REVISED NATIONAL TUBERCULOSIS CONTROL PROGRAMME (RNTCP)

MANUAL FOR LABORATORY TECHNICIANS

Central TB Division, Directorate General of Health Services
Ministry of Health and Family Welfare, Nirman Bhavan, New Delhi 110 011

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1 Aims of sputum microscopy

The aims of sputum microscopy are to:

1. Diagnose patients with infectious tuberculosis
2. Monitor the progress of tuberculosis patients who are on treatment.

2 Advantages of sputum microscopy

- **More reliable diagnostic tool than X-ray for the diagnosis of infectious TB**
- **Simple to perform**
- **Easy to read**
- **Minimal infrastructure required to set up a Microscopy Centre**
- **Inexpensive**
- **Quick results**
- **Necessary to monitor patient progress and to declare the patient as "cured"**

3 Layout of the Microscopy Laboratory

A Microscopy Laboratory should have areas to:

- Receive sputum samples
- Prepare and stain sputum smears
- Examine slides under the microscope
- Temporarily store contaminated materials
- Record results in the Laboratory Form and Laboratory Register
- Store boxes containing positive and negative slides
- Store slides, reagents, forms and other materials.

4 When to collect sputum samples

For diagnosis of tuberculosis, 3 sputum examinations (SPOT — MORNING — SPOT) are performed. For follow-up of a case of tuberculosis, 2 sputum examinations (MORNING — SPOT) are performed. Patients taking different categories of treatment have their sputum examined for follow-up at different times depending on the categories of treatment and results of the first follow-up specimen (see Table 1).

Table 1: Schedule of sputum examinations

Category of treatment	Schedule of follow-up sputum examinations
Smear-positive Category I	At the end of 2, 4 and 6 months of treatment
Smear-positive Category I (If sputum-positive at the end of Month 2)	At the end of 2, 3, 5 and 7 months of treatment
Smear-positive Category II	At the end of 3, 5 and 8 months of treatment
Smear-positive Category II (If sputum-positive at the end of Month 3)	At the end of 3, 4, 6 and 9 months of treatment
Smear-negative Category I or Category III	At the end of 2 and 6 months of treatment

5 How to collect sputum samples

Receive the patient and Laboratory Form

Check Laboratory Form for completeness and accuracy

The Laboratory Technician (LT) can help patients by showing genuine concern and patience. Emphasise that diagnostic facilities and treatment are free and that *tuberculosis (TB) can be cured simply by taking regular and complete treatment as prescribed*.

Record the Laboratory Serial No. on the Laboratory Form and sputum container

Laboratory Serial No.: When the patient comes for diagnosis, all of his 3 sputum samples are given one Laboratory Serial No. When the same patient comes for sputum examination at the end of 2 months, both follow-up samples are given a single new Laboratory Serial No. When the patient comes for the next follow-up sputum examination, both his samples are given another new Laboratory Serial No. **In brief, each patient is given one**

distinct Laboratory Serial No. for each set of samples he submits. The Laboratory Serial No. begins with 1 on 1 January each year and increases by one with each patient until 31 December of the same year.

Write the Laboratory Serial No. on the Laboratory Form, and **on the side of the sputum container, never on the lid.** This is because the lid from one container may be placed on another container, causing incorrect labelling of specimens. Write the Laboratory Serial No. clearly on the container using a permanent marker.

Obtain the sputum specimen from the patient

Give the patient the sputum container with the Laboratory Serial No. written on the side of the container. Demonstrate to the patient how to open and close the container and explain the importance of not rubbing off the number written on the side of the container.

A good sputum container is

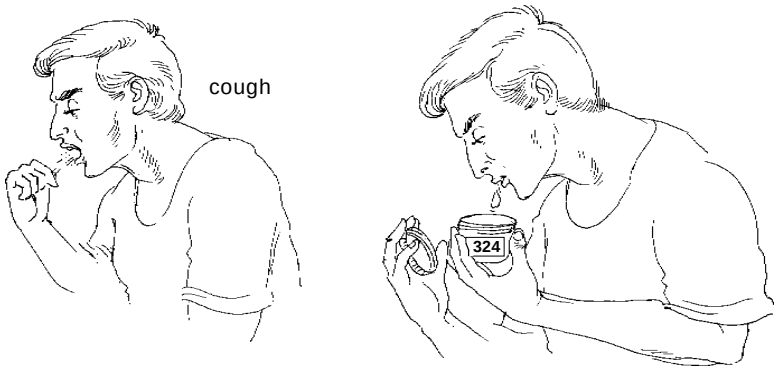
- **Disposable**
- **Clean**
- **Wide-mouthed**
- **Unbreakable**
- **Easily burnt**
- **Made of clear thin plastic**
- **Leak-proof**
- **Provided with tight-fitting lid**

Explain the difference between sputum and saliva, and the importance of bringing out sputum for examination and make sure the patient understands. **All sputum containers should be used only once**.

Spot samples

Give the patient the labelled container, and bring him to the nearby open space far away from other people, and then instruct him by demonstrating with actual actions to:

- Inhale deeply 2–3 times;
- Cough out deeply from the chest;
- Open the container, bring it close to the mouth and bring the sputum out into it;
- Not give saliva or nasal secretions;
- Close the container.



Correct method of bringing out sputum

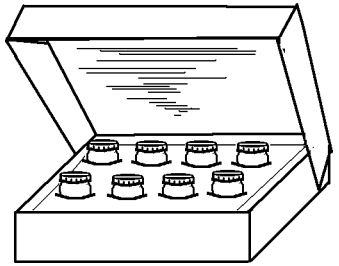
Before the patient leaves the laboratory, visually examine the sputum sample for quality. If the sample is only saliva, ask the patient to cough again until a good quality sample is obtained. A good quality sample may require repetition of the procedure several times.

Give the patient another container with the same Laboratory Serial No. written on its side for an early morning specimen. Repeat the above instructions for bringing out sputum, **adding that the patient should rinse his/her mouth with plain water before bringing up the early morning sputum specimen.** This is to keep the sample free of food particles. Food particles can appear like AFB under the microscope and can give a false-positive result.

Transportation of the sputum

If there is no microscopy centre easily accessible to the patient, sputum can be collected in plastic sputum containers and transported to the nearest designated microscopy centre. For this purpose, a wooden box of appropriate size with space to lodge sputum containers tightly can be made locally.

Wooden box with sputum containers

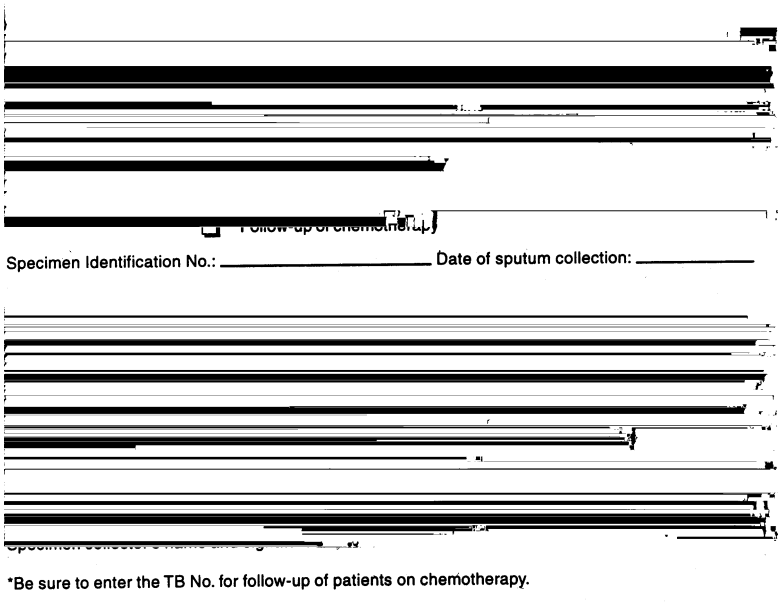


The sputum should be:

- collected in a clean container
- kept in a cool place or in a refrigerator until transported
- protected from excessive heat and direct sunlight
- sent to the nearest designated microscopy centre preferably by the next day and definitely within one week.

The Specimen Identification No. in all such cases is given only by health workers and others who are collecting sputum specimens and transporting the containers to the microscopy centre for examination. However, all patients including these patients, will have a Laboratory Serial No.

The upper portion of a Laboratory Form for sputum examination is reproduced below:


Specimen Identification No.: _____ Date of sputum collection: _____

*Be sure to enter the TB No. for follow-up of patients on chemotherapy.

Assess and record visual appearance of the sample

A good sputum sample is:

- **thick (semi-solid), coughed out deeply from the lungs;**
- **purulent (yellowish mucus);**
- **sufficient in amount (at least 2 ml).**

A poor quality sputum sample:

- **contains only saliva (watery fluid) or nasal mucus;**
- **is small in quantity (less than 2 ml).**

Make sure the sputum sample is of good quality for microscopic examination. A good sample increases the chances of detecting AFB.

The portion of the Laboratory Form where this information must be entered is reproduced on the next page. Indicate the visual appearance by writing M, B or S in the appropriate column. Make sure the Laboratory Serial No. on the form matches the Laboratory Serial No. on the container.

RESULTS (To be completed in the laboratory)

Lab Serial No: _____

Microscopy

Date	Specimen	Visual appearance (M, B, S)*	Results**	Positive (grading)			
				3+	2+	1+	Scanty
[redacted]							

6 How to prepare slides for examination

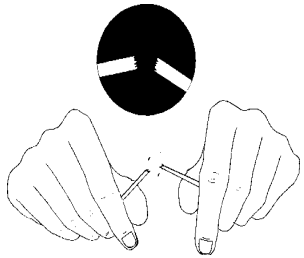
Write the Laboratory Serial No. on the slide with a diamond marker.

Arrange the specimen containers in serial order. Ensure that the Laboratory Serial Nos. on the sputum containers match the Laboratory Serial Nos. on the accompanying Laboratory Forms.

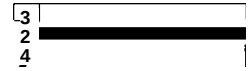
Select new, clean, grease-free, unscratched slides, and be careful not to leave fingerprints on the slide. Inscribe the Laboratory Serial No. with a diamond marker on one end of the slide.

Step 1 **Spread sputum on the slide using a broomstick**

Remove the lid of the container and inspect the sample for solid, purulent or blood-stained particles. Ensure that the appearance of the sample has been noted on the Laboratory Form.

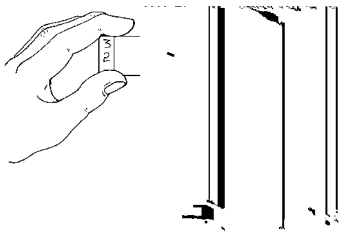


- Break a broomstick (wooden/ bamboo) in two halves with uneven ends.



- Ensure that the number on each slide corresponds to the number on the specimen container.

- Using the jagged ends of the broken stick, select and pick up the larger, yellow, purulent portion and transfer them onto the slide. **Use a separate stick for each sample.**



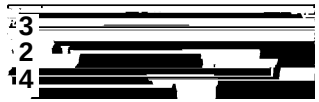
- With one of the sticks, spread the sputum evenly to cover 2/3 of the central portion of the slide, using a continuous, rotational movement as shown here.
- Place the applicators (broken wooden sticks) into a bucket containing disinfectant.
- Place the smeared slide on the drying rack and replace the lid of the sputum container.

The size of the smear should be approximately 3x2 cm. The smear should neither be too thick nor too thin.

A good smear is

- **Made from mucopurulent sputum**
- **Spread evenly**
- **3 cm × 2 cm in size**
- **Not too thick**
- **Thin enough to read newsprint through**
- **Air-dried before being fixed**

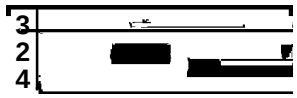
Example of a good smear



A bad smear is

- **Made from saliva**
- **Too small**
- **Too big**
- **Uneven**
- **Not in the centre of slide**
- **Too thick**
- **Too thin**

Examples of bad smears



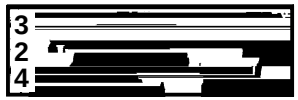
Too small

Not in centre

Too big

Too thick

Uneven



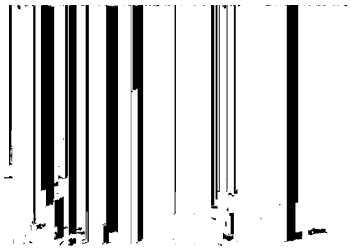
Too thin

Step 2 **Let the slides air-dry for 15–30 minutes.**

Do not use flame for drying.

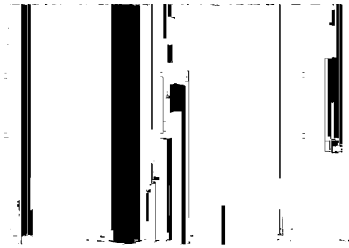
Do not dispose of the specimens until all smears have been examined and results entered.

Step 3 **Fix the dry slide by heating it briefly**

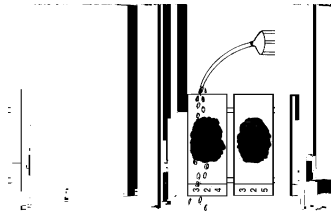


- After the slide is dry, hold the slide using forceps with the smeared side facing upwards.
- Pass the slide over the flame 3–5 times, for 3–4 seconds each time.
- Do not heat the slide for too long or keep it stationary over the flame.
- Place the slide in the clean slide tray.

Step 4 **Stain the slides with carbol fuchsin**



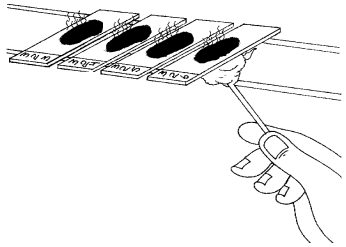
- Place the slides in serial order on the staining rack with the smeared sides facing upwards. Leave space between the slides so that they do not touch each other.



- Pour 1% carbol fuchsin to cover the entire surface of the slide. If the carbol fuchsin solution drains off, pour more to cover the entire slide.
- Do not leave the carbol fuchsin on the slide for a long time. (5 minutes is sufficient time.)

- **Do not allow the carbol fuchsin to drain off the slide.**
- **Do not leave the carbol fuchsin on the slide for a long time or it will dry.**
- **Add more carbon fuchsin if required.**

Step 5 **Heat the slides with the carbol fuchsin on them**

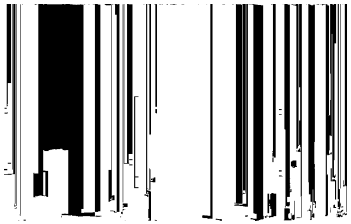


- Heat the slides from underneath until vapours start rising.

- **Do not allow the carbol fuchsin to boil.**
- **Do not keep the flame stationary under the slide.**

Step 6 **Allow the slides to stand for 5 minutes**

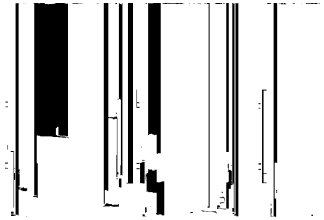
Step 7 **Rinse the slides**



- Gently rinse the slides with tap water to remove excess carbol fuchsin stain.

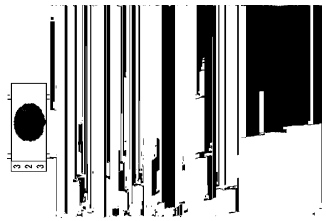
- Tilt the slides to drain off excess water.

  At this point the sputum smears on the slides should look red in colour.



Step 8 **Decolourize the stained slides**

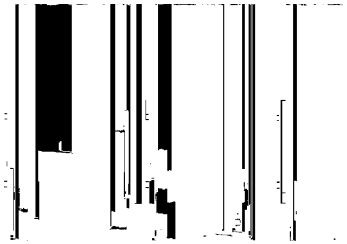
- Pour 25% sulphuric acid onto the slides and let it stand for 2–4 minutes.



- The red colour should have almost completely disappeared from the smears.

Step 9 Gently rinse away excess stain

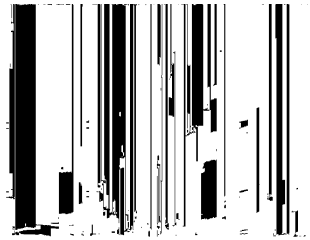
- **Lightly** wash away sulphuric acid and excess stain with tap water making sure that the smear itself is not washed away.



- Tilt the slide to drain off the water.

If the slide is still red, re-apply sulphuric acid for 1–3 minutes until the red colour disappears from the smear and repeat Step 9.

Step 10 Counterstain with 0.1% methylene blue



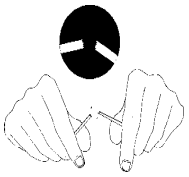
- Pour 0.1% methylene blue onto the slide.
- Let the stain stand for 30 seconds.

- Gently rinse the slide with tap water.

- Tilt the slide to drain off the water and allow to air dry.

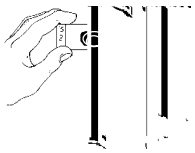
Key steps in the preparation and staining of smears

Step 1



Break a broomstick into two

Pick up the large, yellow purulent portion of sputum

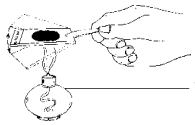


Spread evenly onto 2/3 of central portion of the numbered slide

Step 2

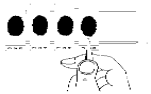
Air-dry the slide for 15–30 minutes

Step 3

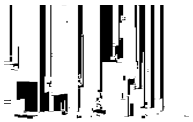


Fix the dry slide by heating briefly 3–5 times for 3–4 seconds each time

Step 4

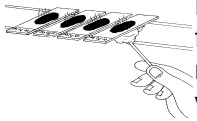


Place the slides in serial order on the staining rack



Stain the slides with 1% carbol fuchsin

Step 5

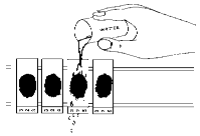


Heat the slides from underneath until vapours rise

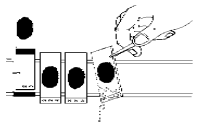
Step 6

Let the slides stand for 5 minutes

Step 7

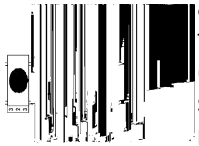


Rinse the slides with tap water



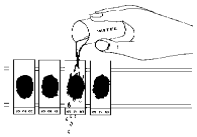
Drain off excess water

Step 8

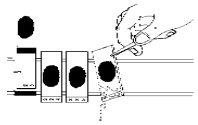


Decolourize with 25% sulphuric acid and let it stand for 2–4 minutes (repeat, letting stand for 1–3 minutes, if necessary)

Step 9

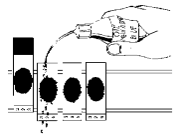


Rinse away excess stain with tap water

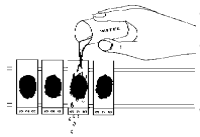


Drain off the water

Step 10



Counterstain with 0.1% methylene blue and let stand for 30 seconds



Gently rinse the slides with tap water, drain the water off, and allow the slide to dry

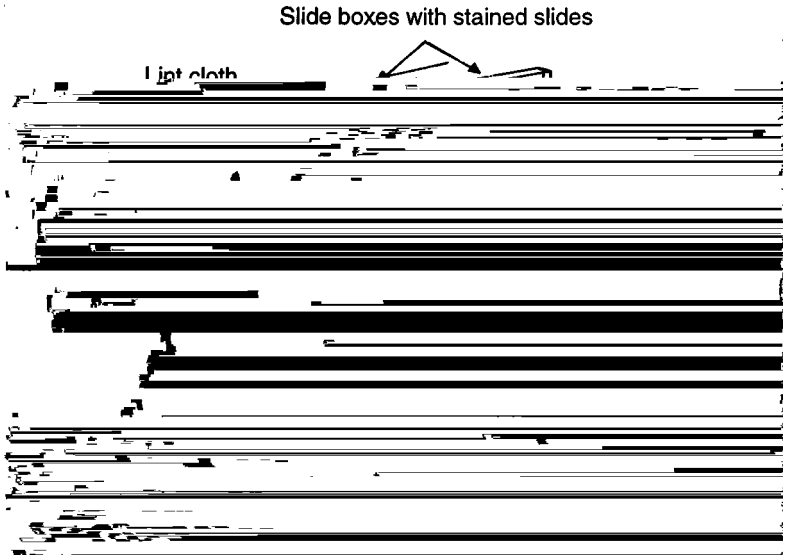
Examine the slides under the microscope

7 How to examine slides under the microscope (see Annexure II)

- **Never examine a slide while it is wet. Examining a wet slide may damage the microscope.**
- **Do not dry the wet slides on a blotting paper.**

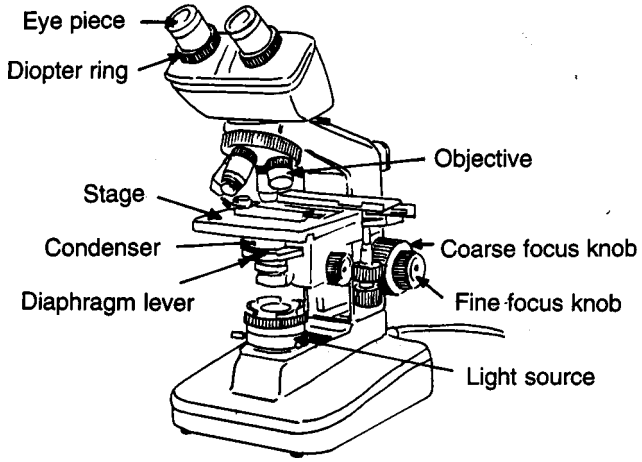
Keep all materials ready

Microscope (preferably binocular) with x40 and x100 lenses and eye piece (usually x5, x10 or x15);



Set up the microscope

- Remove the microscope from the box only at the time of use. The microscope should be kept in the box when not in use.
- Carefully place the microscope on the table.
- Refer to the diagram on the next page to locate specific parts of the microscope. (*Italicized* words are labelled in the diagram.)
- Using lens paper, **gently** clean the lenses (*objectives, eye piece* and *condenser*).
- Clean the remaining exposed non-lens parts of the microscope with a fresh piece of lint cloth.
- Raise the *condenser* to its uppermost position.
- Bring the x40 objective into position.
- Place a stained slide on the *stage* and look through the eye piece to adjust the *light source* for optimal light.



Focus with x40 and then x100 lens

Using the x40 lens, find a suitable area of the slide to examine. The selected area should not be too thick or too thin and should have more pus cells than epithelial cells (see Annexure III).

Add one drop of immersion oil

Place one drop of immersion oil on the stained smear**.**

Never let the immersion oil applicator touch the slide.

Focus with the x100 lens

Slowly change to the x100 lens. The oil will make a thin film between the x100 lens and the slide.

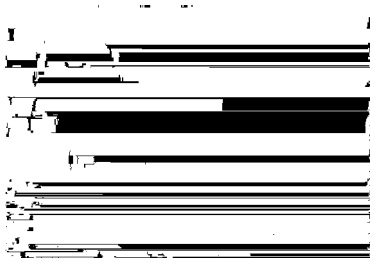
Never let the lens touch the slide.

Use only the fine adjustment knob with the x100 lens.

Examine at least 100 microscopic fields. For a skilled microscopist, this will take at least 5 minutes.

The examination must be systematic and standardized. Begin examining the slide at the left end of the smear. By slight adjustments of the fine focusing knob, systematically examine each field, beginning at the periphery of the field and ending at the centre of the field.

After examining one microscopic field, move the slide longitudinally so that the neighbouring field can be examined. In this manner all the microscopic fields from the beginning to the end of the length of the slide should be examined, as shown in the accompanying diagram on the next page. Move the slide a small distance vertically, then read a second length, from right to left.



Search for and identify tubercle bacilli, which look like thin red rods and are slightly curved. They can appear isolated, in pairs, or in clumps, and they stand out clearly against the blue background (Annexure III).

Count the number of AFB and record the results as: 3+, 2+, 1+, scanty, or negative, as given in Table 2. If 1–9 bacilli are found in 100 oil immersion fields, examine another 100 oil immersion fields.

Table 2: Grading of AFB smears

Examination	Result	Grading	No. of fields to be examined
More than 10 AFB per oil immersion field	Positive	3 +	20
1–10 AFB per oil immersion field	Positive	2 +	50
10–99 AFB per 100 oil immersion fields	Positive	1 +	100
1–9 AFB per 100 oil immersion fields	Scanty	Record exact number seen	200
No AFB per 100 oil immersion fields	Negative	—	100

Smear grading is done for clinical and epidemiological purposes, to find out the load of infection.

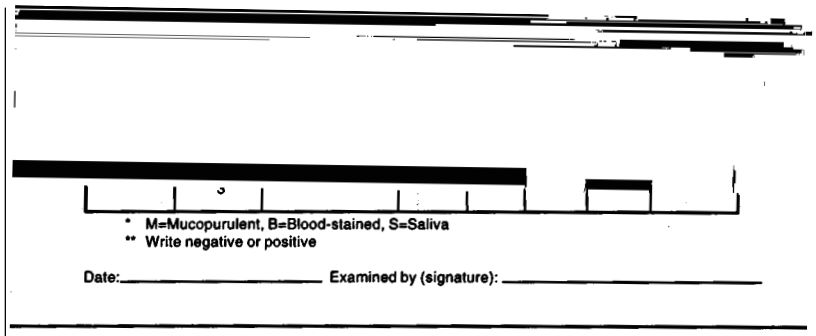
After the slides have been examined, open the sputum containers and put them in a metal bucket which has a foot-operated lid and which contains a sufficient quantity of 5% hypochlorite or 5% phenol so that all containers are fully immersed.

8 How to record and report results

Verify that the Laboratory Serial No. on the slide is the same as that on the Laboratory Form and record the results on the Laboratory Form

Make sure the upper portion of the Laboratory Form is complete and accurate. Fill in the results of smear examination in the lower portion of the form. Refer to Table 2 on page 14 for result and grading.

Write the date on which the report was made and sign the form. The relevant portion of the Laboratory Form is reproduced in the adjacent column:


* M=Mucopurulent, B=Blood-stained, S=Saliva
** Write negative or positive

Date:_____ Examined by (signature): _____

The completed form (with results) should be sent to the Health Centre to record the results on the Treatment Card.

Write results from the Laboratory Form in the Laboratory Register

Record results from the Laboratory Form in the appropriate columns of the Laboratory Register. In ***Reason for examination***, make a tick (✓) under the column "***Diagnosis***" for specimens examined for diagnosis. **Write the patient's TB No. in the "Follow-up" column for specimens examined after the initial diagnosis**. For patients examined for diagnosis, record the TB Number (when known) in the 'Remarks' column.

Every result MUST be entered into the Laboratory Register regardless of where the patient lives or is being treated. **All positive results should be written with red ink.**

Example of a correctly filled page in a Laboratory Register

REVISED NATIONAL TUBERCULOSIS CONTROL PROGRAMME

Laboratory Register

Year 1996

Lab Serial No.	Date	Name (in full)	Sex M / F	Age	Complete address (for new patients)	Name of Referring Health Centre	Reason for Examination*		Results			Signature	Remarks
							Diagnosis	Follow-up	1	2	3		
101	4/9	Parvathi Sinha	F	16	1964, Gali Paranthe Wali, Chandni Chowk	PHI 237	✓		1+	Scanty	1+	Joshi	TB No. 239 Cross-checked Ram, STLS
102	4/9	Lakshmi Kumari	F	46	223, Gandhi Dham Bapu Nagar	PHI 101	✓		2+	2+	1+	Joshi	TB No. 243 Cross-checked Ram, STLS
103	4/9	Lakshmi Pati Rao	M	50	As above	PHI 101	✓		3+	1+	2+	Joshi	TB No. 241 Cross-checked Ram, STLS
104	4/9	Kailash Nath	M	35	225, Gandhi Dham	PHI 101	✓		3+	2+	1+	Joshi	TB No. 247 Cross-checked Ram, STLS
105	4/9	Bhola Ram	M	32	Bapu Nagar 1704, Gali Gobi Wali	PHI 237	✓		2+	1+	Neg	Joshi	TB No. 253 Third smear is 1+ Ram, STLS
106	4/9	Man Bahadur Lal	M	52	Near Mandir 25A, Tilonia	PHI 237		96	Neg	Neg		Joshi	
107	4/9	Lallan Parsad Parmar	M	51	217, Gali Akara	PHI 237	✓		1+	Neg	Neg	Joshi	TB No. 271 Cross-checked Ram, STLS
108	4/9	Kiran Kumar	M	37	Near Rivoli 15, Gulmohar Park	PHI 237	✓		Neg	Neg	Neg	Joshi	
109	4/9	Srinivasa Rao	M	36	WB 2451, Gali Pathan	PHI 237	✓		Neg	Neg	Neg	Joshi	TB No. 314 Cross-checked Ram, STLS
110	4/9	Nanda Kumar	M	24	Wali, Loni Village 54, Khan Market	PHI 237	✓		Neg	Neg	Neg	Joshi	

* If sputum is for diagnosis, put a tick (✓) mark in the space under “Diagnosis”.
If sputum is for follow-up of patients on treatment, write the patient’s TB No. in the space under “Follow-up”.

Send the Laboratory Form with results to the Treatment Centre

Send the completed Laboratory Form back to the treating physician promptly. If the patient was referred from another health unit, give a copy of the completed Laboratory Form to the patient and send the original to the treating physician at the referring health unit. The patient's treatment depends on these results, and any delay reduces the value of all the work done to prepare and report a smear correctly.

Never give the results only to the patient. If the patient fails to bring the results to the Medical Officer or treatment centre, he may not receive treatment.

Clean and store the microscope and slides

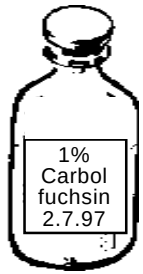
Clean the slides with xylene and preserve them in the appropriate slide box for the supervisor to review. Xylene will not damage the slide or stain, facilitating neat and clean slide storage. Pour a small amount (2–3 ml) of xylene onto the stained side of the slide, and then allow it to air dry. Do not clean too vigorously or the stain itself may come off. All positive slides should be preserved in one box, and negative slides in a different box.

Clean the x100 microscope lens with lens paper. Use xylene if necessary. If the lens is not cleaned it will be damaged. **Never use methylated spirit to clean the lens.** Keep the microscope back in its box, away from dust and vibration.

9 How to prepare stains and reagents

Preparation of 1% carbol fuchsin

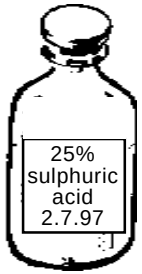
- Weigh 5 grams of basic fuchsin dye in a balance and transfer it to 250 ml Ehrlenmeyer glass flask.
- Add 50 ml of methylated spirit and shake to dissolve the dye.
- Heat 25 grams of phenol to melt it and add it to the above solution.



- Heat the flask containing basic fuchsin dye dissolved in spirit and phenol gently in a water bath at about 60 ºC. **Do not heat directly on a flame**.
- Transfer the contents into a 500 ml measuring cylinder.
- Add distilled water to make up a final volume of 500 ml.
- Pour the solution through filter paper (Whatmann No. 1) and store filtered solution in a glass bottle. Label the bottle as 1% carbol fuchsin and date of preparation.

Any time particles start to form in carbol fuchsin solution, the solution must be filtered again.

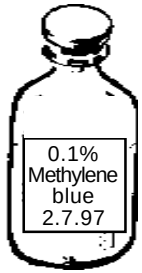
Preparation of 25% sulphuric acid



- Pour 375 ml of distilled water into a 1 litre glass flask.
- Measure 125 ml of concentrated sulphuric acid and transfer it slowly into the flask containing water.
- Always add acid to water. Never add water to acid.
- Store the sulphuric acid solution in a labelled glass bottle.

Preparation of 0.1% methylene blue solution

- Weigh 0.5 grams of methylene blue and transfer to a 1 litre glass flask.
- Add 500 ml of distilled water.
- Shake well to dissolve.
- Store in a glass bottle with the label showing name of the reagent and date of preparation.



After each batch of reagents is made, slides known to be positive and negative should be stained as internal quality control of the reagents.

10 How to ensure an adequate and continuous supply of stains, reagents, and other materials

Approximate quantities of solutions and reagents needed for 1,000 slides are given in Table 3. Solutions should be

Do not reuse old slides for AFB staining. AFB smear-negative slides may be used in other programmes such as malaria and filaria microscopy and haematology.
AFTER THE SUPERVISOR HAS CROSS-CHECKED
THE POSITIVE SLIDES THESE MUST BE BROKEN AND DESTROYED.

made to last no more than one month, and should be made at the district or subdistrict level. Solutions must be protected from light. **If particles have started to form in the carbol fuchsin, filter it again.**

With experience, the actual quantity of reagents needed on a monthly basis will be known.

Table 3: Estimated quantity of reagents required for 1000 smears

Stain/Reagent	For 1,000 smears
Carbol Fuchsin (1%)	5,000 ml
Sulphuric acid (25%)	6,000 ml
Methylene blue (0.1%)	3,000 ml

Various other items which will be needed for sputum microscopy are given in Table 4.

Table 4: Other items needed for sputum microscopy

Item	For 1,000 smears
Filter paper (Whatmann No. 1, packs of 100)	3 packs
Lens paper (books of 50 leaves)	2 books
Fine silk or lint cloth 15 cm × 15 cm	5
Diamond marker (or grease pencils)	1 (or 5)
Sputum containers	1,100
Wooden applicators	1,100
New glass slides	1,100
Immersion oil	50 ml
Xylene	2000 ml

11 How to dispose of contaminated materials safely

Sputum specimens examined in the laboratory are potentially infectious and after examination these must be disinfected and destroyed so that risk of infection is avoided. **All disposable containers are used only once. Positive slides should never be used again and should be destroyed.**

After the smears are examined, remove the lids from all sputum cups and put the cups and removed lids in a bucket containing 5% hypochlorite or 5% phenol solution. The cups and lids should be fully submerged in the solution. Similarly, used wooden sticks should also be put in the same bucket containing 5% hypochlorite or 5% phenol solution. The bin/bucket should have a foot-operated lid. Thereafter, the used sputum cups, lids and wooden sticks can be disposed of by any of the following methods:

1. Autoclaving in an autoclave or in a pressure cooker. At the end of the laboratory work the sputum cups and the removed lids, along with wooden sticks can be placed in a pressure cooker of approximately 7 litre capacity containing adequate amount of water to submerge the contents and boiled for at least 20 minutes using any heating source, electrical or non-electrical. After proper cooling the material can be discarded with other waste.

Keep Your Laboratory Safe
Prevent Spread of Infection

- **Wash your hands with soap and water frequently.**
- **Disinfect all infected materials (e.g. bamboo sticks, sputum containers) before discarding.**
- **Clean laboratory bench tops with a disinfectant (5% phenol) at the end of the day.**
- **Do not eat, drink or smoke in the laboratory area.**
- **Before disposing of positive slides, break them.**

2. If autoclaving cannot be done, use chemicals such as freshly prepared 5% hypochlorite solution or 5% phenol. Caps of the sputum cups must be removed and the cups, caps and wooden sticks submerged in the solution in a secure place overnight. After this, the solution, cups, caps and wooden sticks can be discarded with other waste.

3. As a last resort, if none of the above is available, sputum cups, caps and wooden sticks can be burnt in a pit at a safe distance away from inhabited areas.

12 How to ensure quality of sputum microscopy

Quality control of sputum microscopy is an indispensable part of effective TB control. Quality control involves supervising the processes of sputum collection, smear preparation, smear staining, microscopy and recording. Each step in the process should be reviewed.

The LT preserves all slides after examination. The supervisor cross-checks all the smear-positive slides and 10% of the smear-negative slides selected at random by using the last digit of the Lab Serial No. Discrepancies should be noted in the “Remarks” column of the Laboratory Register and reviewed with the LT. The supervisor should discuss discrepant slides with the LT and provide specific recommendations. The LT should implement the recommendations and make appropriate changes.

The supervisor can also specifically examine negative diagnostic slides of patients who have been placed on treatment, and negative follow-up slides from patients whose initial smears were positive. These two types of slides are more likely to contain errors (false-negative results) than randomly selected slides. If needed, the LT should be given a refresher training course.

All positive and an equal number of negative slides are carried by the STLS to the District TB Centre every month, and a sample of these is sent to the assigned State TB Training and Demonstration Centre for cross-checking.

ANNEXURE I
Care of the Microscope

The microscope is the lifeline of the Revised National Tuberculosis Control Programme. Proper handling and maintenance of the microscope, particularly of its lenses, is very important. The following points should be observed:

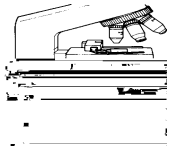
1. Place and store the microscope in a dry, dust-free and vibration-free environment.

- Vibration damages the microscope.
- When the microscope is not being used, cover or keep it in the box so as to keep it free from dust.
- Avoid exposing the microscope to direct sunlight.
- Avoid exposing the microscope to moisture. Humidity may allow fungus to grow on the lens and cause rusting of the metal parts.
- Put plenty of dry blue silica gel into a shallow plate and place it in the box when the microscope is kept in it. Silica gel is blue in colour when it is dry but when it becomes wet it turns pinkish. As soon as the silica gel becomes pink, change or heat it until it turns blue again and then reuse it.

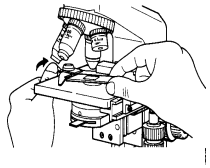
2. Keep the microscope and lenses clean.

- Clean the microscope with lens paper before and after use.
- Do not leave immersion oil on the surface of the immersion lens.
- Never use spirit or alcohol to clean the lenses, as these can damage them.
- Never let the oil immersion lens touch the smear.
- Use the fine focusing knob only while using the oil immersion lens.
- All the lenses should be cleaned with dry lens paper. Lens paper can be moistened with xylene if necessary. Do not clean lenses with an ordinary cloth.

ANNEXURE II
How to Use the Microscope

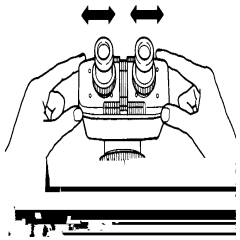


- Switch on the light (adjust the mirror if electric light source is not available).



- Place the slide on the stage.

- Raise the condenser as high as possible. Focus with ×40 lens by turning the coarse focusing knob.



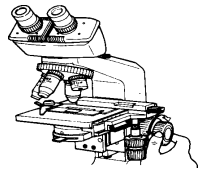
- Adjust the distance between the eyepieces until both the right and left images become one.

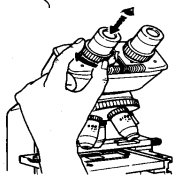
- Focus the image.

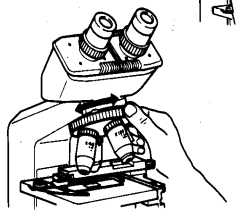
- By turning the diopter ring focus the image with the left eye looking into the left eyepiece.

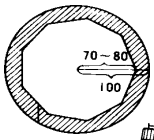
- Change to ×100 lens.

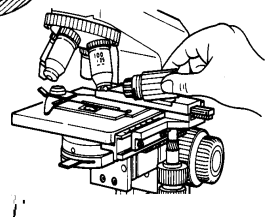
- Open the diaphragm to 70–80% of the objective's numerical aperture.
- Put immersion oil on the slide and use the fine adjustment knob only to focus the image.
- Systematically examine the slides as described in Section 7 on page 13.











ANNEXURE III
Colour Plates of AFB

Fig. 1. Many AFB in a sputum smear. Quality of the sample selected is good, as evidenced by the presence of many pus cells. The smear is well stained, showing good colour contrast. (Zeihl-Neelsen stain, examined at ×1000.)

Fig. 2. Clump of AFB seen in a sputum smear. (Zeihl-Neelsen stain, examined at ×1000.)

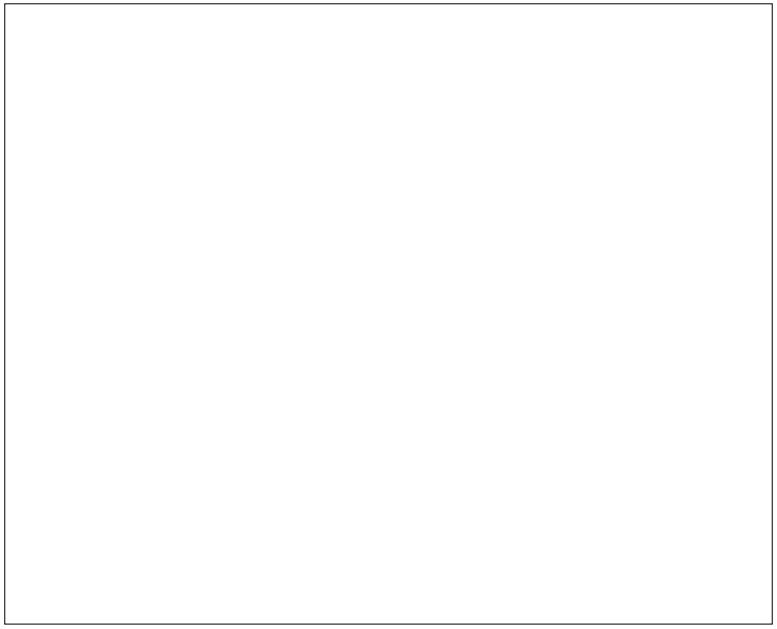


Fig. 3. A well stained, properly selected field, negative for AFB. If at least 100 fields are negative, a slide should be considered negative. (Zeihl-Neelsen stain, examined at ×1000.)

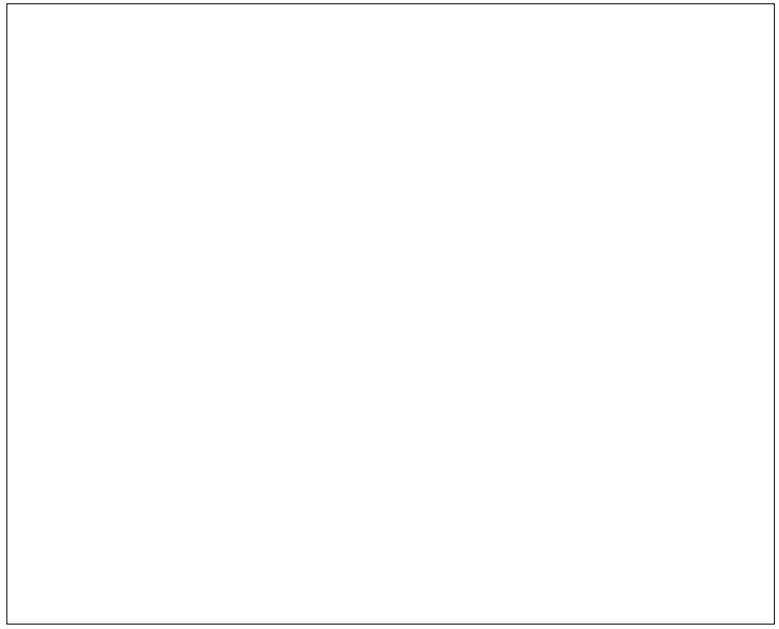


Fig. 4. Sputum smear which has been underdecolourized. There is no evidence of counter stain. No comments can be made on the presence or absence of AFB in such smears. (Zeihl-Neelsen stain, examined at ×1000.)

ANNEXURE IV
Prevention and Consequences of False-positive and False-negative Sputum Results

HOW TO PREVENT FALSE-POSITIVE SPUTUM RESULTS

- **Always use new, unscratched slides**
- **Use a separate wooden stick for each sample**
- **Always use filtered carbol fuchsin**
- **Do not allow the carbol fuchsin to dry during staining**
- **Decolourize adequately with sulphuric acid**
- **Make sure there are no food particles or fibres in the sputum sample.**
- **Never allow the oil immersion applicator to touch a slide**
- **Never allow the oil immersion lens to touch a slide**
- **Label sputum containers, slides, and Laboratory Forms accurately**
- **Cross-check the number on the Laboratory Form and sputum container before recording**
- **Record and report results accurately**

Consequences of false-positive sputum results

- Patients are begun on treatment unnecessarily.
- Treatment is continued longer than necessary, in the case of follow-up examinations.
- Medications will be wasted.
- Patients may lose confidence in the Programme.

HOW TO PREVENT FALSE-NEGATIVE SPUTUM RESULTS

- **Make sure the sample contains sputum, not just saliva**
- **Make sure there is enough sputum (at least 2 ml)**
- **Select thick, purulent particles to make the smear**
- **Prepare smears correctly—not too thick, too thin or too little material**
- **Fix the slide for the correct length of time, not too short or too long**
- **Stain with carbol fuchsin for the full 5 minutes**
- **Do not decolourize with sulphuric acid too intensively**
- **Examine every smear for at least five minutes before recording it as negative**
- **Label the sputum containers, slides and Laboratory Forms carefully**
- **Cross check the number on the Laboratory Form and sputum container before recording**
- **Record and report results accurately**

Consequences of false-negative sputum results

- Patients with TB may not be treated, resulting in suffering, spread of TB and death.
- Intensive phase treatment may not be extended for the required duration, resulting in inadequate treatment.
- Patients may lose confidence in the Programme.

ANNEXURE V
Troubleshooting Guide for Microscopy

Problem	Possible Causes	Solution
Field is dim	Condenser may be too low	Raise the condenser
	Condenser iris may be closed	Open the diaphragm
Dark shadows in the field which move when eye piece is moved	Eye piece may be dirty	Clean the eye piece
	Eye piece or objective may be contaminated with fungus	Eye piece may need repair
	Surface of eye piece may be scratched	A new eye piece may be needed
The image is not clear	The smeared portion of the slide may be upside down	Turn the slide over
	There may be an air bubble in the oil	Move the $\times$100 lens from side to side
	The oil may be of poor quality	Use only good quality immersion oil
	There may be dirt on the lens	Clean the lens
The image through low power is not clear	There may be oil on the lens	Clean the lens
	There may be dust on the upper surface of the lens	Clean the lens
	The lens may be broken	A new lens may be needed

ANNEXURE VI
Job Responsilities of Laboratory Technicians in the Revised National Tuberculosis Control Programme

1. Sputum collection

- Demonstrate to patients how to bring out good quality sputum.
- Label the sputum container properly.
- Before the patient leaves, check the sample to see if it is sputum or only saliva.

2. Sputum processing and examination

- Write the Laboratory No. and visual appearance of the sputum on the Laboratory Form.
- Always use new slides.
- Spread the smear and heat it in order to fix it on the slide.
- Stain the smear by the Ziehl-Neelsen method.
- Examine the stained smear under the microscope.

3. Recording and reporting

- Enter the result of each microscopic examination on the Laboratory Form and in the Laboratory Register.
- Maintain the Laboratory Register properly, including the reason for sputum examination.
- Send the Laboratory Form with results recorded to the treating physician promptly.

4. Quality control

- Preserve all positive and negative slides until they are reviewed by the Supervisor.

5. Safety

- Keep the laboratory clean.
- Do not eat, drink, or smoke in the laboratory.
- Safely dispose of all contaminated materials including sputum cups.
- Break all positive slides after they have been cross-checked by the supervisor.

6. Material management

- Keep the microscope in good working condition.
- Prepare and store solutions and reagents properly.
- Order supplies well in advance to avoid shortages.
- Use freshly prepared reagents.

Wash hands every time you handle contaminated material

ANNEXURE VII
Job Responsibilities of Senior TB Laboratory Supervisors (STLS) in the Revised National Tuberculosis Control Programme

1. Organize smear examination at the microscopy centres of the sub-district

- Maintain a list of all microscopy centres in the sub-district which carry out TB activities, including distribution (map of the area) and staff responsible (name, position and address).
- Arrange for and provide coverage of microscopists in case of leave, so that there is regular and permanent availability of smear examination facility at each microscopy centre.

2. Ensure the quality of sputum microscopy

- Supervise the microscopy centres at least once a month and perform quality control of slides, recording the number of slides checked and the proportion of discordance for positive and negative slides. Assess the reasons for discordance and take remedial measures to ensure that mistakes are not repeated. Arrange for re-training if essential.
- Check the Laboratory Register and compare the number of patients having sputum smears examined with the general outpatient attendance in the health facilities.
- Maintain a diary recording the details of all field visits.

3. Ensure the smooth functioning of laboratory services

- Ensure that microscopes are maintained in good working order.
- Prepare and distribute reagents and ensure regular and sufficient supply of reagents and sputum containers in each health facility.
- Ensure proper storage and transport of sputum specimens.
- Prepare and forward reports on microscopy to the DTO regarding implementation, quality control, supervision, and management of laboratory supplies as per schedule.
- Ensure safety of laboratory staff.

4. Organize regular training and continuing education of laboratory technicians

5. Motivate, coordinate, facilitate and guide all microscopists of the area

6. Perform all job responsibilities of the Laboratory Technician as and when required

REVISED NATIONAL TUBERCULOSIS CONTROL PROGRAMME

Laboratory Form for Sputum Examination

Name of Health Centre: _____ Date: _____

Name of patient: _____ Age: _____ Sex: M ☐ F ☐

Complete address: _____

_____

Patient's TB No.*: _____

Source of specimen: ☐ Pulmonary

☐ Extra-pulmonary Site: _____

Reason for examination: ☐ Diagnosis

☐ Follow-up of chemotherapy*

Specimen Identification No.: _____ Date of sputum collection: _____

Specimen collector's name and signature _____

*Be sure to enter the TB No. for follow-up of patients on chemotherapy.

RESULTS (To be completed in the laboratory)

Lab Serial No: _____

Microscopy

Date	Specimen	Visual appearance (M, B, S)*	Results**	Positive (grading)			
				3+	2+	1+	Scanty
	1						
	2						
	3						

* M=Mucopurulent, B=Blood-stained, S=Saliva

** Write negative or positive

Date: _____ Examined by (signature): _____

The completed form (with results) should be sent to the Health Centre to record the results on the Treatment Card.

ANNEXURE IX

REVISED NATIONAL TUBERCULOSIS CONTROL PROGRAMME

Laboratory Register

Year _____

Lab Serial No.	Date	Name (in full)	Sex M / F	Age	Complete address (for new patients)	Name of Referring Health Centre	Reason for Examination*		Results			Signature	Remarks
							Diagnosis	Follow-up	1	2	3		

* If sputum is for diagnosis, put a tick (✓) mark in the space under “Diagnosis”.
If sputum is for follow-up of patients on treatment, write the patient’s TB No. in the space under “Follow-up”.

Microscopy is key to the diagnosis and cure of TB patients.

Every slide represents a patient's life and the health of his family.

Clean the lens when you have finished reading the slides so that the microscope remains in good working condition.

Never use spirit or alcohol to clean the lens.

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