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Center for Veterinary Biologics
Testing Protocol**

SAM 900

Supplemental Assay Method for Testing Growth Promoting Qualities of Fluid Thioglycollate Medium and Soybean-Casein Digest Medium using *Bacillus subtilis* Spores and *Candida krusei* as the Indicator Organisms

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and Soybean-Casein Digest Medium using *Bacillus subtilis* Spores and *Candida krusei*
as the Indicator Organisms**

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1. Introduction

This is a Supplemental Assay Method (SAM) for testing Fluid Thioglycollate Medium (FTM) and Soybean-Casein Digest Medium (SCDM) for growth promoting qualities, as required in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.25(b). Trypticase Soy Broth (TSB) or SCDM are the same media formulation. Each lot of media that is used in sterility tests (9 CFR 113.26 – 113.27) must be tested to ensure that it will support the growth of contaminants, should they be present in the biologics sample being tested for sterility. FTM and SCDM are two of the media used in codified sterility tests.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

- 2.1.1** 30°- 35°C incubator
- 2.1.2** 20°- 25°C incubator
- 2.1.3** Sterile disposable cotton-plugged pipettes
- 2.1.4** Sterile 10-mL disposable syringes with needles
- 2.1.5** Class II Biosafety cabinet
- 2.1.6** Magnetic stirrer
- 2.1.7** Flasks, sterile, 250-mL and 1000 mL
- 2.1.8** Sterile glass beads
- 2.1.9** Sterile 2-mL serum vials
- 2.1.10** Tubes, 25 x 200-mm, screw-capped
- 2.1.11** 4 x 4-inch sterile gauze pads
- 2.1.12** Sterile funnel

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2.1.13 Vortex mixer

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Indicator Organisms: Use *Bacillus subtilis* (ATCC #6633) and *Candida krusei* (ATCC #6258), or equivalent organisms as specified in the current United States Pharmacopoeia (USP), as the control organisms in order to determine the growth promoting qualities of the medium according to 9 CFR, Part 113.25

2.2.2 Media: Brain Heart Infusion Agar (BHIA), Soybean-Casein Digest Agar (SCDA) or Trypticase Soy Agar (TSA), SCDM or Trypticase Soy Broth (TSB), and FTM (See the **Appendices** for media formulations.)

2.2.3 Stabilizer: Phosphate buffered saline (PBS) with 12% sucrose (**Appendix V**)

2.2.4 Sterile glycerin

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

3.2.1 Turn on biosafety cabinets at least 30 minutes before preparing positive control reagents or testing media for growth promotion.

3.2.2 Monitor incubators, freezers, and coolers daily for temperature.

3.3 Preparation of *Bacillus subtilis* reagent

3.3.1 Place 1 mL of *B. subtilis* spore suspension from a previous stock culture into a 500-mL flask containing 100 mL of BHIA.

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3.3.2 Swirl flask to wet entire agar surface.

3.3.3 Incubate flask at 30°- 35°C for 7 days. Make a Gram stain of the resulting growth and examine microscopically for purity. A valid Gram stain shows uniform Gram positive rods.

3.3.4 If the Gram stain is valid, place about 2 dozen sterile glass beads plus 160 mL of a solution of 50% SCDM and 50% glycerin in the flask.

3.3.5 Swirl the flask until the glass beads have loosened most of the growth from the agar. Filter the culture through sterile gauze to remove the glass beads.

3.3.6 Pour this solution into a sterile 250-mL flask.

3.3.7 Incubate this flask at 20°- 25°C for 7 more days to allow sporulation.

3.3.8 After the second 7 days, mix well and dispense 1.5-mL aliquots of this stock culture (spore suspension) into sterile 2-mL serum vials with rubber stoppers. Label the vials with the organism name, lot number, and date, then freeze at -70°C or less.

3.4 Preparation of *Candida krusei* reagent

3.4.1 Inoculate 2 petri dishes containing BHIA with one loopful (approximately 10 µL) of *C. krusei* from a previous stock culture.

3.4.2 Incubate at room temperature (20°- 25°C) for 7 days. Make a Gram stain of the resulting growth and examine microscopically for purity. A valid Gram stain shows a pure culture of Gram positive budding organisms.

3.4.3 Take 1 loopful of a colony from the BHIA plate and inoculate a 250-mL flask containing 120 mL of SCDM.

3.4.4 Incubate the flask of SCDM for 24 hours at 30°- 35°C.

3.4.5 Check the flask of SCDM for purity with a Gram stain.

3.4.6 If the Gram stain is valid, pipette 1 mL of the culture into each of three to five 500-mL flasks containing 100 mL of SCDA. Swirl the flasks to spread the culture for even growth.

3.4.7 Incubate these SCDA flasks for 5 to 7 days at 20°- 25°C.

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3.4.8 Pour 150 mL of PBS with 12% sucrose (**Appendix V**) and about 2 dozen glass beads into each flask of SCDA. Swirl the flasks to remove the growth from the agar surface.

3.4.9 Recheck each flask for purity with a Gram stain.

3.4.10 Combine the contents of all flasks showing a pure culture into one 1000-mL flask.

3.4.11 Place this flask on a magnetic stirrer for 1 hour.

3.4.12 Filter the culture through sterile gauze to remove the glass beads.

3.4.13 Dispense 1.5 mL of the culture into 2-mL serum vials and lyophilize. Label the vials with the name of the organism, the lot number, and the date, then store at -70°C or colder. Label and fill records are filed according to standard operating procedures.

4. Performance of the Test

4.1 Establishing the working dilutions of the indicator organisms

Titrate each new lot of indicator organism to determine the optimum working dilution. This working dilution will be used to test new batches or lots of media for growth promoting qualities.

4.1.1 Remove a vial of the newly prepared stock culture from the freezer and rapidly thaw. If the culture is lyophilized, rehydrate with 1 mL of SCDM.

4.1.2 Make tenfold dilutions of the stock culture in sterile screw-capped tubes. Use a 1-mL pipette to dispense 1 mL of the stock culture into 9 mL of SCDM.

4.1.3 Mix by inverting the tube several times or vortex mixing.

4.1.4 Using a 1-mL pipette, transfer 1 mL of the diluted culture into another tube containing 9 mL of SCDM. Mix as before and continue the procedure until 10^{-10} dilution is prepared.

4.1.5 Incubate the *B. subtilis* dilution tubes for 24 to 48 hours at 30°- 35°C. Incubate the *C. krusei* dilution tubes for 14 days at 20°- 25°C.

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4.1.6 Examine the tubes visually for growth to establish the growth endpoint (the highest dilution with observable growth) of the stock culture.

4.1.7 The growth endpoint and the next lower dilution are used to test new lots or batches of media.

4.1.8 Collect data to confirm the reproducibility of the growth endpoint in the assay system (**Section 4.1.7**). For each type of media (FTM or SCDM), perform two independent tests (one with *B. subtilis* and one with *C. krusei*) with each of five valid (i.e., already satisfactorily tested for growth promotion) lots of media (total of 10 tests per media type). Use a separate vial of stock culture for each test.

4.1.9 Growth is expected in 9 or 10 tubes inoculated with the lower dilution (**Section 4.1.7**) and in less than 9 tubes inoculated with the higher dilution (**Section 4.1.6**).

4.1.10 If all of the tests (**Section 4.1.8**) give the expected number of tubes with growth (**Section 4.1.9**), then these dilutions will be used to test new media for growth promotion. If these results are not repeatable, adjust the dilutions and retest according to **Section 4.1.8**.

4.2 Testing new lots of media for growth promotion

4.2.1 Test each batch of SCDM and FTM prepared for sterility testing for growth promoting qualities with both the *C. krusei* and *B. subtilis* stock cultures. Thaw the frozen vials of stock culture rapidly. Rehydrate with SCDM those stock cultures that are lyophilized. Transfer 1 mL of each stock culture to 2 separate tubes with 9.0 mL of SCDM (10^{-1} or tenfold dilution). Use a sterile pipette for each transfer.

4.2.2 Mix each of the 10^{-1} dilutions by vortex mixing.

4.2.3 Continue making tenfold dilutions of each indicator organism in SCDM to approach the working dilutions established in **Section 4.1.7**. Mix as before and continue until the last 2 dilutions.

4.2.4 So that a sufficient volume of each working dilution is prepared for each indicator organism, increase the transfer volume to 3 mL into 27 mL of SCDM for the final 2 dilutions.

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4.2.5 Using a sterile 10-mL pipette or syringe with needle, deposit 1.0 mL of the higher working dilution of *B. subtilis* into each of ten 25 x 200-mm tubes containing 40 mL of SCDM. Refill the same syringe with the same dilution of culture and deposit 1.0 mL into each of ten 25 x 200-mm tubes containing 40 mL of FTM.

4.2.6 Using the same 10-mL pipette or syringe, deposit 1.0 mL of the next lower working dilution of *B. subtilis* into each of ten 25 x 200-mm tubes containing 40 mL of SCDM and into each of ten 25 x 200-mm tubes containing 40 mL of FTM. Repeat **Sections 4.2.5 and 4.2.6** for the *C. krusei*.

4.2.7 Incubate all tubes (40) containing the *C. krusei* culture at 20°- 25°C and observe for growth of the organism throughout a 14-day incubation period.

4.2.8 Incubate all tubes (40) containing the *B. subtilis* culture at 30°- 35°C and observe for growth of the organism throughout a 7-day incubation period.

5. Interpretation of the Test Results

Growth is expected in at least 9 or 10 tubes inoculated with the lowest working dilution of each of the organisms and in less than 9 tubes inoculated with the next higher working dilution of each organism. If at least 9 or 10 of the tubes inoculated with the lower working dilution of a stock culture contain growth, the growth promoting quality of that medium is satisfactory (SAT). If less than 9 tubes at the lower working dilution have growth, then the growth promoting qualities of the media are in question and the test must be repeated. If after repeating the test and the media's growth promoting properties are still in question, the media must not be used and all tests already conducted with this media must be considered no tests (NT).

6. Report of Test Results

Report results of the test(s) as described by standard operating procedures.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.25, U.S. Government Printing Office, Washington, DC.

7.2 The U.S. Pharmacopoeia, 1985, Vol. 21, pp 1151-1160, Mack Publishing Co., Easton, PA.

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8. Summary of Revisions

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- The Contact has been changed from Gerald Christianson to Sophia Campbell.
- **1:** Clarification on the use of this SAM and the media designations have been added.
- **2:** The list of equipment/instrumentation has been updated to reflect current procedures.
- **3.3.3:** The status of a valid Gram stain for *B. subtilis* has been clarified.
- **3.3.5:** A filtration step to remove beads has been added.
- **3.4.2:** The status of a valid Gram stain for *C. krusei* has been clarified.
- **4.1:** This section has been rewritten to provide further clarification of current procedures and to add an explanation on titration of each new lot of indicator organisms.
- **4.2:** This section has been rewritten to provide further clarification of current procedures.
- **5:** The interpretation of test results has been clarified.
- **Appendices:** The storage conditions have been added.

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Appendices--Media Formulations

Appendix I

National Veterinary Services Laboratories (NVSL) Media #10204

Brain Heart Infusion Agar (BHIA)

Brain Heart Infusion Agar	52 g
QH ₂ O	1000 mL

Autoclave 20 minutes at 121°C. Media is stored at 20°- 25°C for up to 3 months.

Appendix II

NVSL Media #10487

Trypticase Soy Agar (TSA) or Soybean-Casein Digest Agar (SCDA)

Trypticase Soy Agar	40 g
QH ₂ O	1000 mL

Autoclave 20 minutes at 121°C. Media is stored at 20°- 25°C for up to 3 months.

Appendix III

NVSL Media #10423

Trypticase Soy Broth (TSB) or Soybean-Casein Digest Medium (SCDM)

Trypticase Soy Broth	30 g
QH ₂ O	1000 mL

Autoclave 20 minutes at 121°C. Media is stored at 20°- 25°C for up to 3 months.

TSB and SCDM are 2 names for the same media formulation from different media companies.

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Appendix IV

NVSL Media #10135

Fluid Thioglycollate Medium (BBL)

Fluid Thioglycollate Medium	29.5 g
QH ₂ O	1000 mL

Mix and heat to boiling. Autoclave 20 minutes at 121°C. Media is stored at 20°-25°C for up to 3 months.

Appendix V

NVSL Media #30035

0.15M Phosphate Buffered Saline with 12% Sucrose

Potassium Phosphate Monobasic	4.7359 g
Sodium Phosphate Dibasic	16.3558 g
Sodium Chloride	8.5 g
QH ₂ O, QS to	1000 mL

Adjust pH to 7.3 with 10% NaOH.

Add Sucrose	120 g
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Autoclave 20 minutes at 121°C. Media is stored at 20°- 25°C for up to 3 months.