**NEGATIVE STAIN**

The negative, or indirect stain, is a technique used to stain the background of a slide, leaving the bacterial cells unstained. The dark background provides the necessary contrast to make possible the visualization of the cells. Since the cells remain clear and the background stains a dark gray color, the appearance under the microscope is one of viewing bright stars against a night sky.

The stain used is congo red or nigrosin, which is an acidic stain containing negatively charged (anionic) chromophores. The negatively charged dye is repelled by the negative charge of the bacterial cell wall and therefore does not stain the cell.

Unlike the simple stain, this technique does not require heat-fixing, nor do cells take up any stain. Therefore, cells do not shrink or become distorted with aggressive heating and permeation of stain. This is particularly useful when accurate determination of cell size and shape is needed. It also allows for observation of spirochetes or spirilla that do not stain readily by other staining methods.

**Materials**

* Stock bacteria cultures*.*
* Congo red or nigrosin stain
* Inoculating loop
* Clean, blank microscope slides
* Permanent marking pens
* One bunsen burner and striker
* Immersion oil
* Compound microscope p
* Lens paper
* Lens cleaning solution

**Procedure**

1. Obtain a clean slide for each bacterium. Using your permanent marking pen, label one end of each slide with the initials of the bacterium you will smear on that slide. You will need to prepare one slide for each of the organisms in the materials list.
2. Place a small drop of nigrosin stain at one end of the slide.
3. Using aseptic technique, transfer a small amount of the culture to be stained into the drop of nigrosin and mix well within the small drop. DO NOT spread the drop or let it air dry.
4. Using the edge of a second slide held at a 30° angle, place the edge in front of the drop of stain/culture mix, pull slightly back, and then stop until the drop spreads across the edge of the second slide. Then push forward to the other side of the slide, dragging the drop along the entire surface of the bottom slide, producing a broad, even, thin smear.
5. Allow the smear to air dry completely. Do not heat-fix the cells.   
     
   The smear should appear gray with a feathered edge at the end. Too much dye will cause it to appear black in color and will make it difficult to visualize cell morphology when examining microscopically.
6. Examine the stained slides under the oil immersion objective. Remember, the nigrosin will outline your cells and stain the background. Your results should appear similar to Figure 3, which shows the results of a negative stain performed on a Bacillus bacterium.
7. Photograph your stain results through the occular lens or microscope with attached camera. Describe the cells according to their size, morphology, and arrangements.
8. When you complete this exercise, make sure that you clean up your work area and dispose of your slides in the sharps biohazard container.
9. When you have finished viewing your slides and recording your results, **you must also thoroughly blot the oil off of your 100X objective** with lens paper and clean off any oil that has contaminated other parts of your microscope, such as the 40X objective, the condenser lens, or the stage. Use lens cleaning solution if necessary.

**CAPSULE STAIN**

**ASEPTIC TECHNIQUE**

**Procedure**

1. Label the sterile tube.
2. Hold the loop in your dominant hand.
3. Flame the loop from tip to base.
4. Lift the plate lid slightly using it as a shield.
5. Touch the loop to the growth to be transferred and obtain a small amount.
6. Streal the plate accordingly.
7. Replace the lid.
8. Flame loop from tip to base.

**Quadrant Streak**

* Purpose: to dilute the sample and obtain isolated colonies in the 4th quadrant.
* Important in separating bacteria in a mixed culture.
* Isolated colonies contain one type of bacteria.

Steps

1. Label plate:
   1. Name
   2. Date
   3. Teacher assistant
   4. Class section
   5. Name or bacteria/sample
   6. Quadrants
2. Set up and turn on Bunsen burner.
3. Flame the inoculating loop from tip to base (flashes red) to sterilize.
4. Allow the inoculating loop to cool near the fire.
5. Pass the test tube opening through the flame back and forth.
6. Obtain sample of culture using the inoculating loop.
7. Flame tube before replacing the cap.
8. Open the lid of the plate slightly to avoid contamination and streak 1st quadrant with the inoculating loop (be careful not to damage or cut the agar surface—not much pressure is needed).
9. Replace the lid on the plate and flame the loop from tip to base and allow it to cool.
10. Streak the second quadrant starting in the first quadrant.
11. Repeat for quadrants 3 and 4.

**SIMPLE STAIN**

Simple stain is aka basic stain.

* + There is only a single-staining agent.

Auxochrome found in the staining agent is (+) charged.

* + Opposites attract ~ Attracted to (–) charged on the surface of most bacterial cells.

These stains will readily give up a hydroxide ion or accept a hydrogen ion, which leaves the stain positively charged. Since the surface of most bacterial cells is negatively charged, these positively charged stains adhere readily to the cell surface.

Basic dyes are frequently used.

* + Dyes with positive charges:
    - Ex: Methylene blue (we will use this)
    - Ex: Safranin
    - Ex: Crystal violet

**Steps**

1. Heat fix bacteria on slide.
2. Flood slide with basic stain (methylene blue).
3. Allow stain to sit for 60 seconds.
4. Rinse slide with DI water for 6 seconds.
5. Blot dry using bibulous paper and allow to air dry.
6. View under oil immersion.

**GRAM STAIN**

**Theory**

Microbiologists divide bacteria into two groups—Gram positive bacteria and Gram negative bacteria. This division is based upon how bacteria react when stained using the Gram stain and is due to differences in cell wall chemistry between the two groups. The Gram stain was developed by Hans Christian Gram in 1884 and is the most common differential stain used in microbiology.

Gram positive bacteria have a cell wall that is composed of between 60% to 90% peptidoglycan. Multiple layers of connected layers of peptidoglycan cause the cell wall to be approximately 20–80 nm thick. Negatively charged polyalcohols known as teichoic acids extend throughout the cell wall of these bacteria. Lipoteichoic acids, teichoic acids that are covalently linked to lipids, help anchor the peptidoglycan layer to the cytoplasmic membrane of the organism.

Conversely, Gram negative bacteria have a cell wall that contains only a few layers of peptidoglycan, is only 10–20% peptidoglycan, and does not contain teichoic acids. However, Gram negative bacteria have an outer membrane that is an asymmetrical bilayer membrane. The inner leaflet of this outer membrane is composed of phospholipids and lipoproteins which link the outer membrane to the peptidoglycan. The outer leaflet of the outer membrane is composed of lipopolysaccharide (LPS) which consists of sugars and a lipid known as lipid A. The lipid A portion of LPS is embedded in the outer membrane while the sugars extend outward from the surface. Therefore, while the peptidoglycan cell wall layer of Gram negative bacteria is thinner than that of Gram positive bacteria, the overall cell wall structure of Gram negatives is decidedly more complex. See Figure 1 for a depiction of the cell wall structures of Gram positive and Gram negative bacteria.

There are four basic steps in the Gram stain procedure. In the first step the heat-fixed smear is flooded with the primary stain crystal violet. This step imparts a purple color to all cells in the smear. The addition of the mordant, Gram’s iodine, is the second step. The iodine forms a water-insoluble complex with crystal violet (crystal violet-iodine complex; CV-I) inside all cells, so all cells remain purple during this step. Next, the cells are washed with the decolorizer 95% ethanol. The decolorizer dissolves the outer membrane in Gram negative organisms and enhances the removal of the CV-I complex through the cell wall.  Therefore, Gram negative organisms become colorless following decolorization. On the other hand, Gram positive bacteria contain a thick layer of peptidoglycan and no outer membrane. The addition of 95% ethanol dehydrates these cells and prevents the removal of the CV-I complex through the cell wall. Consequently, Gram positive organisms remain purple following decolorization. The final step in the Gram stain procedure is to counterstain the bacteria with safranin. The safranin stains the cells that are colorless, namely, the Gram negative organisms, but does not affect the color of the Gram positive cells. These procedures are summarized in Table 1 and Figure 2.

The majority of bacteria can be stained using the Gram stain procedure. However, there are times when this procedure is not universally applicable. Some bacterial species are easily over decolorized, meaning that they will appear Gram negative, when, in actuality, they are Gram positive. As was discussed in earlier exercises, some bacteria stain poorly at best and some do not take up stains at all.  Older bacterial cultures do not show typical Gram stain results, so it is best to perform the Gram stain on young, actively growing cultures. Finally, some species are Gram variable, where some cells are Gram positive and other cells from the same pure culture are Gram negative. One method of clarifying indistinct Gram stain reactions is to perform the 10% KOH test. In this test a bacterial colony is touched to a glass slide and a thick paste is made. One drop of 10% KOH is added to this paste. If the organisms are Gram negative, a mucoid string will result when the loop is pulled through the mixture. The KOH breaks down the cell wall of these bacteria and the DNA is released. It is this DNA that causes the strings to form. Conversely, Gram positive cells are not affected by this procedure.

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Gram Stain Reagent** | **Gram + Bacteria** | **Gram – Bacteria** |
| **Primary Stain** | Crystal Violet | Cells stain violet | Cells stain violet |
| **Mordant** | Gram’s Iodine | Crystal violet-iodine complex forms within cells; cells remain violet | Crystal violet-iodine complex forms within cells; cells remain violet |
| **Decolorizer** | 95% Ethanol | Cell walls dehydrate and shrink pores in peptidoglycan; permeability of cell wall decreases and the crystal violet-iodine complex cannot pass out; cells remain violet | Lipids extracted from outer membrane result in looser peptidoglycan structure; permeability of the cell wall increases and the crystal violet-iodine complex is washed from the cells; cells become colorless |
| **Counterstain** | Safranin | Cells are unaffected; cells remain violet | Cells stain red |

**Gram Negative**

Important Features

* Outer membrane
* Cell wall: thin peptidoglycan
* Periplasmic space
* Cell membrane

**Gram Positive**

Important Features

* Cell wall: **thick** peptidoglycan
* Cell membrane
* No outer membrane
* No periplasmic space

**Perform a Gram Stain**

**Materials**

Crystal Violoet           Glass Slides                 Staining Tray          Lens Paper

Gram's Iodine           Inoculating Loop           Forceps                 Lens Cleaner

Decolorizer               Distilled Water              Microscope

Safranin                   Bibulous Paper             Immersion Oil

**Step 1**

1. Take a sterilized loop with your bacteria, and place on a slide.
2. Air dry and heat fix.
3. Apply crystal violet for 1 minute.
4. Rinse with DI water.

**Step 2**

1. Apply Gram's Iodine for 1 minute.
2. Rinse with DI water.
3. Apply decolorizer for 10 seconds.
4. Rinse with DI water.

**Step 3**

1. Apply Safranin for 1 minute.
2. Rinse with DI water.
3. Blot with BILBULOUS paper.

**Step 4**

1. Clean microscope.
2. Scan on 40X for a variety of bacteria.

**Step 5**

1. View on oil.
2. Take a picture

**Crystal Violet-Iodine Complex**

In the gram staining procedure, Gram's Iodine acts as the mordant and forms a complex with the cyrstal violet dye.

Gram (+): Holds the Crystal Violet-Iodine complex (CV-I) during decolorization.

Gram (-): Does not hold the CV-I complex during decolorization.

**Gram Staining Errors**

*What if we forgot decolorizer?*

* Both gram positive and gram negative bacteria will appear purple due to gram negative bacteria not being decolorized.

*What if we forgot Iodine?*

* Both gram positive and gram negative bacteria will appear pink due to the crystal violet complex not forming and crystal violet being washed out.

*What if we excessively apply heat during fixation?*

* Heat fixing the cells, when done to excess, alters the cell morphology and makes the cells more easily decolorized.

*What if we excessively counterstain?*

* As the counterstain is also a basic dye, it is possible to replace the crystal violet-iodine complex in gram-positive cells with an over exposure to the counterstain.

*What if we excessively decolorized?*

* If ethanol is applied excessively, all bacteria in a sample will appear pink due to the counter stain Safranin.

|  |  |
| --- | --- |
| **Stain Type** | **Characteristics** |
| **Simple** | Uses a basic dye to apply color to a cell. This allows for the production of contrast between the cell and the transparent background. |
| **Differential** | Differentiates one group of microorganisms from another group of microorganisms. |
| **Gram Stain** | Allows for distinction and separation of gram negative and gram positive bacteria. |
| **Acid-fast Stain** | Allows for detection of Mycobacterium in a sample. |
| **Special Stains** | Used to stain specific structures on the inside or outside of the cells. |
| **Capsule** | Because of the mucoid type structure of the capsule, the background is stained so the capsule stands out. |
| **Endospore** | Stains endospores found commonly in genera Bacillus and Clostridium. |
| **Flagella** | Staining agent sticks to and coats thin flagella to be seen with light microscopy. |

**STANDARD PLATE COUNT**

*How is a single colony isolated from a mixed culture?*

One method of obtaining isolated pure colonies is the quadrant streak method. The quadrant steak is also a method of diluting an original bacteria sample concentration.  A limitation of the quadrant streak technique is that the original bacteria cell concentration and not be calculated.  The standard plate count method (also known as a viable count assay) uses the spread plate technique to obtain isolated colonies and determines the original cell density of the bacteria cell sample.  A limitation of the standard plate count method is that it only measures the number of viable (living) cells present.  Spectrophotometry would be the method to use to determine the total concentration of a sample that includes viable and dead cells.

**Standard Plate Count Termonology: Dilutions & Serial Dilutions**

The following is a list of terms used with the standard plate count method and the dilution formula used to calculate original cell density.

Colony Forming Unit (CFU)  A single cell or group of cells that forms a colony when grown on agar plates. One CFU is considered a pure culture of bactrium because it is assume that the CFU evolved from the replication of a single bacterium cell.

Countable Number  A range between 30 to 300 CFU is considered a statistically valid population to calculate original cell dentisty. A CFU count less then 30 or greater than 300 are not valid and the plate is considered not countable.

Dilution Lowering the concentration of a sample by adding a diluent to a sample.

Dilution Equation D1V1 = D2V2

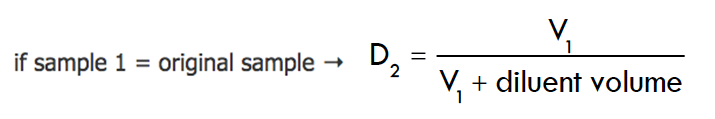
V1= transferred volume of sample 1

V2 = total volume of sample 2 = V1 + diluent volume

D1 = dilution of sample 1

        if sample 1 = original sample → D1= 1

D2 = dilution of sample 2



The equation can be written as: Original Cell Density= (Number of Colony Forming Units)/ (Volume Plated+ Dilution)

                                                               OCD=CFU/(VxD)

Original Cell Density The number of bacteria cells found in a initial sample measured as a ratio of colony formiing units per milliliter. The units are represneted as  CFU/mL = Original Concentration

Plating Factor The volume of sample taken from a serial dilution tube and plated on an agar plate. A plating factor less then one millitier is calculated as an additional dilution.

Serial Dilution  A series of dilutions performed with the standard plate count method.  The serial dilution technique is used when:

* Sample concentration is too high to be diluted in one step.
* Sample concentration is unknown and you want a range of different concentrations to work with (one of them should work).
* You are trying to show a relationship between sample concentration and variable "x" and want a range of different concentrations to demonstrate this relationship (ex: as concentration increases, variable "x" increases too).

|  |
| --- |
|  |

Simple Dilutions

1:1 dilution = ½ = 2-1 = "2-Fold"

* 1 part sample + 1 part diluent = 1 part sample/2 parts total
* Example: 500 μL sample + 500 μL diluent = 500 μL sample/1000 μL total

1:9 = 1/10 = 10-1 = "10-Fold"

* 1 part sample + 9 part diluent = 1 part sample/10 parts total
* Example: 100 μL sample + 900 μL diluent = 100 μL sample/1000 μL total