

Multiple-tube method for thermotolerant (faecal) coliforms

In the multiple-tube method, a series of tubes containing a suitable selective broth culture medium is inoculated with test portions of a water sample. After a specified incubation time at a given temperature, each tube showing gas formation is regarded as “presumptive positive” since the gas indicates the possible presence of coliforms. However, gas may also be produced by other organisms, and so a subsequent confirmatory test is essential. The two tests are known respectively as the *presumptive test* and the *confirmatory test*.

For the confirmatory test, a more selective culture medium is inoculated with material taken from the positive tubes. After an appropriate incubation time, the tubes are examined for gas formation as before. The most probable number (MPN) of bacteria present can then be estimated from the number of tubes inoculated and the number of positive tubes obtained in the confirmatory test, using specially devised statistical tables. This technique is known as the MPN method.

A5.1 Inoculation

Different test portions to provide tenfold serial dilution steps may be used, the dilutions being based on the anticipated number of coliform bacteria in the water sample being tested. The reliability of the result obtained depends on the number of tubes inoculated with each test portion; in certain instances, the number can be reduced to three in each dilution step. Each combination of inoculated tubes will have its own table of MPN values. Typical volumes for analysis are given in Table 4.4 (p. 62).

A5.2 Unpolluted and treated water

Water in or entering the distribution system may generally be assumed to contain little or no pollution. In this case, it is recommended that one 50-ml plus five 10-ml volumes of water sample should be inoculated into the tubes; five tubes should each contain 10 ml and one tube 50 ml of double-strength medium.

A5.3 Polluted water

Water suspected to be more highly contaminated, e.g. untreated water from certain raw water sources, should be examined using different inoculation volumes in tenfold dilution steps. The following inoculations are normally made:

- 10 ml of sample to each of five tubes containing 10 ml of double-strength medium;
- 1.0 ml of sample to each of five tubes containing 10 ml of single-strength medium;
- 1.0 ml of a 1 : 10 dilution of sample (i.e. 0.1 ml of sample) to each of five tubes containing 10 ml of single-strength medium.

If the sample is expected to be highly contaminated, aliquots of 1.0 ml of tenfold serial dilutions from each dilution step are inoculated into five tubes that each contain 10 ml of single-strength medium.

If the workload is very heavy and the time available is limited, the number of tubes can be reduced to three in each series. Statistically, however, inoculation of five tubes with each sample volume produces a more reliable MPN result.

A5.4 Equipment and supplies

The following laboratory equipment and glassware are essential:

- *Autoclave*: required for sterilizing the culture media. Its size should be determined by the number and type of samples to be taken. Operation of the autoclave should be strictly in accordance with the manufacturer's instructions and should ensure that all the air in the chamber is replaced by steam. Sterilization should be achieved in not more than 30 minutes. Strict adherence to recommended sterilization temperatures and times for different types of culture media is essential. Racks are needed to hold tubes and bottles of prepared culture media in the autoclave.
- *Incubator(s) or water-baths*: must each be fitted with a temperature control and should be capable of maintaining a uniform temperature correct to 35 or $37 \pm 0.5^\circ\text{C}$ and/or 44 or $44.5 \pm 0.25^\circ\text{C}$. The choice of temperature depends on the indicator bacteria and the medium used. The temperature of incubators and water-baths fitted with thermometers placed at representative points should be monitored periodically (preferably daily). Stainless-steel racks should be fitted to hold sample tubes.
- *Balance*: needed for weighing powdered culture medium. It should have an accuracy of 0.05 g. A weighing scoop is also required. (No balance is required if culture media are available in suitable preweighed quantities.)
- *Water distillation apparatus, hose, and container*: required to produce non-toxic water, i.e. water free from any substances that can interfere with bacterial growth. The container for the distilled water should have a volume of at least 5 litres and be fitted with a tap.
- *Pipettes*: 1 ml and 10 ml, with cotton plugs at the mouthpiece, are required. The 1-ml pipettes should be graduated in 0.1-ml increments and are used for preparing dilutions; the 10-ml pipettes are used for the addition of samples to tubes containing media. Any pipettes with chipped or broken tips should

be discarded. Glass pipettes can be conveniently stored in a sterilizable metal container; alternatively, disposable plastic pipettes can be used. A separate container should be employed for each size of pipette. Pipettes may also be wrapped individually in paper and heat-sterilized. Pipette canisters and bulbs are also needed, as is a container for discarded pipettes.

- *Test-tubes and racks*: tubes can be 20 × 150 mm in size for 10-ml sample volumes plus 10 ml of culture medium (screw caps are not recommended for fermentation media). The racks should be large enough to accommodate culture tubes of the largest diameter used.
- *Bottles*: used for the larger volumes consisting of 50 ml of sample and 50 ml of culture medium. They should have loose-fitting caps and ideally be calibrated with 50-ml and 100-ml marks.
- *Media preparation equipment*: glass or stainless-steel containers (usually flasks) are required. Any heating equipment and stirrers used in the preparation of media should be clean and free from soluble toxic materials.
- *Gas burner*: a Bunsen or similar burner is adequate.
- *Culture tubes containing inverted vials (Durham tubes)*: each tube should be large enough for a vial, completely filled with medium, to be submerged in it.
- *Inoculation loop and holder*: lengths of 24- or 26-gauge wire (7.5–10 cm) should be used. Nichrome wire is acceptable, but platinum–iridium is better. The wire is set in a handle made of metal or glass, of diameter similar to that of a pencil. To make the inoculation loop, the wire is bent to form a circle 3–4 mm in diameter.
- *Dispenser*: for sodium thiosulfate solution (see below).
- *Cleaning and maintenance equipment*: items such as brushes for cleaning tubes, bottles, etc., a waste bin, and a tool kit are required.
- *Safety equipment*: there should be an adequate first-aid kit and a fire extinguisher or other means of fire control in every laboratory.
- *General laboratory equipment*: various sizes of round and Erlenmeyer flask, beakers, stands, glass or unbreakable plastic measuring flasks, spatulas, etc. are required.

The following equipment is also desirable in a laboratory:

- *Refrigerator*: for the storage of prepared culture media.
- *Hot air sterilizer*: for sterilizing pipettes.

The following consumable items are required:

- *Culture medium*: Table A5.1 describes the uses for the various media; see also section A5.5.
- *Laboratory disinfectant*: for cleaning laboratory surfaces and the pipette discard bin.
- *Detergent*: for washing glassware, etc.
- *Sodium thiosulfate solution*: required when chlorinated supplies are tested.

Sodium thiosulfate neutralizes any residual chlorine in samples at the time of collection, preventing it from acting on any microorganisms present in water samples.

- *Autoclave tape.*
- *Diluent:* typical diluents include Ringer's solution and phosphate-buffered saline.

Table A5.1 Culture media for MPN^a

Medium	Uses	Incubator temperature	Remarks
MacConkey broth	Presumptive isolation of coliform bacteria	35 ± 0.5°C or 37 ± 0.5°C	Traditional medium for the presumptive isolation of coliform bacteria by MPN. The quality of bile salts can vary and may affect the result
Lauryl tryptose (lactose) broth	Presumptive isolation of coliform bacteria	35 ± 0.5°C or 37 ± 0.5°C	—
	Confirmation of thermotolerant coliform bacteria	44°C	—
Improved formate lactose glutamate medium	Presumptive isolation of coliform bacteria	35 ± 0.5°C or 37 ± 0.5°C	This is a selective medium because it contains chemically defined nutrients that can be utilized only by a limited number of bacterial species. The composition of the medium is complex and special care is required during preparation
Brilliant green lactose (bile) broth; EC	Confirmation of coliform bacteria	35 ± 0.5°C or 37 ± 0.5°C	Media for gas production
	Confirmation of thermotolerant coliform bacteria	44°C	
Tryptone water	Production of indole for confirmation of <i>Escherichia coli</i>	44°C	The formation of indole, detected by the addition of Kovacs reagent ^b to tryptone water after incubation, with gas production from lactose at 44°C indicates the presence of <i>E. coli</i>

^a Adapted from ISO 9308-2: 1990. Detection and enumeration of coliform organisms, thermotolerant coliform organisms, and presumptive *Escherichia coli*—Part 2: Multiple tube (most probable number) method.

^b To make Kovacs reagent, dissolve 5g *p*-dimethylaminobenzaldehyde in 75ml amyl (or isoamyl) alcohol, and add 25ml concentrated hydrochloric acid slowly. Store at 4°C in the dark.

A5.5 Culture media and dilution water

Commercially available dehydrated media simplify the preparation of culture broths and are therefore recommended for laboratory work. Various manufacturers produce these media as powders, which can then be easily weighed out, dissolved in distilled water, and dispensed into culture tubes before sterilization.

A5.5.1 Preparation of media

Media should be prepared in accordance with the manufacturer's instructions, as follows:

- (a) Dissolve the stated amount of the dehydrated medium in distilled water to obtain the double-strength or single-strength presumptive medium (for confirmatory analysis, only single-strength medium is used).
- (b) Dispense the requisite volume into culture tubes containing an inverted Durham tube, and cap the culture tubes.
- (c) Sterilize in an autoclave or pressure cooker at 115°C for 10 minutes (or in accordance with the manufacturer's specifications). It is particularly important that media containing disaccharides, e.g. lactose, are not autoclaved at higher temperatures.
- (d) The sterilized medium may be stored at room temperature (approximately 25°C) or, ideally, at 2–8°C. Media should in any case be warmed to room temperature before use to ensure that all components have redissolved. In addition, since several dyes are light-sensitive, the solution should be protected from exposure to light.

A5.5.2 Preparation of dilution water

A special buffered, sterilized water is used to make sample dilutions for inoculation into the culture medium. It is prepared from a concentrated stock solution of phosphate buffer. To make the stock solution, dissolve 34g of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of distilled water. The pH should be checked with a pH-meter; it should be 7.2. It can be increased if necessary by adding a few drops of sodium hydroxide solution (4.0g dissolved in 1000 ml of distilled water). Then add sufficient distilled water to make up to 1 litre. When the stock solution is not in use, it should be stored in a tightly closed bottle at 4–10°C, to prevent microbial growth.

When using the dilution water, add 1.25 ml of stock phosphate solution to 1 litre of distilled water and dispense into bottles for sterilization in the autoclave. Before sterilization, loosen the stoppers of the bottles. Sterilize for 15 minutes at 121°C. Tighten the stoppers after sterilization and store the dilution water in a clean place until needed.

An alternative dilution water can be prepared by the addition of magnesium chloride and has been shown to give a slightly higher recovery rate. Other

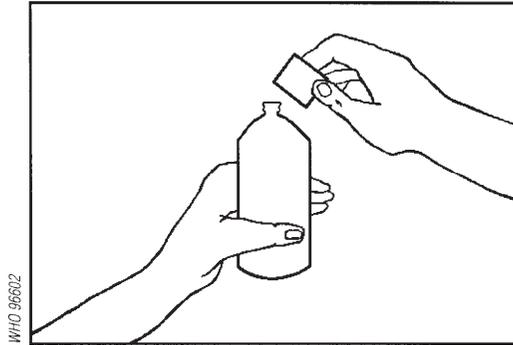
alternatives include a 0.1% solution of peptone in distilled water (final pH 6.8), Ringer's solution, and physiological saline (9g of sodium chloride per litre). These should be sterilized after dispensing into bottles, as described above.

A5.6 Application to unpolluted water

A5.6.1 Procedure

The procedure to be used for testing relatively unpolluted water, such as treated water from waterworks, is described below.

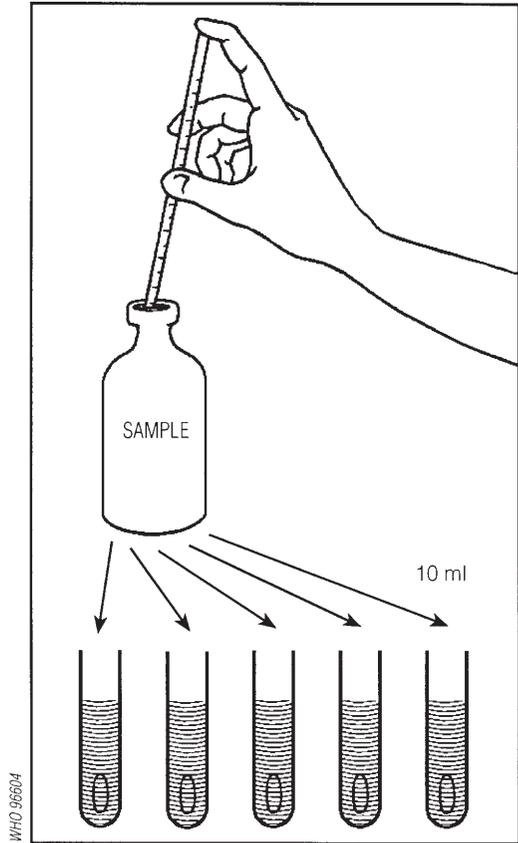
A. Remove the cap from the sample bottle.



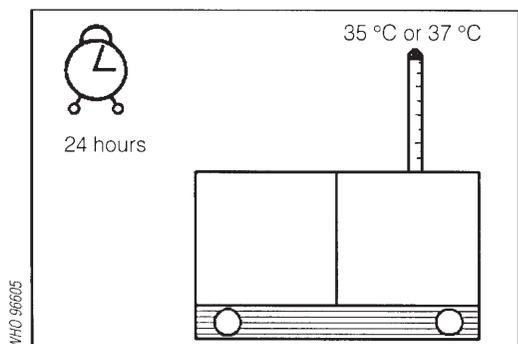
B. With the stopper in position, shake the bottle vigorously to achieve a homogeneous dispersion of bacteria. (If the bottle is completely full, remove the stopper and discard about 20–30 ml of water; then replace the stopper and shake. This ensures thorough mixing.)



C. With a sterile 10-ml pipette, inoculate 10 ml of the sample into each of five tubes containing 10 ml of presumptive broth (double strength). Add 50 ml of sample to a tube containing 50 ml of presumptive broth. It is advisable to shake the tubes gently to distribute the sample uniformly throughout the medium.

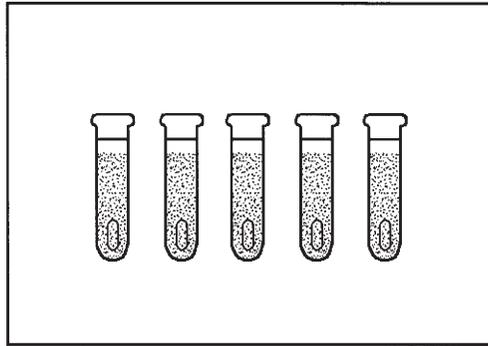


D. Incubate the tubes at 35°C or 37°C for 24 hours.



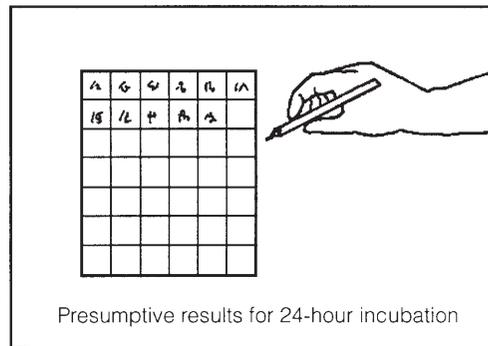
E. At the end of the 24-hour incubation period, examine each tube for the presence of gas. If present, gas can be seen in the Durham tube. If none is visible, gently shake the tube; if any effervescence (streams of tiny bubbles) is observed, the tube should be considered positive.

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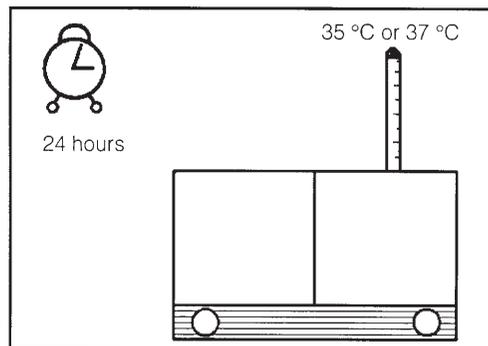
F. Using a table like the one shown here, record the number of positive tubes after 24 hours.

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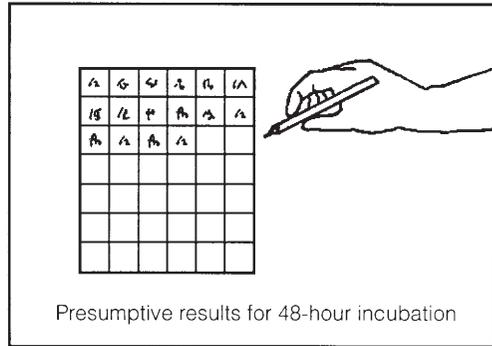


G. Reincubate negative tubes for a further 24-hour period. At the end of this period, check the tubes again for gas production as in E above. Gas production at the end of either 24 or 48 hours' incubation is presumed to be due to the presence of coliforms in the sample.

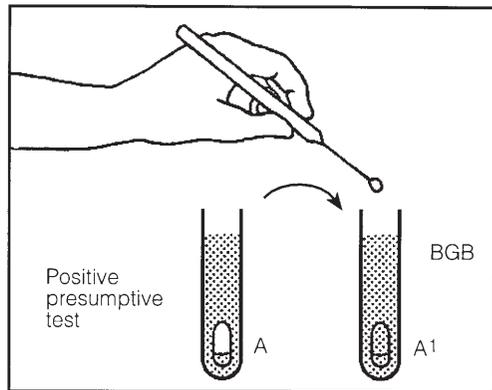
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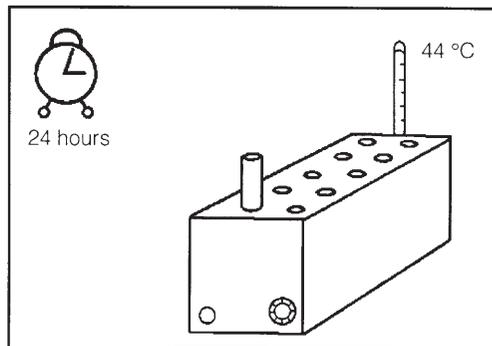
H. Record the number of positive tubes after 48 hours.



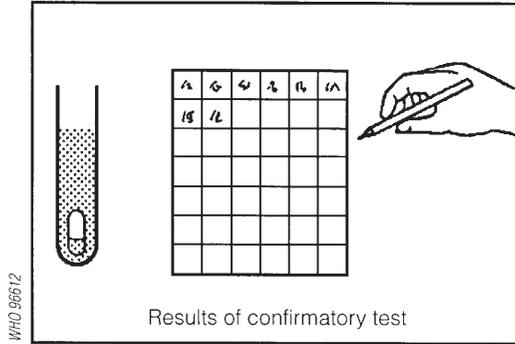
I. The confirmatory test should be carried out at the end of both the 24-hour and the 48-hour incubation. Using a sterile loop, transfer one or two drops from each presumptive positive tube into two tubes containing respectively confirmatory broth and tryptone water. (Sterilize the inoculation loop before each transfer by flaming and allow to cool.)



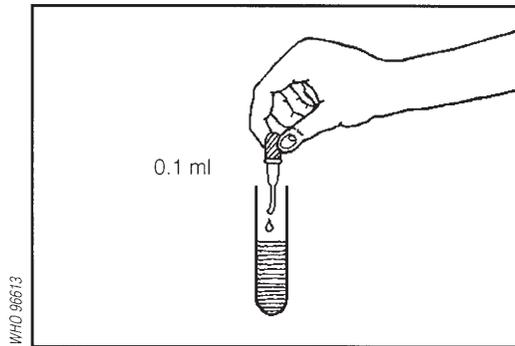
J. To confirm the presence of thermotolerant coliforms, incubate the subculture tubes from each presumptive positive tube for 24 hours at $44 \pm 0.5^\circ\text{C}$.



K. At the end of 24 hours' incubation, examine each broth tube for growth and the presence of gas in the Durham tube. Enter the results on the table as shown.



L. To each tube of tryptone water, add approximately 0.1 ml of Kovacs reagent (see Table A5.1, p. 192) and mix gently. The presence of indole is indicated by a red colour in the Kovacs reagent, forming a film over the aqueous phase of the medium.



M. Confirmatory tests positive for indole, growth, and gas production show the presence of *E. coli*. Growth and gas production in the absence of indole confirms thermotolerant coliforms.

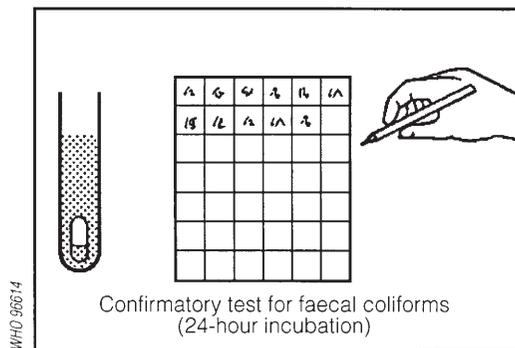


Table A5.2 MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when one 50-ml and five 10-ml test portions are used)

No. of tubes giving a positive reaction		MPN (per 100 ml)	95% confidence limits	
1 of 50 ml	5 of 10 ml		Lower	Upper
0	0	<1	—	—
0	1	1	<1	4
0	2	2	<1	6
0	3	4	<1	11
0	4	5	1	13
0	5	7	2	17
1	0	2	<1	6
1	1	3	<1	9
1	2	6	1	15
1	3	9	2	21
1	4	16	4	40
1	5	>18	—	—

A5.6.2 Determination of MPN

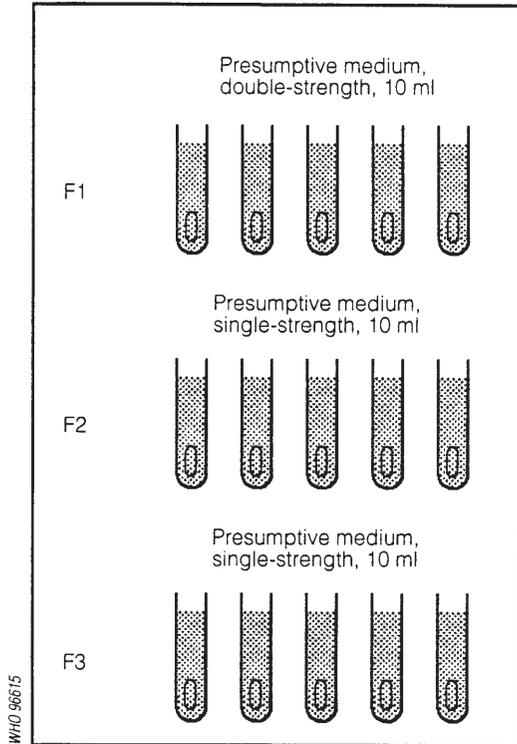
For treated water, where one 50-ml and five 10-ml portions are inoculated, the MPN can be found from the test results by means of Table A5.2.

A5.7 Application to polluted water (full method)

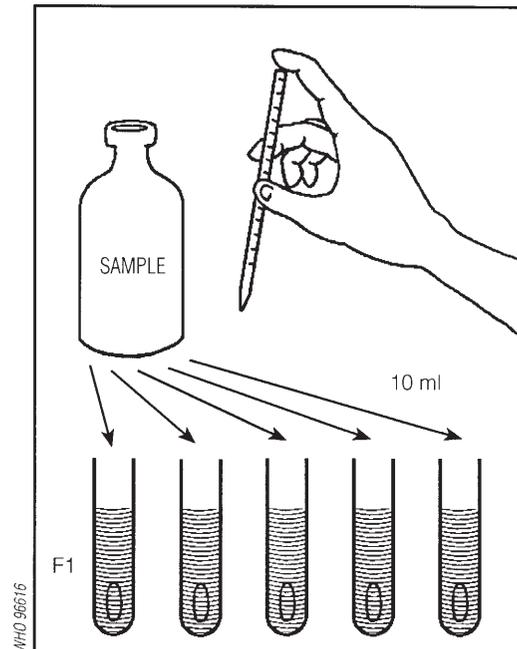
A5.7.1 Procedure

The procedure to be used for the testing of water that is expected to be polluted, even though it may have been treated, is shown below and is essentially similar to that described in section A5.6, with the exception that several dilutions are used.

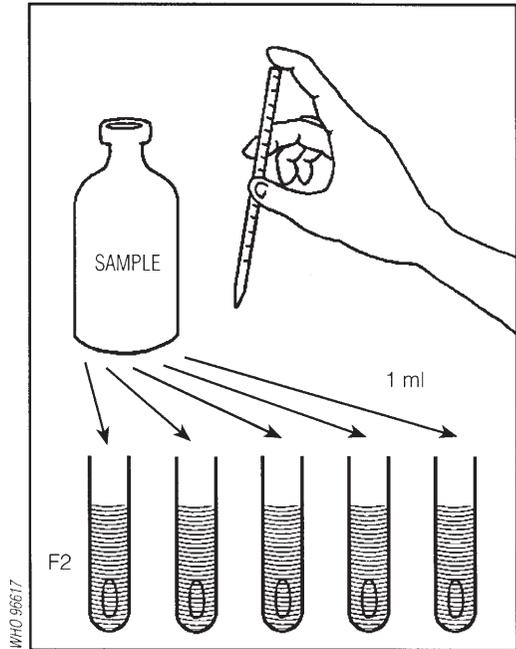
A. Arrange three rows of five tubes each in a test-tube rack. The tubes in the first row (F1) hold 10 ml of double-strength presumptive medium while the tubes in the second and third rows (F2, F3) contain 10 ml of single-strength presumptive medium.



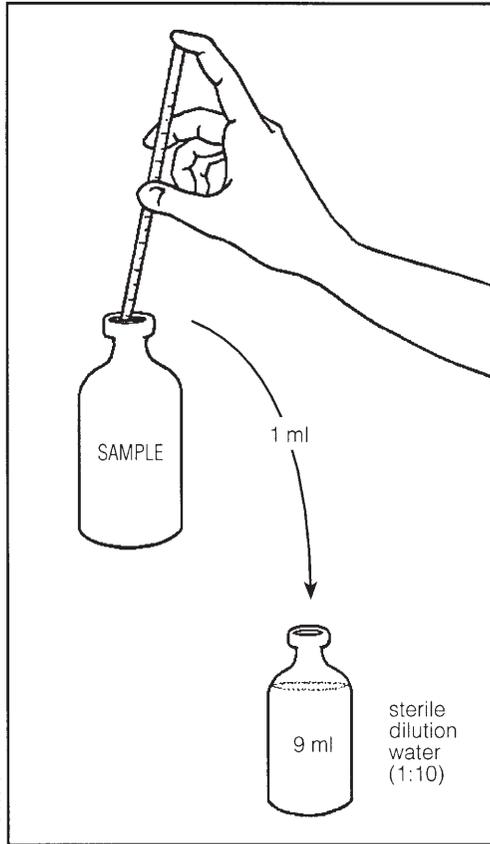
B. With a sterile pipette add 10 ml of sample to each of the five tubes in row F1.



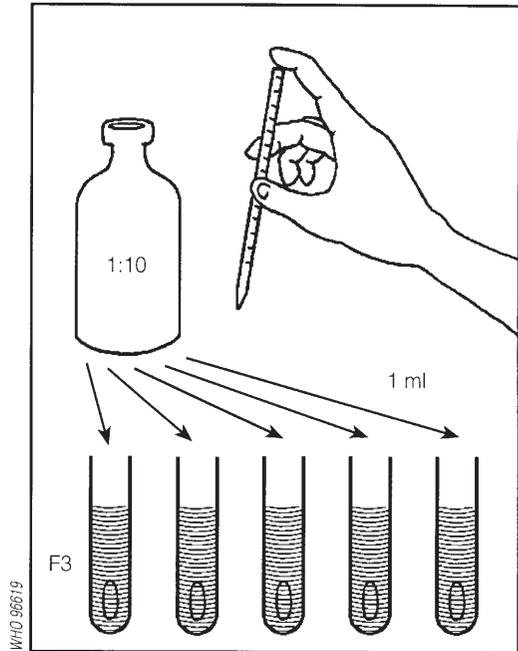
C. With a sterile pipette, add 1ml of sample to each of the five tubes in row F2.



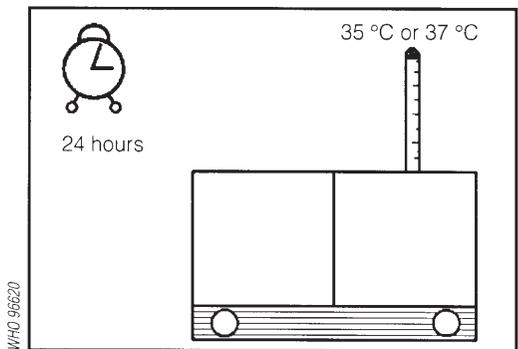
D. Prepare a 1:10 dilution of the sample by adding 1ml of sample to 9ml of dilution water (use a 1-ml sterile pipette). Recap the bottle containing the diluted sample and shake it vigorously.



E. With another sterile pipette add 1 ml of the 1:10 dilution to each of the five tubes in row F3.



F. After gently shaking the tubes to mix the contents, incubate the rack with the 15 tubes at 35°C or 37°C for 24 hours. Then proceed in the same way as for *unpolluted* water.



A5.7.2 Determination of MPN

The MPN is found in a similar way to that described in section A5.6.2 but, because of the large number of tubes involved, a more complicated table—Table A5.3—must be used.

The following example shows how the results are obtained.

Suppose that, after confirmation of the presence of thermotolerant (faecal) coliforms, the following results are obtained:

- 5 positive tubes in row F1 (sample volume inoculated, 10 ml)
- 3 positive tubes in row F2 (sample volume inoculated, 1 ml)
- 1 positive tube in row F3 (sample volume inoculated, 0.1 ml).

Table A5.3 MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when five 10-ml, five 1-ml and five 0.1 ml test portions are used)

No. of tubes giving a positive reaction :			MPN (per 100 ml)	95% confidence limits	
5 of 10ml	5 of 1 ml	5 of 0.1 ml		Lower	Upper
0	0	0	<2	<1	7
0	1	0	2	<1	7
0	2	0	4	<1	11
1	0	0	2	<1	7
1	0	1	4	<1	11
1	1	0	4	<1	11
1	1	1	6	<1	15
2	0	0	5	<1	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46
3	3	0	17	5	46
4	0	0	13	3	31
4	0	1	17	5	46
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78
4	2	0	22	7	67
4	2	1	26	9	78
4	3	0	27	9	80
4	3	1	33	11	93
4	4	0	34	12	93
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	110
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	150
5	2	0	49	17	130
5	2	1	70	23	170
5	2	2	94	28	220
5	3	0	79	25	190
5	3	1	110	31	250
5	3	2	140	37	340
5	3	3	180	44	500

Table A5.3 (continued)

No. of tubes giving a positive reaction :			MPN (per 100 ml)	95% confidence limits	
5 of 10 ml	5 of 1 ml	5 of 0.1 ml		Lower	Upper
5	4	0	130	35	300
5	4	1	170	43	490
5	4	2	220	57	700
5	4	3	280	90	850
5	4	4	350	120	1000
5	5	0	240	68	750
5	5	1	350	120	1000
5	5	2	540	180	1400
5	5	3	920	300	3200
5	5	4	1600	640	5800
5	5	5	>1800	—	—

The results can thus be coded as 5–3–1; they represent the confirmatory test for thermotolerant coliforms. Table A5.3 indicates that a coded result of 5–3–1 (5 × 10 ml positive, 3 × 1 ml positive, 1 × 0.1 ml positive) gives an MPN value of 110, i.e. the water sample contains an estimated 110 coliforms per 100 ml.

Next, consider an example of heavily polluted water. The procedure outlined above may give a coded result of 5–5–5. Such a result does not give a definite MPN value. When such heavy contamination is suspected it is usual to inoculate more than three dilutions in a series of factors of 10. This series of 10-fold dilutions should be made in such a way that a negative result is likely for at least the highest dilution incubated. If 5 × 1.0 ml, 5 × 0.1 ml, 5 × 0.01 ml, and 5 × 0.001 ml are initially inoculated and a confirmed coded result of 5–5–4–1 is obtained, only three of these results should then be used to obtain the MPN value from Table A5.3. These should be selected by choosing the smallest sample volume (in this case, 0.1 ml) for which all the tubes give a positive result, and the two next succeeding higher dilutions. The coded result of these three volumes is then used to obtain the MPN value from Table A5.3. In the above example, the result 5–4–1 would be chosen, representing volumes of 0.1, 0.01, and 0.001 ml of the sample. The MPN value obtained from Table A5.3 should be multiplied by 100 to obtain the MPN for this particular sample (see below); in this case, the result is 17 000 per 100 ml.

Sometimes the laboratory worker may find it difficult to determine the multiplying factor to be used to obtain the appropriate MPN for the sample tested. A simple way to determine the MPN is to divide the MPN value obtained from Table A5.3 by the sample volume represented by the middle number in the chosen code. For example, consider a chosen code of 5–2–0, in which the 2 represents a sample volume of 0.01 ml (see Table A5.4). From Table A5.3, MPN for a code of 5–2–0 is 49. The MPN value for the sample tested will therefore be:

$$(49/0.01) = 49 \times 100 = 4900.$$

Table A5.4 Example of multiplying factors for determination of the MPN for different dilutions of sample

Example	No. of tubes giving a positive reaction					Coded result chosen	Multiplying factor for MPN
	5 of 1 ml	5 of 0.1 ml	5 of 0.01 ml	5 of 0.001 ml	5 of 0.0001 ml		
1	5	5	2	0	0	5-2-0	100
2	5	5	4	1	0	5-4-1	100
3	5	3	0	0	0	5-3-0	10
4	5	5	5	3	1	5-3-1	1000
5	0	1	0	0	0	0-1-0	10

Table A5.5 MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when three 10-ml, three 1-ml, and three 0.1-ml test portions are used)

No. of tubes giving a positive reaction			MPN (per 100 ml)	95% confidence limits	
3 of 10 ml	3 of 1 ml	3 of 0.1 ml		Lower	Upper
0	0	1	3	<1	9
0	1	0	3	<1	13
0	0	0	4	<1	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	49
2	2	0	21	4	47
2	2	1	28	10	149
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	379
3	1	0	48	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800

Examples are given in Table A5.4 of the factors to be used to multiply the MPN value found in order to obtain the appropriate MPN for different dilutions.

A5.8 Application to polluted water: “shorter method”

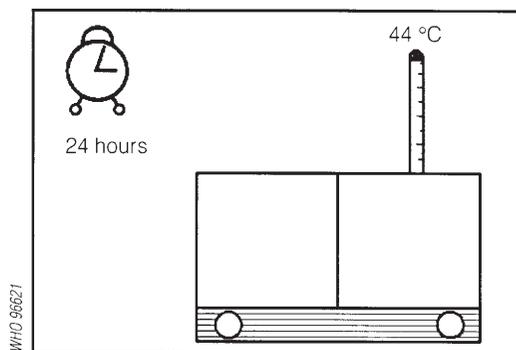
The procedure for the shorter method is almost identical to that described in section A5.7, with the single difference that only three tubes of each sample volume are inoculated, instead of five. This requires the use of a different table—Table A5.5—for determining the MPN.

A5.9 Direct thermotolerant coliform method

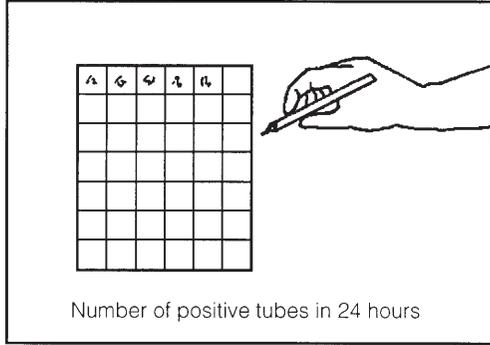
If *unchlorinated* water from small-community water supplies is tested and only the number of thermotolerant coliforms is of interest, a direct multiple-tube method can be used. This is recommended for use where the total coliform result is not of great significance, e.g. in small-community supplies in developing countries or where space, time, or facilities are limited. The method is based on the normal MPN procedure, but the tubes are incubated directly in a water-bath at $44.5 \pm 0.2^\circ\text{C}$, without previously incubating at 35 or 37°C for 24 hours and testing for total coliforms.

The procedure is similar to that described for the examination of polluted water, except that MacConkey broth is used as the presumptive medium. Prepare 15 tubes of sample and medium, as described on pp. 199–203, and then proceed as shown below.

A. After gently shaking the tubes to mix the contents, incubate the 15 tubes at 44°C for 24 hours.

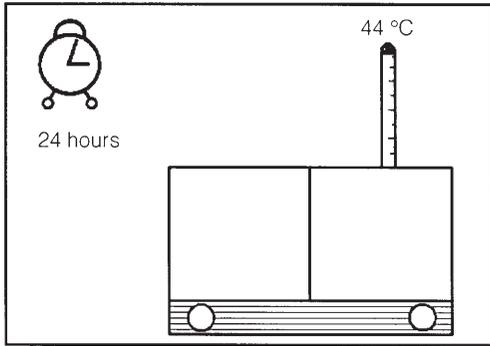


B. Observe each tube for the presence of gas and enter the number of positive tubes after 24 hours in the appropriate table.



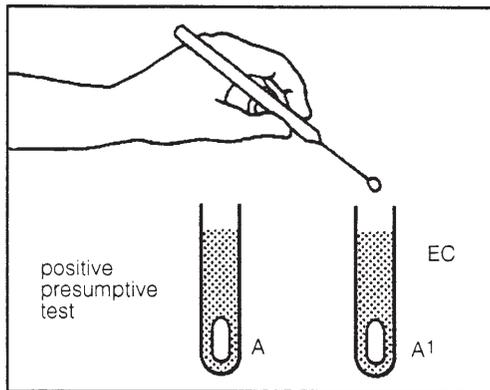
WHO 96622

C. Negative tubes should be reincubated for a further 24-hour period, after which they should be observed for the presence of gas.



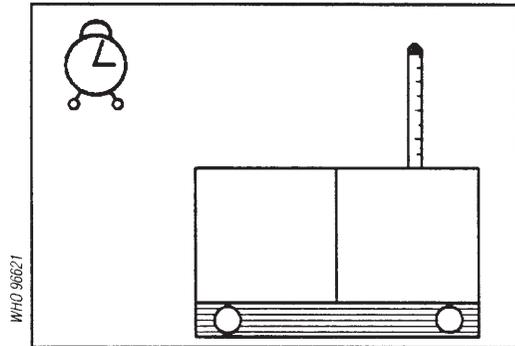
WHO 96621

D. Confirm the presumptive results after 24 and 48 hours by transferring a loopful of broth to a confirmatory broth and incubating at 44 °C for 24 hours.



WHO 96623

E. The presence of thermotolerant coliforms is confirmed if gas is present in the confirmatory broth after 24 hours at 44°C. Determine the MPN from Table A5.3 as before.



A5.10 Selection of tubes for confirmatory test

Any bacteriological analysis should always include the confirmatory test. If only five 10-ml portions are tested, the confirmatory test for coliforms and thermotolerant coliforms must be carried out on all tubes showing gas production. However, if the inoculation involved five (or three) tubes for each of, or more than, three sample volumes (e.g. 10, 1.0, 0.1, 0.01, and 0.001 ml), it is not necessary to carry out confirmatory tests on all the positive tubes.

If all five (or three) tubes of two or more consecutive dilutions are positive, the set of tubes should be selected that presents the smallest sample volume for which all the tubes are positive. The confirmatory test should be carried out on all these tubes and on all the positive tubes corresponding to subsequent and lower volumes. The following example should help to illustrate this procedure. After 24 hours' incubation, five tubes with 10 ml, five with 1.0 ml, five with 0.1 ml, four with 0.01 ml, and one with 0.001 ml gave positive results. Thus the confirmatory test should be carried out on the positive tubes initially inoculated with 0.1, 0.01, and 0.001 ml of sample.

A5.11 Record forms

The analysis of a given sample will provide several results. The form drawn up for recording these results, although it should not be complicated, must be completed. The completed form should contain the data on the sampling, which will also serve to identify the samples, those entered on the sample dispatch form, and data on the bacteriological analysis itself. A suggested comprehensive form is shown in Fig. A5.1. Once the analysis is completed, the laboratory carrying out the work should record the results obtained in a standardized form (protocol); this should follow the recommendations given in Chapter 3. The protocol can be a very simple report, which records the sample identification information together with the result of the analysis and the appropriate classification of the water. An example of such a protocol is shown in Fig. A5.2.

Fig. A5.2 Suggested protocol for results of bacteriological analysis

	WATER-QUALITY CONTROL PROGRAMME	BACTERIOLOGICAL WATER ANALYSIS
[..... Authority]		
COMMUNITY:		SAMPLE NO.
SAMPLE SITE:		
PLACE:		
SOURCE:		
SENDER:		
DATE OF SAMPLING	____ / ____ / ____	TIME:
DATE OF ANALYSIS	____ / ____ / ____	TIME:
	RESIDUAL FREE CHLORINE	<div style="border: 1px solid black; width: 40px; height: 20px; display: inline-block;"></div> mg/litre
<u>RESULTS</u>		
TOTAL COLIFORMS	/100 ml	
FAECAL COLIFORMS	/100 ml	
WATER BACTERIOLOGICALLY		
GOOD – BAD	 Laboratory Technician
	 Chief (Signed)

WHO 96624

Membrane filtration method for thermotolerant (faecal) coliforms

A6.1 Principle

In contrast to the multiple-tube method, the membrane-filtration method gives a direct count of total coliforms and thermotolerant coliforms present in a given sample of water. The method is based on the filtration of a known volume of water through a membrane filter consisting of a cellulose compound with a uniform pore diameter of 0.45 or 0.2 µm; the bacteria are retained on the surface of the membrane filter. When the membrane containing the bacteria is incubated in a sterile container at an appropriate temperature with a selective differential culture medium, characteristic colonies of thermotolerant coliforms develop, which can be counted directly.

A6.2 Volume of water sample for filtration

Since the filtration area is relatively small, it can support the growth of only a limited number of colonies: the optimum number is between 20 and 80, with a maximum of 200. If this figure is exceeded, very small atypical colonies or superimposed colonies may develop, or there may be growth inhibition due to overpopulation. The choice of the volume of sample to be filtered will depend on the type of water. Examples of typical volumes are provided in Table 4.3 (p. 61).

A6.3 Equipment and glassware

In addition to the basic equipment and glassware used in the multiple-tube method (see Annex 5), the following items are needed for the membrane-filtration technique:

- *Membrane-filtration apparatus*: including an electric or hand-powered vacuum pump, a vacuum flask (e.g. an Erlenmeyer side-arm flask), and a filter support.
- *Reusable Petri dishes*: made from glass or metal (disposable plastic Petri dishes may also be used).
- *Blunt-ended forceps*: for picking up membrane filters.
- *Reusable (autoclavable) bottles*: for culture media (e.g. 25-ml polypropylene bottles).

- *A magnifying lens*: with $\times 4$ or $\times 5$ magnification for examining and counting the colonies on the membrane filters.
- *A boiling bath/pan*: if filtration apparatus is to be disinfected in boiling water between analyses.
- *Sterile pipettes*: 1 ml and 10 ml.
- *A graduated cylinder*: 100 ml.

In addition to the consumables needed for the MPN, the following are required:

- *Membrane filters*: 47–50 mm in diameter, with a pore diameter of $0.45\ \mu\text{m}$. Singly packed, presterilized membrane filters are very convenient. Unsterilized membrane filters can also be used, however, and should be wrapped in paper packets in convenient numbers (depending on the number of water samples to be tested). These can then be sterilized in the autoclave and dried by rapid exhaustion of the steam.
- *Nutrient absorbent pads*: filter-paper discs about 1 mm thick, with the same diameter as the membrane filters.
- *Culture media*: different types are available (see section A6.4).
- *Wax pencils*: for labelling Petri dishes.
- *Polythene bags*: for wrapping Petri dishes if a dry incubator is used, to prevent drying of the sample and media.

A6.4 Culture media and dilution water

Various media can be used for the examination of coliform organisms by the membrane-filtration method. Of these, lactose Tergitol¹ agar, lactose TTC Tergitol¹ agar, and membrane lauryl sulfate lactose broth may be used for coliform organisms at 35 or 37°C and for thermotolerant coliform organisms at 44°C or 44.5°C. Membrane faecal coliform (MFC) broth should be used only at 44 or 44.5°C for thermotolerant coliform counts. Although the use of all these media for the detection of presumptive coliform organisms is based on the fermentation of lactose, the characteristic reaction varies with each medium, as shown in Table A6.1.

Although it is possible to prepare the media from the basic ingredients, this may be impractical in a small laboratory. The use of dehydrated media is therefore recommended. The media can be prepared as a broth and used together with nutrient absorption pads, or as solid agar plates. The broths may be solidified by the addition of 1.2–1.5% agar before boiling.

A6.5 Procedure

The procedure generally used is described here, but different types of filtration units and equipment exist.

¹ Tergitol 7 is an example of a suitable product available commercially. This information is given for the convenience of the user and does not constitute an endorsement of this product by WHO.

Table A6.1 Colony characteristics following analysis by the membrane-filtration method^a

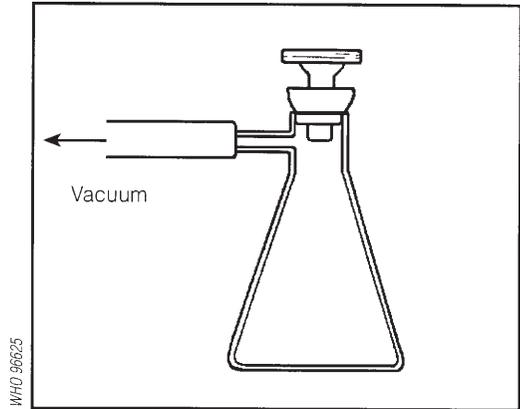
Medium ^b	Colony characteristics	
	Total coliforms at 35/37 °C	Thermotolerant coliforms at 44/45.5 °C
Lactose TTC ^c agar with Tergitol 7	Yellow, orange or brick-red coloration with yellow central halo in the medium under the membrane	As for total coliforms at 35/37 °C
Lactose agar with Tergitol 7	Yellow central halo in the medium under the membrane	As for total coliforms at 35/37 °C
Membrane-enriched Teepol broth	Yellow colour extending on to the membrane	As for total coliforms at 35/37 °C
Membrane lauryl sulfate broth	Yellow colour extending on to the membrane	As for total coliforms at 35/37 °C
Endo agar or broth	Dark red colour with golden-green metallic sheen	—
LES–Endo agar	Dark red colour with golden-green metallic sheen	—
Membrane faecal coliform (MFC) broth	—	Blue colonies

^a Adapted from ISO 9308-1: 1990, Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive *Escherichia coli*—Part 1: Membrane filtration method.

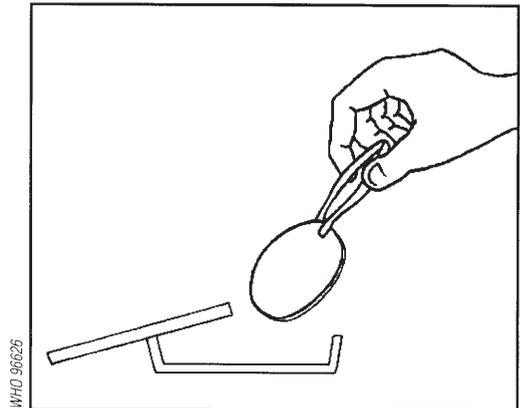
^b Tergitol 7 and Teepol are examples of suitable products available commercially. This information is given for the convenience of the user and does not constitute an endorsement of these products by ISO or WHO.

^c 2,3,5-Triphenyltetrazolium chloride.

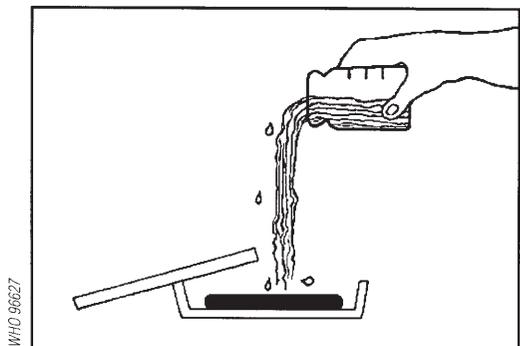
A. Connect the Erlenmeyer flask to the vacuum source (turned off) and place the porous support in position. If an electric pump is used, it is advisable to put a second flask between the Erlenmeyer flask and the vacuum source; this second flask acts as a water trap, and thus protects the electric pump.



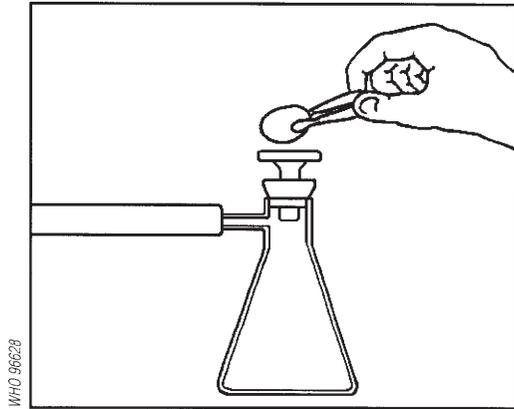
B. Open a sterile Petri dish and place a sterile absorbent pad in it.



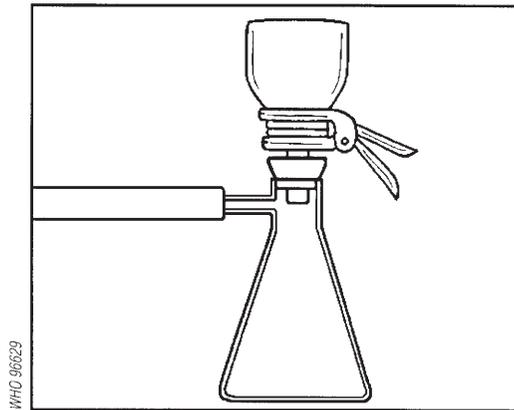
C. Add broth medium to saturate the pad; remove excess broth.



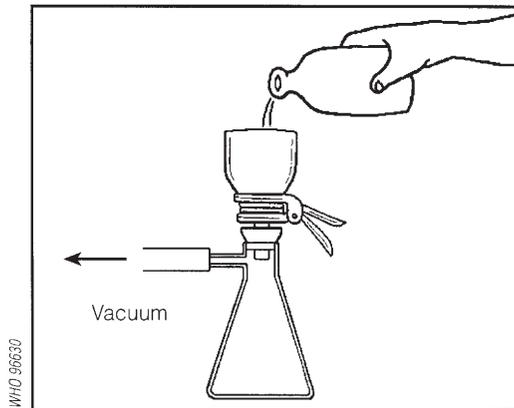
D. Assemble the filtration unit by placing a sterile membrane filter on the porous support, using forceps sterilized by flaming.



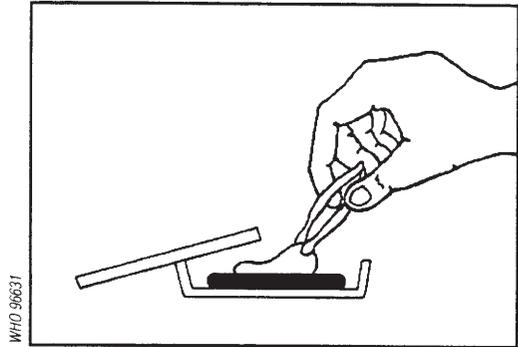
E. Place the upper container in position and secure it. (The type of clamp used will depend on the type of equipment.)



F. Pour the volume of sample chosen as optimal for the type of water (see Table 4.3, p. 61), into the upper container. If the test sample is less than 10 ml, at least 20 ml of sterile dilution water should be added to the top container before filtration. Apply the vacuum.

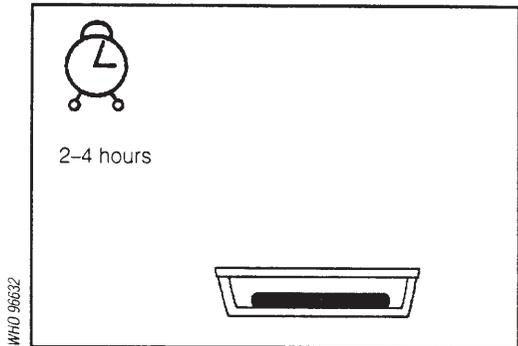


G. Take the filtration unit apart and, using the sterile forceps, place the membrane filter in the Petri dish on the pad with the grid side up. Make sure that no air bubbles are trapped between the pad and the filter.



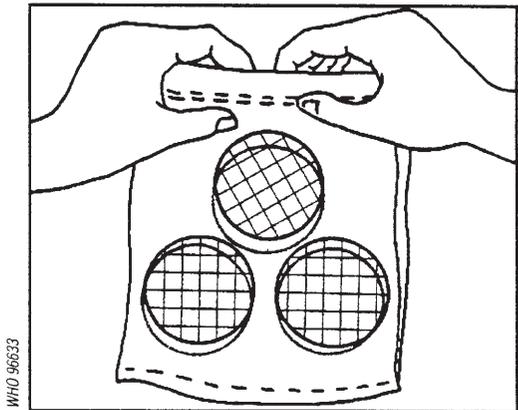
WHO 96631

H. Leave the Petri dish at room temperature or at 35 or 37 °C for 2–4 hours, for resuscitation of stressed microbes.



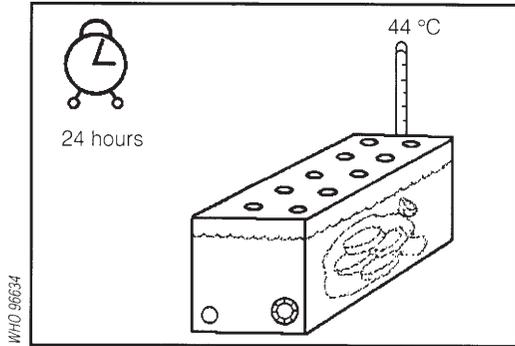
WHO 96632

I. Place the dishes in an incubator at $44 \pm 0.5^\circ\text{C}$ for 18–24 hours with 100% humidity. Alternatively, tight-fitting or sealed Petri dishes may be placed in waterproof plastic bags for incubation.



WHO 96633

J. Submerge the bags in a water-bath maintained at $44 \pm 0.5^\circ\text{C}$ for 18–24 hours. The plastic bags must be below the surface of the water throughout the incubation period. They can be held down by means of a suitable weight, e.g. a metal rack.



The colonies of thermotolerant coliform bacteria should be identified from their characteristics on the medium used. The number of thermotolerant coliforms per 100 ml is then given by:

Thermotolerant coliforms per 100 ml

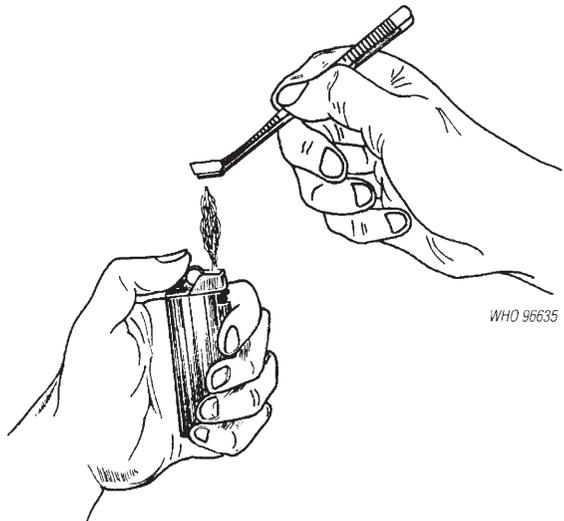
$$= \frac{\text{no. of thermotolerant coliform colonies counted}}{\text{no. of ml of sample filtered}} \times 100$$

Annex 7

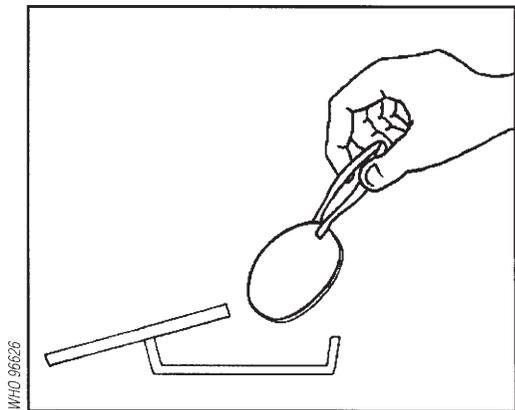
Field test method for thermotolerant coliforms

The field test method for thermotolerant coliforms involves the following:

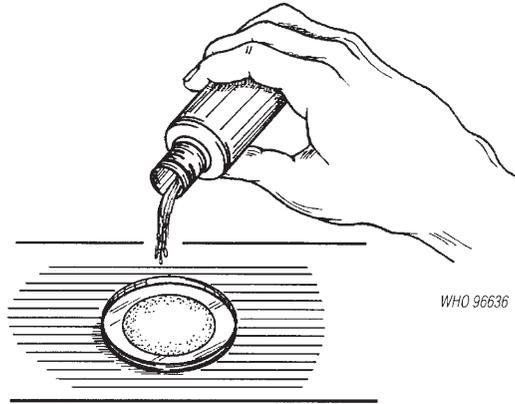
A. Flame-sterilize the tips of blunt-ended forceps and allow to cool between successive manipulations of the filters.



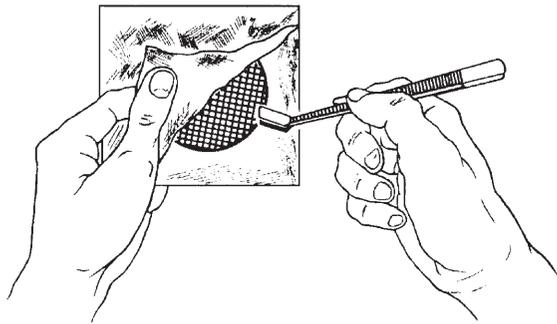
B. Place a sterile absorbent pad in a sterile Petri dish.



C. Add broth medium to saturate the pad and remove the excess broth.



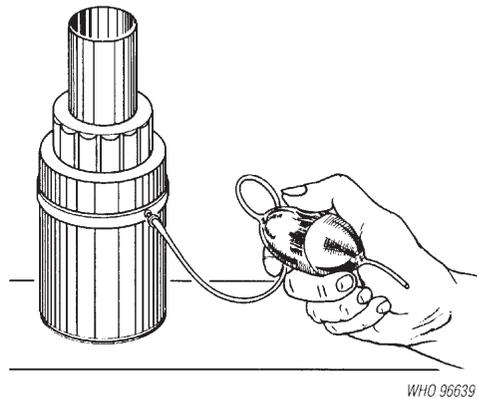
D. Sterilize the filter apparatus and assemble by placing a sterile filter membrane on the membrane support.



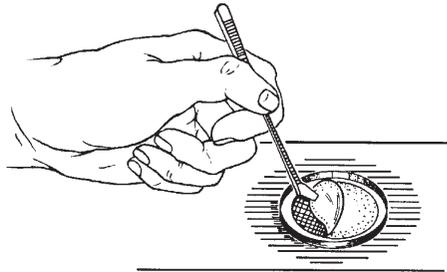
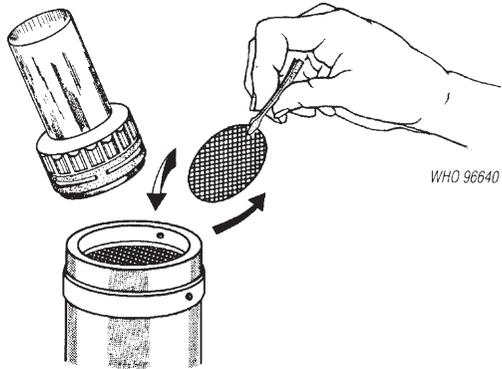
E. Mix the sample thoroughly by inverting the sample bottle several times, and put the volume to be tested into the previously sterilized filtration apparatus. The appropriate volume of sample should be selected in accordance with the type of water being tested (see Table 4.3, p. 61).



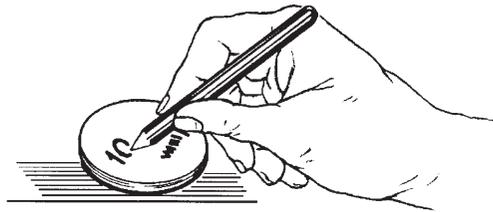
F. Apply a vacuum to the filter apparatus to draw the sample through the filter membrane. Disconnect the vacuum and dismantle the apparatus.



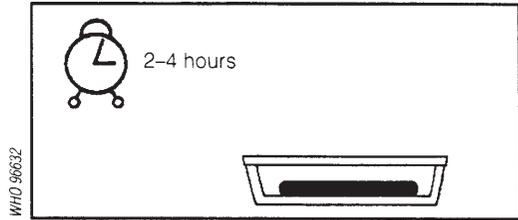
G. Using sterile forceps, remove the membrane filter from the filter apparatus and transfer it to the nutrient pad in the Petri dish. Lower the membrane, grid side uppermost, carefully onto the nutrient pad, making sure that no air bubbles are trapped between the pad and the filter.



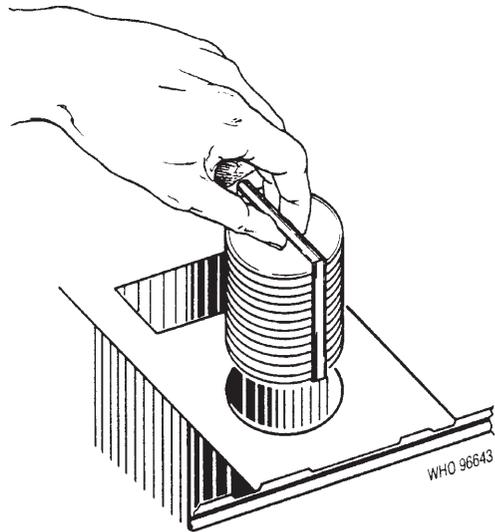
H. Replace the lid on the Petri dish and label with the sample identification code using a wax pencil or waterproof pen.



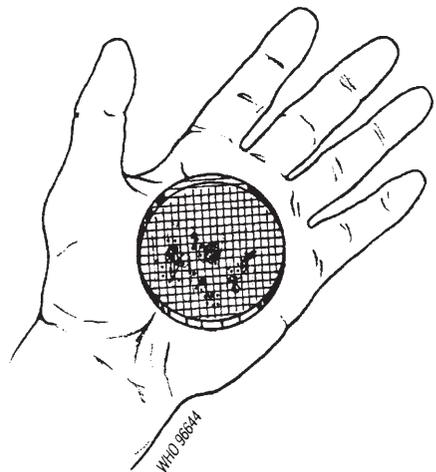
I. Incubate the Petri dish at ambient temperature for 2–4 hours to allow stressed bacteria to resuscitate.



J. Incubate the Petri dish at the selected temperature for 18–24 hours.



K. Following incubation, count all colonies with a morphology typical of the bacterium and the medium used. Calculate and express the result in colony-forming units (CFU) per 100 ml of sample.



Presence–absence test for total coliform bacteria

Presence–absence tests may sometimes be appropriate where positive results are known to be rare. They are not quantitative and, as their name suggests, they indicate only the presence or absence of the indicator sought. Such results are of little use in countries or situations where contamination is common and the purpose of the analysis is then to determine the degree of contamination rather than simply to indicate its presence. Thus presence–absence tests are *not recommended* for use in the analysis of surface waters, untreated small-community supplies, or larger water supplies in countries where operation and maintenance difficulties may occasionally occur.

Before a decision is taken to use the presence–absence test for the analysis of a water source, the results obtained by the test should be compared with those obtained with a recognized, quantitative method of analysis. Approximately 100 samples should be examined by both methods.

A8.1 Preparation of medium

The constituents of the medium used for the presence–absence test for coliform bacteria are as follows:

lactose broth (dehydrated)	13.0 g
lauryl tryptose broth (dehydrated)	17.5 g
bromocresol purple	0.0085 g
distilled water	1 litre

Make this formulation triple-strength when examining 100-ml samples.

The medium is prepared in the following stages:

- Dissolve the dehydrated lactose broth and lauryl tryptose broth sequentially in water, without heating.
- Dissolve the bromocresol purple in 10 ml of sodium hydroxide solution (4 g of NaOH in 1 litre of water). Sodium hydroxide pellets are caustic and great care should be taken during the preparation of the solution; in particular, gloves and eye protection should be worn.
- Add the bromocresol purple solution to the broth solution.
- Dispense 50 ml of the medium into screw-cap glass dilution bottles of capacity 250–300 ml. A fermentation tube is not necessary.

- (e) Autoclave for 12 minutes at 121 °C, limiting the total time in the autoclave to 30 minutes or less.
- (f) Measure the pH of the medium after autoclaving; it should be 6.8 ± 0.2 .

A8.2 Procedure

- (a) Mix the sample thoroughly by inverting the sample bottle several times.
- (b) Add 100 ml of the sample to the dilution bottle.
- (c) Incubate at 35 ± 0.05 °C and examine after 24 and 48 hours.
- (d) A positive result (acid production) is indicated by a distinct yellow colour in the medium. Shake the bottle gently and examine for foaming, which indicates the production of gas. Any test in which gas and/or acid is produced should be regarded as a positive presumptive test.
- (e) Positive presumptive tests should be confirmed by inoculating a tube of brilliant-green lactose–bile (BGLB) broth with cultures that show acid and/or gas production and incubating at 35 ± 0.5 °C. Growth and the production of gas in the BGLB broth culture within 48 hours confirm the presence of coliform bacteria.

Other indicator bacteria can be detected by the presence–absence test by selecting the appropriate confirmatory medium.

Residual free chlorine test

The method recommended for the determination of chlorine residual in drinking-water employs *N,N*-diethyl-*p*-phenylenediamine, more commonly referred to as DPD. Methods employing orthotolidine and starch–potassium iodide were formerly also recommended. The first of these reagents is a recognized carcinogen and the method is not reliable. The method based on the use of starch–potassium iodide is not specific for free chlorine, but measures directly the total of free and combined chlorine; it is not recommended except in countries where DPD cannot be obtained or prepared. In this Annex, therefore, only the DPD method is considered.

In the laboratory, photolorimetry or spectrophotometry may both be used for the determination of chlorine by means of DPD. However, it is common practice and highly recommended for field measurements using simple colour-match comparators to be done on site. The colour is generated following the addition of DPD to the water sample and is matched against standard coloured discs or tubes. The method can be used by staff without extensive specialized training. The reagent may be solid (e.g. individually wrapped tablets) or in the form of a solution; the former is more stable. If the solution is used, it should be stored in a brown bottle and discarded as soon as it starts to become discoloured.

A9.1 Commercial visual comparator technique

A9.1.1 Equipment

Commercial comparators are of two basic types—the disc type, containing a wheel of small coloured glasses, and the slide type, containing liquid standards in glass ampoules. However, both consist of the same components: a box with an eye-piece in front and two cells, the whole arranged so that both cells are in the field of vision of the eye-piece.

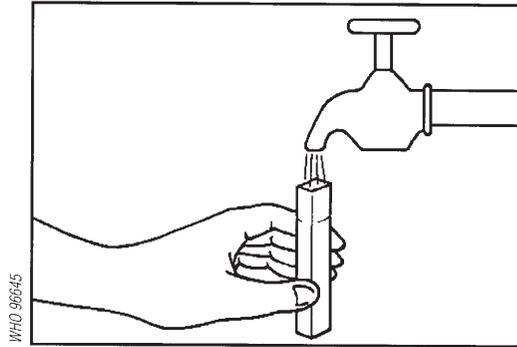
One cell, containing a water sample without the reagents, is placed in line with the rotating coloured glasses or the ampoules containing the standards. The water sample containing the reagent is placed in another cell. If free chlorine is present, a colour will develop. The concentration of chlorine is estimated by matching the colours in both cells, as seen through the eye-piece. Each colour of the disc or ampoule corresponds to a certain quantity of chlorine in the water; different calibration discs or ampoules are needed for each of the reagents specified.

A9.1.2 Reagents

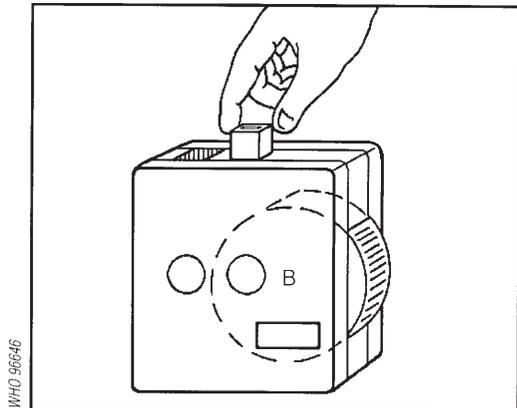
Most comparators are intended for use with the manufacturer's own reagents, and care must therefore be taken to keep a good stock of these. This is a disadvantage, since it involves dependence on the local supplier, and importation problems may occasionally arise. On the other hand, it is not necessary to prepare solutions of standards, which makes the technique very easy to use.

A9.1.3 Method

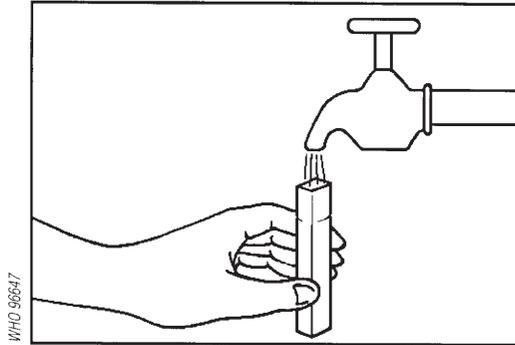
A. Rinse a comparator cell two or three times, and then fill it up to the mark with the water sample.



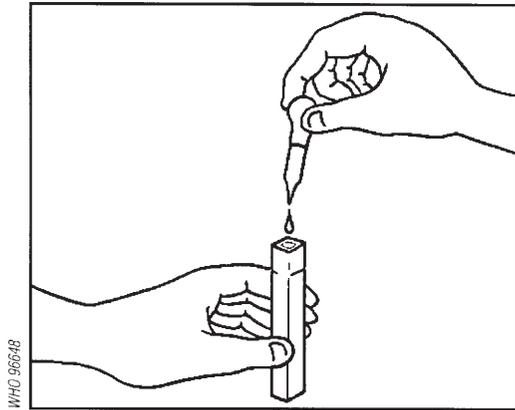
B. Place the cell in the cell carrier of the comparator, which is in line with the coloured standards (B).



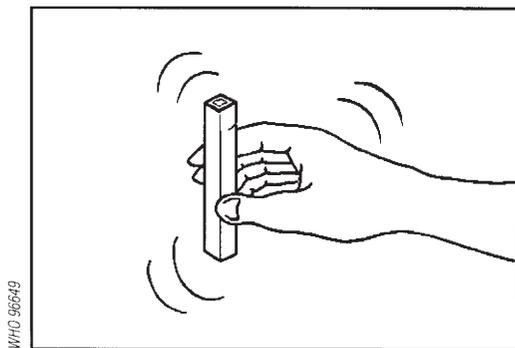
C. Rinse the second cell and fill it with the same water.



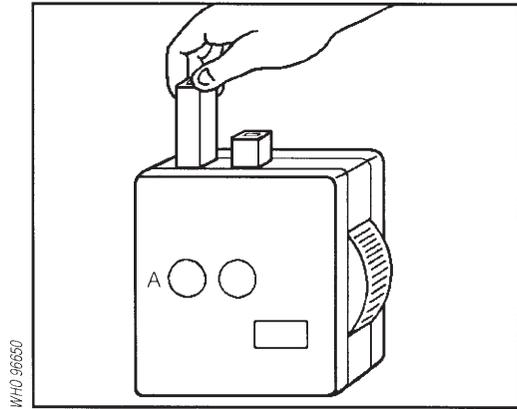
D. Add reagent to the second cell, in accordance with the manufacturer's instructions.



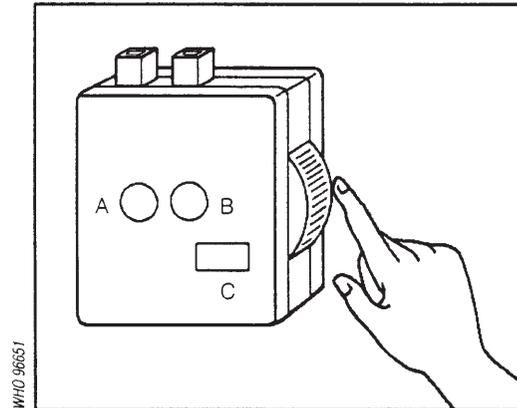
E. Shake the cell (for not more than 3–5 seconds) to mix the reagent.



F. Place the cell in the comparator (A).



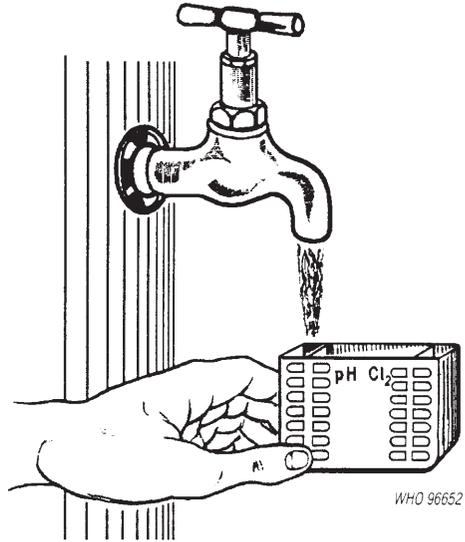
G. While holding the comparator facing good natural light, rotate the disc until the colour of a standard (B) is the same as that developed by the reagent (A). Immediately (i.e. in less than 20 seconds) read at C the value of free chlorine in mg/litre.



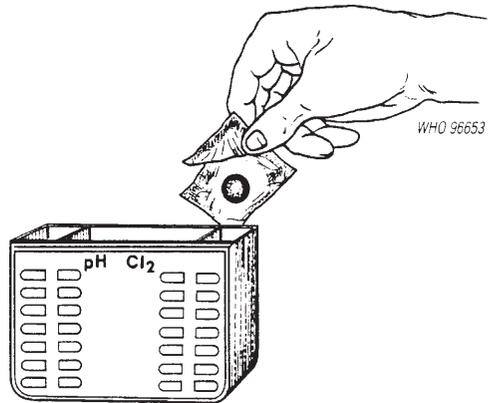
A9.2 Colour match comparator method

The procedure employed when a colour-match comparator is used is summarized below. Some comparators employ tubes or discs with the standard colours; the procedure is similar in all cases.

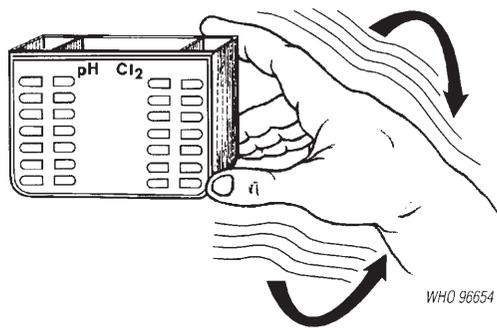
A. Rinse the comparator thoroughly in the water to be tested and then fill to the specified lines on the test and control tubes.



B. Add tablet or liquid reagent and mix thoroughly to dissolve. This may require the crushing of the tablet with a clean glass rod.



C. Compare the pink colour in the test compartment with the standards in the control compartment by viewing the comparator in good, transmitted natural light. Express the result as mg/litre of free residual chlorine.

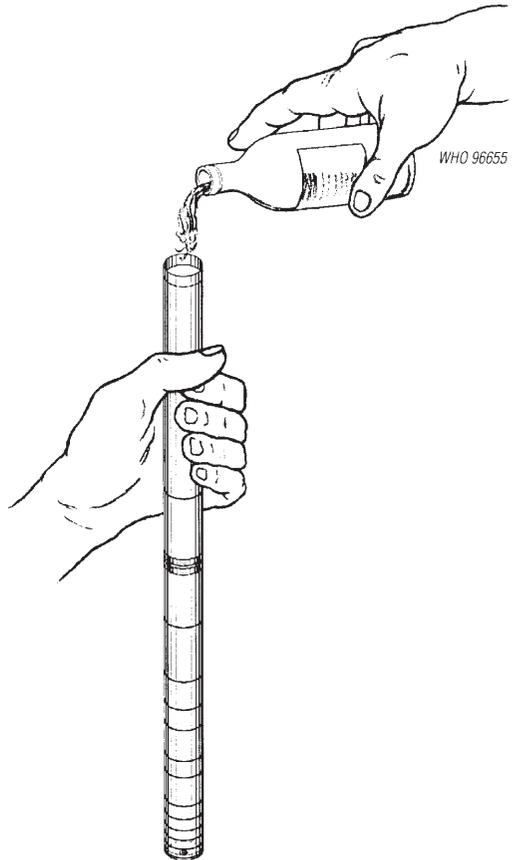


Turbidity and pH

A10.1 Measurement of turbidity

High levels of turbidity can protect microorganisms from the effects of disinfection, stimulate the growth of bacteria, and exert a significant chlorine demand. Where disinfection is practised, the turbidity must always be low, e.g. below 5 NTU/JTU, and ideally below 1 NTU for effective disinfection. Measurement of turbidities lower than 5 NTU will generally require electronic meters. However, turbidities of 5 NTU upwards can be measured by simple extinction methods, which are far cheaper and require no consumables. In the monitoring of small-community supplies in developing countries, such methods may be preferable. The sequence of steps involved in turbidity determination by an extinction method is shown below.

A. Add water slowly to the turbidity tube, taking care not to form bubbles. Fill until the mark at the bottom of the tube just disappears.



- (c) Using ready-prepared pH buffer solutions (pH buffer powder mixed with distilled water according to the manufacturer's instructions), place the pH electrode in a pH 7.0 buffer and adjust the meter if necessary.
- (d) Rinse the electrode in distilled water and transfer it to pH 4.0 buffer; adjust the meter if necessary.
- (e) Rinse the electrode in pH 9.0 buffer and adjust the meter if necessary.
- (f) Check the meter in all three buffer solutions. If it does not read true, repeat the above process. If it cannot be adjusted to read correctly in all buffers, suspect a faulty or damaged electrode.

The meter is now ready for use in testing the water sample; calibration of the meter must be carried out daily.

A10.2.2 Comparator disc method

The comparator disc method for measuring pH requires a comparator, colour discs—depending on the range required (see below)—and the following reagents:

universal	pH 4–11
phenol red	pH 6.8–8.4
bromothymol blue	pH 6.0–7.6
bromothymol purple	pH 5.2–6.8
thymol blue	pH 8.0–9.6

For most natural waters; the universal reagent and phenol red will be sufficient. Where greater accuracy in a particular range is required, the appropriate disc and reagents should be purchased.

The comparator unit is generally suitable for all the discs and so only one such unit is required. The method of use is similar for all pH ranges:

- (a) Place a water sample in the glass or plastic cuvettes provided.
- (b) Add the reagent tablets, powders, or drops according to the manufacturer's instructions.
- (c) Select the appropriate colour disc and place it in the comparator unit.
- (d) Place the cuvettes in the comparator unit.
- (e) Hold the comparator unit up to the eye, facing good daylight (but not direct sunlight).
- (f) Rotate the disc and observe until the colour matches that of the water sample.
- (g) Read the pH value from the disc.

If the pH is not within the range of the disc, select the appropriate reagents and disc and repeat the above procedure.

Examples of regional and national monitoring report forms for water supplies and for coverage with basic sanitary facilities

This annex contains examples of report forms for a national rural water-supply component inventory (Fig. A11.1), sanitary inspections of gravity-fed supply systems from protected spring sources without treatment (Fig. A11.2), surveillance of rural water-supply quality (Fig. A11.3), and regional and national rural coverage with sanitary facilities (Figs A11.4 and A11.5).

Fig. A11.1 National rural water-supply component inventory

Component	National totals
Number of systems	
Number of protected springs	
Number of surface-water intakes	
Treatment plants:	
— number of sedimenters	
— number of systems with coagulant dosing	
— number of systems with a flocculator	
— number of systems with slow sand filtration	
— number of slow sand filters	
— number of systems with rapid sand filtration	
— number of rapid sand filters	
— number of storage tanks	

Fig. A11.2 Sanitary inspections of gravity-fed supply systems from protected spring sources without treatment

Inspection	National totals
<i>Springs:</i>	
— with protection	
— with sanitary lid	
— locked	
— with fence or wall	
— with surface-water diversion ditch	
— with excreta disposal nearby	
<i>Conduction lines:</i>	
— with visible leaks	
<i>Reservoirs:</i>	
— with sanitary lid	
— locked	
<i>Disinfection:</i>	
— with equipment	
— with chlorine stock	
— operating when inspected	
<i>Adduction lines:</i>	
— with visible leaks	
<i>Distribution networks:</i>	
— with visible leaks	
— with constant pressure	
<i>Mean risk score:</i>	

Fig. A11.3 Surveillance of rural water-supply quality

Department: Province:		Community	System type	Source type	Total population	Quality (mean thermotolerant coliforms per 100 ml)	Monthly cost for domestic use ^a	Continuity ^b (overall %)	Quantity ^c (mean litres/day)
Subtotals/ means									

^a Cost is the tariff paid for domestic connection.

^b Continuity is the overall percentage of time for which water is supplied.

^c Quantity data are derived from intake volume and do not necessarily reflect the volume devoted to domestic use.

Fig. A11.4 Regional rural coverage with sanitary facilities

Department: Province:	Community	Total population	Water		Excreta disposal				
			Domestic connection	Public standpost	Private latrine	Communal latrine	Septic tank	Sewerage	
Province subtotals									
Department subtotals									

Fig. A11.5 National rural coverage with sanitary facilities

	National totals
<i>Water:</i>	
— by domestic connection	%
— by public standpost	%
<i>Excreta disposal:</i>	
— by private latrine	%
— by communal latrine	%
— by septic tank	%
— by sewerage	%