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CRISPR-Cas12a-based detection and differentiation of Mycobacterium spp.

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CRISPR-Cas12a-based detection and differentiation of *Mycobacterium spp.*

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การตรวจหาและจำแนกเชื้อ Mycobacteria ในระดับสปีชีส์ ด้วยระบบ CRISPR-Cas12a

น.ส.พีระพรรณ คัมภิโร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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เชื้อมัยโคแบคทีเรีย (*Mycobacterium* spp.) ก่อโรคในมนุษย์ที่สำคัญหลายชนิดเช่น วัณโรค (TB) และโรคติดเชื้อ Non-tuberculous mycobacteria (NTM) โดยวัณโรคเป็นโรคติดต่อเรื้อรังที่มีสาเหตุจากเชื้อแบคทีเรียในกลุ่ม *Mycobacterium tuberculosis* complex (MTBC) นอกจากนี้โรคติดเชื้อ Non-tuberculous mycobacteria เกิดจากการติดเชื้อแบคทีเรีย NTM ที่สำคัญทางการแพทย์ ได้แก่ *Mycobacterium abscessus*, *Mycobacterium avium*, *Mycobacterium fortuitum*, *Mycobacterium kansasii*, *Mycobacterium gordonae* และ *Mycobacterium intracelulare* ซึ่งทั้งสองกลุ่มพบการแพร่ระบาดในผู้ป่วยที่มีภูมิคุ้มกันบกพร่องและผู้ป่วยโรคเอดส์ (AIDS) อย่างไรก็ตามการติดเชื้อที่เกิดจากเชื้อมัยโคแบคทีเรียทั้งสองกลุ่มนี้นำไปสู่การรักษาที่แตกต่างกัน แต่การวินิจฉัยเบื้องต้นนั้นมีข้อจำกัดในด้านระยะเวลาและเครื่องมือในการวินิจฉัย ดังนั้นหากมีการวินิจฉัยที่ถูกต้องและรวดเร็ว จะทำให้การรักษาเป็นไปได้อย่างมีประสิทธิภาพ และสามารถควบคุมการแพร่กระจายของโรคได้ ในงานวิจัยนี้ ได้ประยุกต์ใช้เทคนิค CRISPR-Cas12a มาประยุกต์ใช้ในการพัฒนาการตรวจหาและจำแนกเชื้อมัยโคแบคทีเรียในระดับสปีชีส์ ผลการทดสอบพบว่า การใช้เทคนิค RPA ในการเพิ่มจำนวนสารพันธุกรรมร่วมกับเทคนิค CRISPR-Cas12a ในการตรวจหาเชื้อมัยโคแบคทีเรียในระดับสปีชีส์สามารถตรวจพบปริมาณต่ำสุดที่สามารถวัดได้ (Limit of Detection) ที่ 1 และ 10 copies/µl และให้ผลลัพธ์ในการตรวจจับตัวอย่างที่เพาะแยกได้จากสิ่งส่งตรวจสอดคล้องกับวิธีการตรวจมาตรฐาน โดยสรุปเทคนิคการตรวจเชื้อมัยโคแบคทีเรียในระดับสปีชีส์ด้วยวิธี CRISPR-Cas12a เป็นวิธีที่มีประสิทธิภาพในการตรวจจับคัดกรอง ใช้เครื่องมือไม่ซับซ้อน และให้ผลลัพธ์อย่างรวดเร็วภายในหนึ่งชั่วโมง

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Peeraphan Compiro : CRISPR-Cas12a-based detection and differentiation of *Mycobacterium spp.*. Advisor: PORNCHAI KAEWSAPSAK, Ph.D. Co-advisor: Asst. Prof. SUWATCHAREEPORN ROTCHEEWAPHAN, M.D.

Mycobacterium species cause several vital human diseases, including tuberculosis (TB) and non-tuberculous mycobacterial (NTM) infections. TB is a chronic infectious disease caused by the *Mycobacterium tuberculosis* complex (MTBC). Additionally, non-tuberculous mycobacteria infections are caused by major NTM species, including *Mycobacterium abscessus*, *Mycobacterium avium*, *Mycobacterium fortuitum*, *Mycobacterium kansasii*, *Mycobacterium gordonae*, and *Mycobacterium intracelulare*, are medically necessary. Both groups of infections can lead to outbreaks in immunocompromised individuals and AIDS patients. It is crucial to differentiate between these two groups of mycobacterial infections, as they require distinct treatment approaches. However, initial diagnosis poses limitations in terms of time and diagnostic tools. Therefore, accurate and rapid diagnosis is essential for effective treatment and controlling the spread of diseases. This research, the CRISPR-Cas12a technique was applied to develop a detection and classification method for mycobacteria at the species level. The testing results showed that the combination of the RPA technique with CRISPR-Cas12a for mycobacterial detection at the species level achieved a Limit of Detection (LOD) of 1 and 10 copies/µl, providing results consistent with standard detection methods for cultured samples. In summary, the CRISPR-Cas12a technique for the detection *Mycobacterium* spp. proved to be an efficient screening method, utilizing simple tools and delivering rapid results within one hour.

Field of Study:	Medical Biochemistry	Student's Signature .....
Academic Year:	2023	Advisor's Signature .....
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Peeraphan Compiro

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CHAPTER 1

INTRODUCTION

Background and Rationale

Mycobacteria are a large group of microorganisms that inhabit a wide range of natural environments. Some species are capable of infecting humans and causing disease [1]. The most clinically important species is *Mycobacterium tuberculosis*, which causes tuberculosis in humans. This infectious disease is one of the leading causes of illness and mortality worldwide. Prior to the COVID-19 pandemic, tuberculosis was the leading cause of mortality due to a single infectious agent, surpassing HIV/AIDS. Additionally, both tuberculosis in cattle and humans is commonly caused by *Mycobacterium bovis*. Although these pathogenic species exhibit some phenotypic differences, they are genetically closely related. Therefore, both *M. tuberculosis* and *M. bovis* belong to the *Mycobacterium tuberculosis* complex (MTBC) [2] [3].

In addition to *Mycobacterium tuberculosis*, other pathogenic mycobacteria include the *Mycobacterium avium* complex (MAC) and other non-tuberculous mycobacteria (NTM), also known as atypical mycobacteria or environmental mycobacteria. The main members of the MAC group causing diseases in humans are *Mycobacterium avium* and *Mycobacterium intracellulare*. Other common NTM isolated from clinical specimens are *Mycobacterium abscessus*, *Mycobacterium fortuitum*, *Mycobacterium gordonae*, and *Mycobacterium kansasii* [2]. Studies have revealed an increase in the incidence of MAC and NTM infections. Both MTBC and NTM are now known to be important opportunistic pathogens that cause public health problems [4-6]. The diagnosis of NTM infections is challenging and often confused with infections of the MTBC. Standard TB treatments are often ineffective

against NTM infections, and misdiagnosis and mistreatment have significant implications for patient outcomes [7, 8].

In general, the identification of mycobacteria is based on conventional methods, including examination under a microscope for bacterial morphology and acid-fast bacilli (AFB) staining using Ziehl-Neelsen (Z-N), Kinyound, or fluorescence staining [9]. Z-N staining is a quick, easy, and inexpensive method but has low specificity and cannot distinguish between the MTBC and NTM. Additionally, the gold standard for laboratory diagnosis is isolation of *Mycobacterium* from clinical samples, but culture using Lowenstein-Jensen (LJ) medium is a slow and difficult process that typically takes 4-8 weeks for confirmation. Biochemical tests, such as niacin, catalase, nitrate reduction, and urease tests, are also often used to identify species, but they are time-consuming and cannot differentiate between closely related species. These testing methods are complex and labor-intensive. For these reasons, conventional methods have been developed to detect and differentiate mycobacteria in clinical samples that are rapid, simple, sensitive, and specific [10-12].

In recent decades, molecular approaches have been developed to diagnose *Mycobacterium* infections. Nucleic acid amplification-based tests such as PCR, GeneXpert MTB/RIF assay, loop-mediated isothermal amplification (LAMP), and line probe assay have greatly improved the sensitivity, specificity, and accuracy of mycobacterial diagnosis. However, despite their widespread use, these techniques require expensive laboratory equipment and the chemicals used are costly and must also be imported from abroad. Furthermore, the amplification and detection steps of the assay involve multiple steps which may introduce the potential for error and sample contamination [13, 14].

Recently, a clustered regularly interspaced short palindromic repeat (CRISPR-Cas) system has been successfully developed and demonstrated. This system

consists of a Cas effector, CRISPR RNA (crRNA), nucleic acid amplification mixture, CRISPR-Cas buffer, and reporter molecules (single-strand nucleic acid). For example, CRISPR-Cas12a-based HOLMES (One-Hour-Low Cost Multipurpose Highly Efficient System) and CRISPR-Cas13a-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) assays have been developed for the diagnosis of SARS-CoV-2, HPV (human papillomavirus), ZIKV (Zika virus), and DENV (Dengue virus) with attomolar sensitivity. In these target gene assays, crRNAs must be designed to match the target nucleotide sequence, which recruits Cas proteins to the target site and triggers nonspecific nucleotide cleavage. The activated Cas proteins then cleave a nucleotide reporter containing fluorescence and quencher. The cleavage unleashes a fluorescence signal that enables detection of the target gene. The difference between the two systems is that Cas12 recognizes double-strand DNA (dsDNA) as an activator and cleaves single-strand DNA (ssDNA), while Cas13 recognizes ssRNA and cleaves ssRNA. However, CRISPR-Cas12a and CRISPR-Cas13 both provide high sensitivity and specificity [15-18]. In the preparation of DNA for detection, the CRISPR technique primarily relies on the utilization of PCR to amplify the target DNA [17]. However, PCR necessitates precise temperature control throughout its 3-step amplification cycle, which is time-consuming, and the thermocycler is not portable. Therefore, recent studies have shown interest in recombinase polymerase amplification (RPA) for the amplification of DNA targets. RPA operates at a single constant temperature and can control the DNA amplification time within 30 minutes, allowing for easy operation and a rapid turnaround time [19-21].

Therefore, this study aims to develop a CRISPR-Cas12a system combined with the RPA system to detect and differentiate *Mycobacterium* spp. The goals are to improve the sensitivity and specificity of the analysis and to create easy-to-use tools that can be developed into a rapid test kit.

Research Question

Can CRISPR-Cas12a be used in combination with RPA to detect and differentiate *Mycobacterium* spp.?

Hypothesis

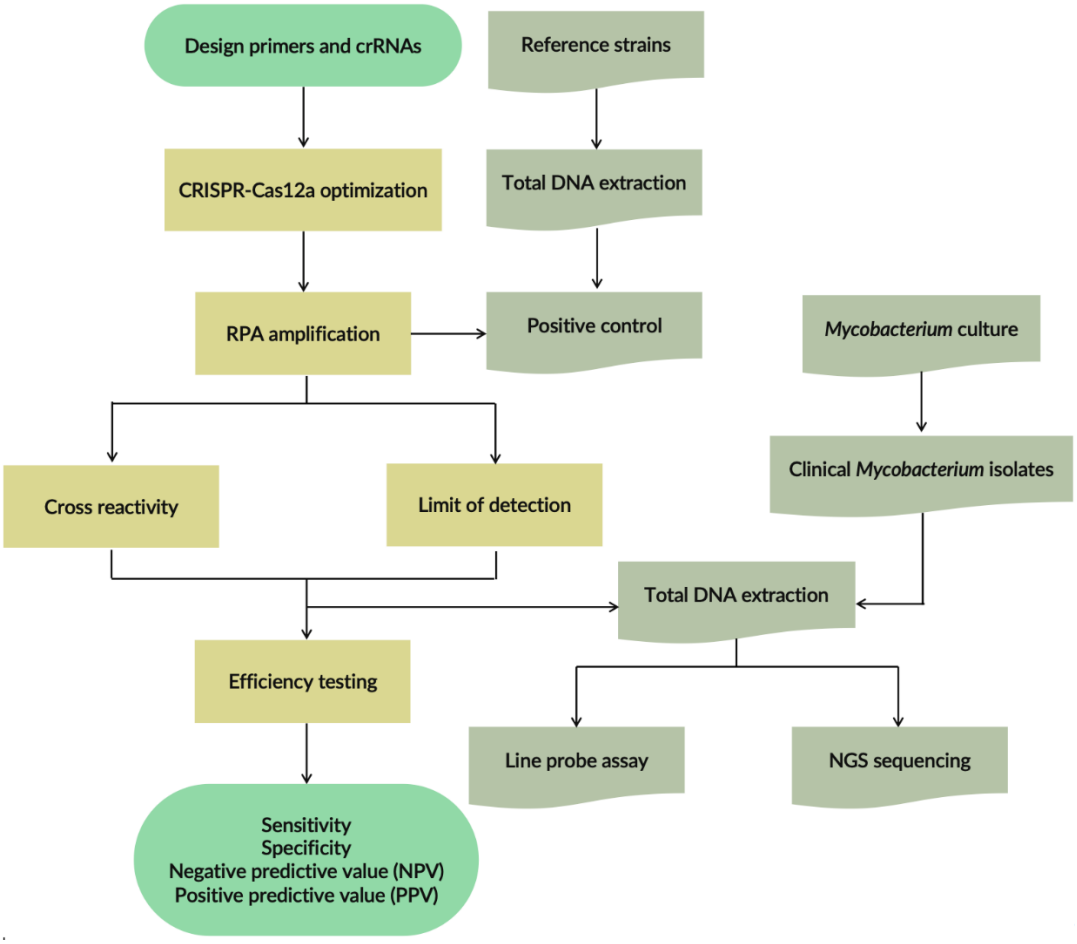
Mycobacterium spp. can be differentiated using a combination of CRISPR-Cas12a and RPA. This technique is expected to have high sensitivity and specificity, similar to those of the standard assay.

Objectives

1. To develop a CRISPR-Cas12a protocol in combination with RPA for the detection and differentiation of *Mycobacterium* spp.
2. To evaluate the effectiveness of CRISPR-Cas12a in combination with RPA for the detection and differentiation of *Mycobacterium* spp. from DNA of clinical isolates.

Research Workflow

The detection and differentiation of *Mycobacterium* spp. using a combination of CRISPR-Cas12a and RPA were performed in reference strains and clinical isolates with the *rpoB* gene.



Keywords

- CRISPR-Cas12a
- *Mycobacterium*
- Tuberculosis
- Recombinase Polymerase Amplification
- CRISPR-based diagnostic test

CHAPTER 2

LITERATURE REVIEW

Mycobacterium

The genus *Mycobacterium* is a group of aerobic bacteria that are Gram-positive, catalase-positive, nonmotile, and nonspore-forming. They are rod-shaped and range in size from 0.2 to 0.6 x 1 to 10 μm. Occasionally, they form branched filaments. Their cell walls are very thick, consisting of four layers, with the innermost layer being made up of peptidoglycan and other lipids, making the surface hydrophobic and resistant to many disinfectants and common laboratory stains. Because of this, they are known as acid-fast bacteria, as the rods cannot be decolorized with acid solution. Due to the complexity of their cell walls and fastidious nature, most mycobacteria grow slowly, dividing every 12 to 24 hours and requiring up to 8 weeks before growth is detected in laboratory cultures [2, 22, 23]. Mycobacteria are a significant cause of morbidity and mortality, particularly in countries with limited medical resources. Currently, the genus *Mycobacterium* contains more than 200 described species, of which only 30 have been reported to cause human disease (Table 1) [24]. Notably, pathogens in the MTBC, such as *M. tuberculosis* and *M. bovis*, can cause tuberculosis in humans and animals, respectively. Although these organisms are highly similar in genetic makeup, they differ greatly in epidemiology, pathogenicity, and host spectrum [21, 25]. Additionally, other mycobacteria in addition to *M. tuberculosis* can also be human pathogens, and the incidence of infection appears to be on the rise. These organisms are commonly found in soil and water and are generally less harmful to humans than *M. tuberculosis*. These organisms are referred to atypical, environmental, and non-tuberculous mycobacterial (NTM), including *M. abscessus*, *M. fortuitum*, *M. gordonae*, and *M. kansasii*. These organisms also induce a wider range of clinical symptoms in individuals living with HIV and those who are immunosuppressed. Among NTM, the *Mycobacterium avium* complex (MAC), consisting of *M. avium* and *M. intracellulare*, is the leading cause of pulmonary disease in humans [26].

Table 1 Principal types of mycobacterial disease in humans and causative species

Disease	Agent	Reference
Tuberculosis	*M. tuberculosis*	[21]
	M. bovis	[25]
Pulmonary disease	*M. avium* complex	[26]
	M. kansasii	[27]
	M. abscessus	[28]
	M. fortuitum	[29]
	M. gordonae	[30]
Disseminated disease	*M. avium* complex	[31]
AIDS-related	*M. kansasii*	[32]
Non-AIDS related	*M. avium* complex	[33]
Lymphadenopathy	*M. avium* complex	[34]
	M. gordonae	[35]

Standard Methods and Diagnostic Techniques for the Detection of *Mycobacterium* species

The currently accepted methods for diagnosing tuberculosis are as follows: First, staining the acid-fast bacilli from a sample and then growing the bacterium from the sample. The diagnosis of tuberculosis is based on the microbiological characteristics of the bacterium to identify the pathogen and determining its susceptibility to treatment drugs (Table 2) [36, 37].

Microscopic Examination and Acid-Fast Staining

The initial laboratory diagnosis of tuberculosis continues to rely on a smear examination. The traditional diagnostic methods for detecting mycobacterial cells include histopathological examination and microscopic detection using a Ziehl-Neelsen or Kinyoun stain. Smear microscopy is an affordable and effective way to

detect the most infectious cases of pulmonary tuberculosis, particularly in resource-limited settings where the quality of smear microscopy is crucial in the fight against tuberculosis. However, smear microscopy has several limitations, including limited sensitivity (25-75% compared to culture) and a high number of bacilli required for a positive result (5 x 10^3–10^4 bacilli per ml). The sensitivity and positive predictive value of smear microscopy can be affected by various factors, such as the frequency and severity of the disease, the type and quality of the sample, the number of mycobacteria present and the quality of smear preparation, staining, and interpretation. Additionally, smear microscopy does not identify the mycobacterial species or provide information about the viability of the mycobacteria in the sample. Patients with tuberculosis co-infected with HIV may have a paucibacillary form of the disease with fewer acid-fast bacilli, making smear microscopy less reliable in these cases. As a result, smear microscopy may require more scrutiny or may be negative in the screening for fewer acid-fast bacilli [38].

Mycobacterial Culture and Identification

Culture identification is still considered the gold standard, although most *Mycobacterium* species require oxygen and have slow growth rates. For example, *M. tuberculosis* divides every 15-20 hours, which is extremely slow compared to other bacteria that have division times measured in minutes, such as *Escherichia coli*, which divides every 20 minutes. *M. tuberculosis* is typically grown in a selective medium known as Löwenstein-Jensen medium. However, this method is relatively slow, as it takes 6-8 weeks for the organism to grow, resulting in delayed results. To address this limitation, liquid medium culture has emerged as a more sensitive and faster technique for detecting bacilli growth and simultaneously testing for drug susceptibility. Liquid systems are more sensitive for detecting mycobacteria, and thus can increase the case yield by 10% over solid media and reduce the time to get result. Positive cultures require some form of confirmatory identification to confirm the presence of the MTBC, this can be done by microscopy of an aliquot of the medium for acid-fast bacilli, molecular or biochemical testing, or recognition of characteristic phenotypic growth in the medium. Cultures containing both *M. tuberculosis* and non-tuberculous mycobacteria (NTM) may occur more frequently in liquid media than in solid media and can cause confusion during drug susceptibility testing (DST) as NTM growth can be mistaken for highly resistant strains of *M. tuberculosis* [39-41].

Table 2 Comparison of the standard methods for the detection of Mycobacterium species

Methods	Advantages	Limitations
AFB stain	Simple, rapid, inexpensive, and available for all laboratories	Low sensitivity, unable to differentiate between MTBC, NTM, live, and dead cells
Culture	Standard method, allowing for the phenotypic determination of DST	The time-consuming requirement of BSL3 (for *M. tuberculosis*), requires trained staff

Molecular testing for the detection of *Mycobacterium* species

DNA amplification

Real-Time PCR

RT-PCR is the amplification and quantification of the tuberculosis genetic material from the sample by attaching a fluorescent label to the original genetic material. To measure the increased amount of genetic material during analysis, the detection of *M. tuberculosis* is based on the detection of IS6110 and MPB64 genes, specific to stable and conserved TB genes. The sensitivities of the individual IS6110 and MPB64 targets were 90.3% and 64.5%, respectively, and the specificities were 88.4% and 97.7%, respectively. The identification of NTM can be included based on the 16S rRNA sequence [42, 43]. The *rpoB* was used for the diagnosis of tuberculosis multidrug-resistant organisms. For detecting rifampicin-resistant and Isoniazid-resistant TB, *katG* and *inhA* were used respectively. The sensitivities of RT-PCR in detecting INH resistance using *katG* and *inhA* targets individually were 55% and 25%, respectively, and 73% when combined [44]. Although this method can detect drug resistance and provide high sensitivity, it requires a huge and expensive thermocycler, preventing for use in areas with limited resources [45].

GeneXpert MTB/RIF assay

The Xpert/MTB RIF assay is a cartridge-based, automated, real-time polymerase chain reaction analyzer that amplifies the amount of DNA in the *rpoB* gene of the specimen. This assay detects mutations in the *rpoB* gene that cause resistance to rifampicin. It has a sensitivity of 94.4% and a specificity of 98.3% [46]. The test takes approximately 2 hours to detect in the sputum samples, regardless of

whether the AFB smear results are positive or negative [47]. However, when choosing to use the Xpert MTB/RIF test, it is important to consider that the operation of the detector requires a constant power supply for at least 2 hours, that the detector can only be used at a temperature not exceeding 30 ℃, and the storage cartridges should be stored at a temperature between 2-8 ℃. Additionally, it is not suitable for monitoring a patient's response to treatment and the costs of maintenance and calibration are expensive [48].

Loop-mediated isothermal amplification (LAMP)

For the detection of TB, LAMP amplifies the 16S rRNA gene at a constant temperature. It does not require a thermal cycler, and the results can be read with the naked eye using a pH-sensitive dye. The sputum test takes about 1 hour to detect, and the materials can be stored at room temperature. This method has a high detection sensitivity [49]. TB-LAMP is a point-of-care test (POC) that can diagnose tuberculosis but cannot be used to test for drug resistance. Therefore, it should not be used in areas with a high prevalence of multidrug-resistant TB (MDR-TB) or where the Xpert MTB/RIF test is already well established [50].

Amplification and reverse hybridization

Line probe assay (LPA)

LPA is a technique based on the polymerase chain reaction (PCR) and reverse hybridization method that detects *M. tuberculosis* DNA in specimens and can identify nucleotide mutations (single nucleotide polymorphisms: SNPs) in the case of drug resistance. The molecular LPA can be divided into three steps: DNA extraction, DNA amplification, and hybridization as shown in Figure 1. However, this method requires at least 160 cells. Additionally, it necessitates a laboratory and equipment capable of performing molecular techniques, and personnel must be skilled to use this method effectively [51, 52].

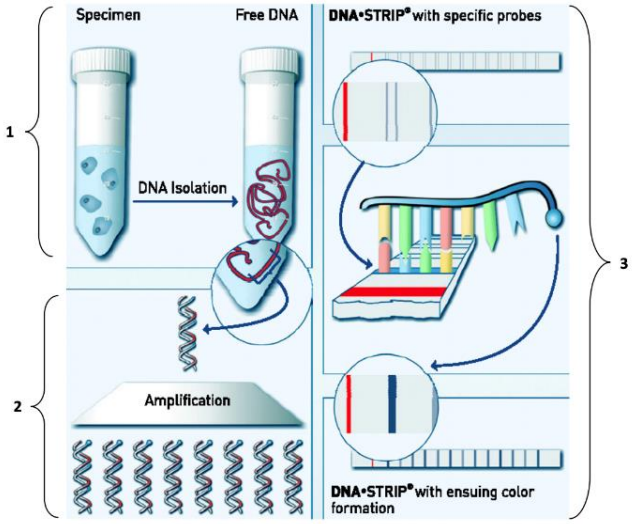


Figure 1 Schematic representation of three LPA steps

The first step is DNA extraction, followed by the amplification of the gene with biotinylated primer. Subsequently, the amplified DNA is hybridized to probes, and the bands are visually detected using a color-forming enzymatic reaction involving streptavidin adhering to biotinylated primers [52].

Clustered regularly interspaced short palindromic repeat (CRISPR-Cas) system

The CRISPR-Cas systems composed of clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins play a role in the adaptive immunity of archaea and bacteria against foreign nucleic acids. In recent years, CRISPR/Cas technology has emerged as a powerful tool for editing genomes in all forms of life [53]. It has been adapted for use in molecular engineering for genome editing and transcription regulation. The CRISPR-Cas system uses guide RNA to direct Cas effector proteins, such as Cas9, Cas12, and Cas13, to target sites where they cleave the target sequence, allowing manipulation and research of the target gene function. Additionally, some Cas effector proteins, such as Cas12a, Cas12b, Cas13, and Cas14, have been shown to have potential for the development of next-generation nucleic acid detection technologies due to their ability to cleave both target sequences and non-specific single-stranded nucleic acids [54, 55]. In addition to the different homologs of Cas, endonucleases also vary in their substrate preferences and target recognition mechanisms. For example, Cas12 recognizes double-stranded DNA as an activator and cleaves single-stranded DNA, Cas13 recognizes single-stranded RNA and cleaves it, and Cas14 recognizes single-stranded DNA and cleaves it [21]. These endonucleases can generally cleave sequences that

meet certain requirements. The target site must have a short nucleic acid sequence called the protospacer adjacent motif (PAM) near it, and the 20-28 bp sequence located next to the PAM must be complementary to the guide RNA. Guide RNA is a short RNA that is bound to the Cas protein and plays a crucial role in the target recognition [56].

For example, the function of Cas12a is to cleave the target double-stranded DNA, which then proceeds to cleave single-stranded DNA in a nonspecific manner, known as trans cleavage. By incorporating a pair of fluorophore-quencher linked by single-stranded DNA (FQ reporter), trans cleavage events release the fluorophore from its quencher, activating fluorescence that can be measured using a microplate reader or by eye [57] as shown in Figure 2.

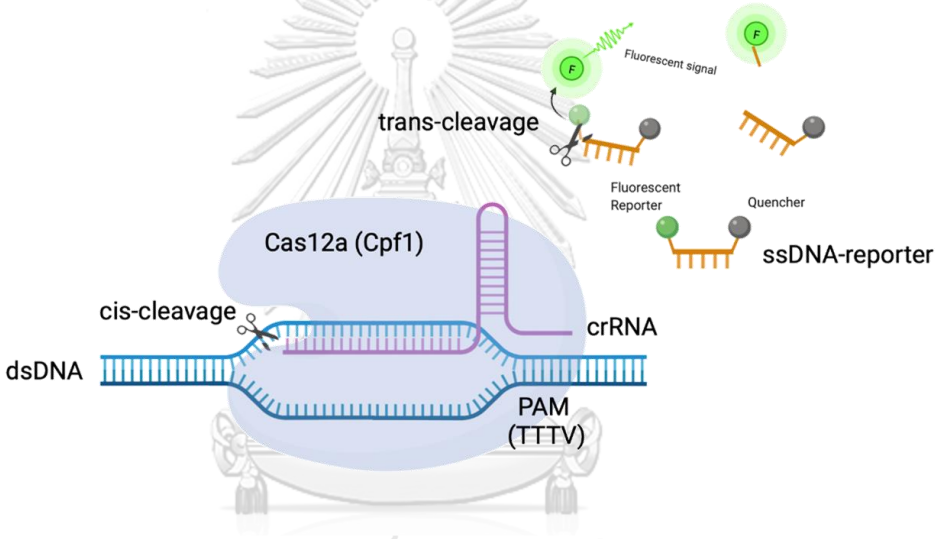


Figure 2 Schematic representation of Cas12a-crRNA with the DNA association of the target strand

This image is modified from Haipo Xu et al. [58] and created by BioRender.com

Recent advancements in CRISPR technology have expanded its applications to include nucleic acid detection using Cas12a. CRISPR-Cas12a is being used to detect a variety of viral strains, such as SARS-CoV-2, influenza viruses, HPV, pseudorabies virus, and Japanese encephalitis virus [18, 59].

CRISPR-based diagnostics have been listed as one of the seven technologies to watch in 2022 in Nature's fifth annual roundup of technologies with the potential to revolutionize science. The CRISPR/Cas platforms, when combined with nucleic acid amplification strategies, can detect nucleic acids rapidly, accurately, and cost-effectively at attomolar concentration levels, which is promising for addressing the diagnostic challenges of tuberculosis [60]. Thus, CRISPR-Cas12a has generated interest

in the detection of *Mycobacterium* compared to other methods, as shown in Table 3.

Table 3 Comparison of molecular methods for the detection of Mycobacterium species

Methods	Advantages	Disadvantages	Reference
Real-Time PCR	High sensitivity and specificity, fast (result in hours)	Expensive, require specialist training and equipment	[13-15]
GeneXpert assay	Automatic one-step process, quick (result in<2hrs)	Expensive, require specialist training and equipment	[16-18]
Loop-mediated isothermal amplification (LAMP)	High sensitivity and specificity	Primer design is complex (utilizes 4-6 primers), is not capable of multiplex amplification, cannot be used to test drug resistance	[22, 23]
Line probe assay (LPA)	Can perform multiple tests at once, fast (result in less than 48 h.)	Expensive and requires well-trained staff in a professional laboratory	[24]
CRISPR-Cas	Rapid testing, point-of-care, and lower cost	Nonspecific binding of sgRNA to the genome of the organism (off-target phenomenon)	[1, 21]

To increase the sensitivity of Cas12a in detecting low levels of nucleic acids, an upstream amplification step can be incorporated. Some research groups have used PCR and LAMP for amplification, however, these methods require multiple cycles of heating and cooling to denature and anneal DNA strands, and require at least four primers, and up to six, to amplify a target DNA sequence, respectively. To reduce costs and diagnostic time, the Recombinase Polymerase Amplification (RPA) technique, an isothermal amplification method similar to LAMP, is of particular

interest because it requires only two primers and can be completed within 10-30 minutes [61].

Recombinase polymerase amplification (RPA)

RPA is an isothermal DNA amplification technique, it can be performed isothermally between 37 and 42 ℃ without the need for the thermal cycling used in PCR, close to the optimal temperature for the Cas12a cleavage assay (37 ℃). RPA relies on a combination of enzymes, including recombinases and polymerases, to facilitate the amplification of target DNA. These enzymes work together to initiate strand exchange and DNA synthesis. The amplification mechanism of the RPA reaction commences with the binding of recombinase proteins to oligonucleotide primers. These recombinase proteins facilitate the formation of a complex between the primer and the target DNA. Subsequently, a single-stranded DNA binding (SSB) protein binds to the displaced DNA strand, stabilizing the resulting D loop. DNA amplification by polymerase is initiated from the primer, but only if the target sequence is present (Figure 3). Once initiated, the amplification reaction progresses rapidly, so that starting with just a few target copies of DNA, highly specific DNA amplification reaches detectable levels within minutes. The entire process carried out isothermally is an efficient and rapid method for DNA amplification. Therefore, RPA has been used in various diagnoses and applied in clinical settings with limited resources because it has advantages such as being simple to use, low time-consuming, low-cost, and focused on point-of-care techniques [20, 21, 62, 63].

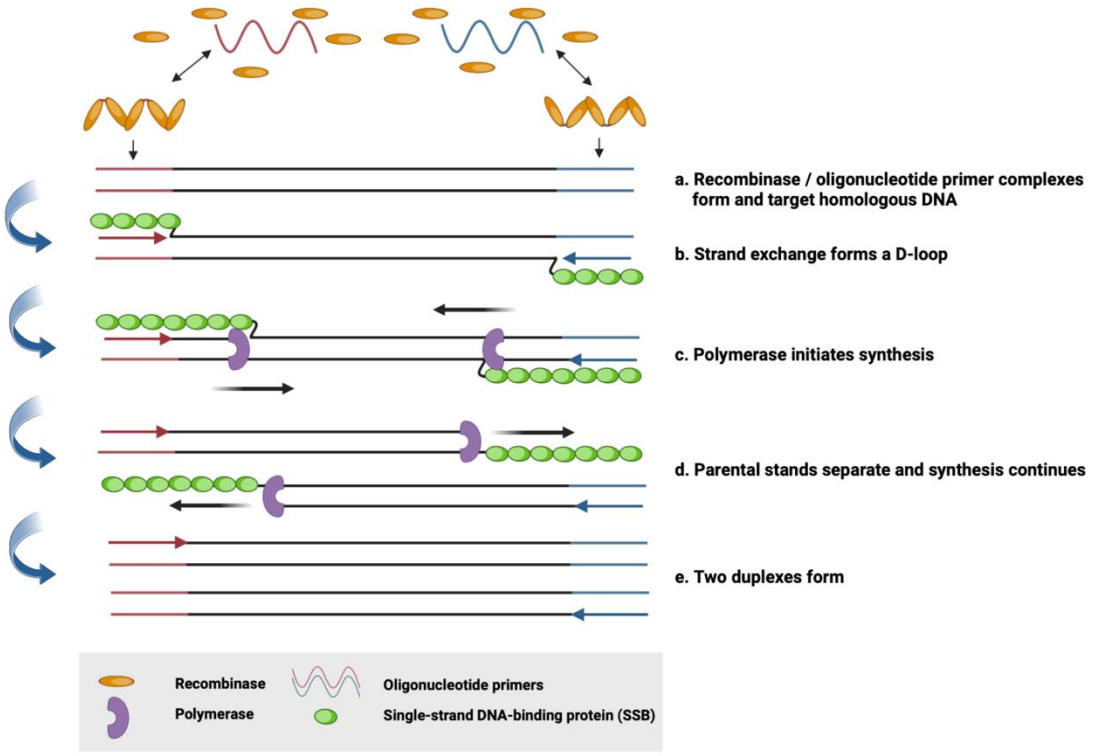


Figure 3 The amplification mechanism of the RPA reaction

The three core proteins are recombinase, single-strand DNA binding protein (SSB), and polymerase. This process works at a constant temperature (37°C). This image is modified from Liu et al. [63] and created by BioRender.com

Currently, most detection assays have been used only for the detection or quantification of specific *Mycobacterium* species, such as *M. tuberculosis*, or the detection of drug-resistance [19-21, 58, 64]. Therefore, this study aims to develop a CRISPR-Cas12a protocol combined with RPA for the rapid detection and differentiation of *Mycobacterium* spp. This method allows for easy operation, and rapid turnaround time and can be used as a point-of-care clinical diagnostic test and can be used in areas with limited budgets for tuberculosis and NTM isolation in Thailand, allowing doctors to plan specific and timely treatment.

CHAPTER 3

RESEARCH METHODOLOGY

Research Design

Experimental Research

Materials and equipments

Balance (Precisa, UK)
ChemiDoc Imaging Systems (Bio-Rad, USA)
Biosafety cabinet class 2a (Esco, Singapore)
BluPAD Dual LED Blue/White Light Transilluminator (Bio-Helix, Taiwan)
Centrifuge (1.5mL) (Eppendorf, Germany)
Centrifuge (50mL) (Hettich, Singapore)
Easypet® 3-Pipette controller (Eppendorf, Germany)
Electrophoresis chamber set (Major Science, USA)
Freezer -20 °C (Sanyo, Japan)
Freezer -80 °C (Panasonic, Japan)
Gel Doc 1000 (Vilber lourmat, Hong Kong)
Heat block (Eppendorf, Germany)
incubator shaker (Biosan, Lattvia)
Mastercycler® Thermocycler (Eppendorf, Germany)
Qubit™ Fluorometric Quantitation (Thermo Fisher Scientific, USA)
Refrigerator 4 °C (Panasonic, Japan)
Vortex mixer (Scientific industry, USA)
0.2 mL microcentrifuge tube (Kirgen, China)
0.65 mL microcentrifuge tube (Axygen, USA)
1.5 mL microcentrifuge tube (Eppendorf, Germany)
Conical flask 1 L (Pyrex, Germany)
Serological pipette (SPL, Korea)
TissueLyser LT (QIAGEN, USA)
NanoPhotometer® (IMPLEN, Germany)
MINI-6KS centrifuge (GIPTHAI, Thailand)

Chemicals

miRNA isolation kit (GeneAid, Taiwan)
pGEM®-T Easy Vector (Promega, USA)
Riboprobe® in vitro Transcription Systems (Promega, USA)
T4 DNA Ligase (5 U/µl) (Thermo Scientific, USA)
T7 promoter (Macrogen, Korea)
TURBO™ DNase (Ambion, USA)
dNTPs (Thermo Fisher Scientific, USA)
Taq DNA polymerase 5 unit/µl (Biotechrabbit, Germany)
10X reaction buffer (Biotechrabbit, Germany)
Qubit™ HS DNA Assay Kit (Invitrogen TM, USA)
Qubit™ microRNA Assay Kit (Invitrogen TM, USA)
Q5® High-Fidelity DNA Polymerase (New England Biolab, USA)
TwistAmp® Basic Kit (TwistDx, UK)
Agarose (Invitrogen, USA)
100 bp DNA ladder (GeneDirex, Taiwan)
Primer 10 µM (U2Bio, Korea)
ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, California, USA)
Agar (Becton, Dickinson and company (BD), USA)
Ampicillin (M&H manufacturing, Thailand)
EDTA (Invitrogen, USA)
Glucose (Cat No.783, Ajex Finechem, New Zeland)
Glycerol (Merck, Germany)
$MgCl_2$ (Invitrogen, USA)
NaCl (Merck, Germany)
Sodium chloride (Merck, Germany)
Tris (2-carboxyethyl) phosphine hydrochloride, TCEP (Aldrich, USA)
10X NEBuffer 2.0 Reaction Buffer (New England Biolab, USA)
EnGen® *Lba* Cas12a, Cpf1 (New England Biolab, USA)
ssDNA fluorescent-quencher reporter probe (Macrogen, Korea)
QIAquick PCR Purification Kit (QIAGEN, USA)

Preparation of reference and clinical mycobacteria isolates

Assistant Professor Suwatchareeporn Rotcheewaphan from the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok provided the extracted DNA of the following heat-killed mycobacteria;

- *M. tuberculosis* strain ATCC 27294, *M. bovis* strain ATCC 19210*, M. abscessus* ATCC 19977, *M. avium* strain ATCC 700898, *M. fortuitum* strain ATCC 6841, *M. kansasii* strain ATCC 12478, *M. gordonae* strain ATCC 14470, *M. intracellulare strain* ATCC 13950.
- Clinical isolates of *M. tuberculosis* complex and *M. avium* complex under project Genotypic and phenotypic characteristics of isoniazid resistant *Mycobacterium tuberculosis* in Thai population (IRB 699/62, CU-IBC 004/2023) and Genomic diversity and drug-susceptibility profiles of *Mycobacterium avium* complex clinical isolates in Thailand (IRB 0840/65, CU-IBC 003/2023).

Additionally, the NTM including, *M. abscessus*, *M. fortuitum, M. kansasii* and *M. gordonae* were obtained from the Department of Microbiology, King Chulalongkorn Memorial Hospital. The document for the usage of clinical isolates will be submitted to Excellence Clinic Center for approval.

A minimum of 19 samples per species were used in this study, which was calculated based on the sample size estimation formula as shown below.

$$n = \frac{(Z_{\alpha/2})^2 PQ}{d^2} = \frac{(1.96)^2(0.95)(0.05)}{0.1^2} = 18.2476$$

n stands for the clinical specimens of patients diagnosed with mycobacteria infection
Z stands for the confidence level at 95% equal to 1.96
P stands for the sensitivity of the predicted assays equal to 95% [58]
Q stands for 1-P
d stands for acceptable error equal to 10%

Genomic DNA extraction from reference strains and clinical isolates

Heat-killed mycobacteria isolates were centrifuged at 10,000g for 10 minutes to obtain pellets. The pellets was used to extract genomic DNA using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, California, USA) following the manufacturer's instructions or the CTAB method [65]. The extracted DNA was stored at -80°C until use.

Preparation of positive control DNA sequences from reference strains

Primer Design for amplification target *rpoB* gene

To design a PCR primer targeting specific *rpoB* gene for *Mycobacterium* spp., DNA sequences of known variable genes from *Mycobacterium* spp. were aligned and conserved regions were identified for amplification by BioEdit program version 7.0.5.3. The target gene loci and primer names, primer sequence, and amplification product size are listed in Table 4.

Table 4 The sequences of the primers for amplification *rpoB* gene

Target	Primer name	Nucleotide Sequence	Size(bp)
rpoB gene	RpoB F1	5'- CTCCGTACCCGGAGCGCC-3'	18
	RpoB F2	5'- CTCCGTACCCGGAGCGCCAAACCG-3'	24
	RpoB F1.v1	5'- AATAACTCCGTACCCGGAGCGCC-3'	23
	RpoB F1.v2	5'- AATAACTCCGTACCCGGAGCSCC-3'	23
	RpoB F1.v3	5'- AATAACTCCGTACCCGGRGCSCC-3'	23
	RpoB R1	5'- GGTCAGAGAACGACAACGACATCGAC-3'	26
	RpoB R2	5'- CGAAACGAGGGTCAGAGAACGACAACGACATCGAC-3'	35
	RpoB R3	5'- GCCGCGTACGTCATGTCCTTGTCTTTGCACTCGTCG-3'	36
	RpoB R4	5'- AACCTGGGAATCAAYYTGTCSCGCA -3'	22

PCR condition for amplification target *rpoB* gene and addition of 3' A overhangs to PCR products

The master mix was prepared using the following reagents listed in the table below (Table 5). The master mix for the amplification target *rpoB* gene was incubated under the following conditions: 95 ° C for 1 min, 95 ° C for 15 sec, 58-62° C for 15 sec, 72 ° C for 12 min, repeat 35-40 cycles and 72 ° C for 4 min. Then, the PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, USA) according to the manufacturer's protocol. After that, the product PCR was added adenosine

nucleotide overhang using terminal transferase activity of certain thermophilic DNA polymerases, such as *Thermus aquaticus* (Taq) polymerase. Taq DNA polymerase catalyzes the non-template directed addition of an adenine residue to the 3´-end of both strands of DNA molecules to make it suitable for TA cloning [66]. The reagent used in the addition of 3' A overhangs to PCR products was prepared using the following reagents listed in the table below (Table 6). Then, the reaction was incubated at 72 ℃ for 20 min.

Table 5 The reagents used in the PCR condition

Reagent	Volume (µl)
5x Q5 buffer	10
5x GC enhancer	10
Forward primer (RpoB F1.v2) (10 mM)	1
Reverse primer (RpoB R4) (10 mM)	1
Q5 polymerase	0.5
dNTP mix (10 mM)	1
DNA template 10 ng (Reference strains)	2
distilled water	24.5
Total	**50**

Table 6 The reagents used in the addition of 3' A overhangs to PCR products

Reagent	Volume (µl)
Purified PCR product (300 - 3000 ng)	variable
dATP (10 mM)	1
10x PCR Buffer with Mg	5
Taq DNA Polymerase (5 U/µl)	0.2
distilled water	Up to 50 µl
Total	**50**

Ligation

The table below provides the reagents used in the ligation of the pGEM®-T Easy vector and insert gene (Table 7). The molar ratio calculation from http://www.insilico.uni-duesseldorf.de/Lig_Input.html (vector: insert = 1:3-1:5 molar ratio). Then, the reaction was incubated at 22 ℃ for 60 min.

Table 7 The reagents used in the ligation

Reagent	Volume (µl)
5X Rapid Ligation buffer	2.5
3 Weiss units/µl T4 DNA ligase	1
50 ng pGEM-T Easy (TA) vector	1
DNA insert	variable
distilled water	Up to 10 µl
Total	**10**

Transformation

Fifty µl competent *E. coli* strain DH5-α cells were added to 10 µl of pre-chilled ligation reaction before incubating on ice for 30 minutes. The mixture was then heat-shocked at 42°C for 30 seconds and incubated on ice for the next 5 minutes. To finalize the volume to 1 ml, SOC growth media was added to the mixture followed by incubating at 37°C and shaking at 200 rpm for 90 minutes. Then, centrifuge at 4,000 rpm for 5 min and discard the supernatant. The pellet was mixed with X-gal 15 µl and IPTG 5 µl, spread plated onto a pre-warmed LB/ampicillin plate, and incubated at 37°C overnight.

Positive colony selection PCR

An isolated colony was selected for PCR. The table below provides the reagents used in this process (Table 8). The primers for detection were listed (5'-3') M13-forward primer: GTTTTCCCAGTCACGAC and M13-Reverse primer: TCACACAGGAAACAGCTATGAC. After the master mix was prepared, the colony was picked as a template. Then, the master mix for colony selection PCR was incubated under the following conditions: 95 ° C for 2 min, 95 ° C for 30 sec, 55 ° C for 15 sec, 72 ° C for 4 min, repeat 35 cycles and 72 ° C for 5 min.

Table 8 The reagents used in the colony selection PCR

Reagent	Volume (µl)
10X Reaction buffer (Rabbit Biotech)	2.5
50 mM $MgCl_2$ (Rabbit Biotech)	0.75
10 mM dNTPs mix	0.5
10 µM M13-forward primer	0.5
10 µM M13-reverse primer	0.5
5U/µl Taq DNA polymerase (Rabbit Biotech)	1
distilled water	7.15
Total	**12**

Then, the PCR product was subjected to gel electrophoresis. Any colony that showed the size of the product matched the expected size would be chosen for further propagation. A selected colony was inoculated in 2 ml µl Luria Broth (LB) media and incubated at 37°C overnight at 200 rpm.

Plasmid extraction and purification

The bacteria culture was extracted and purified using ZymoPURE™ Plasmid Miniprep Kit. Centrifugation of 1 ml of bacterial culture in a 1.5 ml microcentrifuge tube was operated at full speed for 15-20 seconds and the remaining supernatant was discarded. The pellet was resuspended by 250 µl of ZymoPURE™ P1 before adding 250 µl of ZymoPURE™ P2. The tube with the two mixtures of ZymoPURE™ and pellet was then immediately inverted for 8-10 times and incubated for 3 minutes at room temperature. Following the addition of 250 µl of ZymoPURE™ P3 and mix thoroughly by inversion, the tube was inverted for 5 times or until the sample turns thoroughly yellow. The tube was subjected to the centrifugation of the neutralized lysate for 5 minutes at 16,000g to the transfer of 600 µl of centrifuged lysate to a new clean 1.5 ml microcentrifuge tube. Addition of 260 µl of ZymoPURE™ Binding Buffer to the lysate was done before resuspending for 15 seconds using a vortex. Zymo-Spin™ II-PX Column was placed in a collection tube. All the mixture was transferred to the Zymo-Spin™ II-PX Column/Collection Tube and sit at room temperature for 1 minute before the centrifugation at max speed for 1 minute, any remaining flow-through was discarded. Addition of 800 µl of ZymoPURE™ Wash 1 to the column was proceeded prior to the centrifugation at max speed and discarding the flow-through, respectively. Eight hundred µl of ZymoPURE™ Wash 2 was next subjected to proceed with the exact same procedure as ZymoPURE™ Wash 1. Before the centrifugation at max speed for 1 minute and discarding of flow through, 200 µl

of ZymoPURE™ Wash 2 was added to the column. The column was further centrifuged for 3 minutes at max speed to dry any remaining residual wash buffer. The column was placed in a new 1.5 ml microcentrifuge tube and prewarmed 25 µl ZymoPURE™ Elution Buffer at 50°C was directly added to the column. After the incubation of 10 minutes at room temperature, the column/tube was centrifuged at 10,000g for 1 minute in a microcentrifuge and eluted plasmid DNA was collected at -20 °C, respectively. The plasmid DNA was quantified using NanoPhotometer® (IMPLEN, Germany) and diluted to a concentration of 20 ng/µl. The diluted sample was sequenced using BTSeq™ barcode-tagged sequencing (U2Bio, Thailand) to confirm the sequence.

Primer Design for RPA Reaction

To design an RPA primer targeting the highly specific *rpoB* gene for *Mycobacterium* spp., DNA sequences of known variable genes from *Mycobacterium* spp. were aligned and conserved regions were identified for amplification. A primer pair for the amplification of target sequences were designed based on the conserved sequences flanking the crRNA probe targeting regions. The designed primer was tested using genomic DNA extracted from *Mycobacterium* spp. The target gene loci and their primer names, primer sequences, amplification product sizes, and the *rpoB* sequence used to amplify are listed in Table 4 and Table 9.

Table 9 Sequences of the *rpoB* gene from NGS base BTSeq™ barcode-tagged sequencing

Name	Size (bp)
***Mycobacterium tuberculosis* strain ATCC 27294**	3419
***Mycobacterium bovis* strain ATCC 19210**	3421
***Mycobacterium abscessus* strain ATCC 19977**	3418
***Mycobacterium fortuitum* strain ATCC 6841**	3412
***Mycobacterium avium* strain ATCC 700898**	3423
***Mycobacterium kansasii* strain ATCC 12478**	3424
***Mycobacterium gordonae* strain ATCC 14470**	3412
***Mycobacterium intracellulare* strain ATCC 13950**	3424

Preparation of Cas12a crRNA

The crRNA for Cas12a utilizes a 20-24 base pair spacer sequence to bind to the target DNA, which must be preceded by the PAM sequence 5'-TTTV-3' (where V is either A, C, or G). The crRNA was designed to target the highly specific *rpoB* gene for each *Mycobacterium* species. Each oligonucleotide for the crRNA template for *in vitro* transcription was prepared via overlap extension PCR. Briefly, two short DNA oligos, one containing the T7 promoter and the other containing the spacer were designed (Figure 4). The T7 promoter was used as the starting point for the formation of crRNA strands by the enzyme T7 RNA polymerase, which was initiated by adding the nucleotide sequence of the T7 promoter (T7 LbCpf1 crRNA Top; 5' TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGAT '3) into the scaffold. The master mix of overlap extension PCR was prepared using the following reagents listed in the table below (Table 10).

Table 10 The reagents used in the overlap extension PCR

Reagent	Volume (µl)
5x Q5 polymerase buffer	10
T7 LbCpf1 crRNA (10 mM)	1
crRNA template (10 mM)	1
Q5 polymerase	0.5
dNTP mix (10 mM)	1
distilled water	36.5
Total	**50**

The oligonucleotides for the crRNA templates primer were annealed to the T7 promoter primer and incubated under the following conditions: 95°C for 1 min, 95°C for 30 sec, 58°C for 15 sec, 72°C for 15 sec, repeat 35-40 cycles and 72°C for 1 min. The annealed crRNA template was quantified using NanoPhotometer® (IMPLEN, Germany). The crRNA was then transcribed using RiboMAX® in vitro transcription systems (Promega, USA). A total volume of 20 µL of master mix contain 4 µL of 5x T7 transcription buffer, 6 µL of 25 nM rNTPs, 8 µL of crRNA template and 2 µL of T7 enzyme mix. Then, gentle pipeted the reaction to mix and incubated at 37°C overnight in a heat box, followed by treatment with 2 µL of RQ1 DNase at 37 ° C for 10 min, and 5 µL of enzyme DNase I (Sigma) and 5 µL of 10x Reaction buffer (Sigma) at room temperature for 5 hr to remove DNA template. Transcribed crRNA was purified using the miRNA isolation kit (Geneaid, Taiwan) following the manufacturer's

instructions and quantified using the Qubit TM microRNA Assay Kit (Thermo Scientific, USA).

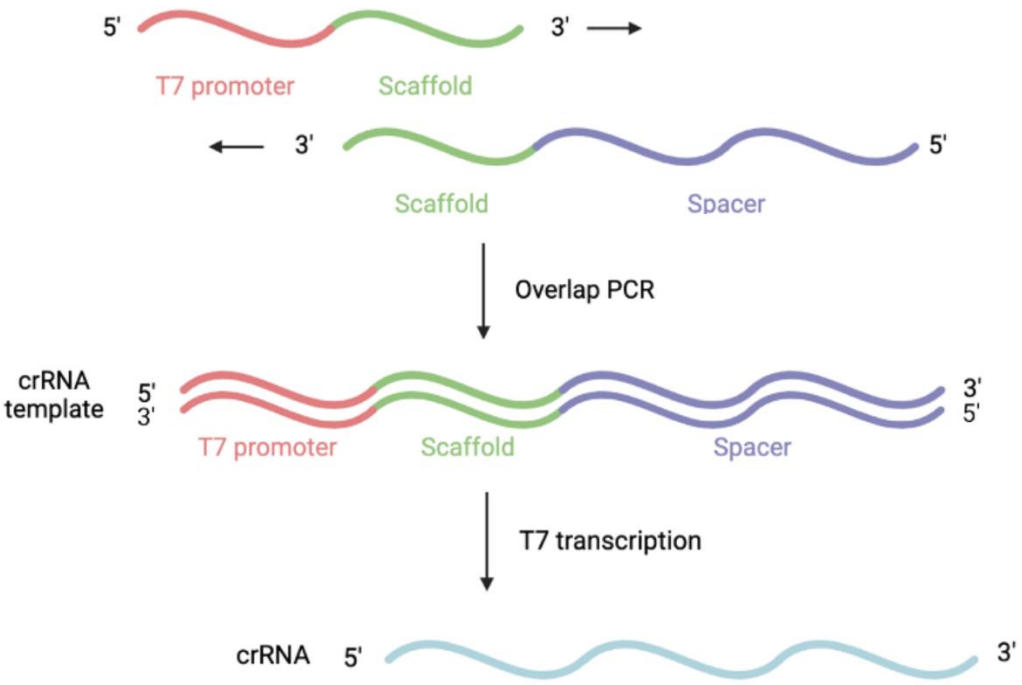


Figure 4 Schematic representation of the composition and orientation of template DNA for crRNA and crRNA construction

This image is modified from Zheng Hu et al. [67] and created by BioRender.com

Table 11 Sequences of the crRNA template

Name	Nucleotide Sequence (5'-3')	Size(bp)
RpoB TB 1	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATgacgatgtcaaggcacccgt	59
RpoB TB 2	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATgacgatgtcacggcacccgt	59
RpoB Abs 1	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctgcgtgaac	59
RpoB Abs 2	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATaatggctggttggatcgccg	59
RpoB Abs 3	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATgccgatcgaagacttctccg	59
RpoB Abs 4	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctgcatgaac	59
RpoB Abs 5	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctgcgtaaac	59
RpoB Abs 6	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcggttcccggcctgctcgat	59
RpoB Abs 7	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATgaatggctggttggatcgcc	59
RpoB Abs 8	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATaggagatcctcacggagctt	59
RpoB Abs 9	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcgccgatcgaagacttctcc	59
RpoB AvH 1	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctgcgcgaac	59
RpoB AvH 2	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATtccgacccccgcttcgacga	59
RpoB AvH 3	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctgcgcaaac	59
RpoB AvH 4	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctgcgttaac	59
RpoB AvH 5	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctgcgataac	59
RpoB AvH 6	TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGATcttcgccaagctgcacgaac	59
RpoB AvH 7	TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGATcttcgccaagctgcacaaac	59
RpoB AvH 8	TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGATcttcgccaagctgcgcggac	59
RpoB AvH 9	TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGATgccaagctgcgcgaaccgct	59
RpoB AvH 10	TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGATgccaagctgtgcgaaccgct	59
RpoB AvH 11	TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGATgccaagctgcacgaaccgct	59
RpoB MF 1	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATatttgccaagctccgtgaac	59
RpoB MF 2	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATccaagctccgtgaaccgctt	59
RpoB MF 3	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATtcgggctcgatgtcgctgag	59
RpoB MF 4	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATtcgggctcgatgtcgctaag	59
RpoB MF 5	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATtcgggctcgatgtcgccgag	59
RpoB MK 1	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctccgcgaac	59
RpoB MK 2	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATacgtgcagaccgactcgttc	59
RpoB MG 1	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATTttcgccaagctccgtgaac	59
RpoB MG 2	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATgccaagctccgtgaaccgct	59
RpoB MI 1	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctccgcgagc	59
RpoB MI 2	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctccgcgggc	59
RpoB MI 3	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagctccgccagc	59
RpoB MI 4	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATcttcgccaagttccgcgagc	59
RpoB MI 5	TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGATgagctctcgccgatcgagga	59
RpoB MI 6	TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGATacgtgcagatcgactccttc	59

The capital letter shows the sequence of scaffold, and the small letter shows the sequence of spacer.

RPA amplification of *rpoB* gene

RPA amplification of *rpoB* gene which contains a region of deletion. These were used TwistAmp® Basic kit (TwistDx, Cambridge, UK). A total volume of 10 µL of RPA contained 5.9 µL of rehydration buffer with RPA powder, 0.48 µL of 10 µM of each forward and reverse primer, and 2.14 µL of distilled water. Then, the solution was mixed and added to freeze-dried reagent lyophilize. After that, the mixture was vortexed and added 0.5 µL of 280 mM magnesium acetate on the tube lid before adding 1 µL DNA template. Finally, the RPA reaction was incubated at 39°C for 30 min followed by heat inactivation at 75 °C for 5 min in a heat box.

The presence and size of each RPA product were electrophoretically separated on 2% agarose gels. A 100-bp DNA ladder (GeneDirex, Taiwan) was used as a size marker.

Limit of detection (LOD)

The limit of detection was performed in triplicate by using a 10-fold serial dilution of each standard DNA target ranging from 1 to 10^5 copies/µl as templates for RPA with CRISPR-Cas12a. The limit of detection was observed from reaction tubes containing the lowest concentration of the DNA template that yielded the fluorescent signal.

CRISPR-Cas12a detection

The reaction of CRISPR-Cas12a-based nucleic acid detection was consisted of 50 nM crRNA (Table 11), 330 nM EnGen Lba Cas12a (Cpf1) (New England Biolabs, USA), 660 nM fluorescent reporter/quencher probe, 1x NEBuffer 2.0 reaction buffer (New England Biolabs, USA), and 1 µL of RPA product in a final volume of 15 µL (Table 12). The Cas reaction was incubated at 39 °C for 15 min and then the fluorescent signal was visualized with a BluPAD dual LED blue/white light transilluminator (BIO-HELIX, Taiwan). Negative controls (water, DNase, and RNase-free) were used in parallel each time.

Table 12 The reagents used in the CRISPR-Cas12a reaction

Reagent	Final concentration	Volume (µl)
10X NEBuffer 2.0	1X	1.50
500 nM crRNA	50 nM	1.50
6 µM ssDNA fluorescent-quencher probe	660 nM	1
1 µM Cas12a (Cpf1)	33 nM	0.75
30 nM DNA substrate	1 nM	1
distilled water	-	9.25
Total	**-**	**15**

Data analysis and interpretation

The interpretation of the CRISPR-Cas assay was recorded by three independent individuals and the results obtained from a minimum of two of these interpreters were used as the final outcome. The collected data was then analyzed.

After ensuring that the test kit can work with the genetic material of the clinical isolates, the next step is to evaluate its correct identification. The line probe assay was used as the gold standard method for performance evaluation in terms of concordance with this assay.

CHAPTER 4

RESULTS

The workflow of the overall process assay

The assay process shown in Figure 5 involves a sequential combination of RPA reaction and CRISPR-Cas12a detection. In this platform, the target DNA from an extracted DNA is first amplified using RPA. Subsequently, the amplified DNA is incubated with Cas12a, a crRNA probe, and a quenched fluorescence reporter ssDNA. If the specific crRNA probe in the reaction mixture detects the target DNA, the Cas12a/gRNA/target DNA ternary complex is formed, leading to the cleavage of the reporter ssDNA, and generating fluorescence. Both reference and clinical Mycobacteria isolates were tested to evaluate the assay performance.

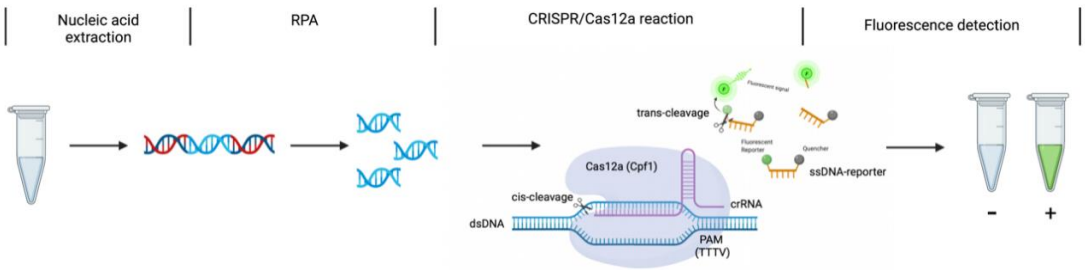


Figure 5 The workflow of the overall process for rapid detection based on RPA and CRISPR-Cas12a assay

This image was created by BioRender.com

Preparation of positive control DNA sequences

To create the DNA template as positive control, the *rpoB* DNA were amplified by PCR. The *rpoB* gene sequences for each mycobacterium obtained from NCBI were used to design primers that amplifies the whole *rpoB* gene for all eight *Mycobacterium* reference strains, including *M. tuberculosis* ATCC 27294, *M. bovis* ATCC 19210, *M. abscessus* ATCC 19977, *M. avium* ATCC 700898, *M. fortuitum* ATCC 6841, *M. kansasii* ATCC 12478, *M. gordonae* ATCC 14470, and *M.* intracellulare ATCC 13950. The PCR amplicon were confirmed by gel electrophoresis, as shown in Figure 6.

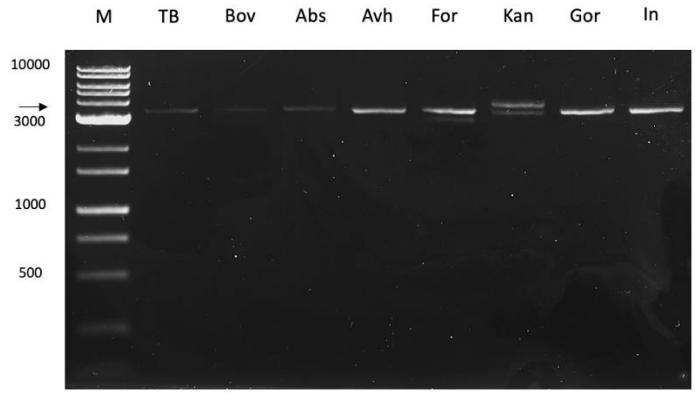


Figure 6 Illustration showing PCR products of positive control of *rpoB* gene from reference *Mycobacterium* strain DNA bands separated on a gel

PCR product size was 3,400 bp which is compared to OmniMark 1K (100-10,000 bps) (M). (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs) *M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare*

The DNA sequences of *rpoB* amplicons were confirmed by BT-seq and subsequently utilized for the design of RPA primers and crRNA.

RPA amplification of *Mycobacterium* spp. *rpoB* gene

To test the performance of RPA using primer pair as shown in Table 4. First, we designed three forward primers and three reverse primers in the conserve region of the *Mycobacterium* spp. *rpoB* gene. These primers are located in the same region but have different lengths. After RPA amplification and gel electrophoresis, the primer pair composed of a forward primer (F1V1) and a reverse primer (R3) can amplify *rpoB* in most strains except *M. abscessus* (Figure 7). To cover more species, the nucleotide in position 18 and 21 of F1V1 primer were replaced by degenerate bases, which allows more matching with DNA sequence. As shown in Figure 8, the modified primer of a forward primer (F1V2) and a reverse primer (R3) could amplify *rpoB* gene in all species with better band intensity. Hence, the forward primer (F1V2) and the reverse primer (R3) were selected to amplify DNA from all eight *Mycobacteriu*m species in subsequent experiments.

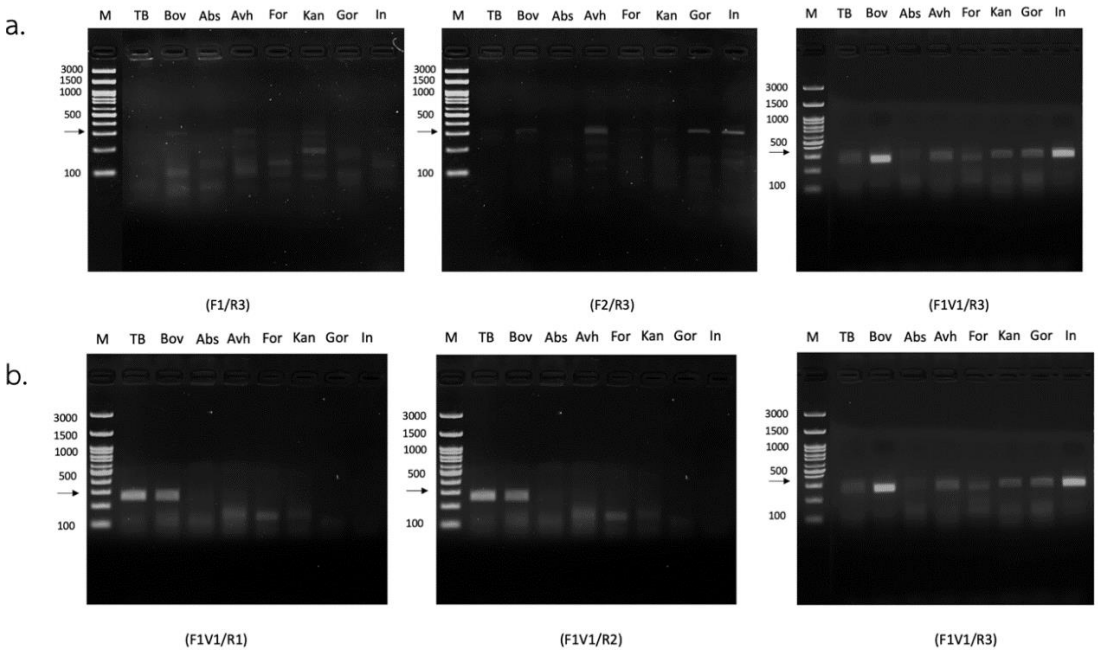


Figure 7 Gel electrophoresis of RPA products for *Mycobacterium* spp. *rpoB* partial gene (308 bp)

The brackets below display the primer pair names, and the arrows indicate the positions of the target RPA products. (M) marker DNA ladder 100 bp (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs) *M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare*.

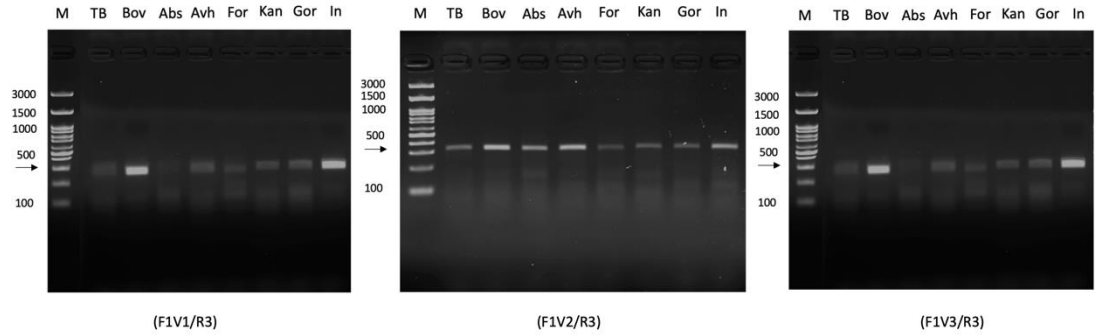


Figure 8 Gel electrophoresis of RPA products of partial *rpoB* gene from the modified primers (F1V1, F1V2 and F1V3 with R3)

The brackets below display the primer pair names, and the arrows indicate the positions of the target RPA products. (M) marker DNA ladder 100 bp. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs) *M. abscessus* (AvH) *M. avium* (For*) M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare*.

CRISPR-Cas12a detection of *Mycobacterium* spp. *rpoB* gene

To test the CRISPR-Cas12a detection system, the specific crRNAs targeting the *rpoB* gene of each *Mycobacterium* species were designed. Cross-reactivity tests were performed to assess the specificity of the crRNA with the target template using reference strain in the CRISPR assay. Finally, we conducted a limit of detection tests to evaluate the sensitivity of the assay.

Designing specific crRNA of *Mycobacterium* spp.

To design crRNAs, the *rpoB* sequences of *Mycobacterium spp.* were aligned and the sequences within the region located inside the RPA product containing the 5'-TTTV-3' protospacer adjacent motif (PAM) for Cas12a were chosen. The crRNA sequences are shown in Supplementary Figure 1.

The crRNAs for M. tuberculosis and M. bovis

The MTBC group includes *M. tuberculosis* and *M. bovis.* share identical DNA sequences within *rpoB* region. To target the common sequence, the first crRNA was chosen and called "TB 1". The TB 1 crRNA contains a 24 bp spacer sequence to bind to the target DNA, which had preceded by the PAM sequence 5'-TTTV-3' (Figure 9a). When the *rpoB* targeted gene fragment from RPA was present in the CRISPR-cas12a detection system, the amplified genes for eight *Mycobacterium* species of DNA samples including *M. tuberculosis*, *M. bovis*, *M. abscessus*, *M. avium*, *M. fortuitum*, *M. kansasii*, *M. gordonae*, *M. intracellulare*, and Negative control (no template), the specificity of CRISPR detection system was verified at the same time as shown in Figure 9b. The result demonstrated that this crRNA of CRISPR-Cas12a yielded specificity with MTBC but had cross-reactivity with *M. fortuitum*. Therefore, a mismatch was introduced into the TB 1 crRNA at the 11th position of spacer (A to C) to enhance the specificity of the crRNA (TB 2) towards MTBC and differentiate it from *M. fortuitum*, reducing its binding to the target position. The sequence and CRISPR-Cas result were shown in Figure 9b and 9c.

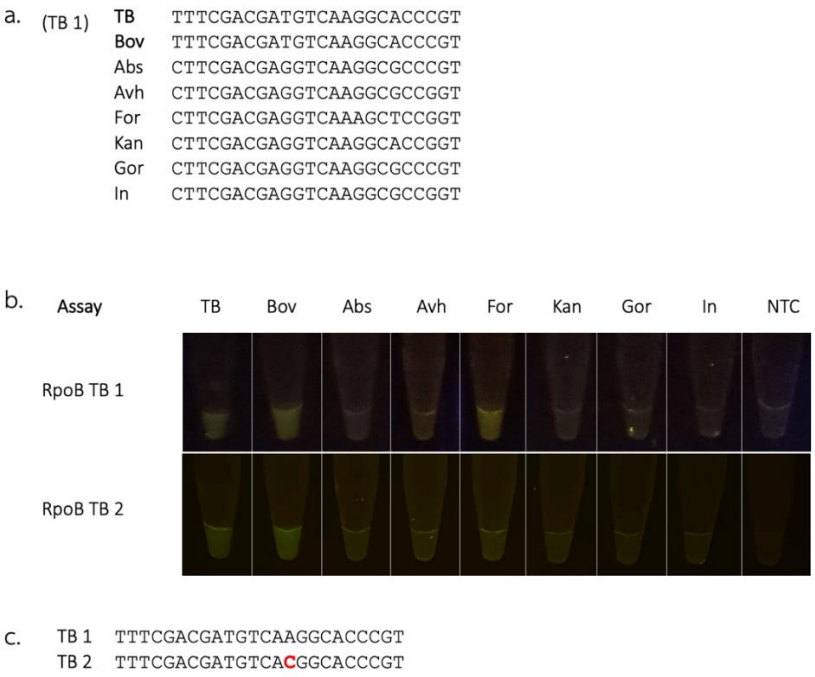


Figure 9 The detection of MTBC based on CRISPR-Cas12a

(a) DNA alignment of *rpoB* region specific to MTBC targets. (b) Cross-reactivity testing of CRISPR-Cas12a assay with TB 1 crRNA and TB 2 against other species. (c) PAM and spacer sequences of crRNA sequences for MTBC. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs) *M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare*. (NTC) Negative control (no template).

The crRNAs for M. abscessus

To design specific crRNA of *M. abscessus*, three regions in the RPA target containing PAM sequence 5'-TTTV-3' were chosen (Figure 10a). The result showed that Abs 1 crRNA of CRISPR-Cas12a provided the signal for *M. abscessus* but had also cross-reactivity with *M. avium* and *M. fortuitum* (Figure 10b). While other crRNAs (Abs 2 and Abs 3) had specificity with *M. abscessus* but had cross-reactivity with *M. gordanae* and *M. tuberculosis*, respectively (data not shown). To enhance the efficiency of crRNA binding to the target sequence, mismatches were introduced at positions 15th (guanine to adenosine) and 17th (guanine to adenosine) of the Abs 1 crRNA spacer, respectively (Figure 10c). The modified crRNAs called Abs 4 and Abs 5 showed low fluorescence yield and had cross-reactivity with *M. fortuitum* (Figure 10b, Abs 5 data not shown in the figure).

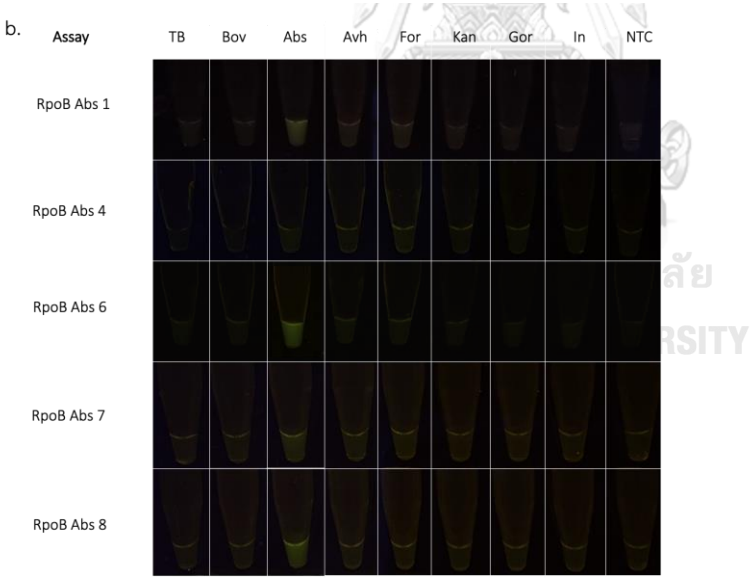
Upon experimental observation, spacers with PAM 5'-CTTV-3' sequence can cross-react with the assay. Hence, to identify additional positions for new crRNAs, the

regions with the PAM 5'-CTTV-3' sequence in the target sequence were selected. Three new crRNAs (Abs 6, 7, and 8) yield high fluorescence signals without cross-reactivity (Figure 10a and Figure 10b). Eventually, Abs 6 crRNA was chosen for the detection of *M. abscessus.*

a.

(Abs 1)	TB	TCTCCTTCGCTAAGCTGCGCGAAC	(Abs 2)	TB	TTCGAGTGGCTGATCGGTTCGCCG
	Bov	TCTCCTTCGCTAAGCTGCGCGAAC		Bov	TTCGAGTGGCTGATCGGTTCGCCG
	Abs	TTTCCTTCGCCAAGCTGCGTGAAC		**Abs**	TTTGAATGGCTGGTTGGATCGCCG
	Avh	TTTCCTTCGCCAAGCTGCGCGAAC		Avh	TTCGAGTGGTTGATCGGCGCGCCG
	For	TTTCATTTGCCAAGCTCCGTGAAC		For	TTCGACTGGCTCGTCGGTGCGGAT
	Kan	TTTCCTTCGCCAAGCTCCGCGAAC		Kan	TTCGAGTGGCTGATCGGTTCCCAG
	Gor	TTTCATTCGCCAAGCTCCGTGAAC		Gor	TTCGAGTGGCTGATCGGCTCGCAG
	In	TTTCCTTCGCCAAGCTCCGCGAGC		In	TTCGAGTGGTTGATCGGCGCGCCG
(Abs 3)	TB	TGTCTCCGATCGAGGACTTCTCCG	(Abs 6)	TB	CTTGAGGTTCCGGGACTCCTTGAC
	Bov	TGTCTCCGATCGAGGACTTCTCCG		Bov	CTTGAGGTTCCGGGACTCCTTGAC
	Abs	TTTCGCCGATCGAAGACTTCTCCG		**Abs**	CTTGCGGTTCCCGGCCTGCTCGAT
	Avh	TGTCGCCGATCGAGGACTTCTCGG		Avh	CTCGAGGTTCCCGGCCTGCTGGAC
	For	TCTCCCCGATCGAGGATTTCTCGG		For	CTTGAGGTTCCGGGGCTACTCGAC
	Kan	TCTCACCGATCGAGGACTTCTCCG		Kan	CTGGAGGTTCCGGGGTTGCTTGAC
	Gor	TCTCACCGATCGAGGACTTCTCCG		Gor	CTCGAGGTTCCGGGGCTGCTTGAC
	In	TCTCGCCGATCGAGGACTTCTCCG		In	CTCGAGGTTCCGGGCCTGCTTGAC
(Abs 7)	TB	GTTCGAGTGGCTGATCGGTTCGCC	(Abs 8)	TB	CTGGAAGAGGTGCTCTACGAGCTG
	Bov	GTTCGAGTGGCTGATCGGTTCGCC		Bov	CTGGAAGAGGTGCTCTACGAGCTG
	Abs	CTTTGAATGGCTGGTTGGATCGCC		**Abs**	CTTGAGGAGATCCTCACGGAGCTT
	Avh	CTTCGAGTGGTTGATCGGCGCGCC		Avh	CTGGAAGAGGTGCTCGACGAGCTG
	For	CTTCGACTGGCTCGTCGGTGCGGA		For	CTCGAAGAGGTGCTCGAAGAGCTC
	Kan	GTTCGAGTGGCTGATCGGTTCCCA		Kan	CTGGAAGAGGTGCTCGACGAGCTC
	Gor	ATTCGAGTGGCTGATCGGCTCGCA		Gor	CTCGAAGAGGTGCTCGACGAGCTC
	In	CTTCGAGTGGTTGATCGGCGCGCC		In	CTCGAAGAGGTGCTCTTGGAGCTC

b.



c.

```
Abs1  TTTCCTTCGCCAAGCTGCGTGAAC
Abs4  TTTCCTTCGCCAAGCTGCATGAAC
Abs5  TTTCCTTCGCCAAGCTGCGTAAAC
```

Figure 10 The detection of *M. abscessus* based on CRISPR-Cas12a

(a) DNA alignment of 6 *rpoB* regions containing PAM and spacer sequences. (b) Cross-reactivity test of the CRISPR-Cas12a assay with Abs 1, 4, 6, 7, and 8 crRNA against

other species. (c) Two additional crRNA sequences for *M. abscessus*. The red letters indicate the mismatch nucleotides. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs) *M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare*. (NTC) Negative control (no template).

The crRNAs for M. avium

To design crRNAs for *M. avium*, two regions in the RPA target that contained PAM sequence 5'-TTTV-3' were selected (Figure 11a). The Avh 1 crRNA of CRISPR-Cas12a yielded specificity with *M. avium* but had cross-reactivity with *M. abscessus* and *M. fortuitum* (Figure 11b). In contrast, the Avh 2 crRNA had specificity with *M. avium* but cross-reactivity with *M. gordonae, M. kansasii,* and *M. intracellulare* (data not shown in the figure). To reduce non-specificity, mismatches were introduced at the end of the Avh 1 crRNA sequence. A total of six different crRNAs with mismatches are illustrated in Figure 11c. The new modified crRNA is still showing cross-reactivity with other species, despite the mismatches (Figure 11b). As a final alternative, three crRNAs (Avh 9-11) in the new positions with the PAM 5'-CTTV-3' sequence were designed. The Avh 9 crRNA had high specificity with *M. avium* without cross-reactivity to other species (Figure 11b). While two crRNAs (Avh 10 and Avh 11) showed no fluorescent signal (data not shown in the figure).

a.

(Avh 1)

TB	TCTCCTTCGCTAAGCTGCGCGAAC
Bov	TCTCCTTCGCTAAGCTGCGCGAAC
Abs	TTTCCTTCGCCAAGCTGCGTGAAC
Avh	TTTCCTTCGCCAAGCTGCGCGAAC
For	TTTCATTTGCCAAGCTCCGTGAAC
Kan	TTTCCTTCGCCAAGCTCCGCGAAC
Gor	TTTCATTCGCCAAGCTCCGTGAAC
In	TTTCCTTCGCCAAGCTCCGCGAGC

(Avh 2)

TB	GTTCTCTGACCCTCGTTTCGACGA
Bov	GTTCTCTGACCCTCGTTTCGACGA
Abs	GTTCAGCGACCCGCGCTTCGACGA
Avh	TTTCTCCGACCCCCGCTTCGACGA
For	CTTCTCCGACCCGCGCTTCGACGA
Kan	CTTCTCCGACCCCCGCTTCGACGA
Gor	GTTCTCCGACCCTCGCTTCGACGA
In	GTTCTCCGACCCGCGCTTCGACGA

(Avh 9)

TB	CTTCGCTAAGCTGCGCGAACCACT
Bov	CTTCGCTAAGCTGCGCGAACCACT
Abs	CTTCGCCAAGCTGCGTGAACCGCT
Avh	CTTCGCCAAGCTGCGCGAACCGCT
For	ATTTGCCAAGCTCCGTGAACCGCT
Kan	CTTCGCCAAGCTCCGCGAACCGCT
Gor	ATTCGCCAAGCTCCGTGAACCGCT
In	CTTCGCCAAGCTCCGCGAGCCCCT

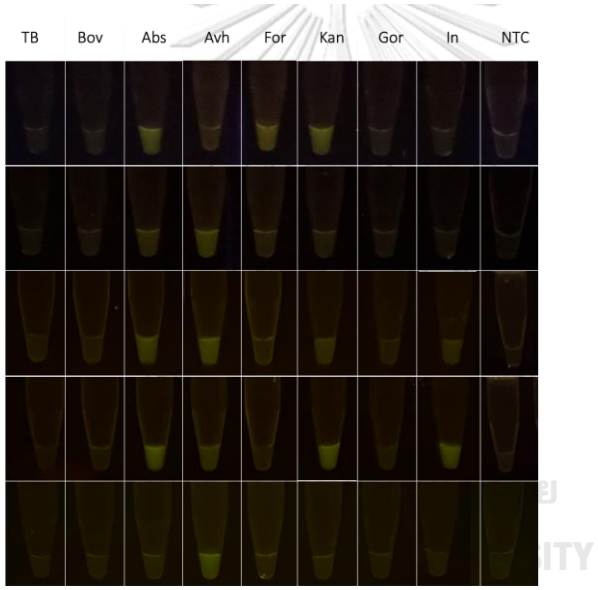
(Avh 10)

TB	CTTCGCTAAGCTGCGCGAACCACT
Bov	CTTCGCTAAGCTGCGCGAACCACT
Abs	CTTCGCCAAGCTGCGTGAACCGCT
Avh	CTTCGCCAAGCTGTGCGAACCGCT
For	ATTTGCCAAGCTCCGTGAACCGCT
Kan	CTTCGCCAAGCTCCGCGAACCGCT
Gor	ATTCGCCAAGCTCCGTGAACCGCT
In	CTTCGCCAAGCTCCGCGAGCCCCT

(Avh 11)

TB	CTTCGCTAAGCTGCGCGAACCACT
Bov	CTTCGCTAAGCTGCGCGAACCACT
Abs	CTTCGCCAAGCTGCGTGAACCGCT
Avh	CTTCGCCAAGCTGCACGAACCGCT
For	ATTTGCCAAGCTCCGTGAACCGCT
Kan	CTTCGCCAAGCTCCGCGAACCGCT
Gor	ATTCGCCAAGCTCCGTGAACCGCT
In	CTTCGCCAAGCTCCGCGAGCCCCT

b.



c.

Avh1	TTTCCTTCGCCAAGCTGCGCGAAC
Avh3	TTTCCTTCGCCAAGCTGCGC**A**AAC
Avh4	TTTCCTTCGCCAAGCTGCG**TT**AAC
Avh5	TTTCCTTCGCCAAGCTGCG**AT**AAC
Avh6	TTTCCTTCGCCAAGCTGC**A**CGAAC
Avh7	TTTCCTTCGCCAAGCTGC**A**C**A**AAC
Avh8	TTTCCTTCGCCAAGCTGCGCG**G**AC

Figure 11 The detection of *M. avium* based on CRISPR-Cas12a

(a) DNA alignment of 5 *rpoB* regions containing PAM and spacer sequences. (b) Cross-reactivity testing of CRISPR-Cas12a assay with Avh 1, 3, 6, 8, and 9 crRNA against other species. (c) Six additional crRNA sequences for *M. avium*. The red letters indicate the mismatch nucleotides. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs*) M. abscessus* (AvH) *M.*

avium (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare.* (NTC) Negative control (no template).

The crRNAs for M. fortuitum

To design crRNAs for *M. fortuitum,* three regions within the RPA product were chosen (Figure 12a). The MF 1 and MF 2 crRNAs were not specific to *M. fortuitum* but *were* only specific with *M. gordonae (*data not shown in the figure). However, MF 3 had flurescence signal to *M. fortuitum* with low fluorescence signals with *M. avium*, indicating cross-reactivity. Therefore, the mismatches at positions 17th and 18th were introduced to MF 3 crRNA to increase the difference between *M. fortuitum* and *M. avium* (Figure 12c). The MF4 crRNA showed a low fluorescence signal for both of *M. fortuitum* and *M. avium*. In addition, MF 5 crRNA showed a low fluorescence signal-specific *M. fortuitum* without cross-reactivity with *M. avium* (Figure 12b).

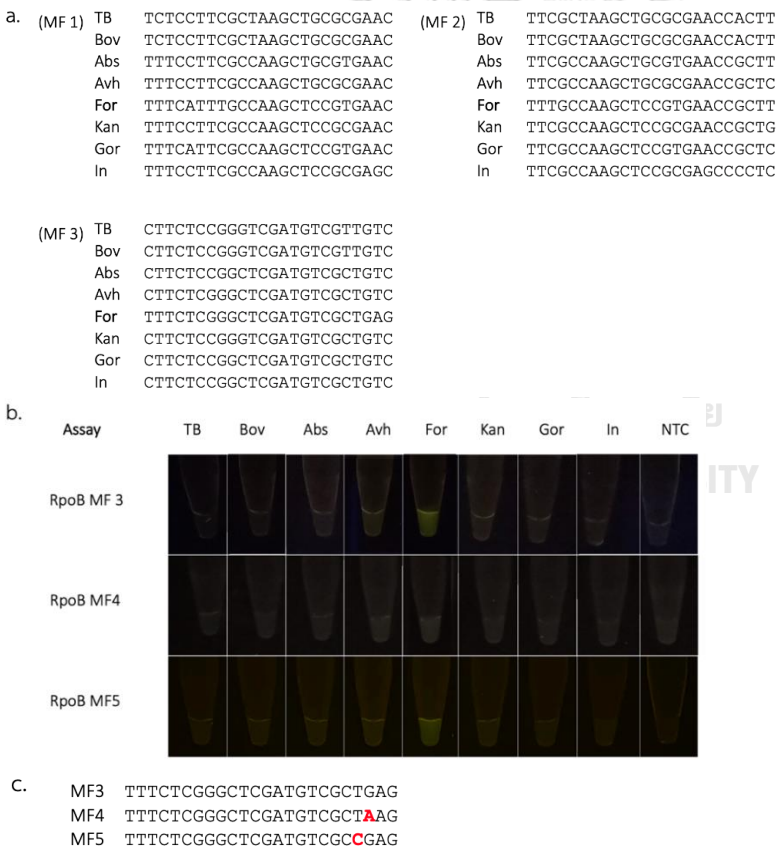


Figure 12 The detection of *M. fortuitum* on CRISPR-Cas12a

(a) DNA alignment of 3 *rpoB* regions containing PAM and spacer sequences. (b) Cross-reactivity testing of CRISPR-Cas12a assay with MF crRNAs against other species. (c)

Two additional crRNA sequences for *M. fortuitum.* The red letters indicate the mismatch nucleotides. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs*) M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare.* (NTC) Negative control (no template).

The crRNAs for M. kansasii

To design crRNAs for *M. kansasii,* the first crRNA was chosen and called "MK 1" (Figure 13a). The results demonstrate that this crRNA of CRISPR-Cas12a yielded specificity with *M. kansasii* but had cross-reactivity with *M. fortuitum, M. gordonae,* and *M. intracellulare* (data not shown in the figure). Therefore, a new crRNA (MK 2) in the new position with the PAM 5'-CTTV-3' sequence was designed. The MK 2 crRNA had high specificity with *M. kansasii* without cross-reactivity other species (Figure 13b).

Figure 13 The detection of *M. kansasii* on CRISPR-Cas12a

(a) DNA alignment of 2 *rpoB* regions containing PAM and spacer sequences. (b) Cross-reactivity testing of CRISPR-Cas12a assay with MK 2 a crRNA against other species. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs*) M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare.* (NTC) Negative control (no template).

The crRNAs for M. gordonae

To design crRNAs for *M. gordonae,* two regions within the RPA product were chosen (Figure 14a). The MG 2 crRNA had high specificity with *M. gordonae* without cross-reactivity other species (Figure 14b). While MG 1 crRNA showed no fluorescent signal (data not shown in the figure).

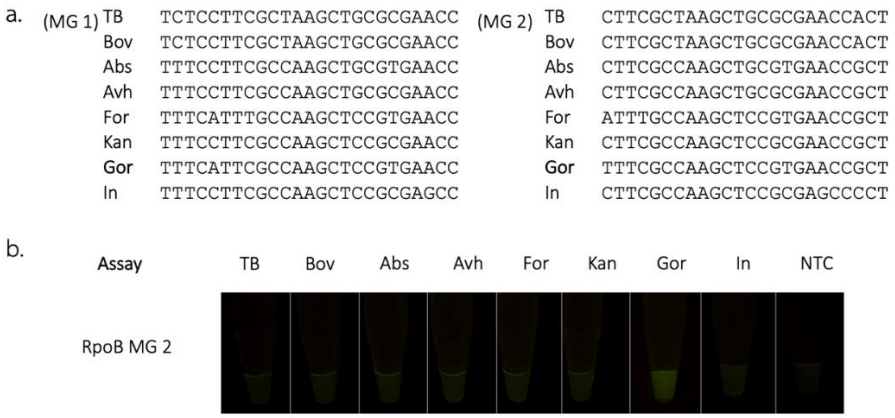


Figure 14 The detection of *M. gordonae* on CRISPR-Cas12a

(a) DNA alignment of 2 *rpoB* regions containing PAM and spacer sequences. (b) Cross-reactivity testing of CRISPR-Cas12a assay with MG 2 a crRNA against other species. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs) *M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare.* (NTC) Negative control (no template).

The crRNAs for M. intracellulare

To design crRNAs for *M. intracellulare*, the first crRNA was chosen and called "MI 1" (Figure 15a). The MI 1 crRNA of CRISPR-Cas12a yielded specificity with *M. intracellulare* but had high cross-reactivity with *M. kansasii* (Figure 15b). To reduce non-specificity, mismatches were introduced at the MI 1 crRNA sequence. A total of three different crRNAs with mismatches are illustrated in Figure 15c. The new modified crRNA is still showing cross-reactivity with other species, despite the mismatches (Figure 15b). As a final alternative, two crRNAs (MI 5 and MI 6) in the new positions with the PAM 5'-CTTV-3' sequence were designed. Both MI 5 and MI 6 crRNAs yield high fluorescence signals without cross-reactivity (Figure 15b). Eventually, MI 6 crRNA was chosen for the detection of *M. intracellulare.*

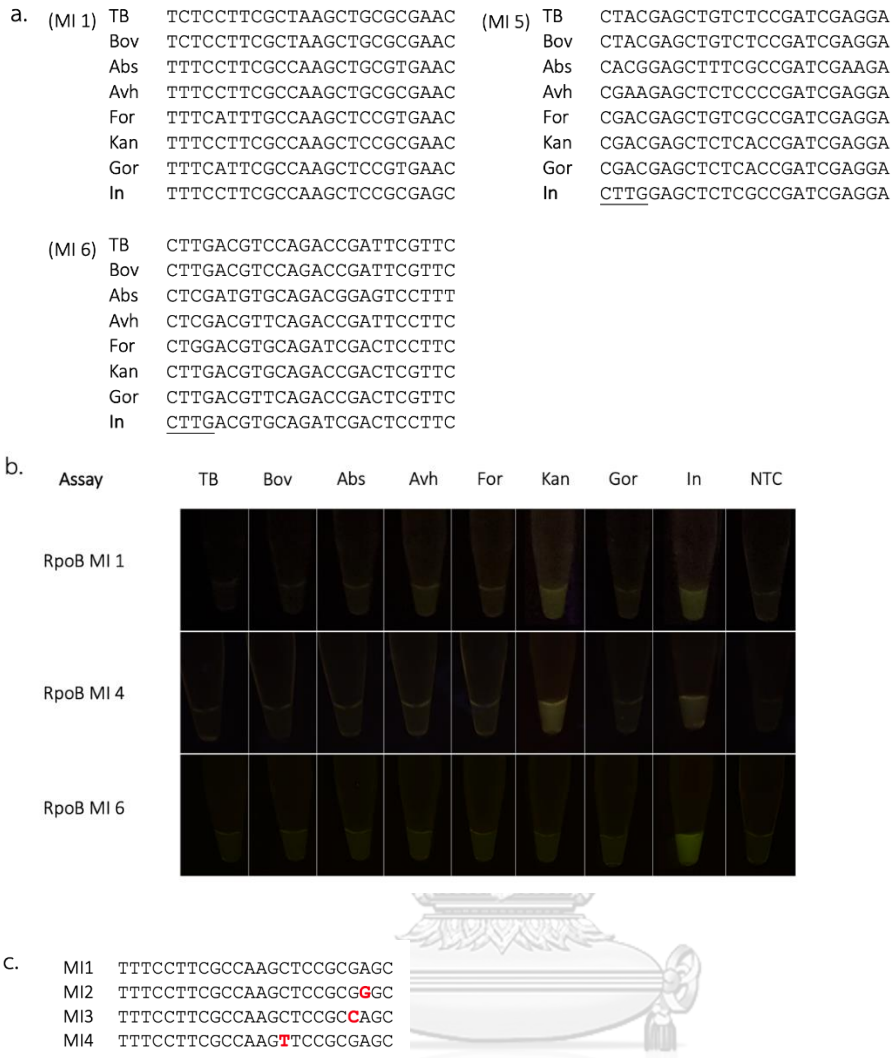


Figure 15 The detection of *M. intracellulare* on CRISPR-Cas12a

(a) DNA alignment of 3 *rpoB* regions containing PAM and spacer sequences. (b) Cross-reactivity testing of CRISPR-Cas12a assay with MI 1, 4, and 6 a crRNA against other species. (c) Three crRNA sequences for *M. intracellulare*. The red letters indicate the mismatch nucleotides. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs*) M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare.* (NTC) Negative control (no template).

Finally, we selected the best crRNA for each mycobacterial species to be analyzed. From the fluorescence detection results shown in Figure 16, each designed crRNA was able to identify the specific mycobacterial species without cross-reaction. This suggested that the assays can be applied for discrimination between mycobacterial species.

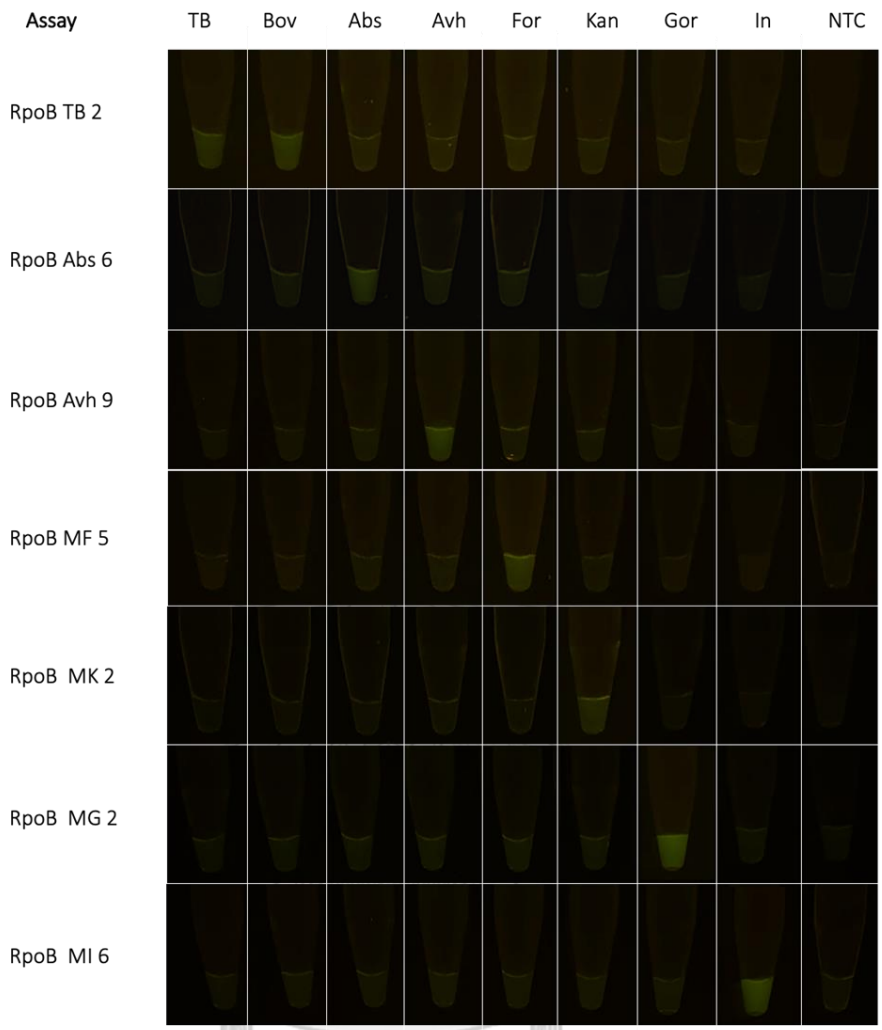


Figure 16 Determination of the specificity of the designed crRNAs using reference *Mycobacterium* species

The final list of all crRNAs consists of TB 2, Abs 6, Avh 9, MF 5, MK 2, MG 2, and MI 6. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs*) M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare.* (NTC) Negative control (no template).

Limit of detection tests

To evaluate the limit of detection (LOD), each standard DNA were diluted to the concentration ranging from 1 to 10^5 copies/µl and were used as a template for RPA reaction with CRISPR-Cas12a. The results revealed that the limit of detection for *Mycobacterium* spp. was approximately 1-10 copies/µl as shown in Figure 17. The summary for the LOD of each mycobacterial species is illustrated in Table 13.

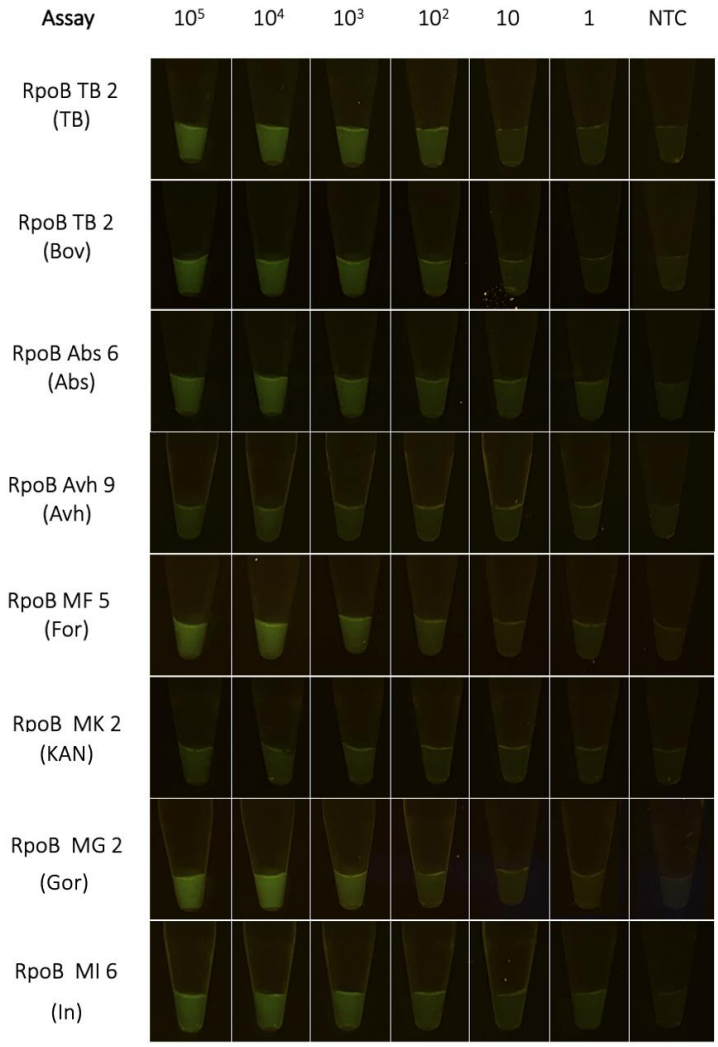


Figure 17 Evaluation of LOD for RPA-CRISPR Cas system for *Mycobacterium* spp. The standard DNA ranging from 1 to 10^5 copies/µl and TB 2, Abs 6, Avh 9, MF 5, MK 2, MG 2, and MI 6 crRNAs were used in this experiment. (TB) *M. tuberculosis* (Bov) *M. bovis* (Abs*) M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare.* (NTC) Negative control (no template).

Table 13 Summary of the results for the limit of detection

crRNAs	Limit of detection (copies)
TB 2	10
Abs 6	1
Avh 9	10
MF 5	1
MK 2	10
MG 2	1
MI 6	1

Performance evaluation of detection in clinical isolates

Evaluation of the performance of CRISPR-Cas12a-based diagnosis clinical isolates of 74 samples. Due to the limited number of samples collected, the number of clinical isolates used in the assay was fewer than the initial sample size calculated value. This clinical testing *rpoB* gene detection of each mycobacterial species. Reading the test results involved observing the fluorescence within the reaction tubes that indicated the occurrence of detection. The presence of fluorescence indicates a positive reaction, as depicted in Figure 18.

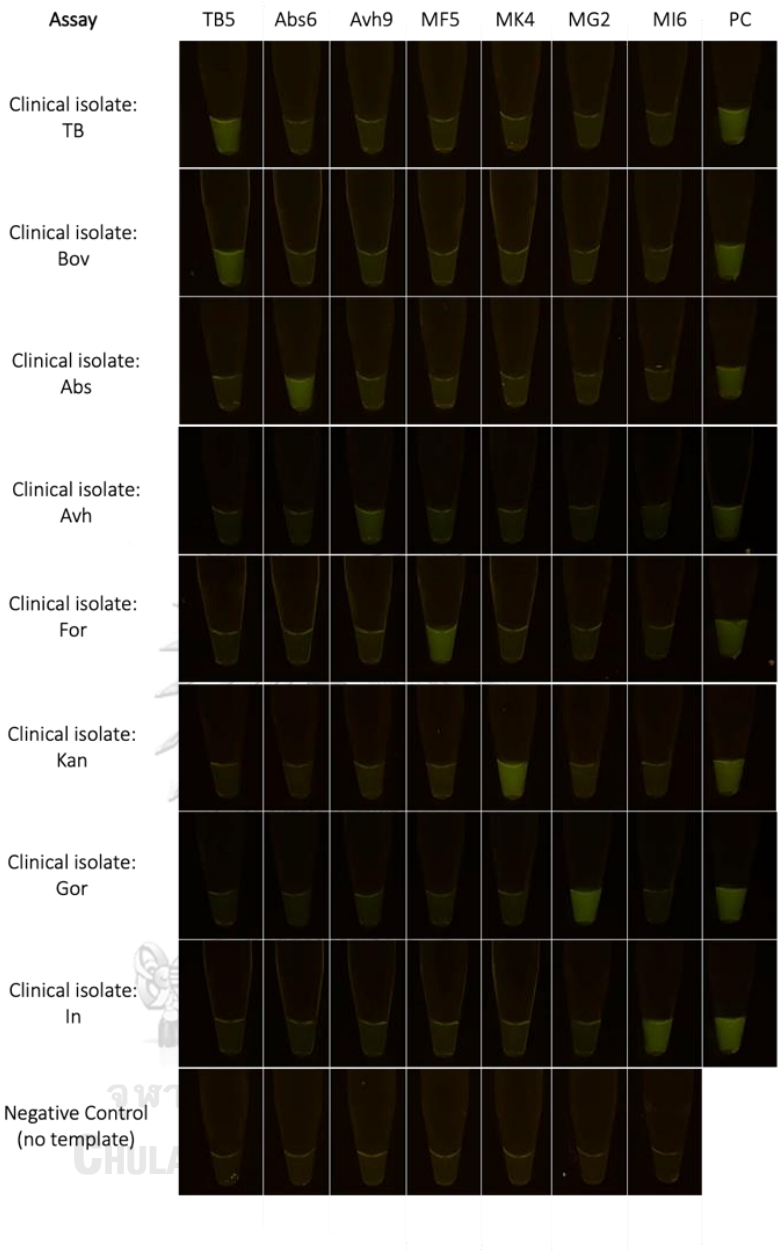


Figure 18 Example evaluation of the performance of CRISPR-Cas12a-based diagnosis in 8 clinical isolates

The crRNAs consist of TB 2, Abs 6, Avh 9, MF 5, MK 2, MG 2, and MI 6. (TB) *M. tuberculosis* (Bov) *M. bovis* BCG (Abs*) M. abscessus* (AvH) *M. avium* (For) *M. fortuitum* (Kan) *M. kansasii* (Gor) *M. gordonae* (In) *M. intracellulare.* (NTC) Negative control (no template).

The clinical testing involved 74 samples using line probe assay and RPA-CRISPR Cas12a assay in the identification *Mycobacterium* species. The samples will be divided into species, each consisting of 10 samples per species, except *M. bovis*

bacillus Calmette-Guérin (BCG) had 2 samples, and *M. gordonae* had 9 samples, *M. avium* had 6 samples and *M. intracellulare* had 17 samples. The results of the testing are presented in Table 14.

Table 14 Number of samples tested and the performance of RPA-CRISPR-Cas12a and LPA assay

Clinical isolates	No. of clinical isolates (%)	LPA No. correctly Identified (%)	RPA-CRISPR-Cas12a No. correctly Identified (%)	RPA-CRISPR-Cas12a No. misidentified (%)	crRNA name
M. tuberculosis	10 (13.51)	10 (100)	10 (100)	0	TB 2
M. bovis BCG	2 (2.70)	2 (100)	2 (100)	0	TB 2
M. abscessus	10 (13.51)	10 (100)	10 (100)	0	Abs 6
M. fortuitum	10 (13.51)	10 (100)	10 (100)	0	MF 5
M. kansasii	10 (13.51)	10 (100)	10 (100)	0	MK 2
M. gordonae	9 (12.61)	9 (100)	8 (88.89)	1	MG 2
M. avium	6 (8.10)	6 (100)	4 (66.67)	2	Avh 9
M. intracellulare	17 (22.97)	17 (100)	12 (70.59)	5	MI 6
Total	**74**	**74**	**66**	**8**	**-**

The results showed that the performance of the RPA-CRISPR-Cas12a assay had a total of 66 out of 74 clinical isolate samples correctly identified. Seven samples thought to be other species were misidentified by the Avh 9 and MI 6 crRNA. Then, one *M. gordonae* sample had not been detected by the MG 2 crRNA. Additionally, the test showed that two samples of seven samples thought to be *M. kansasii* were identified by MI 6 crRNA. However, we further investigated using whole genome sequencing data of two *M. kansasii* samples. The sequencing data showed contamination levels exceeding 100%, indicating the presence of two species in that sample, which might have resulted in false positives.

CHAPTER 5

DISCUSSION

Tuberculosis (TB) and *Mycobacterium* infections are global health concerns that leading causes of death among infectious diseases. Therefore, early detection and identification of the *Mycobacterium* spp. causing the disease would have a significant clinical impact since the treatment of the infection caused by MTB complex is different from that of non-tuberculosis species for helping diagnosis, would be an important factor, which could lead to early treatment and stop the disease [68]. The standard *Mycobacterium* detection and identification method is a combination of culture-based methods and molecular techniques, such as mycobacterial culture and the Xpert MTB/RIF assay, respectively [69-71]. However, this assay requires specialized facilities and instrument that is not portable and only available with a high capital investment [72]. Here, the present assay was developed based on RPA combined with the CRISPR-Cas12a system for portable detection of *Mycobacterium* spp. (Figure 4). This technique is a portable method for detecting *Mycobacterium* that requires a few basic instruments comprising a heat box for RPA isothermal amplification and a BluPAD Dual LED Blue/White Light Transilluminator for the fluorescent signal observed by the naked eye and provided the turn-around time within 1 hour, which was obviously shorter than the standard method.

This study demonstrated the Limit of Detection (LOD) of *Mycobacterium* spp. was approximately 1 and 10 copies/µl. In contrast, Kim et al. (2021) reported a LOD of *M. tuberculosis* at 10^4 copies/µl using LAMP assay [73]. Consequently, the combination of RPA for amplification and CRISPR-Cas12a for detection in this assay outperformed the sole use of LAMP assay. Additionally, Ai et al. (2019) reported a LOD of *M. tuberculosis* at 5 copies/µl using RPA combined CRISPR-Cas12a assay. These favorable results align with our research findings, in which the same technique was employed for the detection of *Mycobacterium* spp. [21]. Therefore, the improved LOD achieved by incorporating the CRISPR-Cas12a detection step highlights its efficacy in identifying target DNA even at low genetic material concentrations [21]. These findings have significant implications and offer a promising alternative for the diagnosis and early detection of *Mycobacterium* spp.

In addition, this RPA reaction showed the *rpoB* target gene was successfully amplified by designing primers in the conserved region of *Mycobacterium* spp. Generally, the primers for RPA amplification were designed with appropriate lengths (30 to 35 nucleotides) and specific to the target gene [74]. However, this study

demonstrates that RPA primer was designed with a length of 23 bp forward primer and 36 bp reverse primer. These primers can be utilized to amplify the *rpoB* target gene (product size 308 bp) of *Mycobacterium* spp. Adding multiple bases can also increase amplified target DNA in some *Mycobacterium* species. Consequently, this design enables efficient and accurate amplification of the target gene in various *Mycobacterium* species. This capability has significant implications in the field of molecular diagnostics and research, as it allows for the detection and identification of different *Mycobacterium* species using a common target gene. The conservation of the amplified region ensures the specificity and reliability of the assay [75].

The designed primers provide a valuable tool for studying the *rpoB* gene across various *Mycobacterium* species. Moreover, these primer pairs can also aid in studying the specificity of detection using *Mycobacterium*-specific crRNA of eight clinically relevant species. During the specificity testing of CRISPR-Cas12a for *Mycobacterium* spp. detection, it was observed that the first designed crRNA exhibited specificity towards each *Mycobacterium* species but showed cross-reactivity with some other species. To improve the specificity of each *Mycobacterium* species crRNA, a mismatch strategy based on the work of Ooi et al. (2021) were implemented [76]. The modified crRNA has less binding affinity to the non-target compared to the target, minimizing the occurrence of cross-reactivity with closely related species. Therefore, the development of the modified crRNA demonstrates the potential for tailored crRNA designed to enhance the accuracy and reliability of *Mycobacterium* spp. detection using CRISPR-Cas12a technology. In addition to the standard PAM 5'-TTTV-3', the PAM 5'-CTTV-3' can also be an alternative [77]. This research provides another option for crRNA design.

In this detection system, the diagnostic performance for this assay through clinical isolates (n=74) yielded were observed to identify *Mycobacterium* spp. by specific crRNAs targeting *rpoB* of each species. This performance showed that 66 of the 74 clinical isolates were correctly identified. Out of these eight misidentified clinical isolates, seven clinical isolates were false positives by *M. intracellulare* crRNA (MI 6) and *M. avium* crRNA (Avh 9), and one clinical isolate was false negative by *M. gordonae* crRNA (MG 2). However, the errors leading to false positives and false negatives have been preliminarily investigated using whole-genome sequencing. It was observed that some issues arose from sample contamination. For example, the contamination genome data in the sample identified as false positive by MI 6 crRNA in *M. kansasii* clinical isolates showed contamination levels exceeding 100%, indicating the presence of two species in that sample. However, further verification is

necessary to confirm if the identified species align with the experimental results. In addition, our results should be interpreted cautiously; due to the small number of samples analyzed, no following sample size analyses could be performed. Moreover, the RPA combined with the CRISPR-Cas12a assay with MI crRNA bring about to false positive because was unable to differentiate between *M. intracellulare*, *M. chimaera* and *M. yongonense*, three closely related potentially pathogenic species of NTM that are members of the *M. avium* complex (MAC group) [78]. In the future, a detailed investigation of errors will be necessary using techniques such as NGS sequencing to confirm the actual efficiency of this assay.

Despite some disconcordance between the RPA combined with the CRISPR-Cas12a assay results and LPA for *M. gordonae*, *M. avium* and *M. intracellulare*, this assay showed concordance between LPA correctly identified 100 % in *M. tuberculosis*, *M. bovis* BCG, *M. abscesses*, *M. fortuitum* and *M. kansasii*, a major *Mycobacterium* species. Moreover, the method shows reduced time required by the RPA combined with the CRISPR-Cas12a assay (less than 1 hour). While the current clinical reference method requires at least 5 hours for the Line Probe Assay GenoType CM, the Line Probe GenoType *Mycobacterium* AS or NTM-DR (Hain LifeScience, Nehren, Germany). In comparison, the time required to run this study assay analysis is more rapid than the standard method.

Nevertheless, this study also has other limitations. One significant limitation is the absence of direct clinical sample evaluation for the RPA combined with the CRISPR-Cas12a assay in the detection and identification of *Mycobacterium* infections. The use of clinical samples, including sputum, and bronchoalveolar lavage fluid, is crucial for assessing the performance and reliability of the assay in real-world scenarios [79]. To address these limitations, future investigations should prioritize the evaluation of the RPA combined with the CRISPR-Cas12a assay using clinical samples. This will provide more comprehensive and clinically relevant data on the assay's performance and feasibility in diagnosing and monitoring *Mycobacterium* infections.

In conclusion, the RPA combined with CRISPR-Cas12a technology offers several advantages for *Mycobacterium* detection. The assay is portable, user-friendly, and provides results within a short timeframe, typically within one hour. These features make it a promising tool for point-of-care testing and rapid diagnosis of *Mycobacterium* infection.

Suggestions

The utilization of CRISPR-Cas technology in genetic testing is an intriguing proposition. Not only does it present a promising method for genetic testing, but it also holds potential for further development as a diagnostic tool. However, the interpretation of results based on fluorescence signals observed by the naked eye may introduce discrepancies in result interpretation. Therefore, future enhancements could focus on the development of portable fluorescence readers capable of providing precise numerical values, facilitating easier result interpretation. Furthermore, ongoing improvements should address the speed and accuracy of genetic testing processes to ensure reliable and efficient results.

Appendixes

5X Tris-Borate buffer (TBE), 1 L

Tris-base (M.W. = 121.14)	54 g
Boric acid (M.W. = 61.83)	27.5 g
EDTA (M.W. = 372.24)	4.16 g
ddH_2O	to 1 L

1% agarose gel

Agarose	1 g
1X TBE	100 ml
Red safe	5 µl

2% agarose gel

Agarose	2 g
1X TBE	100 ml
Red safe	5 µl

LB agar with ampicillin, 100 ml

Agar	1.5 g
Yeast extract	0.5 g
Tryptone	1 g
5 M NaCl	0.5 g
ddH_2O	100 ml
Ampicillin (100 mg/ml)	100 µl

LB broth with ampicillin, 100 ml

Yeast extract	0.5 g
Tryptone	1 g
5 M NaCl	0.5 g
ddH_2O	100 ml
Ampicillin (100 mg/ml)	100 µl

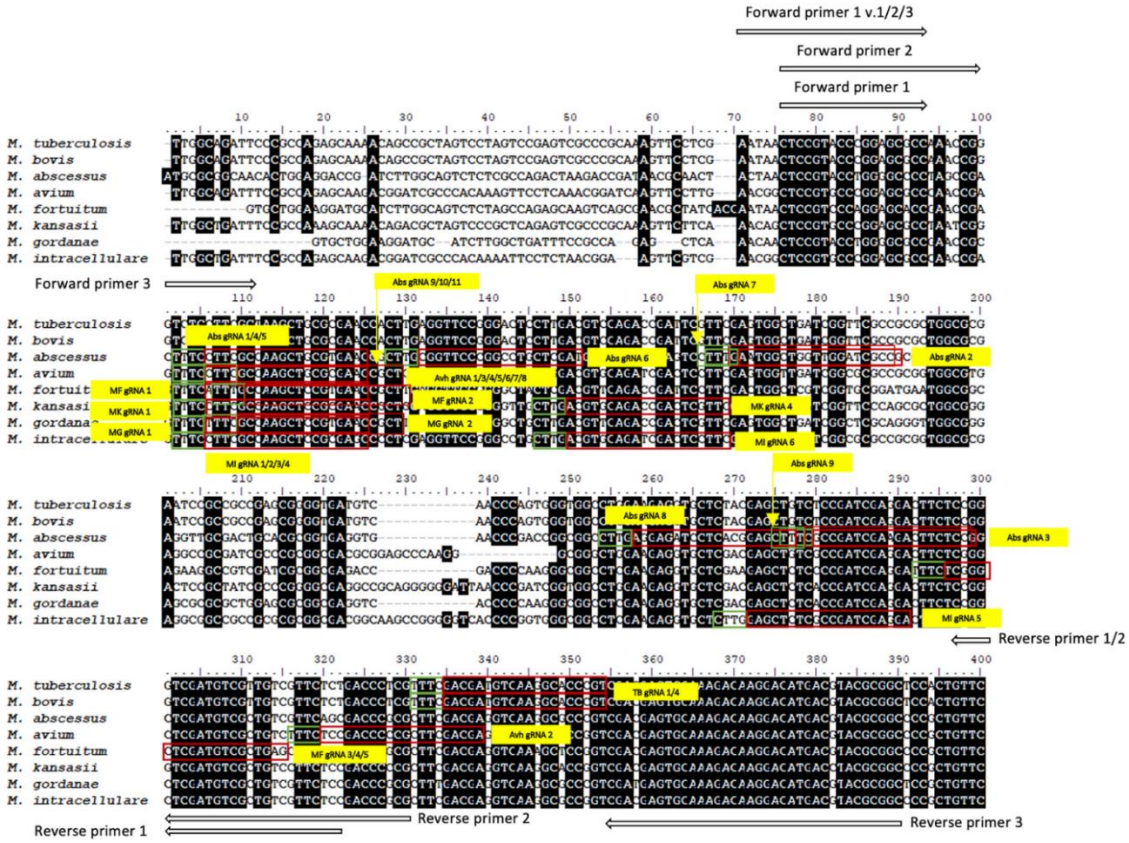
SOC media, 100 ml

Yeast extract	0.5 g
Tryptone	1 g
5 M NaCl	0.5 g

ddH_2O	100 ml
2 M Glucose	10 µl
2 M Magnesium	10 µl

Supplement

Supplementary Figure 1. Nucleotide sequences from BTSeq™ barcode-tagged sequencing with RPA primers and crRNAs.



Alignment of *rpoB* sequences from eight *Mycobacterium* spp. Conserved sequences are highlighted in black. Red boxes represent crRNA-targeted species regions. Green boxes represent the PAM motif sequences. The yellow boxes represent the names of crRNAs. The arrows indicate the positions of the RPA primers.

REFERENCES

1. Bartram, J., *Pathogenic Mycobacteria in Water: A guide to public health consequences, monitoring and management*. 2004.
2. Percival, S.L. and D.W. Williams, *Chapter Nine - Mycobacterium*, in *Microbiology of Waterborne Diseases (Second Edition)*, S.L. Percival, et al., Editors. 2014, Academic Press: London. p. 177-207.
3. Bagcchi, S., *WHO's Global Tuberculosis Report 2022.* Lancet Microbe, 2023. **4**(1): p. e20.
4. Uchida, Y., et al., *Safety and Efficacy of Nontuberculous Mycobacteria Treatment among Elderly Patients.* Medicina (Kaunas), 2020. **56**(10).
5. Dahl, V.N., et al., *Global trends of pulmonary infections with nontuberculous mycobacteria: a systematic review.* Int J Infect Dis, 2022.
6. Nasiri, M.J., et al., *Antibiotic therapy success rate in pulmonary Mycobacterium avium complex: a systematic review and meta-analysis.* Expert Rev Anti Infect Ther, 2020. **18**(3): p. 263-273.
7. Raju, R.M., et al., *Leveraging Advances in Tuberculosis Diagnosis and Treatment to Address Nontuberculous Mycobacterial Disease.* Emerg Infect Dis, 2016. **22**(3): p. 365-9.
8. Saxena, S., H.P. Spaink, and G. Forn-Cuní, *Drug Resistance in Nontuberculous Mycobacteria: Mechanisms and Models.* Biology (Basel), 2021. **10**(2).
9. Koch, M.L. and R.A. Cote, *COMPARISON OF FLUORESCENCE MICROSCOPY WITH ZIEHL-NEELSEN STAIN FOR DEMONSTRATION OF ACID-FAST BACILLI IN SMEAR PREPARATIONS AND TISSUE SECTIONS.* Am Rev Respir Dis, 1965. **91**: p. 283-4.
10. Levidiotou, S., et al., *Detection of mycobacteria in clinical specimen using the mycobacteria growth indicator tube (MGIT) and the Lowenstein Jensen medium.* Microbiol Res, 1999. **154**(2): p. 151-5.
11. Pai, M., et al., *Tuberculosis.* Nature Reviews Disease Primers, 2016. **2**(1): p. 16076.
12. Wu, X., et al., *Comparison of three methods for rapid identification of mycobacterial clinical isolates to the species level.* J Clin Microbiol, 2007. **45**(6):

p. 1898-903.

13. Nurwidya, F., et al., *Molecular Diagnosis of Tuberculosis.* Chonnam Med J, 2018. **54**(1): p. 1-9.
14. Neonakis, I.K., et al., *Molecular diagnostic tools in mycobacteriology.* J Microbiol Methods, 2008. **75**(1): p. 1-11.
15. Chen, J.S., et al., *CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity.* Science, 2018. **360**(6387): p. 436-439.
16. Myhrvold, C., et al., *Field-deployable viral diagnostics using CRISPR-Cas13.* Science, 2018. **360**(6387): p. 444-448.
17. Feng, W., et al., *CRISPR technology incorporating amplification strategies: molecular assays for nucleic acids, proteins, and small molecules.* Chemical science, 2021. **12**(13): p. 4683-4698.
18. Mayuramart, O., et al., *Detection of severe acute respiratory syndrome coronavirus 2 and influenza viruses based on CRISPR-Cas12a.* Exp Biol Med (Maywood), 2021. **246**(4): p. 400-405.
19. Liu, P., et al., *A Recombinase Polymerase Amplification-Coupled Cas12a Mutant-Based Module for Efficient Detection of Streptomycin-Resistant Mutations in Mycobacterium tuberculosis.* Frontiers in Microbiology, 2022. **12**.
20. Sam, I.K., et al., *TB-QUICK: CRISPR-Cas12b-assisted rapid and sensitive detection of Mycobacterium tuberculosis.* J Infect, 2021. **83**(1): p. 54-60.
21. Ai, J.-W., et al., *CRISPR-based rapid and ultra-sensitive diagnostic test for Mycobacterium tuberculosis.* Emerging microbes & infections, 2019. **8**(1): p. 1361-1369.
22. Cook, G.M., et al., *Physiology of mycobacteria.* Adv Microb Physiol, 2009. **55**: p. 81-182, 318-9.
23. Murray, P.R., K.S. Rosenthal, and M.A. Pfaller, *Medical microbiology*. 5th ed. 2005, Philadelphia: Elsevier Mosby Philadelphia.
24. Radulski, Ł., et al., *Infection of a Free-Living Wild Boar (Sus scrofa) with a Bacterium from the Mycobacterium kansasii Complex.* Animals (Basel), 2022. **12**(8).
25. Borham, M., et al., *Review on Bovine Tuberculosis: An Emerging Disease*

Associated with Multidrug-Resistant Mycobacterium Species. Pathogens, 2022. **11**(7).

26. Henkle, E. and K.L. Winthrop, *Nontuberculous mycobacteria infections in immunosuppressed hosts.* Clin Chest Med, 2015. **36**(1): p. 91-9.
27. Mogami, R., et al., *Pulmonary infection caused by Mycobacterium kansasii: findings on computed tomography of the chest.* Radiol Bras, 2016. **49**(4): p. 209-213.
28. Griffith, D.E. and C.L. Daley, *Treatment of Mycobacterium abscessus Pulmonary Disease.* Chest, 2022. **161**(1): p. 64-75.
29. Park, S., et al., *Clinical significance of Mycobacterium fortuitum isolated from respiratory specimens.* Respir Med, 2008. **102**(3): p. 437-42.
30. Mazumder, S.A., A. Hicks, and J. Norwood, *Mycobacterium gordonae pulmonary infection in an immunocompetent adult.* N Am J Med Sci, 2010. **2**(4): p. 205-7.
31. Horsburgh, C.R., Jr., *The pathophysiology of disseminated Mycobacterium avium complex disease in AIDS.* J Infect Dis, 1999. **179 Suppl 3**: p. S461-5.
32. Burman, W.J., et al., *AIDS-related Mycobacterium kansasii infection with initial resistance to clarithromycin.* Diagn Microbiol Infect Dis, 1998. **31**(2): p. 369-71.
33. Ramirez, J., et al., *Mycobacterium avium complex pulmonary disease: management options in HIV-negative patients.* J La State Med Soc, 2008. **160**(5): p. 248-54; quiz 254, 293.
34. Itano, J., et al., *A case of axillary lymphadenitis caused by Mycobacterium intracellulare in an immunocompetent patient.* Respiratory Medicine Case Reports, 2019. **28**: p. 100947.
35. Youssef, D., et al., *Pulmonary infection with caseating mediastinal lymphadenitis caused by Mycobacterium gordonae.* Int J Mycobacteriol, 2014. **3**(3): p. 220-3.
36. Che Engku Chik, C.E.N., et al., *Detection of Tuberculosis (TB) using Gold Standard Method, Direct Sputum Smears Microscopy, PCR, qPCR and Electrochemical DNA Sensor.* 2016. **4**: p. 16-21.
37. Sitthidet Tharinjaroen, C., *Review article: Tuberculosis diagnosis: From knowledge to innovation in public health.* Journal of Associated Medical

Sciences, 2017. **50**(1): p. 1.

38. Tagliani, E., et al., *Handbook on tuberculosis laboratory diagnostic methods in the European Union Handbook on tuberculosis laboratory diagnostic methods in the European Union*. 2023.
39. Yang, C.S., J.M. Yuk, and E.K. Jo, *The role of nitric oxide in mycobacterial infections.* Immune Netw, 2009. **9**(2): p. 46-52.
40. Lee, B.H., et al., *Impact of Implementation of an Automated Liquid Culture System on Diagnosis of Tuberculous Pleurisy.* J Korean Med Sci, 2015. **30**(7): p. 871-5.
41. Ryu, Y.J., W.J. Koh, and C.L. Daley, *Diagnosis and Treatment of Nontuberculous Mycobacterial Lung Disease: Clinicians' Perspectives.* Tuberc Respir Dis (Seoul), 2016. **79**(2): p. 74-84.
42. Raveendran, R. and C. Wattal, *Utility of multiplex real-time PCR in the diagnosis of extrapulmonary tuberculosis.* The Brazilian Journal of Infectious Diseases, 2016. **20**(3): p. 235-241.
43. Morais, F.C.L., et al., *Detection of non-tuberculosus mycobacteria (NTMs) in lung samples using 16S rRNA.* Mem Inst Oswaldo Cruz, 2022. **117**: p. e220031.
44. Gonçalves, M.G., et al., *Fast test for assessing the susceptibility of Mycobacterium tuberculosis to isoniazid and rifampin by real-time PCR.* Mem Inst Oswaldo Cruz, 2012. **107**(7): p. 903-8.
45. Panpradist, N., et al., *Simpler and faster Covid-19 testing: Strategies to streamline SARS-CoV-2 molecular assays.* EBioMedicine, 2021. **64**: p. 103236.
46. Sailo, C.V., et al., *Distribution and frequency of common mutations in rpoB gene of Mycobacterium tuberculosis detected by Xpert MTB/RIF and identification of residential areas of rifampicin resistant-TB cases: A first retrospective study from Mizoram, Northeast India.* J Clin Tuberc Other Mycobact Dis, 2022. **29**: p. 100342.
47. Horne, D.J., et al., *Xpert MTB/RIF and Xpert MTB/RIF Ultra for pulmonary tuberculosis and rifampicin resistance in adults.* Cochrane Database Syst Rev, 2019. **6**(6): p. Cd009593.
48. Gidado, M., et al., *Unsuccessful Xpert(®) MTB/RIF results: the Nigerian*

experience. Public Health Action, 2018. **8**(1): p. 2-6.

49. Grandjean Lapierre, S. and M. Drancourt, *rpoB Targeted Loop-Mediated Isothermal Amplification (LAMP) Assay for Consensus Detection of Mycobacteria Associated With Pulmonary Infections.* Front Med (Lausanne), 2018. **5**: p. 332.
50. Zijenah, L., *The World Health Organization Recommended TB Diagnostic Tools*. 2018.
51. Nguyen, T.N.A., et al., *Molecular Diagnosis of Drug-Resistant Tuberculosis; A Literature Review.* Front Microbiol, 2019. **10**: p. 794.
52. Barnard, M., et al. *Molecular Detection of Drug-Resistant Tuberculosis by Line Probe Assay. Laboratory Manual for Resource-Limited Settings*. 2012.
53. Parsaeimehr, A., R.I. Ebirim, and G. Ozbay, *CRISPR-Cas technology a new era in genomic engineering.* Biotechnol Rep (Amst), 2022. **34**: p. e00731.
54. Aman, R., A. Mahas, and M. Mahfouz, *Nucleic Acid Detection Using CRISPR/Cas Biosensing Technologies.* ACS Synthetic Biology, 2020. **9**(6): p. 1226-1233.
55. Li, J., et al., *Application of CRISPR/Cas Systems in the Nucleic Acid Detection of Infectious Diseases.* Diagnostics (Basel), 2022. **12**(10).
56. Kanitchinda, S., et al., *CRISPR-Cas fluorescent cleavage assay coupled with recombinase polymerase amplification for sensitive and specific detection of Enterocytozoon hepatopenaei.* Biotechnology Reports, 2020. **27**: p. e00485.
57. Lv, H., et al., *Definition of CRISPR Cas12a T rans-Cleavage Units to Facilitate CRISPR Diagnostics.* Front Microbiol, 2021. **12**: p. 766464.
58. Xu, H., et al., *An Isothermal Method for Sensitive Detection of Mycobacterium tuberculosis Complex Using Clustered Regularly Interspaced Short Palindromic Repeats/Cas12a Cis and Trans Cleavage.* J Mol Diagn, 2020. **22**(8): p. 1020-1029.
59. Gao, H., et al., *Recent advances in the use of the CRISPR-Cas system for the detection of infectious pathogens.* J Zhejiang Univ Sci B, 2022. **23**(11): p. 881-898.
60. Qi, Y., et al., *CRISPR-Based Diagnostics: A Potential Tool to Address the Diagnostic Challenges of Tuberculosis.* Pathogens, 2022. **11**(10).
61. Kellner, M.J., et al., *SHERLOCK: nucleic acid detection with CRISPR nucleases.* Nat Protoc, 2019. **14**(10): p. 2986-3012.

62. Piepenburg, O., et al., *DNA detection using recombination proteins.* PLoS Biol, 2006. **4**(7): p. e204.

63. Boyle, D.S., et al., *Rapid Detection of Mycobacterium tuberculosis by Recombinase Polymerase Amplification.* PLoS ONE, 2014. **9**.

64. Ao, W., et al., *Rapid Detection of rpoB Gene Mutations Conferring Rifampin Resistance in Mycobacterium tuberculosis.* Journal of Clinical Microbiology, 2012. **50**: p. 2433 - 2440.

65. Larsen, M.H., et al., *Genetic Manipulation of Mycobacterium tuberculosis.* Curr Protoc Microbiol, 2007. **Chapter 10**: p. Unit 10A.2.

66. Zhou, M.Y. and C. Gomez-Sanchez, *Universal TA cloning.* Current issues in molecular biology, 2000. **2**: p. 1-7.

67. Hu, Z., et al., *Customized one-step preparation of sgRNA transcription templates via overlapping PCR Using short primers and its application in vitro and in vivo gene editing.* Cell & Bioscience, 2019. **9**(1): p. 87.

68. Gopalaswamy, R., et al., *Of tuberculosis and non-tuberculous mycobacterial infections - a comparative analysis of epidemiology, diagnosis and treatment.* J Biomed Sci, 2020. **27**(1): p. 74.

69. Campelo, T.A., et al., *Revisiting the methods for detecting Mycobacterium tuberculosis: what has the new millennium brought thus far?* Access Microbiol, 2021. **3**(8): p. 000245.

70. Caulfield, A.J. and N.L. Wengenack, *Diagnosis of active tuberculosis disease: From microscopy to molecular techniques.* Journal of Clinical Tuberculosis and Other Mycobacterial Diseases, 2016. **4**: p. 33-43.

71. Diriba, G., et al., *Performance of Mycobacterium Growth Indicator Tube BACTEC 960 with Lowenstein-Jensen method for diagnosis of Mycobacterium tuberculosis at Ethiopian National Tuberculosis Reference Laboratory, Addis Ababa, Ethiopia.* BMC Res Notes, 2017. **10**(1): p. 181.

72. Shapiro, A.E., et al., *Xpert MTB/RIF and Xpert Ultra assays for screening for pulmonary tuberculosis and rifampicin resistance in adults, irrespective of signs or symptoms.* Cochrane Database Syst Rev, 2021. **3**(3): p. Cd013694.

73. Kim, J., et al., *Development and evaluation of a multiplex loop-mediated*

isothermal amplification (LAMP) assay for differentiation of Mycobacterium tuberculosis and non-tuberculosis mycobacterium in clinical samples. PLoS One, 2021. **16**(1): p. e0244753.

74. Daher, R.K., et al., *Recombinase Polymerase Amplification for Diagnostic Applications.* Clin Chem, 2016. **62**(7): p. 947-58.

75. Huh, H.J., et al., *Recent advances in molecular diagnostics and understanding mechanisms of drug resistance in nontuberculous mycobacterial diseases.* Infect Genet Evol, 2019. **72**: p. 169-182.

76. Ooi, K.H., et al., *An engineered CRISPR-Cas12a variant and DNA-RNA hybrid guides enable robust and rapid COVID-19 testing.* Nature Communications, 2021. **12**(1): p. 1739.

77. Yamano, T., et al., *Structural Basis for the Canonical and Non-canonical PAM Recognition by CRISPR-Cpf1*. Molecular Cell, 2017. **67**(4): p. 633-645.e3.

78. Rindi, L., et al., *Rapid and Accurate Identification of Nontuberculous Mycobacteria Directly from Positive Primary MGIT Cultures by MALDI-TOF MS.* Microorganisms, 2022. **10**(7).

79. Kalawat, U., et al., *Study of bronchoalveolar lavage in clinically and radiologically suspected cases of pulmonary tuberculosis.* Lung India, 2010. **27**(3): p. 122-4.

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