Ministry of Health

Document Title	Gram Stain Microscopy Test SOP
Document Type	Procedure
Directorate/Institution	Diagnostic Laboratories Services at Directorate General of Specialized Medical Care (DGSMC) at Ministry of Health (MOH)
Targeted Group	Medical laboratories
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Release Date	May 2023
Review Frequency	Three Years

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Date	May 2023	Date	June 2023

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Acknowledgment

The diagnostic laboratories services at the Directorate General of Specialized Medical Care (DGSMC) at Ministry of Health (MOH) would like to thank and appreciate the great effort of the Microbiology documents development team. Participated and contributed personnel are:

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Acronyms:

ATCC	American Type Culture Collection
SOP	Standard operating procedure
GNB	Gram Negative Bacilli
GPB	Gram Positive Bacilli
GNC	Gram Negative Cocci
GPC	Gram Positive Cocci
YST	Yeast
EPI	Epithelial Cells

1. **Purpose:**

   This document describes the procedure for gram stain.

2. **Scope:**

   This document is applicable for all medical laboratories under MOH and other collaborative governmental and non-governmental health institutions.

3. **Definitions:**

   3.1 Gram stain: is the differential staining procedure most commonly used for microscopic examination of bacteria. This procedure was first described by Hans Christian Joachim Gram.

4. **Procedure**

   4.1.Clinical background:

   Gram stain is considered one of the most important diagnostic tests in microbiology which is quick and easy to preform to give presumptive identification of microorganisms:

   4.1.1 In cases of Meningitis, Septicemia and Sterile site infection support in the treatment choice.

   4.1.2 Presences of numerous polymorphonucleocytes are indicative of infection or presence of clue cells (indicate bacterial vaginosis), or presence or absence of epithelial cells may give an indication of quality of specimen collection (e.g. sputum).

   4.1.3 Morphology of organisms may aid identification of bacteria as it helps to differentiate between gram positive, gram negative, cocci, bacilli, and its arrangement shape (single, pairs, group, chain, spiral etc.).

   4.1.4 Gram stain observation should correlate with cultures. However if organisms were seen in gram microscopy but there was no growth detected this may indicate that either the patient is on antimicrobial therapy or growth requirement is not met.

   4.1.5 This technique has also been used for staining of certain fungi such as *Candida* and *Cryptococcus* which are observed as Gram positive yeasts.

   4.2. Principle of the test:

   Gram positive bacteria have a thicker layer of peptidoglycan in their cell wall which retains the primary stain appearing purple when finished. Gram negative bacteria allow

the primary stain to be flushed away due to a thinner peptidoglycan layer leaving the final stain the chance to counterstain the bacteria leaving a red appearance when viewed under the microscope.

4.3.Pre – analytical stage:

4.3.1. Sample:

4.3.1.1 Sample type: Clinical specimen (e.g. CSF, pus, sputum…. etc.), Smear, colonies & Fungus.

4.3.1.2 Amount of sample required, including minimum requirements: 0.05 ml of clinical specimen or separate colonies.

4.3.1.3 Transportation media: all clinical samples containers or broths.

4.3.2. Material:

Reagents	Consumables/Supplies	Equipment
➢ Crystal violet/ Methylene blue. ➢ Lugol's iodine. ➢ Ethanol 95 - 100%. ➢ Acetone. ➢ Safranin or Basic Fuchsin.	➢ Microscopic twin frosted slides. ➢ Oil immersion. ➢ Sterile loops. ➢ Lens tissue or Gauze. ➢ Wood Sticks. ➢ Xylene.	➢ Microscope. ➢ Slide dryer or heater. ➢ Safety cabinet class II.

4.3.3. Safety precaution:

4.3.3.1 All specimens need to be treated as potentially infectious.

4.3.3.2 Standard procedures for handling of biohazard material must be followed at all times. Universal Precautions must be practiced at all stages of these procedures.

4.3.3.3 Iodine is toxic and therefore avoids the following: inhalation, ingestion, or skin contact.

4.3.3.4 Ethanol and acetone are flammable. They both cause irritation to skin, eyes and intoxication when ingested or inhaled for a long period of time.

4.3.3.5 COSHH and risk assessments shall be followed when performing staining procedures.

4.3.4. Quality control:

4.3.4.1 Check the expiry dates of all media, reagents and stains before use.

4.3.4.2 All reagents and stains MUST be quality controlled before use.

4.3.4.3 Gram stain test should be run with appropriate controls.

4.3.4.4 For blood culture smears, run the gram stain control slide once in every shift or / and in case of changing the stains reagents. Refer to Check blood culture QC sheet in Annexes 8.2.

4.3.4.5 Record the quality control results in the appropriate QC sheet.

4.3.4.6 Prepare a smear using known gram positive and gram negative bacteria (for example, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922).

4.3.4.7 Stain the slide.

4.3.4.8 Let air dry, check under microscope with oil immersion for staining color of each bacteria and quality of staining.

Bacteria Strain Name	Expected result
Staphylococcus aureus ATCC 25923	Purple color
Escherichia coli ATCC 25922	Pink/ red color

4.4. Analytical stage:

4.4.1 Make smear from the specimen or colonies inside the safety cabinet class II. The smear shall not be too thick or two thin and locate the position of the material by lead pencil if needed.

4.4.2 Let the smear air dry and then fix it by heat by Bunsen burner or by slide heater.

4.4.3 Flood the fixed smear with crystal violet and allow the stain to remain for 30 second.

4.4.4 Decant the crystal violet, and rinse slide gently with running tap water. Avoid excessive rinsing in this step which may causes the crystal violet to be washed from the Gram-positive cells.

4.4.5 Rinse off excess water with iodine, and flood the slide with fresh iodine solution and allow the iodine to remain for 30 second.

4.4.6 Rinse gently with tap water.

4.4.7 Decolourize with 50/50 acetone alcohol by letting the Reagent flow over the smear for 1-5 seconds, depending on thickness of smear while the slide is held at an angle.

4.4.8 Remove excess decolourizer with gentle flow of tap water.

4.4.9 Flood slide with Safranin /dilute carbol fuchsin, and allow counter-stain to remain for 30 second.

4.4.10 Remove excess counter-stain with a gentle flow of tap water.

4.4.11 Drain slide and allow to air dry in an upright position, or use a commercial slide drier.

4.4.12 Examine the slide by low power to locate the area for examination and then move to high power examination.

4.4.13 Spend adequate time to examine the slide carefully examine the slide using an oil immersion objective to observe cell morphology and Gram reaction in details.

4.5.Post – analytical stage:

4.5.1. Results:

4.5.1.1 Gram Positive: stain deep blue/purple.

4.5.1.2 Gram Negative: stains pink/red.

4.5.2. Interpretation:

Gram Stain Codes	**Quantitation**	**Number per oil immersion field**
GPC = Gram positive cocci	(NS) (SC) (+) (++) (+++)	(0) <(5) (5-10) (10-25) (>25)
GPB = Gram positive bacilli	(NS) (SC) (+) (++) (+++)	(0) <(5) (5-10) (10-25) (>25)
GNC =Gram negative cocci	(NS) (SC) (+) (++) (+++)	(0) <(5) (5-10) (10-25) (>25)
GNB = Gram negative bacilli	(NS) (SC) (+) (++) (+++)	(0) <(5) (5-10) (10-25) (>25)
YST = Yeast	(NS) (SC) (+) (++) (+++)	(0) <(5) (5-10) (10-25) (>25)
Polymorphonuclear	(NS) (SC) (+) (++) (+++) (Or % for sterile fluids only E.g. CSF)	(0) <(5) (5-10) (10-25) (>25) (Or % for sterile fluids only E.g. CSF)
Mononuclear (restrict to sterile fluid specimens)	%	%
EPI = Epithelial cells	(NS) (SC) (+) (++) (+++)	<(5) (5-10) (10-25) (>25)
CLUE CELLS (restricted to vaginal specimens)	(NS) (SC) (+) (++) (+++)	<(5) (5-10) (10-25) (>25)

4.5.3. Reporting:

4.5.3.1 Records results of the quality control on the QC worksheet.

4.5.3.2 Check that the culture correlates with the Gram stain and if not matching re-check the Gram film and culture plates.

4.5.3.3 Test results should be transcribed into the computer and released.

4.5.4. Limitation of the test: The most common errors in gram stain are due to:

4.5.4.1 Smear preparations being too thick.

4.5.4.2 Excessive heat during fixation.

4.5.4.3 Low concentration of crystal violet.

4.5.4.4 Excessive rinsing between steps during the staining procedure. This could cause the step of the crystal violet or the dye-iodine complex to be washed off from the Gram positive cells.

4.5.4.5 Insufficient iodine exposure.

4.5.4.6 Prolonged decolourisation. Over-decolourising will lead to an erroneous result where Gram positive cells may stain pink to red indicating a Gram negative result, and under-decolourising will lead to an erroneous result where Gram negative cells may appear blue to purple indicating a Gram positive result. The degree of decolourising required is determined by the thickness of the smear.

4.5.4.7 Excessive counterstaining.

4.5.4.8 Uneven staining or decolourisation due to insufficient reagent being used for staining.

4.5.4.9 Decolourising step missed or need to increase time of decolourising step

5. Responsibilities:

5.1.Responsible staff:

- To ensure the adherence to critical result communication procedure
- To facilitate the alternative channels once needed

5.2.Quality manager /officer

- To follow up the implementation of the procedure

- To monitor regularly communication of critical results and raise non-conformance with corrective action once needed.

5.3.All lab staff:

- To adhere to the procedure.
- To document record and release results as recommended
- To report test failures or incident

6. Document History and Version Control:

Version	Description	Review Date
1	Initial Release	May 2026

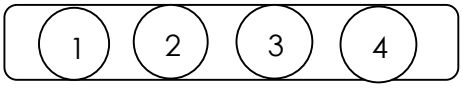
7. References:

Title of book/ journal/ articles/ Website	Author	Year of publication	Page
UK Standards for Microbiology Investigations Staining procedures	Standards Unit, National Infection Service, Public Health England	2019	17
Quantitative Gram Stain Interpretation Criteria Used by Microbiology Laboratories in Alberta, Canada, *Journal of Clinical Microbiology*, vol.38	DEIRDRE CHURCH, ELIZABETH MELNYK, and BARBARA UNGER	2000	4266–4268
Gram Stain protocols, *American Society for Microbiology*,	Ann C. Smith & Marise A. Hussey	2016	

8. Annexes: 8.1. Grams stain quality control record:

Gram stain reagents	Primary Stain		Gram Iodine		Decolourizer		Counter stain
Used Ingredients for Reagent preparation	Crystal violet	Ethanol / Methanol	Lugol's iodine	Potassium iodide	Acetone	Ethanol/ methanol (absolute)	Safranin or Basic Fuchsin
Reagent Lot no.							
Reagent Expiry Date							
Prepared By							
Reagent Quantity used							
Expiry Date Of Prepared Reagent							
Positive control							
Negative Control							
QC Status (Pass/Fail)							
QC Done by							
Comments							

Annexes 8.2. Blood culture gram stain – quality control record sheet Month/year……

QC slide: (1) GPC (2) GPB (3) GNB (4) GNC 

Date prep.	Morning shift				Afternoon shift				Night shift				Supervisor sign
	1	**2**	**3**	**sign**	**1**	**2**	**3**	**Sign**	**1**	**2**	**3**	**sign**	
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Gram QC slide must be stained daily with patient samples slides.