# Aim

To describe the preparation and use of stains in routine use in the microbiology laboratory.

# Principle

Microbiological stains make use of the principle of selectivity: certain groups of bacteria may be stained by virtue of their cell wall / membrane / intracellular characteristics. Use of the light microscope to view slides is discussed in Appendix 1.

# References

1. Health Protection Agency, UK SOP T39: Staining Procedures (Issue 1.1; October 2011).
2. Cheesbrough, M. District Laboratory Practice in Tropical Countries, Part 2. 2nd Edition Update (2006). Cambridge University Press.
3. Standard Operating Procedures from LOMWRU, SMRU and AHC.

# Gram’s stain

Gram’s stain differentiates bacteria into “Gram positive” or “Gram negative” based on their cell wall composition. Gram positive bacteria have a thick layer of peptidoglycan in comparison to Gram negative bacteria which only have a thin layer. After primary stain with crystal violet, iodine is added and forms a complex with the primary stain. During the decolourisation step, the acetone-alcohol interacts with lipid components of the bacterial cell membrane, and the outer membrane of the Gram negative bacteria is removed. Therefore, the crystal violet-iodine complex is lost in Gram negative bacteria, but retained by Gram positive bacteria, which stain violet or purple. The Gram negative organisms may then be detected with a counterstain (safranin).

## Method

In-house reagents are used (see Appendix 2 for preparation details).

Prepare a smear, allow to dry, and heat gently to fix (using the hotplate).

Place the slide on the staining rack with the smear side facing upwards.

Flood the slide with crystal violet and leave for 30 seconds.

Tilt the slide, pour on sufficient Lugol’s iodine to wash away the stain, cover with fresh iodine and allow to act for 30 seconds.

Tilt the slide and wash off the iodine with acetone-alcohol until colour ceases to run out of the smear (up to 10 seconds).

Rinse with water.

Pour on safranin and leave to act for about 2 minutes.

Wash with water and blot dry.

## Interpretation

Positive result: Gram positive organisms stain deep blue/purple.

Negative result: Gram negative organisms stain pink/red.

## Quality assurance

Smears prepared from known organisms (e.g. *S. aureus* ATCC25923 and *E. coli* ATCC25922) may be used to QC Gram reagents and staining procedures. QC should be performed weekly and whenever a new batch of stain is made.

## Limitations

Some Gram positive organisms (e.g. *Bacillus* spp., *Clostridium* spp., rapidly growing streptococci) may appear Gram variable or Gram negative.

Certain Gram negative organisms, especially *Haemophilus* spp., can easily be missed if the background is slightly pink.

Gram stain of old cultures may be easily over-decolourised.

# Ziehl-Neelsen stain for mycobacteria

Ziehl-Neelsen (ZN) stain renders mycobacteria pink (carbol fuchsin) as a result of the mycolic acid in their cell wall. Most other bacteria have a lower fat content and hence the carbol fuchsin is washed away by the acid-alcohol, resulting in their being stained blue by the counterstain (methylene blue).

## Method

Prepare a smear and allow to air dry for 15-30 minutes in the class II biosafety cabinet.

Fix the smear by passing the slide over a spirit flame 3-5 times or by flooding with methanol for 2 minutes.

Place the slide on the staining rack with the smear side facing upwards.

Flood the slide with 0.3% carbol fuchsin.

Heat with a flaming alcohol-soaked swab until steaming (DO NOT BOIL / ALLOW TO DRY OUT) and leave for 5 minutes.

Rinse with distilled water and flood with 3% acid alcohol (use 1% H2SO4 (not acid alcohol) for identification of *Nocardia* spp*.*) for 3 minutes.

Rinse again with distilled water: If the slide is still red, add more acid alcohol for 1-3 minutes and rinse again with distilled water.

Flood with 0.3% methylene blue for 30 seconds.

Rinse and dry.

## Interpretation

Positive result: acid fast bacilli (AFB) vary from 0.5-10 µm in length and stain red. Some may appear beaded.

Negative result: all other organisms and background material stain blue (if methylene blue counterstain is used).

## Quality assurance

Proven positive and negative smears or a commercial ZN QC slide (e.g. BD BBL AFB slide (cat no. 231931)) may be used for QC.QC should be performed weekly and whenever a new ZN staining kit is opened.

## Limitations

Ziehl-Neelsen’s staining is less sensitive than Auramine-phenol staining, but provides more morphological details. Care should be taken because spores and artefacts may stain with Ziehl-Neelsen’s stain and appear as positive to untrained eyes.

# Modified Ziehl-Neelsen for *Cryptosporidium* species

This technique is used for the demonstration of oocysts of Cryptosporidium species in faeces.

## Method

Prepare a medium to thick smear and air dry.

Fix in methanol for 3 minutes and air dry.

Flood the slide with modified Kinyoun’s acid fast stain (3% carbol fuchsin) and leave for

approximately 15 minutes.

Rinse with tap water.

Flood the slide with 1% acid methanol to decolourise and leave for 15-20 seconds.

Rinse with tap water.

Counterstain with 0.3% methylene blue and leave for 30 seconds.

Rinse with tap water and air dry.

## Interpretation

Positive result: *Cryptosporidium* species oocysts are pink, round or oval and 4-5 µm in diameter.

Negative result: parasite not detected.

## Quality assurance

Proven positive and negative specimens may be used as controls.

## Limitations

Care should be taken because spores and artefacts may stain with Ziehl-Neelsen’s stain and appear as positive to untrained eyes.

# Lugol’s iodine for parasites

1% Lugol’s iodine, when diluted, is used to stain ova and protozoan cysts in wet mounts to enhance their internal structures.

## Method

Mix a small amount of faeces with 1 drop of saline on the left hand side of the slide and one drop of the iodine reagent on the right hand side of the slide.

Cover with coverslip and examine.

## Interpretation

Positive result: protozoan nuclei take up the iodine and stain pale brown while cytoplasm remains colourless.

Negative result: parasites not detected.

## Quality assurance

Proven positive and negative specimens may be used as controls.

## Limitations

For this method to work effectively the 1% Lugol’s iodine solution should be a fresh preparation (10-14 days).

# India ink preparation

India ink (nigrosin) staining is a negative staining technique because the background is stained whereas the organisms remain unstained. Capsules displace the dye and appear as halos surrounding the organism. This technique is useful for demonstration of the capsule of *Cryptococcus neoformans* but can also be used to demonstrate the presence of bacterial and yeast capsules.

## Method

Place a drop of India ink on to a clean slide.

Add 1 drop of specimen or liquid culture or rub a small amount of material on the slide surface just beside the ink before mixing it into the ink.

Cover with a cover slip, press it down through a sheet of tissue paper so that the film becomes very thin and pale in colour, and examine.

## Interpretation

Positive result: organisms possessing a capsule appear highly refractile (bright), surrounded by a clear zone against a dark background.

Negative result: no clear zone around the organism is observed.

## Quality assurance

Proven positive and negative specimens (or cultured organisms) may be used as controls.

## Limitations

Use of the correct concentration of India ink is critical.

# **Lactophenol cotton blue stain for identification of moulds**

This stain is used to distinguish the microscopic morphology of cultured moulds (fungi).

## Method

Place one drop of Lactophenol cotton blue onto a clean slide.

Prepare a length of clear sellotape (similar length as the slide).

Gently overlay the sellotape on the fungal culture (if the culture is old overlay on the outer edge of the growth).

Place the sellotape over the slide so the Lactophenol blue covers the sellotape.

## Interpretation

Macroscopic and microscopic appearances should be compared to identification keys (e.g. PHLS “Identification of Pathogenic Fungi”).

## Quality assurance

None

## Limitations

Multiple preparations may be required to obtain a preparation of sufficient quality to enable identification.

Over vigorous application of sellotape often results in a preparation yielding only spores.

# Synopsis / Bench aids





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# Risk assessment

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| **COSHH risk assessment - University of Oxford COSHH Assessment Form** |
| **Description of procedure**Staining of specimens / cultures | **Substances used**Absolute methanol for fixationGram stain reagents (crystal violet, Lugol’s iodine, acetone-alcohol, safranin)ZN stain reagents (carbol fuchsin, 3% hydrochloric acid in isopropyl alcohol, methylene blue)Lactophenol blue |
| **Quantities of chemicals used**Small | **Frequency of SOP use**Daily |
| **Hazards identified**1. Potentially infectious material in sample 2. Stain reagents can cause burns, are harmful by inhalation, skin contact and ingestion | **Could a less hazardous substance be used instead?** No |
| **What measures have you taken to control risk?** 1. Training in good laboratory practices (GLP)2. Appropriate PPE (lab coat, gloves, eye protection)3. Handling of specimens or cultures is done in the Class II biosafety cabinet until the presence of HG3 organisms (e.g. *B.* pseudomallei) have been excluded3. Small amounts of stain are in use only (stocks kept in a closed chemical cupboard)4. Laboratory is well ventilated |
| **Checks on control measures**Observation and supervision by senior staff |
| **Is health surveillance required?**No | **Training requirements:**GLP |
| **Emergency procedures**:1. Report all incidents to Safety Adviser2. In event of any solution coming in contact with the eyes, immediately flush with running water for at least 15 minutes3. If contact with skin, remove contaminated clothing and wash skin thoroughly4. If any substance inhaled, move to fresh air and seek medical attention if any respiratory symptoms.5. If ingestion of any substance, do NOT induce vomiting. If conscious, drink copious amounts of water, and seek medical attention if any gastrointestinal symptoms | **Waste disposal procedures**:All stains are washed down the sink with copious amounts of water |

# Appendix 1. Microscopy procedure

## Oil immersion microscopy

A stained smear can only be examined by oil immersion microscopy.

## The microscope

Before starting, identify the following on the microscope:

* Objective lens for oil immersion: this has a black and white band and is marked 100x/1.25 oil.
* The focusing controls (coarse and fine).
* The tension control: if this is too tight the stage can-not be raised or lowered, too loose and it drops down under its own weight.
* Stage movement controls.
* Sub stage condenser: this needs to have its central hole in the light path and is racked to the top of its travel and then lowered 1/8//.
* Iris diaphragm: this can be opened / closed to improve contrast as required.
* Light control.

## Examining a Smear

* Switch on microscope and adjust the light to almost maximum.
* Lower the stage using the coarse focus.
* Put one drop of immersion oil onto the stained smear and place slide smear side uppermost onto the stage with a well-stained area in the light path.
* Watching carefully from the side, raise the stage using the coarse focus until the lens just touches the slide. Be careful or the slide may break.
* Looking carefully through the eyepiece use the coarse focus to rack downwards slowly until the material on the slide is in focus. The focal distance is short and the plane of focus on the slide will quickly flash into and out of focus if not careful.
* When the plane of focus is located adjust the fine focus, tension control, light, and stage movement as appropriate and carefully examine the slide.
* Never rack the stage upwards for any considerable distance whilst looking down the eyepiece as the slide will crack and the lens will be damaged.
* When finished, remove the slide and keep it in the slide tray at least until the end of the day.
* Clean the oil from the lens using lens tissue, switch off and place the protective cover over microscope.

# Appendix 2. Preparation of Gram stain reagents

## Crystal Violet

### Reagents

* Crystal violet powder
* Ammonium oxalate powder
* Absolute ethanol (or methanol)
* Distilled water

### Method

* Add 10g crystal violet to 47.5ml absolute ethanol (or methanol)
* Mix until dissolved
* Dissolve 4.5g ammonium oxalate in 100ml distilled water
* Add to the stain solution
* Make up to 500ml with distilled water
* Mix well
* Store at room temperature in a brown bottle
* Filter before use

## Lugol’s Iodine

### Reagents

* Iodine
* Potassium Iodide
* Distilled water

### Method:

* Wear an N95 mask and work in a well-ventilated room (iodine is toxic)
* Add 10g potassium iodide to a clean/dry brown glass bottle
* Add 200ml distilled water and mix thoroughly until the potassium iodide is *completely* dissolved
* Weigh out 5g iodine and add to the potassium iodide solution and mix well until the iodine is dissolved
* Make up to 500ml with distilled water
* Store in a dark place at room temperature: label the bottle “*toxic*”
* Prepare a new batch if the colour has faded

## Alcohol/Acetone Decolouriser

### Reagents

* Acetone
* 95% ethanol

### Method

* Mix 250ml acetone with 250ml 95% ethanol in a glass bottle
* Label the bottle “*Highly flammable*”
* Store at room temperature

## Safranin

### Reagents

* Safranin O powder
* 95% ethanol
* Distilled water

### Method

* **Stock solution**
	+ Dissolve 1.25g safranin O in 50ml 95% ethanol
* **Working solution**
	+ Dilute 50 ml stock solution with 450ml of distilled water
	+ Filter before use
	+ Store in a brown bottle