AN Microplate Instructions For Use

For Research Use Only

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Intended Use

The AN Microplate test panel provides a standardized method using 95 biochemical tests to profile and identify a broad range of anaerobic and microaerophilic bacteria. Biolog's software is utilized to identify the bacterium from its metabolic pattern in the AN Microplate. The AN Microplate is not for human in vitro diagnostic use. It is intended for research use only.

Description

Biolog Microplates™ test the ability of a microorganism to utilize or oxidize compounds from a preselected panel of different carbon sources. The test panel provides a phenotypic fingerprint of the microorganism for making a species level identification.

All necessary nutrients and biochemicals are prefilled and dried into the 96 wells of each plate. Tetrazolium redox dyes are used to colorimetrically indicate the utilization of the carbon sources or resistance to inhibitory chemicals.

The isolate to be identified is cultured on an agar medium and later suspended in an inoculating fluid[3] (AN-IF) with gelling properties at the recommended cell density. Then, the cell suspension is inoculated into the AN Microplate at 100 µl per well. Each well appears colorless when inoculated. During incubation, a burst of respiration occurs in the wells containing chemicals that oxidize. The cells reduce the tetrazolium dye, forming a purple color. Negative wells remain colorless, as does the reference well (A-1) with no carbon source.

The AN Microplates are incubated for 20-24 hours, allowing a pattern to form. The pattern of purple wells is then analyzed by Biolog software, which automatically cross-references the data to an extensive library of species. If an adequate match is found, the isolate is identified.

Upon Receipt

1. Inspect each pouch containing the AN Microplates for damage from shipping.
2. Each foil pouch should appear "vacuum packed" due to the presence of an oxygen absorbing packet. If the packet fails, oxygen will enter, and the pouch will appear "pillowed." Do not immediately use AN Microplates whose packaging has been affected in this manner. If the packaging has developed small holes in shipping, the plate performance can be recovered overnight by sealing it inside an anaerobe box or resealable plastic bag with a small handful of oxygen absorbers. Do not incubate in an anaerobic chamber containing hydrogen as the hydrogen will abiotically reduce the dye in the Microplates.
3. Store the AN Microplates at 2-8° C inside their pouches to preserve full shelf life.
4. Ensure each AN Microplate is within the expiration date printed on each pouch. *DO NOT USE THE AN Microplates AFTER THE EXPIRATION DATE.*

Materials Required for Test Procedure

Materials Included

1. 10 Biolog AN Microplates (Biolog Catalog #1007)

Materials Not Included

1.	BUA Agar	BUA (Biolog Universal Anaerobe) Agar dehydrated medium (Biolog Catalog #70007), or plated BUA Agar with 5% sheep blood (Biolog Catalog #71007). Use sheep blood with a hematocrit of at least 40%.
2.	Kanamycin disks	1 mg.
3.	AN-Inoculating Fluid (AN-IF)	Screw capped test tubes (20 x 113 mm) containing 14-14.5 ml of anaerobically prepared, sterile "gelling" inoculating fluid (0.40% NaCl, 0.03% Pluronic F-68, 0.02% Gellan Gum, 0.084% $NaHCO_3$, 0.000075% methylene green, 0.0015% sodium thioglycolate, final pH of AN-IF should be 7.0-7.2, Biolog Catalog #72007).
4.	LongSwabs™	Sterile 7-inch disposable cotton-tipped swabs (Biolog Catalog #3023).
5.	Streakerz™	Sterile 6-inch tapered wooden streaking sticks (Biolog Catalog #3026).
6.	Transfer Pipets	Sterile disposable 9-inch transfer pipets (Biolog Catalog #3019).
7.	Reservoirs	Sterile disposable reservoirs for multichannel pipettors (Biolog Catalog #3102).
8.	Pipettor	8-Channel Repeating Pipettor (Biolog Catalog #3711).
9.	Pipettor Tips	Sterile disposable pipettor tips (Biolog Catalog #3201).
10.	Turbidimeter	Biolog Catalog #3587.

AN Microplate Map

A-1 Water	A-2 N-Acetyl-D-Galactosamine	A-3 N-Acetyl-D-Glucosamine	A-4 N-Acetyl-β-D-Mannosamine	A-5 Adonitol	A-6 Amygdalin	A-7 D-Arabitol	A-8 Arbutin	A-9 D-Cellobiose	A-10 α-Cyclodextrin	A-11 β-Cyclodextrin	A-12 Dextrin
B-1 Dulcitol	B-2 i-Erythritol	B-3 D-Fructose	B-4 L-Fucose	B-5 D-Galactose	B-6 D-Galacturonic Acid	B-7 Gentiobiose	B-8 D-Gluconic Acid	B-9 D-Glucosaminic Acid	B-10 α-D-Glucose	B-11 α-D-Glucose-1-Phosphate	B-12 D-Glucose-6-Phosphate
C-1 Glycerol	C-2 D,L-α-Glycerol Phosphate	C-3 m-Inositol	C-4 α-D-Lactose	C-5 Lactulose	C-6 Maltose	C-7 Maltotriose	C-8 D-Mannitol	C-9 D-Mannose	C-10 D-Melezitose	C-11 D-Melibiose	C-12 3-Methyl-D-Glucose
D-1 α-Methyl-D-Galactoside	D-2 β-Methyl-D-Galactoside	D-3 α-Methyl-D-Glucoside	D-4 β-Methyl-D-Glucoside	D-5 Palatinose	D-6 D-Raffinose	D-7 L-Rhamnose	D-8 Salicin	D-9 D-Sorbitol	D-10 Stachyose	D-11 Sucrose	D-12 D-Trehalose
E-1 Turanose	E-2 Acetic Acid	E-3 Formic Acid	E-4 Fumaric Acid	E-5 Glyoxylic Acid	E-6 α-Hydroxybutyric Acid	E-7 β-Hydroxybutyric Acid	E-8 Itaconic Acid	E-9 α-Ketobutyric Acid	E-10 α-Ketovaleric Acid	E-11 D,L-Lactic Acid	E-12 L-Lactic Acid
F-1 D-Lactic Acid Methyl Ester	F-2 D-Malic Acid	F-3 L-Malic Acid	F-4 Propionic Acid	F-5 Pyruvic Acid	F-6 Pyruvic Acid Methyl Ester	F-7 D-Saccharic Acid	F-8 Succinamic Acid	F-9 Succinic Acid	F-10 Succinic Acid Mono-Methyl Ester	F-11 m-Tartaric Acid	F-12 Urocanic Acid
G-1 Alaninamide	G-2 L-Alanine	G-3 L-Alanyl-L-Glutamine	G-4 L-Alanyl-L-Histidine	G-5 L-Alanyl-L-Threonine	G-6 L-Asparagine	G-7 L-Glutamic Acid	G-8 L-Glutamine	G-9 Glycyl-L-Aspartic Acid	G-10 Glycyl-L-Glutamine	G-11 Glycyl-L-Methionine	G-12 Glycyl-L-Proline
H-1 L-Methionine	H-2 L-Phenylalanine	H-3 L-Serine	H-4 L-Threonine	H-5 L-Valine	H-6 L-Valine plus L-Aspartic Acid	H-7 2'-Deoxy Adenosine	H-8 Inosine	H-9 Thymidine	H-10 Uridine	H-11 Thymidine-5'-Mono-phosphate	H-12 Uridine-5'-Mono-phosphate

Procedure Precautions

TO OBTAIN ACCURATE AND REPRODUCIBLE RESULTS, THE INSTRUCTIONS BELOW MUST BE FOLLOWED.

1. Read the entire "Instructions for Use" manual prior to using the AN Microplates.
2. Pure cultures must be used to obtain identifications. The system is not designed to identify individual bacterial strains within mixed cultures.
3. Culture media selection and repeated subculturing prior to testing are very important. Many strains will produce different metabolic patterns depending upon how they are cultured prior to inoculation. Refer to sections "Step 2. Specimen Preparation and Characterization" and "Limitations" for details.
4. Sterile components and aseptic techniques must be used in set-up procedures. Contamination will affect results.
5. Disposable glassware should be used to handle all cell suspensions and solutions. Washed glassware may contain trace amounts of soap or detergent that will tamper results.
6. Prewarm the AN-IF and the AN Microplates to room temperature before use. Some species may be sensitive to cold shocks.
7. Calibrate your turbidimeter carefully and always prepare your inoculum within the specified density range. Refer to section "Step 3. Inoculum Preparation."
8. Biolog's chemistry contains components that are sensitive to temperature and light. Dark brown wells in the AN Microplates indicate deterioration of the carbon source. Wells with an inherent yellow or pink hue are normal.

ALWAYS KEEP IN MIND that you are testing the metabolic properties of live cells. Some species can lose their metabolic vigor when subjected to stresses (e.g., temperature, pH, and osmolarity) for even a few seconds.

Test Procedure

A. Preparations

— Prewarm the AN Microplates and tubes of AN-IF to at least 25°C before use.

B. Steps

Step 1. Culture Isolation on Biolog Recommended Media

1. Isolate a pure culture on agar media.
2. Use Biolog recommended media (BUA Agar with 5% sheep blood) and incubate anaerobically at 26°, 30°, or 35-37° C. All species identifiable with the AN Microplate will grow under these conditions.
3. For best results, always streak for isolated colonies.
4. Subculture anaerobic bacteria twice on BUA and prepare a suspension from the second plate only.

Step 2. Specimen Preparation and Characterization

1. It could be helpful to perform a Gram stain on your isolate and observe the cell morphology in the Gram stain: coccus or rod. This will allow you to categorize the bacterium into one of the following groups: gram-negative coccus (GNC), gram-negative rod (GNR), gram-positive

coccus (GPC), or gram-positive rod (GPR). Categorization can aid the identification procedure by directing the Biolog ID database to search the appropriate class. A Gram stain is not strictly required.

2. Grow the bacterium using the recommended conditions. The choice of the agar medium is critical since it supports growth and promotes retention of full metabolic activity. All species that can be identified should be grown anaerobically on BUA with 5% sheep blood (BUA+B) and in most cases should be incubated at 35-37° C. Only a few species require 30° C (*Lactobacillus malefermentans, Pectinatus cerevisiiphilus and frisingensis, Tetragenococcus halophilus, Zymomonas mobilis, Zymophilus raffinosivorans and paucivorans*) or 26° C (*Lactobacillus collinoides and hilgardii, Leuconostoc gelidum*). For gram-negative rapidly growing rods, a 1 mg kanamycin disk should also be included in the first quadrant of the culture on the agar plate. All species are cultured strictly under anaerobic conditions.
3. The cells must be freshly grown since many strains lose viability and metabolic vigor in the stationary phase. The recommended incubation period for most organisms is 24-48 hours.
4. If insufficient growth is obtained to inoculate the panel, continue to incubate the plate to obtain more growth, or restreak heavily (as a lawn) onto one or more agar plates. Incubate for 24-72 more hours. This should give sufficient growth to inoculate the panel.

STEP 3. INOCULUM PREPARATION

1. Examine your AN-IF to verify it is anaerobic. The inoculating fluid contains methylene green as an indicator. If anaerobic, it will be colorless. If oxidized, the indicator will turn a faint blue-green color, and the AN-IF should not be used unless the oxygen is removed and the fluid returns to the colorless state.
2. Establish the acceptable turbidity range on your turbidimeter. First, set the transmittance adjustment to 100% by using an uninoculated AN-IF tube. Then determine the desired turbidity with the AN Turbidity Standard. Using the Biolog turbidimeter and a 20mm tube of the Standard, the transmittance value will be approximately 65% with slight variations per Biolog turbidimeter. With other instruments or tubes, the transmittance readings may vary substantially.
3. Blank the turbidimeter (transmittance = 100%) with a clean tube containing uninoculated AN-IF. Because the tubes used are not optically uniform, they should be blanked individually and not rotated in the light path of the turbidimeter.
4. Prepare a uniform suspension while maintaining anaerobic conditions. If several AN Microplates are being inoculated, prepare the suspensions inside an anaerobic chamber. If only a few AN Microplates are being inoculated, the suspensions can be prepared and inoculated quickly under aerobic conditions. Preparation of all suspensions should be completed within a 5-minute period. Therefore, to obtain optimum results, no more than six suspensions should be prepared at any one time. To prepare the suspensions, remove cells from the anaerobic agar plate with a sterile cotton swab to prevent contamination of nutrients from the agar medium. Twirl and press the swab against the inside surface of the tube on the dry glass above the fluid line to break up clumps and release cells. Move the swab up and down the wall of the tube into the fluid until the organism becomes a homogenous, clump-free suspension. Use the swab with a vertical stirring motion to generate a uniform suspension all the way to the bottom of the tube. A sterile transfer pipet may also be used to mix the suspension without creating an aerosol. If clumps remain, let the tube stand for several minutes, allowing them to settle.
5. Adjust the inoculum density. Watch as the turbidimeter needle moves toward the acceptable turbidity range. The acceptable range is defined by the turbidity standard ±2% transmittance. This must be done with precision since it establishes the oxygen concentration for the cells and for the redox chemistry. The density can be lowered by adding more inoculating fluid or raised by adding more cells.

6. Inoculate the cell suspension into the AN Microplate promptly. Some strains lose metabolic activity if held too long (no more than 20 minutes) in inoculating fluid without nutrients.

Step 4. Inoculation of the AN Microplate

THE FOLLOWING STEPS MUST BE PERFORMED QUICKLY UNDER AEROBIC CONDITIONS.

1.1 For Kanamycin resistant gram-negative rods that have grown well enough to prepare a suspension overnight, the pouches should be opened to expose the AN Microplates to air 20 minutes before inoculation.

1.2 For all other bacteria, after all suspensions have been prepared, open the pouch, and remove the AN Microplate. Inoculate the AN Microplate within 5 minutes.

2. Label the AN Microplate with the organism's name and number.
3. Pour the cell suspension into the multichannel pipet reservoir.
4. If you are using an electronic pipettor, fasten 8 sterile tips securely onto the 8-Channel Repeating Pipettor. Refer to the manufacturer's instructions. Fill the tips and ensure all tips are filling equally. If not, refasten any loose tips.
5. If you are using a manual pipettor, prime the tips by dispensing the first delivery back into the reservoir.
6. Fill all wells with precisely 100 µl. Be careful not to carry over chemicals or splash from one well into another.
7. The inoculating fluid will form a soft gel shortly after inoculation.
8. Cover the AN Microplate with its lid.
9. Allow the AN Microplate to sit in aerobic conditions for 10-15 minutes after inoculation with the lid on.

Step 5. Incubation

1. Incubate the AN Microplate under anaerobic conditions in an oxygen impermeable jar, box, or bag; using an appropriately sized anaerobic sachet that generates a hydrogen-free anaerobic environment. Use indicator strips containing resazurin (Oxoid®) rather than methylene blue. *DO NOT INCUBATE THE AN Microplate IN A STANDARD ANAEROBIC CHAMBER*. Many anaerobic bacteria have strong hydrogenase activity, reducing the tetrazolium dye. This forms a purple color in all wells due to the hydrogen gas in the atmosphere.
2. Incubate the AN Microplates for 20-24 hours.

The following table summarizes the testing procedures:

	Microbe type	Anaerobe
Step 1	Culture medium	BUA+B
Step 2	Temperature	35-37° C *EXCEPTION:* Use 30° C or 26° C for certain species.
	Atmosphere	Anaerobic (must use a hydrogen-free anaerobic atmosphere for incubating the AN Microplates)
Step 3	Inoculating fluid	AN-IF
	Inoculum turbidity	65% T
Step 4	Exposure of AN Microplate to air	• Less than 5 minutes of exposure to air before inoculation. *EXCEPTION:* 20 minutes of exposure to air before inoculation for a) Gram-negative rods that are kanamycin resistant and that produce enough growth to prepare a suspension after overnight incubation, b) Strains that produce a high "false positive" purple color in the AN Microplate. • 10-15 minutes exposure to air after inoculation.
	Inoculum per well	100 µl
Step 5	Incubation	20-24 hours anaerobically without hydrogen gas.

Results

Reading and Interpretation

1. Read the AN Microplates using an Odin™ system, the MicroStation™ Reader, or the MicroLog™ System "False positive" color is typically defined as a purple color forming in the control well (A-1) and in other "negative" wells. This problem is caused by the bacterial suspension reducing the buffer and subsequently reducing the tetrazolium dye. It is typically seen in genera strains *Actinomyces, Bacteroides*, and *Propionibacterium*. In most cases, the "false positive" color is faint and not a problem so long as the "true positive" reactions are discernible. If the strain produces a high background color making it difficult to determine which wells are positive, repeat the setup and inoculation of the AN Microplate after exposure to air for 20 minutes (refer to Step 4 and summary table on page 5).
2. Most species give dark, clearly discernible "positive" reactions. However, it is normal for the "positive" reactions of some genera strains including *Clostridium*, *Eubacterium*, *Fusobacterium*, *Prevotella*, and *Porphyromonas* to be light or faint purple.
3. For AN Microplates read at 20-24 hours of incubation, the similarity index must be at least 0.50 to be considered acceptable.

Performance Characteristics

The AN Microplate performance characteristics have been determined by establishing a database from a large collection of well characterized organisms, including species type strains. The database is designed to identify all species in accordance with current taxonomic nomenclature. TO OBTAIN ACCURATE AND

REPRODUCIBLE RESULTS, ALL PROCEDURES AND RECOMMENDATIONS IN THE INSTRUCTIONS FOR USE MUST BE FOLLOWED PRECISELY.

Limitations

The AN Microplate is designed to identify pure cultures of anaerobic and certain microaerophilic bacteria. The panel will only recognize members of the species in the current database. Other species will be reported with the message "No identification." Atypical strains may also yield a similarity index that is less than 0.5 at 20-24 hours and, therefore, will report "No identification."

Troubleshooting

If you experience a problem utilizing the AN Microplate, start by rereading the Instructions for Use and review whether you have deviated from the recommended procedures. Then refer to the table below.

If all wells are positive, ensure:	**If all wells are negative, ensure:**
1. You are incubating the bacteria in an anaerobic atmosphere that is hydrogen-free. Many anaerobes have a strong hydrogenase activity and will reduce the tetrazolium dye and form a purple color in all wells due to the presence of hydrogen gas. 2. You have accurately prepared your AN-IF. If additional sodium thioglycolate was added, excessive levels of thioglycolate can cause "false-positive" reactions. 3. You are not carrying over any nutrients from the agar growth medium into the inoculating fluid. 4. Your inoculum density is not excessive – check the calibration of your turbidimeter. 5. You have exposed the AN Microplate to aerobic conditions for 10-15 minutes following inoculation. 6. Your inoculum is free of clumps.	1. The AN Microplate packaging maintained anaerobic conditions. If not, oxygen will be absorbed into the plastic and can inhibit growth. 2. Your cells are freshly grown and you have used the recommended BUA+B agar medium. 3. The inoculating fluid was anaerobic (i.e. the methylene green indicator was colorless) before the tube was opened. 4. The inoculating fluid was prewarmed, prepared appropriately, has the correct pH and salinity, and does not contain preservatives. 5. You are handling the cells with disposable hardware (soap residues are toxic). 6. Your inoculum density is sufficient – check the calibration of your turbidimeter. 7. You did not leave the cells exposed to aerobic conditions for more than 20 minutes during the inoculation process.

Quality Control

See the User Guide for the specific software or system you are using, for further assistance in interpreting identification results.

Biolog Microplates are tested to meet internal quality control standards before being released for sale. However, some laboratories may desire or may be required to perform independent validations on each manufacturing lot. To test the performance of the AN Microplate use the 4 strains specified below. These are available from ATCC®.

1. *Bifidobacterium breve* ATCC 15700™

2. *Paeniclostridium sordellii*	ATCC 9714™
3. *Lacticaseibacillus casei*	ATCC 393™
4. *Parvimonas micra*	ATCC 33270™

Inoculate each bacterium following the specified test procedures. When lyophilized or frozen cultures are used, they should be subcultured at least twice before being tested.

Read the AN Microplates at 20-24 hours of incubation. The resulting identification should correctly correspond to the identity of the quality control strain.

If the identification does not match, review the test procedures and check the purity of your culture. Repeat the test procedure. Call Biolog Technical Service if you have further problems or questions.

Technical Service

For help or to **report problems** with this product contact Biolog Technical Service during business hours (8 A.M. to 5 P.M. Pacific Time) or contact your local Biolog Distribution Partner.

phone 510-785-2564
email tech@biolog.com

General information, Certificates of Analysis, or SDS documents may be found at www.biolog.com.

References

1 Bochner, BR 1989. Sleuthing out Bacterial Identities. Nature 339:157-158.
2 Bochner, BR 1989. "Breathprints" at the Microbial Level. ASM News 55:536-539.
3 Biolog, Inc., US Patent # 5,627,045.