IFU

HARDY DIAGNOSTICS
A Culture of Service™

Instructions for Use

NITRATE SUBSTRATE BROTH

Cat. no. R97	Nitrate Substrate Broth, 13x100mm Tube, 2ml	20 tubes/box
Cat. no. Z126	Concentrated HCl Solution, 50%, 0.5oz. Polyethylene Bottle with Dropper Tip, 5ml	1 bottle
Cat. no. Z124	AFB Nitrate Reagent A* (Sulfanilamide, 0.2%)	15ml
Cat. no. Z125	AFB Nitrate Reagent B* (Naphthylethylenediamine Dihydrochloride, 0.1%)	15ml
Cat. no. Z73	Nitrate Reagent C* (Zinc Dust)	5.0gm
* These products are sold separately		

INTENDED USE

Hardy Diagnostics Nitrate Substrate Broth is recommended for use in the determination of nitrate utilization by *Mycobacterium* species.

SUMMARY

The nitrate test is used to determine if a member of the genus *Mycobacterium* has the enzyme nitrate reductase. The nitrate reduction procedure using chemical reagents is recommended by the Centers for Disease Control, Bureau of Laboratories, Training and Consultation Division for the testing of mycobacteria for nitrate reduction.[1]

There are numerous end products of nitrate reduction and the resulting end products depend upon the species of bacteria.[2] Presence of a catabolic end product or the absence of nitrate in the medium is evidence that nitrate reduction occurred. If the nitrate has been reduced to nitrite, a pink to red color will develop after the addition of the three reagents; hydrochloric acid, sulfanilic acid and naphthylethylenediamine dihydrochloride. The red coloring is due to the formation of a diazonium compound. A negative reaction is confirmed by the addition of zinc dust. The zinc reduces the diazonium salt in the presence of acetic acid and produces the colored compound arhydrazine.

FORMULA

Ingredients per liter of deionized water:*

Nitrate Substrate Broth:	
Sodium Nitrate	0.85gm
Disodium Phosphate	4.85gm
Monopotassium Phosphate	1.17gm

Final pH 7.0 +/- 0.2 at 25ºC.

AFB Nitrate Reagent A (Sulfanilamide 0.2%):	
Sulfanilamide	2.0gm

AFB Nitrate Reagent B (Naphthylethylenediamine Dihydrochloride, 0.1%):	
n-1-Naphthylethylenediamine Dihydrochloride	1.0gm

Nitrate Reagent C (Zinc Dust):	
Zinc Dust	5.0gm

* Adjusted and/or supplemented as required to meet performance criteria.

STORAGE AND SHELF LIFE

Storage: Upon receipt store Nitrate Substrate Broth, AFB Nitrate Reagent A and AFB Nitrate Reagent B at 2-30ºC. away from direct light. Nitrate Reagent C can be stored at 15-30 degrees C. away from direct light. Media should not be used if there are any signs of deterioration (shrinking, cracking, or discoloration), contamination, or if the expiration date has passed. Product is light and temperature sensitive; protect from light, excessive heat, moisture and freezing.

The expiration date on the product label applies to the product in its intact packaging when stored as directed. The product may be used and tested up to the expiration date on the product label and incubated for the recommended incubation times as stated below.

Refer to the document "Storage" for more information.

PRECAUTIONS

This product may contain components of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not guarantee the absence of transmissible pathogenic agents. Therefore, it is recommended that these products be treated as potentially infectious, and handle observing the usual Universal Precautions for blood. Do not ingest, inhale, or allow to come into contact with skin.

This product is for *in vitro* diagnostic use only. It is to be used only by adequately trained and qualified laboratory personnel. Observe approved biohazard precautions and aseptic techniques. All laboratory specimens should be considered infectious and handled according to "standard precautions." Refer to the document "Guidelines for Isolation Precautions" from the Centers for Disease Control and Prevention.

For additional information regarding specific precautions for the prevention of the transmission of all infectious agents from laboratory instruments and materials, and for recommendations for the management of exposure to infectious disease, refer to CLSI document M29: *Protection of Laboratory Workers from Occupationally Acquired Infections.*

Sterilize all biohazard waste before disposal.

Refer to the document "Precautions When Using Media" for more information.

PROCEDURE

1. Place 3 or 4 drops (0.2ml) of sterile distilled or deionized water in each sterile screw-cap test tube.

2. Suspend 2 loopfulls of a mature, 2-4 week old culture from a solid medium in the water. A heavy suspension yields the best results.[3]

3. Add 2ml of Nitrate Substrate Broth to each test tube.

4. Shake by hand to mix and incubate in a 37ºC. waterbath for 2 hours.

5. Add 1 drop of 50% concentrated hydrochloric acid and shake by hand to mix.

6. Add 2 drops of AFB Nitrate Reagent A (Sulfanilamide, 0.2%).

7. Add 2 drops of AFB Nitrate Reagent B (Naphthylethylenediamine Dihydrochloride, 0.1%).

8. Examine immediately for a pale pink (+/-) to deep red (5+) color development within 30-60 seconds. Positive color controls used for comparison in the nitrate procedure are outlined in the CDC manual.[3]

9. Confirm negative results (nitrate not reduced) by adding a pinch of Nitrate Reagent C (zinc dust) to the tube. The development of a red color following the addition of Nitrate Reagent C confirms the negative result, nitrate was not reduced initially. If no color change occurs upon adding Nitrate Reagent C, the result was positive, nitrate was reduced beyond nitrite to a colorless compound. In such cases, the test should be repeated to confirm the observation.

ALTERNATE PROCEDURE: COMBINED NIACIN-NITRATE TEST

1. Add 2.0-2.5ml of Nitrate Substrate Broth (Cat. no. R97) to an actively growing 3-5 week old subculture with at least 3+ growth on a Lowenstein Jensen slant.[9]

2. Cut the slant with a loop over the confluent growth. Using a loop or a swab, emulsify the growth into the broth making a turbid suspension. Heavy suspensions will yield better results.[3]

3. Incubate the slant in an upright position at 37 degrees C. overnight.

4. Transfer 1.0ml of this suspension into a sterile screw-cap tube and perform Niacin Test. Please refer to the Niacin Test technical insert (Cat. no. 231741) for procedure and interpretation of results.

5. To the remaining broth in the LJ slant, add nitrate reagents in the following order.

   a. 1 drop of 50% HCl Solution (Cat. no. Z126)
   b. 2 drops of 0.2% Sulfanilamide (Cat. no. Z124)
   c. 2 drops of 0.1% Naphthylethylenediamine Dihydrochloride (Cat. no. Z125)

6. Examine immediately for a pale pink (+/-) to deep red (4+) color development within 30-60 seconds. Positive color controls used for comparison in the nitrate procedure are outlined in the CDC manual.[3]

7. Confirm negative results (nitrate not reduced) by adding a pinch of Nitrate Reagent C (zinc dust, Cat. no. Z73) to the slant. The development of a red color following the addition of Nitrate Reagent C confirms the negative result, nitrate was not reduced initially. If no color change occurs upon adding Nitrate Reagent C, the result was positive, nitrate was reduced beyond nitrite to a colorless compound. When this occurs, the test should be repeated to confirm the observation.

INTERPRETATION OF RESULTS

Positive Test	3+ to 5+ pink/red color development after the addition of AFB Nitrate Reagent A and Reagent B (refer to CDC manual for color controls).
Negative Test	No color change after the addition of AFB Nitrate Reagent A and Reagent B.

LIMITATIONS

It is recommended that biochemical, immunological, molecular, or mass spectrometry testing be performed on colonies from pure culture for complete identification of bacteria and/or fungi.

A positive test for nitrate reduction (red color development) may flash instantly or quickly fade.

The ability of acid-fast bacilli to reduce nitrate is influenced by age of the colonies, temperature, pH and enzyme inhibitors. Rapid growers can be tested within 2 weeks, slow growers should be tested after 3 to 4 weeks of luxuriant growth.

MATERIALS REQUIRED BUT NOT PROVIDED

Standard microbiological supplies and equipment such as loops, other culture media, swabs, applicator sticks, incinerator, incubators, AFB Nitrate Reagent A, AFB Nitrate Reagent B and Nitrate Reagent C, etc., are not provided.

Refer to the document "Limitations of Procedures and Warranty" for more information.

QUALITY CONTROL

Hardy Diagnostics tests each lot of commercially manufactured media using appropriate quality control microorganisms and quality specifications as outlined on the Certificate of Analysis (CofA) and the CLSI document M22-A3 *Quality Assurance for Commercially Prepared Microbiological Culture Media*. The following microorganisms are routinely used for testing at Hardy Diagnostics:

Test Organisms	Inoculation Method*	Incubation			Results
		Time	Temperature	Atmosphere	
Mycobacterium tuberculosis H37Ra ATCC® 25177	See Procedure	2hr	35°C	Aerobic	Positive; pink/red color development after the addition of AFB Nitrate Reagents A and B
Mycobacterium intracellulare Group III ATCC® 13950	See Procedure	2hr	35°C	Aerobic	Negative; no color development after the addition of AFB Nitrate Reagents A and B

USER QUALITY CONTROL

End users of commercially prepared culture media should perform QC testing in accordance with applicable government regulatory agencies, and in compliance with accreditation requirements. Hardy Diagnostics recommends end users check for signs of contamination and deterioration and, if dictated by laboratory quality control procedures or regulation, perform quality control testing to demonstrate growth or a positive reaction and to demonstrate inhibition or a negative reaction, if applicable. Hardy Diagnostics quality control testing is documented on the certificate of analysis (CofA) available from Hardy Diagnostics Certificate of Analysis website. Also refer to the document "Finished Product Quality Control Procedures," and the CLSI document M22-A3 *Quality Assurance for Commercially Prepared Microbiological Culture Media* for more information on the appropriate QC procedures. See the references below.

PHYSICAL APPEARANCE

- Nitrate Substrate Broth, AFB Nitrate Reagent A and AFB Nitrate Reagent B should appear clear and colorless.
- Nitrate Reagent C should appear as a homogeneous, free-flowing powder, and gray in color.

Showing Positive Nitrate Test
Mycobacterium tuberculosis H37Ra (ATCC® 25177) suspension of four drops of deionized water and 2mL of Nitrate Substrate Broth (Cat. no. R97). Incubated aerobically for 2 hours in a 37ºC. waterbath. One drop of Concentrated HCl solution (Cat. no. Z126) was added and mixed by shaking. Next, two drops of AFB Nitrate Reagent A (Cat. no. Z124) and two drops of AFB Nitrate Reagent B (Cat. no. Z125) were added. The pink/red color development was indicative of a positive reaction for the reduction of nitrate to nitrite.

Showing Negative Nitrate Test
Mycobacterium intracellulare Group III (ATCC® 13950) suspension of four drops of deionized water and 2mL of Nitrate Substrate Broth (Cat. no. R97). Incubated aerobically for 2 hours in a 37ºC. waterbath. One drop of Concentrated HCl solution (Cat. no. Z126) was added and mixed by shaking. Next, two drops of AFB Nitrate Reagent A (Cat. no. Z124) and two drops of AFB Nitrate Reagent B (Cat. no. Z125) were added. No pink/red color development indicated that nitrate was not reduced to nitrite (presumptive negative). To ensure nitrate was not reduced to an end product other than nitrite, all negatives should be confirmed by adding Nitrate Reagent C (zinc dust, Cat. no. Z73). A pink/red color development is indicative of a "true negative" for nitrate reduction.

Showing confirmation reaction for *M. intracellulare* .
Since there was not red color development after the addition of the development reagents, a small amount of Nitrate Reagent C (zinc dust, Cat. no. Z73) was added using the end of a sterile wooden stick. The pink/red color development was indicative of a "true negative" for nitrate reduction.

REFERENCES

1. Vestal, A. 1975. *Procedures for the Isolation and Identification of Mycobacteria*, U.S. Dept. of HEW, Pub. No. (CDC); 76-8230, Centers for Disease Control, Atlanta, GA.

2. MacFaddin, J.F. *Biochemical Tests for Identification of Medical Bacteria*,, Lipincott Williams & Wilkins, Philadelphia, PA.

3. Kent, P.T. and G.P. Kubica. 1985. *Public Health Mycobacteriology A Guide for the Level III Laboratory* , U.S. Dept. of Health & Human Services, CDC, Atlanta, GA.

4. Anderson, N.L., et al. *Cumitech 3B; Quality Systems in the Clinical Microbiology Laboratory*, Coordinating ed., A.S. Weissfeld. American Society for Microbiology, Washington, D.C.

5. Jorgensen., et al. *Manual of Clinical Microbiology*, American Society for Microbiology, Washington, D.C.

6. Tille, P., et al. *Bailey and Scott's Diagnostic Microbiology*, C.V. Mosby Company, St. Louis, MO.

7. Isenberg, H.D. *Clinical Microbiology Procedures Handbook*, Vol. I, II & III. American Society for Microbiology, Washington, D.C.

8. Koneman, E.W., et al. *Color Atlas and Textbook of Diagnostic Microbiology*, J.B. Lippincott Company, Philadelphia, PA.

9. Isenberg, H.D. Clinical Microbiology Procedures Handbook, Long Island Campus for Albert Einstein College of Medicine, New Hyde Park, New York.

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The Hardy Diagnostics manufacturing facility and quality management system is certified to ISO 13485.

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