Preparation of a Gram stain: Manual Method

SOP 22137 **Rev. 06**

Table of Contents

1.0 Purpose

This procedure describes how to prepare a Gram stain.

2.0 Scope

This procedure applies to Process Analytics/Quality Control (PA/QC) personnel who perform the preparation of a Gram Stain to identify microbial colonies.

3.0 Authority and Responsibility

3.1 The Director of Process Analytics**/**Quality Control (PA/QC) has the authority to establish this procedure.

3.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA/QC personnel are responsible for the performance of this procedure.

3.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.

3.5 Biopharmaceutical Quality Assurance (BQA) is responsible for quality oversight of this procedure.

4.0 Materials and Equipment

- Gram Stain Check Slide, Fisher, Catalog Number 08-801 or equivalent (BDP PN 30188).
- Microscope Slides (BDP PN 20568).
- Gram Stain Kit containing: Crystal Violet, Gram Iodine, Decolorizer, and Safranin (BDP PN 30048).
- Wright’s Stain (BDP PN 31195)

- Plastic, disposable inoculating loops, sterile pipettes, or equivalent.
- Bunsen Burner or equivalent.
- Slide Forceps
- Microscope
- Slide Warmer
- Biological Safety Cabinet
- Chemical Safety Cabinet
- Immersion Oil, BDP PN 30176
- Bibulous Paper, BDP PN 20658
- High Purity Water

5.0 Sample Fixation

5.1 Label a Gram check slide with the QCTR number, date and the identification number for each sample using a black laboratory marker.

NOTE: A single Gram check slide provides 6 sample spaces and 2 fixed controls. Additional controls are not required.

5.2 Aseptically, using either a sterile pipet or inoculating loop, place a drop of the liquid sample on the microscope slide. Smear the sample over the surface of the slide, using the pipet or inoculating loop.

When sampling colonies from solid media, place a small loopful of Type 1 water on the slide section. Touch a sterile inoculating loop to a colony and mix thoroughly into the drop of water on the slide to obtain an even suspension.

NOTE: It is recommended that a fresh colony be used, approximately 18-24 hours old. If the sample is labeled “Biohazard” or “Potentially Infectious”, all slide inoculation and fixation operations are to be performed in a biological safety cabinet (BSC). The samples are **NOT** considered safe (no longer a biohazard/non-infectious) for staining until the fixation process is complete. Follow ***SOP 17109 - Procedures for Safe Handling, Decontamination, and Spill Cleanup of Infectious or Potentially Infectious Materials*** for guidance on safe handling and disposal of any hazardous material.

5.3 Allow the specimen sample(s) to air dry on the slide.

5.4 Optional heat fixation may be performed using a Bunsen burner and a chemical safety cabinet. The specimen slide should be passed (smear side up) through a Bunsen burner flame at least three times. Avoid overheating; the slide should be just hot to the touch. Cool to room temperature before staining.

5.5 Alternatively, a slide warmer set to 60.0°C can be used to heat fix samples to the slide. Samples labeled “Biohazard” or “Potentially Infectious” should be fixed only on a slide warmer in a BSC.

5.5.1 If a slide warmer is utilized, place the inoculated slide(s) on the surface of the slide warmer after the temperature setpoint has stabilized. Allow the slide to remain on the heated surface until all liquid has visibly evaporated. The slide warmer is the only apparatus to be used within a biological safety cabinet as needed per Step 5.2.

5.5.2 Once the sample(s) are visibly dry, the slide is ready for the staining process.

6.0 Gram Stain Procedure

6.1 Flood the fixed Gram check slide(s) with crystal violet. Allow the slide to sit for at least 1 minute.

6.2 Rinse the slides with high purity water, and then remove excess water by gently tapping the slide on its side.

6.3 Flood the Gram check slide and the sample slide(s) with Gram Iodine. Allow the slide to sit for at least 1 minute.

6.4 Rinse the slides with high purity water.

6.5 Decolorize by flooding the slides with decolorizer and rocking them back and forth, approximately 3 to 5 seconds.

6.6 Quickly rinse the slides with high purity water.

6.7 Flood the Gram check slide and the sample slide(s) with Safranin. Allow the slide to sit for 1 minute.

6.8 Rinse with high purity water.

6.9 Gently air dry or blot dry with bibulous paper.

7.0 Gram Stain Results and Interpretation

7.1 The slides may now be examined under the microscope starting at a 10x magnification. Place a drop of oil on the slides and examine them using the oil immersion objective (100X).

7.1.1 If the slide has been heated too much, the cells will appear disrupted. Repeat steps 5.1 – 5.4 using a new Gram check slide.

7.1.2 If the gram-positive and gram-negative control specimens on the check slide do not exhibit the expected stain characteristics repeat steps 5.1-5.4 using a new Gram check slide. The expected organism shape and gram stain results are listed on the check slide packaging.

7.1.2.1 Gram-Positive: *S. aureus*, blue/purple cocci.

7.1.2.2 Gram-Negative: *E. coli*, red/pink rod.

7.1.3 If the test specimen retained the blue/purple stain, it is classified as Gram positive.

7.1.4 If the test specimen retained the red/pink stain, it is classified as Gram negative.

7.1.5 If the slide has not been heated enough to "fix" the cells, they may wash off during staining.

7.1.6 If the results are inconclusive due to under/over decolorization or poor fixation, repeat steps 5.1 - 5.4 using a new Gram check slide.

7.2 In addition to ascertaining the stain color fixed to the sample cells, microbial morphology should be categorized, if possible.

7.2.1 Overall shape: cocci, rods, filaments, hyphae, yeast-like.

7.2.2 Appearance of ends: rounded, tapered, flattened, or clubbed (swollen).

7.2.3 Appearance of sides: parallel, ovoid(bulging), concave, or irregular.

7.2.4 Nature of axis: straight or curved.

7.2.5 Pleomorphism: variation in microbial shape.

7.3 Mixed cell type samples (i.e. blood derived samples) will exhibit multiple staining characteristics and cell morphologies. Gram-positive and Gram-negative classifications should only be assigned to microbial cells. All other cells from expected cell populations (such as mammalian cells) within the fixed specimen should be excluded from reported results unless otherwise specified by the QCTR or area supervisor.

7.4 If atypical results are categorized (when compared to the QCTR specified criteria) contact the area supervisor and test requestor immediately.

7.5 Additional staining may be performed to better characterize blood derived cell types and delineate those from possible microbial contamination. The requirement for additional staining should be made by the area supervisor and or requestor. The area

supervisor/requestor will assess each PA/QC sample where interference from metazoan cells (including WBCs/PBMCs and eukaryotic cells in general) may affect the clear identification of potential Gram-positive microbes in the sample.

7.5.1 Wright's Stain (for blood derived specimens)

7.5.1.1 Place 1-2 mL of Wright Stain over the specimen slide.

7.5.1.2 Allow stain to pool on the specimen sample for 30 seconds.

7.5.1.3 Add an equal volume of high purity water to the pool of Wright Stain.

7.5.1.4 Allow the mixture to sit for 1 minute. Rinse with high purity water and allow to air dry.

7.5.1.5 Wright's Stain criteria found below should be used when assessing specimens. These results should be recorded in the comments field of the Gram stain datasheet, Form 22137-01.

7.5.1.5.1 Red blood cells (if present) will stain pink to orange.

7.5.1.5.2 Nuclei of lymphocytes will stain varying shades of purple.

7.5.1.5.3 Cytoplasmic staining of lymphocytes stain blue to light pink.

7.5.1.5.4 Cytoplasmic granules will stain dark blue for B lymphocytes.

7.5.1.5.5 Cytoplasmic granules will stain bright orange for Eosinophils.

7.5.2 For cell therapy assays, if a microscope camera and associated software is available, the fixed specimen(s) should have digital images captured. Step 7.1 should be exclusively used when making any morphological assessment on the fixed specimen(s).

7.5.2.1 Using the same specimen slide(s) from Steps 7.1 – 7.2, a minimum 50x oil immersion objective is required in order to provide sufficient image detail and clarity.

7.5.2.2 Three representative locations on the fixed specimen(s) should be collected digitally. Each image file should be labeled using the following format.

7.5.2.2.1 QCTR number

7.5.2.2.2 Sample number (id number found on the associated QCTR)

7.5.2.2.3 Capture number (camera software id number assigned to image)

7.5.2.2.4 Example: QC 063073 Sample-1 Capture 0

7.5.2.3 All three digital images should be saved on the BDP VLAN under the Gram Stain folder, the calendar year the samples were processed, and using the image file format listed in Step 7.5.2.2.

7.5.2.4 The three representative images should be attached to the QCTR package and submitted with all required documentation listed in this SOP using the same labeling criteria described in Step 7.5.2.2.

8.0 Documentation

8.1 Document control and sample results on the BQC Test Request, Form 22002-01 and the Gram Stain Datasheet, Form 22137-01. Then submit for review per ***SOP 22002 - Request for Quality Control Testing***.

9.0 References and Related Documents

BDP SOP 17109 *Procedures for Safe Handling, Decontamination, and Spill Cleanup of Infectious or Potentially Infectious Materials*

BDP SOP 22002 *Request for Quality Control Testing*

Form 22137-01 *Gram Stain Datasheet,*

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