| KCMC Biotechnology Laboratory, Microbiology | STANDARD OPERATING PROCEDURE | Effective Date | SOP-Number MIC.021 |
|---|---|---|---|
| | | Page 1 of 6 | Date |

**Title: Gram Stain Procedure**

**Author/Date: Anne Morrissey, 9 May 2006**

**Approvals/Date:**

_____

**John A. Crump, MB, ChB, Microbiology Laboratory Director**

_____

**Anne Morrissey, MS, MT(ASCP)SM, Microbiology Lab Supervisor**

**This SOP has been read and understood by:**

| Name | Date |
|---|---|
| 1. | |
| 2. | |
| 3. | |
| 4. | |
| 5. | |
| 6. | |
| 7. | |
| 8. | |
| 9. | |
| 10. | |

| Annual Review | |
|---|---|
| By | Date |
| | |
| | |
| | |
| | |
| | |

**Title: Gram Stain Procedure**

**Document History:**

| Version Number | Reason for Changes | Date |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |

**Copies distributed to:**

| Name | Date |
|---|---|
| | |
| | |
| | |

**PURPOSE**
For staining bacteria, yeasts and aerobic actinomycetes.

**PRINCIPLE**
The composition of the cell wall of a microorganism determines whether it will retain crystal violet dye or be decolorized and made visible only with the counterstain, safranin. Those organisms that retain the crystal violet dye will stain blue (Gram Positive), those that do not are stained red with safranin (Gram Negative).

**CLINICAL APPLICATIONS**
The Gram stain is useful in the diagnosis of bacterial and some fungal infections by demonstrating the causative agent in smears prepared from clinical material or positive blood cultures. Smears prepared from growing cultures demonstrate microscopic morphology tha is helpful in organism identification.

**SCOPE**
This Standard operating Procedure applies to the performance of the Gram stain by all technical staff in the microbiology laboratory that have been trained and are competent in performing this test.

**SAFETY PRECAUTIONS**
- Always wear gloves when handling patient specimens.
- Aliquot blood cultures and prepare slides from blood cultures in a biohazard safety hood.
- Use care when handling unstained slides to avoid touching infectious material.

**MATERIALS**

Glass slides
Syringe with needle (for aliquoting blood cultures)
Pasteur pipettes (for preparing slides from clinical material and blood culture aliquots)
Loop (1 ul for preparing slides of colonies)
Staining rack
Slide warmer set at position #7 ($70^{o}$ C)
Light microscope
Staining reagents:
Crystal Violet
95% ethanol/acetone:
Acetone 400 ml
5% ethanol 1200 ml
`
Safranin
Store stains at ambient/room temperature.

## QUALITY CONTROL

**Frequency:** Once per week

**Controls**: Prepared slides with Gram positive and Gram negative organisms.

Gram Positive: *Staphylococcus aureus*

Gram Negative: *E. coli*

**Acceptable Results**:

Gram Positive: blue cocci

Gram negative: red bacilli

**Corrective Actions:**

1. Restain new control slide.
2. If still unacceptable, have a different technologist restain the slide.

**Documentation:**

1. Record QC results on GRAM STAIN QC sheet.
2. Document all unacceptable QC results on QC DEVIATION form.
3. Review QC results monthly.

## PREPARATION OF SMEARS

**From blood culture:**

1. Remove an aliquot of blood culture into sterile test tube.
2. Using a Pasteur pipette or a loop, remove a small aliquot and place a drop or loopful onto a glass slide.
3. Place on slide warmer to dry and fix slide for approximately 30 minutes.

**From colonies:**

1. Place a drop of sterile distilled water or saline onto a glass slide.
2. Using a 1 ul loop, remove a colony and emulsify in the droplet.
3. Place on slide warmer to dry and fix slide for approximately 30 minutes.

## STAIN PROCEDURE

1. Flood slide with CRYSTAL VIOLET.
2. Let stand one minute.
3. Rinse with tap water and drain off excess water.
3. Flood slide with GRAM'S IODINE and let stand for one minute.
4. Rinse with tap water and drain off excess water.
5. Decolorize with 95% ethyl alcohol/acetone until most of the crystal violet is removed in thin areas (length of decolorizing time depends on thickness of smear).

6. Rinse with tap water and drain off excess water.
7. Counterstain with SAFRANIN for 10 seconds.
8. Rinse with tap water and drain off excess water.
9. Place on slide warmer until dry or blot gently on paper towel.

**EXAMINATION OF GRAM STAINS**

1. Place a drop of immersion oil on the slide.
2. Examine using oil immersion (100x) objective.
3. Focus using coarse and fine adjustment knobs until objects are in focus.

**RESULTS**

Blue organisms – Gram Positive

Red organisms – Gram Negative

Yeasts stain Gram Positive

Background material, cell cytoplasm stain red

**REPORTING**

**1.** Describe organisms by their Gram reaction (Gram Positive - blue, Gram negative – red) and their microscopic morphology and arrangement (e.g. cocci in pairs, chains, clusters; bacilli, small, large, filamentous, yeasts).

Sample Report: "Gram positive cocci in chains"

2. Record findings on Positive Culture Result form.
3. Notify ISAAC Study Coordinator of all positive Gram stains on blood cultures.

**TEST LIMITATIONS**

1. The length of time of the decolorizing step (ethanol/acetate) is critical. Thin smears require less time than thick. Too much decolorizing will render everything on the slide red; not enough, blue.
2. Gram positive organisms, especially bacilli, from cultures that are not fresh (>48 hrs) may not retain the crystal violet and stain red.
3. Some species of bacteria are described as "Gram variable" i.e. may stain blue or red or show both colors (e.g. *Gardnerella vaginalis*).

**REFERENCE**

Chapin, KC, Lauderdale, T. 2003. Reagents, Stains and Media. In: Manual of Clinical Microbiology, 8th Ed. ASM Press, Washington, DC.

APPENDIX A – Gram Stain QC sheet

APPENDIX A

## KCMC Biotechnology Laboratory — Quality Control

**Microbiology** — **Gram Stain Reagents**

**ACCEPTABLE RESULTS: GRAM POS/*S. aureus*/blue (Gram +)**
**GRAM NEG/*E. coli*/red (Gram -)** **Year: _____**

| QC DATE | LOT # CRYSTAL VIOLET | LOT# IODINE | LOT # SAFRANIN | RESULT | | BY |
|---|---|---|---|---|---|---|
| | | | | GRAM + | GRAM - | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

**Document all unacceptable results on QC Deviation form.**

**Supervisor Review**

| | | | | | |
|---|---|---|---|---|---|
| | | | | | |

**GRAM STAIN QC/QC SHEETS**