ANALYTICAL PROCEDURES

EPA Approved* Method 10029

Coliforms: Membrane Filtration (simultaneous detection)

For detection of: total coliforms, *E. coli*
Media: m-ColiBlue24® Broth
Sample Type: potable water, nonpotable water, wastewater

Simultaneous Total Coliform and *E. coli* Screening

Hach's new m-ColiBlue24®** Broth allows for the simultaneous detection of total coliform bacteria and *Escherichia coli (E. coli)* within 24 hours. An enzymatic indicator in the medium causes non-fecal total coliform colonies grown on the m-ColiBlue24 medium to be red, while the *E. coli* (fecal coliform) colonies are blue.

The selectivity of the enzymatic indicator eliminates the need for confirmation. The low false positive and false negative rates allow for the detection of at least 95% of all *E. coli.*

m-ColiBlue24 Broth enhances the growth rate of coliform bacteria and optimizes the recovery of stressed or injured organisms. Special inhibitors in the medium efficiently minimize the growth of non-coliform bacteria.

Convenient Packaging

Hach's m-ColiBlue24 Broth comes in ready-to-use PourRite™ Ampules; no need to measure, mix, or autoclave. The ampules have a large, unrestrictive opening that allows the medium to pour easily. Simply break the top off the ampule, using the Hach ampule breaker, and pour the medium onto an absorbent pad in a petri dish.

Each ampule contains enough selective medium for one test. Medium in PourRite ampules has a shelf life of one year. Ampules are shipped with a Certificate of Analysis and have an expiration date printed on the label. m-ColiBlue24 Broth is also available in plastic ampules and 100 mL bottles.

Specifications

Incubation: 24 hours at 35 ± 0.5 °C

Sensitivity: 1 CFU/100 mL

Selectivity: Detects total coliforms and *E. coli*

Shelf Life: 1 year when refrigerated at 2 to 8 °C and protected from light

Technique is Important

Good laboratory technique is essential when accuracy is important, particularly in microbiological laboratory procedures. Care in sample collection and preservation, a clean laboratory or work surface, proper sterilization and inoculation practices, and close temperature control help assure reliable results.

* This method is USEPA approved for drinking water
** U. S. Patents 5,650,290 and 5,849,515

Preparing Materials

Start the incubator while preparing other materials. Adjust the incubator temperature setting to 35 °C.

Using Presterilized Equipment and Media

To simplify techniques and minimize the possibility of contamination, use presterilized equipment and media. Hach offers presterilized and disposable membrane filters, pipets, petri dishes, absorbent pads, inoculating loops, buffered dilution water in 99-mL bottles, sampling bags, and prepared growth media. MEL Portable Labs include presterilized consumables and field filtration assembly.

Using Field Filtration Apparatus

1. Flame-sterilize the top surface of the stainless steel Field Vacuum Support.
2. Attach the syringe tip to the vacuum support tubing.
3. Using sterile forceps, place a membrane filter, grid side up, onto the center of the vacuum support.

Note: *To sterilize forceps, dip forceps in alcohol and flame in an alcohol or Bunsen burner. Let forceps cool before use.*

4. Open a package of funnels (start at the bottom of the package). Remove a funnel (base first) from the package.
5. Place the funnel onto the vacuum support. Do not touch the inside of the funnel. Push evenly on the funnel's upper rim to snap it onto the vacuum support.
6. Pour the sample into the funnel.
7. Pull on the syringe plunger to draw the sample through the filter apparatus.

Note: *See specific procedures for the sample volume required.*

8. Remove the funnel.
9. Press the lever on the vacuum support stem to lift the membrane filter from the vacuum support surface.
10. Use sterile forceps to remove the membrane filter.
11. Place the membrane filter into a prepared petri dish and incubate according to the appropriate procedure.
12. Disconnect the syringe tip from the vacuum support tubing. Dispose of the liquid in the syringe.
13. Follow *steps 1–12* to filter remaining samples.

Using Autoclavable Equipment
When numerous samples must be run on a routine basis, you may prefer to use an autoclave for reusable materials.

1. Wash sample bottles, pipets, petri dishes, filter holder with stopper, and graduated cylinder (if needed) with hot water and detergent.
2. Rinse several times with tap water and then with deionized water. Dry thoroughly.
3. Prepare all equipment for autoclaving.
    - Loosely thread caps on sample bottles and cover caps and bottle necks with metal foil or paper.
    - Cover the openings of graduated cylinders with metal foil or paper.
    - Insert the filter funnel base into an autoclavable rubber stopper that will fit the filter flask.
    - Wrap the two parts of the filter funnel assembly separately in heavy wrapping paper and seal with masking tape.
    - Wrap petri dishes (borosilicate glass) in paper or place in aluminum or stainless cans.
4. Sterilize equipment in an autoclave at 121 °C for 15 minutes. Borosilicate glass items may be sterilized with dry heat at 170 °C for a minimum of one hour.

Preparing Autoclavable Filter Assembly
Disinfect the work bench or work area with a germicidal cloth, dilute bleach solution, or dilute iodine solution. Wash hands thoroughly with soap and water.

1. After sterilization, remove the filter funnel assembly from the wrapping paper.
2. Do not contaminate the funnel by touching the inner surfaces that will be exposed to the sample.
3. Insert the funnel with rubber stopper into the filtering flask or filter funnel manifold and connect to the water trap and aspirator with rubber tubing.

Note: *A vacuum pump may be used in place of the water trap and aspirator.*

4. Using sterile forceps, place a sterile membrane filter on the filter base and attach the filter funnel top.
5. Before filtering the sample, filter a small quantity of sterile buffered dilution water through the funnel to assure a good seal on the filter and connections.

Sample Size

Sample size is governed by bacterial density and turbidity.

The ideal sample volume of nonpotable water or wastewater for coliform testing yields 20–80 coliform colonies per filter.

Where the coliform numbers are uncertain, three different volumes should be filtered and cultured. *Table 1* lists recommended volumes for samples from various sources.

When the sample is less than 20 mL (diluted or undiluted), add 10 mL of sterile dilution water to the filter funnel before applying the vacuum. This aids in distributing the bacteria evenly across the entire filter surface.

Diluting Samples

Very small sample volumes may be required for samples with large coliform populations or for very turbid samples. These volumes are obtained by making serial dilutions of the sample. One method of doing this is described below.

Dilution Technique

1. Wash hands.
2. Open a bottle of sterile Buffered Dilution Water.
3. Shake the sample collection container vigorously, approximately 25 times.
4. Use a sterile transfer pipet to pipet the required amount of sample into the sterile Buffered Dilution Water.
5. Recap the buffered dilution water bottle and shake vigorously 25 times.
6. If more dilutions are needed, repeat *steps 3–5* using clean, sterile pipets and additional bottles of sterile Buffered Dilution Water.

Dilution Series

A. If a 10-mL sample is required:
Transfer 11 mL of sample into 99 mL of sterile, buffered dilution water. Filter 100 mL of this dilution to obtain the 10-mL sample.

B. If a 1-mL sample is required:
Transfer 11 mL of the 10-mL dilution from *step A* into 99 mL of sterile dilution water. Filter 100 mL of this dilution to obtain the 1-mL sample.

C. If a 0.1-mL sample is required:
Transfer 11 mL of the 1-mL dilution from *step B* into 99 mL of sterile dilution water. Filter 100 mL of this dilution to obtain the 0.1-mL sample.

D. If a 0.01-mL sample is required:
Transfer 11 mL of the 0.1-mL dilution from *step C* into 99 mL of sterile dilution water. Filter 100 mL of this dilution to obtain the 0.01-mL sample.

E. If a 0.001-mL sample is required:
Transfer 11 mL of the 0.01-mL dilution from *step D* into 99 mL of sterile dilution water. Filter 100 mL of this dilution to obtain the 0.001-mL sample.

F. If a 0.0001-mL sample is required:
Transfer 11 mL of the 0.001-mL dilution from *step E* into 99 mL of sterile dilution water. Filter 100 mL of this dilution to obtain the 0.0001-mL sample.

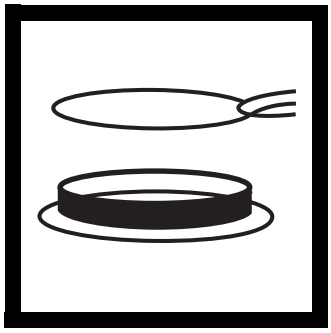
Table 1 Suggested Sample Volumes for MF Total Coliform Test*

	Volume to be Filtered (mL)							
Water Source	**100**	**50**	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**
Drinking water	X							
Swimming pools	X							
Wells, springs	X	X	X					
Lakes, reservoirs	X	X	X					
Water supply intake			X	X	X			
Bathing beaches			X	X	X			
River water				X	X	X	X	
Chlorinated sewage				X	X	X		
Raw sewage					X	X	X	X

* *Standard Methods for the Examination of Water and Wastewater*, 18th ed., pp. 9–56.

Using m-ColiBlue24 Broth PourRite Ampules

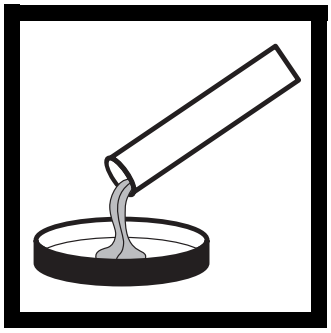
The m-ColiBlue24 Broth can be used to analyze drinking water; bottled water; beverages; surface, well, and groundwater; waste water; recreational waters; and process water for ultrapure, chemical processing and pharmaceutical applications.



1. Use sterilized forceps to place a sterile, absorbent pad in a sterile petri dish. Replace the lid on the dish.

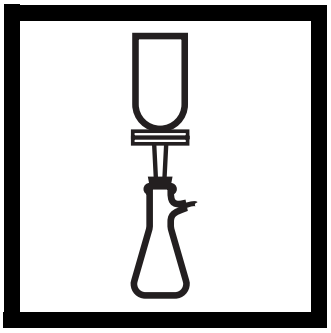
Note: *Do not touch the pad or the inside of the petri dish.*

Note: *To sterilize the forceps, dip them in alcohol and flame in an alcohol or Bunsen burner. Let the forceps cool before use.*



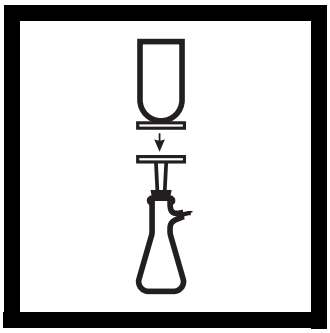
2. Invert ampules two or three times to mix broth. Break open an ampule of m-ColiBlue24 Broth using an ampule breaker. Pour the contents evenly over the absorbent pad. Replace the petri dish lid.

3. Set up the Membrane Filter Apparatus; see *Preparing Materials* on page *2*. With sterile forceps, place a membrane filter, grid side up, into the assembly.

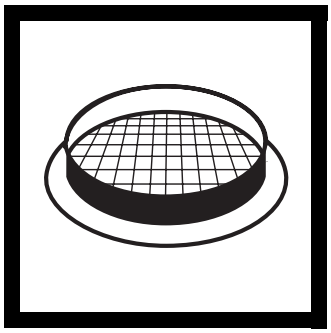


4. Shake the sample vigorously to mix. Pour 100 mL of sample or diluted sample into the funnel. Apply vacuum and filter the sample. Rinse the funnel walls three times with 20 to 30 mL of sterile buffered dilution water.

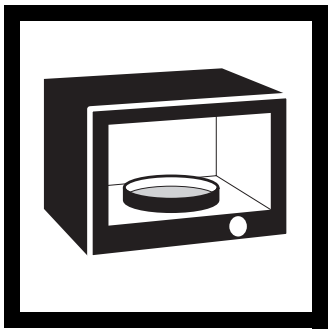
Coliforms: Membrane Filtration (simultaneous detection), continued



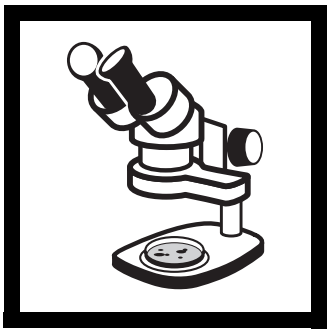
5. Turn off the vacuum and lift off the funnel top. Using sterile forceps, transfer the filter to the previously prepared petri dish.



6. With a slight rolling motion, place the filter, grid side up, on the absorbent pad. Check for trapped air under the filter and make sure the filter touches the entire pad. Replace the petri dish lid.



7. Invert the petri dish and incubate at 35 ± 0.5 °C for 24 hours.



8. Remove the petri dish from the incubator and examine the filters for colony growth. Colonies are typically readily visible; however, a stereoscopic microscope or other 10–15X magnifier may be useful. Red and blue colonies indicate total coliforms and blue colonies specifically indicate *E. coli*.

Note: *Sometimes only the center of a colony will be colored. Therefore, a colony with any amount of red color should be counted as red and a colony with any amount of blue should be counted as a blue colony. Red colonies may vary in color intensity. Blue colonies may appear blue to purple. Count all the red and blue colonies as total coliforms. Count all the blue to purple colonies as* E. coli.

Interpreting Results

Coliform density is reported as the number of colonies per 100 mL of sample. Use samples that produce 20–80 coliform colonies per filter to compute coliform density. Drinking water samples should produce very few colonies.

Use Equation A to calculate coliform density. Note that "mL sample" refers to actual sample volume, and not the volume of the dilution.

Equation A—Coliform density on a single membrane filter

$$\text{Coliform colonies per 100 mL} = \frac{\text{Coliform colonies counted}}{\text{mL of original sample filtered}} \times 100$$

- If growth covers the entire filtration area of the membrane or a portion of it, and colonies are not discrete, report results as "Confluent Growth With or Without Coliforms."

- If the total number of colonies (coliforms plus non-coliforms) exceeds 200 per membrane or the colonies are too indistinct for accurate counting, report the results as "Too Numerous to Count (TNTC)."

In either case, a new sample must be run using a dilution that will give 20–80 coliform colonies per filter.

When testing nonpotable water, if no filter meets the desired minimum colony count, calculate the average coliform density with *Equation B*.

Equation B—Average coliform density for 1) duplicates, 2) multiple dilutions, or 3) more than one filter/sample

$$\text{Coliform colonies per 100 mL} = \frac{\text{Sum of colonies in all samples}}{\text{Sum of volumes (in mL) of all samples}} \times 100$$

Equations A and **B** can also be used to calculate the *E. coli* density by substituting *E. coli* (blue colony counts) for total coliform (red and blue counts).

Optional Testing of Red Colonies

The m-ColiBlue24 Broth is formulated so that coliforms other than *E. coli* grow as red colonies. The percentage of red colonies that are false positives (non-coliforms) is comparable to the percentage of sheen colonies grown on m-Endo Broth that are false positives (non-coliforms); therefore, confirmation is not required.

A few varieties of the non-coliform bacteria *Pseudomonas, Vibrio,* and *Aeromonas* spp. may grow on m-ColiBlue24 Broth and form red colonies. Such bacteria can be readily distinguished from total coliforms by the oxidase test. *Pseudomonas, Vibrio,* and *Aeromonas* spp. are oxidase-positive. Total coliforms and *Escherichia coli* are oxidase-negative. If your sample contains high levels of interfering bacteria, you can perform an oxidase test to confirm which red colonies are total coliforms.

Two oxidase procedures are provided. Count the red and blue colonies on the m-ColiBlue24 Broth membrane filter before starting the oxidase test.

Oxidase Method 1

This method enables you to conveniently and rapidly evaluate membrane filters that have numerous colonies. Use this method after 24 hours of incubation on m-ColiBlue24 Broth.

Research* shows that the oxidase test cannot be performed on media that undergoes acidification during bacterial growth. The m-ColiBlue24 Broth is formulated so that the medium does not undergo such acidification. Consequently, many colonies can be simultaneously tested for their oxidase reaction using the following procedure.

1. Remove the lid from the petri dish containing the m-ColiBlue24 Broth membrane filter, invert the lid, and place it on the bench top.

   Controls: Positive and negative controls are important. *Pseudomonas aeruginosa* is recommended for positive controls and *Escherichia coli* for negative controls. Hach offers Aqua QC-Stiks™ for quality control procedures.

2. Drop approximately 0.5 mL of Difco SpotTest™ Oxidase Reagent into the center of the inverted lid.

* A.H. Havelaar et al. 1980. False-negative oxidase reaction as a result of medium acidification. *Antonie van Leeuwenhoek.* 46, 301-312.
L.K. Hunt et al. 1981. Role of pH in oxidase variability of *Aeromonas hydrophila. Journal of Clinical Microbiology.* 13: 1054-1059.

3. Using sterile forceps, transfer the membrane filter from the pad and place the filter upright in the inverted lid.

4. Within 10 to 15 seconds, the oxidase reagent will soak into the filter and cause the oxidase-positive colonies to turn purple. This purple color may be visible in the colony itself or adjacent to the colony. Oxidase-negative colonies will retain the red color they developed when incubated on m-ColiBlue24 Broth.

5. After the initial 10 to 15 second reaction time, start counting the red colonies that turn purple. Count individual colonies by using a microscope with 10X to 15X magnification

   Note: *To simplify colony counting place a spare lid on the lid containing the oxidase reagent and membrane filter. Use a felt-tip pen to mark the lid as you identify the purple colonies. After 30 seconds, you can count marks that indicate purple (oxidase-positive) colonies.*

6. Stop counting 30 seconds after initial 10 to 15 second reaction time, because oxidase-negative colonies will start to develop a purple color.

   Note: *Bacteria are not killed with this procedure, so colonies may be selected for streaking and for additional testing.*

Colonies that are blue after the initial 24-hour incubation on m-ColiBlue24 Broth are almost always *E. coli* and do not need confirmation with the oxidase procedure.

Oxidase Method 2

This method is the official oxidase test described in *Standard Methods for the Examination of Water and Wastewater,* 18th edition, 1992.

1. Select red colonies from an m-ColiBlue24 Broth membrane filter and streak onto Tryptic Soy Agar.

2. Incubate Tryptic Soy Agar plates at 35 °C for 18 to 24 hours or until isolated colonies are obtained.

   Controls: Positive and negative controls are important. *Pseudomonas aeruginosa* is recommended for positive and *Escherichia coli* for negative controls. Hach offers Aqua QC-Stiks for quality control procedures.

3. Saturate a piece of filter paper with Difco SpotTest Oxidase Reagent. (This reagent contains a stabilized solution of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride.)

   Note: *Alternatively, oxidase reagent can be dropped directly onto colonies growing on Tryptic Soy Agar. Oxidase-positive colonies will turn pink to purple.*

4. Using a sterile nichrome inoculating needle, transfer cellular material from an isolated Tryptic Soy Agar colony to the moist filter paper.

   Note: *Do not use iron or other reactive needles for inoculation, because they will cause false-positive results. Wooden applicator sticks work well.*

5. Oxidase-negative colonies will not react with the reagent, but oxidase-positive colonies will cause the reagent to turn dark purple within 10 seconds.

6. Oxidase-negative colonies should be counted as total coliform bacteria.

REQUIRED MEDIA AND REAGENTS

Description	Quantity	Cat. No.
m-ColiBlue24®* Broth, glass ampules	20/pkg	26084-20
OR		
m-ColiBlue24®* Broth, plastic ampules	20/pkg	26084-10
OR		
m-ColiBlue24®* Broth, 100 mL bottle	1 bottle	26084-42

REQUIRED APPARATUS

Ampule Breaker, PourRite™*	1	24846-00
Bags, Whirl-Pak with dechlorinating agent, 180-mL	100/pkg	20753-33
Counter, hand tally	1	14696-00
Dish, Petri, with pad, 50 mm, sterile, disposable	100/pkg	14717-99
Filter Holder, magnetic coupling (use with 24861-00)	1	13529-00
Filter Funnel Manifold, aluminum, 3-place (use with 13529-00)	1	24861-00
Filtering Flask, 1000-mL	1	546-53
Filters, Membrane, 47 mm, 0.45 µm, gridded, sterile	200/pkg	13530-01
Forceps, stainless steel	1	21411-00
Incubator, Culture, 120 Vac, 50/60 Hz	1	26192-00
OR		
Incubator, Culture, 220 V ac, 50/60 Hz	1	26192-02
Pump, vacuum/pressure, 115 V ac, 60 Hz	1	14697-00
OR		
Pump, vacuum/pressure, 220 V ac, 50 Hz	1	14697-02
Stopper, rubber, one-hole, No. 7	6/pkg	2119-07
Tubing, rubber, 0.8 cm (5/16 in.) ID	3.7 m (12 ft.)	559-19

OPTIONAL MEDIA AND REAGENTS

Dechlorinating Reagent Powder Pillows	100/pkg	14363-69
Dilution Water, buffered, sterile, 99-mL bottles	25/pkg	14305-98
Dilution Water, buffered, sterile, 99-mL bottle	1 bottle	14305-72
Escherichia coli Aqua QC-Stik™* Device	3/pkg	27063-03
Peptone Powder Pillows, 1 g	30/pkg	21429-64
Potassium Dihydrogen Phosphate and Magnesium Chloride Powder Pillows for buffered dilution water (25 of each)	50/pkg	21431-66
Pseudomonas aeruginosa Aqua QC-Stik™* Device	3/pkg	27065-03
Tryptic Soy Agar	100 g	25659-26

OPTIONAL APPARATUS

Alcohol Burner	1	20877-42
Aspirator, water	1	2131-02
Autoclave, Automatic, 120 V ac, 50/60 Hz	1	24630-00
Autoclave, Automatic, 240 V ac, 50/60 Hz	1	24630-02
Beaker, 600 mL	1	500-52
Bottles, sample, sterilized, 100-mL fill-to line, disposable	12/pkg	24950-12
Bottles, sample, sterilized, 100-mL fill-to line, disposable	50/pkg	24950-50
Bottles, sample, sterilized, 100-mL fill-to line, disposable with dechlorinating agent	12/pkg	25991-12
Bottles, sample, sterilized, 100-mL fill-to line, disposable with dechlorinating agent	50/pkg	25991-50
Dish, Petri, 50 mm, sterile, disposable	500/pkg	14852-00
Dish, Petri, 50 mm, sterile, disposable	100/pkg	14852-99
Filter Holder, reusable (use with 26566-00)	1	26567-00

* m-ColiBlue24 and PourRite are trademarks of Hach Company; AQUA QC-STIK is a trademark of MicroBioLogics.

OPTIONAL APPARATUS (continued)

Description	Quantity	Cat. No.
Filter Unit, sterile, disposable with gridded membrane (use with 26567-00)	12/pkg	26566-00
Filtration Support (for field use), stainless steel	1	25862-00
Funnels, Push-Fit and membrane filters (use with 25862-00)	72 of each/pkg	25863-00
Germicidal Cloths	50/pkg	24632-00
Graduated Cylinder, 100-mL	1	508-42
Incubator, portable, 12 V dc	1	25699-00
Incubator, Water Bath, 120 V ac, 50/60 Hz, with gable cover	1	26163-00
Incubator, Water Bath, 240 V ac, 50/60 Hz, with gable cover	1	26163-02
Inoculating Loop, nichrome wire, with handle	1	21121-00
Inoculating Needle, nichrome, 76 mm	1	21779-00
Magnifier, illuminated, 10X, portable	1	25853-00
Microscope Illuminator (use with 23174-00)	1	23175-00
Microscope, Stereo Binocular, 10X (15X available)	1	23174-00
Pad, absorbent, with dispenser	1000/pkg	14918-00
Pen, colony counter (Felt-tip pen attached to a counter, that marks, beeps, and registers accumulative count on an LCD display)	1	26132-00
Pen, laboratory	1	20920-00
Pipet, serological, 10–11 mL, sterile, disposable	25/pkg	2097-98
Syringe, 140-mL, polypropylene (use with 25862-00)	1	25861-00

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