Part 1: Performing the Gram Stain

Theory:

One of the most frequently used differential staining techniques is the **Gram stain**. Like other **differential stains**, it is used to detect the presence of a difference between two different organisms — for the Gram stain, the difference detected is the cell wall's structural type. In addition to the cell wall type, the Gram stain can also allow for determination of a bacteria's morphology by allowing a visual assessment of a cell's shape, size, and arrangement. A structural stain, such as the Capsule stain, Endospore stain, and Flagella stain are primarily looking for those specific structures after which they are named.

The Gram stain has many subtile variations, but the basic sequence of steps stays the same: First, a **primary stain** is added, which colorizes all cells on the slide. The primary stain in the Gram stain is crystal violet. Iodine is added, which, acting as a **mordant**, adds more covalent bonds between the Iodine and the peptidoglycan layer of the Gram-positive cell wall to enhance the crystal violet's hold just before the decolorization step. The primary stain is then **decolorized** (i.e., removed) from the Gram-negative cell wall with the use of an acid alcohol, ethanol or another similar chemical. The alcohol and acetone in the decolorization step extracts lipids resulting in a Gram-negative wall that is more porous and now incapable of retaining the crystal violet-iodine complex. The thicker peptidoglycan layer and greater degree of cross-linking (due to the teichoic and lipoteichoic acids) of the Gram-positive cell wall can trap the crystal-violet-iodine complex more effectively. The now colorless Gram-negative cells are colored a reddish color with Safranin — this second color is the **counterstain**. The bluish Gram-positive cells also pick up the red Safranin, resulting in a more purple appearance.

Lab Materials:

Amount per Student	Material
1	Microscope (Stored in cabinet in the lab. Okay to share with 1 lab partner. Return it when done.)
as needed	Blank Slides and Coverslips (at your station, or nearby. Discard in the glass waste receptacle when done.)
as needed	Immersion Oil
1	mixed bacterial sample (with Gram positive & Gram negative organisms)
1	wooden clothespin
shared (at the sinks)	Crystal Violet dye (aka Gram's Crystal Violet)
shared (at the sinks)	Iodine (aka Gram's Iodine)
shared (at the sinks)	95% ethanol or Acid Alcohol solution
shared (at the sinks)	Bottle of distilled water
shared (at the sinks)	Bilbous Paper (located near the sinks and used for drying slides)
1 (at your station)	Bunsen Burner
1 (at your station)	Inoculation Loop

Gram Stain procedure

(Memorize this procedure! *Troubleshooting tips are in italics.* Key parts are in **bold typeface**.):

1. If starting with a **non-broth source of bacteria**, place a small drop of distilled water on a clean slide before or after adding and mixing the emulsion with an inoculation loop. *Spread the bacterial emulsion out as it is mixed in order to "thin" the sample out.* Go to step 3. (Start here if you are using a toothpick sample as in the demo.)
2. If starting with a **broth source of bacteria**, then just aseptically add the liquid bacterial sample to the slide with an inoculation loop. *Spread the sample out to thin it and enhance air drying.* Go to step 3.
3. Using a wooden clothespin, hold the slide at one end and pass it through the upper part of a bunsen burner flame to **heat-fix** the sample by slowly evaporating the rest of the moisture from the slide. *Note that this step can take "minutes", so don't rush it! Only keep the slide in the open flame for a few seconds at a time to avoid overheating or burning. 100% water evaporation is ideal but not necessary — however, keep in mind that any area which is not heat-fixed will be washed away and lost in the subsequent rinse steps. Once heat-fixed, there is no rush to get to the staining steps. In fact, you can heat-fix more than one slide then take them over to the staining racks and Gram stain them as a group to save time.*
4. Place the slide on the rack over the sink and flood the smear with **Crystal Violet** (the "**primary stain**"), then let it sit for **1 minute**.
5. Hold the slide with the clothespin and **rinse the slide with distilled water**.
6. Place the slide on the rack over the sink and flood the smear with **Iodine** (the "**mordant**"), then let it sit for **1 minute**.
7. Hold the slide with the clothespin and **rinse the slide with distilled water**.
8. Still holding the slide with the clothespin, **decolorize** the slide with **95% ethanol** or the **acid alcohol solution** till the run-off becomes "**nearly clear**". This step usually takes about **5 to 10 seconds** to accomplish. *Do NOT overdo this step—it is possible to decolorize all the Crystal Violet out!*
9. Keep holding the slide with the clothespin and **rinse the slide with distilled water**.
10. Place the slide on the rack over the sink and flood the smear with **Safranin** (the "**counterstain**"), then let it sit for **1 to 2 minutes** (longer is better).
11. Hold the slide with the clothespin and **rinse the slide with distilled water**.
12. Gently **blot to a "partly dry" state** with bibulous paper or paper towels. *Do not rub the slide. A little moisture left on the slide will help the cover slip to stick better.*
13. Apply a clean **cover slip** and observe with a microscope under **oil immersion** (1000 X) & interpret your findings.

**** The times are just estimates. There are plenty of slides that turn out just fine despite all sorts of mistakes. Most of the more critical mistakes are missing a step, getting the steps out of order, or difficulty with the decolorization step (Acid Alcohol).*

Part 2: Interpreting the Gram Stain

Theory:

Bacterial cell shape and arrangement is not dependent on the result on the Gram stain, but should be interpreted anyway. **Gram-positive cells will be a blue-purple color** and **Gram-negative cells will be a pink-red color**. However, problems can occur during the Gram stain to interfere with the interpretation. Assuming the staining sequence was followed correctly, the decolorization step is typically the cause of most problems when learning the Gram stain. If under-decolorized, then parts of the slide may have Gram-negative cells which still hold onto the crystal violet. Typically, these slides have a variable type of quality to them with some portions partially decolorized and others not at all decolorized. Decolorizing too long, or waiting too long to rinse the decolorizing solution off can result in the loss of all the crystal violet from the Gram-positive cells as well. Old and poorly filtered crystal violet stain can have crystals precipitate that can be mistaken for bacteria or hamper interpretation overall. Also, the older the Gram-positive bacteria is, the harder it is for it to resist the decolorization solutions — thus, they can appear artificially Gram-negative. For this reason, fresh cultures (24 hours or younger) are best when working with an unknown.

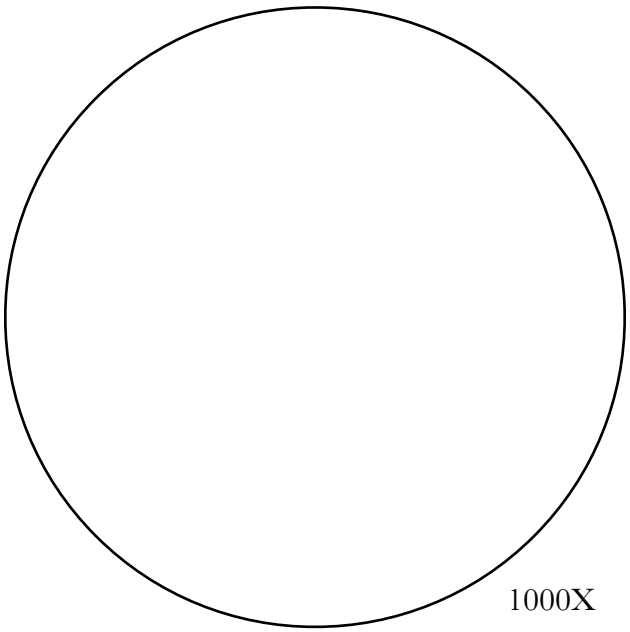
Lab 7 Questions (Due at the end of lab)

Name: _____ **Grade:** _____ **of 10 points**

1. (8 points) Predict what a Gram-positive cell and a Gram-negative cell would look like if the following "mistakes" were made. **Assume the rest of the Gram stain procedure was done correctly after the mistake.** Fill in the chart with the color due to the mistake.

"Mistake"	Color of the G+ cell due to the mistake	Color of the G– cell due to the mistake
Failure to add the Mordant (Iodine).	Color of Gram-Positive Cell: _____ Tip: This mistake keeps enough Crystal Violet from sticking to all the cell walls.	Color of Gram-Negative Cell: _____ Tip: This mistake keeps enough Crystal Violet from sticking to all the cell walls.
Forgot to decolorize with 95% Ethanol or Acid Alcohol.	Color of Gram-Positive Cell: _____ Tip: This mistake means the Crystal Violet is never removed from the all the cell walls.	Color of Gram-Negative Cell: _____ Tip: This mistake means the Crystal Violet is never removed from the all the cell walls.
Decolorized with 95% Ethanol or Acid Alcohol too long.	Color of Gram-Positive Cell: _____ Tip: This mistake removes all the Crystal Violet from every location.	Color of Gram-Negative Cell: _____ Tip: This mistake removes all the Crystal Violet from every location.
Failure to apply the counterstain (Safranin).	Color of Gram-Positive Cell: _____ Tip: This mistake means you just don't add any of the red Safranin color at the end of the gram stain steps.	Color of Gram-Negative Cell: _____ Tip: This mistake means you just don't add any of the red Safranin color at the end of the gram stain steps.

2. (2 points) **Draw** a simple sketch of just some of the **bacterial cells** your best Gram stain (if you did more than one) at oil immersion (1000X) and **label** any bacterial cells as Gram-positive or Gram-negative. Also, **describe** the ***cell morphology shapes*** *of what you see (coccus, bacillus, etc…). Also,* ***draw*** *any human cells and comment on their Gram stain result.* The field diameter of the following 1000X view is **180 µm**. Provide some measurements in micrometers (µm) for the bacterial and human cells in your drawing.



1000X