PROTOCOL FOR ALTERNATE TEST PROCEDURES FOR COLIFORM BACTERIA IN COMPLIANCE WITH WATER AND WASTEWATER REGULATIONS

QUANTITATIVE MEMBRANE FILTER METHODS

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NATIONAL EXPOSURE RESEARCH LABORATORY
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I. INTRODUCTION

1.1 Regulatory Background

1.1.1 The Administrator, U.S. Environmental Protection Agency (EPA), approves analytical methods for contaminants regulated under the Clean Water Act (CWA) and the Safe Drinking Water Act (SDWA). When EPA publishes a regulation under these Acts, which sets permit or maximum contaminant levels, it generally approves at least one method for detection and/or quantification of that contaminant. After a regulation is published, the Administrator may approve additional methods or modifications of approved methods that have satisfactorily completed a comparability study under the Alternate Test Procedure (ATP) Program.

1.1.2 Although the June 29, 1989 and January 8, 1991 regulations on the microbiological characterization of finished drinking water samples require the determination of the presence or absence of coliforms rather than their quantitative enumeration.[1,2] The quantitation of these organisms is still needed for source water, other ambient waters and wastewaters under the Surface Water Treatment Rule of the SDWA[3] and the 304(h) regulations of the CWA[4]. The quantitative MF methods for the evaluation of proposed quantitative microbiological methods for total and fecal coliform bacteria are in this protocol.

1.1.3 If the data evaluation demonstrates that the applicant's method performs at least as well as the currently approved method, the National Exposure Research Laboratory at Cincinnati (NERL-Cincinnati) will recommend approval to the Office of Water, which begins the regulation development process. Regulation development includes a Federal Register notice proposing to approve an ATP, public comment on the proposed method, and depending on public comment, a final rule published in the Federal Register that approves the method. The regulation development process can take one year or more.

1.2 Comparability Determination

1.2.1 This protocol describes the method description and Comparability Study data which EPA needs to evaluate an ATP for quantitative membrane filter (MF) methods in microbiology. The ATP program is intended to be flexible, consequently, EPA's NERL-Cincinnati may modify the study design for a particular proposed method. For this reason, before beginning the comparability testing, the applicant is required to contact the ATP Coordinator, Ecological Exposure Research Division, NERL-Cincinnati, U.S. Environmental Protection Agency, 26 West Martin Luther King Drive, Cincinnati, Ohio 45268, for approval of the test design.

1.2.2 Generally the reference method selected by EPA for use in the Comparability Study will be the same type of test as the proposed method. Consequently, if an MF method were proposed for the target organism, the corresponding MF reference method for the target organism would be used in the Comparability Study. However, for ATP applications in which a new technology is

proposed and for which there is no reference method counterpart, the ATP Program will use professional judgment in determining which other method would be the most appropriate as the reference method for the ATP Comparison Study.

2. APPLICATION FOR ATP

2.1 General Requirements

2.1.1 The general requirements for an application for nationwide approval of a new or revised method for total coliforms, fecal coliforms, and/or *E. coli* currently include the name and address of the applicant and/or authorized representative; the microbiological analyte and EPA regulation for which the new procedure is proposed; justification for the proposed new method; the title, company identification number, the date of submission, and a complete, stand-alone description of the proposed method in the required format (see Section 2.3 below).

2.1.2 If the applicant believes the proposed method is very similar to an Agency method, and/or represents a minor optional change to an Agency method, the applicant should also prepare a two-column side-by-side description of the sections of the Agency reference method and the proposed method and highlight differences between the methods. If the method is a proposed commercial version of a previously approved method, differences in reagents, interferences, test conditions, etc. should be presented with available performance data from the proposed and reference methods.

2.1.3 NERL-Cincinnati will judge the proposed method to be: 1) an acceptable version of or an optional minor modification of a previously promulgated method, which does not require approval as an ATP or 2) a significantly different method which requires an application for an ATP approval.

2.2 Every application for approval of a method must be made in triplicate (original + 2 copies) and forwarded to the Director, Ecological Exposure Research Division, NERL-Cincinnati, USEPA, Cincinnati, OH 45268. Upon receipt of the application, the ATP Coordinator will assign it an identification number, which should be used in all future communications. NERL-Cincinnati staff will initiate its technical reviews. The initial review will concentrate on the clarity and completeness of the description of the proposed method, the applicability of the proposed microbiological principles and reactions and the performance characteristics described for the method. The ATP staff will evaluate the submitted information and advise the applicant whether or not a Comparability Study is required.

2.3 Method Description

2.3.1 Each method description must include the following topics, listed in the EMMC method format[5], in the order given. The purpose of the description is to: 1) permit a fair comparison of the proposed and reference methods and 2) provide a clean, clear description of the method that can be easily used by laboratories. The method should read like a scientific paper.

2.3.1.1 Scope and Application

Include target organisms, type of test, e.g., membrane filter, chromogenic test, fluorogenic test, etc. and the sample types to which it is applicable.

2.3.1.2 Summary of Method

Include a brief outline of the method that describes its essential features without extraneous details.

2.3.1.3 Definitions

Include special terms or unique usage of terms. Do not include common microbiological terms.

2.3.1.4 Interferences

Include information and data generated by applicant during method development using typical samples containing a specific quantity of an interference such toxic materials, particulates, non-target organisms, etc.

2.3.1.5 Safety

Refer to good laboratory practices and use of a hood, goggles, and/or protective clothing, if appropriate. Emphasize any special procedure or precaution.

2.3.1.6 Instrumentation, Equipment and Supplies

Describe the necessary instrumentation, equipment and supplies and reference applicable manuals.

2.3.1.7 Reagents, Standards and Media

Describe reagent, standard and media formulations and preparation. Indicate shelf life of packaged materials and special storage requirements.

2.3.1.8 Sample Collection, Dechlorination, Preservation, Shipment, and Storage

Detail sample collection and handling requirements. Consider the sample collector, sample containers, dechlorination (if applicable), sample holding times and temperature as specified in Standard Methods[6].

2.3.1.9 Quality Control (QC)

Indicate the specific QC procedures and the frequency of performance required for the proposed method. They should include sterility checks, positive and negative controls, verification/confirmation of the target organism, media performance checks, duplicate analyses, etc. Document that a general QA/QC program is in operation, that routine QC checks are recorded and that actions are taken if a problem is indicated[7,8].

2.3.1.10 Calibration and Standardization

If applicable, include the calibration steps that are performed on pH meter, analytical balance, thermometer, autoclave, etc.

2.3.1.11 Procedure

Detail the sample preparation and analytical steps in the proposed method write-up. Exceptions are those routine microbiological procedures, such as membrane filtration of samples, that are known to professionals and that may be incorporated by reference.

2.3.1.12 Data Analyses and Calculations

Describe the procedures for data analyses, calculations, interpretation and reporting of results.

2.3.1.13 Method Performance Characteristics (sensitivity, specificity, recovery, and precision)

Provide available information on the performance characteristics of the proposed method and the procedures by which they were determined. Specificity data should demonstrate the ability of the proposed method to recover and distinguish the target organism from other organisms in the sample. The proposer should have data on method sensitivity, the variability of replicate analyses and data on recoveries of known numbers of target and non-target organisms by the proposed method. Summaries of these evaluative data should be included.

(However, in addition to the data provided above in the method description, a separate formal Specificity Study will be required as part of the ATP process. The method for determining the specificity of a method is presented in the Appendix.)

2.3.1.14 Pollution Prevention

Pollution prevention is any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. It is the environmental management tool preferred over waste disposal or recycling. When feasible, laboratory staff should use a pollution prevention technique such as preparation of the smallest practical volumes of reagents, standards and media or downsizing of the test units in a method.

If the proposed method prevents or reduces exposure to toxicity, pollution of the laboratory or the general environment including reduced generation of wastes, cite here. Also indicate non-applicability.

2.3.1.15 Waste Management

The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. Excess reagents and samples and method process wastes should be characterized and disposed of in an acceptable manner. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and

spirit of any waste discharge permit and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult "The Waste Management Manual for Laboratory Personnel," available from the American Chemical Society.

Describe the proper disposal methods for waste reagents, materials, supplies and samples.

2.3.1.16 References

Cite those source documents and publications which are necessary sources of information to properly perform the method.

2.3.1.17 Tables, Diagrams, Flowcharts, and Validation Data

Provide as needed.

2.3.1.18 Proprietary Information

Mark proprietary information in the proposed method description as "Confidential". EPA staff will treat proprietary information according to the regulations outlined in Subparts A and B in Part 2 of Title 40 of the CFR.

2.4 Study Approval

2.4.1 Method comparability data and quality control data will be required for each application of a new or significantly modified method. The applicant is urged not to initiate the Comparability Study until EPA has completed an evaluation of the method description and the preliminary performance characteristic information and has approved the Comparability Study design.

3. COMPARABILITY STUDY DESIGN

3.1 Summary of Study Design

3.1.1 Typically, the Comparability Study data are generated from replicate analyses of ten or more ambient waters and/or wastewaters from geographically-dispersed areas, which contain the target organism or organisms in varying numbers and different water or wastewater compositions. To simplify the tests, preliminary screening analyses are recommended to establish approximate numbers and reduce the number of dilutions and analyses in the formal study. The volumes or dilutions of water and wastewater samples tested must generate data within the acceptable range of the Agency reference method.

3.1.2 A minimum of twenty (20) replicate analyses are performed by each method on the 3 or more dilutions selected for each of the 10 samples. The replicate filtrations on the three or more dilutions of each sample must be performed on the same day for both the reference and proposed methods.

3.1.3 See the following example of a Comparability Study design.

Example of ATP Study Design

	Natural Sources of Organisms	No. Samples/ Source
Reference Method and Proposed Method	10	1

	Replicate MF Filtrations per Organism* and Sample Dilution	Minimum Comparability Results Required per Method
Reference Method	20	200
Proposed Method	20	200

*Total Coliform, Fecal Coliform, etc.

3.2 Laboratory Participation

3.2.1 Since the purpose of the study is to compare the proposed method to the reference method with minimal variability due to individual analyst error, the number of laboratories participating in the study should be minimized. It is strongly recommended that only one laboratory perform the analyses. This laboratory must be certified under the drinking water laboratory certification program. An applicant having a vested interest in the method, instrumentation, apparatus, reagents, media, or associated kits, may not perform the Comparability Study analyses in the applicant's laboratory.

3.3 Quality Control Data

3.3.1 Conduct QC checks on each day of analyses including temperature of incubators, analyses of known positive and negative response cultures, sterility controls and verification of colonies. Maintain records of QC checks performed. See the Manual for the Certification of Laboratories Analyzing Drinking Water[7] and the Intralaboratory Quality Control Guidelines in Standard Methods[8] for general guidance.

3.4 Sample Collection and Handling

3.4.1 Each sample should be collected and held in a single sterile, wide-mouth bottle of non-toxic heat-resistant plastic or borosilicate glass with a leak-proof screw cap or ground-glass stopper. The container must be resistant to the solvent action of water and survive sterilization without deformity or production of toxic materials. Screw caps must not produce bacteriostatic, toxic or nutritive products during sterilization. Bottles and closures should be checked for these effects before use in the study[9].

3.4.2 Samples should be maintained at 1-4 C during transit and holding time.

4. PREPARATION FOR COMPARABILITY STUDY

4.1 Selection and Collection of Water and Wastewater Samples

4.1.1 The reason for requiring ten different water/wastewater samples is to obtain, as much as is practical, a good presentation of the wide range of water types with their even wider range of target organisms, to which a method must respond appropriately. The ten samples can be collected over time as needed to complete the Comparability Study, with the understanding that all testing of a water or wastewater must be completed in a single day for both the reference and proposed methods.

4.1.2 Collect each water or wastewater sample in sufficient volume to complete all replicate analyses of sample or dilution volumes by both the reference and proposed methods.

4.2 Sample Volumes and Dilutions for the Comparability Study

4.2.1 Since the study is intended to compare the performance of the proposed method to the reference method, the data for the comparison must be from the same sample volumes or dilutions for both methods based on those sample volumes or dilutions that produce plate counts within the appropriate range for the reference method.

4.2.2 The sample volumes or dilutions are prepared by one of two options:

Option A utilizes preliminary analyses to establish target organism densities before beginning the Comparability Study. This approach reduces the number of dilutions needed to assure a bracketing of the range count but requires the additional effort of these analyses and a 24 h holding period for samples.

Option B proceeds directly with the Comparability Study. The samples are not held for 24 h and this option does not require preliminary analyses. Instead, the analyst gambles on her/his ability to "guess-timate" the sample volumes or dilutions necessary to bracket the reference method's counting range. This usually requires an expanded series of dilutions.

4.3 Option A. Determination of Bacterial Density

4.3.1 After collecting a water or wastewater sample(s), determine the density of the target organism using the MF procedure and media designated for the target organism:

TABLE 1

Target Organism	Method	Medium
Total coliforms	SM 9222B[10]	M-Endo LES Agar
Fecal coliforms	SM 9222D[11]	M-FC

4.3.2 Count the colonies of target organisms after 24 h to determine their approximate density in each water or wastewater sample. Use these results to

establish the sample volumes and/or dilution series needed to bracket the desired counting range for the reference method(s) in the Comparative Study. For the total coliform method using M-Endo LES agar, the range is 20-80 colonies/plate and for the fecal coliform method using M-FC agar, the range is 20-60 colonies/plate. Sample volumes of less than 1 mL must be added as a dilution. Filtration volumes of less than 10 mL must be proceeded in the filtration process by approximately 10 mL of sterile dilution water.

5. COMPARABILITY STUDY

5.1 Test Methods

5.1.1 The description of the reference methods and guidelines for use of the proposed method follow. For valid comparisons, the samples, sample volumes and dilutions must be the same for both methods and be based on the appropriate dilutions for the reference method. The spiked samples must be stirred continuously while portions are withdrawn for filtration and preparation of dilutions.

5.1.2 Include the testing of pure cultures of organisms of known positive and negative responses for total coliform and fecal coliform cultures to insure a proper interpretation of the results.

5.2 Reference Methods

5.2.1 The current reference methods that will be used in the comparison studies are listed in Table 2.

Table 2

Target Organism	Reference Method	Medium
Total coliforms	SM 9222B[10]/9221B[12]	M-Endo LES/LTB/BGLB
Fecal coliforms	SM 9222B[10]/9221E.1[13]	M-FC/LTB/EC

5.3 Reference Method for Total Coliform Bacteria

5.3.1 Filter the appropriate volumes of water or wastewater and/or dilutions to yield 20-80 total coliform bacteria on M-Endo LES agar.

5.3.2 Incubate at 35 C for 22-24 h. Golden green metallic sheen colonies are coliforms. Count and record sheen colonies.

5.3.3 For each sample, randomly pick 10 sheen colonies from each of 20 replicate plates, into LTB tubes. Incubate for 24-48 h at 35 C and examine. Growth, growth and gas, or growth and acid are positive presumptive tests.

5.3.4 Transfer growth from positive tubes into BGLB tubes and incubate at 35 C for 24-48 h. Growth and gas verify total coliform bacteria. Adjust original count based on verified count, and record.

5.4 Reference Method for Fecal Coliform Bacteria

5.4.1 Filter the appropriate volumes of water or wastewater and/or dilutions to yield 20-60 fecal coliform bacteria on M-FC agar plates.

5.4.2 Incubate M-FC agar plates for 24 h at 44.5 C. Count and record blue colonies as fecal coliform bacteria.

5.4.3 From each sample randomly pick 10 blue colonies on each of the 20 replicate plates into LTB tubes. Incubate for 24-48 h at 35 C. Growth, growth with gas, or growth and acid are presumptive positive tests.

5.4.4 Transfer growth from the positive LTB tubes into EC tubes and incubate at 44.5 C for 24 h. Growth and gas in EC medium verify fecal coliform bacteria. Adjust original count based on positive verification and record results.

5.5 Proposed Method

5.5.1 Filter the appropriate amounts of water or wastewater and/or dilutions and follow developer's directions for analyses.

5.5.2 Incubate tests units as directed. Follow developer's instructions for identifying and enumerating target colonies.

5.5.3 Complete verification steps according to the developer's directions.

5.6 Recording Results

Record the colony counts for all volumes tested by the reference and proposed methods.

6. DATA REPORTING

6.1 All the data from the comparability study, i.e., the replicate observations of the samples by the EPA reference method and the proposed method and the quality control observations should be forwarded to the Director, Ecological Exposure Research Division, National Exposure Research Laboratory - Cincinnati (address on page 1).

6.2 The results from the analyses of the ten samples should be recorded in the formats suggested in Attachments 1 and 2. Note that the forms have only one entry space for LTB and BGLB verification results for each pick from M-Endo LES of M-FC agar. The values entered should be the sums of the 24 and 48 h responses. The evaluation of the application can be accomplished more quickly by the EERD, NERL-Cincinnati ATP program if the information is also forwarded on disks compatible with an IBM-PC computer. The text on the disks should be presented in the latest version of WordPerfect (currently 5.1) and the data presented in WordPerfect or in ASCII. Confirm the current formats with EPA before generating study data.

7. DATA REVIEW

7.1 Upon receipt of an applicant's data sets, NERL-Cincinnati staff will initiate its technical and statistical reviews. Appropriate criteria will be

used to determine the acceptability of the reference method data as a basis in the evaluation of the analyses by the proposed method. If this evaluation is favorable, the proposed method will be recommended for approval by the program office and the applicant so notified. If the results of the analyses by the approved method are not satisfactory, the applicant will be notified that the application has not been approved.

7.2 The statistical review will consist of two parts: an examination of the data including descriptive statistics and a comparability analysis of the data. First, the data will be screened for outliers and unusual patterns. Descriptive statistics, such as the mean, standard deviation and coefficient of variation, will then be calculated for each set of replicates. The second part of the statistical review will compare the precision and recovery of the proposed method with that of the reference method using appropriate statistical techniques. The precision of the proposed method will be compared with that of the reference method by means of a nonparameteric statistical procedure attributable to Scheffe[14]. An analysis of variance (ANOVA) will be used to compare the recovery of the proposed method to that of the approved method.

8. METHOD RECOMMENDATION AND APPROVAL

8.1 After completion of the technical and statistical reviews, NERL-Cincinnati will prepare its recommendation for approval/disapproval of the new or significantly revised method. It will notify the applicant of its recommendation, and forward the recommendation to the Office of Water (OW), which has the responsibility for proposing the method in the Federal Register. Following a three-month public comment period, OW will review submitted comments and prepare the final nationwide approval/disapproval decision and promulgation notice in the Federal Register.

8.2 Upon approval of the method, the applicant will be responsible for the publication and distribution of the approved method to anyone requesting a copy.

9. REFERENCES

1. U.S. Environmental Protection Agency. Drinking Water; National Primary Drinking Water Regulations; Total Coliforms (Including Fecal Coliforms and *E. coli*); Final Rule. Federal Register 54(124):27562-27568, June 29, 1989. -

2. U.S. Environmental Protection Agency. National Primary Drinking Water Regulations; Analytical Techniques; Coliform Bacteria, Federal Register 56(5):636-643, January 8, 1991.

3. 40 CFR Part 141 and 142, Drinking Water: National Primary Drinking Water Regulations; Filtration, Disinfection, Turbidity, *Giardia lamblia*, Viruses, *Legionella* and Heterotrophic Bacteria; Final Rule June 29, 1989, pp. 27530-27531.

4. 40 CFR Part 136, Guidelines Establishing Test Procedures for the Analysis of Pollutants Under the Clean Water Act; Final Rule and Interim Final Rule and Proposed Rule, October 26, 1984, p. 19.

5. Villa, O. and L. Reed, Co-Chairs, EMMC Methods Integration Panel. Final Version of Approved EMMC Format (Memorandum to Members of EMMC Steering Committee, Methods Integration Panel, and Work Group, Tri-Chairs). U.S. Environmental Protection Agency, February 14, 1992. pp. 1-2.

6. Standard Methods for the Examination of Water and Wastewater, 18th Ed., APHA, Washington, DC, 1992, Section 9060A and 9060B, pp. 9-18 thru 9-20.

7. U.S. Environmental Protection Agency. Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures, Quality Assurance, 3rd ed. EPA/570-9-90-008A. Chapter 5, Microbiology (revised). Office of Drinking Water, Washington, D.C., October 1991, pp. 37-48.

8. Standard Methods..., Section 9020B, pp. 9-3 thru 9-12.

9. Standard Methods..., Section 9020B, p. 9-5.

10. Standard Methods..., Section 9222B, pp. 9-54 thru 9-58.

11. Standard Methods..., Section 9222D, pp. 9-60 thru 9-61.

12. Standard Methods..., Section 9221B, pp. 9-46 to 9-50.

13. Standard Methods..., Section 9221E.1, pp. 9-52 and 9-53.

14. Scheffe, Henry. The Analysis of Variance, John Wiley and Sons, New York, NY, 1959.

ATTACHMENT 1. EXAMPLE FORMAT

RESULTS OF ATP COMPARABILITY STUDY OF QUANTITATIVE MF METHOD FOR TOTAL COLIFORM BACTERIA

Title of Proposed Method: _____ **Lab Name & Location:** _____

Analyst: _____ **Supervisor's Signature:** _____

RECORD COLONY COUNTS and VERIFIED COUNTS by REFERENCE and PROPOSED METHODS

Sample # and Description: _____

Date of Analysis: __/__/__

REFERENCE METHODS

Replicate	M-ENDO LES (Selected Dilutions)			Replicate	LTB	BGLB
1				1		
2				2		
3				3		
4				4		
5				5		
6				6		
7				7		
8				8		
9				9		
10				10		
11				11		
12				12		
13				13		
14				14		
15				15		
16				16		
17				17		
18				18		
19				19		
20				20		
Sum of Results:				Sum of Results:		

PROPOSED METHOD

Replicate	(Selected Dilutions)			Replicate	PROPOSED METHOD VERIFICATION (If required)
1				1	
2				2	
3				3	
4				4	
5				5	
6				6	
7				7	
8				8	
9				9	
10				10	
11				11	
12				12	
13				13	
14				14	
15				15	
16				16	
17				17	
18				18	
19				19	
20				20	
Sum of Results:				Sum of Results:	

ATTACHMENT 2. EXAMPLE FORMAT

RESULTS OF ATP COMPARABILITY STUDY OF QUANTITATIVE MF METHOD FOR FECAL COLIFORM BACTERIA

Title of Proposed Method: _____ **Lab Name & Location:** _____

Analyst: _____ **Supervisor's Signature:** _____

RECORD COLONY COUNTS and VERIFIED COUNTS by REFERENCE and PROPOSED METHODS

Sample # and Description: _____

Date of Analysis: ___/___/___

REFERENCE METHODS

Replicate	M-FC Selected Dilutions			Replicate	LTB	EC
1				1		
2				2		
3				3		
4				4		
5				5		
6				6		
7				7		
8				8		
9				9		
10				10		
11				11		
12				12		
13				13		
14				14		
15				15		
16				16		
17				17		
18				18		
19				19		
20				20		
Sum of Results:				Sum of Results:		

PROPOSED METHOD

Replicate	Selected Dilutions			Replicate	PROPOSED METHOD VERIFICATION (If required)
1				1	
2				2	
3				3	
4				4	
5				5	
6				6	
7				7	
8				8	
9				9	
10				10	
11				11	
12				12	
13				13	
14				14	
15				15	
16				16	
17				17	
18				18	
19				19	
20				20	
Sum of Results:				Sum of Results:	