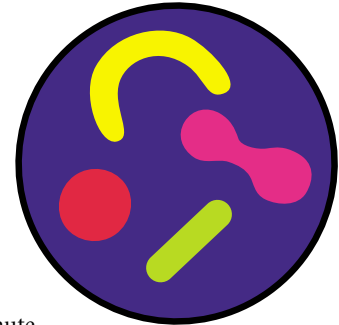


## DIFFERENTIATING GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

The Gram-Stain is the single most common and cost effective staining technique used in identifying bacterial organisms. The method was originally devised in 1844 by a Danish bacteriologist, Hans Christian Gram.

### I. THE BASIC STAINING PROCEDURE

1. Make a thin layer of bacterial cells on a clean glass slide from a fresh (18-24 hours of growth), single isolated colony off a nutrient agar growth plate. Allow to air dry.
2. Fix with 95% methanol for 2 minutes, air dry. This is the current recommended fixing method as it does not distort cells. Heating the slide (which is the traditional method) may cause artifacts and does not fix the specimen to the slide well.
3. Flood the slide with crystal violet staining reagent for one minute.
4. Wash the slide with a gentle and indirect stream of tap water for two seconds. Tilt the slide to drain off the excess water.
5. Flood the slide with iodine staining reagent for one minute.
6. Repeat step 4.
7. Add the decolorizer to the slide with it slanted until no color washes out, about 10 – 30 seconds.
8. Repeat step 4.
9. Flood the slide with safranin counterstaining reagent for one minute.
10. Wash the slide in a gentle, indirect stream of tap water until no color washes out. Air dry or blot the slide dry with absorbent paper.
11. View the slide with the light microscope under oil-immersion. Gram-positive bacteria appear blue or violet and gram-negative bacteria appear pinkish red.



### INSIDE

- 1 Differentiating gram-negative and gram-positive bacteria
- 3 Culture Purification
- 4 Handling Difficult to Grow Bacteria
- 4 Where Can I find the Information?

### THINGS TO LOOK FOR ON YOUR SLIDE:

Is your smear too thick?

Did you overheat the slide?

Is your decolorizer too strong?

How old is the culture you are Gram-staining?

See page two for troubleshooting tips and solutions.

Gram staining is based on the ability of the bacterial cell wall to retain crystal violet dye during decolorizing treatment with a decolorizing agent. The cell walls of gram-positive bacteria have a higher peptidoglycan and lower lipid content than gram-negative bacteria. The bacterial cell walls are initially stained with crystal violet. Iodine is added as a fixative to form a complex so that the dye cannot be easily removed. This step is commonly referred to as fixing the dye. Subsequent treatment with a decolorizer, a mixture of ethanol and acetone, dissolves the lipid layer from the gram-negative cells. The removal of the lipid layer allows the leaching of the crystal violet stain from the cells. When the counterstain of safranin is added the decolorized gram-negative bacteria stain pink. In contrast, the solvent dehydrates the thicker gram-positive cell walls, closing the pores as the cell wall shrinks. As a result, the violet-iodine complex is retained and the cells remain purple.

### II. TROUBLESHOOTING THE GRAM STAIN

#### 1. SMEAR PREPARATION

**problem:** If the smear is too thick, the cells can appear Gram-positive in very thick area. You may see Gram-variability from the thick to the thin areas.

**solutions:** Try to prepare a single cell layer of organism. Running positive and negative controls can help (pre-prepared slides with controls are available for purchase).

*problem:* If cells are prepared in hyper or hypotonic solutions, morphology may be disturbed.

*solutions:* Smear the cells onto the slide dry with a sterile toothpick.

*problem:* Over warming the smear (this happens most often when smears are warmed prior to being completely air dried, or when flaming too much to fix the slides) will cause all cells to appear Gram-negative.

*solutions:* Dry slide thoroughly prior to "heat fixing", be extremely careful when using flames.

*problem:* Old culture smears can cause cells to appear Gram-negative (weak walls).

*solutions:* Prepare smears only from fresh, log-phase growth (usually overnight culture).

*problem:* The medium from which the colonies are taken is important. Often, if you take colonies from liquid media or from selective media, cells will appear Gram-negative and more coccoid.

*solutions:* Take sample from fresh colonies from nutrient agar medium.

## 2. STAINING

*problem:* Over-decolorization: This is an extremely common problem, often caused by using a strong decolorizer or by leaving the decolorizer on the slide for too long.

*solutions:* Use decolorizer manufactured by the same company as your stains, and follow their protocol.

- Run positive and negative controls with your daily stains.
- Test your decolorizer solution using different times (e.g. 5, 10, 15 seconds) with positive, negative and some weakly positive cultures; until you find a method that works well for you.

*Problem:* Gram variability: This can be due to the organism itself, and not to the staining method.

*solutions:* The vast majority of Gram-variable organisms are Gram-positive. Characteristically Gram variable organisms (e.g. *Corynebacterium variabilis*) or those whose membranes alter with age and appear Gram-variable (e.g. *Arthrobacterium spp.*) are grouped with the Gram-positive organisms. Therefore, they are treated as Gram-positive organisms.

## III. GRAM STAINING OPTIONS

The conventional preparation for Gram's iodine is relatively unstable and may lose up to 60% of the available iodine in 30 days when stored at 25°C. Gram-positive organisms may stain poorly when 40% of the available iodine is lost. The iodine solution is stabilized by some manufactures by the addition of sodium bicarbonate to the solution (REMEL), or creating a stabilized iodine-polyvinylpyrrolidone iodine (BD Biosciences, DIFCO).

REMEL also offers "enhanced" gram staining reagents in which the concentrations of the reagents and the staining times are different. After the typical decolorizing step the slide is stained with GRAM ENHANCER solution to somewhat suppress red color in the background area, which may stain from rust or light violet to blue-green. This aids in the differentiation of gram-negative bacteria from the background.

Bacto TM 3-Step Gram Stain Sets by DIFCO are identical to the 4-step procedure described above, except the decolorizing and counterstaining steps have been combined into one reagent.

## IV. ADDITIONAL METHODS TO DISTINGUISH BETWEEN GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Gram stain results are not always a conclusive test to indicate the structure of the cell wall of bacteria. Certain gram-positive bacteria lose some of their cell wall properties with age or exposure to harsh or bacterial static/ bactericidal agents causing them to appear gram-negative or gram-variable. Also many *Bacillus* sp. and *Clostridium* sp. stain falsely gram-negative. Several additional tests can be performed to aid in the determination of gram reactivity, although none are foolproof.

1. *L-alanine-4-nitroanilide* (LANA) can distinguish aerobic and facultative anaerobes. The reagent is sold commercially impregnated in cotton swabs, which turn yellow when touched to the colony of a gram-negative bacterium.

2. *Potassium hydroxide* (KOH) test also detect gram-negative bacteria. A loop of growth from a colony is emulsified on the surface of a glass slide in a suspension of 3% KOH and stirred continuously for 60 seconds. Gram-negative cell walls break down and when the loop is gently pulled from the suspension it will be thick and stringy.

3. *Vancomycin* is an antimicrobial agent that acts on the wall of most gram-positive bacteria. Heavily inoculate the organism onto the surface of a sheep blood agar plate and place a 5-ug vancomycin disk on the inoculated area. Any zone of inhibition of growth after overnight incubation usually indicates a gram-positive bacterium. Many lactic acid bacteria are typically resistant to vancomycin. Some gram-negative organisms, especially *Moraxella* and *Acinetobacter*, may be sensitive to vancomycin. Conversely, most gram-negative bacteria are susceptible to polymyxin at 10ug/ml.
4. *Maconkey agar* is selective for the growth of gram-negative bacteria. Gram-positive organisms are inhibited by the crystal violet and bile salts.
5. *Columbia colistin-nalidixic acid agar (CNA)* is selective for the growth of gram-positive cocci.

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## CULTURE PURIFICATION

"ALTHOUGH A FEW BACTERIA ARE SO MORPHOLOGICALLY REMARKABLE AS TO MAKE THEM IDENTIFIABLE WITHOUT ISOLATION, PURE CULTURES ARE NEARLY ALWAYS A NECESSITY BEFORE ONE CAN ATTEMPT IDENTIFICATION OF AN ORGANISM. IT IS IMPORTANT TO REALIZE THAT THE SINGLE SELECTION OF A COLONY FROM A PLATE DOES NOT ASSURE PURITY."

*Bergey's Manual of Systemic Bacteriology, vol. I*

Testing pure cultures is essential to the success of the any microbial identification system including the Microlog System. Very often live but non-growing contaminants may be present in, or near a colony and can be sub-cultured along with the chosen organism. This is the reason that non-selective media are preferred for final isolation. A big difficulty occurs with bacteria that form extracellular slime; many contaminants often become firmly embedded or entrapped in this slime/mucus and are very difficult to separate. Mixed cultures that are processed as though they are pure most often produce poor results and cause much frustration and extra work. Using pure cultures when inoculating Biolog MicroPlates™ cannot be stressed enough. There are some key elements that can reduce the likelihood of using a mixed culture when processing a bacterial sample.

*How can I be sure that I have a pure culture and species?*

It has been said that a mistake in determination of shapes, Gram stain, colony morphology, pigment and many other characteristics of the species is the most frequent cause of mistaken identity of bacteria. There are three preliminary actions to follow in order to aid in the classification of bacteria.

- A: be sure that one colony has been selected when streaking on the appropriate media.
- B: perform a Gram stain.
- C: perform other tests required by the manufacturer in order to classify the organism in the corresponding category.

*Key #1: How do I proceed when cultures are not pure?*

- A. Be sure to select one colony and transfer it into 5 ml of sterile water or saline solution.
- B. Mix well, place a very small drop of the suspension onto BUG+B media and proceed to streak the plate to obtain isolated colonies.
- C. Incubate streaked plate to the appropriate temperature for the genus and species as indicated in Biolog MicroLog User Guide. If you are doing unknowns, you may be required to incubate the bacteria at many temperatures.
- D. After twenty-four hours of incubation check again for purity. **Never assume that the culture is pure.** Repeat the procedure as many times as necessary until a pure culture has been obtained before processing the bacteria. This may seem like more work than it is necessary but it will save time and materials if tests do not have to be repeated.

*Key #2: After you have a pure culture.*

Select colonies from the last (third or fourth) quadrants of the agar plate to inoculate the MicroPlate. This will minimize the chance of a contaminant being carried over in the final step of inoculation. There will be times when bacteria from the second quadrant must be used but this should be done only when necessary (slow growing organisms).

*Key #3: If you are attempting to identify environmental organisms, you may find several different species of bacteria in our initial sample.*

Many bacteria utilize the nutrients of other bacteria to grow and synergistically stick to each other. When your sample is initially grown on rich media, numerous types of bacteria grow even if your plate appears to be pure. This is where Key #1 is extremely important because isolating one colony and subculturing it three times will drastically increase the odds for a pure culture and a correct identification.

*Key #4: Check the plate for several different types of bacteria.*

Frequently one finds that it is necessary to leave cultures for more than two days at room temperature in order to see differences in morphology. Look for different colonies using the colony magnifier lamp that comes with the Microlog system. Look for colonies growing within the colony in question, different colors, sizes, colony edges, and textures.

*Key #5: Remember that bacteria are everywhere, including the bench top, doorknobs, hands, hair and air.*

To reduce contamination, all aspects of processing a specimen should be done using aseptic technique. Everything that touches the bacteria should be sterile. Every time the top of the petri dish containing the sample is opened, contamination is possible. Try not to talk when the agar plate is opened, as respiratory isolates are often contaminants. Using these keys will help to achieve a correct identification with the MicroLog System. While they may seem time-consuming and

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tedious, they greatly reduce the chance of having to repeat the test, which costs money as well as time. Bacteria isolated from the environment can sometimes grow slowly and may need to acclimate to the media for MicroLog identification. The optimal temperature for growing the organism also needs to be found. Once the organism is growing on the initial isolation media it needs to be subcultured for both purity and growth on the proper media for MicroLog identification.

Sometimes that first subculture will not grow the organism as expected. Things you can try:

- Try initial isolation on plain BUG or TSA media and take the second subculture to BUG with blood.
- If the organism grows after first subculture on BUG w/ blood, but the growth is poor, let it grow enough to make sure you have a pure culture. Subculture a single colony using a swab that has been dipped in sterile saline. This will produce a "lawn" of growth on your agar plate. You may need to subculture to more than one plate to get sufficient growth.  
NOTE: It will not be streaked for isolation.
- Try growing the organism at different temperatures to find optimum growth temperature. Try using 26°C, 30°C or 35-37°C. Remember that the optimum temperature used for growing the organism is the same temperature that should be used for incubating the MicroPlate.
- If the organism does not grow on BUG or BUG with blood it may be an organism that is not in the MicroLog database. Organisms that grow well on R2A such as *Methylobacterium* and *Rhizobium* are not in the current MicroLog database, but can be added as a User Database. These organisms will not usually grow on BUG agar.
- The other possibility is that the organism is fastidious. Organisms such as streptococci and some gram negatives require CO<sub>2</sub> for growth. Try sub-culturing these organisms on BUG w/ Blood and growing them at 35-37°C with 6.5% CO<sub>2</sub> atmosphere. If it's a Gram-negative organism and it still grows poorly on BUG with blood agar try growing it on chocolate agar and under the same fastidious conditions. Organisms that are in our GN-FAS group were all grown on chocolate agar when building our database. Multiple growth plates may be needed to obtain the appropriate inoculum density.

## WHERE CAN I FIND THE INFORMATION?

Many of the references we use at Biolog can be found directly through our website. We can be found at [www.biolog.com](http://www.biolog.com) and the links can be found by clicking on [Microbiology Links](#). There are also many reference books that can be useful in any microbiology laboratory. Here is a list of some:

1. *Bergey's Manual of Systematic Bacteriology*, 4 volumes, Noel R. Krieg & John H. Holt, 1984, Williams & Wilkins, Baltimore, MD.
2. *Bergey's Manual of Determinative Bacteriology*, Ninth Edition, J. Holt, N. Krieg, P. Sneath, J. Staley, S. Williams, 1994, Williams & Wilkins.
3. *Manual of Clinical Microbiology*, Seventh Edition, P. Murray, E.J. Barron, M. Pfaller, F. Tenover, R. Tenover, 1999, ASM Press, Washington D.C.
4. *Medical Tests for the Identification of Medical Bacteria*, Third Edition, Jean MacFaddin, 2000, Lippincott, Williams & Wilkins.
5. [www.dsmz.de/bactnom](http://www.dsmz.de/bactnom), this is a good site for current bacterial nomenclature. You can also link to this site through Biolog's web page.
6. [www.cbs.knaw.nl/htbin2/search\\_ydb.com](http://www.cbs.knaw.nl/htbin2/search_ydb.com), this is a good site for current yeast nomenclature and information. We hope you found our first newsletter useful and informative.

We would appreciate any feedback. If you have any suggestions or ideas for future topics please e-mail us at [tech@biolog.com](mailto:tech@biolog.com).

### BiOLOG

Biolog, Inc.  
3938 Trust Way  
Hayward, CA 94553  
Phone: (510) 785-2564  
Fax: (510) 782-4639  
[www.biolog.com](http://www.biolog.com)