

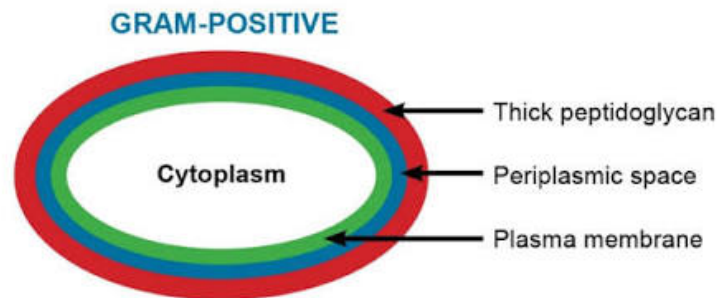
Lab 3: Gram Stains

Components

- Aseptic Technique
- Bacterial Smears
- Crystal Violet (Primary Stain)
- Sodium Bicarbonate, NaCO_3^- (Stain Facilitator)
- Gram's Iodine (Mordant)
- Acetone (Decolorizer)
- Sargranin (Secondary Stain)

Lab Concepts

❖ Gram Positive



- Cell walls are very thick, soaking up more stain
- Purple stain rinses out much slower
- Still purple after decolorization
- Includes *staphylococcus* and *streptococcus*

1 *Staphylococcus aureus*

2 *Staphylococcus saprophyticus*

3 *Staphylococcus epidermidis*

4 *Streptococcus pyogenes*

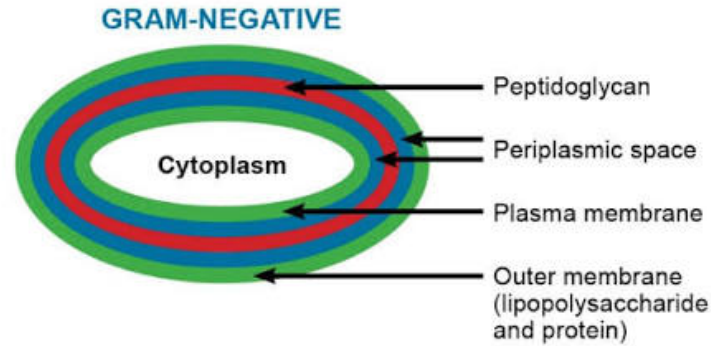
5 *Streptococcus agalactiae*

6 *Streptococcus salivarius*

- Vulnerable to too much decolorizing

❖ Gram Negative

Lab 3: Gram Stains



- Cell walls are very thin
- Crystal violet stain rinses out faster
- After decolorization, Gram negative cells appear clear and free to soak up the pinkish/reddish safranin
- Includes the seven enterics (*Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter cloacae*, *Citrobacter freundii*, *Proteus mirabilis*, *Salmonella enterica*, *Shigella flexneri*) and the three As: *Aeromonas caviae*, *Acinetobacter baumannii*, *Alcaligenes faecalis*
- Vulnerable to thick smears
- Vulnerable to too little decolorizing

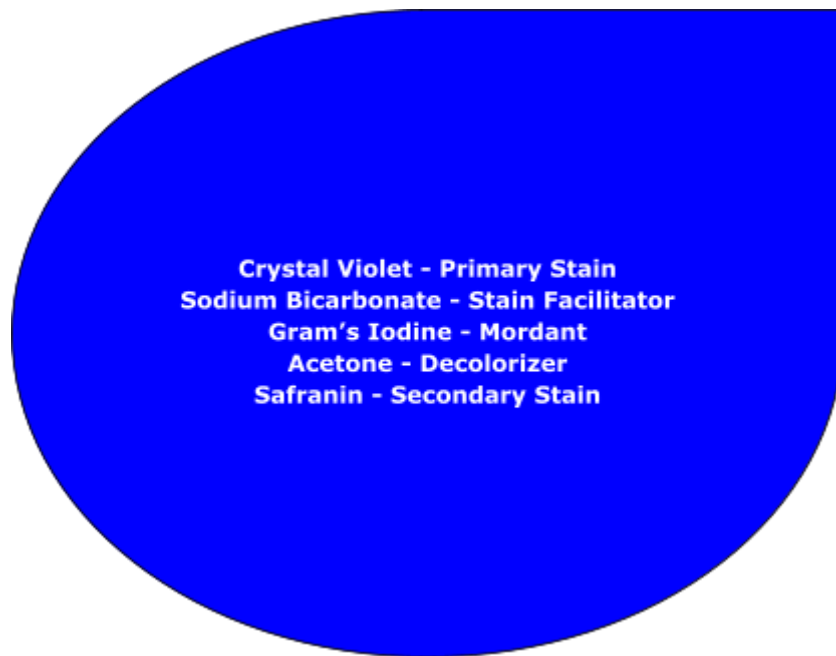
Steps

Preparing the Stains

1. Wash the slide with water and detergent until it's squeaky clean; dry with a paper towel but try to avoid getting lint on the slide
2. Place a small droplet on the clean slide
3. Use aseptic technique to pick up some of the sample and create smears; Gram negative, Gram positive, and Gram negative orientation
 - a. Take care not to get stain too close to the edge of the slide
 - b. Try to avoid having too many cells—this interferes with observing the morphology
 - c. Focus on a thick part, but navigate to a thin spot to examine morphology
 - d. Stirring too much will destroy the cellular arrangement
4. Dry and heat fix (kill, stick, stain)
5. Drop crystal violet stain (**primary stain**) on each stain and allow to sit for at least 10 seconds
6. Drop sodium bicarbonate (NaCO_3^-), a **stain facilitator** (acts as an accelerator/intensifier), into the crystal violet and wiggle the slide to mix the sodium bicarbonate and crystal violet
 - a. Sodium bicarbonate changes the surface of the cell and allows it to soak up crystal violet

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- b. Sodium bicarbonate is an alkaline (basic) chemical; adding it raises the pH of the dye solution, which increases the negative charge on the bacterial cell wall
 - i. Draws in more of the positively charged crystal violet dye
 - c. Makes the primary stain significantly darker and more vibrant, making it easier to see under a microscope
 - d. Reduces the time needed for the crystal violet to bond with the bacterial cells
7. Dump the stain—do not rinse
 8. Tilt the slide and add Gram's iodine to rinse the slide
 9. Add more Gram's iodine (a **mordant**) on a flat slide and let it sit 15 to 20 seconds
 - a. **Mordant** - a chemical substance used to bind, set, or intensify a stain so it adheres permanently to a biological specimen
 - i. Increases the margin of time it takes to stain a specimen
 10. Dump Gram's iodine and rinse the slide thoroughly with water
 11. Tilt the slide again and add watered-down acetone (the **decolorizer**) to rinse the slide for just 1 to 2 seconds, just until the runoff is no longer purple
 12. Shake off the excess acetone
 13. Let the slide dry
 14. Add the safranin, the **secondary stain** (red) by adding 1 to 2 drops per smear
 15. Allow the safranin to sit, 15 to 20 seconds
 16. Dump the safranin, rinse the slide well, and dry by blotting with the bibulous paper a few times (until dry)



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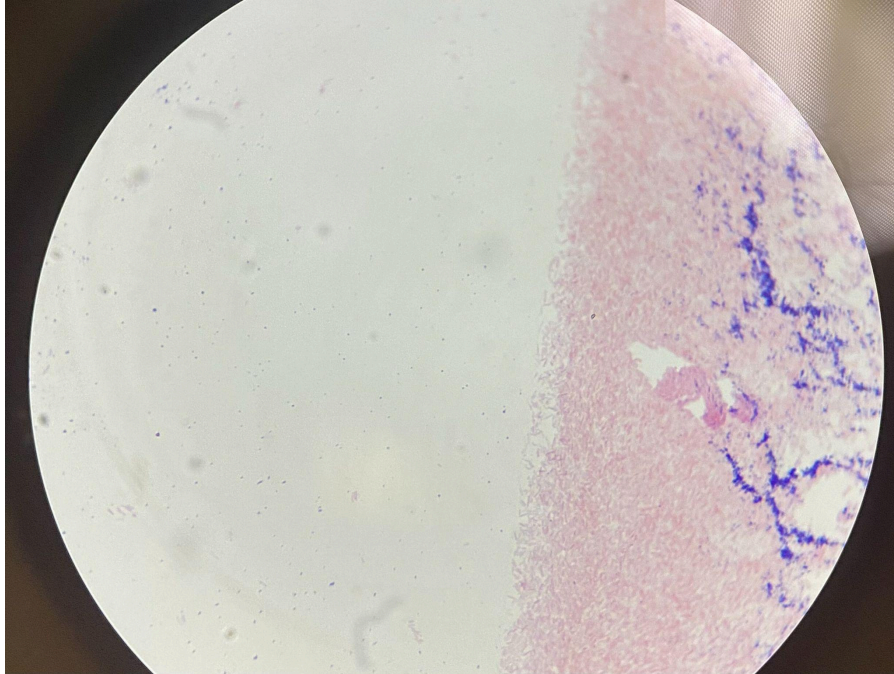
Microscopy

17. Load the slide into the microscope
18. Use the yellow (10 power) lens and coarse adjustment knob until the space between the lens and slide is about the diameter of a pencil; you may need to adjust the light to a dimmer setting
19. Use the fine focus knob to bring the sample into focus
20. Switch to the red lens, and add two to three drops of oil, enough to form a dome of oil over the sample
21. Switch from red to the oil immersion lens (black and white); you may need to dim the light
 - a. Avoid using the blue lens at all; not compatible with oil
 - b. Switch between red, yellow, and the oil immersion lens
22. Use the stage adjusting knobs to move the slide until you see something to focus on
23. Use the fine adjustment knobs to focus the lens
24. Observe the first sample
25. Switch to the red lens
26. Adjust the slide to orient the second sample under the lens
27. Add oil to the second sample, and switch back to the oil immersion lens
28. Repeat steps 21 and 23
29. Repeat steps 19 through 27 for the third sample
30. Dispose of the slide in the bucket under the fumigation hood
31. Wipe down the microscope stage with a paper towel
32. Use the microscope lens wipe on: yellow, ocular pieces, and then the oil immersion lens
 - a. Must complete in this order to prevent spreading oil to clean lenses
33. Lower the stage
34. Unplug the microscope, and wrap the power cord around the storage piece
35. Place the microscope back in the cabinet

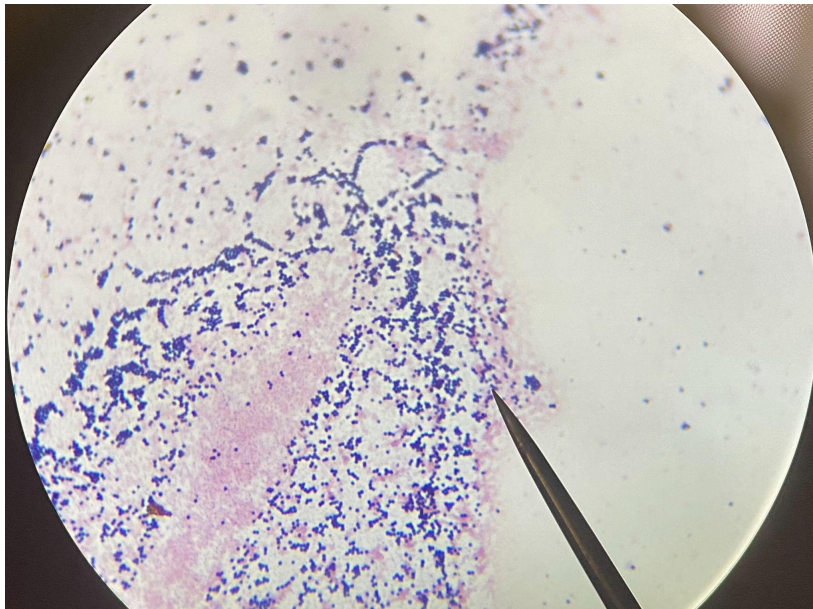
Observations

1. *Seromonas caviae* (17)

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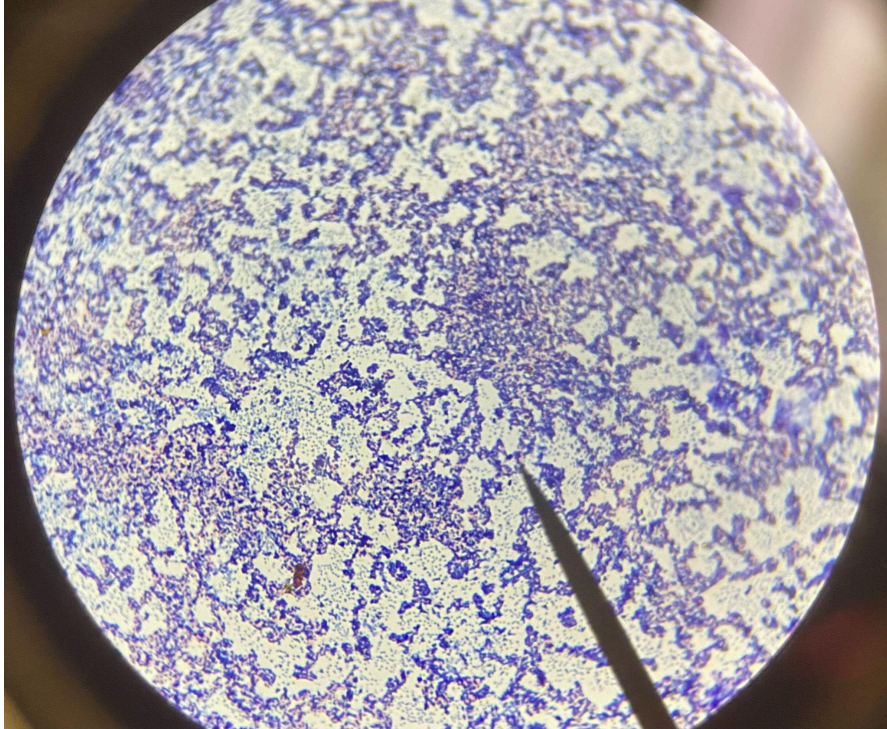


- a. Gram negative oxidase fermenting rods
 - b. Appears pink
 - c. Very small cells—difficult to focus on
- Note: first sample had very few cells due to either heat fixing a wet sample or failing to cool loop before inoculating*
2. Staphylococcus aureus (1)



- a. Gram positive cocci
 - b. Appears violet
3. Acinobacter baumannii (18)

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a. Gram negative non-fermenting rods

b. Appears pink

Note: not effectively decolorized by the acetone and appears violet and pink in observations