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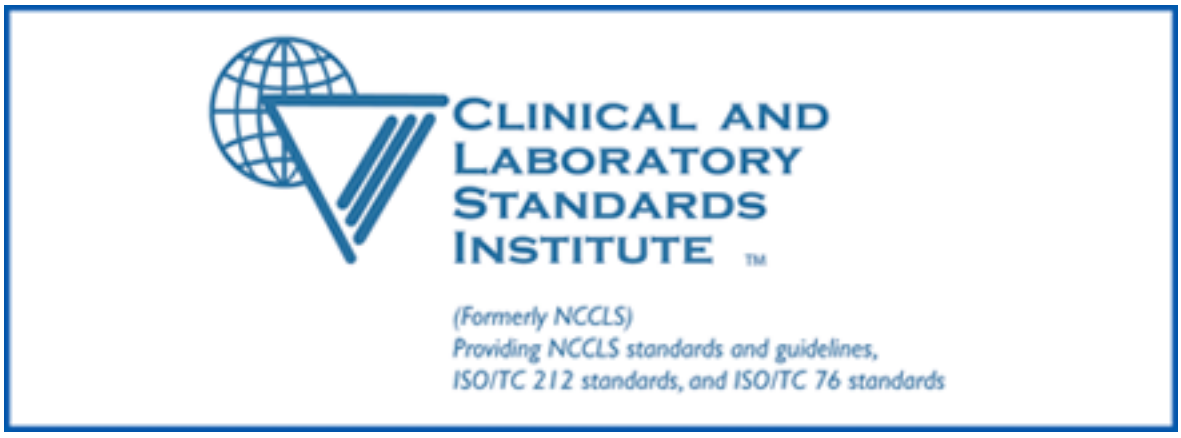
Protocols for Evaluating Dehydrated Mueller–Hinton Agar; Approved Standard



This document provides procedures for evaluating production lots of Mueller–Hinton agar and for the development and application of reference media.

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Protocols for Evaluating Dehydrated Mueller–Hinton Agar; Approved Standard

Abstract

This document describes three protocols for the evaluation of dehydrated Mueller–Hinton agar in the disk diffusion procedure for antimicrobial susceptibility testing. The first protocol is for use by manufacturers to evaluate production lots of Mueller–Hinton agar. The second and third are for selection and stability testing of primary and secondary reference lots of Mueller–Hinton agar.

Performance of production lots is determined by comparison with the primary standard lot, which is provided to manufacturers upon request. The secondary standard lot may be used when the supply of the primary standard is threatened or its performance becomes unsatisfactory. Acceptable lots may be labelled with a statement indicating that the lot was tested according to the protocol found in this document and meets the acceptance limits of that protocol. These acceptance criteria apply only to disk diffusion procedures. They do not qualify Mueller–Hinton agar for other methods, such as agar dilution or antibiotic gradient.

Performance of new reference lots is determined by comparison with the current primary standard lot. The original primary reference standard lot was selected in 1983 and the secondary standard lot in 1984. Replacement reference standards were selected in 1992. Replacement primary and secondary reference standard lots are expected to be selected in 1996.

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George L. Evans, Ph.D.
Richard H. Bell, Ph.D.
Leslie V. Cunningham, Ph.D.
Mary Jane Ferraro, Ph.D.
Anthony E. Maltese, M.S.
Phyllis A. Pienta, M.S.
Robert P. Rennie, Ph.D.
Roxanne G. Shively, M.S.
Jana M. Swenson, M.M.Sc.

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Committee Membership

Area Committee on Microbiology

J. Allan Waitz, Ph.D. Chairholder	Eugene, Oregon

Subcommittee on Culture Media

George L. Evans, Ph.D. Chairholder	Becton Dickinson Microbiology Systems Hunt Valley, Maryland
Richard H. Bell, Ph.D.	Difco Laboratories Detroit, Michigan
Leslie V. Cunningham, Ph.D.	Acumedia Manufacturers, Inc. Baltimore, Maryland
Mary Jane Ferraro, Ph.D.	Massachusetts General Hospital Boston, Massachusetts
Anthony E. Maltese, M.S.	Remel Lenexa, Kansas
Phyllis A. Pienta, M.S.	American Type Culture Collection Rockville, Maryland
Robert P. Rennie, Ph.D.	University of Alberta Hospitals Edmonton, Alberta, Canada
Roxanne G. Shively, M.S.	Center for Devices and Radiological Health, Food & Drug Administration Rockville, Maryland
Jana M. Swenson, M.M.Sc.	Centers for Disease Control and Prevention Atlanta, Georgia

Advisors

Arthur L. Barry, Ph.D.	Clinical Microbiology Institute Tualatin, Oregon
Janet Hindler, MCLS, M.T.(ASCP)	UCLA Medical Center Los Angeles, California
Gerald A. Moore	Unipath Co. (Oxoid Division) Ogdensburg, New York
Mary E. Nichols	Remel Lenexa, Kansas

David A. Power, Ph.D.	Becton Dickinson Microbiology Systems Hunt Valley, Maryland
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Contents

Foreword

This standard is the result of the comprehensive efforts of the NCCLS Subcommittee on Culture Media. The project had four objectives:

- To evaluate the extent of variation of current manufactured lots of Mueller–Hinton agar
- To select a primary reference lot of medium that would become a "gold standard" for the evaluation of Mueller–Hinton agar and the selection of secondary reference standard lots
- To select a reference lot of medium that could be used directly by manufacturers for the standardization of production lots of Mueller–Hinton agar
- To develop a protocol that manufacturers could use to evaluate the performance of Mueller–Hinton agar in comparison with the reference medium.

The subcommittee addressed the first two of these objectives in 1982 by organizing a controlled, blinded, multicenter study involving five independent laboratories. For the study, seven manufacturers each provided 25 kg of Mueller–Hinton agar in bottles containing 100 grams. These lots were coded and distributed by the Biological Products Division, Centers for Disease Control (CDC; now Centers for Disease Control and Prevention). The control organisms were supplied from a single lot by the American Type Culture Collection. Technologists from the participating laboratories were trained to perform standard disk diffusion testing in a course given at the CDC to ensure uniform methodology. In addition to the five independent laboratories, five manufacturers' laboratories also participated, but in the analysis of the results, only data from the independent laboratories were used.

The data from the study were statistically analyzed at the Cleveland Clinic. The variables included:

- Precision of the zone diameter readings
- Daily variation in zone readings
- Interlaboratory variations
- The performance of each lot versus the criteria in the NCCLS standard M2 (Performance Standards for Antimicrobial Disk Susceptibility Tests).

After examining the analyzed data, the subcommittee selected a single lot of Mueller–Hinton agar as a primary reference medium. The following criteria were used for selecting the primary reference medium:

- Disk diffusion testing yielded zone diameters with mean values that were close to the midpoint of the control ranges recommended in the NCCLS standard M2.
- Minimal variability was detected.
- The medium met all other physical and chemical criteria set forth in the NCCLS standard M2.

Samples of the primary reference medium were then submitted to the participating media manufacturers to provide them with a benchmark for producing production lots that would then become candidates for the secondary reference medium. These candidate lots were tested at the CDC and a secondary reference standard was selected on the basis of guidelines established by T.L. Gavan, M.D., and G.W. Williams, Ph.D., at the Cleveland Clinic. A comprehensive report of this project was presented at an American Society for Microbiology symposium.[1,2] An historical review

of the subcommittee's development of reference standards for Mueller–Hinton agar has been published.[3]

The secondary reference standard was used in conjunction with the manufacturers' protocol discussed in this document to standardize production lots of Mueller–Hinton agar. Initially, each participating manufacturer submitted data to the subcommittee showing satisfactory performance of three production lots of Mueller–Hinton agar. When this phase was completed, the manufacturers could then label their products with a statement indicating that their Mueller–Hinton agar conforms to NCCLS requirements (see Section 3.4). After submitting the results of three lots that meet the acceptance criteria of the protocol, the manufacturers could continue to use the statement on the label of any lot meeting the acceptance criteria of the protocol without submitting the results to the subcommittee.

In 1992, the subcommittee selected new primary and secondary reference standards from four candidate lots.[3] The primary standard was labeled lot C-90 and the secondary standard was labeled A-90. Since that time, A-90 has been in use by dehydrated media manufacturers who incorporate the label statement in this standard onto their product labels.

In 1994, the subcommittee organized a project to select replacement standards. This time there were five participating manufacturers and three test sites. Each of the five manufacturers submitted a candidate lot in the form of 350 x 100 g glass jars, and testing was completed at the three test sites in February 1995. A statistical analysis has been completed and the data are being reviewed.

The definitions and applications of the primary and secondary reference standards have been changed in this edition, as indicated in Section 2. In addition, there is a clarification of the reading of the inhibition zone for the methicillin-resistant *Staphylococcus aureus* (MRSA) strain (ATCC® 43300) and an increase in the incubation times of cultures during inoculum preparation. There has also been a clarification of the acceptance criteria for those drug–organism combinations with only four replicates.

George L. Evans, Ph.D.
Chairholder, Subcommittee on Culture Media

Universal Precautions

Because it is often impossible to know which might be infectious, all patient blood specimens are to be treated with universal precautions. Guidelines for specimen handling are available from the U.S. Centers for Disease Control and Prevention [*MMWR* 1987; 36 (suppl 2S): 2S-18S]. NCCLS document M29-T2, *Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue—Second Edition; Tentative Guideline*, deals specifically with this issue.

Key Words

Antimicrobial susceptibility testing, disk diffusion, Mueller–Hinton agar.

Acknowledgments

The subcommittee acknowledges the efforts of Aaron Lane, who was a member of the subcommittee until his retirement from Difco Laboratories. The encouragement, support, and sharing of knowledge he provided to the subcommittee from the beginning of this project contributed significantly to its success. The subcommittee also acknowledges the leadership and expertise of Harry A. Frankel, M.A., S.M. (AAM) (Pfizer Pharmaceuticals, New York, New York) and Helen M. Pollock, Ph.D. (University of South Alabama Medical Center, Mobile, Alabama), who were co-chairholders of the subcommittee at the time of the development of the proposed standard.

The subcommittee is grateful to Phyllis Pienta and the American Type Culture Collection for the coding and storage of candidate lots, as well as shipping of these lots to the test sites. The subcommittee is also grateful to the American Type Culture Collection for the donation of test cultures and to Becton Dickinson Microbiology Systems and Difco Laboratories for the donation of susceptibility testing disks.

The subcommittee acknowledges the technical assistance of Dr. George Williams and Sarah B. Forsythe of the Cleveland Clinic Foundation for statistical analysis of the data generated in the selection of the first reference standards and Betty Stephenson of Becton Dickinson for the analysis of data generated in the selection process for the new reference media.

Protocols For Evaluating Dehydrated Mueller–Hinton Agar; Approved Standard

1 Introduction

The NCCLS disk diffusion standard M2 (Performance Standards for Antimicrobial Disk Susceptibility Tests) recommends the use of Mueller–Hinton agar for routine susceptibility testing for the following reasons:

- It shows fairly good batch-to-batch reproducibility
- It is low in sulfonamide, trimethoprim, and tetracycline inhibitors
- It gives satisfactory growth of most nonfastidious pathogens
- A large amount of data have been collected from antimicrobial susceptibility tests with this medium.

A report of a multicenter, international collaborative study by Ericsson and Sherris presents data on world-wide antibiotic susceptibility testing using Mueller–Hinton agar and other culture media.[4] This study discloses many variables that affect the size of zones of inhibition. Among the critical elements that contribute to variation are the culture media used in agar diffusion tests. Recent studies add considerable support for developing more exacting performance criteria for Mueller– Hinton agar.[5-8] These studies indicate that an objective evaluation of the extent of variation of Mueller–Hinton agar is needed. This document provides protocols for evaluating production lots of Mueller–Hinton agar and for the selection of standard reference lots of this medium. These acceptance criteria apply only to disk diffusion procedures. They do not qualify Mueller–Hinton agar for other methods, such as agar dilution or antibiotic gradient. Details of the disk diffusion procedure appear in several publications,[9-11] including NCCLS document M2 (Performance Standards for Antimicrobial Disk Susceptibility Tests).

2 Definition of Reference Media

2.1 Primary Reference Medium

The primary reference medium is a coded lot of Mueller–Hinton agar selected by the NCCLS Subcommittee on Culture Media. It is stored at a facility selected by the subcommittee and is tested at one-year intervals to determine stability. Past experience with the first primary reference standard (lot 5) indicates that the dehydrated medium has excellent stability (about 10 years) when stored in sealed glass jars at controlled room temperature.

The primary reference medium is provided to manufacturers of dehydrated Mueller–Hinton agar for evaluation of production lots. The primary reference medium is also distributed for stability testing and for the development of new primary and secondary reference media. The manufacturer of this lot is only known by the coding laboratory. New reference media will be selected whenever appropriate; i.e., when the current supply nears depletion (<5,000 g), or shows signs of deterioration or unsatisfactory performance.

2.2 Secondary Reference Medium

The secondary reference medium is a coded lot of Mueller–Hinton agar with performance characteristics that are similar to the primary reference medium, as determined by the protocol in this standard (Section 5.2). The main purpose of this medium is for use as a back-up for the primary reference standard when its supply is threatened, or performance becomes unsatisfactory. This medium is provided to manufacturers of dehydrated Mueller– Hinton agar for their use in evaluating production lots only when the primary reference medium is not available. The stability testing is performed at the same intervals as for the primary reference medium (see Section 4). The manufacturer of this lot is only known by the coding laboratory.

3 Manufacturers' Protocol for Testing Production Lots of Mueller–Hinton Agar

Upon request, manufacturers of dehydrated Mueller–Hinton agar are provided with adequate amounts of the primary reference medium for evaluating the performance of production lots, according to the following protocol.

3.1 Preparation of Control Cultures

3.1.1 Stock cultures for this procedure are prepared from lyophilized cultures obtained from ATCC®.* Reconstitute these cultures according to the directions obtained from ATCC. The cultures required are *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 33186 (or ATCC 29212), *S. aureus* ATCC 43300, and *E. coli* ATCC 35218.

3.1.2 Using a sterile loop, inoculate by streaking two or three soybean-casein digest agar (tryptic soy agar; TSA) with 5% sheep blood plates with the suspension in the reconstituted vial for each control culture. Incubate the inoculated plates for 18–24 hours at 35 °C in ambient air.

3.1.3 After incubation, check for purity and harvest the entire growth from the plates and suspend it in soybean-case-in digest broth (tryptic soy broth; TSB) containing 15% glycerol. To prepare this medium, dissolve 30 g of the dehydrated broth medium in approximately 500 mL of deionized water and add 150 mL of glycerol. Adjust the volume to 1L, mix well, and sterilize it at 121 °C for 15 minutes. The suspension may be adjusted to the turbidity of a 0.5 McFarland standard (about 1 to 2 x 10^8 CFU/mL), if a known viability is desired. Dispense 0.5 mL of the suspension into small, sterile vials. Store the vials at –60 °C, or a lower temperature. With this method, cultures should be viable for at least 1 year. Other methods of preparing stock cultures may be used if they provide adequate viability and stability.

3.1.4 The day before the inoculation of the plates (day 1), thaw a vial of each of the control cultures that will be needed. Inoculate each culture onto a plate of TSA with 5% sheep blood and incubate it for 18–24 hours at 35 °C in ambient air. After incubation, check for purity. If satisfactory, these plates are then used to prepare standardized inoculum, as described in M2 (Performance Standards for Antimicrobial Disk Susceptibility Tests).

NOTE: The incubation period for plate cultures used to prepare inoculum is not critical for the organisms used in this protocol. It may be from 16 to 24 hours. A sufficient incubation period might be needed so that the test plates (containing disks) can be read during normal working hours after a 16- to 18-hour incubation.

3.1.5 Periodically renew stock cultures from fresh, lyophilized cultures obtained from ATCC.

3.2 Performance of the Test

3.2.1 Perform the standard disk diffusion test, while adhering strictly to the procedure and time restrictions as described in the NCCLS standard M2 (Performance Standards for Antimicrobial Disk Susceptibility Tests).

3.2.2 Perform testing according to the following schedule:

*ATCC is a registered trademark of the American Type Culture Collection.

Day 1

1. Medium preparation: Unless instructed otherwise on the label, add 38.0 g of the dehydrated medium to 1 L of deionized or purified (USP) water. Boil for 1 minute and sterilize at 121 °C for 15 minutes. Cool to approximately 50 °C before pouring plates.

2. Pour plates to achieve a depth of 4 to 5 mm (usually 70 mL for 150-mm-style plates). Prepare three plates of each medium (production lot and the primary reference standard) for each of the first three control cultures listed in Section 3.1.1. Prepare one plate of each medium for each of the remaining three control cultures and one plate for pH determination.

3. Measure the pH of both media at 25 °C as described in M2. The pH should be 7.2 to 7.4. Record the pH of the test lot and the reference lot on the manufacturers' protocol report form (Appendix A).

4. Thaw frozen stock cultures and subculture them to TSA with 5% sheep blood plates, as described in Section 3.1.4.

Day 2

1. Examine the plates prepared on Day 1. If excess surface moisture is present, the plates should be placed in an incubator (35 °C) or a laminar flow hood at room temperature with lids ajar until the excess surface moisture is lost by evaporation (usually 10 to 30 minutes). The surface should be moist, but no droplets of moisture should be on the surface of the medium or on the petri dish covers when the plates are inoculated.

2. Pick several colonies from the plate cultures of the control organisms and prepare a standardized inoculum by suspending the growth in TSB and adjusting to the turbidity of a 0.5 McFarland standard (approximately 1 to 2 x 10^8 CFU/mL) as described in the NCCLS standard M2.

3. Inoculate the three replicate plates of each medium with the standardized inoculum of each of the following control cultures: *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853. Inoculate one plate with each of the following control cultures: *E. faecalis* ATCC 33186 (or ATCC 29212), *S. aureus* ATCC 43300, and *E. coli* ATCC 35218. Inoculate plates so that no more than 15 minutes elapse between the time the suspension is adjusted and all plates are inoculated. Each control organism may be tested on different days.

4. Dispense the appropriate antimicrobial disks onto each plate according to the protocol described in Appendix A.

5. To the plate with *E. faecalis* ATCC 33186 (or ATCC 29212), apply two disks of trimethoprim/sulfamethoxazole for the thymidine test. To the plate with *S. aureus* ATCC 43300, apply two disks of oxacillin for the MRSA test. To the plate with *E. coli* ATCC 35218, apply two disks of amoxicillin/clavulanic acid.

6. Incubate the plates agar side up (lid side down) for 16 to 18

hours (24 hours for *S. aureus* 43300) at 35 °C in ambient air.

Day 3

1. After incubation, measure the diameter of each zone of inhibition to the nearest 0.1 mm using calipers (those with a digital readout are recommended) held against the back of the culture plate, which is illuminated with a light that is above the plate (reflected light), and with a black, nonreflective surface in the background. The plate may be laid over a black, nonreflective surface with a light source that is above and behind the plate at a 45 °C angle opposite from the reader. Since this protocol measures the difference in the mean zone diameters of two lots of media, the important factor is that all the zones are measured in precisely the same manner. For *S. aureus* ATCC 43300, the zone of inhibition should be carefully examined with *transmitted* light, to detect hazy growth or small colonies within the zone.

2. Record results on the data sheet (Appendix A) as described in Sections 3.3.1 and 3.3.2. If, in a particular antimicrobial agent–organism combination, three replicates are not obtained because a disk failed to dispense, fell off the agar when inverted, etc., the testing must be repeated for that combination.

3.3 Interpretation of Results

3.3.1 For each medium (production and reference standard), calculate the mean of the three zone diameters for each antimicrobial agent–organism combination with *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853. Calculate the difference between the mean values for the production lot and the mean values for the reference medium, and record the results on the data sheet (Appendix A).

3.3.2 For *E. faecalis* ATCC 33186 (or ATCC 29212), *S. aureus* ATCC 43300, and *E. coli* ATCC 35218, calculate the mean of the two zone diameters for the production lot and the reference standard. Record the results on the data sheet (Appendix A).

3.3.3 For acceptable lots, the difference in the mean zone diameters determined in Section 3.3.1 must not be greater than 2.0 mm for 90% of the antimicrobial agent–organism combinations and none may exceed a 3.0-mm difference. For *E. faecalis* ATCC 33186 (or ATCC 29212) and trimethoprim/sulfamethoxazole, the inhibition zone must be equal to, or greater than, 20 mm, with clarity comparable to the reference medium. For *S. aureus* ATCC 43300, there should be no zone, or a very hazy zone with growth up to the oxacillin disk, after 24-hour incubation. For *E. coli* ATCC 35218, the mean zone diameter with amoxicillin/clavulanic acid should be 17 to 21 mm. If the reference medium does not produce satisfactory results with *E. faecalis* ATCC 33186 (or ATCC 29212), *S. aureus* ATCC 43300, or *E. coli* ATCC 35218, then that test must be repeated.

3.3.4 For acceptable lots, the pH value must be 7.2 to 7.4.

3.4 Label Statement

For acceptable lots, the following statement may be added to the product label:

"This lot of Mueller–Hinton agar has been tested according to, and meets the acceptance limits of, the current M6 protocol published by the NCCLS."

The basis for approval of the label statement for each manufacturer is data from three consecutive production lots. Review of these data by the subcommittee is required. This process is necessary only for the initial submission.

4 Storage and Control of Primary and Secondary Reference Media, and Stability Studies

4.1 Storage and Control of Primary and Secondary Reference Media

The primary and secondary reference media are packaged in 100-g quantities in glass jars; they are stored at a facility approved by the NCCLS Subcommittee on Culture Media. Inventory of these reference materials is made semi-annually and reported to the Chairholder of the NCCLS Subcommittee on Culture Media, or to the NCCLS staff liaison. Shipments of either the primary or secondary reference medium can be made only with the approval of the Chairholder of the NCCLS Subcommittee on Culture Media.

4.2 Stability Studies

The primary and secondary reference media are tested every twelve months or until test data indicate that a different schedule is feasible. The testing is carried out according to the procedures described below and includes the determination of moisture (loss on drying), gel strength, and pH. Specifications for gel strength and moisture have not been established at this time.

4.2.1 For stability testing, lyophilized vials of the ATCC control cultures shall be reconstituted, and stock cultures shall be prepared, as described in the manufacturers' protocol. The control strains are *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 33186, *S. aureus* ATCC 43300, and *E. coli* ATCC 35218. For stability testing of reference media, ATCC 29212 cannot be substituted for ATCC 33186 (see Section 5.4.1.7).

4.2.2 Samples of the primary and secondary reference lots shall be sent to two manufacturers for determination of moisture, pH, and gel strength. The procedures for these tests are the same as those used by the manufacturers' laboratory.

4.2.3 Disks to be used for the stability tests shall be within 95 to 130% of labelled potency and shall be supplied by one manufacturer, who will determine the disks' potency using the FDA-recommended assays just before shipping them. The disk content is the same as that in Table 3 of M2. The disks shall be stored at –20°C or lower and shalll be used until their respective expiration dates. If discrepancies are observed during the stability program, disks shall be re-assayed to determine the level of antimicrobial agent.

4.2.4 The stability testing shall be conducted by a laboratory approved by the subcommittee. The procedure is the same as for the manufacturers' protocol, except that a total of six replicates of each antimicrobial agent–organism combination is tested. If, for any reason, six replicate zones are not obtained for a given antimicrobial agent– organism combination, the test should be repeated.

4.2.5 After 16 to 18 hours of incubation (24 hours for *S. aureus* ATCC 43300), the zones shall be measured by two persons. One of the readers, insofar as is possible, shall be the same for each annual stability check.

4.2.6 Data from the stability tests shall be reviewed by the NCCLS Subcommittee on Culture Media. Any significant change in the performance, moisture, pH, or gel strength shall be reported to users of the reference media.

5 Selection of New Reference Media

The procedure that follows is used to evaluate candidate lots for primary and secondary reference media when the supply of existing media is close to being depleted, or when results of the stability tests indicate a significant change. Changes in physical quality of the medium are indicated by caking of the medium, or a pH below 7.2 or above 7.4. An increase in moisture content, or a change in gel strength can suggest deterioration. Although no values for gel strength have been established at this time that would be a reason for rejection, a change of ± 10% in a given twelve-month period would signal the need for replacement media. The process of selecting new lots may be initiated every five years for either of the reference media, or sooner, if necessary.

5.1 General Process

5.1.1 When new primary or secondary reference lots are needed, at least three manufacturers are asked to participate in the process. They are provided with at least 600 g of the primary reference medium and asked to prepare at least 35 kg of a new lot or select 35 kg from a current production lot that produces satisfactory results when tested in parallel with the NCCLS primary reference medium. The candidate lot selected is dispensed in amounts of 100 g into 350 jars. The jars are provided by one of the manufacturers. No labels are placed on the jars. Only the outer shipping container indicates the name and address of the manufacturer. The candidate lots are shipped to an independent facility designated by the NCCLS Subcommittee on Culture Media.

5.1.2 The candidate lots of Mueller–Hinton agar are logged in and appropriately coded at the designated facility. The jars from each of the manufacturers are then labeled with the appropriate information indicated in Section 5.1.3. Codes are confidential and are known only by the facility receiving the candidate lots.

5.1.3 Labels applied by the designated facility convey the following information:

- Mueller–Hinton agar
- NCCLS code no.
- Candidate Reference Standard
- Date
- Use 38.0 g/L

5.1.4 At least 500 g of each of the candidate lots and an adequate supply of the current primary reference medium are then sent to the independent investigators to be tested using the procedure described below. At least two independent investigators are solicited and selected by the NCCLS Subcommittee on Culture Media.

5.1.5 Antimicrobial susceptibility disks are supplied by one manufacturer at the request of the subcommittee. The potency and storage are as indicated in Section 4.1.3.

5.1.6 Using the protocol in Section 5.2, the independent laboratories then test the candidate lots in parallel with the primary reference lot.

5.1.7 A final decision on the selection of a primary and secondary reference medium is then made by the subcommittee, using the selection criteria given in Section 5.4.

5.2 Protocol for Evaluating New Reference Media

5.2.1 The control cultures for this procedure are prepared in the same manner as is described in Section 3.1. The following control cultures are used in this protocol:

S. aureus ATCC 25923
E. coli ATCC 25922
P. aeruginosa ATCC 27853

E. faecalis ATCC 33186[**]
S. aureus ATCC 43300
E. coli ATCC 35218.

5.2.2 For *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853, thirty replicate zone diameters with each candidate lot and the primary reference lot are required. For *E. faecalis* ATCC 33186, *S. aureus* ATCC 43300, and *E. coli* ATCC 35218, only four replicates are needed.

5.2.3 Performance of the Test

The general procedure is the same as is described in Section 3.2, but with an expanded test battery. Because of the need for thirty replicates with the first three control cultures with each medium (several candidate lots and the reference lot), it is recommended that only one culture be tested in each three-day period. If more than three candidate lots are to be tested, the testing may require additional three-day periods and an additional amount of primary reference medium.

Day 1

1. For thirty replicate plates that are required for the first three cultures, approximately 2.5 L of each of the candidate lots and the primary reference lot are prepared. Increase the volume of primary reference medium, as needed, if more than three candidate lots are to be tested. Boil the media for 1 minute and autoclave for 15 minutes at 121 °C. Cool the media to 45 to 50 °C and pour 70 mL into each of at least thirty large petri dishes (150 mm-style) for each medium.

2. For the four replicates required for the next three cultures and an extra plate for pH determination, prepare 500 mL of each of the candidate lots and the reference lot. If more than three candidate lots are to be tested, increase the volume of the primary reference medium proportionately.

3. Measure the pH of each of the candidate lots and the reference lot at 25 °C, as described in the NCCLS standard M2. Round off the readings to the nearest 0.1 pH units. The pH should be 7.2 to 7.4.

4. Thaw a frozen vial of the control culture to be used and subculture it to blood agar plates, as described in Section 3.1.4.

Day 2

1. Examine the plates prepared on Day 1. If excess surface moisture is present, the plates should be allowed to dry as described in Section 3.2.2, Day 2(1).

2. For the first three cultures in Section 5.2.1, pick ten well-isolated colonies from the blood agar plates and suspend in each of three 10-mL tubes of TSB. Adjust the suspension to the turbidity of a 0.5 McFarland standard as described in the NCCLS standard M2. This should provide sufficient inoculum for 120 plates (thirty of each of the candidate lots and thirty of the primary reference lot). If more than three candidate lots are to be tested, prepare additional inoculum, as needed, or test on separate days. For the remaining three cultures, only one tube of inoculum is needed.

[**] ATCC 29212 may not be substituted (see Section 5.4.1.7).

3. Because of the time required to inoculate 120 plates, refrigerate the adjusted suspension or readjust the turbidity after every sixty plates are inoculated.

4. When inoculating thirty replicates of each medium for the first three cultures, alternate inoculation of the primary reference lot and each of the candidate lots. Label the plates in the order of inoculation (from 1 to 30).

5. Dispense the appropriate antimicrobial disks as follows (the disk content is the same as in Table 3 of the NCCLS standard M2):

   For *S. aureus* ATCC 25923, use the following antimicrobial disks:

   - Amoxicillin/clavulanic acid
   - Erythromycin
   - Ampicillin/sulbactam
   - Oxacillin
   - Cephalothin
   - Tetracycline
   - Ciprofloxacin
   - Vancomycin

   For *E. coli* ATCC 25922, use the following antimicrobial disks:

   - Ampicillin
   - Chloramphenicol
   - Cefotaxime
   - Gentamicin
   - Cefoxitin
   - Sulfisoxazole
   - Cephalothin
   - Tetracycline

   For *P. aeruginosa* ATCC 27853, use the following antimicrobial disks:

   - Amikacin
   - Gentamicin
   - Cefoperazone
   - Imipenem
   - Cefotaxime
   - Piperacillin
   - Ceftazidime
   - Ticarcillin/clavulanic acid
   - Ciprofloxacin
   - Tobramycin

   For *E. faecalis* ATCC 33186, use the following antimicrobial disks:

   - Trimethoprim/sulfamethoxazole
   - Trimethoprim
   - Vancomycin

   For *S. aureus* ATCC 43300, use the following antimicrobial disks:

   - Methicillin
   - Oxacillin

   For *E. coli* ATCC 35218, use the following antimicrobial disks:

   - Amoxicillin/clavulanic acid
   - Ampicillin/sulbactam
   - Ticarcillin/clavulanic acid

6. Incubate all plates, agar side up, at 35 °C for 16 to 18 hours (24 hours for *S. aureus* ATCC 43300).

Day 3

1. To prevent changes in zone diameters that might occur because of the long time required to measure them, remove the plates from the incubator and place at 2–8 °C.

2. For the cultures with thirty replicates, remove the first ten plates of each medium (forty plates) that were inoculated from the refrigerator and leave

them at room temperature for 15 minutes. Measure the zones of inhibition to the nearest 0.1 mm with the digital readout calipers, alternating the reading as was done for the inoculation; i.e., read the forty plates in the same sequence as they were inoculated. Remove the next forty plates from the refrigerator, allow them to come to room temperature, and continue as previously described. Continue with the last set of forty plates.

3. Record the thirty replicate zone diameters for the first three cultures for each of the candidate lots and the reference lot, and calculate the means. If less than thirty replicate zones are obtained because a disk was not dispensed, or it fell off the agar when the plate was inverted, it is acceptable to use the mean of at least twenty-eight replicates. Otherwise, all thirty replicates for that particular drug-organism combination must be repeated for that lot.

4. For the last three cultures, all plates may be removed from the refrigerator at one time for measurement of zone diameters.

5. Record the zone diameters of the last three cultures and calculate the means. For *E. faecalis* ATCC 33186, record the clarity of the zone, as well as the diameter.

5.3 Selection Process for New Reference Media

5.3.1 The subcommittee selects a new primary and secondary reference medium based on the criteria in Sections 5.4 and 5.5. The primary and secondary reference media are retained at the same facility and are distributed as described in Section 2 of this document.

5.3.2 Disposition of rejected lots of Mueller–Hinton agar that have been submitted by manufacturers as candidate reference lots is determined by the subcommittee. They are not returned to the manufacturers because this would compromise the confidentiality of the process.

5.4 Criteria for the Selection of the Primary Reference Medium

5.4.1 The mean zone diameters for *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 27853 should be within 2.0 mm of the midpoint of the quality control ranges in Table 3 of M2 and within the range of these zone diameters, insofar as they are not the subject of investigation.

5.4.2 The variability of the zone diameters should not exceed two standard deviations from the mean. The variance (σ^2) shall be $\leq$ 0.5 mm.

5.4.3 The medium must have a pH of 7.2 to 7.4.

5.4.4 When compared with the primary reference medium, 90% of the mean zone diameters of the candidate lot must be within 2.0 mm of the mean values of the reference lot and all must be within 3.0 mm.

5.4.5 The Student *t* values should be within +2.663 and –2.663 as determined by the calculation in Appendix B. This critical value of *t* assumes equal samples of thirty each (reference lot and test lot) and $\alpha = 0.01$.

5.4.6 There must be no discernable zone of inhibition (hazy growth or tiny colonies up to the disk) for the *S. aureus* ATCC 43300 culture with oxacillin after a 24-hour incubation

period. A zone of inhibition may be observed with methicillin.

5.4.7 The medium must be relatively free of thymidine, as shown by zone diameter with trimethoprim/sulfamethoxazole and *E. faecalis* ATCC 33186 that are of comparable clarity to the zones produced by the primary reference medium and are at least 20 mm in diameter. Because it is less sensitive in detecting thymidine, *E. faecalis* ATCC 29212 is not used for the selection and stability testing of reference media.

5.4.8 All of the mean zone diameters obtained with *E. coli* ATCC 35218 shall be within 3.0 mm of the mean values of the reference medium.

5.5 Criteria for the Selection of the Secondary Reference Medium

5.5.1 The secondary reference medium lot ranks second best according to the criteria in Section 5.4 and it also meets the following criteria:

5.5.2 The pH is 7.2 to 7.4.

5.5.3 Satisfactory trimethoprim/sulfamethoxazole zones are produced with *E. faecalis* ATCC 33186.

5.5.4 No more than three mean zone diameters (thirty replicates) have a difference greater than 3.0 mm compared with the new primary reference medium.

5.5.5 The same results are obtained with *S. aureus* ATCC 43300 as is stated in Section 5.4.6.

5.5.6 If there is no suitable candidate for a secondary reference medium, another study shall be conducted. In this study, the candidate lots are compared with the new reference medium. A secondary reference medium is then selected using the criteria in Section 5.4.

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Appendix A: Manufacturers' Protocol—Data Sheet

Manufacturer _____ Date _____

Mueller–Hinton Agar, Lot No. _____ Expiration Date _____ NCCLS Lot No. _____

pH: NCCLS Lot _____ Test Lot _____ (Specification = 7.2 to 7.4)

Antimicrobial Agent	Disk Content (μg)	NCCLS Lot				Test Lot				
		1	2	3	Mean	1	2	3	Mean	Diff*
S. aureus 25923										
Cephalothin	30									
Ciprofloxacin	5									
Erythromycin	15									
Oxacillin	1									
Tetracycline	30									
Vancomycin	30									
E. coli 25922										
Ampicillin	10									
Amoxicillin/clavulanic acid	20/10									
Cefotaxime	30									
Cefoxitin	30									
Cephalothin	30									
Chloramphenicol	30									
Gentamicin	10									
Trimethoprim/ sulfamethoxazole	1.25/23.75									
P. aeruginosa 27853										
Amikacin	30									
Cefotaxime	30									
Ceftazidime	30									
Gentamicin	10									
Imipenem	10									
Piperacillin	100									

E. faecalis - SXT Test: NCCLS Lot: _____ mm; Test Lot: _____ mm (Specification = ≥ 20 mm).
E. coli 35218 with amoxicillin/clavulanic acid: NCCLS Lot: _____ mm; Test Lot: _____ mm (Specification = 17–21 mm).
S. aureus 43300 with oxacillin (MRSA test): NCCLS Lot: _____ mm; Test Lot: _____ mm (Specification = very hazy to no zone).

*Diff = Difference, in mm, between the mean zone diameter of the test lot and the NCCLS lot. No more than 10% (two means) may exceed a difference of 2.0 mm and none shall exceed a difference of 3.0 mm.

Appendix B: Statistical Calculation for the Selection of New Reference Media

This method is based on the assumption that the maximum variance of zone diameters (s^2) is 0.5 mm. A difference of 1 mm or more in the mean zone diameters of the test lot and the reference lot can be detected with a probability of 99% for each antimicrobial agent–organism combination by a two-sample *t* test with $\alpha = 0.01$ (two-sided). If more than one candidate lot is evaluated in comparison with the reference lot, an adjustment must be made by dividing the probability by the number of lots tested.

1. Using the protocol in Section 5, determine the thirty replicate zone diameters for the reference medium (*r*) and each of the test media (*t*) for the three cultures requiring 30 replicates for each of the antimicrobial agents indicated for those organisms.

2. Calculate the mean ($\bar{x}$) and variance (s^2) for each set of thirty tests ($n = 30$, or the actual number of replicates) for each of the drug/organism combinations:

$$\bar{x}_{r \text{ or } t} = \frac{\sum x_{r \text{ or } t}}{n_{r \text{ or } t}} \tag{1}$$

$$s^2_{r \text{ or } t} = \frac{\left(\sum x_{r \text{ or } t}\right)^2 - \frac{\left(\sum x_{r \text{ or } t}\right)^2}{n_{r \text{ or } t}}}{n_{r \text{ or } t} - 1} \tag{2}$$

3. Calculate the pooled variance (s_p^2):

$$s_p^2 = \frac{(n_r - 1)\, s_r^2 + (n_t - 1)\, s_t^2}{n_r + n_t - 2} \tag{3}$$

4. Calculate *t*:

$$t = \frac{\bar{x}_r - \bar{x}_t}{\sqrt{(1/n_r + 1/n_t)\, s_p^2}} \tag{4}$$

Summary of Comments and Subcommittee Responses

M6-T: *Protocols for Evaluating Production Lots of Mueller–Hinton Agar; Tentative Standard*

General

1. Performance testing for dehydrated media does not include testing for the detection of pneumococcal resistance to penicillin. Because this form of resistance is relatively new in the United States, I believe that the performance of Mueller–Hinton agar at the manufacturers' level should be tested for the ability to detect it. One could extend this logic to include testing for all of the recently recognized forms of non-beta-lactamase-mediated resistance to the penicillin/ampicillin group of drugs. One should also consider testing for the ability to detect vancomycin resistance in enterococci.

- **Antimicrobial susceptibility testing of pneumococci requires the addition of 5% sheep blood to Mueller–Hinton agar. Therefore, the complete medium must be quality-controlled. This type of quality control is covered by NCCLS document M2. Special procedures for testing fastidious and problem bacteria, such as vancomycin-resistant enterococci, appear in Table 7 of NCCLS document M7.**

2. It should be emphasized that the performance tests described in this standard apply only to Mueller–Hinton agar that is intended to be used with disk-susceptibility testing. Nothing in this method validates Mueller–Hinton agar for other purposes, such as agar-dilution testing or the E-test. I think this should be stated explicitly in the protocol so that laboratories will continue to do performance testing for agar dilution and antibiotic gradient as appropriate.

- **This is stated in the Introduction of this edition.**

3. Section 3.3.2 allows a manufacturer to use either *E. faecalis* ATCC 33186 or ATCC 29212. The following sections, in particular Section 5.2.3, do not define which antibiotic should be tested against ATCC 29212. Please clarify.

- **This point is clarified in this edition. ATCC 29212 may be substituted for ATCC 33186 in the manufacturers' protocol (Section 3.1.1) but not in the selection or stability testing of reference media (Sections 4.1.1 and 5.2.1).**

Related NCCLS Publications

M2-A5 **Performance Standards for Antimicrobial Disk Susceptibility Testing–Fifth Edition; Approved Standard (1993).** *American National Standard.* M2-A5 includes the current recommended techniques for disk susceptibility testing, criteria for quality control testing, and updated tables for interpretive zone diameters.

M7-A3 **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically–Third Edition; Approved Standard (1993).** *American National Standard.* M7-A3 discusses reference methods for the determination of minimum inhibitory concentrations (MIC) of aerobic bacteria by broth macrodilution, broth microdilutioin, and agar dilution.

M22-A **Quality Assurance for Commercially Prepared Microbiological Culture Media; Approved Standard (1990).** M22-A addresses quality assurance procedures for manufacturers and users of prepared, ready-to-use microbiological media.

M100-S6 **Performance Standards for Antimicrobial Disk Susceptibility Testing; Sixth Informational Supplement (1995).** M100-S6 provides updated tables for NCCLS documents M2, M7, and M11.