GEN III Microplate Instructions For Use

For Research Use Only

Biolog,Inc.
21124 Cabot Blvd.
Hayward, CA 94545
www.biolog.com

Technical Assistance:
tech@biolog.com
510-785-2564

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Description

The Biolog GEN III Microplate analyzes a microorganism with 94 phenotypic tests: 71 carbon source utilization assays (GEN III Microplate Map, columns 1-9) and 23 chemical sensitivity assays (GEN III Microplate Map, columns 10-12). 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 utilization of the carbon sources or resistance to inhibitory chemicals.

The isolate to be identified is grown on an agar medium and later suspended in a special gelling inoculating fluid[3] (IF) at the recommended cell density. Then the cell suspension is inoculated into the GEN III Microplate at 100 µl per well. The GEN III Microplate is incubated to allow the phenotypic fingerprint to form. Each well appears colorless when inoculated. During incubation, an increase in respiration in the wells occurs allowing cells to utilize a carbon source and/or divide. Increased respiration causes reduction of the tetrazolium redox dye, forming a purple color. Negative wells remain colorless as does the negative

control well (A-1) with no carbon source. There is also a positive control well (A-10) used as a reference for the chemical sensitivity assays in columns 10-12. After incubation, the phenotypic fingerprint of purple wells is compared to Biolog's extensive species library. If a match is found, the isolate is identified.

Intended Use

The GEN III Microplate test panel provides a standardized method using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria[1,2]. Biolog software is used to identify the bacterium based on its phenotypic pattern in the GEN III Microplate. The GEN III Microplate is not for human in vitro diagnostic use. It is intended for research use only.

Upon Receipt

1. Inspect each pouch containing the GEN III Microplates for damage from shipping. *DO NOT USE THE GEN III Microplates IF THE PACKAGING INDICATES SIGNS OF DAMAGE.*
2. Store the GEN III Microplates at 2-8° C inside their pouches to preserve full shelf life.
3. Ensure each GEN III Microplate is within the expiration date printed on each pouch. *DO NOT USE THE GEN III Microplates AFTER THE EXPIRATION DATE.*
4. During shipment, the GEN III Microplates may be maintained at room temperature for a period of up to 12 days.

Materials Required for Test Procedure

Materials Included

1. 10 GEN III Microplates (Biolog Catalog #1030)

Materials Not Included

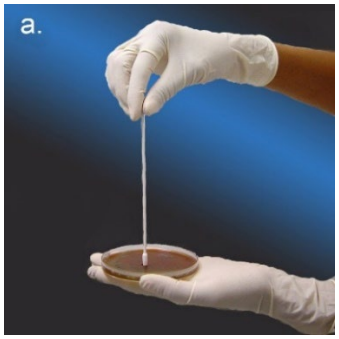
1. Agar Culture Media	— BUG Agar with 5% sheep blood (BUG+B) (Biolog Catalog #71102) — Chocolate Agar (Biolog Catalog Bio-M1012) — Biolog Dehydrated Growth Agar (BUG Agar) (Biolog Catalog #70101) — or comparably performing agar media
2. Inoculating Fluid	— IF-A (Biolog Catalog #72401) — IF-B (Biolog Catalog #72402) — IF-C (Biolog Catalog #72403)
3. Inoculatorz™	— Sterile disposable inoculator swabs (20x50) (Biolog Catalog #3321) — (100x1) (Biolog Catalog #3323)
4. Streakerz™	— Sterile disposable wooden agar plate streakers (50x20) (Biolog Catalog # 3026)

5. Transfer Pipets — Sterile disposable 9-inch transfer pipets (Biolog Catalog #3019)
6. Reservoirs — Sterile disposable reservoirs for multichannel pipettor (Biolog Catalog #3102)
7. Pipettor — 8-Channel Repeating Pipettor (Biolog Catalog #3711)
8. Pipettor Tips — Sterile disposable pipettor tips (Biolog Catalog #3201)
9. Turbidimeter — with Universal Power Adapter (Biolog Catalog #3587)
10. Turbidity Standards — 85% T (Biolog Catalog #3441)
    — 65% T (Biolog Catalog #3440)

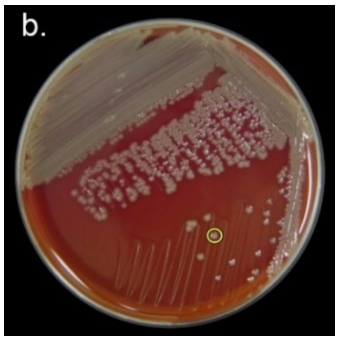
GEN III Microplate Map

A-1 Negative Control	A-2 Dextrin	A-3 D-Maltose	A-4 D-Trehalose	A-5 D-Cellobiose	A-6 Gentiobiose	A-7 Sucrose	A-8 D-Turanose	A-9 Stachyose	A-10 Positive Control	A-11 pH 6	A-12 pH 5
B-1 D-Raffinose	B-2 α-D-Lactose	B-3 D-Melibiose	B-4 β-Methyl-D-Glucoside	B-5 D-Salicin	B-6 N-Acetyl-D-Glucosamine	B-7 N-Acetyl-β-D-Mannosamine	B-8 N-Acetyl-D-Galactosamine	B-9 N-Acetyl Neuraminic Acid	B-10 1% NaCl	B-11 4% NaCl	B-12 8% NaCl
C-1 α-D-Glucose	C-2 D-Mannose	C-3 D-Fructose	C-4 D-Galactose	C-5 3-Methyl Glucose	C-6 D-Fucose	C-7 L-Fucose	C-8 L-Rhamnose	C-9 Inosine	C-10 1% Sodium Lactate	C-11 Fusidic Acid	C-12 D-Serine
D-1 D-Sorbitol	D-2 D-Mannitol	D-3 D-Arabitol	D-4 myo-Inositol	D-5 Glycerol	D-6 D-Glucose-6-PO4	D-7 D-Fructose-6-PO4	D-8 D-Aspartic Acid	D-9 D-Serine	D-10 Macrolide	D-11 Rifamycin SV	D-12 Minocycline
E-1 Gelatin	E-2 Glycyl-L-Proline	E-3 L-Alanine	E-4 L-Arginine	E-5 L-Aspartic Acid	E-6 L-Glutamic Acid	E-7 L-Histidine	E-8 L-Pyroglutamic Acid	E-9 L-Serine	E-10 Lincomycin	E-11 Guanidine HCl	E-12 Niaproof 4
F-1 Pectin	F-2 D-Galacturonic Acid	F-3 L-Galactonic Acid Lactone	F-4 D-Gluconic Acid	F-5 D-Glucuronic Acid	F-6 Glucuronamide	F-7 Mucic Acid	F-8 Quinic Acid	F-9 D-Saccharic Acid	F-10 Vancomycin	F-11 Tetrazolium Violet	F-12 Tetrazolium Blue
G-1 p-Hydroxy-Phenylacetic Acid	G-2 Methyl Pyruvate	G-3 D-Lactic Acid Methyl Ester	G-4 L-Lactic Acid	G-5 Citric Acid	G-6 α-Keto-Glutaric Acid	G-7 D-Malic Acid	G-8 L-Malic Acid	G-9 Bromo-Succinic Acid	G-10 Nalidixic Acid	G-11 Lithium Chloride	G-12 Potassium Tellurite
H-1 Tween 40	H-2 γ-Amino-Butyric Acid	H-3 α-Hydroxy-Butyric Acid	H-4 β-Hydroxy-D,L-Butyric Acid	H-5 α-Keto-Butyric Acid	H-6 Acetoacetic Acid	H-7 Propionic Acid	H-8 Acetic Acid	H-9 Formic Acid	H-10 Aztreonam	H-11 Sodium Butyrate	H-12 Sodium Bromate

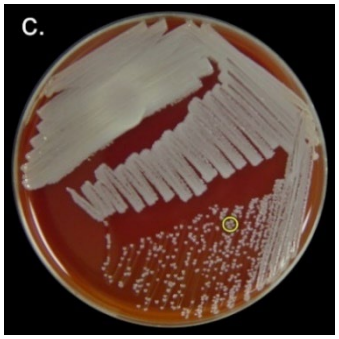
The GEN III Microplate test procedure:



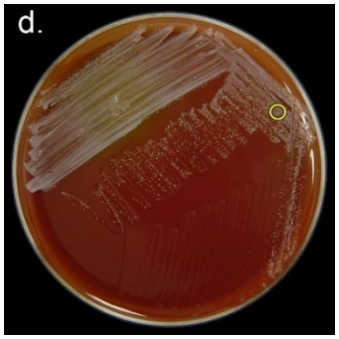
a. Swab cell growth from agar medium



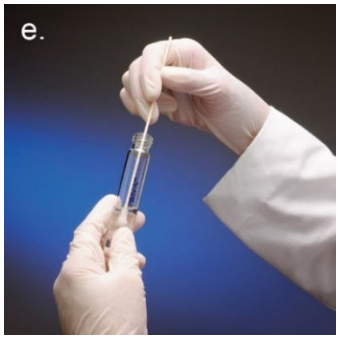
b. Example of fast-growing bacteria



c. Example of medium-growing bacteria



d. Example of slow-growing bacteria



e. Release bacteria into IF

f. Read IF tube in turbidimeter

g. Fill GEN III Microplate wells with cell suspension

h. Place GEN III Microplate into Odin™ Instrument

Procedure Precautions

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

1. Read the "Instructions for Use" prior to using the GEN III Microplate and follow the procedures.
2. Pure cultures must be used to obtain identifications. The system is not designed to identify individual bacterial strains from within mixed cultures. The most common problem in identification is that microbiologists are not aware that they have a mixed culture. Streaking for isolated colonies may not be sufficient because isolated colonies can arise from a clump of cells as well as a single cell. Bacteria have sticky surfaces and they tightly adhere to other bacteria. This is particularly a problem with mucoid bacteria, fresh environmental isolates, and staphylococci. First, examine cultures with care, using a dissecting microscope or colony magnifying lens, to make sure that only one colony morphology is present in the culture. If no species identification is obtained, you may still have a mixed culture. In that case, re-streak the cells onto a multi-chromogenic agar medium and let the original agar plate and the chromogenic agar plate sit at room temperature for 3 or 4 days. Examine both plates carefully, looking for the outgrowth of "bumps" or non-uniform growth in the areas of confluent growth. On the chromogenic agar plate, look for more than one color. If necessary, re-isolate the colony types that are present and perform the identification assay a second time.
3. Culture media and repeated subculturing may affect the results. Strains may produce different phenotypic patterns depending upon how they are cultured prior to inoculation.
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 affect results.
6. Prewarm the IF and the GEN III Microplates to room temperature before use. Some species may be sensitive to cold shocks (e.g., *Neisseria sp.*).
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. Store the inoculating fluid in the dark with refrigeration. Brown or yellow wells in the GEN III Microplate indicate potential deterioration of the chemistry.

ALWAYS KEEP IN MIND that you are testing 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 GEN III Microplates and tubes of IF to room temperature and review the entire protocol including precautions.

— Determine the Appropriate Protocol to Use (Inoculating Fluid and Cell Density)

- All protocols are performed in the same manner, the only difference being the choice of inoculating fluid (IF) and cell density for inoculation.
- Protocol A is used for the vast majority of species—it is the default protocol.
    - IF-A
    - 95% T Cell Density. Note that 95-98% T is best for fast-growing bacteria while 92-95% T is best for slow-growing bacteria.
- Protocol B is used for a small number of strongly reducing species and capsulated species (primarily some strains of *Aeromonas, Vibrio,* and spore-forming Gram-positive rods). These species will give a false positive result in the A-1 well with Protocol A. If this occurs, simply repeat the test using Protocol B.
    - IF-B
    - 95% T Cell Density.
    - Strongly reducing and capsule-producing GN (e.g., some *Aeromonas, Vibrio,* and mucoid enterics) and some GP spore-forming species, (e.g., *Bacillus, Aneurinibacillus, Brevibacillus, Fictibacillus, Lysinibacillus, Rummeliibacillus, Solibacillus, Viridibacillus*) and some non-spore forming species (e.g., *Arthrobacter, Janibacter, Sinomonas*)
- Protocol C1 is used for slow growing bacteria that typically form pinpoint-sized colonies (less than 1 mm in diameter) on BUG+B Agar in 24 hours of growth (see example in Figure d.). These are primarily microaerophilic and capnophilic Gram-positive cocci and tiny rods.
    - IF-C
    - 92-95% T Cell Density
    - Microaerophilic, capnophilic GP (e.g., *Dolosicoccus, Dolosigranulum, Eremococcus, Erysipelothrix, Gemella, Globicatella, Helcococcus, Ignavigranum, Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Tetragenococcus, Trueperella, Turicella, Weissella,* and some *Aerococcus, Mycobacterium, Nocardia, Pediococcus*)
- Protocol C2 is used for fastidious, capnophilic, and very oxygen-sensitive bacteria that grow very slowly or not at all on BUG+B Agar. For example, it is used for fastidious Gram-negative species that would most likely be encountered from respiratory tract specimens after cultivation on Chocolate Agar with 6.5% CO_2. Some very oxygen-sensitive Gram-positive bacteria also require the higher inoculation density of Protocol C2.
    - IF-C
    - 65% T Cell Density
    - Fastidious, capnophilic, oxygen sensitive GN (e.g., *Actinobacillus, Aggregatibacter, Alysiella, Avibacterium, Basfia, Bergeriella, Bergeyella, Biberstenia, Bordetella, Brackiella, Campylobacter, Capnocytophaga, Cardiobacterium, Dysgonomonas, Eikenella, Francisella, Gallibacterium, Haemophilus, Histophilus, Kingella, Methylobacterium, Moraxella, Neisseria, Nicoletella, Oligella, Ornithobacterium, Pasteurella, Simonsiella, Suttonella,Taylorella, Volucribacter. N*ote that *Campylobacter* strains will give a stronger pattern if tested at a higher cell density of 40%T.) and GP (*Actinomyces, Aerococcus, Alloiococcus, Arcanobacterium, Facklamia, Gardnerella, Granulicatella,* and some *Lactobacillus, Pediococcus.*)
    - If unsure of the appropriate test protocol, use protocol A. If the result fails to yield an identification because of a false-positive A-1 well, then use Protocol B. If the result fails because of insufficient positive carbon source reactions, in succession, follow Protocols C1 and C2.

- Special Protocol for Identifying Plant Pathogenic Bacteria: Plant pathogenic bacteria often require lower temperatures for culture and incubation in the GEN III Microplate.

- A universal protocol for plant pathogenic bacteria is to culture them on YNA agar medium (Yeast Extract Nutrient Agar) at 26° C, inoculate the GEN III Microplate, and incubate the GEN III Microplate at 30° C instead of 33° C. Refer to the document "*GEN III Database Species and Their Characteristics*" to determine which species require 30° C.

B. Steps

Step 1. Culture Organism on Biolog Recommended Media

1. Isolate a pure culture on agar media.
2. Use Biolog recommended media (BUG+B or Chocolate Agar) and incubate at 33°C. Some species may require special culture conditions, for example lower or higher temperatures (26–37 °C) and elevated CO_2 (6.5% - 10%). Refer to the document "GEN III Database Species and Their Characteristics" for additional guidance on the recommended culture conditions for each species.
3. Use of alternative media should be validated. For laboratories that need to use agar media without blood, we recommend BUG Agar. However, some species will grow extremely slowly or not at all if blood is omitted. See Protocols C1 and C2 for examples of some genera. R2A Agar and Tryptic Soy Agar with or without blood (TSA, TSA+B) are substitutes, but may not culture as wide a range of bacteria as BUG+B. Furthermore, their recipes and performance characteristics from different vendors may vary.
4. The cells must be freshly grown since many strains lose viability and metabolic vigor in stationary phase. The recommended incubation period for most organisms is 4-24 hours. Spore-forming gram-positive bacteria (*Bacillus* and related genera) should be grown for less than 16 hours to minimize sporulation while on the agar medium.
5. If insufficient growth is obtained to inoculate the panel, re-streak heavily (as a lawn) onto one or more agar plates. Incubate for 4-48 hours. This should give enough growth to inoculate the panel.

Step 2. Inoculum Preparation

1. The inoculum preparation depends on the protocol selection. Refer to the section above "*How to Determine Appropriate Protocol to Use,"* and the document "*GEN III Database Species and Their Characteristics*" for additional guidance on the protocol recommended for each species.
2. Check the calibration of the turbidimeter periodically. Use the appropriate turbidity standard (85% T or 65% T) and refer to the turbidimeter manual instructions to verify the turbidimeter is calibrated and operating properly.
3. Blank the turbidimeter with a clean tube (wiped clean with an alcohol prep pad of dirt and fingerprints) containing uninoculated IF. Because the tubes used are not optically uniform, they should be blanked individually. Set the transmittance adjustment to 100% by using an uninoculated tube containing IF.
4. Prepare the inoculum at the desired turbidity. The target cell density is 95% T for Protocols A, B, and C1. Protocol C2 requires a higher cell density of 65% T for species that are sensitive to oxygen. Use a cotton tipped Inoculatorz swab to acquire a 3 mm diameter area of cell growth from the surface of the agar plate. As shown in Figure a, grasp the swab at its tip and, holding the swab vertically, touch it to the cell growth. Figures b, c, and d show examples of fast, medium, and slow growing bacteria. The yellow circle indicates where to touch the end of the cotton swab. For fast-growing bacteria, touch a single colony; for medium-growing bacteria, touch a cluster of colonies; for slow-growing bacteria touch the first area of confluent growth. Release the bacteria into the IF by rubbing the swab tip against the bottom of the tube containing IF as shown in Figure e. Crush any cell clumps against the tube wall or remove them from the IF by catching

them on the swab. Stir the IF with the swab to obtain a uniform cell suspension. Then, read it in the turbidimeter, as shown in Figure f. If the cell density is too low, add more cells. If the cell density is too high, add more IF.

For extremely clumpy bacteria that cannot be dispersed directly, use the following procedure.

a. Prepare a dense suspension in 2 ml of IF as follows. Use a sterile wooden Streakerz stick to remove a clump of cell mass from the agar surface without gouging the agar. If the bacteria are extremely dry and embedded in the agar, use the edge of a sterile glass microscope slide to gently scrape a mass of cells onto the glass slide, again, without gouging the agar. The cells can then be scraped off the glass slide with a sterile Streakerz stick.
b. Use the Streakerz stick to deposit the cell mass onto the inner wall of a dry tube. Use the Streakerz stick to crush, break up, and spread the clumps of cells against and along the inner wall of the tube.
c. Add 2 ml of IF, and gradually slide the dispersed cells into the IF. The resulting cell suspension will be a mixture of suspended cells and residual clumps. Stand the tube in a rack for about 5 minutes and allow the clumps to settle to the bottom.
d. Use a small pipet and transfer the suspended cells at the top into a fresh tube of IF to achieve the target cell density.

Step 3. Inoculation of the GEN III Microplate

1. Pour the cell suspension into the multichannel pipet reservoir.
2. Fasten 8 sterile tips securely onto the 8-Channel Repeating Pipettor and fill the tips by drawing up the cell suspension from the reservoir.
3. Fill all wells with 100 µl as shown in Figure g. Be careful not to carry over chemicals or splash from one well into another.
4. The inoculating fluid will form a soft gel shortly after inoculation.
5. Cover the GEN III Microplate with its lid.

Step 4. Incubation

1. Place the GEN III Microplate into the Odin incubator/reader, as shown in Figure h, or into a microbiological incubator for 4 to 36 hours. Incubate at 33°C or use specific incubation conditions for the bacterium found to be optimal in Step 1.

Results

Reading and Interpretation

1. Read the GEN III Microplates using the Odin Platform, the MicroStation™ Reader, or the MicroLog™ System. Refer to the User Guide for each system for more detailed instructions. Odin automatically reads the GEN III Microplates every 20 minutes until an identification result is called. When using the MicroStation and/or the Microbial Identification software, the GEN III Microplates can be read at any time after there are at least 3 carbon source wells that have turned purple. For optimal results, we recommend performing readings at 8, 16, and 22 hours. Refer to the document "*GEN III Database Species and Their Characteristics*" with MTI (median time to identification) guidance for all species in the database. This information can also provide guidance on the best or earliest time to read the GEN III Microplate and obtain an identification result. Software on Odin and the MicroStation automatically perform all analysis and interpretation of results.
2. For manual identification determinations: the color densities in wells of the carbon source utilization assays in columns 1-9 are referenced against the negative control well, A-1.

2.1. All wells visually resembling the A-1 well should be scored as "negative" (-).
2.2. All wells with a noticeable purple color (greater than well A-1) should be scored as "positive" (+).
2.3. Wells with extremely faint color, or with small purple flecks or clumps are best scored as "borderline" (\).

3. "False positive" color is defined as purple color forming in the negative control well (A-1) and in other "negative" wells. This is seen with only a few species such as from the genera *Aeromonas, Vibrio,* and *Bacillus*. If such a result occurs, the cells can be retested with Protocol B and IF-B.
4. The color densities in wells of the chemical sensitivity assays in columns 10-12 are referenced against the positive control well, A-10.
   4.1. All wells showing significant sensitivity to the inhibitory chemical, with less than half the color of the A-10 well are considered "negative" (-) for growth.
   4.2. All other wells showing normal or near normal purple color (similar to well A-10) are considered "positive" (+).
   4.3. If there is uncertainty about the interpretation, it is best to score the well as "borderline" (\).
5. Most species give dark, clearly discernible "positive" reactions. However, it is normal for the "positive" reactions of certain genera to be light or faint purple.

See Biolog's Microbial Identification Systems User Guide for further assistance in interpreting identification results.

Performance Characteristics

The GEN III Microplate performance characteristics have been determined by establishing a database using a large collection of microorganisms from diverse sources. The database is designed to give identifications of all species accordance with current standards of classical identification methods and current taxonomic nomenclature. *TO OBTAIN ACCURATE AND REPRODUCIBLE RESULTS, ALL PROCEDURES AND RECOMMENDATIONS IN THE INSTRUCTIONS FOR USE MUST BE FOLLOWED PRECISELY.*

Limitations

The GEN III Microplate is designed to identify pure cultures of Gram-negative and Gram-positive bacteria. The panel will only recognize members of the species in the current database. Other species will usually be reported with the message "No identification." Atypical strains may also yield a low similarity index and, therefore, will result in the message "No identification." Some bacterial species are reportable to government and public health agencies under certain circumstances. For any isolate that is identified as *Salmonella* or *Shigella or E. coli O157:H7,* we recommend confirmation by serology. *Neisseria gonorrhoeae* identifications should also be confirmed. Appropriate caution and confirmation should be used for isolates suspected of being dangerous pathogens.

Troubleshooting

If you experience a problem utilizing the GEN III 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 in columns 1-9 are positive, ensure that:	If all wells in columns 1-9 are negative, ensure:
1. You are using a microorganism appropriate for the GEN III Microplate. If the bacterium is a strongly reducing or capsulated species causing false-positive color in the A-1 well, repeat the test using Protocol B and IF-B. 2. You are not carrying over any nutrients from the agar growth medium into the inoculating fluid. 3. Your inoculum is free of clumps. 4. Your inoculum density is not excessive – check the calibration of your turbidimeter. 5. The A-1 well is not under-filled, as it is used as a reference well.	1. You are using a microorganism appropriate for the GEN III Microplate. Oligotrophic species or extremely slow-growing or oxygen-sensitive bacteria may produce all negative wells. 2. Your cells are freshly grown, and you have used the recommended BUG+B or Chocolate agar culture medium. 3. Your incubation temperature and atmosphere are correct for the organism being tested. 4. The inoculating fluid was stored correctly and prewarmed prior to use. 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. The A-1 well is not over-filled, as it is used as a reference well.

Quality Control

Biolog Microplates must meet internal quality control standards before being released for sale. However, some laboratories may desire or may be required to perform independent quality control checks on each manufacturing lot.

To test the performance of the GEN III Microplate, use the 2 gram-negative and 2 gram-positive strains specified below using Protocol A. They are available from ATCC®.

1. *Escherichia coli* 1	ATCC 11775™
2. *Paenibacillus polymyxa*	ATCC 842™
3. *Staphylococcus epidermidis*	ATCC 12228™
4. *Stenotrophomonas maltophilia*	ATCC 13637™

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

Read the GEN III Microplates after appropriate 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 any 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. PST) 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.