IDEXX
Literature Cover Sheet

IDEXX Library #: 4D

Topic: It's easier to get certified with Colilert than MF

Title: US EPA Manual For The Certification Of Laboratories Analyzing Drinking Water

Author(s): US EPA, EPA 815-B-97-001

Date: March 1997

Source: Excerpts from USEPA Website:
http://www.epa.gov/OGWDW/labindex.html

Highlights:
- The certification audit section for membrane filtration section is three times longer than the Colilert section.
- Underlined MF portions can be avoided by switching to Colilert and IDEXX vessels.
- Colilert is on pages 2-5 of this document.
- Membrane filtration in on pages 6-17.

COLILERT SECTION
Chapter V
Critical Elements for Microbiology

Note: This chapter uses the term "must" to refer to certification criteria that are required by the National Primary Drinking Water Regulations which include the approved drinking water methods. The term "should" is used for procedures that, while not specifically required by the regulations, are considered good laboratory practice for quality assurance. To assure the validity of the data, it is critical that laboratories observe both the regulatory and non-regulatory criteria. Certification Officers have the prerogative to refuse certification if the quality control data are judged unsatisfactory or insufficient.

Note: References to Standard Methods for the Examination of Water and Wastewater are to the 18th edition (1992)

1. Personnel

1.1 Supervisor/Consultant

The supervisor of the microbiology laboratory should have a bachelor's degree in microbiology, biology, or equivalent. Supervisors who have a degree in a subject other than microbiology should have had at least one college-level microbiology laboratory course in which environmental microbiology was covered. In addition, the supervisor should have a minimum of two weeks training at a Federal agency, State agency, or academic institution in microbiological analysis of drinking water or, 80 hours of on-the-job training in water microbiology at a certified laboratory, or other training acceptable to the State or EPA. If a supervisor is not available, a consultant having the same qualifications may be substituted, as long as the laboratory can document that the consultant is acceptable to the State and is present on-site frequently enough to satisfactorily perform a supervisor's duties.

The laboratory supervisor has the responsibility to insure that all laboratory personnel have demonstrated their ability to satisfactorily perform the analyses to which they are assigned and that all data reported by the laboratory meet the required quality assurance and regulatory criteria.

1.2 Analyst (or equivalent job title)

The analyst should perform microbiological tests with minimal supervision, and have at least a high school education. In addition, the analyst should have a minimum of at least three months of bench experience in water, milk, or food microbiology. The analyst should also have training acceptable to the State (or EPA for non-primacy States), in microbiological analysis of drinking water and a minimum of 30 days of on-the-job training under an experienced analyst. Analysts should take advantage of workshops and training programs that may be available from State regulatory agencies and professional societies. Before analyzing compliance samples, the analyst must demonstrate acceptable results for precision, specificity and satisfactory analysis on unknown samples.

1.3 Waiver of Academic Training Requirement

The certification officer may waive the need for the above specified academic training, on a case-by-case basis, for highly experienced analysts.

1.4 Personnel Records

Personnel records which include academic background, specialized training courses completed and types of microbiological analyses conducted, should be maintained on laboratory analysts

2. Laboratory Facilities

Laboratory facilities should be clean, temperature and humidity controlled, and have adequate lighting at bench tops. They should have provisions for disposal of microbiological waste. Laboratory facilities should have sufficient bench-top area for processing samples; storage space for media, glassware, and portable equipment; floor space for stationary equipment (incubators, water baths, refrigerators, etc.); and associated area(s) for cleaning glassware and sterilizing materials.

3. Laboratory Equipment and Supplies

The laboratory must have the equipment and supplies needed to perform the approved methods for which certification has been requested.

3.3 ***Temperature Monitoring Device***

3.3.1 Glass, dial, or electronic thermometers must be graduated in 0.5C increments (0.2C increments for tests which are incubated at 44.5C) or less. The fluid column in glass thermometers should not be separated. Dial thermometers that cannot be calibrated should not be used.

QC **3.3.2** Calibrations of glass and electronic thermometers should be checked annually and dial thermometers quarterly, at the temperature used, against a reference National Institute of Standards and Technology (formerly National Bureau of Standards [NBS]) thermometer or one that meets the requirements of NBS Monograph SP 250-23. The calibration factor should be indicated on the thermometer. Also, the laboratory should record the date the thermometer was calibrated and the calibration factor in a QC record book

QC **3.3.3** If a thermometer differs by more than 1C from the reference thermometer, it should be discarded. Reference thermometers should be recalibrated every three years.

QC **3.3.4** Continuous recording devices that are used to monitor incubator temperature should be recalibrated at least annually. A reference thermometer that meets the specifications described in paragraph 3.3.2 should be used for calibration.

3.4 ***Incubator Unit***

3.4.1 Incubator units must have an internal temperature monitoring device and maintain a temperature of 35 ±0.5C, and if used, 44.5 ±0.2C. For non-portable incubators, thermometers should be placed on the top and bottom shelves of the use area with the thermometer bulb immersed in liquid (except for electronic thermometers). If an aluminum block incubator is used, culture dishes and tubes should fit snugly. Laboratories which use the chromogenic/fluorogenic substrate tests with air-type incubators should note the caution indicated in 5.6.8.

QC **3.4.2** Calibration-corrected temperature should be recorded for days in use at least twice per day with readings separated by at least 4 hours.

3.15 ***Sample Containers***

3.15.1 Sample containers must be wide-mouth plastic or non-corrosive glass bottles with non-leaking ground glass stoppers or caps with non-toxic liners that should withstand repeated sterilization, or sterile plastic bags containing sodium thiosulfate. Other appropriate sample containers may be used. The capacity of sample containers should be at least 120 mL (4 oz.).

3.15.3 Glass and plastic bottles that have not been presterilized should be sterilized by autoclaving or, for glass bottles, by dry heat. Empty containers should be moistened with several drops of water before autoclaving to prevent an "air lock" sterilization failure.

3.15.4 If chlorinated water is to be analyzed, sufficient sodium thiosulfate (Na2S2O3) must be added to the sample before sterilization to neutralize any residual chlorine in the water sample. Dechlorination is addressed in Section 9060A of Standard Methods.

3.16 ***Glassware and Plasticware***

QC **3.16.2** Graduated cylinders for measurement of sample volumes must have a tolerance of 2.5% or less. In lieu of graduated cylinders, precalibrated containers that have clearly marked volumes of 2.5% tolerance may be used. The calibration of each new lot of precalibrated containers should be validated by selecting at least one container at random and checking the calibration using a previously verified graduated cylinder.

4. General Laboratory Practices

Although safety criteria are not covered in the laboratory certification program, laboratory personnel should be aware of general and customary safety practices for laboratories. Each laboratory is encouraged to have a safety plan available.

4.2 ***Sample Containers***

4.2.1 See Section 6.2 for sample preservation.

QC **4.2.2** At least one sample container should be selected at random from each batch of sterile sample bottles or other containers, and sterility confirmed by adding approximately 25 mL of a sterile non-selective broth (e.g., tryptic soy, trypticase soy, or tryptone broth). The broth should be incubated at 35 ±0.5oC for 24 hours and checked for growth. Resterilize if growth is detected.

5. Analytical Methodology

5.6 ***Chromogenic/fluorogenic substrate tests (MMO-MUG test [Colilert test] for total coliforms in source water and total coliforms and E. coli in drinking water; Colisure test for total coliforms and E. coli in drinking water)***

5.6.1 Media

5.6.1.1 These media must not be prepared from basic ingredients, but rather purchased from a commercially available source.

5.6.1.2 The media must be protected from light. Colisure medium must be refrigerated until use and brought to room temperature before adding the sample.

5.6.1.3 Some lots of fluorogenic media have been known to autofluoresce. Therefore, each lot of medium should be checked before use with a 366-nm ultraviolet light with a 6-watt bulb. If the media exhibit faint fluorescence, the laboratory should use another lot that does not fluoresce. If the samples plus a medium exhibit a color change before incubation, it should be discarded and another batch of medium used.

QC **5.6.1.4** For each lot of medium, a quality control check must be performed by inoculating sterile water containing the medium with a MUG-positive E. coli strain, a MUG-negative coliform, and a non-coliform and analyzing them.

QC **5.6.1.5** Laboratories may also use Quanti-Tray test or Quanti-Tray 2000 test for drinking water and source waters. Both tests use the Colilert medium. If the Quanti-Tray or Quanti-Tray 2000 test is used, the sealer should be checked monthly by adding a dye (e.g., bromcresol purple) to the water. If dye is observed outside the wells, another sealer should be obtained.

5.6.2 A glass bottle that contains inoculated medium should be checked with a 366-nm ultraviolet light source with a 6-watt bulb. If fluorescence is observed before incubation, do not use.

5.6.3 For enumerating total coliforms in source water with the Colilert test, 5 or 10 tube MTF, Quanti-Tray or Quanti-Tray 2000 must be used for each sample dilution tested. Dilution water (for the chromogenic/fluorogenic substrate test only), if used, must be sterile dechlorinated tap water, deionized water, or distilled water.

5.6.4 For determining the presence of total coliforms in drinking water by a chromogenic/fluorogenic substrate test, laboratories must use 10 tubes, each containing 10 mL of water sample, or a single vessel containing 100 mL of water sample.

5.6.5 For the Colilert test, samples must be incubated at 35 ±0.5oC for 24 hours. A yellow color in the medium equal to or greater than the reference comparator indicates the presence of total coliforms and must be reported as a total coliform positive. If the sample is yellow, but lighter than the comparator, it must be incubated for another four hours (do not incubate more than 28 hours total). If the color is still lighter than the reference comparator at 28 hours, the sample should be reported as negative. Laboratories that use the Colilert-18 test must incubate for 18 hours.

5.6.6 For the Colisure test, samples must be incubated at 35 ±0.5oC for 28 hours. If an examination of the results at 28 hours is not convenient, then results may be examined at any time between 28 and 48 hours. If the medium changes from a yellow color to a magenta color, the sample must be reported as E. coli positive.

5.6.7 For E. coli testing, the laboratory must place all total coliform-positive bottles/tubes under an ultraviolet lamp (366 nm, 6-watt) in a darkened room. If E. coli is present, the medium will emit a blue fluorescence.

QC **5.6.8** Air-type incubators, especially small ones, may not bring a cold 100-mL water sample(s) to the specified incubation temperature of 35C for several hours. This problem may be further aggravated if several cold water samples are placed in the incubator at the same time. The problem may cause false-negative results with the chromogenic/fluorogenic substrate tests. Therefore, laboratories with air-type incubators should check the time it takes for a 100-mL water sample (or a set of 100-mL water samples, depending on normal use) to reach 35C, and ensure that the specified incubation period at that temperature is followed. This check should be repeated whenever there is a significant change in the sample load.

5.6.9 The Colilert/Colisure tests must not be used to confirm total coliforms on membrane filters. The filtration step not only concentrates coliforms, but also non-coliforms and turbidity, which at high levels, can suppress coliforms or cause false-positive results in the chromogenic/fluorogenic substrate test.

5.6.10 The Colilert/Colisure tests must not be used to confirm total coliforms in the MTF or Presence-Absence (P-A) coliform test. High densities of non-coliforms in the inoculum may overload the chromogenic/fluorogenic substrate test suppressant reagent system and cause false positive results.

MEMBRANE FILTRATION SECTION
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The analyst should perform microbiological tests with minimal supervision, and have at least a high school education. In addition, the analyst should have a minimum of at least three months of bench experience in water, milk, or food microbiology. The analyst should also have training acceptable to the State (or EPA for non-primacy States), in microbiological analysis of drinking water and a minimum of 30 days of on-the-job training under an experienced analyst. Analysts should take advantage of workshops and training programs that may be available from State regulatory agencies and professional societies. Before analyzing compliance samples, the analyst must demonstrate acceptable results for precision, specificity and satisfactory analysis on unknown samples.

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3. Laboratory Equipment and Supplies

The laboratory must have the equipment and supplies needed to perform the approved methods for which certification has been requested.

3.1 ***pH Meter***

3.1.1 Accuracy and scale graduations must be within ±0.1 units.

3.1.2 pH buffer aliquots should be used only once.

3.1.3 Electrodes should be maintained according to the manufacturer's recommendations.

QC **3.1.4** pH meters should be standardized before each use period with pH 7.0 and either pH 4.0 or 10.0 standard buffers, whichever range covers the desired pH of the media or reagent. The date and buffers used should be recorded in a log book.

QC **3.1.5** Commercial buffer solution containers should be dated upon receipt, and when opened. Buffers should be discarded before the expiration date.

3.2 ***Balance (top loader or pan)***

3.2.1 Balances should have readability of 0.1 g

QC **3.2.2** Balances should be calibrated monthly using ASTM type 1, 2, or 3 weights (minimum of three traceable weights which bracket laboratory weighing needs). (ASTM, 1916 Race St.., Philadelphia, PA 19103) Non-reference weights should be calibrated every six months with reference weights.

QC **3.2.3** Service contracts or internal maintenance protocols and maintenance records should be available. Maintenance should be conducted annually at a minimum. A record of the most recent calibration should be available for inspection. Correction values should be on file and used. A reference weight should be re-certified if it is damaged or corroded.

3.3 ***Temperature Monitoring Device***

3.3.1 Glass, dial, or electronic thermometers must be graduated in 0.5C increments (0.2C increments for tests which are incubated at 44.5C) or less. The fluid column in glass thermometers should not be separated. Dial thermometers that cannot be calibrated should not be used.

QC **3.3.2** Calibrations of glass and electronic thermometers should be checked annually and dial thermometers quarterly, at the temperature used, against a reference National Institute of Standards and Technology (formerly National Bureau of Standards [NBS]) thermometer or one that meets the requirements of NBS Monograph SP 250-23. The calibration factor should be indicated on the thermometer. Also, the laboratory should record the date the thermometer was calibrated and the calibration factor in a QC record book

QC **3.3.3** If a thermometer differs by more than 1C from the reference thermometer, it should be discarded. Reference thermometers should be recalibrated every three years.

QC **3.3.4** Continuous recording devices that are used to monitor incubator temperature should be recalibrated at least annually. A reference thermometer that meets the specifications described in paragraph 3.3.2 should be used for calibration.

3.4 ***Incubator Unit***

3.4.1 Incubator units must have an internal temperature monitoring device and maintain a

temperature of 35 ±0.5C, and if used, 44.5 ±0.2C. For non-portable incubators, thermometers should be placed on the top and bottom shelves of the use area with the thermometer bulb immersed in liquid (except for electronic thermometers). If an aluminum block incubator is used, culture dishes and tubes should fit snugly. Laboratories which use the chromogenic/fluorogenic substrate tests with air-type incubators should note the caution indicated in 5.6.8.

QC 3.4.2 Calibration-corrected temperature should be recorded for days in use at least twice per day with readings separated by at least 4 hours.

3.4.3 An incubation temperature of 44.5 ±0.2C can best be maintained with a water bath equipped with a gable cover and a pump or paddles to circulate water.

3.5 Autoclave

3.5.1 The autoclave should have an internal heat source, a temperature gauge with a sensor on the exhaust, a pressure gauge, and an operational safety valve. The autoclave should maintain a sterilization temperature during the sterilizing cycle and complete an entire cycle within 45 minutes when a 12-15 minute sterilization period is used. The autoclave should depressurize slowly enough to ensure that media will not boil over and bubbles will not form in inverted tubes.

3.5.2 Because of safety concerns and difficulties with operational control, pressure cookers should not be used.

QC 3.5.3 The date, contents, sterilization time and temperature, total time for each cycle, and analyst's initials should be recorded each time the autoclave is used. A copy of the service contract or internal maintenance protocol and maintenance records should be kept. Maintenance should be conducted annually at a minimum. A record of the most recent service performed should be available for inspection.

QC 3.5.4 A maximum-temperature-registering thermometer or continuous recording device should be used during each autoclave cycle to ensure that the proper temperature was reached, and the temperature recorded. Overcrowding should be avoided. Spore strips or ampules should be used monthly to confirm sterilization.

QC 3.5.5 Automatic timing mechanisms should be checked quarterly with a stopwatch or other accurate timepiece or time signal.

3.5.6 Autoclave door seals should be clean and free of caramelized media. Also, autoclave drain screens should be cleaned frequently and debris removed.

3.6 Hot Air Oven

3.6.1 The oven should maintain a stable sterilization temperature of 170-180C for at least two hours. Only dry items should be sterilized with a hot air oven. Overcrowding should be avoided. The oven thermometer should be graduated in 10C increments or less, with the bulb placed in sand during use.

QC 3.6.2 The date, contents, sterilization time and temperature of each cycle, and analyst's initials should be recorded.

QC 3.6.3 Spore strip or ampule should be used on a monthly basis to ensure sterility of items.

3.7 Colony Counter

A dark field colony counter should be used to count Heterotrophic Plate Count colonies.

3.8 Conductivity Meter

3.8.1 Meters should be suitable for checking laboratory reagent-grade water and readable in appropriate M units (micromhos or microsiemens per centimeter). Use an instrument capable of measuring conductivity with an error not exceeding 1% or 1 micromho per centimeter, whichever is more lenient.

QC 3.8.2 Cell constant should be determined monthly using a method indicated in Section 2510, "Conductivity," in Standard Methods. Monthly calibration checks using an appropriate certified and traceable low-level standard may be substituted for determining the cell constant.

3.8.3 If an in-line unit cannot be calibrated, it should not be used to check reagent-grade water.

*3.9 **Refrigerator***

3.9.1 Refrigerators should maintain a temperature of 1-5C. Thermometers should be graduated in at least 1C increments and the thermometer bulb immersed in liquid.

QC 3.9.2 The temperature should be recorded for days in use at least once per day.

*3.10 **Inoculating Equipment***

Sterile metal or disposable plastic loops, wood applicator sticks, sterile swabs, or sterile plastic disposable pipet tips should be used. If wood applicator sticks are used, they should be sterilized by dry heat. The metal inoculating loops and/or needles should be made of nickel alloy or platinum. (For the coliform test and any other oxidase test used for the verification of membrane filter colonies, nickel alloy loops must not be used because they may interfere with the oxidase test).

*3.11 **Membrane Filtration Equipment** (if MF procedure is used)*

3.11.1 MF units must be stainless steel, glass, or autoclavable plastic, not scratched or corroded, and must not leak.

QC 3.11.2 If graduation marks on clear glass or plastic funnels are used to measure sample volume, their accuracy should be checked with a standard graduated cylinder, and a record of this calibration check retained. Tolerance should be ì?cÿÿëÜ2.5%.

3.11.3 A 10X to 15X stereo microscope with a fluorescent light source must be used to count sheen colonies.

3.11.4 Membrane filters must be approved by the manufacturer for total coliform water analysis. Approval is based on data from tests for toxicity, recovery, retention, and absence of growth-promoting substances. Filters must be cellulose ester, white, gridmarked, 47 mm diameter, and 0.45 m pore size, or alternate pore sizes if the manufacturer provides performance data equal to or better than the 0.45 m pore size. Membrane filters must be purchased presterilized or autoclaved for 10 minutes at 121C before use.

QC 3.11.5 The lot number for membrane filters and the date received should be recorded.

*3.12 **Culture Dishes (loose or tight lids)***

3.12.1 Presterilized plastic or sterilizable glass culture dishes must be used. To maintain sterility of glass culture dishes, stainless steel or aluminum canisters, or a wrap of heavy aluminum foil or char-resistant paper, must be used.

3.12.2 Loose-lid petri dishes should be incubated in a tight-fitting container, e.g., plastic vegetable crisper containing a moistened paper towel to prevent dehydration of membrane filter & medium.

3.12.3 Opened packs of disposable culture dishes should be resealed between use periods.

3.13 Pipets

3.13.1 To sterilize and maintain sterility of glass pipets, stainless steel or aluminum canisters should be used, or individual pipets should be wrapped in char-resistant paper or aluminum foil.

3.13.2 Pipets must have legible markings and should not be chipped or etched.

3.13.3 Opened packs of disposable sterile pipets should be resealed between use periods.

3.13.4 Pipets delivering volumes of 10 mL or less must be accurate within a 2.5% tolerance.

3.13.5 Calibrated micropipetters may be used if tips are sterile. Micropipetters should be calibrated annually and replaced if the tolerance is greater than 2.5%

3.14 Culture Tubes and Closures

3.14.1 Tubes should be made of borosilicate glass or other corrosion-resistant glass or plastic.

3.14.2 Culture tubes and containers should be of sufficient size to contain medium plus sample without being more than three quarters full.

3.14.3 Tube closures should be stainless steel, plastic, aluminum, or screw caps with non-toxic liners. Cotton plugs should not be used.

3.15 Sample Containers

3.15.1 Sample containers must be wide-mouth plastic or non-corrosive glass bottles with non-leaking ground glass stoppers or caps with non-toxic liners that should withstand repeated sterilization, or sterile plastic bags containing sodium thiosulfate. Other appropriate sample containers may be used. The capacity of sample containers should be at least 120 mL (4 oz.).

3.15.2 Glass stoppers must be covered with aluminum foil or char-resistant paper for sterilization.

3.15.3 Glass and plastic bottles that have not been presterilized should be sterilized by autoclaving or, for glass bottles, by dry heat. Empty containers should be moistened with several drops of water before autoclaving to prevent an "air lock" sterilization failure.

3.15.4 If chlorinated water is to be analyzed, sufficient sodium thiosulfate ($Na_2S_2O_3$) must be added to the sample before sterilization to neutralize any residual chlorine in the water sample. Dechlorination is addressed in Section 9060A of Standard Methods.

3.16 Glassware and Plasticware

3.16.1 Glassware must be borosilicate glass or other corrosion-resistant glass and free of chips and cracks. Markings on graduated cylinders and pipets must be legible. Plastic items must be clear and non-toxic to microorganisms.

QC 3.16.2 Graduated cylinders for measurement of sample volumes must have a tolerance of 2.5% or less. In lieu of graduated cylinders, precalibrated containers that have clearly marked volumes of 2.5% tolerance may be used. The calibration of each new lot of precalibrated containers should be validated by selecting at least one container at random and checking the calibration using a previously verified graduated cylinder.

3.17 Ultraviolet lamp (if used)

3.17.1 The unit should be disconnected monthly and the lamps cleaned by wiping with a soft cloth moistened with ethanol.

QC 3.17.2 If a UV lamp (254 nm) is used for sanitization, the lamp should be tested quarterly with a UV light meter or agar spread plate. The lamp should be replaced if it emits less than 70% of its initial output or if an agar spread plate containing 200 to 250 microorganisms, exposed to the UV light for two minutes, does not show a count reduction of 99%. Other methods may be used to test a lamp if data demonstrate that they are as effective as the two suggested methods.

4. General Laboratory Practices

Although safety criteria are not covered in the laboratory certification program, laboratory personnel should be aware of general and customary safety practices for laboratories. Each laboratory is encouraged to have a safety plan available.

4.1 Sterilization Procedures

4.1.1 Required times for autoclaving at 121oC are listed below. The items must be at temperature for this required amount of time. Except for membrane filters and pads and carbohydrate-containing media, indicated times are minimum times which may necessitate adjustment depending upon volumes, containers, and loads.

Item	Time (min)
Membrane filters & pads	10
Carbohydrate containing media	12-15
Contaminated test materials	30
Membrane filter assemblies	15
Sample collection bottles	15
Individual glassware	15
Dilution water blank	15
Rinse water (0.5 - 1 L)	15-30*

* time depends upon water volume per container and autoclave load

4.1.2 Autoclaved membrane filters and pads and all media should be removed immediately after completion of the sterilization cycle.

4.1.3 Membrane filter equipment must be autoclaved before the beginning of the first filtration series. A filtration series ends when 30 minutes or longer elapses after a sample is filtered.

4.1.4 Ultraviolet light (254 nm) may be used as an alternative to sanitize equipment, if all supplies are presterilized and QC checks are conducted as indicated in paragraph 3.17.2. Ultraviolet light may also be used to control bacterial carry-over between samples during a filtration series.

4.2 Sample Containers

4.2.1 See Section 6.2 for sample preservation.

QC 4.2.2 At least one sample container should be selected at random from each batch of sterile sample bottles or other containers, and sterility confirmed by adding approximately 25 mL of a sterile non-selective broth (e.g., tryptic soy, trypticase soy, or tryptone broth). The broth should be incubated at 35 ±0.5oC for 24 hours and checked for growth. Resterilize if growth is detected.

4.3 Reagent-Grade Water

4.3.1 Only satisfactorily tested reagent water from stills or deionization units may be used to prepare media, reagents, and dilution/rinse water for performing bacteriological analyses.

QC 4.3.2 The quality of the reagent water should be tested and should meet the following criteria:

Parameter	Limits	Frequency
Conductivity	<2 micromhos/cm (microsiemens/cm) at 25°C	Monthly
Pb, Cd, Cr, Cu, Ni, Zn	Not greater than 0.05 mg/L per contaminant. Collectively, no greater than 0.1 mg/L	Annually
Total Chlorine Residual	1< 0.1 mg/L	Monthly
Heterotrophic Plate Count	2< 500/mL	Monthly
Bacteriological Quality of Reagent Water3	Ratio of growth rate 0.8:3.0	Annually

1 DPD Method should be used. Not required if source water is not chlorinated.
2 Pour Plate Method. See Standard Methods 9215B.
3 See Standard Methods, Section 9020B. This bacteriological quality test is not needed for ASTM (ASTM, 1916 Race St.., Philadelphia, PA 19103) Types 1 reagent water, as defined in Standard Methods, Section 1080.

4.4 Dilution/Rinse Water

4.4.1 Stock buffer solution or peptone water should be prepared, as specified in Standard Methods, Section 9050C.

4.4.2 Stock buffers should be autoclaved or filter-sterilized, and containers should be labeled and dated. Stock buffers should be refrigerated. Stored stock buffers should be free from turbidity.

QC **4.4.3** Each batch of dilution/rinse water should be checked for sterility by adding 50 mL of water to 50 mL of a double strength non-selective broth (e.g., tryptic soy, trypticase soy or tryptose broth). Incubate at 35 ±0.5oC for 24 hours and check for growth. Discard if growth is detected.

4.5 Glassware Washing

4.5.1 Distilled or deionized water should be used for final rinse.

QC **4.5.2** A glassware inhibitory residue test (Standard Methods, Section 9020B) should be performed before the initial use of a washing compound and whenever a different formulation of washing compound, or washing procedure, is used. In addition, batches of dry glassware should be spot-checked occasionally for pH reaction, especially if glassware is soaked in alkali or acid (Standard Methods, Section 9020B). These tests will ensure that glassware is at a neutral pH and is free of toxic residue.

4.5.3 Laboratory glassware should be washed with a detergent designed for laboratory use.

5. Analytical Methodology

5.1 General

5.1.1 For compliance samples, laboratories must only use the analytical methodology specified in the Total Coliform Rule (40 CFR 141.21(f)) and the Surface Water Treatment Rule (40 CFR 141.74(a)).

5.1.2 A laboratory must be certified for all analytical methods, indicated below, that it uses for compliance purposes. At a minimum, the laboratory must be certified for one total coliform method and one fecal coliform or E. coli method. A laboratory should also be certified for a second total coliform method if one method cannot be used for some drinking waters (e.g., where

the water usually produces confluent growth on a plate). In addition, for principal State laboratories and other laboratories that may enumerate heterotrophic bacteria (HPC) for compliance with the Surface Water Treatment Rule, the laboratory must be certified for the Pour Plate Method, the only method approved for heterotrophic bacteria.

5.1.3 Absorbent pads must be saturated with a liquid medium (at least 2 mL of broth) and excess medium removed by "decanting" the plate.

5.1.4 Water samples should be shaken vigorously about 25 times before analyzing.

QC 5.1.5 If no total coliform-positive result occurs during a quarter, the laboratory should perform the coliform procedure using a known coliform-positive, fecal coliform and/or E. coli- positive control to spike the sample.

5.1.6 Sample volume analyzed for total coliforms in drinking water must be 100 mL ±2.5 mL.

5.1.7 **Media**

5.1.7.1 The use of dehydrated or prepared media manufactured commercially is strongly recommended due to concern about quality control. Dehydrated media should be stored in a cool, dry location. Caked or discolored dehydrated media should be discarded.

QC **5.1.7.2** For media prepared in the laboratory, the date of preparation, type of medium, lot number, sterilization time and temperature, final pH, and the technician's initials should be recorded.

QC **5.1.7.3** For liquid media prepared commercially, the date received, type of medium, lot number, and pH verification should be recorded. Medium should be discarded by manufacturer's expiration date.

QC **5.1.7.4** Each new lot of dehydrated or prepared commercial medium should be checked before use with positive and negative culture controls. In addition, each batch of laboratory-prepared medium should include positive and negative culture controls. These control organisms can be stock cultures (periodically checked for purity) or commercially available disks impregnated with the organism. Results should be recorded.

5.1.7.5 Prepared plates may be refrigerated in sealed plastic bags or containers. Because of potential evaporation, they may not be kept for more than two weeks. Each bag or container should include the date prepared or an expiration date. Broth in loose-cap tubes should be stored at <30C no longer than two weeks. Broth in tightly capped tubes should be stored at <30C no longer than three months.

When ready to use, the refrigerated sterilized medium should be incubated overnight at room temperature; media with growth should be discarded.

QC **5.1.8** Laboratories are encouraged to perform parallel testing between a newly approved test and another EPA-approved procedure for enumerating total coliforms for at least several months and/or over several seasons to assess the effectiveness of the new test for the wide variety of water types submitted for analysis. During this testing, spiking the samples occasionally with sewage or a pure culture may be necessary to ensure that some of the tests are positive.

5.2 Membrane Filter (MF) Technique (for total coliforms in drinking water)

5.2.1 Media

5.2.1.1 M-Endo Medium broth or agar (also known as M-Endo broth MF and M-Coliform Broth) or LES Endo agar (also known as M-Endo Agar LES) must be used in the single step or enrichment techniques. Ensure that ethanol used in the rehydration procedure is not denatured. Medium must be prepared in a sterile flask and a boiling water bath must be used or, if constantly attended, a hot plate with a stir bar may be used, to bring the medium just to the boiling point. The medium must not be boiled. pH must be 7.2 ±0.2 for LES Endo agar and 7.2 ±0.1 for M-Endo medium.

5.2.1.2 MF broth must be refrigerated no longer than 96 hours, poured MF agar plates no longer than two weeks, and ampuled M-Endo broth in accordance with the manufacturer's expiration date. Broth, plates, or ampules should be discarded earlier if growth or surface sheen is observed.

QC 5.2.1.3 MF sterility check should be conducted on each funnel in use at the beginning and the end of each filtration series by filtering 20-30 mL of dilution water through the membrane filter and testing for growth. If the control indicates contamination, all data from affected samples must be rejected and an immediate resampling should be requested. A filtration series ends when 30 minutes or more elapse between sample filtrations.

5.2.2 To prevent carry-over, the filtration funnels must be rinsed with two or three 20-30 mL portions of water after each sample filtration.

5.2.3 Inoculated medium must be incubated at 35±0.5C for 22-24 hours.

5.2.4 All samples resulting in confluent or TNTC (too numerous to count) growth must be invalidated unless total coliforms are detected. If no total coliforms are detected, record as "confluent growth" or "TNTC" and request an additional sample from the same sampling site. Confluent growth is defined as a continuous bacterial growth covering the entire membrane filter without evidence of sheen colonies (total coliforms). TNTC is defined as greater than 200 colonies on the membrane filter in the absence of detectable coliforms. Laboratories must not invalidate samples when the membrane filter contains at least one sheen colony. (Before invalidation, the laboratory may perform a verification test on the total coliform-negative culture, i.e., on confluent or TNTC growth, and a fecal coliform/E. coli test. If the verification test is total coliform-positive, the sample must be reported as total coliform-positive. If the test is total coliform-negative, the sample must be invalidated. A fecal coliform/E. coli-positive result is considered a total coliform-positive, fecal coliform/E.coli-positive sample, even if the initial and/or verification total coliform test is negative.)

5.2.5 All sheen colonies (pick all sheen colonies up to a maximum of five) must be verified using either single strength lactose broth (LB) or lauryl tryptose broth (LTB) and single strength brilliant green lactose bile broth (BGLBB), or EPA-approved cytochrome oxidase and ß-galactosidase rapid test procedure. Individual colonies can be transferred with a sterile needle or loop, or applicator stick. When picking individual colonies, different morphological types of up to five red questionable sheen colonies and/or red non-sheen colonies per sample must be verified to include different types. Alternatively, wipe the entire surface of the membrane filter with a sterile cotton swab.

5.2.6 When EC Medium or EC Medium + MUG is used, the colonies must be transferred by employing one of the options specified by paragraph 141.21(f)(5). For the swab technique, a single swab can be used to inoculate a presumptive total coliform-positive culture into up to three different media (e.g., EC or EC-MUG Medium, LTB, and BGLBB, in that order).

5.3 (Multiple Tube fermentation Technique (MTF or MPN) (for total coliforms in drinking water)
-Section has been omitted for the purposes of this document-

5.4 Presence Absence (P-A) Coliform Test (for drinking water)
-Section has been omitted for the purposes of this document-

5.5 Fecal Coliform Test (using EC Medium for fecal coliforms in drinking water or source water, or A-1 Medium for fecal coliforms in source water only)

5.5.1 EC medium must be used to determine whether a total coliform-positive culture taken from the distribution system contains fecal coliforms, in accordance with the Total Coliform Rule. The laboratory must transfer each total coliform-positive culture from a presumptive tube/bottle, or each presumptive total coliform-positive colony (five such colonies minimum) unless a cotton swab is used, to at least one tube containing EC Medium with an inverted vial, as specified by §141.21(f)(5).

5.5.2 EC Medium may be used to enumerate fecal coliforms in source water, in accordance with the Surface Water Treatment Rule. Initially, conduct an MTF test (presumptive phase). Three sample volumes of source water (10, 1 and 0.1 mL), 5 or 10 tubes/sample volume, should be used. A culture from each total coliform-positive tube must be transferred to a tube containing EC Medium with an inverted vial.

5.5.2.1 Medium must be autoclaved for 12-15 minutes at 121C. The pH must be 6.9 ±0.2.

5.5.2.2 Inverted vials should be examined to ensure that they are free of air bubbles. The inverted vial must be at least one-half to two-thirds covered after the sample is added.

5.5.2.3 If prepared medium is stored, it should be maintained in the dark at <30C. Prepared medium stored in tubes with loose-fitting closures should be used within two weeks. Prepared medium stored in tightly closed screw type tubes may be kept up to three months. If the medium is stored in a refrigerator, it should be incubated overnight at room temperature before use; tubes that show growth and/or bubbles should be discarded.

5.5.3 A-1 Medium may be used as an alternative to EC Medium to enumerate fecal coliforms in source water, in accordance with the Surface Water Treatment Rule. A-1 Medium must not be used for drinking water samples. Three sample volumes of source water (10, 1 and 0.1 mL), 5 or 10 tubes/sample volume, should be used. Unlike EC Medium, A-1 Medium can be directly inoculated with a water sample.

5.5.3.1 Medium must be sterilized by autoclaving at 121C for 10 minutes. The pH must be 6.9 ±0.1.

5.5.3.2 Inverted vials should be examined to ensure that they are free of air bubbles.

5.5.3.3 Loose-cap tubes should be stored in dark at room temperature not more than two weeks. A-1 Medium may be held up to three months in a tightly closed screwcap tube in the dark at <30C.

5.5.4 The water level of the water bath must be above the upper level of the medium in the culture tubes.

5.5.5 EC Medium must be incubated at 44.5 ±0.2C for 24 ±2 hours. A-1 Medium must be incubated at 35 ±0.5C for three hours, then at 44.5 ±0.2C for 21 ±2 hours.

5.5.6 Any amount of gas detected in the inverted vial of a tube that has turbid growth must be considered a fecal coliform-positive test.

5.6 Chromogenic/fluoregenic substrate tests (MMO-MUG test (Colilert test) for total coliforms in source water and total coliforms and E.coli in drinking water; Colisure test for total coliforms and E. coli in drinking water)

-Section has been omitted for the purposes of this document-

5.7 EC Medium + MUG Test (for E. coli)

5.7.1 If EC medium + MUG is used, a total coliform-positive culture must be transferred from a presumptive tube/bottle or colony to EC medium + MUG, as specified by §141.21(f)(5).

5.7.2 Medium

5.7.2.1 MUG may be added to EC Medium before autoclaving. EC Medium + MUG is also available commercially. The final MUG concentration must be 50 g/mL. The pH must be 6.9 ±0.2.

5.7.2.2 The inverted vial may be omitted, because gas production is not relevant to the test, and the use of an inverted vial may cause confusion on test interpretation.

5.7.2.3 Test tubes and autoclaved medium should be tested before use with a 366-nm ultraviolet light to ensure they do not fluoresce. If fluorescence is exhibited, non-fluorescing tubes or another lot of medium that does not fluoresce should be used; alternatively, a MUG-positive E. coli and MUG-negative (e.g., uninoculated) control should be performed for each analysis.

5.7.2.4 If prepared medium is stored, it should be maintained in the dark at <30C. Prepared medium stored in tubes with loose-fitting closures should be used within two weeks. Prepared medium stored in tightly closed screw type tubes may be kept up to three months. Tubes with growth should be discarded.

QC 5.7.2.5 In accordance with paragraph 5.1.7.5, control cultures should be incubated at 35 ±0.5C for 24 hours in LTB. A loopful should be transferred to EC Medium + MUG and then incubated at 44.5±0.2C for 24 hours. The results should be read and recorded.

5.7.3 The water level of the water bath must be above the upper level of the medium.

5.7.4 The medium must be incubated at 44.5±0.2C for 24 ±2 hours.

5.7.5 Fluorescence must be checked using an ultraviolet lamp (366 nm) with a 6-watt bulb in a darkened room. Laboratories should ensure that a weak auto-fluorescence of medium, if present, is not misinterpreted as positive for E. coli. (If uncertain, a MUG-positive E. coli and MUG negative (e.g., uninoculated) control for each analysis should be used whenever the medium autofluoresces.)

5.8 Nutrient Agar + MUG Test (for E. coli)

5.8.1 Medium

5.8.1.1 Medium must be autoclaved in 100-mL volumes at 121C for 15 minutes. MUG may be added to Nutrient Agar before autoclaving. Nutrient Agar + MUG is also available commercially. The final MUG concentration must be 100 g/mL. The pH must

be 6.8 ±0.2.

5.8.1.2 If sterile medium is stored, the medium should be refrigerated in petri dishes, in a plastic bag or tightly closed container, and used within two weeks. Before use, refrigerated sterilized medium should be incubated overnight at room temperature; plates with growth should be discarded.

QC 5.8.1.3 Positive and negative controls should be tested as stated in paragraph 5.1.7.5. Filter or spot-inoculate control cultures onto a membrane filter on M-Endo LES agar or M-Endo broth or agar, and incubate at 35C for 24 hours. Then transfer the filter to Nutrient Agar + MUG and incubate at 35C for another four hours. The results should be read and recorded.

5.8.2 The membrane filter containing coliform colony(ies) must be transferred from the total coliform medium to the surface of Nutrient Agar + MUG medium. Each sheen colony should be marked with a permanent marker on the lid. Also, the lid and the base should be marked with a line to realign the lid should it be removed. A portion of the colony may be transferred with a needle to the total coliform verification test before transfer to Nutrient Agar + MUG or after the four-hour incubation time. Another method is to swab the entire membrane filter surface after the 4-hour incubation time onto the Nutrient Agar + MUG medium, with a sterile cotton swab, and transfer to a total coliform verification test.

5.8.3 Inoculated medium must be incubated at 35±0.5C for four hours.

5.8.4 Fluorescence must be checked using an ultraviolet lamp (366 nm) with a 6-watt bulb in a darkened room. Any amount of fluorescence in a halo around a sheen colony should be considered positive for E. coli.

-The remainder of sections in Chapter V, "Critical Elements for Microbiology", have been omitted for the purposes of this document-