

Concentration of Mycobacteria in Clinical Samples using the Thermo Scientific General Purpose Centrifuge with 8x50 mL Individually Sealed Rotor

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KEY WORDS

- *M. tuberculosis*
- Sputum
- Clinical Samples
- General Purpose Centrifuge
- 8x50 mL Individually Sealed Rotor
- 50 mL Conical Tubes

Introduction

Tuberculosis (TB), a contagious and airborne disease affecting mostly young adults, remains a major global public health problem. It has been cited as the leading cause of death in many resource-poor and developing countries.¹ The World Health Organization (WHO) estimates that 1.7 million people died from TB in 2009, equal to 4700 deaths a day². Infections caused by *M. tuberculosis* complex organisms are classically pulmonary.

Failure to detect TB cases rapidly and accurately, shortages of resources for TB control programs in the developing world, failure to ensure that patients complete their therapy, emergence of multi-drug-resistant strains of tubercle bacilli, and the impact of HIV infection on the incidence of TB, have resulted in TB being the leading global cause of death from infectious diseases. The WHO estimates that unless better precautions are taken to tackle this disease, nearly 1 billion people will be infected between 2000 and 2020.

Mycobacteria Specimen Processing

The *Mycobacterium tuberculosis* complex includes *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* that cause tuberculosis in humans. *M. tuberculosis* grows slowly, requiring three weeks for formation of colonies on solid media. The organism has a thick, lipid-rich cell wall that renders the bacilli resistant to harsh treatments, including alkali and detergents, and allows them to stain acid-fast. The diagnosis of pulmonary Tuberculosis currently relies upon the detection of Mycobacterium Tuberculosis Complex (MTC) organisms in sputum.

Specimen Collection

Specimen should be collected in a sterile 50 mL screw-cap centrifuge tube or graduated, disposable sputum

cup showing the 10 mL volume. If a larger volume of sputum is collected, the specimen should be first separated into 10 mL volumes. Also avoid any contamination of the specimen with oral or nasal secretions. Since TB is airborne and contagious, it is essential to transport specimens to the lab without delay in sealed containers dedicated for this purpose, lid firmly secured and properly labelled. Also, it is crucial to place specimens in a special container which can withstand leakage of contents, shocks, and other conditions pertaining to ordinary handling practices. Specimen should be processed immediately and refrigerated if processing is delayed.

Three main steps are usually required to process specimen:

Liquefaction: Many specimens submitted for mycobacterial isolation contain mucus such as sputum. Mycobacteria, as well as contaminating flora, are often present, but trapped within the mucus. Liquefaction is achieved by adding chemicals which, when vortexed with the specimen, break down the mucus and release the organisms.

Several agents can be used to liquefy a clinical specimen, including NALC and enzymes. In most procedures, liquefaction (release of the organisms from mucin or cells) is enhanced by vigorous mixing in a closed container. Following mixing, the container should be allowed to stand for 15 minutes before opening, to prevent dispersion of fine aerosols generated during mixing³.

Decontamination: Most specimens received for mycobacterial culture contain various amounts of organic debris and a variety of contaminating, normal, or transient bacterial flora. A chemical decontamination process



Figure 1: Thermo Scientific 1-Liter General Purpose Centrifuge and Thermo Scientific 8x50 mL Individually Sealed Rotor (part number: 75003694)

is usually effective in killing the contaminants, while allowing recovery of the mycobacteria. The high lipid content of the Acid Fast Bacilli cell wall makes the mycobacteria more resistant to both acid and alkaline decontaminating agents. Strict adherence to the timed killing period is necessary to maximize recovery⁴. Sodium hydroxide (NaOH), the most commonly used decontaminant, also serves as a mucolytic agent but must be used cautiously as it is slightly less harmful to tubercle bacilli than to contaminating organisms⁵.

Generally, a combination of liquefaction-decontamination mixture is used. The N-Acetyl-L-Cysteine Sodium Hydroxide (NALC-NaOH)



Figure 2: Preferred centrifuge configuration with 8x50 mL individually sealed fixed angle rotor in non-refrigerated Thermo Scientific 1-Liter general purpose centrifuge



Figure 3: Alternative centrifuge configuration with individually sealed adapters for TX-750 swinging bucket rotor in Thermo Scientific 3-Liter refrigerated general purpose centrifuge

method^{6, 7, 8, 9} utilises a mucolytic agent, NALC for digestion, and NaOH for decontamination. The NALC-NaOH method is the most commonly used method in clinical laboratories.

Concentration: Mycobacteria are often present in clinical specimens in very small numbers; therefore it is essential to concentrate them by centrifugation before inoculating to cultures. The centrifuge must be fast enough to attain a minimum relative centrifugal force (RCF) of 3000 g .^{8, 9} If the RCF is not high enough, many tubercle bacilli could remain in suspension following centrifugation, and poured off with the discarded supernatant fluid. Several studies have shown that 3000 g for 15 minutes would sediment 95% of mycobacteria in a digested sputum specimen. The specific gravity of tubercle bacilli ranges from 1.07 to 0.79, making centrifugal concentration of specimens ineffective if the RCF is not 3000 g .^{10, 11}

Choosing the Right Centrifuge

Centrifuges are essential in laboratories where tubercle bacilli are cultured. Methods involving the use of a centrifuge are more efficient than simple decontamination and culture of sputum directly onto medium.

The recommended centrifuge for use in tuberculosis culture laboratories is one with a fixed angle rotor – to minimize heat build-up due to air friction – containing sealed centrifuge buckets, such as the Thermo Scientific 1-Liter general purpose centrifuge (Figure 1). This

centrifuge can spin 8x50 mL conical screw-cap tubes, each of them in an individually sealed container certified by HPA, Porton Down, UK (formerly CAMR). The Thermo Scientific centrifuge with 8x50 mL individually sealed rotor (Figures 1 and 2) attains the minimum RCF (3,000 g or greater) in only 15 seconds (in the ventilated unit, profile 9, rotor full loaded, at 230V and 120V) to concentrate mycobacteria in clinical specimens.

Alternatively, a Thermo Scientific 3-Liter refrigerated general purpose centrifuge can be used with the Thermo Scientific TX-750 swinging bucket rotor, accommodating 12 individually sealed containers for 50 mL conical tubes per run (Figure 3). Centrifugation at high speed using swing-out buckets may result in the generation of heat that could injure or kill mycobacteria. As a result, a refrigerated centrifuge is required, not to cool, but to maintain room temperature for the sample. It is, therefore, important to keep the spinning time low (15 minutes) and the RCF high (3,000 g) to achieve 95% sedimentation.

Procedure

Treat all sputum samples as potentially infectious and **use leak proof containers for collection, transportation and centrifugation** like those supplied with the Thermo Scientific 8x50 mL individually sealed fixed angle rotor.

Additionally, use a biological safety cabinet and wear gloves while carrying out all procedures involving sputum.

1. Use a 50 mL conical screw-cap tube containing precise volumes of decontamination reagent, to prevent both the addition of excess reagent

that will require pH neutralization later, and to avoid the possibility of cross-contamination between specimens.

2. Vortex for a minimum of 20 seconds up to a maximum of 30 seconds, using a timer.
3. Invert the tube a few times during the vortexing process to insure decontamination of all surfaces of the specimen tube.
4. If the NALC-NaOH method is used, extremely mucoid or bloody specimens may require additional NALC powder for complete digestion.

Note: Observe timing of decontamination step. Longer exposure times can cause destruction of the mycobacteria, so it is best to not process too many samples at one time.

5. Pour the neutralization buffer, stopping the action of the digestion agent, from an individual tube to avoid the possibility of cross-contamination between specimens.
6. Add buffer to the 50 mL mark on the centrifuge tube to maximize buffering capability of this step.
7. Mix by inversion to ensure all digestion agent is neutralized within the sample tube.
8. Concentrate the sample by spinning at a minimum of 3000 g for 15 minutes in the individually sealed rotor using a Thermo Scientific 1-Liter general purpose centrifuge.
9. Open the safety sealed centrifuge bucket and remove the tubes only in a biological safety cabinet.
10. Pour off supernatant completely into a splash-proof discard container; this allows for better pH control after resuspension of the pellet.

11. Carefully wipe the lip of the tube with an individual gauze pad dampened with disinfectant, being careful to avoid getting the disinfectant into the sample tube.
 12. Re-suspend the pellet in 1-2 mL of sterile phosphate buffer, pH 6.8, using individual sterile transfer pipettes. Lower resuspension volumes (less dilution) increase the numbers of organisms in the 0.5 mL sample to be inoculated into the bottle.
- Note:** Do not put other additives, such as albumin, into the sample pellet prior to inoculation.
13. Periodically check the final pH of the re-suspended pellet. It should be between pH 6.8 and 7.5. Higher pH may cause false positives, and can be detrimental to the mycobacteria, delaying the time to detection. Lower pH may also cause false positives.
 14. Sample is ready to be inoculated into medium culture.

Containment Recommendations

BSL-2 practices and procedures, containment equipment, and facilities are required for non-aerosol-producing manipulations of clinical specimens, such as preparation of acid-fast smears. All aerosol-generating activities must be conducted in a biological safety cabinet.

BSL-3 practices, containment equipment, and facilities are required for laboratory activities in the propagation and manipulation of cultures of any of the subspecies of the *M. tuberculosis* complex.

Conclusion

After collection of specimen for TB tuberculosis culture, specimen must be placed immediately in a special container. Then, after chemical liquefaction-decontamination, the concentration of *Mycobacterium tuberculosis* cells from sputum is almost universally performed by centrifugation using biocontainment buckets or even better the individually sealed containers. These methods are dependent on the effective sedimentation of mycobacterial cells, and as a result, the buoyant density of these cells

relative to sputum is of critical importance.

Optimum relative centrifugal force (RCF) and centrifugation time to concentrate mycobacteria in clinical specimens are determinant for processing samples of sputa containing mycobacteria. It is essential to reach required RCF rapidly. An RCF of at least 3,000 xg must be applied for 15 minutes to achieve 95% sedimentation rate and effectively concentrate mycobacteria in clinical specimens. Extended centrifugation time may have adverse effects on the viability of mycobacteria in partially neutralized specimens, such as those treated by the NALC-NaOH procedure.

The Thermo Scientific 1-Liter general purpose centrifuge with its **unique 8x50 mL individually sealed rotor** certified by HPA, Porton Down, UK (formerly CAMR), is ideal to:

- Sediment and concentrate 95% of mycobacteria in clinical sputum specimens
- Rapidly attain the required RCF of 3,000 xg in only 15 seconds
- Perform the whole protocol in maximum safety conditions with the unique, individually sealed containers for 50 mL conical tubes, certified by HPA, Porton Down UK.

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