

Protocol for processing specimens for culture

Note: The LJ medium containing pyruvate is needed only if *M. bovis* is suspected.

DEFINITIONS AND ABBREVIATIONS

MW	molecular weight
NALC	<i>N</i> -acetyl- <i>L</i> -cysteine
CPC	cetylpyridinium chloride
BSC	biological safety cabinet
ID	patient's specimen identification, usually laboratory number
LJ	Löwenstein–Jensen
RCF	relative centrifugal force

EQUIPMENT AND MATERIALS NEEDED

- BSC, with exhaust air ducted or vented to the outside.
- Safety Bunsen burner, with device to light on demand, or micro-incinerator.
- Microscope slides.
- Refrigerated centrifuge with safety shield, a minimum RCF of 3000g and an operating temperature of 8 - 10 °C. (Not necessary for the Kudoh method described below.)
- Centrifuge tubes, clear plastic or thick-walled glass tubes, with covers, resistant to >3000g RCF and ideally of 50-ml capacity. (Not necessary for the Kudoh method described below.)
- Rack for tubes.
- Pipettes for 1.0 ml, preferably sterile, single-use, plastic, Pasteur pipettes (with graduation).
- Pipetting aids.
- Mortar or blender (for biopsy).
- Sterile forceps (for swabs).
- Disinfectants.
- Separate autoclavable waste containers for pipettes and disposables
- Autoclave.
- Buckets, stainless steel or polypropylene.
- Vortex mixer.
- Timer.
- Incubator.
- General laboratory glassware.
- Refrigerator.
- Decontamination reagents and solutions.
- McFarland standard solution.

REAGENTS AND SOLUTIONS

The necessary reagents and solutions are indicated for each method described below. Avoid use of previously opened reagent bottles. Use aliquot stock solutions, one vial per specimen.

SPUTUM PROCESSING – DETAILED STEPWISE INSTRUCTIONS

Sputa should not be processed in batches of more than 6–8 because the following methods are strictly time-dependent; larger batches cannot be handled in time except in the trisodium phosphate and CPC procedures. For these less time-dependent methods, the first step can be performed in microscopy laboratories, outside a BSC; transport to a laboratory of biosafety level 2 (BSL-2) must then be organized within 1 day for the trisodium phosphate method and within 1 week for the CPC method.

Decontamination using Sodium hydroxide (modified Petroff) method

NaOH is toxic, both for contaminants and for tubercle bacilli; strict adherence to the indicated timings is therefore required.

Reagents

1. Sodium hydroxide (NaOH) solution, 4%
2. Phosphate buffer, 0.067 mol/litre, pH 6.8

Procedure

1. Transfer the sputum (at least 2 ml, not more than 5 ml) into a centrifuge tube. Add an equal volume of 4% NaOH.
2. Tighten cap of centrifuge tube and vortex mix to digest.
3. Let stand for 15 minutes at room temperature.
4. Fill the tube to within 2 cm of the top (e.g. to the 50-ml mark on the tube) with phosphate buffer. Vortex mix.
5. Centrifuge at 3000g for 15 minutes.
6. Carefully pour off the supernatant into a discard can containing 5% phenol or other disinfectant.
7. Inoculate the deposit onto two slopes of LJ medium (and one slope of LJ with pyruvate if needed), labelled with the ID number. Using a pipette (not a loop), inoculate each slope with 3–4 drops (=0,2-0,4 ml). Smear on a slide (marked with the identity number) for microscopic examination.

Decontamination using NALC–NaOH

The mucolytic agent *N*-acetyl-*L*-cysteine (NALC) enables the decontaminating agent, sodium hydroxide, to be used at a lower final concentration. Sodium citrate is included to bind the heavy metal ions that may be present in the specimen and could inactivate NALC. The method is suitable for inoculation in liquid media.

Reagents

1. Sodium hydroxide, 4%
2. Trisodium citrate·3H₂O, 2.94%
3. *N*-acetyl-*L*-cysteine (NALC)
4. NALC–NaOH solution, freshly prepared for daily use only:
 - mix (1) and (2);
 - add 0.5 g NALC just before use;
 - aliquot in 4-ml amounts.
5. Phosphate buffer, 0.067 mol/litre, pH 6.8

Procedure

1. Transfer the sputum (at least 2 ml, not more than 5 ml) into a centrifuge tube. Add an equal volume of NALC–NaOH solution.
2. Tighten the cap of the tube and shake or vortex-mix for no more than 20 seconds.
3. Keep at 20–25 °C for 15 minutes for decontamination.
4. Fill the tube to within 2 cm of the top (e.g. the 50-ml mark on the tube) with phosphate buffer or distilled water. Vortex mix.
5. Centrifuge at 3000g for 15 minutes.
6. Carefully pour off the supernatant into a discard can containing 5% phenol or other disinfectant.
7. Resuspend the deposit and inoculate onto two slopes of LJ medium (and one slope of LJ with pyruvate if needed) or into liquid medium. Using a pipette (not a loop), inoculate each slope with 3–4 drops (0.2–0.4 ml). Smear on a slide (marked with the ID number) for microscopic examination.

Decontamination using trisodium phosphate

Interestingly, the timing of this process is not critical for the viability of tubercle bacilli and it is therefore especially suitable for simultaneous preservation, transportation and decontamination of sputum specimens collected at distant locations.

Reagents

Trisodium phosphate solution, 10%

Procedure

1. Transfer the sputum into a centrifuge tube. Mix with an equal volume of trisodium phosphate solution.
2. Tighten the cap of the tube and let stand at room temperature for 12–18 hours.
3. Fill the tube to within 2 cm of the top (e.g. the 50-ml mark on the tube) and vortex-mix.
4. Centrifuge at 3000g for 15 minutes.
5. Carefully pour off the supernatant into a discard can containing 5% phenol or other disinfectant.

6. Inoculate the deposit onto two slope of LJ medium (and one slope of LJ with pyruvate if needed). Using a pipette (not a loop), inoculate each slope with 3–4 drops. Smear onto a slide, marked with the ID number, for microscopic examination.

Decontamination using 5% oxalic acid

This method is suitable for sputum or respiratory samples from patients who are likely to be colonized with *Pseudomonas aeruginosa*, such as those with cystic fibrosis and bronchitis.

Reagent

5% oxalic acid
(or Kit OxATM Oxalic Acid Reagent Kit (Alpha-Tec Systems) ref Biocentric 048-05)

Procedure

1. Transfer the sputum into a centrifuge tube. Add an equal volume of 5% oxalic acid.
2. Tighten the cap of the tube. Shake to digest.
3. Allow the acid to act at room temperature for 15 minutes. Shake intermittently to aid homogenization and decontamination.
4. Fill the tube to within 2 cm of the top (e.g. the 50-ml mark on the tube) with sterile water or buffer. Vortex-mix.
5. Centrifuge at 3000g for 15 minutes.
6. Carefully pour off the supernatant into a discard can containing 5% phenol or other disinfectant.
7. Inoculate the deposit onto two slopes of LJ medium (and one slope of LJ with pyruvate if needed). Using a pipette (not a loop), inoculate each slope with 3–4 drops. Smear onto a slide, marked with the ID number, for microscopic examination.

Decontamination using CPC/NaCl method

This soft decontamination method is a means of digesting and decontaminating specimens in transit. Duration of transportation should not exceed 7 days, otherwise the probability of survival of TB bacilli is low.

CPC is used to decontaminate the specimen. CPC is bacteriostatic for mycobacteria and this effect is not neutralized in the digestion process. The centrifugation step is needed for CPC removal, and sediments should be inoculated to egg-media only and not in liquid media.

Centrifugation must be carried out at room temperature because CPC precipitates at lower temperatures.

Reagent

1% CPC–2% NaCl solution

Procedure

1. Add an equal volume of CPC–NaCl solution to the specimen. Transport to a processing laboratory *within 7 days*.

2. Transfer the liquefied specimen into a centrifuge tube (if not delivered in one).
3. Fill the tube to within 2 cm of the top (e.g. the 50-ml mark on the tube) with sterile water. Vortex-mix.
4. Centrifuge at 3000g for 15 minutes.
5. Carefully pour off the supernatant into a discard can containing 5% phenol or other disinfectant.
6. Inoculate the deposit onto two slope of LJ medium (and one slope of LJ with pyruvate if needed). Using a pipette (not a loop), inoculate each slope with 3–4 drops. Smear onto a slide, marked with the ID number, for microscopic examination.

Precautions during processing of CPC-containing samples:

The specimens should be processed according to the collection date (older specimens first).

Decontamination using the simple culture method (modified Kudoh method)

The simple culture method (modified Kudoh method) is not technically demanding (no centrifugation, no neutralization) but less sensitive than processing methods that use centrifugation.

Reagent

Sodium hydroxide, 4%

Procedure

1. Transfer the specimen into a screw-capped container and add an equal volume of sodium hydroxide solution.
2. Tighten the cap of the container. Vortex mix for no more than 20 seconds.
3. Keep at 20–25 °C for 15 minutes for decontamination.
4. Inoculate directly onto two slopes of Kudoh modified Ogawa medium. Using a pipette (not a loop), inoculate each slope with 3–4 drops. Smear onto a slide, marked with the ID number, for microscopic examination.

SPECIMENS OTHER THAN SPUTUM

Processing of laryngeal swabs

Smear examination is not done for laryngeal swabs.

1. Culture swabs on the same day that they are received.
2. Use sterile forceps to transfer the swab to a sterile centrifuge tube.
3. Add 2 ml of sterile distilled water.
4. Decontaminate.
5. Before adding the phosphate buffer solution, remove the swab from the tube with forceps.

6. Fill the tube to within 2 cm of the top (e.g. the 50-ml mark on the tube) with phosphate buffer, 0.067 mol/litre, pH 6.8. Mix the contents by inversion.
7. Centrifuge at 3000g for 15 minutes.
8. Carefully pour off the supernatant into a discard can containing 5% phenol or other disinfectant.
9. Inoculate deposit on two slopes of LJ medium (and one slope of L-J with pyruvate if needed) or in liquid medium. Using a pipette (not a loop), inoculate each slope with 3–4 drops.

Processing of gastric lavages

Gastric lavage specimens should be processed as soon as possible after collection.

MIND that collection has to be performed in a tube containing 100 mg of sodium bicarbonate

Proceed as for sputum.

If the specimen is watery, centrifuge at 3000g for 15 minutes, pour off the supernatant, resuspend the sediment in 5 ml of sterile distilled water and proceed as for sputum.

Processing of other body fluids

Mucopurulent materials

Handle as for sputum when the volume is 10 ml or less. Handle as for mucoid gastric lavage when the volume is more than 10 ml.

Fluid materials

If the specimen has been collected aseptically, centrifuge and inoculate sediment directly onto culture media, preferably liquid medium.

If the specimen was not aseptically collected:

- handle as for sputum when the volume is 10 ml or less;
- handle as for fluid gastric lavage when the volume exceeds 10 ml.

Warning: *To maximize the recovery rate, the entire CSF volume (or other small volume of aseptically collected fluid) should be cultured, preferably in liquid medium.*

Processing of tissue

If a biopsy needs to be processed for smear and culture, it is necessary to homogenize the biopsy in a sterile porcelain mortar or preferably in a small non-reusable tissue grinder with 2-5 ml of sterile saline.