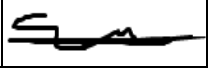
National Public Health Laboratory	Code:	A1
	Version:	V1
	Effective per:	1-8-2013
	Retrieve per:	1-8-2015
	Pages:	4
	Signature authorizer:	

1. Gram Staining

2. Objectives & Scope

Gram staining technique is used for staining bacteria, yeasts and aerobic actinomycetes.

This Standard Operating Procedure is applicable to all technical staff in the microbiology section that have been trained in performing this procedure and are competent and authorized to perform this procedure.

3. Abbreviations and definitions

For general abbreviations, definitions and terms refer to quality manual chapter 1 “General”.

- SOP Standard Operating Procedure
- QM Quality Manual

4. Tasks, responsibilities and accountabilities

For general authorizations refer to the Authorization Matrix.

Task	Authorized	Responsible
Preparation of reagents	LA	LM
Preparation of slide	LT	LM
Staining of slide	LT	LM
Interpretation of results	LT	LM
Reporting of results	DC	LM

5. 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, those that do not are stained red with safranin. Blue micro-organisms are Gram positive. Red micro-organisms are Gram negative.

6. Safety and environment

Safety rules must be taken into consideration when working with potentially infectious patient materials. For general safety rules refer to the biosafety manual.

Aliquot blood cultures and prepare slides from blood cultures in a biosafety cabinets.

7. Sample

Gram stains can be made from blood culture and solid culture. Of both types of samples only a small quantity is needed:

Sample type:	Minimal quantity	Storage conditions
Blood culture:	1 drop	4°C, room A2, refrigerator 1
Solid culture:	1 colony	4°C, room A2, refrigerator 1

After incubation cultures are stored at 4°C in a refrigerator.
Stains are stored at room temperature.

8. Equipment and supplies

Supplies:	Storage location:
Glass slides	Room A3, cabinet 1
Syringe with needle	Room A3, cabinet 1
Pasteur pipettes	Room A3, cabinet 1
Loop	Room A3, cabinet 1
Staining rack	Room A1, safety hood

Equipment (equipment code):	Storage location:
Slide warmer (B001)	Room A1, safety hood
Light microscope (B005)	Room A1, table

9. Reagents and chemicals

Item (SOP for reference)	Storage location	Storage condition
Crystal Violet (P23)	Room A3, safety cabinet 3	Room temperature
Iodine (P23)	Room A3, safety cabinet 3	Room temperature
95% ethanol/acetone (P23)	Room A3, safety cabinet 3	Room temperature
Safranin (P23)	Room A3, safety cabinet 3	Room temperature
Immersion oil	Room A1, table	

10. Quality Control

Perform the quality control below once per week. The quality control consists of reading prepared slides with Gram positive and Gram negative organisms. For Gram positive *Staphylococcus aureus* is used, for Gram negative *E. coli* is used.

Interpretation and acceptable limits:

- Gram Positive *S. aureus* are visible as blue cocci
- Gram negative *E. coli* are visible as red bacilli

Processing of the results:

- Record the QC results on the Gram stain QC sheet provided in Annex 1.
- Record unacceptable QS results also on a nonconformity form to initiate problem analysis and corrective action.
- Review the QC results every month.

Actions in case results are outside acceptable limits (performed by the laboratory technologist):

- Stain new control slides
- If still unacceptable, check the quality of the reagent, check if the staining procedure was followed correctly and check if the correct reference strains were used.

11. Procedure

11.1 Preparation of smears

From blood culture:

1. Using a Pasteur pipette or a loop, remove a small aliquot and place a drop or loop ful of blood on a glass slide
2. 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 µl loop, remove a colony and emulsify in the droplet
3. Place on slide warmer to dry and fix slide for approximately 30 minutes

11.2 Stain procedure

1. Flood dried slide with crystal violet and let stand one minute
2. 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

11.3 Examination

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

11.4 Results

Interpretation of results:

- Blue organisms – Gram Positive
- Red organisms – Gram Negative
- Yeasts stain Gram Positive
- Background material, cell cytoplasm stain red

11.5 Reporting of results

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). For example: "Gram positive cocci in chains"
2. Record the findings in the Result Register
3. Follow the normal reporting procedure as described in SOP P4v2 "Reporting of Results"

11.6 Limitations

1. The length of time of the decolorizing step (ethanol/acetate) is critical. Thin smears require less time than thick smears. Too much decolorizing will render everything on the slide red; not enough decolorizing will render everything on the slide 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” and may stain blue or red or show both colors (e.g. Gardnerella vaginalis)

11.7 Cleanup

1. Used staining liquid should be discarded in the liquid waste chemical containers
2. Gram smears should be discarded in the autoclave bin

12. Related document

- QM1 “General”
- Biosafety Manual, present in every laboratory room and the administration office
- SOP P23 “Preparation of Gram staining reagents
- SOP E4 “Use and maintenance of light microscope”
- SOP P4 “Reporting of results”

13. Related Forms

- P4F1 “Result report”
- Patient register, secretariat

14. References

- Manual of Clinical Microbiology, Chapin, KC, Lauderdale, T., ASM Press, 2003, Washington, DC. Chapter: Reagents, Stains and Media.
- Patient Safety Monitoring & International Laboratory Evaluation portal. Accessed on February 20, 2013. Available at: http://resources.psmile.org/resources/process-control/section-specific-information/microbiology/bacteriology/Pro6.7-A-12%20Gram%20Stain%20%20.doc/view

15. Attachments

- Annex 1 “Quality Control Sheet Gram Stain”

Annex 1: Quality Control Sheet Gram Stain

Acceptable results:

- Gram positive: blue cocci (*Staphylococcus aureas*)
- Gram negative: red bacilli (*E. coli*)

Record all unacceptable quality control results on a nonconformity to start the problem analysis and corrective action process.

Year: _____

Quality control date	Lot number Crystal violet	Lot number Iodine	Lot number Safranin	Result		Quality control done by:
				Gram positive	Gram negative	

Signature of laboratory manager for review: **Date:** _____ **Signature:** _____