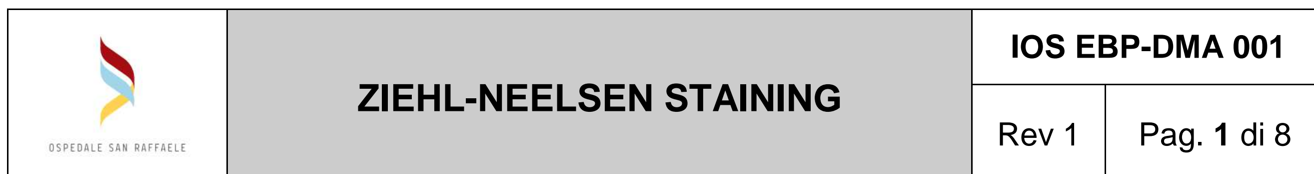



**Destinatari: Coordinatore, Tecnici e Studenti del Settore Micobatteri - EBP**

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## 1. SCOPE

This instruction describes the Ziehl–Neelsen (ZN) staining technique for the detection of acid-fast bacilli (AFB) by microscopy.

## 2. APPLICATION

The ZN staining technique is used with ordinary bright-field microscopes. The present instruction is applicable within the whole Advanced Mycobacterial Diagnosis (DMA) area and whenever positive cultures or possible sources of contamination must be checked out for the presence of mycobacteria.

## 3. DEFINITIONS AND ABBRAVIATIONS

*Microscope magnification:* individual objective magnification x eyepiece magnification

AFB: Acid-Fast Bacilli
QC: Quality Control
ZN: Ziehl–Neelsen method
TB: Tuberculosis
USP: U.S. Pharmacopeia

## 4. RESPONSIBILITIES

Supervision and the correct application of the following instruction is the responsibility of the area coordinator. The execution of the test is responsibility of area technicians, master students and coordinator, while the reading of the slides and the reporting are a coordinator's and technician's duties.

## 5. EQUIPMENT AND MATERIALS

### *5.1 General materials*

- √ Quaternary ammonium compounds jar (only if a loop is used, not needed with disposable sticks).
- √ Wooden swabs
- √ Diamond pencil or lead pencil (if frosted-end slides are available)
- √ Filter paper, small, appropriate for funnel size
- √ Funnels, small, for filtering solutions in use (when necessary)
- √ Forceps
- √ Lens paper or soft tissue paper
- √ Plastic containers for waste disposal
- √ Disposable loops or wooden sticks
- √ Microscope, electric light source, mechanical stage, 100x objective, 10x eyepiece (see Annex 1)
- √ Immersion oil for microscopy
- √ Slide staining rack
- √ Slide boxes
- √ Slides supports
- √ New, clean slides
- √ Timer

- √ Absolute methanol; denatured alcohol
- √ Beaker for rinsing water
- √ Sink and water supply
- √ Oil-absorbing paper
- √ Disinfectant solutions

### *5.2 TB Ziehl-Neelsen staining solutions (Becton and Dickinson – Code 212520)*

a) 250 ml bottle TB Carbolfuchsin ZN (Approximate formula per liter)

| | | |
|---|---|---|
| Basic Fuchsin | 1.7 | g |
| Phenol, USP | 50.0 | g |
| Isopropanol | 95.0 | ml |
| Distilled Water | 905.0 | ml |

b) 250 ml bottle TB Decolourizer (Approximate formula per liter)

| | | |
|---|---|---|
| Hydrochloric acid | 30.0 | ml |
| Ethanol/Methanol | 970.0 | ml |

c) 250 ml bottle Methylene Blue (Approximate formula per liter)

| | | |
|---|---|---|
| Methylene Blue, USP | 2.4 | g |
| Ethanol/Methanol | 300.0 | ml |
| Distilled Water | 700.0 | ml |

## 6. PROCEDURES

### *6.1 Principle*

The property of acid-fastness is based on the presence of mycolic acids in the cell wall of mycobacteria. Primary stain (fuchsin) binds to cell-wall mycolic acids. Intense decolourization (strong acid or acid/alcohol) does not release the primary stain from the cell wall and the mycobacteria retain the red colour of fuchsin – hence acid-fastness. Counterstaining (with methylene blue) provides a contrasting background. While mycobacteria are AFB, very few other bacteria possess the property of weak acid-fastness. They include *Nocardia spp, Rhodococcus spp*, cysts of *Cryptosporidium spp.* or *Isospora spp.* spores.

### *6.2 Specimen*

Ziehl-Neelsen staining is performed on the following biological samples:

#### *6.2.1 Sputum*

- Spontaneous sputa

  Sputa from suspects should be rejected only if they are liquid and clear as water, with no particles or streaks of mucous material. However, they should be accepted if the patient cannot produce a better specimen on a repeated attempt. Sputa from follow-up patients should be accepted and examined even if they look like saliva, since these patients often cannot produce mucoid specimens.

- Induced sputa

  These specimens resemble saliva but have to be processed as adequate specimens.

- Decontaminated sputa, concentrated by centrifugation.

*6.2.2 Other specimens*

- Laryngeal swabs, gastric lavages, bronchial washings, brushings and transtracheal aspirates.
- Urine and urine from sovra-pubic puncture.
- Body fluids (spinal, pleural, pericardial, synovial, fluids from ascites, blood, pus, bone marrow).
- Tissue biopsies.
- All the above mentioned specimen after decontamination (when necessary), and concentration by centrifugation.

### *6.3 Detailed instructions*

*6.3.1 Preparation of smears*

- Disinfect the working area with an adequate disinfectant solution.
- Label the slides properly with a lead pencil using the Country Database or any unique identification number
- Place each slide in front of the corresponding 50ml-Falcon Tube used previously for the concentration step, including digestion and decontamination for non sterile biological material (see IOS EBP-DMA 004)
- Proceed to smearing, taking the labelled slides and opening containers one by one;
  - for a direct sputum smear, select a small portion of purulent or mucopurulent material with the stick/loop and transfer it to the slide;
  - if a smear is prepared after specimen decontamination, the concentrated material must be transferred to the slide with a sterilized loop to avoid splashing.
- Spread the material carefully over an area equal to about 2–3 cm x 1–2 cm using repeated circular movements, without touching the edge of the slide. Make the smear as even as possible by continuing this process until no thick parts remain. The thickness of the smear should be such that a newspaper held under the slide can barely be read through the dried smear.
- Disinfect the working area after smear preparation.
- Let the smears air-dry at room temperature under the safety cabinet; do not use heat to speed the drying.
- When dry, take the slides and put them on the staining rack 1 cm apart and place them under the chemical hood if the methanol fixing method is chosen
- Fix them by one of these options: cover the slides with absolute methanol and let them stand 10 minutes and then let them air-dry or pass them three times quickly through the flame of a Bunsen burner, smear upwards. Do not overheat or AFB will be poor. Do not use the burner under the chemical hood, but on the workbench nearby.
- Take the fixed slides on the staining rack and put them under a chemical hood in order to start staining
- Always keep smears out of direct sunlight.

*6.3.2 Staining method*

- Place the slides, smear upwards, on the staining rack over a basin, about 1 cm apart.
- Prepare the wooden cotton swabs dipping the cotton part in denatured alcohol. Use a lighter to light them up apart from any kind of flammable solutions or potential source of fire.

- Flood with TB Carbolfuchsin ZN. Heat gently to steaming and allow to steam for 5 min. Do not allow the staining solution to dry on the slides – add fresh solution as required.
- Using forceps, tilt each slide to drain off the staining solution.
- Rinse the slides well with distilled water or clean tap water from a beaker (not directly from the tap).
- Decolourize with TB Decolourizer with two changes of reagent 1-2 minutes until no more red colour appear in washing.
- Wash gently in running water from a beaker
- Counterstain with TB Methylene Blue for 30 seconds
- Using forceps, tilt each slide to drain off the methylene blue solution. Rinse the slides well with distilled water or clean tap water from a beaker (not directly from the tap).
- Using forceps, take each slide from the rack and let the water drain off. Stand the slide on an absorbent paper and let them air dry.
- Once dried, remove the slides from the absorbent paper and put them on a slide support in order to be transported out to the microscopy room (Room n. 24)

**Note:** According to manufacturer’s package insert, the staining protocol may vary in terms of time of exposure of the slides to staining solutions or decolourizers.

### *6.4 Quality Control*

- Include positive and negative controls with each batch's reading. Read control slides before other smears.
- If results are unacceptable, re-stain smears of that day together with new controls, paying attention to correct technique; if these controls are also unacceptable, change new staining solutions, repeat the staining and detail all in the “Registro Non Conformità” (EBP 004)

**Be aware!** Among the possible reasons for *false-positive results* are:

- re-use of containers or positive slides;
- contaminated staining solutions;
- use of scratched slides;
- AFB floated off one slide and became attached to another during the staining procedure because there was no space between adjacent slides;
- inadequate decolourization;
- lack of experience, confusion with artefacts;
- microscope (lamp) in poor condition or poorly adjusted: interpreting glitter as AFB;
- poor quality of staining solutions.

Among the possible reasons for *false-negative results* are:

- poor quality of specimen;
- not taking proper portion of specimen for smear preparation;
- excessive decolourization;
- too little time staining with Carbol fuchsin;
- over-staining with methylene blue;
- overheating during fixing;
- reading less than one length;
- slide exposed to daylight for too long;
- too long an interval between staining and reading, particularly if slides were poorly stained or not kept in the dark.

## 7. RECORDING AND REPORTING

### *7.1 Reading (see Annex 1)*

- Set the variable voltage regulator to minimum and switch the power on.
- Slowly adjust the light until the desired intensity is reached.
- Ensure that the lenses, mirrors and other light-conducting surfaces are clean
- Turn the coarse adjustment knob to move the stage away from the objective lens.
- Place a stained slide on the stage, smear upwards.
- Rotate the nosepiece to the 10x objective and adjust the light intensity as required.
- Adjust the inter-pupillary distance until the right and left images merge.
- Focus the image with the right eye by looking into the right eyepiece and adjusting with the fine focus knob.
- Focus the image with the left eye by looking into the left eyepiece and turning the dioptre ring.
- Open the condenser iris diaphragm so that the field is evenly lit (about 80% open).
- Turn the coarse focus knob to bring the 10x objective lens close to the slide; *do not allow the objective lens to touch the smear*.
- While looking into the eyepieces, slowly turn the coarse focus knob to separate the objective lens and the stage. The smear should come into focus within a few turns. Then turn the fine focus knob until the smear is seen most clearly. *Always use the focusing adjustment knobs to lower the stage away from the lens.*
- Place a drop of immersion oil on the smear; do not touch the slide with the oil applicator but allow the drop of oil to fall freely onto it. Then rotate the 100x objective into place. *It is a professional error to focus directly with this objective.*
- With parfocal lenses, the immersion objective will now be in the oil; if not lower it slightly until it just touches the oil (looking from the side).
- Raise the condenser as high as possible. Increase the brightness of the light until the field is well-lit but still comfortable for the eye.
- Focus by adjusting with the fine focus knob. Use a maximum of one turn in one direction; if this is not successful, repeat in the other direction.
- Scan the stained smear systematically from left to right side, covering one length (100–150 microscopic high-power fields, depending on the length of the smear – 2 or 3 cm). This is the minimum that must be scanned before reporting a negative result; the process should take about 5 minutes. Count AFB in positive smears for quantification. Always search for useful areas, i.e. those containing mucous threads and pus cells; do this by moving up or down when arriving at an almost empty area, until another useful zone has been found, then continue moving to the right.

  Acid-fast bacilli appear bright red against the background material counterstained in blue. Report as positive for AFB when the background is bluish and at least one red AFB is seen in a well de-stained smear (even if the AFB may be mycobacteria other than tubercle bacilli). Tubercle bacilli are quite variable in shape, from very short fragments to elongated types. The typical appearance is usually of rather long and slender, slightly curved rods. They may be uniformly stained or with one or many gaps, or even granular. They occur singly or in small groups, and rarely in large clumps.

- Once the smear has been read, rotate the 100x objective away, without changing focus, and remove the slide.
- Place the slide smear-down on a piece of absorbent paper (e.g. folded toilet paper, newspaper) to soak up the oil; do not move the slide once it is on the absorbent paper.
- Take the next slide, put a drop of oil on one end of the smear, fix the slide on the stage, turn the 100x objective into the oil and continue reading. If slides are of the same thickness, focusing this (and subsequent) smears should require only slight adjustment of the fine focusing knob.

- When all slides have been examined, reset the voltage regulator control to minimum and turn the power off.
- Store the slides in a slide box in order and mark the identification number and position in the paper insert of each slide box;
- Clean the objective lens at every new reading by lean on absorbent lens paper.
- Keep the slide in the dark inside the slide box in microscopy room.

### *7.2 Recording*

Two reporting methods can be used relying on the meaning why this method has been asked for. The recording for preliminary diagnosis from direct or concentrated specimen is the following semi-quantitative grading:

| Finding | Recording |
|---|---|
| No AFB found in at least 100 fields | Negative (NEG) |
| 1–9 AFB per 100 fields | exact figure/100 |
| 10–99 AFB per 100 fields | + |
| 1–10 AFB per field (count at least 50 fields) | ++ |
| More than 10 AFB per field (count at least 20 fields) | +++ |

The recording for the detection of contamination or for processes that do not require such a precise semi-quantification is the following:

| Finding | Recording |
|---|---|
| No AFB found in at least 100 fields | Negative (NEG) |
| 1-9 AFB per 100 fields | Scanty |
| More than 9 AFB seen | Positive (POS) |

### *7.3 Reporting*

Results must be reported in the specific Country Database and in the Workflow Summary module (EBP-SM 004)

- For a positive result: report quantification of AFB seen. (It should not be assumed that AFB are tubercle bacilli.)
- Never report “No TB” (or equivalent wording).

## 8. RELATED DOCUMENTS

Angra P et al. Ziehl-Neelsen staining: strong red on weak blue, or weak red under strong blue? *International Journal of Tuberculosis and Lung Disease*, 2007, 11:1160–1161.

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## 9. ANNEXES

*Annex 1*