Rapid Detection of Pyrazinamide Resistant *Mycobacterium tuberculosis* by High Resolution Melting Curve Analysis

Worawan Watcharasamphankul MSc*,
Eric R Houpt MD**, Suporn Foongladda DVM, PhD*

** Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
*** Department of Internal Medicine, Faculty of Medicine, University of Virginia, USA*

Objective: *To develop a high-resolution melting curve analysis (HRM) assay for detection of PZA resistance.*
Material and Method: *Thirty samples of PZA-susceptible M. tuberculosis and eight isolates of PZA-resistant M. tuberculosis were included in the experiment. Five sets of primers were designed to cover the pncA gene and its upstream nucleotides. The pncA gene fragments were amplified by the PCR method. Determination of pncA mutation in the sample by comparing their melting behavior of the PCR products with the M. tuberculosis wild type by using Gene scanning software of the LightCycler® 480 instrument.*
Results: *Mutations were clearly detected in all PZA resistant samples by the HRM, whereas all PZA susceptible samples showed no mutation in the pncA gene. Results were concordant with the drug susceptibility testing by using BACTEC™ MGIT™ 960 PZA kit and mutation detection by the DNA sequencing method.*
Conclusion: *This HRM method offers a rapid and reliable screen for PZA resistant M. tuberculosis.*

Keywords: *Mycobacterium, Pyrazinamide, Drug resistant, pncA gene, High resolution melting curve analysis*

J Med Assoc Thai 2013; 96 (9): 1218-23
Full text. e-Journal: *http://jmat.mat.or.th*

Inclusion of pyrazinamide (PZA) in the treatment of tuberculosis (Tb) is necessary to reduce the duration of Tb treatment to 6 months. PZA resistant tuberculosis has traditionally been considered rare or limited to *Mycobacterium bovis*, however recent findings of PZA-monoresistant Tb have emerged[1]. These features make susceptibility testing for this agent increasingly important.

However, susceptibility testing for PZA is difficult. PZA is active only at low pH thought to be presented in vivo. Since a low pH is inhibitory to the in vitro growth of *M. tuberculosis*, conventional drug susceptibility testing (DST) of PZA on solid medium is of limited value. The newer liquid culture-based methods, such as the BACTEC MGIT 960 (Becton Dickinson Biosciences, Sparks, MD) and the BacT/ALERT 3D (bioMérieux Inc., Durham, NC) methods, utilize protocols adapted for PZA that utilize an acidified culture medium (pH 6.0). This makes DST of PZA time-consuming and high rates of discrepancy have been observed due to the poor growth of *M. tuberculosis* in this acidic condition[2,3].

Correspondence to:
Foongladda S, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkoknoi, Bangkok 10700, Thailand.
Phone: 0-2419-9811, Fax: 0-2411-3106
E-mail: suporn.foo@mahidol.ac.th

PZA is a prodrug that requires conversion into its active form pyrazinoic acid by the bacterial enzyme pyrazinamidase (PZase), which is encoded by the 561-nucleotide (nt) *pncA* gene. Mutations in the *pncA* gene or its putative promoter result in lost or reduced PZase activity, and such mutations are considered to be a major mechanism of PZA resistance in Tb[1-4]. Specifically it has been shown that 72% to 95% of PZA-resistant clinical isolates of Tb carry *pncA* mutations[5,6]. Recently a target of pyrazinoic acid has been described[7].

Mutations in *pncA* are diverse and scattered throughout the gene, thus a molecular approach must interrogate substantial distance. PCR-SSCP, heteroduplex formation, and amplification refractory mutation system (ARMS-PCR) are possible methods but they are expensive and detect only known mutations in defined regions[8-11]. Thus, the authors designed a high resolution melting curve analysis (HRM) for detecting sequence variation in *pncA*.

Material and Method

Mycobacterium tuberculosis samples and DNA extraction

M. tuberculosis used in this work included *M. tuberculosis* H37Rv (ATCC 27294), 22 *M. tuberculosis* clinical isolates (14PZA susceptible and 8 PZA

resistant), and 20 sputum samples of Tb patients with positive smear that their isolates are PZA susceptible. The isolates were determined for PZA susceptibility by BACTEC™ MGIT™ 960 PZA Kit (Becton, Dickinson and company, USA) according to manufacturer's instruction. They were cultured on Lowenstein-Jensen (L-J) medium. Cell suspensions were prepared in phosphate-buffered saline (PBS). DNA was extracted from the isolates and decontamination-digestion sputum samples using High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer's instruction. Extracted DNA was stored at -20°C.

Primers

Five pairs of primers were designed to amplify a 593 bp including the coding region and the -28 upstream region of the *pncA* gene as shown in Table 1 and Fig. 1. Primers were tested for optimal features using Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Specificity of the primers was checked using the 'Primer Pair Specificity Checking Parameters' function of Primer-BLAST (NCBI).

Real time PCR and HRM assay

PCR and HRM were operated in a single white 96-well plate (Roche, Germany) on a LightCycler® 480 Real-Time PCR System (Roche, Germany). The reaction mixture consisted of 20 ng of DNA, 10 micromolar of each primer set (PF1-PR1 or PF2-PR2 or PF3-PR3 or PF4-PR4 or PF5-PR5) and 2.5 mM $MgCl_2$ in 1x LightCycler® 480 High Resolution Melting Master mixture containing ResoLight dye (Roche, Germany) in a final volume of 10 microliters. The testing included five well of each primer set for sample, five for *M. tuberculosis* H37Rv DNA as wild type controls, and five for heteroduplex sample. For heteroduplex sample preparation, *M. tuberculosis* H37Rv genomic DNA (wild type) was spiked to the final concentration at 1/10 of the sample DNA in the reaction mixture. Nuclease-free water replaced DNA template for negative control. Thermal cycling condition was 94°C for 10 minutes followed by 40 cycles with denaturation at 95°C for 10 seconds, annealing at 64°C for 15 seconds, and extension at 72°C for 30 seconds. After PCR, HRM analysis was performed by heating the PCR products at 95°C for two minutes and then cooling to 40°C for five minutes.

Table 1. Primers used for the real-time PCR and sequencing

Primer name	Oligonucleotide sequence (5'-3')	Position on *pncA* gene	Product size (bp)
PF1	GCAGTCGCCCGAACGTAT	-28 to -11	135
PR1	GCCAGGTAGTCGCTGATGG	89 to 107	
PF2	GCGACTACCTGGCCGAAG	95 to 112	136
PR2	GGAGTACCGCTGACGCAAT	212 to 230	
PF3	CATTGCGTCAGCGGTACTC	211 to 229	128
PR3	CCGTTCTCGTCGACTCCTT	320 to 338	
PF4	TCGAAGGAGTCGACGAGAAC	317 to 336	130
PR4	ATTGCGTACCGCGTCCT	430 to 446	
PF5	CGGTACGCAATGGCTTG	436 to 452	130
PR5	GCCATCAGGAGCTGCAAAC	547 to 565	

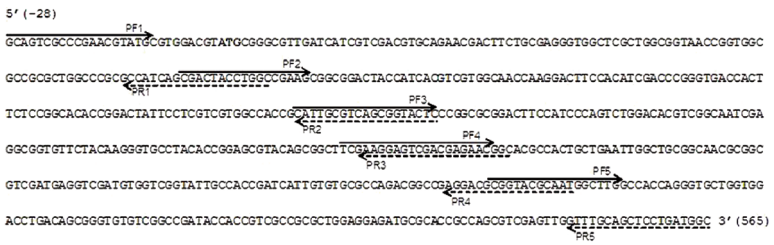

Fig. 1 Schematic representation of the -28 bases upstream and 561 bases of complete nucleotide sequences of *pncA* gene (GenBank accession number NC_000962 GeneID: 888260, gi|57116681:2288681-2289241) of *M. tuberculosis* H37Rv. Five primer pairs and their direction namely, PF1_PR1, PF2_PR2, PF3_PR3, PF4_PR4, and PF5_PR5, are indicated in the arrow line.

The melting temperature analysis was from 65°C to 99°C with temperature ramped at 1°C/sec with 25 acquisitions per degree. Fluorescence data were subsequently visualized and analyzed using the automated grouping functionality provided by the LightCycler® 480 Gene scanning software version 1.5 (Roche, Germany). PCR product melting curves were normalized and temperature shifted to allow samples to be directly compared. Different plots were generated by selecting the wild type control as a base curve control in horizontal line. At that time, the melting curves of the other samples were normalized against this base curve control. Mutations in any tested samples were demonstrated by significant differences in fluorescence signal from the horizontal line.

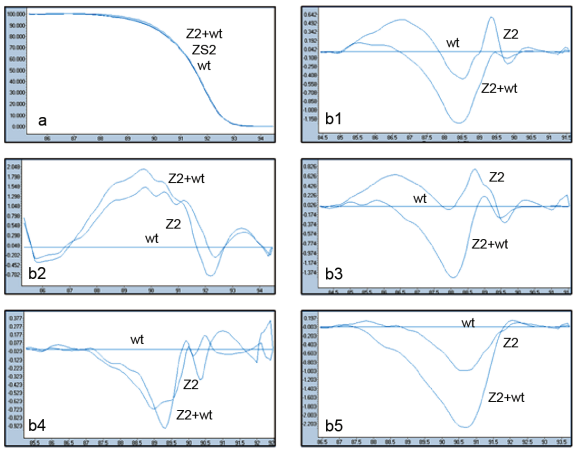

Fig. 2 Normalized and temperature-shifted melting curves (a) and difference plots (b1-b5: at position -28 to 107, 95 to 230, 211 to 338, 317 to 466 and 436 to 565, respectively) of HRM analyses of the 5 *pncA* amplicons of clinical isolate Z2, obtained by spiking unknown samples with wild-type (wt) DNA. Formation of the sample/wt heteroduplex DNA after PCR are not significant different from others, indicative of PZA susceptible *M. tuberculosis*. Per LightCycler® 480 Gene scanning software version 1.5, different color lines indicate significantly different HRM curves from control, while same blue lines indicate no significant difference from the horizontal line control of wild type.

DNA sequencing

The nucleotide sequences of the PCR products were analyzed to determine and confirm the presence of mutation within the desired genes. PCR products were purified using High Pure PCR Product Purification Kit (Roche, Germany) and DNA sequencing was performed by fluorescence-labeled dideoxynucleotide technology in an automated DNA sequencer.

Results

Five areas of the *pncA* gene amplicon were performed, such as -28 to 107, 95 to 230, 211 to 338, 317 to 446, and 436 to 565 (namely, p1, p2, p3, p4, and p5, respectively) in different wells. In the HRM analysis, melting curves of amplicons, such as sample, wild type and heteroduplex sample with wild type, were compared and presented in the same chart of Normalized and Temp-shifted Different Plot of LightCycler® 480 Gene scanning software using wild type control as baseline. In case of no difference in amplicon, melting curve of samples, and wild type control presented in the same color (blue), whereas different colors (red and green) demonstrated as different detections (Fig. 2, 3).

Difference of amplicon between sample, wild type and heteroduplex sample was not observed in all

Table 2. *pncA* mutation detection of eight PZA resistant isolates by HRM assay compared with DNA sequences

Isolate No.	Mutation detection by HRM assay in position of *pncA* areas					Mutation position detected by sequencing
	-28 to 107	95 to 230	211 to 338	317 to 446	436 to 565	
Z4	No	No	Yes	No	No	312 (AGC→AGG)
Z9	No	No	Yes	No	Yes	269 (ATC→AGC)
Z10	No	Yes	No	No	No	195 (TCC→TCT) 329 (A deletion)
Z13	Yes	No	No	No	No	64 (A deletion)
Z25	No	No	No	Yes	No	389 (GTG→GGG)
Z36	No	No	No	No	Yes	472 (GTG→GGG)
Z37	No	Yes	No	Yes	No	200 (TCG→TTG) 390 (GG insertion)
Z48	No	No	Yes	No	No	282 (TTC→TTG)

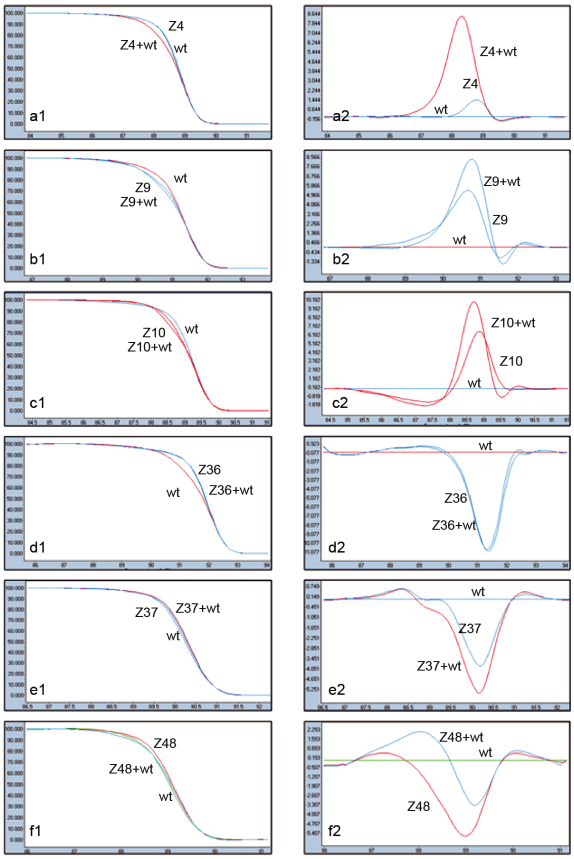

Fig. 3 Normalized and temperature-shifted melting curves (a1-f1) and difference plots (a2-f2) of HRM analyses of the *pncA* amplicons of PZA-resistant *M. tuberculosis* clinical isolates: Z4 (a1, a2), Z9 (b1, b2), Z10 (c1, c2), Z36 (d1, d2), Z37(e1, e2), and Z48 (f1,f2) (at position 211 to 338, 436 to 565, 95 to 230, 436 to 565, 317 to 446, and 211 to 338, respectively). Formation of the sample/wt heteroduplex DNA after PCR is distinguished from wild-type, indicative of PZA resistant *M. tuberculosis*. Per LightCycler® 480 Gene scanning software version 1.5, different color lines indicate significantly different HRM curves from the horizontal line control of wild type.

PZA susceptible isolates and clinical samples. Fig. 2 demonstrates the same blue color in melting curve of a PZA susceptible sample as wild type and heteroduplex sample.

Mutation of the *pncA* gene was found in 10 positions of eight PZA resistant isolates as summarized in Table 2. The assay showed different amplicon of sample from wild type, in area p5 of samples Z9 and Z36, in p2 of Z10 and Z37, and in p3 of Z48. However, the other five positions were found in heteroduplex samples. The areas of mutations were in concordance with the sequencing results and PZA susceptibility testing by Mycobacteria Growth Indicator Tube (MGIT) 960 system as shown in Fig. 2 and Table 2.

One sample, Z9 showed mutation detection in p3 and p5 areas, whereas sequencing results confirmed only in p3 area with one base substitution mutation at T269G. This result may have been due to unreadable sequence at the 5' region on the PR5 primer. On the other hand, mutation in sample Z10 was detected in p2 area by HRM assay, whereas this sample contained two positions of mutations, which were C195T substitution mutation and A329 deletion in PR3primer region.

Discussion

The present study is the first development of HRM for detecting *pncA* mutations as a tool to determine PZA resistance. HRM analysis technique is a rapid molecular method for investigating a region suspected to contain variants of known sequence. The use of this method allows discrimination down to single nucleotide changes by analysis of the melting behavior of the whole amplification product. In the present study, five sets of primer were used to amplify five DNA fragments that cover the *pncA* gene and its 28 base pair upstream region. *M. tuberculosis* H37Rv was used as the wild type control in our study. Nucleotide transversions, such as A:T, and G:C, resulted in very small Tm difference (<0.2°C for A:T, and <0.4°C for C:G) and are difficult to detect. To circumvent this limitation and enhance the separation of meting curves, known genotype of the wild type *M. tuberculosis* was spiked into all DNA samples and tested in the final concentration of 1/10.

Analysis of PZA-susceptible clinical isolates showed no significant difference when plotted against the baseline wild type control. In contrast, all eight PZA-resistant *M. tuberculosis* clinical isolate showed at least one mutation along the *pncA* gene. Of 8 PZA-resistant clinical isolates used in the present study, some previously reported mutations were found such as a deletion at nucleotide position 64[3,5], a base substitution at nucleotide position 269, 312, and 389[12,13], and an insertion at nucleotide 391[12-15].

The HRM analysis method was also applied for direct detection of *pncA* mutation in *M. tuberculosis* from clinical specimens. The authors did not observe any difference in the HRM between *M. tuberculosis* H37Rv and H37Rv/sample, suggesting we amplified

both the specimen Tb and H37Rv. Their HRM results were similar to that of wild type susceptible *M. tuberculosis*. These were compatible with their isolate that were performed PZA susceptibility testing by BACTEC™ MGIT™ 960 PZA Kit. A limitation of this method is that mutation in a primer area might not show distinction. In addition, as same as DNA sequence analysis, it can detect silent point mutations that may not confer resistance. This is admittedly a limitation of many molecular methods that do not employ complete sequencing.

The results of PZA mutation detection by this technique are available four hours after receiving specimen. The cost of HRM is approximately 15 USD per sample. The authors propose utilization of this *pncA* HRM method as a fast and inexpensive method to determine PZA resistance.

Acknowledgement

This work was supported by Siriraj Research Development Fund, Mahidol University.

What is already known on this topic?

Pyrazinamide (PZA), one of the important first-line drugs for tuberculosis treatment, has received increased attention in susceptibility test due to its important role in shortening the time course for TB treatment. However, the current PZA susceptibility test is difficult, time-consuming, and potentially exhibits high discrepancy results among laboratories due to the need of an acidic pH for drug activity and the poor growth of *M. tuberculosis* in this acidic condition. Mutations in the *pncA* gene, encoding nicotinamidase/pyrazinamidase (PZase) and its putative promoter, appear to be a major mechanism of PZA resistance in *M. tuberculosis*. Various molecular methods were used for detection of PZA-resistant *M. tuberculosis* strains, such as PCR-single-strand conformation polymorphism (PCR-SSCP), dideoxy fingerprinting, heteroduplex formation, and amplification refractory mutation system (ARMS-PCR), however some of these methods are costly, time-consuming, and have to note that these methods can only detect mutations in a defined area. Considering that mutations in *pncA* are highly diverse and found scattered throughout the gene, rapid molecular method to detect any mutation along the gene should be useful. High resolution melting curve analysis (HRM) has been reported as a potential rapid, simple, reliable, and cost effective PCR technique for detecting unknown sequence variation such as single base substitution, deletion, and insertion at any position within the amplification products. Therefore, it should be useful for *pncA* mutation detection.

What this study adds?

This is the first study that used the HRM for detection gene mutation associated PZA resistant. The results from this study showed that mutations in PZA-resistant *M. tuberculosis* detection by the HRM method concordant with both of traditional PZA susceptibility test by MGIT960 and the DNA sequencing method. Therefore, HRM is a rapid, reliable, and cost effective screening method for *pncA* mutation detection that indicates PZA resistant in *M. tuberculosis*. Rapid diagnosis of drug-resistant *M. tuberculosis* means rapid and success to control TB spreading.

Potential conflicts of interest

None.

References

1. Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis 2003; 7: 6-21.
2. Hannan MM, Desmond EP, Morlock GP, Mazurek GH, Crawford JT. Pyrazinamide-monoresistant *Mycobacterium tuberculosis* in the United States. J Clin Microbiol 2001; 39: 647-50.
3. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2011; 55: 2032-41.
4. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, et al. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 1997; 41: 540-3.
5. Cheng SJ, Thibert L, Sanchez T, Heifets L, Zhang Y. *pncA* mutations as a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*: spread of a monoresistant strain in Quebec, Canada. Antimicrob Agents Chemother 2000; 44: 528-32.
6. Davies AP, Billington OJ, McHugh TD, Mitchison DA, Gillespie SH. Comparison of phenotypic and genotypic methods for pyrazinamide susceptibility testing with *Mycobacterium tuberculosis*. J Clin Microbiol 2000; 38: 3686-8.
7. Cole ST. Microbiology. Pyrazinamide—old TB

drug finds new target. Science 2011; 333: 1583-4.
8. Mohamed AM, Bastola DR, Morlock GP, Cooksey RC, Hinrichs SH. Temperature-mediated heteroduplex analysis for detection of *pncA* mutations associated with pyrazinamide resistance and differentiation between *Mycobacterium tuberculosis* and *Mycobacterium bovis* by denaturing high-performance liquid chromatography. J Clin Microbiol 2004; 42: 1016-23.
9. Felmlee TA, Liu Q, Whelen AC, Williams D, Sommer SS, Persing DH. Genotypic detection of *Mycobacterium tuberculosis* rifampin resistance: comparison of single-strand conformation polymorphism and dideoxy fingerprinting. J Clin Microbiol 1995; 33: 1617-23.
10. Fan XY, Hu ZY, Xu FH, Yan ZQ, Guo SQ, Li ZM. Rapid detection of rpoB gene mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates in shanghai by using the amplification refractory mutation system. J Clin Microbiol 2003; 41: 993-7.
11. Thomas GA, Williams DL, Soper SA. Capillary electrophoresis-based heteroduplex analysis with a universal heteroduplex generator for detection of point mutations associated with rifampin resistance in tuberculosis. Clin Chem 2001; 47: 1195-203.
12. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuber Lung Dis 1997; 78: 117-22.
13. Louw GE, Warren RM, Donald PR, Murray MB, Bosman M, Van Helden PD, et al. Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. Int J Tuberc Lung Dis 2006; 10: 802-7.
14. Lee KW, Lee JM, Jung KS. Characterization of *pncA* mutations of pyrazinamide-resistant *Mycobacterium tuberculosis* in Korea. J Korean Med Sci 2001; 16: 537-43.
15. Lemaitre N, Sougakoff W, Truffot-Pernot C, Jarlier V. Characterization of new mutations in pyrazinamide-resistant strains of *Mycobacterium tuberculosis* and identification of conserved regions important for the catalytic activity of the pyrazinamidase *PncA*. Antimicrob Agents Chemother 1999; 43: 1761-3.

การตรวจหาเชื้อวัณโรคดื้อยา pyrazinamide อย่างรวดเร็วด้วยวิธี high resolution melting curve analysis

วรวรรณ วัชระสัมพันธ์กุล, เอริค อาร์ ฮอพท์, ศุภร ฟุ้งลัดดา

วัตถุประสงค์: *เพื่อพัฒนาวิธี high resolution melting curve analysis เพื่อใช้ในการตรวจหาเชื้อวัณโรคดื้อยา pyrazinamide*
วัสดุและวิธีการ: *การทดลองประกอบด้วยตัวอย่างเชื้อวัณโรคไวต่อยา PZA จำนวน 34 ตัวอย่าง และเชื้อวัณโรคดื้อยา PZA จำนวน 8 ตัวอย่าง ออกแบบไพรเมอร์จำนวน 5 ชุด ซึ่งครอบคลุมจีน pncA และส่วนหน้าของจีน เพิ่มจำนวนชิ้นส่วนของจีน pncA ในตัวอย่างโดยวิธี PCR ตรวจหาการกลายพันธุ์ของจีน pncA โดยเปรียบเทียบลักษณะ melting curve ของผลผลิต PCR จากตัวอย่างตรวจกับเชื้อวัณโรคสายพันธุ์ปกติโดยใช้โปรแกรม gene scanning ของเครื่อง LightCycler® 480*
ผลการศึกษา: *วิธี HRM สามารถตรวจพบการกลายพันธุ์ของจีน pncA ในทั้ง 8 ตัวอย่างของเชื้อวัณโรคดื้อยา PZA ในขณะที่ทุกตัวอย่างของเชื้อวัณโรคไวต่อยา PZA ตรวจไม่พบการกลายพันธุ์ ผลที่พบนี้สอดคล้องกันกับผลจากการทดสอบความไวต่อยาด้วยชุดตรวจ BACTEC™ MGIT™ 960 PZA และการตรวจหาการกลายพันธุ์โดยการตรวจหาลำดับเบส*
สรุป: *วิธี HRM นี้เป็นวิธีตรวจคัดกรองเชื้อวัณโรคดื้อยา PZA ที่รวดเร็วและเชื่อถือได้*