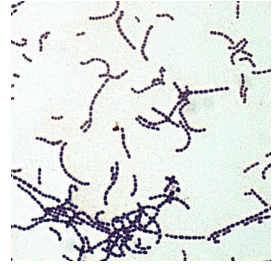


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Gram staining

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We use this protocol and it's working

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Abstract

This protocol is performed as described by the ASM (American Society of Microbiologists) in their document **Gram Staining Protocol, 1981**. Our aim is to include all instructions that could be useful for the absolute beginner.

Procedure:

- The first step of the procedure is the primary stain uses crystal violet, which is then fixed with iodine (the mordant), by forming crystal violet-iodine complexes. This prevents the easy removal of the dye.
- After this, the dye is removed using a decolorizer.
- A second dye (such as safranin or fuchsin) is added to stain the decolorized cells.

Theory:

The Gram stain was developed by Hans Christian Gram in 1882. It lets the experimentator separate microorganisms into two groups, based how well their cell-membranes can retain the crystal violet dye.

At first, most bacteria cells will take up the crystal violet. The lugol solution stabilizes the previous stain with the peptidoglycan wall in bacteria containing one cellular membrane and a thick wall of peptidoglycan (called monoderms or Gram-positives). When the solvent (ethanol) is added, the diderm organisms (or bacteria containing two cytoplasmic membranes, or gram-negative) lose their outer lipid layer and alongside most of the stain, as they contain a very small peptidoglycan layer unable to capture the crystal violet - iodide complexes. To be able to see them under microscope, a counter-stain is added making diderm organisms visible again, with the pink color of the safranin reagent (see Gram Staining protocol, 1981).

There are bacteria classified as "acid-fast" which are not stained with this protocol. If you need more information about them, you can read the paper from Bayot, Mirza and Sharma (2023).

Further reading:



gram-stain-protocol-2886.pdf

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Guidelines

- As good microbiological practice, all containers used for components should be labeled with the name of the solution, the date of creation / aliquote, and initials of the person who did it, in order to ask questions if something is needed.
- If you have doubts about how to perform a step in the protocol, consult your supervisor or someone who has prepared the solution before.
- For any questions, feel free to email us (exotoxinslab-(at)- gmail.com). We are more than happy to help you.
- Some of the protocols will be posted in our YouTube channel (www.youtube.com/@exotoxinslab).

Materials

The following protocol uses the  Gram Staining kit **Merck Catalog #77730**

which includes:

- Reagent 1: Crystal violet solution
- Reagent 2: Lugol's solution stabilised
- Reagent 3: Decolorization solution (two containers)
- Reagent 4: Safranin solution

For the bacterial smear and for microscopy:

- Glass microscopy slides
- Lent-free paper wipes.
- Pipette (10 - 100 µl)
- Pipette tips.
- Sterile eyelet for microbiological work.



Safety warnings

- ! The Gram Stain Kit includes some dangerous chemicals, such as the Crystal violet solution, Lugol's solution stabilised, the Decolorization solution and safranine solution. Some are partially flammable and harmful. For more detailed warnings, consult the safety data-sheet linked here:

 111885_SET_SDS_EU_EN (1).PDF 161.9KB

For **important details and tips**: Here are some from the discussions held at the ASM Conference for Undergraduate Educators 2005 on page 6 of the following gram staining protocol:

 gram-stain-protocol-2886.pdf

A short summary:

1. The bacterial spread should not be too thick and not have any inconsistencies
2. Use young, actively growing cultures.
3. Freshly made reactants are recommended

When choosing containers for the staining, keep in mind that this stains are difficult to remove from plastic surfaces. We use only glass surfaces and containers to wash and/or retain the washed components. Make sure you know the disposal regulations in your country and follow them.

Before start

1. Be aware of your lab's safety protocols and check the attached Warnings (Guidelines and Warnings) to see what steps are necessary to protect yourself, such as protocol-appropriate personal protective gear, like lab coat, gloves and glasses.
2. Make sure to read the whole protocol before starting and have all the materials at hand.

Bacterial smear

1h

- 1 Clean the microscopy slides by wiping them down with a paper towel soaked in 70% ethanol

Note

Microscopy slides are coated with oil during the production process. To be able to fix the cells to the slide, you need to remove any residues of this coating.

- 2 Label the slides with pencil. Do not use a pen or marker. They will wash off during the de-staining with the third solution (ethanol).

- 3 In case you have grown bacteria on agar plates, here we describe how to gather bacteria from the colonies for staining:

Note

Young actively growing cultures are recommended for Gram-staining. Older cultures may have ruptured cell walls, making them unsuitable for staining.

- 3.1 Add a small drop of distilled water onto your prepared slide (~5–10 μ l). You can use a pipette, or trap some water with the inoculation loop. Make sure you do not have more than 100 μ l or you will have to wait much longer until the sample dries.
 - 3.2 Transfer a small trace of a colony of bacteria grown on solid media to the slide with a sterile loop or pipette tip, and carefully place it in the droplet.
 - 3.3 While dissolving the bacteria colony in the water, spread the solution into a thin film over the slide using your loop or pipette tip. Stay within the slide confinements and avoid to get closer than 0.5 cm to the edges or markings.

Note

The thickness of the spread of bacteria will affect the quality of the decolorizing / destaining step. We recommend to avoid clumps or accumulation of material on the slide.

- 4 If the bacteria cultures are in liquid media, these are the steps describing how to gather bacteria for staining. Since they are already in solution, you can skip the use of water



described for bacteria grown on agar plates.

4.1 The media needs to have a high concentration of bacteria (around OD_{550/600} higher than 0.7) for this volume to work. As a guide, the liquid has to have turned visibly opaque/cloudy, enough so you cannot see clearly the markings of the tube's side when looking through.

4.2 Transfer  10 µL to  50 µL µl of the bacteria suspension (culture) onto the slide using a sterile eyelet/drop or by pipetting.

4.3 Spread the droplet into a thin film over the slide using a pipette tip or a sterile loop.

Note

Thickness of the spread will determine the degree of decolorizing. A thin spread is recommended.

5 Once the bacteria are on the slide, allow the solutions to air dry completely.

6 With a pair of tweezers, take the microscope slide and heat fix the smear by exposing the slide briefly to a flame of a Bunsen burner ( 00:00:01). Make sure you do not expose the surface with the dried bacteria suspension to the fire. The flame should only heat the glass enough for bacteria to get heat-fixed to the surface of the glass. Make the movements swift and do not stay too long on a position on the flame or the glass will brake.

1s

Note

Please take care of using your Personal Protection Equipment (safety glasses, lab coat, gloves). If the glass gets too hot, it will split and the shards can fly around the working area.

Make sure to not stay in the flame too long or too short. Too high temperatures potentially damage the cells, and underfixing will lead to the cells washing off in the staining step.

7 Place the slides to be stained on a metal or glass rack where they can lie horizontally and all staining solutions can be captured. We use 30 cm diameter glass petri dishes (or any flat glass container) with glass rods as racks. These allow us to collect the staining solutions inside the petri dish for correct disposal, and allow us to tip over the slides when we wash them.



Gram staining (as described by the American Society of Microbiologists)

2m 2s

- 8 Once fixed, pour the crystal violet stain reagent onto the smear and let it stain for 00:01:00 1m
- 9 With the tweezers, tip the slides and wash it with a gentle and indirect stream of tap water for 00:00:02 2s
- 10 Add the second solution (Lugol) onto the slide and wait 00:01:00 1m
- 11 Repeat wash as described in step 9
- 12 Add the decolorizing agent (3rd solution) onto the slide and wait 00:00:15 . 15s
Optional: You could add drop by drop to slide until decolorizing agent runs down from the sides with no stain visible. We have not tried the optional way to add the decolorizing agent but it is mentioned in the original document.

Note

This is the most consequential step for the final outcome. Both over- AND under-decolorizing will lead to a misinterpretation of the results.
- 13 Tip the slide to remove the decolorizing agent from the slide and add the counter stain (safarin or fuchsin). Wait 00:00:30 to 00:01:00 . 1m 30s
- 14 Repeat wash as described in step 9.
- 15 Blot the slide dry with absorbent paper, without removing the sample. Wait until it is completely dry before advancing to the next step.
- 16 Observe the results of the staining procedure under oil immersion at 1000x magnification using a brightfield microscope.



Protocol references

Gram Stain Protocol (Gephardt et al, 1981, Feedback from ASMCUE participants, ASMCUE , 2005)

For the bacterial smear preparation of bacteria **grown in liquid media**

For more information about acid-fast bacteria, look at Bayot ML, Mirza TM, Sharma S. Acid Fast Bacteria. [Updated 2023 Aug 7]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK537121/>