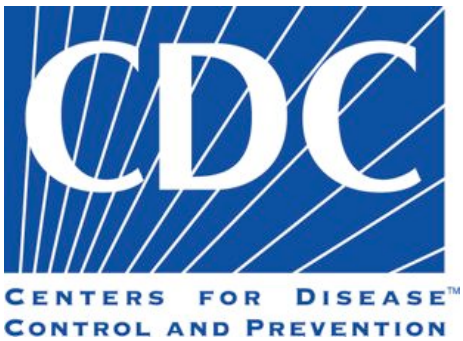
Microbiological Indicator Testing in Developing Countries:

A Fact Sheet for the Field Practitioner



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Microbiological Indicator Testing in Developing Countries

Microbiological indicator testing is a crucial tool for household water treatment and safe storage (HWTS) program implementation, monitoring, and evaluation. Absence of microbiological contamination is an indication that water is safe to drink, and, correspondingly, presence of microbiological contamination indicates drinking the water may cause diarrheal disease.

This fact sheet is intended to provide guidance for researchers, practitioners, evaluators, and other parties interested in testing for microbiological contaminants in developing countries. This fact sheet begins with why we test for microbiological contaminants, and continues through to recommendations for selecting a testing method for specific circumstances. Sample testing procedures are appended at the end of the document.

If you have any questions about this fact sheet, or requests for further information, please feel free to contact safewater@cdc.gov.

1. Why we test for microbiological contaminants

The goal of household water treatment programs, like the CDC Safe Water System, is to reduce diarrheal disease in users by improving the microbiological quality of stored household water. Thus, testing for microbiological contaminants is useful to determine if:

- household drinking water is contaminated before program initiation; and,
- an intervention improves the microbiological quality of stored household water.

Microbiological indicators are bacteria shown to be associated with disease-causing organisms, but do not cause disease themselves. The three common microbiological indicators are: 1) total coliform bacteria; 2) fecal (thermotolerant) coliform bacteria; and, 3) *Escherichia coli.* A fourth indicator, production of hydrogen sulfide, has recently been used as well.

The World Health Organization (WHO) and United States Environmental Protection Agency (USEPA) both use microbiological indicators as the guideline value or standard for safe drinking water. The WHO guideline value is that *E. coli* and thermotolerant (fecal) coliform bacteria "must not be detectable in any 100-ml sample" of water intended for drinking (1). The Guidelines also note that "immediate investigative action must be taken if *E. coli* are detected", and that "medium-term targets for the progressive improvement of water supplies should be set" in developing countries having difficulties meeting the standards.

One example of progressive improvement targets is to use a risk classification scheme for fecal coliform or *E. coli*, where 0 colony forming units (CFU)/100 mL is "in conformity with WHO guidelines", 1-10 is "low risk", 10-100 is "intermediate risk", 100-1000 is "high risk", and >1000 is "very high risk" (2).

The USEPA maximum contaminant level for total coliforms is 0 CFU/100 mL of drinking water (3).

2. Types of microbiological indicators tested

The three common microbiological indicators are: 1) total coliform bacteria; 2) thermotolerant (fecal) coliform bacteria; and, 3) *Escherichia coli (E. coli)*. A fourth, production of hydrogen sulfide, has recently been recommended and used.

Total Coliform Bacteria. Disease-causing organisms can be present in water in small numbers and pose a human health risk. Because of this, indicators of disease-causing organisms present in higher concentrations were initially developed to assess drinking water safety. Because there are numerous coliform bacteria in the intestinal tracts of humans, and each person discharges between 100-400 billion per day, this group was initially chosen as the indicator organism for drinking water safety.

The total coliform test is defined by the laboratory method, and not the biology. In the United States, total coliform bacteria are species of Gram-negative rod bacteria that, at 35 ± 0.5 degrees Celsius, either: 1) ferment lactose with gas production (for most probable number [MPN] and presence/absence [P/A] testing); or, 2) produce a distinctive colony on a suitable medium (for membrane filtration [MF] testing). This definition includes members of the *Escherichia, Klebsiella, Citrobacter,* and *Enterobacter* families. Of these families, *Escherichia* is most commonly associated with waterborne disease.

Although total coliform bacteria have historically been the standard used for drinking water safety, the WHO has moved away from the use of this indicator to assess human health risk. This is because total coliform bacteria are naturally present in the environment, especially in tropical countries, and thus does not always indicate presence of human and animal wastes. However, total coliform bacteria are still a valuable indicator for some purposes, including: 1) routine sampling in a treatment process with a history of compliance to regulations; 2) determination of the efficiency of a treatment process if both pre- and post-treatment waters are collected; and, 3) risk assessment in lower-risk waters when *E. coli* is not present.

Fecal (Thermotolerant) Coliform Bacteria. To provide a more accurate indicator of human health risk, the fecal coliform group was developed. This group is also defined by the laboratory method, and includes those Gram-negative rod bacteria that, at 44 ± 0.2 degrees Celsius, either: 1) ferment lactose with gas production (for MPN and P/A testing), or 2) produce a distinctive colony on a suitable medium (for MF testing). This subgroup includes the genus Escherichia, and some species of Klebsiella, Enterobacter, and Citrobacter. The terms fecal coliform bacteria and thermotolerant coliform bacteria are used interchangeably.

The fecal coliform test has also fallen into some disfavor for the assessment of human health risk for many of the same reasons as the total coliform group, primarily their presence in the normal

environment in tropical countries. However, fecal coliform bacteria are still a valuable indicator for some purposes, including: 1) routine sampling in a treatment plant with a history of compliance to regulations; 2) determination of the efficiency of a treatment process; and, 3) secondary assessment of human health risk after *E. coli*. The WHO guideline values consider both *E. coli* and thermotolerant coliform acceptable tests for drinking water safety.

E. coli. *Escherichia coli (E. coli)* is a bacteria that colonizes the gastrointestinal tract of humans and other mammals shortly after birth and is considered part of our normal intestinal flora. Some types of *E. coli*, such as *E. coli* O157:H7 possess virulence factors and can cause diarrheal disease in humans, but most types of *E. coli* are harmless. A single gram of fresh feces may contain as many as 1,000,000,000 *E. coli*. The mammalian gut is the normal habitat for *E. coli*, and, unlike other coliform bacteria, they are not normally found in uncontaminated waters. This makes *E. coli* an ideal indicator for human health risk. WHO states, "The presence of *E. coli* in water always indicates potentially dangerous contamination requiring immediate attention" (4). Due to its high prevalence and disease-causing properties, *E. coli* is a solid microbiological indicator. However, in some less contaminated environments, there is not enough *E. coli* present to calculate treatment process efficiency. When sampling for both human health risk and treatment efficiency a combined total coliform/fecal coliform bacteria test and *E. coli* test may need to be completed.

Hydrogen Sulfide Production. A relatively new microbiologic indicator test is measuring hydrogen sulfide production. Some bacteria excrete hydrogen sulfide in their metabolic processes. Because hydrogen sulfide is easy and inexpensive to measure, this has been suggested as a new indicator. However, hydrogen sulfide can be produced via other mechanisms than bacterial metabolism, and so hydrogen sulfide production is, in effect, measuring an indicator (hydrogen sulfide presence of bacteria) of an indicator (bacteria of human health risk). In addition, there is currently no quantitative test for hydrogen sulfide production, although some are currently being investigated (5). A comprehensive review of the hydrogen sulfide production test concluded that (6):

> There are good reasons to support the further investigation and use of H_2S tests under certain circumstances and in certain settings. In particular, if the alternative to H_2S testing is no water quality testing at all for fecal contamination, the H_2S test is recommended for use, with caution. The caution concerns possible false positive results due to H_2S presence or formation in water from sources other than fecal contamination. In addition, H_2S testing also is recommended with caution for educational and motivational purposes to promote water sanitation and hygiene education in outreach and dissemination programs. Again, it must be established or verified that the test will give correct results with respect to water classification as suitable or unsuitable when applied to the treated or untreated drinking waters being tested.

In summary, currently the test is worth investigating further, although not enough is known to recommend it as a replacement for the total or fecal coliform bacteria test, or the *E. coli* test.

Growth Media. In order to test for microbiological indicators, a growth media specific to the indicator is purchased or made in the laboratory. Media can be purchased from suppliers in: 1) individual use liquid in plastic sachets or glass ampules: 2) individual use in powdered form sachets; 3) bulk-use liquid; or, 4) bulk-use powder to be mixed with laboratory-grade water and autoclaved before use. Bulk-use powders are significantly less expensive, but much easier to contaminate in developing country circumstances, where an autoclave and sterile procedures may be harder to obtain and maintain. Images below are from www.hach.com.

The incubation temperature for total coliform bacteria is 35°C, while the incubation temperature for fecal coliform bacteria is 44.5°C. Depending on the media used, *E. coli* can be incubated at either temperature. Thus, it is possible to purchase combined total coliform/*E. coli* or fecal coliform /*E. coli* media to measure both indicators in one sample.

3. Laboratory methods for testing microbiological indicators

There are three different laboratory methods used to test for microbiological indicators: 1) presence/absence (P/A), 2) most probable number (MPN), and 3) membrane filtration (MF). The handbook *Standard Methods for the Examination of Water and Wastewater* (7) provides detailed laboratory procedures for each method, and is the standard reference for microbiological testing. All three laboratory methods include: 1) sample collection; 2) sample processing including addition of a specific growth media; and 3) sample incubation for 24-72 hours to await indicator growth.

Presence/Absence (P/A) testing is the simplest testing method for microbiological indicators, which, concurrently, provides the least amount of data. As the name implies, this method provides information on whether the bacteria are present in a sample or not. The procedure involves adding a liquid or powdered media to 100 mL of water and incubating for 24-72 hours at 25-35°C. A color change (for total coliform or fecal coliform bacteria) or UV-fluorescence (for *E. coli)* indicates the presence of bacteria. "P/A testing was developed for and is applicable where most tests provide a negative result. Where a significant proportion of tests provide a positive reaction quantitative testing is preferred in order to determine relative health risk and therefore relative priority of need for correction, such as by improved or greater treatment or by finding a higher quality source water for supply" (6).

Most probable number (MPN) testing uses statistical tables to provide quantitative microbiological data by completing multiple presence/absence tests. In this method, multiple vials or wells are filled with the sample water and media. The vials or plates are incubated for 24-48 hours, and each vial or well is assessed for color change (for total coliform/fecal coliform) or UV-fluorescence (for *E. coli)*. The number of positive and negative vials or wells is compared to a table and a numerical contamination value (in MPN/100 mL) is assigned. The number of vials or wells determines the range of the test - for example, five tubes can have a results range of 0-84 MPN/100 mL. Some commercial tests have significantly higher ranges, up to 2,419 MPN/100 mL.

Membrane filtration (MF) testing has traditionally been the gold standard for microbiological testing and provides quantitative data on the number of colony forming units (CFU) of the indicator bacteria in sample water. To complete the test, a measured sample water volume is filtered through a 0.45 micron (0.00000045 meter) filter. The filter is placed in a petri dish over a pad impregnated with a specific growth media and incubated at a specific temperature for 18-24 hours. Colonies grow in specific colors, and are manually counted.

Incubation. All three testing methods require incubation for the indicator bacteria to grow. In general, the requirements for incubation are more stringent for MF than for MPN and P/A testing. Many of the MPN and P/A tests have a wide incubation temperature range, for example 25-35° Celsius, which can be achieved at ambient temperature depending on the testing environment. MF testing is more dependent on the maintenance of an exact temperature, depending on the specific test.

Incubation can be challenging in areas with poor electricity. A number of incubation methods can be used, depending on the resources available. Temperature should always be measured throughout the incubation period with a non-mercury thermometer to ensure adherence to requirements.

For tests requiring consistent incubation. If electricity is available, a commercially available portable incubator can be used. If electricity is not available, some commercial incubators can be powered by a car battery or generator. It is also possible to use a car battery to heat a cooler with a heat sink, or a chicken incubator, or another 'jury-rigged' design. In all cases, temperature should be regularly monitored. Another option is to use a phase-change material (PCM) incubator developed at MIT. This methodology uses a material whose phase-change temperature is constant at 35 Celsius. Bags of the material are boiled, placed in a cooler, and the temperature is held for 24-48 hours. More information can be found about this incubator at mmadinot@mit.edu. It is of note that in resource limited environments, it is easier to maintain 35°C than 44°C incubation, which may influence the choice of a total coliform versus fecal coliform media.

For tests not requiring consistent incubation. The first option in these cases is to see if the ambient temperature is appropriate for the incubation range required. If that is the case, then simply incubate at ambient temperature. If temperature varies (as it will normally from night to day), it is recommended to incubate for 48, as opposed to 24 hours, to account for variation. If ambient temperature is not sufficient, a jury-rigged local design (placing near the stove, etc) could be sufficient.

Recently, there have been investigations into the reliability of common commercial *E. coli* tests and media at non-standard incubation temperatures. These results have indicated some tests produce reliable and consistent *E. coli* results at constant incubation temperature ranges from 25-40°C (8). If non-standard incubation temperatures are used, it is recommended to verify the results in the specific circumstance and specific test with standard incubation temperatures.

4. Quality Assurance/Quality Control

The importance of quality assurance/quality control (QA/QC) procedures in microbiological sampling in the field can not be overestimated. In addition to normal variability in concentrations of microbiological indicators between samples from the same location, there exists the possibility of contamination in every step of a microbiological sampling procedure. The following techniques will ensure that the data generated are reliable:

1. One blank sample (using boiled dilution water) should be with completed for every 10-20 samples. If the blank samples show bacteria, contamination has occurred during the procedure, and data must be discarded. Dilution water can be: 1) commercially purchased and imported; or, 2) made locally by boiling low-turbidity water and adding (if necessary for the media used) buffer solution available from commercial companies in plastic sachets.
2. Positive controls (using unclean water at hand) should be run each time the possibility that all results will show no bacterial growth. If the positive controls do not show bacterial growth, then either the media is ineffective or the incubation temperature is incorrect and inconsistent, and all data from that run must be discarded.
3. If financially possible, all samples should be duplicated. There is normal variability in the microbiological concentration between one 100 mL sample and the next from the same source. In addition, duplicates provide additional quality assurance and allows for averaging of two samples for more accurate results. At an absolute minimum, 10 percent of all samples should be duplicated. A step-wise duplication process can also be implemented. For example, in the first runs all samples should be duplicated to ensure the procedure is completed correctly. If duplicate samples are accurate, the percentage of duplicate samples can be reduced in subsequent runs. If duplicates begin to disagree, then 100 percent duplication should again be implemented.
4. If at all possible, a small percentage of samples should be split, with one portion sent to an independent laboratory to verify the results.

Sufficient quality assurance/quality control procedures (duplications, blank samples, and positive controls) could more than double the number of samplings (and the cost) of the testing. In addition, if water is highly contaminated, multiple dilutions may be necessary for accurate results. However, this is a small price to pay when compared to the cost of disregarding your entire sampling program because of questionable results and inadequate quality assurance.

5. Specific testing methods

In the following pages, some of the more common testing methodologies used in developing countries are described, including: 1) how they are used; 2) benefits and drawbacks; and, 3) cost considerations. The methods described include:

1. Presence/absence testing
2. Petrifilm™ combined with presence/absence testing
3. Emerging Semi-Quantitative Method – Aquatest
4. Emerging Semi-Quantitative Method #2
5. Most Probable Number with IDEXX
6. Membrane Filtration with Field Kit
7. Membrane Filtration with Laboratory Equipment

Presence/Absence Testing

The first option for microbiological testing is presence/absence (P/A) testing. To complete P/A testing, a known sample water volume (generally 100 mL) is added to a liquid or powder media in a sterile container (such as a plastic bottle or plastic bag).

Benefits of P/A testing are:

- Simple testing procedure
- Minimal equipment
- Simple analysis procedure
- Ability to test total coliform, fecal coliform, *E. coli,* depending on media used
- Incubator not required as there is a wide temperature ran

The IDEXX Colilert Test www.idexx.com
(yellow total coliform +, fluoresce *E. coli* +)

Drawbacks of P/A testing are:

- At low contamination levels (<10 CFU/100 mL), the tests can report a false negative
- The results are not quantitative
- Not appropriate for use with known contaminated waters; more appropriate to confirm lack of contamination with negative results

Cost and Other Information

Pre-prepared, disposable, sterile plastic bottles pre-filled with media, such as Hach P/A with MUG broth or IDEXX Colilert, both of which test for total coliform and *E. coli*, are ~3-5 USD per test. Powdered H2S-production media, such as Hach Pathoscreen, is generally less than 1 USD per powdered media sachet. An inexpensive (~20 USD) handheld UV-light is used to measure fluorescence for identifying the presence of *E. coli*. Costs can be saved in P/A testing by autoclaving or sterilizing reusable bottles or using WhirlPak™ bags, and adding powdered media. Any sample suspected of having chlorine residual should be collected in a bag with thiosulfate to inactivate the residual before completing the test.

A WhirlPak™ sample collection bag (with a thiosulfate tablet to inactivate any chlorine present) www.hach.com

Petrifilm™ combined with Presence/Absence Testing

Recently, a combined method for making P/A testing semi-quantitative has been developed. Petrifilm™ is a commercial product meant for testing coliform contamination in food. To complete a Petrifilm™ test: 1) one milliliter of sample is placed on the pink circle; 2) the film cover is placed over the sample; and, 3) the flat Petrifilm™ is incubated for 24 hours. The lowest contamination level possible to measure is 100 CFU/100 mL, because only 1 mL of sample is tested. When used in combination with a P/A test, which indicates greater than or less than 10 CFU/100 mL, Petrifilm™, which indicates greater than or less than 100 cfu/100 mL, can provide a semi-quantitative risk level result.

Benefits of Petrifilm™ / P/A testing are:

- Simple testing procedure
- Simple analysis procedure
- Small size of Petrifilm™ leads to ease of incubation (either in incubator or in a belt attached to the body)
- Semi-quantitative risk level based results (<10, 10-100, >100 cfu/100 mL)
- Measures both total coliform and *E. coli*

http://multimedia.3m.com

Drawbacks of Petrifilm™ / P/A testing testing are:

- Petrifilm™ is not currently approved for water testing
- The use of only 1 mL of water for testing can lead to inaccuracies, particularly when there are few colonies that develop on the Petrifilm™.

Cost and Other Information

The cost per Petrifilm™ is about 1 USD. To use the risk level testing, this cost is in addition to the P/A testing cost.

This methodology is credited to Susan Murcott and Robert Metcalf.

Emerging Semi-Quantitative Method – Aquatest

A team of developers organized at the University of Bristol has Gates Foundation funding to develop a new field test for *E. coli* with the following parameters: "It will be a small, single-use device for testing water quality. It will be used in the field, without electricity or skilled technicians and will cost around US$1 per test. It is being designed for use in developing countries."

www.bristol.ac.uk/aquatest

The current design is a single-use plastic container. To complete the test, users fill the container with 100 mL of water, incubate in a reusable single-sample incubator for 24 hours, and read using a portable UV light. The results will indicate risk levels of *E. coli* contamination: 0 col/100 mL (no risk); 1-10 col/100 mL (low risk); 11-100 col/100 mL (medium risk); 101-1000 col/100 mL (high risk); and, >1000 col/100 mL (very high risk). The incubator is based on a phase-change material. To use the incubator, boiling water is poured into the incubator and left there for 4 hours. The water is then poured out and the Aquatest container placed in the incubator for 24-48 hours. The Aquatest can also be incubated in a normal incubator.

Benefits of the Aquatest are:

- Simple testing procedure
- Minimal equipment
- Simple analysis procedure
- Semi-quantitative results (based on risk levels)

Drawbacks of the Aquatest are:

- Relatively large-size disposable container for each test
- One incubator needed for each test (if not using alternate indicator)
- Not yet commercially available
- Tests only for *E. coli*

Cost and Other Information

The Aquatest team is currently seeking interested organizations in developing countries to verify the technology in pilot projects. More information can be found at www.bristol.ac.uk/aquatest.

Emerging Semi-Quantitative Method #2

Another team of researchers, based on the University of North Carolina (UNC) Chapel Hill, is developing a second low-cost, easy-to use, portable field test for *E. coli* or H_2S-producing bacteria.

The current UNC design is a modified WhirlPak™ bag, segmented into five internal compartments or chambers of different volumes, totally to 100 mL. To complete the test, users fill the WhirlPak™ bag with 100 mL of water to which *E. coli* or H_2S medium has been added. The bags can incubated between 25 and 40°C (for H_2S bacteria) or between 25 and 45 °C for *E. coli* for 24-48 hours. To read the test, users count the chambers that change color, which is dark blue for the *E. coli* test or black for the H_2S test. The arrays of positive and negative chambers yield MPN estimates per 100 mL of water in the range of 0 to 100 MPN/100 ml. When all 5 chambers are positive, the water is >100 MPN/100 mL.

Benefits of the modified-WhirlPak are:

- Simple testing procedure
- Minimal equipment required
- Simple analysis procedure
- Incubation not required
- Tests for *E. coli* or H_2S production
- Semi-quantitative MPN results for decimal risk levels
- Collection container is analysis container

Drawbacks of the modified-WhirlPak™ are:

- MPN semi-quantitative results up to only 100 MPN/100 mL
- Not yet commercially available

Cost and Other Information

The estimated cost per test is now <1 USD for the H_2S version and <2 USD for the *E. coli* version. Eventually, commercial versions are expected to be <1 USD for either version. The UNC team is currently seeking interested organizations in developing countries to verify the technology in pilot projects. More information can be found at sobsey@email.unc.edu.

Most Probable Number with IDEXX™

Any P/A method can be converted to a most probable number (MPN) method by: 1) running multiple P/A tests of the same sample; 2) counting the positive and negative samples; and, 3) converting the results to numerical reading in MPN/100 mL using a statistical table. The higher the number of tests, the higher the degree of accuracy. However, this method is often unwieldy, as to obtain a high degree of accuracy, a large number of samples vials/bottles is needed.

A sterile, commercial tray with 97 wells of different sizes from IDEXX™ has simplified MPN testing. To use the tray the sample is mixed with a media and poured into the top of the tray. The tray is sealed using a sealer, and incubated for 24 hours. Yellow and fluorescent wells of each size are counted and compared to a statistical chart which converts the counts to numerical total coliform and *E. coli* results.

Benefits of the IDEXX™ Quantitray are:

- Simple testing procedure
- Simple analysis procedure
- Highly reliable quantitative results
- Large range of contamination accurately tested in one sample (from 0-2,419 MPN/100 mL)
- Can be used with a variety of media
- Easy for non-laboratory trained personnel to use
- Fast sample processing, enabling many samples per day

Quantitray
www.idexx.com

Drawbacks of the IDEXX™ Quantitray are:

- Relatively large-size disposable tray for each test
- Relatively large space in incubator for each test
- Need for electricity to run the sealer and large incubator
- Large volume of equipment to transport
- High up-front cost for sealer

Sealer
www.idexx.com

Cost and Other Information

The consumables for each test are approximately 5 USD, in addition to the up-front 1,500 USD cost for the sealer.

Membrane Filtration with Field Kit

Numerous commercial field test kits are available to complete membrane filtration testing of microbiological indicators in emergency (or development) situations. These field kits are self-contained, with all the equipment necessary to collect, process, and incubate samples.

Benefits of Field Kits are:

- One-stop-shopping
- Easily transportable
- Reliable quantitative results
- Few disposable parts
- Ability to be used with a variety of media
- Ability to be used with a variety of power sources (car batteries, batteries, etc)
- Low per test cost if powdered media and reusable petri dishes are used

The Oxfam Delaqua Kit
www.delaqua.com

Drawbacks of Field Kits are:

- Reusable parts and non-pre-mixed powdered media makes sample contamination more likely if not autoclaved / pressure cooked / boiled correctly
- Operators need to understand dilutions and run multiple samples in highly contaminated environments to ensure 20-80 colonies on each plate for accurate results
- Maximum capacity of incubators is ~16 samples – which is limiting in high-contamination contexts necessitating multiple dilutions, while completing sufficient quality control samples
- Slow sample processing, as sterilization requires a 15-minute wait between filtering samples
- High up-front cost for test kit

The Oxfam Delaqua Incubator
www.delaqua.com

Cost and Other Information

Field kits are approximately 2,000 USD, although each test (if reusable petri dishes and powdered media are used) can be quite inexpensive (less than 1 USD).

Membrane Filtration with Laboratory Equipment

In addition to commercial field kits, it is possible to complete high-quality membrane filtration in the field using portable laboratory equipment. To complete the testing, samples are collected in WhirlPak™ bags on ice, and analyzed within 8 hours of collection. A sterile, disposable sample cup and filter are placed on top of a filtration stand, and the appropriate dilution of sample water and buffered dilution water is poured or pipetted into the cup. Water is manually filtered through the stand with a syringe. The cup is removed, and using flamed forceps, the filter is placed in a plastic Petri dish with media-soaked pad, and incubated for 24 hours. Colonies are manually counted.

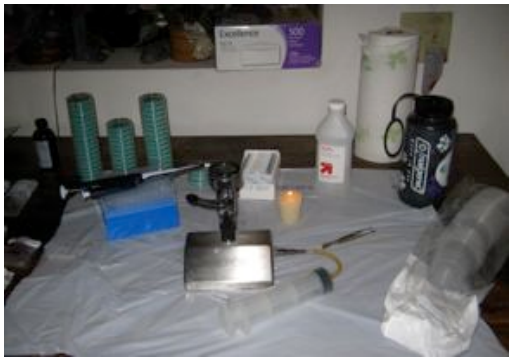
Benefits of Laboratory Testing are:

- Reliable quantitative results
- Laboratory quality membrane filtration testing
- Ability to be used with a variety of media
- Relatively smaller volume of equipment (compared to IDEXX, higher volume compared to Field Kits)
- Fast sample processing, enabling many samples per day
- Difficult to contaminate samples due to disposable equipment
- Small incubation space per test, enabling use of portable incubator
- Ability to complete the testing without electricity

Filtration Stand (www.millipore.com)

Drawbacks of Laboratory Testing are:

- Laboratory-trained personnel needed to complete testing
- Operators need to understand dilutions and run multiple samples in highly contaminated environments to ensure 20-80 colonies on plate for accurate results
- Significant amount of disposable equipment
- Manual processing
- High up-front cost



Sample set-up using equipment to test

Cost and Other Information

The laboratory equipment (filtration stand and accessories and pipettor) are approximately 1,000 USD. The consumables for each test (individual packaged media, sterile disposable cup and petri dish) are approximately 4 USD per test.

6. Recommendations for method selection

Selecting how to measure microbiological indicators in developing countries can be complicated, as there really is no single, easy-to-use, quantitative, inexpensive, self-contained testing method. Selecting the appropriate test for microbiological contamination depends on: 1) the goals of the study; 2) the resources available; 3) the intended purpose of the data; and, 4) operator proficiency. In each case, the program will need to select: 1) the indicator (total coliform, fecal coliform, *E. coli,* hydrogen sulfide production) and the media to measure that indicator; 2) the method (presence/absence, most probable number, membrane filtration); and, 3) the incubation method. General recommendations for which methodology to use based on different programmatic goals follow.

Research, Treatment Efficacy, or High-quality Evaluation:

When high-quality quantitative data are needed – for research studies, to document treatment efficacy by comparing pre-treated and post-treatment waters, or when quantitative results are needed for evaluation – one of the following methods is recommended:

- Membrane filtration using a laboratory equipment system or a field kit with reliable incubation. If >16 samples a day are required, a lab equipment system (or multiple field kits) is indicated.
- Most probable number using a multiple-well tray system.

Program Monitoring:

If the goal is to provide a quick and simple test with little equipment to provide a presence/absence result, the following methods are recommended:

- Use of combined Petrifilm™ and presence/absence test
- Emerging semi-quantitative methods

Spot Sampling / Behavior Change:

If the goal is to provide a quick and simple spot check of an expected negative result or for behavior change communication, a presence/absence or hydrogen sulfide production test is recommended.

If you have any questions about this document or microbiological indicator testing, please feel free to email safewater@cdc.gov.

CDC as a branch of the federal government does not endorse products from specific companies. The above information is for reference purpose only to describe and represents, to the authors' current knowledge, a range of available products.

Sample Procedure – Presence/Absence Testing

This sampling methodology was used with Hach P/A with MUG total coliform and *E. coli* media and Hach Pathoscreen™ hydrogen-sulfide production sachets in rural Nicaragua. No laboratory equipment or electricity was available, and conditions were not sterile. Quality control measures, including duplicate sampling, blank sampling, and comparison with duplicate samples analyzed in a laboratory in Managua, showed no contamination of samples and excellent correlation. Although this method is specific for these two media, the procedures and mentality for conducting microbiological analysis in rural areas of the developing world are accurately represented here, and will be useful no matter the media or specific test that is completed.

Sample collection:

1. Remove WhirlPak™ sampling bag from sealed larger bag.
2. Label WhirlPak™ bag with date, time, and sample identification number in permanent ink.
3. Wash hands with hand alcohol.
4. Open WhirlPak™ bag without touching the lip of the bag.
5. Fill WhirlPak™ bag with sample without touching anything to the lip of the bag.
6. Whirl bag three times quickly, and cinch sides closed.
7. Place WhirlPak™ bag upright in a cooler with ice.
8. Complete analysis of sample within 8 hours.

Cleaning of bottles:

1. If using reusable bottles for the testing (as opposed to sterile disposable bottles or the WhirlPak bag itself (both of which are easier) the bottles should be cleaned.
    a. Inactivate previous sample in bottle by boiling for 10 minutes or adding bleach. Dispose appropriately.
    b. Scrub the vials or bottles with isopropyl alcohol and a scrub brush until no color remains.
    c. Boil vials or bottles and lids in boiling water for 10 minutes.
    d. Remove vials or bottles from boiling water using tongs sterilized with alcohol. Do not touch the inside of the vial, bottle, or cap at any time with any object.
    e. Place vials or bottles (with lids lightly placed on top of vial or bottle) on a garbage bag coated with isopropyl alcohol to cool.
    f. Place clean plastic garbage bag with alcohol on it on top of vials or bottles and lid.
    g. Let vials or bottles dry.
    h. After vials or bottles are dry, close the lids and place vials or bottles in clean ziplock bags and zip.

i. The use of plastic bottles instead of glass vials has the advantage of easier handling and lighter weight. Plastic, however, can retain the smell of the bacteria, although results indicate the retained smell did not influence subsequent tests.

Preparing samples:

1. Wash hands with hand alcohol.
2. Place new clean plastic garbage bag on sampling surface and wipe with isopropyl alcohol.
3. Remove WhirlPak™ bags from cooler with ice.
4. Wipe down WhirlPak™ bags with alcohol soaked paper towel to prevent any cross-contamination if any of the WhirlPak bags have leaked or sweated.
5. Remove and label sterilized bottles from ziplock bag.
6. Aseptically (do not touch your hands to the lip of the WhirlPak™) open WhirlPak™ bags and pour sample into bottle. Do not let any piece of the WhirlPak™ touch the sample bottle.
    a. Place caps facing up on the garbage bag while filling sample bottle. Replace when completed.
7. For P/A with MUG test:
    a. Wash hands with hand alcohol.
    b. Remove glass vial from container not touching the part where you will break it.
    c. Wipe down glass vial with isopropyl alcohol soaked paper towel.
    d. Break vial carefully using paper towel.
    e. Pour media into 100 mL of sample, cap.
8. For hydrogen sulfide (Pathoscreen™) test:
    a. Wash hands with hand alcohol.
    b. Remove powder pillow touching only one end.
    c. Wipe other end with alcohol soaked paper towel.
    d. Snip open powder pillow with clippers sterilized in candle.
    e. Pour into sample bottle, cap, and shake.
9. Incubate for 48 hours at 25-35 C.
10. Read samples at 12, 24, 36, and 48 hours. Record results on data sheet.

Equipment List for Presence/Absence sampling:

Equipment	Laboratory Equipment
Ziploc bags	WhirlPak™ bags
Paper towels	Bottles or vials with lids
Alcohol – Isopropyl and Hand	Growth Media (such as P/A Broth with MUG)
Pot to boil water in	Clippers to open packets
Tongs to remove bottles/vials	Incubator and thermometer
Garbage bags	UV light
Candle and matches	Dilution Water
Data Sheets	
Labeling marker	
Cooler and Ice	

Sample Procedure – Most Probable Number Testing

This sampling methodology was used with the IDEXX system in Kenya and Pakistan, with adequate electricity and access to laboratory resources. Quality control measures, including duplicate sampling or all samples and blank sampling, showed no contamination of samples and excellent correlation.

Preparation

1. All glassware should be autoclaved before use. Do not mix glassware used for sterile water storage and handling with glassware used for sample water storage and handling.
2. Locally purchased distilled water is not reliably free of coliforms. Therefore, all distilled water used for sample dilution should be autoclaved. Approximately 200 mL of sterile water is needed to prepare dilutions of each source water sample. Fill 1000 mL flasks with distilled water to meet expected daily sample load. Autoclave. Cool to room temperature before use (this may take 2 hours). A deionizer can also be used to make sterilize water.

Sample collection and transportation

1. Label each WhirlPak™ bag with the source or household identifiers and date and time of collection. Other data should be recorded according to the study.
2. Collect sample in sterile 125 mL WhirlPak™ bags containing thiosulfate. Thiosulfate neutralizes chlorine activity to prevent ongoing bactericidal activity during transportation. Use sterile technique. In order to collect a representative sample, be careful not to disturb the water during collection.
3. Transport samples to the laboratory in a cooler with ice packs.
4. Process samples completely within 8 hours of collection.

Sample dilution (not needed if results are <2,419 MPN/100 mL)

1. Highly contaminated water (e.g., source water) should be tested both undiluted and diluted 1:10 and 1:100. Less contaminated water (e.g., municipal water, treated water) may require dilution to 1:10, depending on the level of contamination.
2. Label all vessels with sample number and dilution (e.g., 1:10, 1:100) before starting. For each household, there should be three vessels: undiluted, 1:10 dilution, and 1:100 dilution.
3. Lay out two graduated cylinders. Be sure to use sterile technique and wash your hands with hand alcohol in between touching the graduated cylinders and touching other laboratory equipment including household water samples. Fill one graduated cylinder with 90 mL of sterile water. Fill another graduated cylinder with 99 mL sterile water. The graduated cylinders should be used only for sterile water and should be autoclaved daily.

4. For each household, prepare dilutions as follows. Dilutions will be made in the labeled vessels. In each case, the method will result in a final volume of 100 mL for each dilution. To prepare 1:10 solution as final dilution: Pour 90 mL of sterile water from graduated cylinder into appropriately labeled vessel. Using pipette, add 10 mL of undiluted sample to the vessel. To prepare 1:100 solution as final dilution: Pour 99 mL of sterile water from graduated cylinder into appropriately labeled vessel. Using pipette, add 1 mL of undiluted sample to the vessel.
5. Remove excess water from the original sample WhirlPak™ bag until exactly 100 mL remains in the container using the pipette and examining the meniscus from the side at 90º to the sample container. Pour 100mL of this undiluted sample into the appropriately labeled vessel.
6. Check your labeled vessels. There should be there samples: undiluted, 1:10 dilution, and 1:100 dilution.
7. Pipettes should be discarded after preparing dilutions of each sample.

Processing samples for the IDEXX Quanti-Tray/2000™ system

1. Turn on power switch for IDEXX Quanti-Tray Sealer™. The amber power light should illuminate. The sealer will warm up while you are preparing samples. This takes about 10 minutes. The green light will illuminate when the sealer is warmed up.
2. Add one ampoule of Colilert™ test kit reagent to each 100 mL water sample. Shake each sample until the reagent has dissolved.
3. Label a new Quanti-Tray™ with the sample number, collection date, and dilution (e.g., undiluated, 1:10, 1:100). Please use indelible marker only. Do not use ball point pen.
4. Use one hand to hold a Quanti-Tray™ upright with the well side facing the palm.
5. Squeeze the upper part of the Quanti-Tray™ so that the Quanti-Tray™ bends towards the palm.
6. Gently pull the foil tab to separate the foil from the tray. Avoid touching the inside of the foil or tray.
7. Pour the reagent/sample mixture directly into the Quanti-Tray™ avoiding contact with the foil tab. Tap the small wells 2-3 times to release air bubbles. Allow foam to settle.
8. Check that both the amber and green lights are illuminated on the IDEXX Quanti-Tray Sealer™. Place the sample-filled Quanti-Tray™ onto the Quanti-Tray/2000™ rubber insert of the Quanti-Tray Sealer™ with the well side (plastic) of the Quanti-Tray™ facing down.
9. Slide the rubber insert with tray into the sealer until the motor grabs the rubber insert and begins to draw it into the sealer.
10. In approximately 15 seconds, the tray will be sealed and partially ejected from the rear of the sealer. Remove the rubber insert and tray from the rear of the sealer.

11. If at any time you wish to reverse the motor drawing the rubber insert into the sealer (e.g., misaligned tray is accidentally fed into sealer), press and hold the reverse button. However, do not reverse motor once rubber insert has been drawn fully into the input slot.
12. Multiple rubber inserts can be run consecutively without pausing.
13. Turn of sealer when not in use.

Incubation

1. Place labeled and sealed sample tray in a 35ºC air incubator.
2. Results are definitive at 24-48 hours incubation, so may be read at any time during this period according to preference and work flow. If using Colilert-18™, only 18 hours of incubation are required.

Result interpretation

1. For inexperienced technologists, the presence of total coliform bacteria may be interpreted by comparison with an IDEXX P/A Comparator™. The following table may be use for interpretation. Please study the Comparator and become comfortable with it.

Appearance	Result
Less yellow than comparator	Negative for total coliforms and *E. coli*
Yellow equal to or greater than comparator	Positive for total coliforms
Yellow and fluorescence greater than comparator	Positive for *E. coli*

Count the number of large wells positive and the number of small wells positive. Note that the very large well at the end of the tray is counted as a large well. With experience it is not necessary to use a Comparator tray. Use the Quanti-Tray/2000™ Most Probable Number table to calculate the total coliform concentration in most probable number (MPN) per 100 mL.

2. MUG (for *E. coli* although not for the variant O157:H7) is read with a 6-watt 365 nm ultraviolet light source held 5 inches from the sample. Positive wells fluoresce blue. Count the number of large wells positive and the number of small wells positive. Note that the very large well at the end of the tray is counted as a large well. Use the Quanti-Tray/2000™ Most Probable Number table to calculate the *E. coli* concentration in most probable number (MPN) per mL.
3. After reading, dispose of media on accordance with Good Laboratory Practices.

Cleaning of glassware

1. Prepare a solution of detergent (e.g., Pride, OMO).
2. Immerse the glassware in a bath with this solution and soak for 8-12 hours.
3. Remove from solution and rinse with tap water until detergent is removed.
4. Rinse the glassware with distilled water 4 times.
5. Let material dry before sterilization.

Sterilization of glassware

1. Once the glassware is dry, loosely cap bottles and cover glassware without a lid with paper. Fasten with indicator tape.
2. Sterilize in the autoclave for 15 minutes at 121ºC. Label with date of sterilization and store in a dry place.

Equipment Required (for 600 samples)

600 WhirlPak™ bags (with thiosulfate)
600 labels
10 indelible markers
Coolers
Ice packs
600 plastic water vessels (with thiosulfate)
5 graduated cylinders (100 mL)
Hand alcohol
Eye protection
200 pipettes (10mL)
IDEXX Quanti-Tray Sealer™
600 Colilert™ test kit reagent
600 Quanti-Trays/2000™
Quanti-Tray/2000™ rubber insert
35ºC air incubator
IDEXX P/A Comparator™
Quanti-Tray/2000™ Most Probable Number tables
6-watt 365 nm ultraviolet light source
Timer

Sample Procedure – Membrane Filtration Testing

This sampling methodology was used with Millipore mColiBlue24 media with the Millipore filtration stand for enumeration of total coliform and *E. coli* in rural Haiti with no access to laboratory equipment or sterile procedures or electricity. Quality control measures, including duplicate sampling or all samples and blank sampling, showed no contamination of samples and excellent correlation. Although this procedure is specific for this media, the procedures and approach for conducting membrane filtration in rural areas of the developing world are accurately represented here, and will be useful no matter the specific media or specific test that is used.

Sample collection:

1. Remove WhirlPak™ sampling bag from sealed larger bag.
2. Label WhirlPak™ bag with date, time, and sample identification number in permanent ink.
3. Wash hands with hand alcohol.
4. Open WhirlPak™ bag without touching the lip of the bag.
5. Fill WhirlPak™ bag with sample without touching anything to the lip of the bag.
6. Whirl bag three times quickly, and cinch the sides closed.
7. Place WhirlPak™ bag upright in a cooler with ice.
8. Complete analysis within 8 hours.

Fixing samples:

1. Wash hands with hand alcohol.
2. Place new garbage bag on sampling surface and wipe with isopropyl alcohol.
3. Set up filtration apparatus and light candle.
4. Remove WhirlPak™ bags from cooler with ice.
5. Wipe down WhirlPak™ bags with alcohol soaked paper towel before placing on one side of garbage bag to prevent any cross-contamination if any of the WhirlPak™ bags have leaked or sweated.
6. Label disposable plastic petri dishes with date and time of analysis, dilution factor, and sample identification number.
7. Arrange your lab space with samples and petri dishes lined up.
8. Complete analysis of each sample:
    a. Sterilize tweezers in candle, and pick up and sterilize carbon fritt in the candle.
    b. Sterilize tweezers in candle, and carefully open new filter package, pulling away package and paper without touching filter with hands.

i. If you touch filter, discard.

c. Pick up filter with tweezers and carefully center on filtration apparatus.
d. Place new disposable funnel on top of filter and filtration apparatus.
e. Carefully pour sample from WhirlPak™ into the funnel without touching WhirlPak™ to funnel. Close and set aside the rest of the sample in case it is needed later.
    i. Some samples will need to be diluted because the concentration of bacteria is too high to count on the filter. The general guidelines for dilution, from Standard Methods, are included below. In general, it is best to run multiple dilutions at the beginning of the sampling process in order to 'learn' the local contamination situation. As dilutions are learned over the days of sampling, the number of dilutions can be reduced. Ideally, there would be between 20-80 colonies per plate, although up to 200 is acceptable.
f. Use a pipetter and a pipette to add sample to the funnel, and then fill the funnel with buffered dilution water to at least 20 mL in order to not concentrate the bacteria in one part of the filter.
g. Filter sample through the filter, expelling the wastewater into waste bucket or ground.
h. Open media packet and pour entire packet into petri dish, taking care to cover all of the pad with the media.
i. Sterilize tweezers, remove filter funnel from apparatus and discard, pick up filter by the edge with tweezers and place in labeled petri dish. Be careful to avoid air bubbles under the filter.

9. Incubate samples for 24 hours, along with blanks, duplicates, and positive and negative controls.
10. Total coliform colonies appear as red and blue, *E. coli* colonies appear as blue. A maximum of 200 colonies should be present on the filter. Manually count the red and blue colonies and record results on data sheet.

Equipment

Plastic trash bags
Labeling marker
Candle and matches
Alcohol – Isopropyl and Hand
Data sheets
Paper towels
Cooler and Ice

Laboratory Equipment

WhirlPak™ Bags
Sterile dilution water
Filtration apparatus
Petri dishes, Funnels. and Filters
Growth Media
Pipetter and pipettes
Tweezers
Incubator and thermometer
Mask and Magnifying glass

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