Gram Stain Technique

Materials Required:

1. Clean glass slides
2. Inoculating loop
3. Bunsen burner
4. Bibulous paper
5. Microscope
6. Lens paper and lens cleaner
7. Immersion oil
8. Distilled water
9. 18 to 24 hour cultures of organisms

Reagents:

1. Primary Stain - Crystal Violet
2. Mordant - Grams Iodine
3. Decolourizer - Ethyl Alcohol
4. Secondary Stain - Safranin

Procedure:

Part 1: Preparation of the glass microscopic slide

Grease or oil free slides are essential for the preparation of microbial smears. Grease or oil from the fingers on the slides is removed by washing the slides with soap and water. Wipe the slides with spirit or alcohol. After cleaning, dry the slides and place them on laboratory towels until ready for use.

Part 2: Labeling of the slides

Drawing a circle on the underside of the slide using a glassware-marking pen may be helpful to clearly designate the area in which you will prepare the smear. You may also label the slide with the initials of the name of the organism on the edge of the slide. Care should be taken that the label should not be in contact with the staining reagents.

Part 3: Preparation of the smear

- **Bacterial suspensions in broth:** With a sterile cooled loop, place a loopful of the broth culture on the slide. Spread by means of circular motion of the inoculating loop to about one centimeter in diameter. Excessive spreading may result in disruption of cellular arrangement. A satisfactory smear will allow examination of the typical cellular arrangement and isolated cells.
- **Bacterial plate cultures:** With a sterile cooled loop, place a drop of sterile water or saline solution on the slide. Sterilize and cool the loop again and pick up a very small sample of a bacterial colony and gently stir into the drop of water/saline on the slide to create an emulsion.
- **Swab Samples:** Roll the swab over the cleaned surface of a glass slide.

Please note: It is very important to prevent preparing thick, dense smears which contain an excess of the bacterial sample. A very thick smear diminishes the amount of light that can pass through, thus making it difficult to visualize the morphology of single cells. Smears typically require only a small amount of bacterial culture. An effective smear appears as a thin whitish layer or film after heat-fixing.

Part 4: Heat Fixing

Heat fixing kills the bacteria in the smear, firmly adheres the smear to the slide, and allows the sample to more readily take up stains.

- Allow the smear to air dry.
- After the smear has air-dried, hold the slide at one end and pass the entire slide through the flame of a Bunsen burner two to three times with the smear-side up.

Now the smear is ready to be stained.

Please Note: Take care to prevent overheating the slide because proteins in the specimen can coagulate causing cellular morphology to appear distorted.

Part 5: Gram Stain Procedure

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
7. Immediately rinse with water.
8. Gently flood with safranin to counter-stain and let stand for 45 seconds.
9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
10. Blot dry the slide with bibulous paper.
11. View the smear using a light-microscope under oil-immersion.

Differences Encountered in a Real laboratory:

In an actual laboratory setting, there are certain important steps that are not necessarily applicable in a virtual lab:

1. Always wear gloves, and lab coat.
2. Tie your hair properly to prevent any contamination from the culture you are working with.
3. After entering the lab, make sure that the microscope is working properly. Oculars and objective lenses should be cleaned before and after each use with lens paper.
4. Adjust the illumination before using the microscope.
5. Prepare your work space (Laminar Air Flow Cabinet) or lab bench by wiping down the area with disinfectant.
6. Properly adjust the flame of the Bunsen burner. The proper flame is a small blue cone; it is not a large plume, nor is it orange.
7. Wipe the glass slide with spirit and wave the slide over the Bunsen burner to remove any unwanted microorganisms in the slide.
8. Label one side of the glass slide with
    1. Your initials
    2. The date
9. While flaming the inoculation loop be sure that each segment of metal glows orange/red-hot before you move the next segment into the flame.
10. Once you have flamed your loop, do not lay it down, blow on it, touch it with your fingers, or touch it to any surface other than your inoculums. If you do touch the tip to another surface or blow on it, you will have to re-flame the loop before you proceed to your experiment.
11. Allow your loop to cool before you try to pick up your organism. If you pick up organism with a hot inoculation loop, your cells will be killed and will affect your results.
12. When removing the caps from tubes, always keep the caps in your hand. Never set them on the table, as they could pick up contaminants.
13. Always handle open tubes at an angle near to the flame of the burner; never let them point directly up, since airborne or other environmental organisms could fall into the tube and cause contamination.
14. As soon as you transfer the organism into the slide, flame your loop. Never place a contaminated tool on your workbench.
15. Try to prepare a single cell layer of organism (a thin smear). Otherwise the all cells will appear as gram positive in thick area.
16. Do not overwarm the cells. Dry the slide thoroughly prior to heat fixing.
17. Use young, vigorous cultures rather than older cultures for your experiment.
18. Decolorisation step should not exceed the time limit.
19. While washing the slide after staining, do not let the water stream fall directly on the smear. This may disrupt the smear. Let the stream of water flow slowly along the surface, such that only the stain is flooded and the smear is intact.
20. Always prefer to observe under 10X first. This will give you an idea of the location of a good area for observation. After this you may prefer to switch over to 40X.
21. Do not ever observe at a specimen at 100X without oil.
22. While focusing the microscope, glass slides should be handled carefully to avoid the chance of chipping or breaking.
23. After the observation, wipe the microscopic lens with an absorbent paper and cover the microscope properly.
24. Discard all contaminated materials properly and return your supplies to the proper storage locations, and clean up your working area.
25. Always disinfect your work area when you are finished.
26. Ensure proper hand washing before you leave from the laboratory.

Typical Gram-negative bacteria:

1. ***Bordetella pertusis***, the causative agent of whooping cough
2. ***Salmonella typhi***, the causative agent of typhoid
3. ***Vibrio cholera***, the causative agent of cholera
4. ***Escherichia coli***, the normally benign, ubiquitous, gut-dwelling bacteria

Typical Gram-positive bacteria:

1. *Staphylococci* such as ***Staphylococcus epidermidis*** and ***Staphylococcus aureus*** which is a common cause of boils.
2. *Streptococci* such as the many species of oral streptococci, ***Streptococcus pyogenes*** which causes many a sore throat and scarlet fever and ***Streptococcus pneumoniae*** which causes lobar pneumonia.
3. *Clostridia* such as ***Clostridium tetani***, the causative agent of tetanus (lockjaw).
4. *Actinomyces* such as ***Actinomyces odontolyticus*** which is found in mouth.
5. Species of the genus Bacillus such as ***Bacillus subtilis*** which are common microbes living in soil.

Generally cocci are Gram-positive but there are exceptions. The most significant from a clinical point of view is the gonococcus, ***Neisseria gonorrhoea*** which typically appears as a Gram-negative diplococcus looking very much like a pair of kidney bean.