Microbiology Laboratory

Lab 6: Simple Staining- Principle, Procedure and Result Interpretation

Simple staining is one of the conventional staining techniques. From the name, it is quite clear that it is a very simple or **direct staining method** that uses a **single stain** only. The microorganisms are invisible to the naked eye, and to make them visible, staining is performed that gives **divergence** to a microscopic image. Direct staining makes the use of basic dyes like methylene blue, safranin, crystal violet, malachite green etc. called "**simple** or **direct stains**".

The simple stain can be used as a quick and easy way to determine the cell shape, size, and arrangement of bacteria. True to its name, the simple stain is a very simple staining procedure involving a single stain solution. Any basic dye, such as methylene blue, safranin, or crystal violet, can be used to color the bacterial cells. These stains will readily give up a hydroxide ion or accept a hydrogen ion, which leaves the stain positively charged. Since most bacterial cells and cytoplasm surface is negatively charged, these positively charged stains adhere readily to the cell surface. After staining, **bacterial cell morphology** (shape and arrangement) can be appreciated.

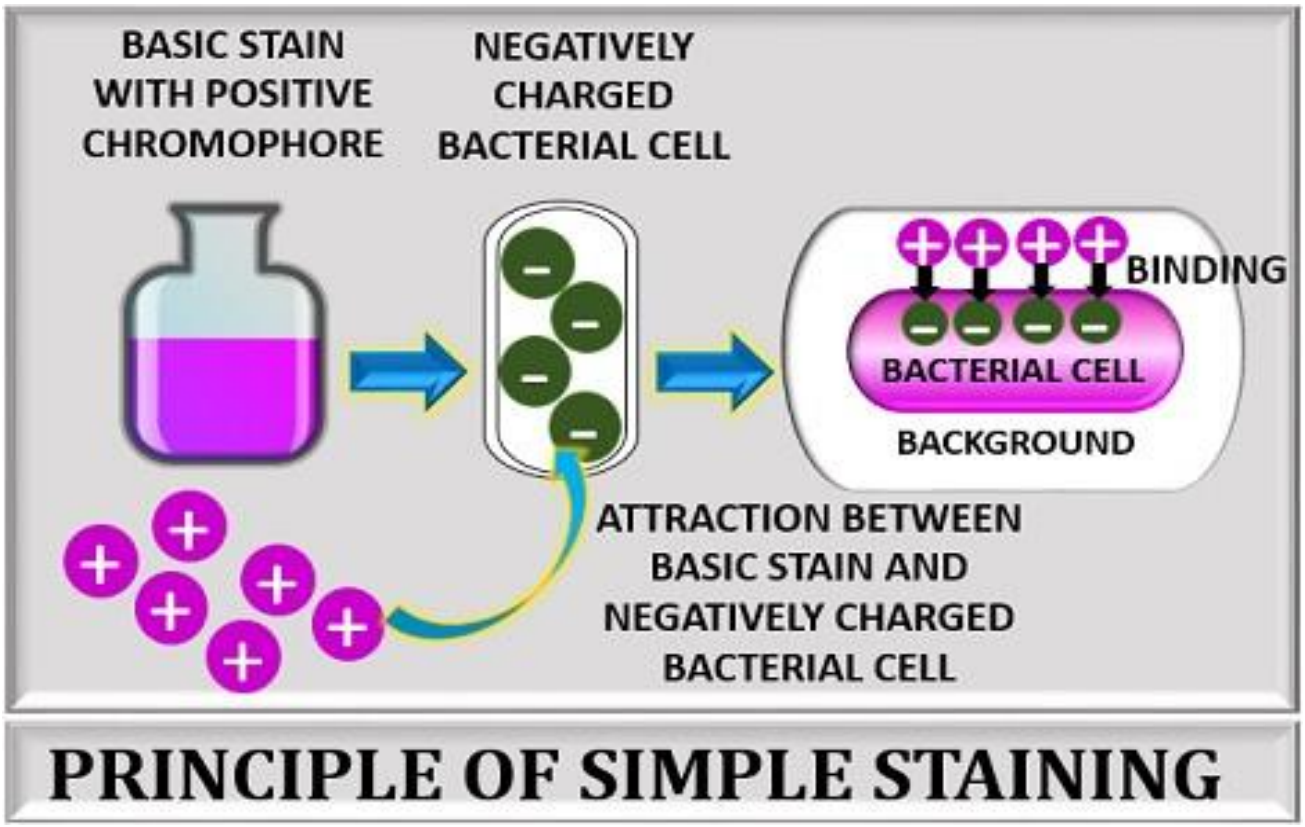
Simple staining is defined as one of the ordinaries yet the popular method used to elucidate the bacterial size, shape and arrangement to differentiate the various bacteria groups. It stains the bacterial cell uniformly **and thus increases the visibility of an organism. The term simple staining sometimes interchangeable with the terms like direct, positive or monochrome staining. Also,** Simple stains can be defined as the **basic dyes**, which are the alcoholic or aqueous solutions (diluted up to 1-2%). These can easily release **OH^-** and accepts **H^+** ion, due to which the simple stains are positively charged. As the simple stains are **positively charged,** they usually termed as positive or cationic dyes. It is commonly used to color most bacteria. As the simple stain carry a positive charge, it firmly adheres to a negative bacterial cell and makes the organism colored by leaving a background colorless. **Examples** of simple stains include safranin, methylene blue, crystal violet etc.

The basic stains have different **exposure times** to penetrate and stain the bacterial cell.

Basic stains	**Exposure time to stain the bacteria**
Methylene blue	1-2 minutes
Crystal violet	20-60 seconds
Carbol fuschin	15-30 seconds
Safranin	30-60 seconds

Principle of Simple staining

In simple staining, the bacterial smear is stained with a single reagent, which produces a distinctive contrast between the organism and its background. Its principle is based on producing a **marked contrast** between the **organism** and its surroundings by using basic stain. A basic dye consists of a positive chromophore, which strongly attracts the negative cell components and charged molecules like nucleic acids and proteins, Basic stains with a positively charged chromogen are preferred because bacterial nucleic acids and certain cell wall components carry a negative charge that strongly attracts and binds to the cationic chromogen Thus, a simple staining technique result in a colored bacterial cell against a colorless background**.** The purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. The most commonly used basic stains are methylene blue, crystal violet, and carbol fuchsin



Reagents and Equipment's for Simple Staining

Methylene blue, crystal violet, and carbon fuchsin, Micro incinerator or Bunsen burner, inoculating loop, staining tray, microscope, lens paper, bibulous (highly absorbent) paper, and glass slides.

Procedure of Simple Staining

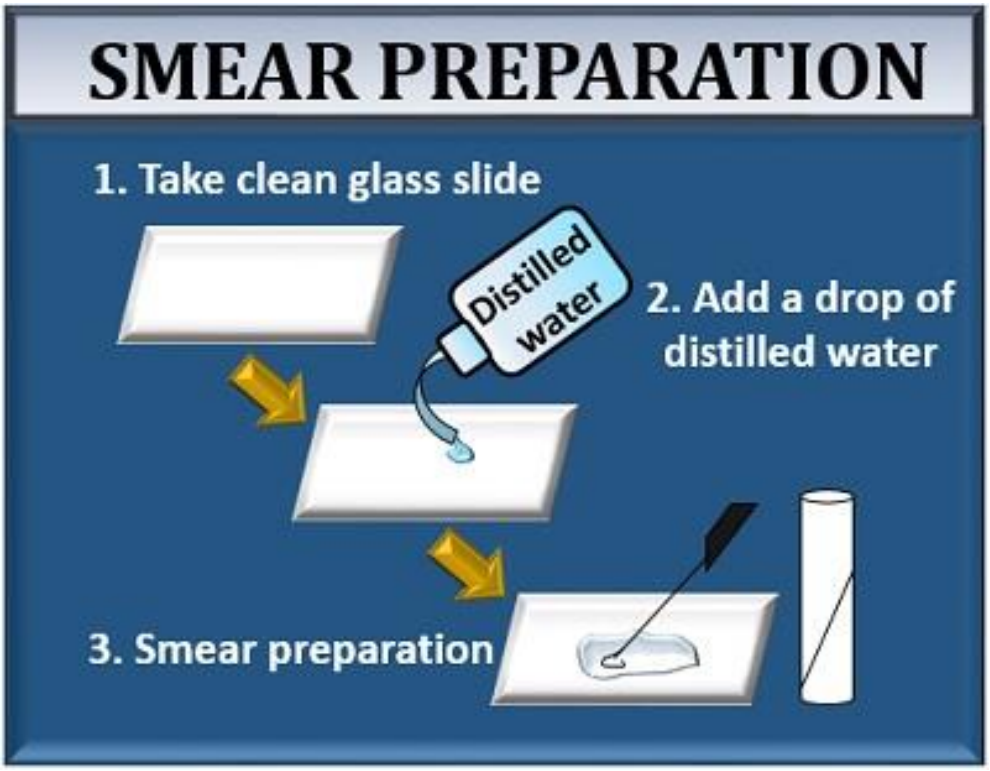
It involves the following three steps:

1. Smear preparation
2. Heat fixing
3. Staining

Smear Preparation

A bacterial smear appears as a thin film of bacterial culture. For the smear preparation, we need to perform the following steps:

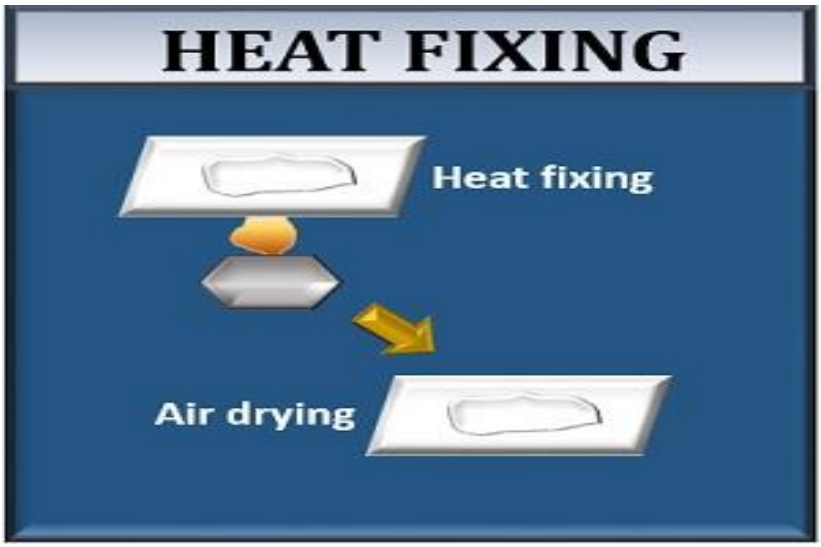
1. Take a clean, grease-free glass slide.
2. Add a drop of distilled water to the center of the glass slide.
3. Then, add inoculum from the bacterial culture with a sterilized inoculating loop on the glass slide.
4. After that, mix the inoculum with a drop of distilled water to make a thin film by uniformly rotating the inoculating loop until a thin bacterial film is formed.



Heat Fixing

After smear preparation, move the prepared slides over the Bunsen burners flame at least three times. Then, allow the slide to air dry. There are many reasons to perform heat fixing, and it cannot be skipped because:

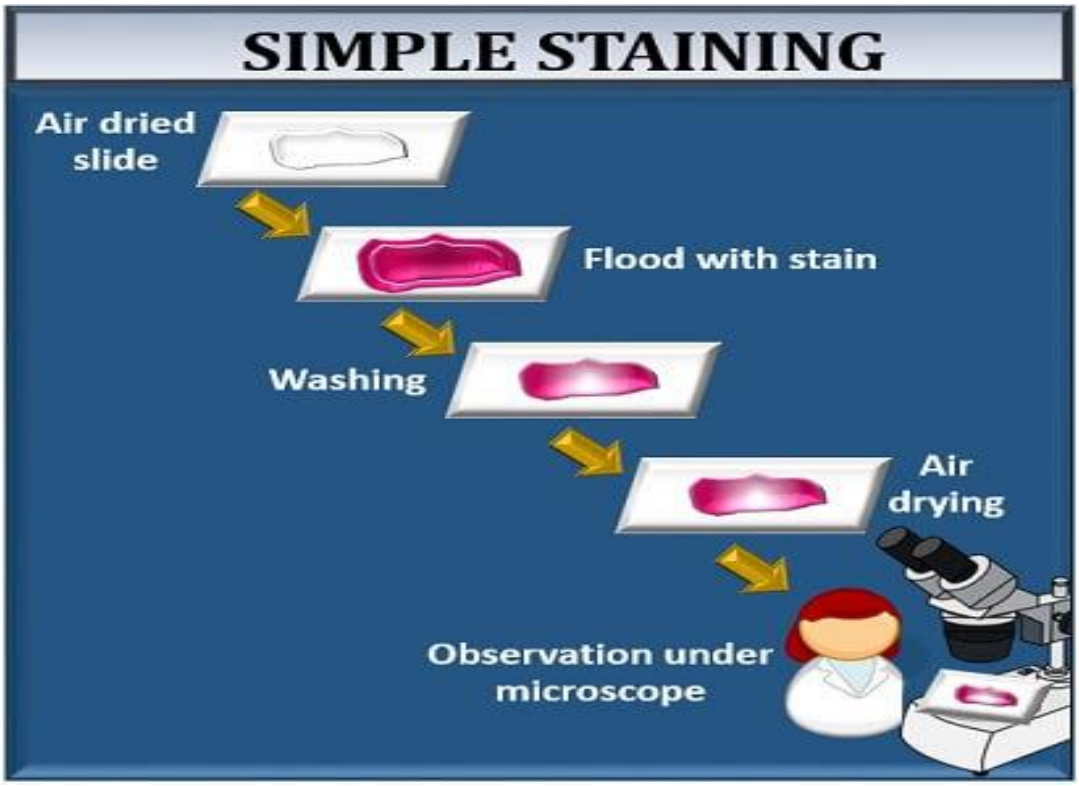
- Heat fixing helps in the fixation of a specimen to the glass slide.
- Heat fixing helps the stain to penetrate the smear.



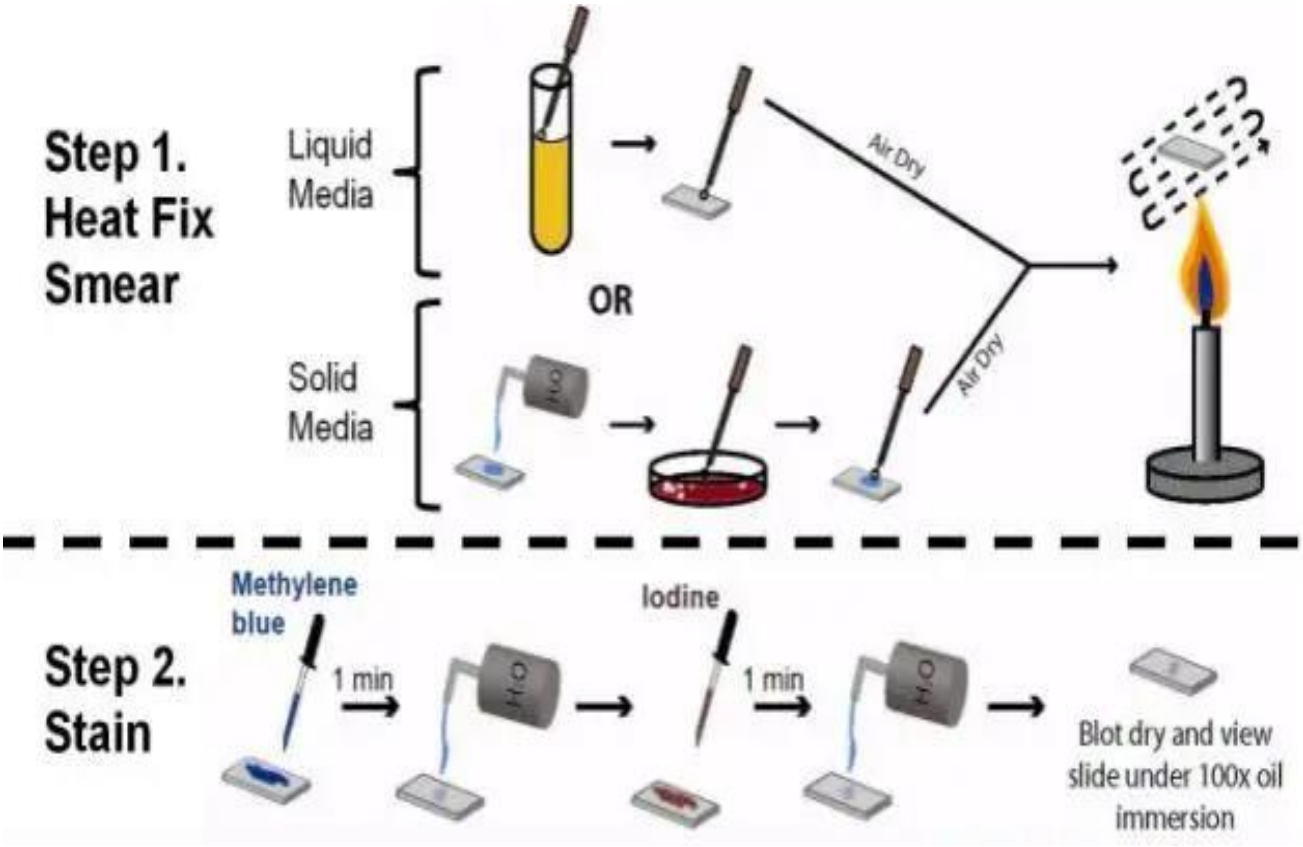
Staining of Bacteria

It is the last and the most crucial step, in which one can identify the morphological characteristics of the bacteria through microscopic examination, once the cells get stained. This stage involves the following steps, which are as follows:

1. Add stain to the heat fixed smear.
2. Allow the stain to stand for at least 1 minute so that it can penetrate between the cells.
3. Wash off the glass slide carefully.
4. Blot dry the slide with absorbent paper (do not wipe the slide).
5. Examine the glass slide under the microscope from low to high power to get a magnified view of the specimen. One can also add a drop of oil



6. Wipe the back of the slide and blot the stained surface with bibulous paper or with a paper towel.
7. Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective
8. Choose an area of the smear in which the cells are well spread in a monolayer. Center the area to be studied, apply immersion oil directly to the smear, and focus the smear under oil with the 100X objective.



What is Gram Staining?

Gram staining is a differential bacterial staining technique used to differentiate bacteria into Gram Positive and Gram-Negative types according to their cell wall composition. It is the most widely used and the most important staining technique in bacteriology, especially in medical bacteriology. It is generally the first test performed on bacteria during their identification and observation process. This staining technique uses two stains; crystal violet as primary stain and safranine as a counterstain. Those bacteria with Gram-positive cell walls will retain primary stain and appear violet or purple. These bacteria are termed Gram-Positive bacteria. The other group of bacteria with Gram-Negative cell wall will lose primary stain and take up the counterstain and

appears pink or red under the microscope. These bacteria are called Gram-Negative bacteria. Using this staining technique, bacteria can be differentiated into two groups hence; it is called the differential staining technique.

History of Gram Staining:

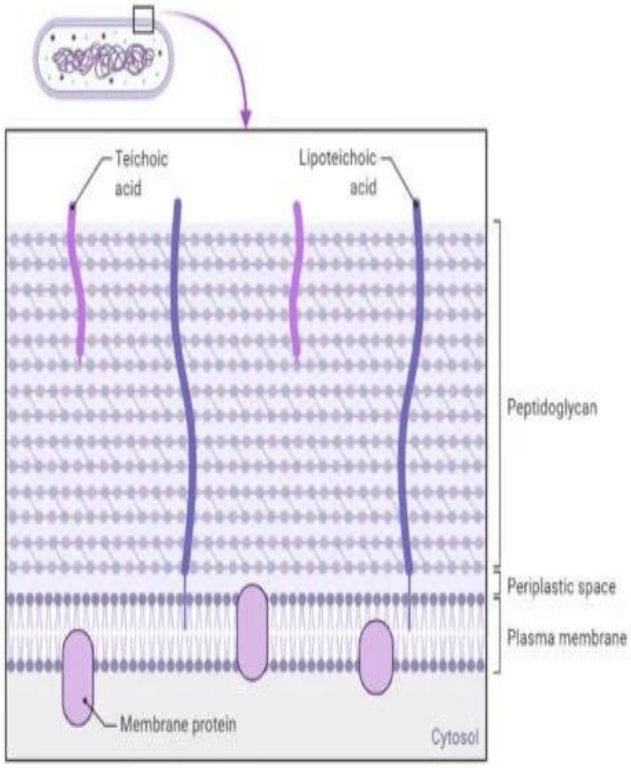
This technique was introduced in 1884 by the Danish Bacteriologist **Hans Christian Gram** (1853 September 13 to 1938 November 14). He developed this staining technique to identify bacteria causing pneumonia. Later it became a popular method to classify bacteria into Gram Positive and Gram-Negative types.

Gram Staining Objectives:

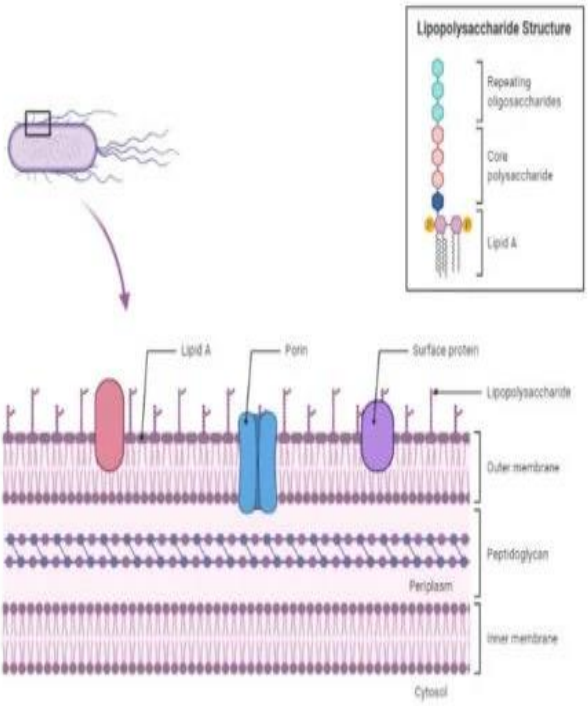
- To differentiate bacteria into Gram-Positive and Gram-Negative.
- To study the morphological structure of bacteria.

Gram Staining Principle:

Gram staining and differentiation are based on the differences in cell wall structure and composition of bacteria. Bacteria having cell walls with a thick layer of peptidoglycan will resist decolorization of primary stain and appear violet or purple. Bacteria having a thin peptidoglycan layer with lesser cross-linkage lose primary stain during decolorizing and gain counter stain appearing pink or red. When counterstain, positively charged safranin, is added, it interacts with the free negatively charged components in Gram-Negative cell wall and membrane and bacteria becomes pink/red. Whereas, there is no space to enter inside the dehydrated Gram-Positive cell wall due to CVI complex and dehydration. Hence, safranin can’t stain them red or pink and Gram-Positive bacteria reveal the purple or violet color.



Gram-Positive Bacteria Cell Wall Structure



Gram-Negative Bacteria Cell Wall Structure

Gram Staining Requirements:

- Sample bacterial colonies or suspension
- Gram Staining Kit (Reagents)
- Glass slide
- Inoculating loop
- Bunsen burner
- Staining rack
- Wash bottle (or Tap water)
- Microscope with 100X objective lens (compound microscope).

Gram Stain Reagents:

1. Primary Stain (Crystal Violet)
2. Mordant (Gram's Iodine)
3. Decolorizing Solution
4. Counter Stain (Safranin)

Gram Staining Protocol:

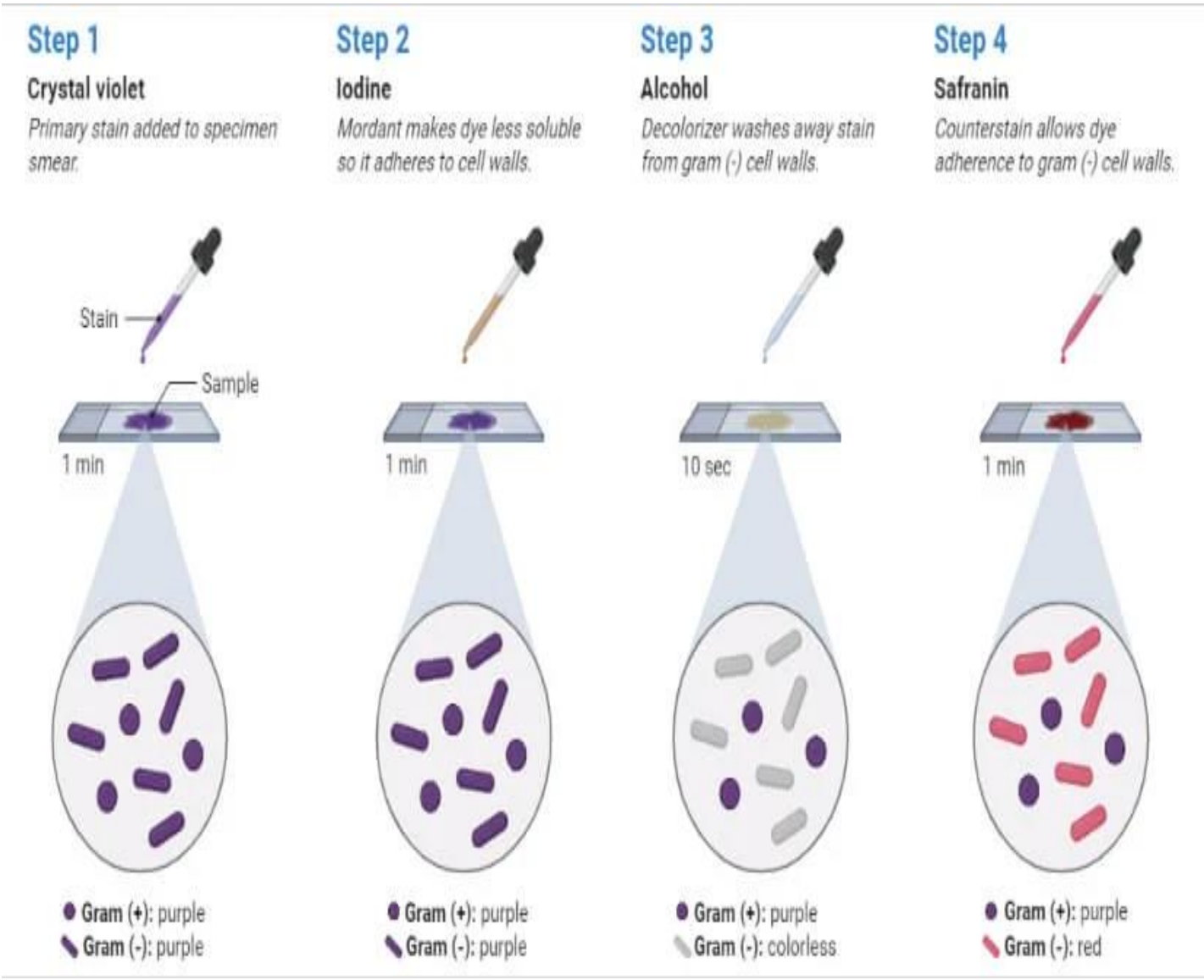
1. Flood crystal violet solution over fixed smear
2. After 30 – 60 seconds, pour off the CV solution and rinse with gentle running water.
3. Flood the Gram's Iodine solution over the smear
4. Leave the iodine solution for 30 – 60 seconds and pour off the excess iodine and rinse with gentle running water
5. Shake off the excess water over the smear
6. Decolorize the smear by passing the decolorizing solution till the solution runs down in clear form. Alternatively, add a few drops of decolorizing solution and shake gently and rinse with distilled water after 5 seconds.
7. Rinse with distilled water to wash decolorizer
8. Shake off the excess water over the smear
9. Pour counter stain over the smear
10. Leave for 30 – 60 seconds and wash with gentle running water
11. Air dry or blow-dry the smear.

Examples of Gram-positive bacteria:

1. **Gram-positive cocci**– *Staphylococcus spp., Streptococcus spp., Enterococcus spp*., etc.
2. **Gram-positive bacilli**– *Bacillus spp., Clostridium spp., Lactobacillus spp., Streptomyces spp.* and other Actinobacteria, *Listeria spp., Corynebacterium spp*., etc.

Examples of Gram-Negative bacteria:

1. **Gram negative cocci**– *Neisseria spp., Moraxella spp., Acinetobacter spp*. etc
2. **Gram negative bacilli-** *E. coli, Klebsiella spp., Salmonella spp., Shigella spp., Pseudomonas spp., Proteus spp.,* etc.



Gram-Positive bacteria appear violet or purple.

Gram-Negative bacteria appear pink or red.