NORTHERN ARIZONA UNIVERSITY

Applied Microbiology
&
Biotechnology Laboratory

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Standard Operating Procedure

AMBL-501-A

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Biological Stain: Differential Gram Stain Technique

METHOD SUMMARY

This method describes a technique for performing the differential Gram stain based on the Hucker modification of the original Gram stain procedure. This procedure is used to determine Gram-positive and Gram-negative bacteria. Gram-positive and Gram-negative bacteria are both stained by the Crystal Violet stain. Adding iodine causes a crystal violet-iodine complex to form in the bacterial cell wall. An alcohol decolorizing solution extracts lipids from the cell wall of gram-negative bacteria, thus causing the porosity of the cell wall to increase. This allows the crystal violet-iodine complex to diffuse back out of the cell well. In Gram-positive bacteria, the decolorizing solution dehydrates the cell wall causing porosity to decrease and thus trapping the crystal violet-iodine complex in the cell well. A counter stain, Safranin, is then used and is able to pass through the porous cell of Gram-negative bacteria.

ENVIRONMENTAL HEALTH AND SAFETY

Hazards Assessment: This method uses the following equipment, samples or chemicals that present a hazard to health and safety. Where specified and before conducting this method, consult the appropriate MSDS for the specific hazards.

a. Gram stain kit or each of the following
    1) Crystal Violet stain
    2) Iodine solution
    3) Decolorizing solution (2-Propanol/Acetone)
    4) Safranin stain
b. Deionized water
c. Bunsen burner

Safety Equipment and Engineering Controls: Knowing the location and being able to use the eye wash and shower station, the fire extinguisher and the first aid kit are required.

Personal Protective Equipment (PPE): When conducting this method, handling samples, working with chemicals or preparing solutions that are associated with conducting this method the following PPE are required.

- Lab coat, eye protection, and gloves (Nitrile or latex).

Review and follow the additional procedures regarding PPE in NAU's Environmental Health and Safety's Chemical Safety Standard Operating Procedure, P16-Personal Protective Equipment (2008) and P25-Safety Glasses (2012).

Analysis-derived Wastes and Disposal: Wastes that are generated by this procedure and the appropriate method to be used for their disposal are summarized in the following table.

Waste Generated	Hazardous (Y / N)	Disposal
Biological staining liquid waste is generated.	Y	Must be collected and disposed according to local hazardous waste handling requirements.

METHOD DESCRIPTION

1.0 Application

The gram stain is a procedure used to differentiate between two groups of bacteria based on the chemical and physical characteristic of the cell wall. Gram-positive bacteria have a cell wall that contains peptidoglycan, which will stain purple. Gram negative bacteria have a much thinner cell wall, lacking peptidoglycan, which will stain pink.

This procedure is intended to be used with bacteria specimens obtained from cultures in liquid or solid media and that have been prepared as a dry mount sample on a glass slide.

2.0 Apparatus

a. Glass microscope slide
b. Microscope with oil immersion objective
c. Wire loop or disposable sterile pipet
d. Bunsen burner
e. Timer
f. Blotter or absorbent paper

3.0 Reagents

a. Gram stain kit or each of the following
   1) Crystal Violet stain
   2) Iodine solution
   3) Decolorizing solution
   4) Safranin stain
b. Deionized water
c. Immersion oil

4.0 Procedure

a. Prepare a dry mount slide sample of the bacteria culture.
   1) Liquid Media: Spread the bacterial suspension onto a glass slide using either a sterile disposable pipet or a sterilized wire loop. Allow the smear to air dry. Heat-fix the smear by holding the slide with the smear-side up and pass it through a Bunsen burner flame 3 or 4 times, being careful not to boil the liquid or overheat the glass. Allow the slide to cool to room temperature before staining.
   2) Solid Media: Place a drop of deionized water onto the glass slide. Touch the sterilized wire loop onto a colony and transfer this to the drop of water on the slide. Suspend the sample in the water while spreading it out on the slide. Allow the smear to air dry. Heat-fix the smear by holding the slide with the smear-side up and pass it through a Bunsen burner flame 3 or 4 times, being careful not to overheat the glass. Allow the slide to cool to room temperature before staining.
b. Flood the prepared slide with Crystal Violet stain and allow this to sit for 60 seconds.
c. Gently rinse the Crystal Violet from the slide using deionized water and gently shake off the excess water.
d. Flood the slide with Iodine solution and allow to sit for 60 seconds.
e. Gently rinse the Crystal Violet from the slide using deionized water and gently shake off the excess water.
f. Gently rinse the slide with Decolorizing solution for 10 seconds or less if the solution runs clear from the slide.
g. Gently rinse the Crystal Violet from the slide using deionized water and gently shake off the excess water.
h. Flood the slide with Safranin stain and allow to sit for 60 seconds.

i. Gently rinse the Crystal Violet from the slide using deionized water and gently shake off the excess water.

j. Carefully blot the slide dry.

k. View the slide with a microscope using the oil immersion technique.

5.0 Interpretation of Results

Gram-positive bacteria will stain purple and Gram negative bacteria will stain pink or red.

6.0 Quality Control

Quality control of this procedure is limited to following the prescribed staining and decolorizing times, and using stains that have not deteriorated.

7.0 Bibliography

Hucker, G.J. and H.J. Conn (1923) Methods of Gram Staining. New York State Agricultural Experiment Station Technical Bulletin No. 93.

Hucker, G.J. and H.J. Conn (1927) Further Studies on the Methods of Gram Staining. New York State Agricultural Experiment Station Technical Bulletin No. 128.

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