

High-sensitive Detection of *Mycobacterium Tuberculosis* from Human Clinical Samples Using the InviMag[®] Bacteria DNA Mini Kit/ KFmL with The Thermo Scientific KingFisher[®] mL Instrument

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Abstract

This application note describes the isolation and detection of *Mycobacterium tuberculosis* from human clinical samples using the InviMag Bacteria DNA Mini Kit/ KFmL (Invitek) and the Thermo Scientific KingFisher mL magnetic particle processor (Thermo Fisher Scientific) combined with real-time PCR detection. In this experiment bacterial cultures were produced by inoculation with clinical samples and up to 15 samples were processed on the KingFisher mL in an automated operation. The results show that the extracted high-quality bacterial DNA can be used in a subsequent real-time PCR application for the detection of the 16S ribosomal RNA gene fragment (16S-rRNA) in *Mycobacterium tuberculosis* and the *Mycobacterium tuberculosis* complex.

Introduction

In bacteriology the limitations of microscopy and culture include poor sensitivity of staining methods; uncultivable, slow-growing (delayed diagnostic) or fastidious

bacteria; and the potential hazard to staff from handling highly dangerous pathogens. Therefore, rapid identification methods using molecular techniques have been developed and used more and more in the clinical laboratory. Particularly with regard to diagnostic purposes, these methods require extremely high efficiency and recovery for isolation and high-sensitive detection of bacterial nucleic acids even from small bacteria titers in the samples. Tuberculosis (TB) remains one of the leading causes of death from infectious disease worldwide and is caused by the mycobacterium *Mycobacterium tuberculosis* or the *M. tuberculosis* complex, which includes 3 further mycobacteria: *M. bovis*, *M. africanum* and *M. microti* [1]. *M. tuberculosis* is a slow-growing obligate aerobic bacterium that divides every 16 to 20 hours. TB most commonly affects the lungs (pulmonary TB) but can also affect the central nervous system, lymphatic system, circulatory system, genitourinary system, bones, joints and even the skin. According to the Centers for Disease Control and Prevention (CDC), nearly one-third of the world's population is infected with the bacteria that cause TB, which kills almost 2 million people per year [2]. About 90% of those infected with *M. tuberculosis* have an asymptomatic, latent TB infection, with only a 10% lifetime chance that a latent infection will progress to active TB disease. However, if untreated,

the death rate for these active TB cases is more than 50% [3]. In patients where TB becomes an active disease, 75% of these cases affect the lungs (pulmonary TB). Symptoms include a productive, prolonged cough of more than 3 weeks duration, chest pain, and coughing up blood [4]. The resurgence of TB in industrialized countries since the mid-1980s, primarily due to the increased incidence of immunocompromised patients with HIV/AIDS, and the emergence of multidrug-resistant strains of *M. tuberculosis*, has accentuated the need for rapid diagnosis of this disease [5]. Conventional detection of mycobacteria is based on a number of protocols, including microscopic examination of smears stained with the Ziehl-Neelson stain or auramine fluorescent dye and selective culture techniques [6]. The key aspect of TB control is rapid diagnosis, which for many years has been based on the staining of smears for the presence of acid-fast bacilli (AFB). Molecular methods, such as DNA probes and nucleic acid amplification tests, offer a rapid, specific and sensitive approach to the detection of the *M. tuberculosis* complex from liquid cultures and for the detection of TB directly from clinical specimens [6]. The real-time polymerase chain reaction (real-time PCR) allows detection of mycobacteria, differentiation of mycobacterial species, quantification of mycobacterial loads, and detection of drug resistance in mycobacte-

rial infection [7]. For this sensitive downstream application, the semi-automatic purification of bacterial DNA from patient samples with a KingFisher mL, from Thermo Fisher Scientific, in combination with the InviMag Bacteria DNA Mini Kit/ KFmL, from Invitex, has already been proven and tested for three years at the Central Institution for Molecular Diagnostic (Clinic Braunschweig GmbH). Compared to using fully automatic machines, this system provides a cost-effective step towards the standardization of nucleic acid purification. In Germany tuberculosis infections are notifiable. Since the implementation of the infection prevention ordinance in 2001, numerous characteristics for every tuberculosis case have to be raised and anonymously forwarded by public health departments to the Robert-Koch-Institut (RKI) [8]. Therefore, rapid identification methods are essential and they are routinely used in public health laboratories.

Material and Methods

Purification of bacterial DNA from *Mycobacterium tuberculosis*

Bacterial DNA from *Mycobacterium tuberculosis* and the *Mycobacterium tuberculosis* complex is purified in 45 minutes using the commercial InviMag Bacteria DNA Mini Kit/ KFmL. The DNA is isolated from bacterial cultures grown for two weeks, which were infected with patient samples (clinical samples: human sputum, bronchial lavage, gastric juice, urine, liquor, sanies, puncture or tissue).

An aliquot of a 100 µl bacterial culture is centrifuged; the pellet is resuspended in 400 µl Resuspension Buffer R and transferred into the Extraction Tube L, provided with the kit. Direct material, such as sputum and

bronchial secretions, can also be used for the DNA isolation, whereas the clinical sample has to be mixed with an equal volume of NAC Buffer (Invitex, order no.: 10332211) to reduce the sample viscosity. After this step the procedure continues as described above. Furthermore, other starting materials, such as small puncture and biopsy samples or swabs, are possible. The tissue biopsy or swab has to be directly placed into the Extraction Tube L that is filled up with 400 µl Resuspension Buffer R. The Extraction Tube L already contains preformulated solid lysis reagents (non-chaotropic lysis buffer, proteinase K and lysozyme), carrier nucleic acids and a precisely calibrated amount of an internal DNA extraction control, coated on the tube wall. The internal control is co-purified with the target sequences and allows to assess the extraction efficiency and PCR amplification, respectively. This monitors the quality of the purified bacterial DNA and excludes false-negative results. After mixing and a short incubation at RT to destroy the bacterial cell walls by hydrolyzing the polysaccharide component (murein) of the cell wall with the help of lysozyme, all samples are placed into a Thermomixer and incubated while continuously shaking for 15 min at 65°C to support proteinase activity for lysis and protein digestion followed by a short incubation at 95°C to disrupt also the last gram-positive bacteria. During lysis an appropriate number of Thermo Scientific KingFisher mL tube strips needed for the samples (one tube strip per sample) are placed onto the tube strip tray. The tube strips B to E are filled with buffers supplied with the kit according to Table 1. After lysis the samples are transferred into

the KingFisher mL tube strips (Tube A) and 400 µl of Binding Buffer B6 and 20 µl MAP Solution A¹ are added and mixed with each lysate to adjust the binding conditions. After filling the tube strips, the tray is placed into the instrument and the tip combs are inserted into the slots. The front lid is closed and the samples processed using the “InviMAG_Bacteria_KFmL” purification protocol. During this process the bacterial DNA is bound to the magnetic particles quantitatively. The beads are transferred through Wash Buffer I and Wash Buffer II to remove all impurities, such as proteins, nucleases, and PCR inhibitors. After removal of ethanol from the beads, the bacterial DNA is eluted in Elution Buffer D. After the program is completed, the tube strip tray is removed from the instrument and the eluates stored for further use.

Table 1 : Pipetting instructions for the InviMag Bacteria DNA Mini Kit/ KFmL

Tube	Content	Sample / Reagent Volume
A	Lysed sample	400 µl
	MAP Solution A ¹	20 µl
	Binding Buffer B6	400 µl
B	Wash Buffer I	800 µl
C	Wash Buffer II	800 µl
D	Wash Buffer II	800 µl
E	Elution Buffer D	120 µl

¹ It is important to mix the bottle with MAP Solution A carefully by vigorously shaking or vortexing before use.

Detection of *Mycobacterium tuberculosis* infections

Routinely a 5 µl aliquot of each eluate is used for the detection of the *Mycobacterium tuberculosis* complex or directly of *Mycobacterium tuberculosis* by a real-time PCR application. Therefore, the species-specific 16S-rRNA sequence is

used as a gene target. The PCR method using FRET probes on a LightCycler® instrument is developed in-house, based on primers and conditions described in Lachnik et al. [9]. In the results, a detailed example of a typical real-time PCR detection of this assay is shown. In this assay a known negative patient sample (patient 1) is used as the negative control. The total sample amount was over 300.

Results

Highly pure bacterial DNA was isolated on the KingFisher mL with the InviMag Bacteria DNA Mini Kit/ KfMl from 100 µl of a bacterial culture of *M. tuberculosis*. The cultures were infected by clinical relevant samples from patients in a routine procedure. The extracted DNA and the controls were quantified by real-time PCR.

Figure 1 shows the amplification of the 16S-rRNA gene fragment of *M. tuberculosis*. The control was used in two different dilutions (control 1 and control 2). The dark green line shows that bacterial DNA of *M. tuberculosis* was detected in patient sample 3. The patient samples 1 (negative control) and 2 do not show any amplification of the 16S-rRNA fragment, that is, these two patients were not infected with *M. tuberculosis*. These results

correlate with the microbiological and staining tests. High-sensitive tests were performed with more than 300 patient samples during the last three years at the Central Institution for Molecular Diagnostic, Clinic Braunschweig GmbH. Also, the direct use of sputum samples, bronchial lavage, liquor or gastric juice has shown high-sensitive mycobacteria detection results.

Conclusion

In combination with the KingFisher mL and the InviMag Bacteria DNA Mini Kit/ KfMl they provide a semi-automated, standardized and economical purification of bacterial DNA from *M. tuberculosis* from 15 patient samples in parallel in 45 minutes. This rapid and convenient method can be adapted to various clinical samples by following the sample preparation protocols for different starting materials. Apart from time-saving and productivity-enhancing aspects, this sample preparation process allows reliable and efficient high-quality bacterial DNA extraction from *M. tuberculosis* for sensitive downstream applications in clinical routine procedures. In combination with microscopy or selective culture techniques, the real-time PCR enhances the detection of mycobacteria by differentiating mycobacterial species, quantifying mycobacterial loads, and detecting drug resistances.

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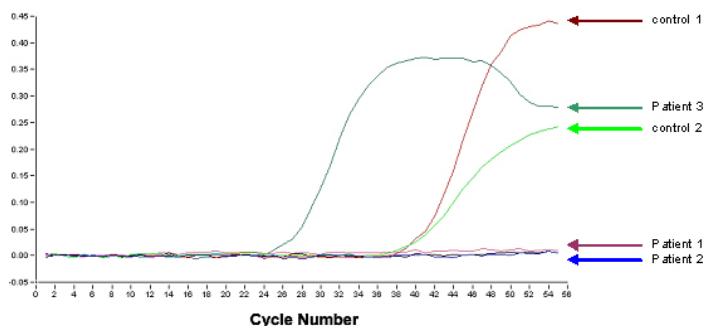


Figure 1: The bacterial DNA was isolated from 100 µl of a two-week old bacterial culture according to the protocol by using the InviMag Bacteria DNA Mini Kit/ KfMl and the KingFisher mL workstation. 5 µl of the eluted DNA was used for the detection of the *Mycobacterium tuberculosis* complex or directly of *Mycobacterium tuberculosis* by a real-time PCR application (16S-rRNA fragment).

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