KCMC Biotechnology Laboratory, Microbiology	STANDARD OPERATING PROCEDURE	Effective Date 25 August 2006	SOP-Number MIC.037
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Title: ACCUPROBE® Culture Identification of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex

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Annual Review	
By	**Date**

Title: ACCUPROBE® Culture Identification of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex

Document History:

Version Number	Reason for Changes	Date

Copies distributed to:

Name	Date

Title: ACCUPROBE® Culture Identification of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex

PURPOSE

For the identification of the *Mycobacterium tuberculosis* complex (MTBc) including *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. canetti* or the *Mycobacterium avium* complex (MAIc) including: *M. avium* and *M. intracellulare.*

PRINCIPLE:

The GEN-PROBE® ACCUPROBE® tests for identification of MTBc and MAIc are rapid DNA probe tests that utilize nucleic acid hybridization for identification. Nucleic acid hybridization tests are based on the ability of complementary nucleic acid strands to specifically align and associate to form stable double-stranded complexes. The ACCUPROBE system uses a single-stranded DNA probe with a chemiluminescent label that is complementary to the ribosomal RNA of the target organism. After the ribosomal RNA is released from the organism, the labeled DNA probe combines with the target organism's ribosomal RNA to form a stable DNA:RNA hybrid. The Selection Reagent allows for the differentiation of non-hybridized and hybridized probe. The labeled DNA:RNA hybrids are measured in a GEN-PROBE Luminometer. A positive result is a Luminometer reading equal to or greater than the cut-off. A value below this cut-off is a negative result.

SCOPE

This Standard Operating Procedure applies to the identification of *M. tuberculosis* complex from culture isolates by technical staff of the microbiology laboratory who are trained and are competent in performing this test.

EQUIPMENT AND MATERIALS:

Equipment:

GEN-PROBE® LEADER® Luminometer
GEN-PROBE® Sonicator
GEN-PROBE® Sonicator Rack (Cat. No. 4027)
Heating block (59.5° to 61°C)
Heating block (95° ± 5°C)
Vortex mixer
Micocentrifuge

Title: ACCUPROBE® Culture Identification of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex

Materials:

ACCUPROBE® MTB Complex Probe Kit (Cat No.2860)
- Probe Reagent – 20 tubes
- Lysing reagent – 20 tubes
- Probe tubes – 20

OR

ACCUPROBE® MAI Complex Probe Kit (Cat No.2845)
- Probe Reagent – 20 tubes
- Lysing reagent – 20 tubes
- Probe tubes – 20

ACCUPROBE® Culture Identification Reagent Kit (Cat. No. 2800)
- Reagent 1 (Lysing reagent – 10 ml)
- Reagent 2 (Hybridization buffer 10 ml)
- Reagent 3 (Selection Reagent 60 ml)

GEN-PROBE® Detection Reagent Kit (Cat. No. 1791)
- Detection Reagent 1 240 ml hydrogen peroxide + nitric acid)
- Detection Reagent 2 (240 ml NaOH)

1μL plastic sterile inoculating loops for selecting colonies
Microcentrifuge tubes (for liquid culture preparation)
Wash buffer (10% sodium dodecyl sulfate in EDTA) – for blood-containing liquid culture preparation)
Control culture strains
Micropipettes (100 μL, 300 μL)
Re-pipettor (100 μL, 300 μL)
Filter pipette tips

STORAGE AND HANDLING REQUIREMENTS:

1. Probe Reagent Tubes must be stored in the foil pouches at 2° to 8°C. Once opened, the pouch should be resealed, and the tubes used within two months and prior to the expiration date.

2. Other reagents used in the ACCUPROBE KITS may be stored between 2° to 25°C and are stable until the expiration date indicated.

DO NOT FREEZE THE REAGENTS.

Title: ACCUPROBE® Culture Identification of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex

PRECAUTIONS:

1. Use standard precautions when performing this assay.
2. Handle cultures and perform all transfer of cultures in a Class II Biological Safety Cabinet.
3. Reagents in this kit contain sodium azide, which may react with lead or copper plumbing to form potentially explosive metal azides. Upon disposal of these reagents, always dilute the material with a large volume of water to prevent azide buildup in the plumbing.
4. Avoid contact of Detection Reagents I and II with skin and mucous membranes. Wash with water if these reagents come into contact with skin. If spills of these reagents occur, dilute with water before wiping dry.

QUALITY CONTROL

Frequency: Each run of patient tests.

Controls:

MTBc Test:
Negative control *M. avium*, ATCC #25291
Positive control *M. tuberculosis*, ATCC #25177

MAIc Test:
Negative control *M. tuberculosis*, ATCC #25177
Positive control *M. avium*, ATCC #25291

Acceptable Results for MTB or MAI tests::

Negative controls: <10,000 Relative Light Units (RLU)
Positive control: >30,000 RLU

Corrective Actions for Unacceptable QC Results:

1. Do not interpret or report patient test results.
2. Examine culture carefully to assess if solid media culture is pure. Mixed or contaminated cultures may cause unacceptable control results. If culture is suspected to be mixed, resubculture for testing.
3. Elevated negative control values greater than 10,000 RLU (Relative Light Units can be caused by insufficient mixing after adding Reagent 3 (Selection Reagent).
4. Low positive control values can be caused by insufficient cell numbers, improper sonication, or by testing mixed or aged cultures.

5. Repeat test on QC organisms and patient isolates.

Documentation:

1. Record control results on ACCUPROBE MTB and/or MAI QC sheets.
2. Document all unacceptable QC results on QC DEVIATION FORM and submit to supervisor for review.
3. Supervisor will review QC results monthly.

SAMPLE PREPARATION

NOTE: Perform culture transfers in a biosafety cabinet.

Solid Media Method. Growth of control organisms and patient isolates from solid media, such as Lowenstein-Jensen slants or Middlebrook 7H10 or 7H11 plates, suggestive of TB complex, may be tested. Samples may be tested as soon as growth is visible and during the subsequent 60 days of incubation.

1. Add 100 ul of Reagent 1 and 100 ul of Reagent 2* to a Lysis tube.

   Note: Reagent 2 may precipitate. Warming and mixing the solution at 35 °to 60 °C will dissolve the precipitate.
2. Remove a loopful of growth with a 1-µL disposable plastic loop. Swabs should not be used.
3. Avoid taking any of the solid media with the cells.
4. Swirl the loop in the Lysis Reagent tube.
5. Vortex to mix.
6. Proceed with sonication

Broth Culture Method. Growth of control organisms or patient isolates in liquid medium may be tested. Positive blood cultures with visible AFB on smear may also be tested after treatment with sodium dodecyl/EDTA wash buffer.

Non-blood containing liquid culture:

1. Mix liquid medium.
2. Transfer 1.5 to 2.0 ml into a microcentrifuge tube.

3. Centrifuge in a microcentrifuge at 10,000xg/5,000 rpm for 6 minutes to pellet bacteria. Check to see that there is a pellet. If not, more liquid culture may be needed. Decant the supernate and add another 1.5 ml of medium and respin.
4. Add 100-µL of Reagent 1 (lysis buffer) to the microcentrifuge tube.
5. Add 100-µL of Reagent 2 (hybridization reagent*) to the microcentrifuge tube.

   **** Note: Reagent 2 may precipitate. Warming and mixing the solution at 35°to 60°C will dissolve the precipitate.***
6. Close cap and vortex thoroughly to suspend pellet.
7. Transfer all contents of the microcentrifuge tube into a Lysis tube from Accuprobe kit.
8. Proceed with sonication.

Blood-containing liquid culture:

Preparation of wash buffer:

Ingredients:

1.86 g EDTA disodium salt

5 N sodium hydroxide (NaOH)

10 g sodium dodecyl sulfate (SDS)

100 ml distilled water

Preparation:

1. Add the EDTA to 100 ml of distilled water.
2. Using 5N NaOH adjust pH to 8.0 to allow EDTA to dissolve.
3. Add the SDS to the EDTA solution.
4. Adjust pH to 7.2 using 1N HCl
5. Filter sterilize.
6. Label container with name of reagent, initials of preparer, date of preparation and expiration date in one month.

Sample Preparation:

1. Remove 1.0 to 1.3 ml of blood culture.
2. Add 100 uL of wash buffer (see below for preparation instructions).
3. Vortex to mix thoroughly.

4. Centrifuge in microcentrifuge at 12,000xg/ 7000 rpm for 10 minutes.
5. Discard supernate.
6. Add 1.0 to 1.5 ml sterile distilled water to the pellet.
7. Vortex thoroughly to resuspend pellet.
8. Centrifuge at 12,000xg/7000 rpm for 10 minutes.
9. Discard supernate.
10. Add 100 uL of Reagent 1 (lysis buffer).
11. Add 100 mL Reagent 2 (hybridization reagent).
12. Close cap and and vortex to resuspend pellet.
13. Transfer all contents of the microcentrifuge tube into a Lysis tube from Accuprobe kit.
14. Proceed with sonication.

EQUIPMENT PREPARATION

1. **Sonicator**: For optimal transfer of sonic energy, water must be thoroughly degassed according to the following procedure:
    a. Add enough hot water to fill the sonicator to within ½ inch of the top of the tank.
    b. Turn on the sonicator and select DEGAS. Let run for 15 minutes to thoroughly degas the water.
    c. Before turning off check that degassing is complete, dip a piece of tinfoil in the water for 20-30 seconds. When removed there should be small holes in the foil.
2. **Heat Blocks**: Adjust one heating block or water bath to 59.5° to 61°C and another heating block or water bath to 95° ± 5°C.

3. **Luminometer**: Prepare the GEN-PROBE Luminometer for operation (Refer to SOP MIC MIC.038 GenProbe Luminometer Operation and Maintenance). Make sure there is a sufficient volume of Detection Reagents I and II to complete the tests.

SAMPLE LYSIS

1. Push the Lyisng Reagent Tubes through the Sonicator Rack so that the reaction mixture in the bottom of the tube is submerged but the caps are above the water. Place the Sonicator Rack on the water bath sonicator. DO NOT ALLOW THE TUBES TO TOUCH THE BOTTOM OR SIDES OF THE SONICATOR.

2. Set the timer on the sonicator for 15 minutes and select the RIGHT ARROW button to begin sonication.
3. Place the Lysing Reagent Tubes, containing the sonicated organisms, in a heat block for 10 minutes at 95° ± 5°C.
4. Carefully remove the Lysing Reagent Tubes from the heating block.

HYBRIDIZATION

1. Open the foil pouch containing the appropriate (MTBc or MAIc) Probe Reagent Tubes by cutting evenly across the top of the pouch. Remove enough Probe Reagent Tubes to test the culture isolates and/or controls. Reseal the pouch by folding the opened edge over several times and securing with adhesive tape or a clip. **Leave the desiccant pillow in the pouch.**
2. Record the date opened on the outside of the pouch. Change the expiration date to 2 months from the date opened.
3. Label a sufficient number of Probe Reagent Tubes to test the culture isolates and/or controls. Remove and retain the caps.
4. Pipette 100 µL of the lysed specimens from the Lysing Reagent Tubes into the corresponding Probe Reagent Tubes and recap the tubes.
5. Incubate Probe Tubes for 15 minutes at 59.5° to 61°C (critical) in a heat block. **Incubation time is critical and may not exceed 20 minutes.**

SELECTION

1. Remove the Probe Reagent Tubes from the heat block. Remove and retain the caps. Pipette 300 µL of Reagent 3 (Selection Reagent) into each tube. Recap the tubes and VORTEX to mix completely.
2. Incubate the **MTBc** Probe Reagent Tubes for **10 minutes** at 59.5° to 61°C (critical) in a heat block. **Incubation time is critical and may not exceed 11 minutes**.
3. Incubate the **MAIc** Probe Reagent Tubes for **5 minutes** at 59.5° to 61°C (critical) in a heat block. **Incubation time is critical and may not exceed 6 minutes**.
4. Remove the Probe Reagent Tubes from the heat block, and leave them at room temperature for at least 5 minutes. Remove and discard the caps. **Read the results in the Luminometer within 1 hour.**

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DETECTION

1. Select Protocol #4 (for Accuprobe Mycobacteria tests – AP MYCOB) from the menu of the Luminometer software.
2. Using a moistened tissue or paper towel, wipe each tube to remove static and to ensure no residue is present on the outside of the tube. Insert the tube into the Luminometer and close the cover.
3. When the analysis is complete, remove the tube(s) from the Luminometer.
4. Continue until all tubes are read.
5. Place a blank tube in the instrument and close the cover.
6. Flush the Detection Reagent tubing with distilled water until wash fluid is free of foam.
7. Place tubing in distilled water.

INTERPRETATION OF RESULTS

The results of the MTBc and MAIc tests are based on the following values. Samples with results in repeat range should be retested.

Positive - $\geq$ 30,000 RLU
Negative - < 20,000 RLU
Repeat Range – 20,000 to 29,999

PROCEDURE LIMITATIONS

1. This method has been tested using fresh growth from solid media and from broth cultures. The efficacy of this test has not been demonstrated on direct clinical specimens.
2. The ACCUPROBE MYCOBACTERIUM TUBERCULOSIS COMPLEX CULTURE IDENTIFICATION TEST does not differentiate between members of the TB complex, i.e., *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. microti* and *M. canetti*. The Probe Reagent does not react with any mycobacteria other than tubercle (MOTT) bacilli.
3. Results from the ACCUPROBE MYCOBACTERIUM TUBERCULOSIS COMPLEX CULTURE IDENTIFICATION TEST should be interpreted in conjunction with other laboratory and clinical data available to the clinician.

REFERENCES:

1. Badak, FZ, etal. 1999. Use of nucleic acid probes for identification of *M. tuberculosis* directly from MB/BacT/ALERT bottles. J. Clin. Micro. 37:1602.
2. Jonas, V. et al. 1990. Detection of *Mycobacterium* species Using Acridinium Ester Labeled DNA Probe. Abstract Annual Meeting ASM, Anaheim, CA.
3. Package insert. 2001. ACCUPROBE® *Mycobacterium tuberculosis* complex Culture Identification kit. Gen-Probe, Inc., San Diego, CA.
4. Package insert. 2001. ACCUPROBE® MAI complex Culture Identification kit. Gen-Probe, Inc., San Diego, CA.

APPENDIX A - ACCUPROBE® MTB QC sheet
APPENDIX B - ACCUPROBE® MAI QC sheet