP Coordinator
Technical Support Center (MS-140)
Office of Ground Water and Drinking Water