CHAPTER IV
RESULTS

1. Result of analyzed specific primers

Ten selected primers were blasted with the 16S-23S rDNA spacer of 39 *Mycobacterium* species by FastPCR computerized program for calculation of product size. The expected amplified product of genus specific was 300-400 bp. The expected amplified products of species specific and genus specific were 95 and 320 bp for *M. avium* complex, 340 and 320 bp for *M. scrofulaceum*, 260 and 320 bp. for *M. tuberculsis* complex, 210 and 370 bp for *M. fortuitum,* 75 and 330 bp for *M. intracellulare*, the last 170 and 335 bp for *M. kansasii*, as shown in **Table 6.**

Additional, these primers were analyzed by Oligo analyzer program for chance of annealing which were considered of the delta G values. The value should be less than 0 kcal/ mol, in each pair of primers. There were two pairs of primers, MACF vs MACF and KAN1 vs INT, which dG were 0.17 kcal/ mol and 0.27 kcal/ mol, respectively as shown in **Table 7.**

Table6. Show the expected amplified products size specific to mycobacteria

Organism	Primers	Product size (bp)
Mycobacteria.	ITS-F1 vs MYCOM-2	300-400
MAC	MACF vs MYCOM-2 and ITS-F1 vs MYCOM-2	95 and 320
M. scrofulaceum	ITS-F1 vs SCOR and ITS-F1 vs MYCOM-2	340 and 320
MTB complex	ITS-F1 vs TBR and ITS-F1 vs MYCOM-2	260 and 320
M. fortuitum	FORF vs MYCOM-2 and ITS-F1 vs MYCOM-2	210 and 370
M. intracellulare	INT vs MYCOM-2 and ITS-F1 vs MYCOM-2	75 and 330
M. kansasii	KAN1,KAN2, mKAN vs MYCOM-2 and ITS-F1 vs MYCOM-2	170 and 335

Table 7. The deltaG (dG) value of each pair primers

dG (kcal/mol)	ITS-F1	MYCOM-2	MACF	FORF	INT	KAN1	KAN2	KANF	SCOR	TBR
ITS-F1	-5.51	-2.51	-1.29	-2.53	-1.66	-2.92	-1.95	-1.56	-3.26	-3.41
MYCOM-2	-2.51	-1.88	-0.62	-2.43	-0.91	-2.16	-2.02	-2.09	-1.71	-1.84
MACF	-1.29	-0.62	0.17	-1.97	-1.57	-0.79	-0.81	-0.91	-1.77	-2.86
FORF	-2.53	-2.43	-1.97	-6.18	-163	-3.19	-1.20	-1.51	-2.36	-1.38
INT	-1.66	-0.91	-1.57	-1.63	-1.98	0.27	-1.81	-0.97	-2.72	-1.25
KAN1	-2.92	-2.16	-0.79	-3.19	0.27	-10.6	-1.39	-6.15	-1.50	-0.18
KAN2	-1.95	-2.02	-0.81	-1.20	-1.81	-1.39	-7.00	-3.33	-1.89	-1.23
mKAN	-1.56	-2.09	-0.91	-1.15	-0.97	-6.15	-3.33	-2.43	-1.10	-1.56
SCOR	-3.26	-1.71	-1.77	-2.36	-2.72	-1.50	-1.89	-1.10	-3.76	-1.45
TBR	-3.41	-1.84	-2.86	-1.38	-1.25	-0.18	-1.23	-1.56	-1.45	-143

2. Preparation of genomic DNA of mycobacteria

The genomic DNA was then quantified by using the UV spectrophotometry at OD 260. The quality of DNA was determined by the ratio of the O. D 260 and 280. The O.D. ratio of 1.8 or more demonstrated the high quality of DNA preparation. The amount of DNA from each preparation was shown in **Table 8**.

Table8. The results of genomic DNA preparation

Genomic DNA	O.D.260	O.D.280	O.D.260/280 ratio	Dilution factor	DNA (ng/ µl)
M. avium	0.296	0.163	1.81	20	296
M. scrofulaceum	0.635	0.345	1.84	20	635
M. tuberculosis	0.200	0.109	1.83	20	200
M. fortuitum	0.430	0.238	1.80	20	430
M. intracellulare	0.176	0.098	1.80	20	176
M. kansasii	0.161	0.086	1.86	20	161

3. The results of Single PCR for detection of each species of Mycobacterium

All 10 selected primers were determined for the specificity to 16S-23S rDNA spacer of mycobacteria by Single PCR. The amplified product of genus specific was 300-400 bp. The amplified products of species specific were 75, 95 bp for *M. avium* and *M. intracellulare*, respectively, 340 bp for *M. scrofulaceum*, and 260 bp for *M. tuberculosis* complex, 210 bp for *M. fortuitum* and 170 bp for *M. kansasii*, as shown in **Figure 2-8.**

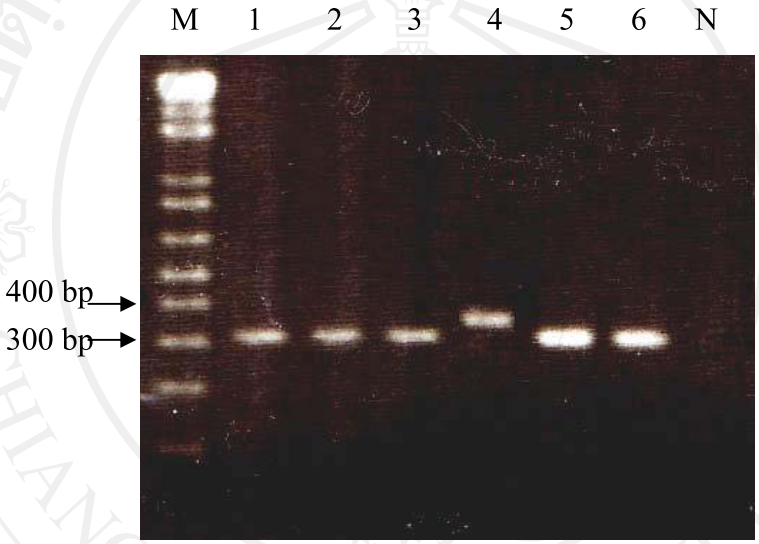

Figure2. The amplified products of genus specific by ITS-F vs MYCOM-2 primers on 2.5% agarose gel. Lane M showed 1 kb DNA ladder marker**;** Lane 1-6 showed the genus specific bands of *M. avium* ATCC 25921, *M. scrofulaceum* ATCC 19982, *M. tuberculosis* ATCC 27294, *M. fortuitum* ATCC 6841, *M. intracellulare* ATCC 13950 *M. kansasii* ATCC 12478 at 320, 320, 320, 370, 330 and 335 bp, respectively.

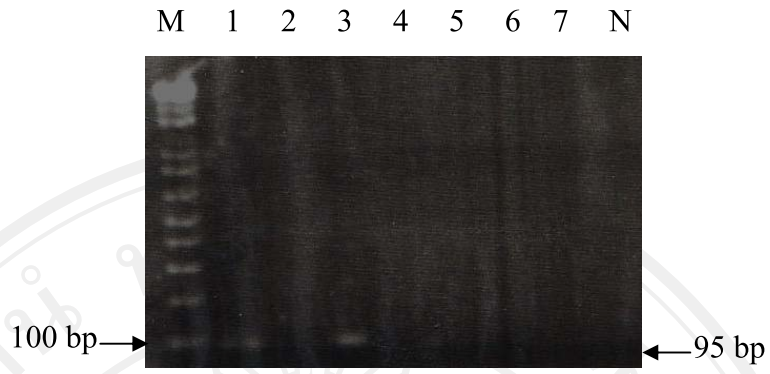

Figure3. The amplified products of MAC by MACF vs MYCOM-2 primers on 2.5% agarose gel. Lane M showed 1 kb DNA ladder marker**.** Lane 1 and 3 showed 95 bp band of *M. avium* ATCC 25291 and *M. intracellulare* ATCC 13950. Lane 2, 4-7 showed no band of amplified product of *M .fortuitum* ATCC 6841, *M. fortuitum* TBD 012-01, *M. scrofulaceum* ATCC 19982, *M. tuberculosis* ATCC 27294 and *M. kansasii* ATCC 12478, respectively. Lane N showed negative control.

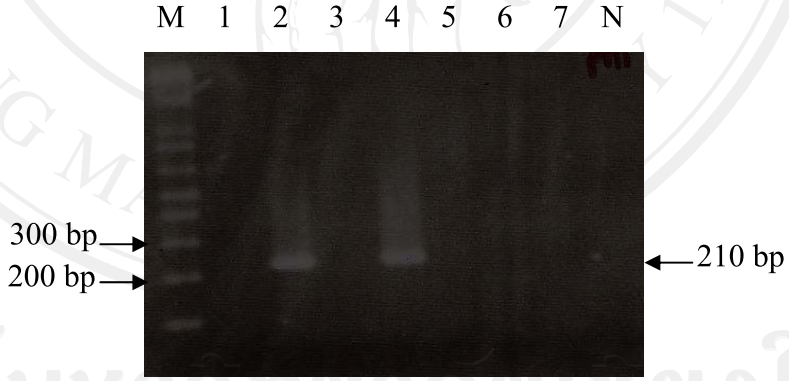

Figure4. The amplified products of *M .fortuitum* by FORF vs MYCOM-2 primers on 2.5% agarose gel. Lane M showed 1 kb DNA ladder marker**;** Lane 2 and 4 showed 210 bp band of *M .fortuitum* ATCC 6841, *M. fortuitum* TBD 012-01. Lane 1, 3, 5-7 showed no band of amplified product of *M. avium* ATCC 25291, *M. intracellulare* ATCC 13950, *M. scrofulaceum* ATCC 19982; *M. tuberculosis* ATCC 27294 and *M. kansasii* ATCC 12478, respectively. Lane N showed negative control

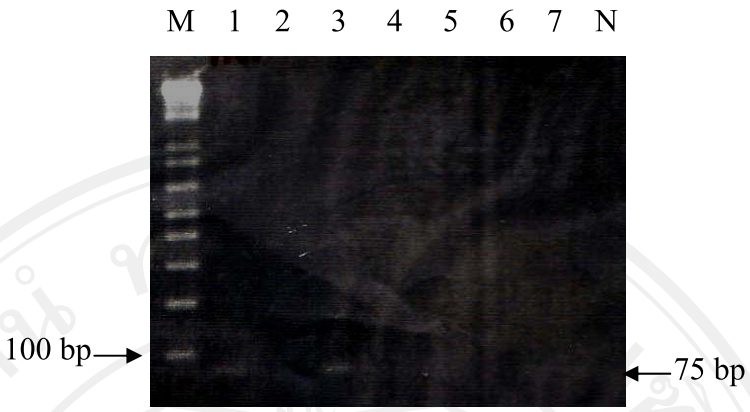

Figure5. The amplified products of *M. intracellulare* by INT vs MYCOM-2 primers on 2.5% agarose gel. Lane M showed 1 kb DNA ladder marker**.** Lane 1 and 3 showed 75 bp band of *M. avium* ATCC 25291 and *M. intracellulare* ATCC 13950. Lane 2, 4-7 showed no band of amplified product of *M .fortuitum* ATCC 6841, *M. fortuitum* TBD 012-01, *M. scrofulaceum* ATCC 19982, *M. tuberculosis* ATCC 27294 and *M. kansasii* ATCC 12478, respectively. Lane N showed negative control.

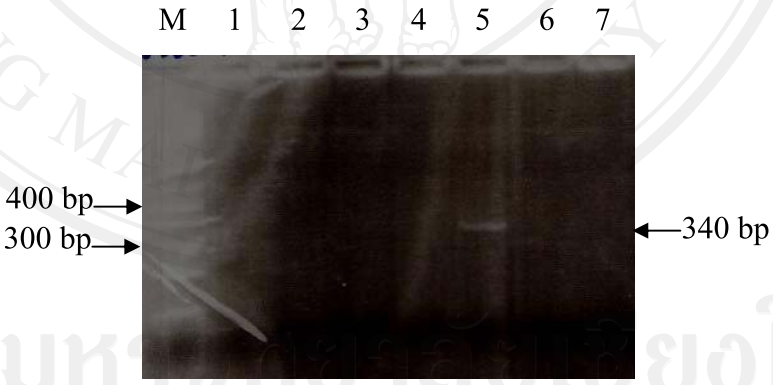

Figure6. The amplified products of *M. scrofulaceum* by ITS-F1 vs SCOR primers on 2.5% agarose gel. Lane M showed 1 kb DNA ladder marker**.** Lane 5 showed 340 bp band of *M. scrofulaceum* ATCC 19982. Lane 1-4 and 6-7 showed no band of amplified product of *M. avium* ATCC 25291, *M .fortuitum* ATCC 6841, *M. intracellulare* ATCC 13950, *M. fortuitum* TBD 012-01, *M. tuberculosis* ATCC 27294 and *M. kansasii* ATCC 12478, respectively.

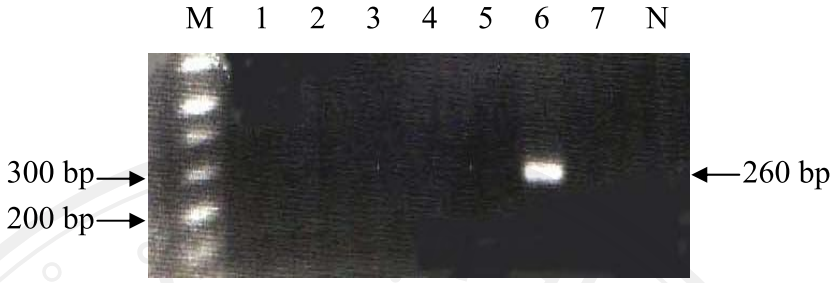

Figure7. The amplified products of *M. tuberculosis* by ITS-F1 vs TBR primers on 2.5% agarose gel. Lane M showed 1 kb DNA ladder marker**.** Lane 6 showed 260 bp band of *M. tuberculosis* ATCC 27294. Lane 1-5, 7 showed no band of amplified product of *M. avium* ATCC 25291*, M .fortuitum* ATCC 6841, *M. intracellulare* ATCC 13950, *M. fortuitum* TBD 012-01, *M. scrofulaceum* ATCC 19982 and *M. kansasii* ATCC 12478, respectively. Lane N showed negative control.

Figure8. The amplified products of *M. kansasii* by KAN1, KAN2 and mKAN vs MYCOM-2 primers on 2.5% agarose gel. Lane M showed 1 kb DNA ladder marker**.** Lane 7 showed 170 bp band of *M. kansasii* ATCC 12478. Lane 1-6 showed no band of amplified product of *M. avium* ATCC 25291*, M .fortuitum* ATCC 6841, *M. intracellulare* ATCC 13950, *M. fortuitum* TBD 012-01, *M. scrofulaceum* ATCC 19982 and *M. tuberculosis* ATCC 27294, respectively. Lane N showed negative control.

4. Optimization of the Single-tube Multiplex PCR

4.1 Optimization of annealing temperature

The reaction was carried out as mentioned in method 3.2.1 with 200 µM dNTP and 1.5 mM $MgCl_2$. The annealing temperature was varied from 55- 66^{o}C. The optimal annealing temperature at 63.2 ^{o}C lane 8 showed the best positive band of amplified products of both genus specific and species specific for all 6 species of mycobacteria, as shown in **Figure 9-14.**

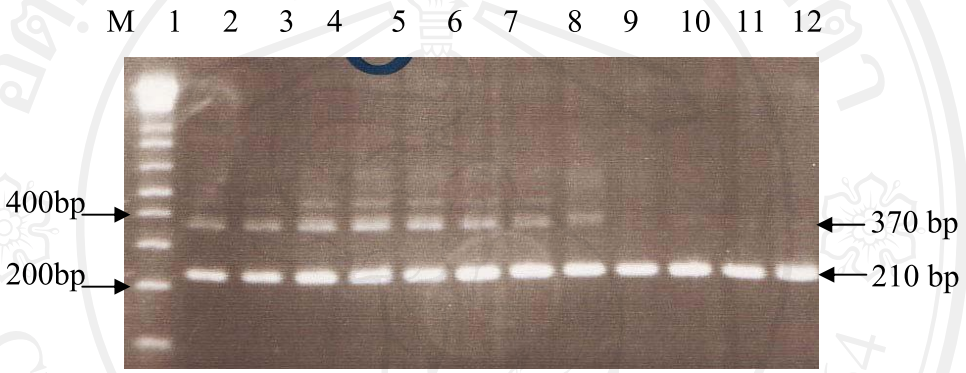

Figure9. The amplified products of genus specific (370 bp) by ITS-F1 vs MYCOM-2 primers and species specific (210 bp) by FORF vs MYCOM-2 primers of *M. fortuitum* ATCC 6841 on 2.5% agarose gel were amplified at Tm 55.0^{o}C, 55.3^{o}C, 56.0^{o}C, 56.8^{o}C, 58.1^{o}C, 59.8^{o}C, 61.6^{o}C, 63.2^{o}C, 64.4^{o}C, 65.2^{o}C, 65.8^{o}C and 66.0^{o}C on lane 1-12, respectively.

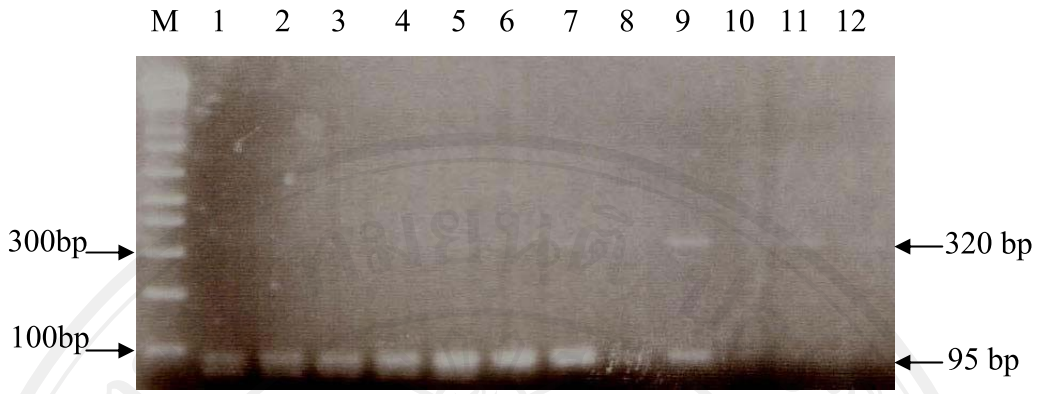

Figure10. The amplified products of genus specific (320 bp) by ITS-F1 vs MYCOM-2 primers and species specific (95 bp) by MACF vs MYCOM-2 primers of *M. avium* ATCC 25291 on 2.5% agarose gel were amplified at Tm 55.0°C, 55.3°C, 56.0°C, 56.8°C, 58.1°C, 59.8°C, 61.6°C, 63.2°C, 64.4°C, 65.2°C, 65.8°C and 66.0°C on lane 1-12, respectively.

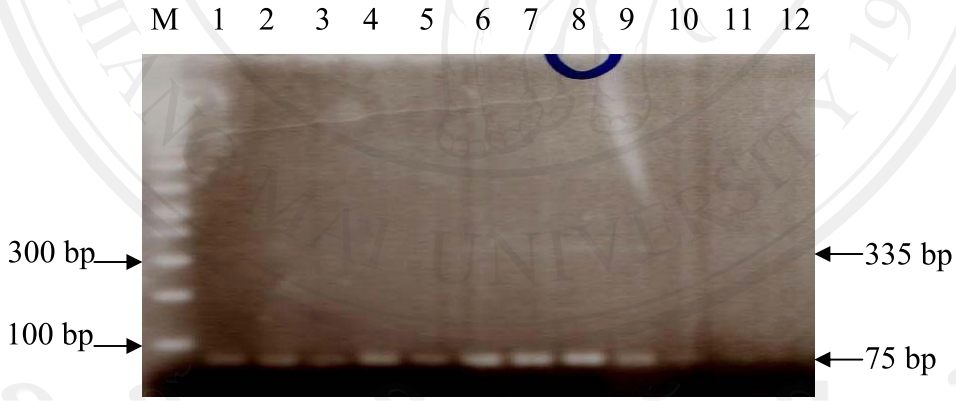

Figure11. The amplified products of genus specific (335 bp) by ITS-F1 vs MYCOM-2 primers and species specific (75 bp) by INT vs MYCOM-2 primers of *M. intracellulare* ATCC 13950 on 2.5% agarose gel were amplified at Tm 55.0°C, 55.3°C, 56.0°C, 56.8°C, 58.1°C, 59.8°C, 61.6°C, 63.2°C, 64.4°C, 65.2°C, 65.8°C and 66.0°C on lane 1-12, respectively.

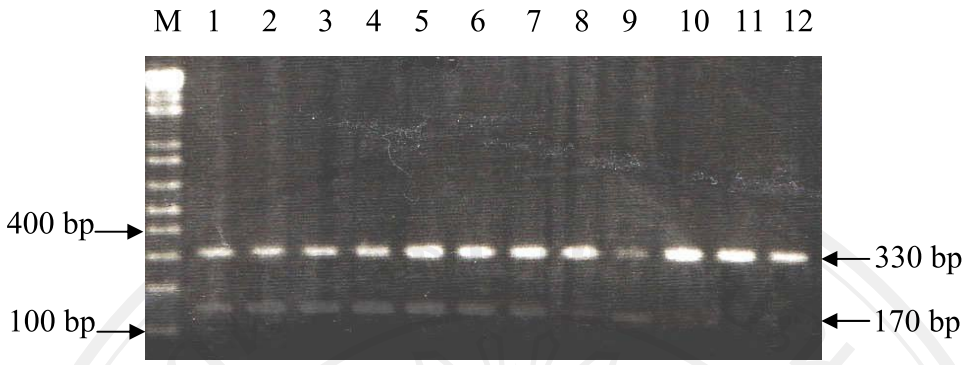

Figure12. The amplified products of genus specific (330 bp) by ITS-F1 vs MYCOM-2 primers and species specific (170 bp) by KAN1, KAN2 and mKAN vs MYCOM-2 primers of *M. kansasii* ATCC 12478 on 2.5% agarose gel were amplified at Tm 55.0°C, 55.3°C, 56.0°C, 56.8°C, 58.1°C, 59.8°C, 61.6°C, 63.2°C, 64.4°C, 65.2°C, 65.8°C and 66.0°C on lane 1-12, respectively.

Figure13. The amplified products of genus specific (320 bp) by ITS-F1 vs MYCOM-2 primers and species specific (340 bp) by ITS-F1 vs SCOR primers of *M. scrofulaceum* ATCC 19982 on 2.5% agarose gel were amplified at Tm 55.0°C, 55.3°C, 56.0°C, 56.8°C, 58.1°C, 59.8°C, 61.6°C, 63.2°C, 64.4°C, 65.2°C, 65.8°C and 66.0°C on lane 1-12, respectively.

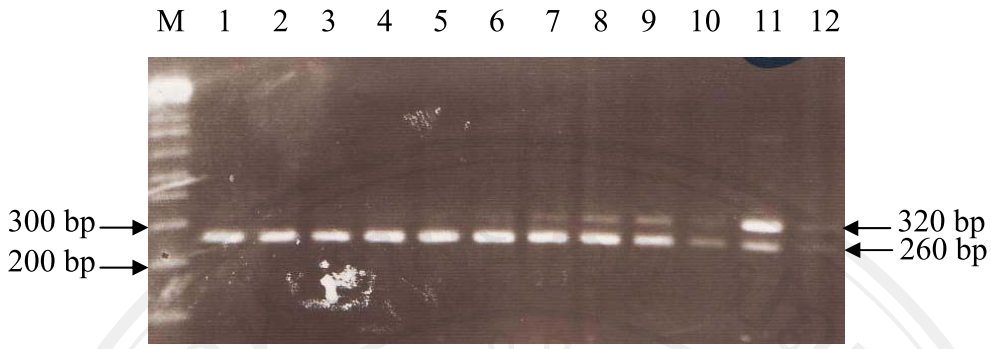

Figure14. The amplified products of genus specific (320 bp) by ITS-F1 vs MYCOM-2 primers and species specific (260 bp) by ITS-F1 vs TBR primers of *M. tuberculosis* ATCC 19982 on 2.5% agarose gel were amplified at Tm 55.0^{o}C, 55.3^{o}C, 56.0^{o}C, 56.8^{o}C, 58.1^{o}C, 59.8^{o}C, 61.6^{o}C, 63.2^{o}C, 64.4^{o}C, 65.2^{o}C, 65.8^{o}C and 66.0^{o}C on lane 1-12, respectively.

4.2 Optimization of $MgCl_2$ concentration

The reaction was carried out as mentioned in method 3.2.2 with annealing temperatures 63.2 ^{o}C and 200 μM dNTPs. The amplified products were analyzed on 2.5% agarose gel electrophoresis as shown in **Figure 15- 20**. The optimal concentration of $MgCl_2$ was selected at 1.5 mM with the best amplified products of Multiplex PCR for all six species.

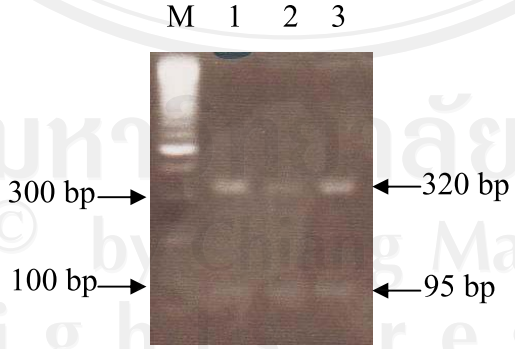

Figure15. Variation of $MgCl_2$ concentration using in Multiplex PCR for *M. avium* ATCC 2529 identification. Lane M indicates 1-kp DNA ladder marker; Lane 1, 1.5 mM $MgCl_2$; Lane 2, 2.0 mM $MgCl_2$; Lane 3, 2.5 mM $MgCl_2$.

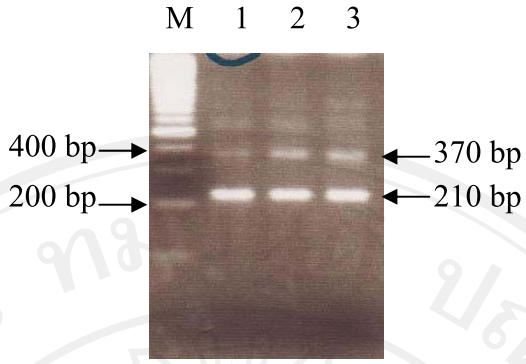

Figure16. Variation of $MgCl_2$ concentration using in Multiplex PCR for *M. fortuitum* ATCC 6841 identification. Lane M indicates 1-kp DNA ladder marker; Lane 1, 1.5 mM $MgCl_2$; Lane 2, 2.0 mM $MgCl_2$; Lane 3, 2.5 mM $MgCl_2$.

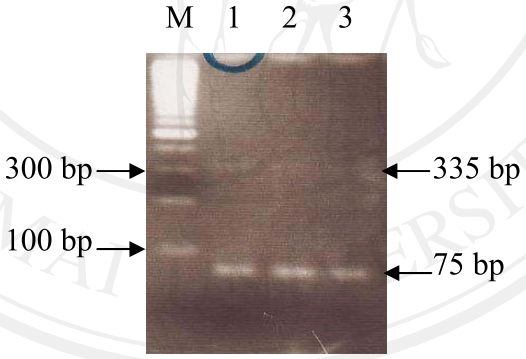

Figure17. Variation of $MgCl_2$ concentration using in Multiplex PCR for *M. intracellulare* ATCC 13950 identification. Lane M indicates 1-kp DNA ladder marker; Lane 1, 1.5 mM $MgCl_2$; Lane 2, 2.0 mM $MgCl_2$; Lane 3, 2.5 mM $MgCl_2$.

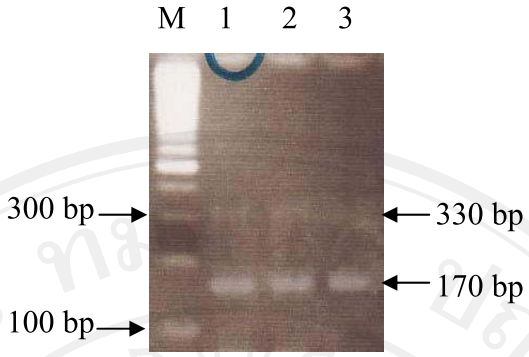

Figure18. Variation of $MgCl_2$ concentration using in Multiplex PCR for *M. kansasii* ATCC 12478 identification. Lane M indicates 1-kp DNA ladder marker; Lane 1, 1.5 mM $MgCl_2$; Lane 2, 2.0 mM $MgCl_2$; Lane 3, 2.5 mM $MgCl_2$.

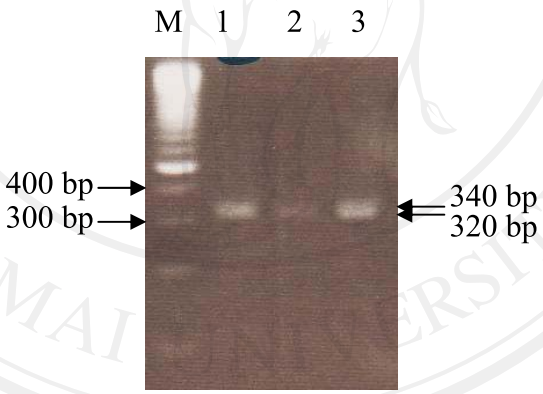

Figure19. Variation of $MgCl_2$ concentration using in Multiplex PCR for *M. scrofulaceum* ATCC 19982 identification. Lane M indicates 1-kp DNA ladder marker; Lane 1, 1.5 mM $MgCl_2$; Lane 2, 2.0 mM $MgCl_2$; Lane 3, 2.5 mM $MgCl_2$.

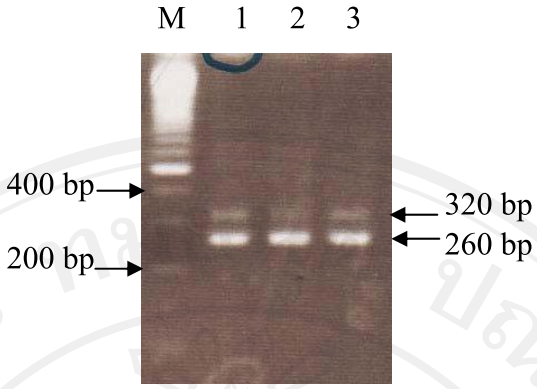

Figure20. Variation of $MgCl_2$ concentration using in Multiplex PCR for *M. tuberculosis* ATCC 27294 identification. Lane M indicates 1-kp DNA ladder marker; Lane 1, 1.5 mM $MgCl_2$; Lane 2, 2.0 mM $MgCl_2$; Lane 3, 2.5 mM $MgCl_2$.

4.3 Optimization of dNTPs concentration

The optimal concentration of dNTPs is usually important for the success of Multiplex PCR for mycobacteria detection. The results showed that the optimal concentration of dNTPs was 200 µM with the best amplified product detection of each reaction, as shown in **Figure 21-23**.

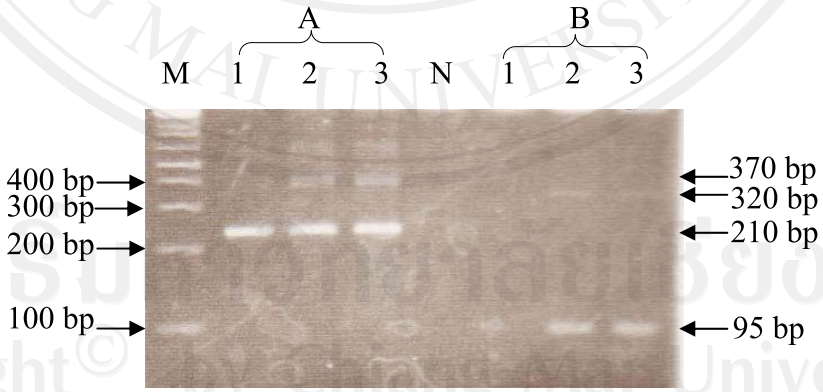

Figure21. Variation of dNTPs concentration using in Multiplex PCR for detection of 210/ 370 bp of *M. fortuitum* ATCC 6841 (panel A) and 95/ 320 bp of *M. avium* ATCC 25291 (panel B). Lane 1 = 100 µM; Lane 2 = 200 µM; Lane 3 = 300 µM

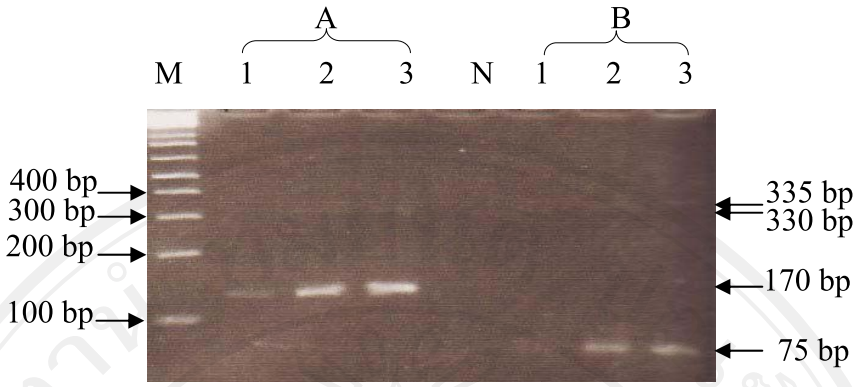

Figure22 Variation of dNTPs concentration using in Multiplex PCR for detection of 170/ 330 bp of *M. kansasii* ATCC 12478 (panel A) and 75/ 335 bp of *M. intracellure* ATCC 13950 (panel B). Lane 1 = 100 µM; Lane 2 = 200 µM; Lane 3 = 300 µM

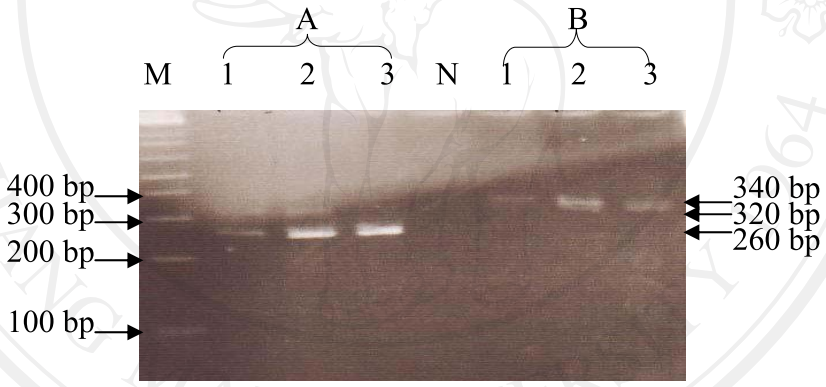

Figure23 Variation of dNTPs concentration using in Multiplex PCR for detection of 260/ 320 bp of *M. tuberculosis* ATCC 27294 (panel A) and 320/ 340 bp of *M. scrofulaceum* ATCC 19982 (panel B). Lane 1 = 100 µM; Lane 2 = 200 µM; Lane 3 = 300 µM

The optimized conditions for detection and identification of mycobacteria by Multiplex PCR for this study were shown in **Table9** and **Table10**.

Table 9. Optimization of master mix for Multiplex PCR in detection and identification of mycobacteria optimized master mix

Reagent	Final concentration	1 Reaction (µl)
10X buffer	1X buffer	5
25 mM $MgCl_2$	1.5 mM	3
10 mM dNTP	0.2 mM	1
20 µM each primers	10 pM	0.5 (10 each primers)
5u Taq DNA polymerase	1u	0.2
Extracted DNA	-	5
Distilled water	-	31
Total	-	50

Table 10. Multiplex PCR program for detection and identification of mycobacteria

Initial denaturation	Amplification (30 cycles)			Final extension
	Denaturation	Annealing	Extension	
94°C, 5 min	94°C, 1 min	63.5°C, 1 min	72°C, 1 min	72°C, 10 min

5. Determination of sensitivity or lower detection limit of Single-tube Multiplex PCR in detection of mycobacteria DNA

The 10-fold serial dilutions of *M. avium, M. fortuitum, M. intracellulare, M. kansasii, M. scrofulaceum* and *M. tuberculosis* DNA varied from 100 pg/µl to 1 fg/ µl were used for determination of the lower detection limit of the assays. Five microliters of each dilution was amplified by Single-tube Multiplex PCR and using agarose gel electrophoresis for amplified product detection. The results were shown in **Figure 24-29.**

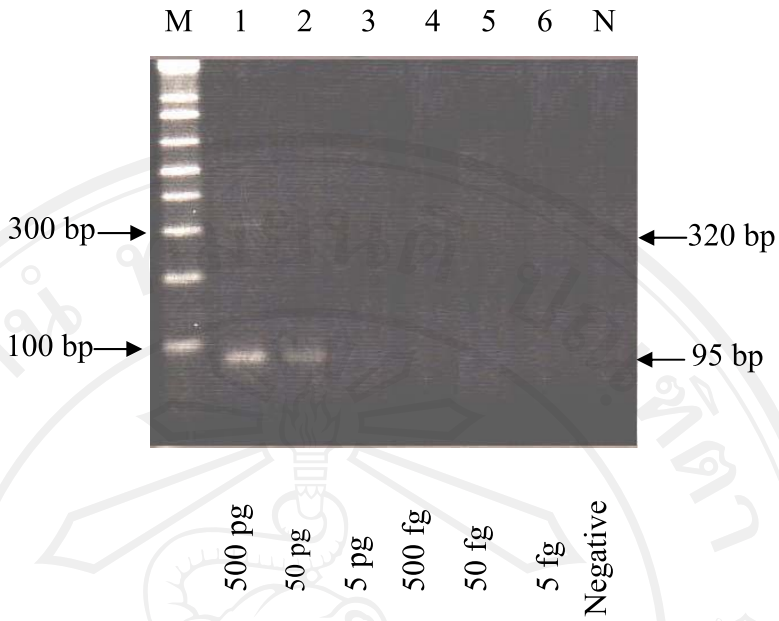

Figure24. Variation of DNA template concentration for *M. avium* ATCC 25291 detection by Multiplex PCR. Lane M = 1Kb plus DNA ladder; Lane N = Negative; Lane 1 = 500 pg; Lane 2 = 50 pg; Lane 3 = 5 pg; Lane 4 = 500 fg; Lane 5 = 50 fg; Lane 6 = 5 fg

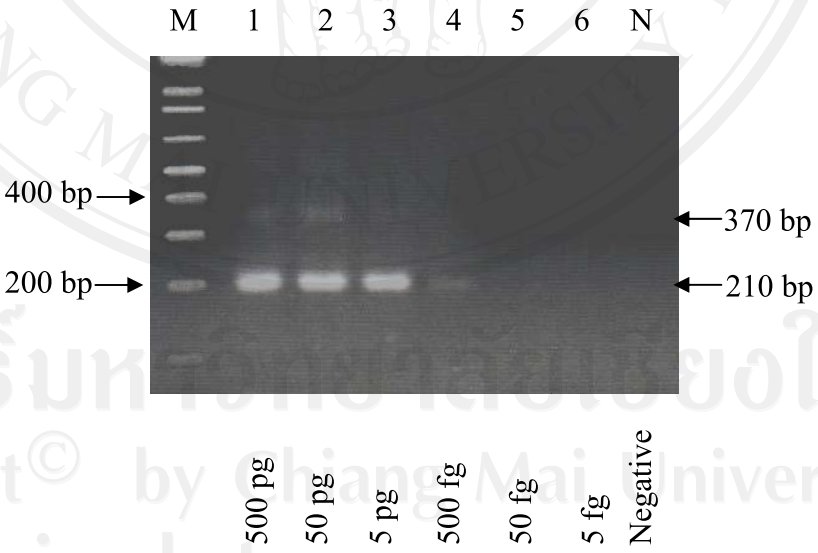

Figure25. Variation of DNA template concentration for *M. fortuitum* ATCC 6841 detection by Multiplex PCR. Lane M = 1Kb plus DNA ladder; Lane N = Negative; Lane 1 = 500 pg; Lane 2 = 50 pg; Lane 3 = 5 pg; Lane 4 = 500 fg; Lane 5 = 50 fg; Lane 6 = 5 fg

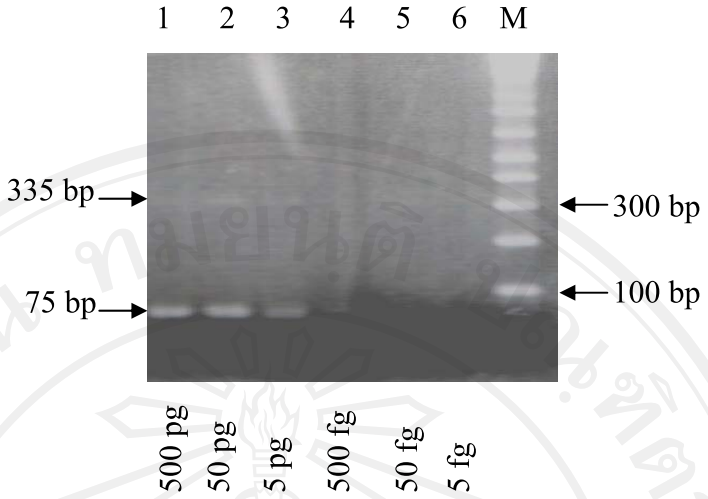

Figure26. Variation of DNA template concentration for *M. intracellulare* ATCC 13950 detection by Multiplex PCR. Lane M = 1Kb plus DNA ladder; Lane N = Negative; Lane 1 = 500 pg; Lane 2 = 50 pg; Lane 3 = 5 pg; Lane 4 = 500 fg; Lane 5 = 50 fg; Lane 6 = 5 fg

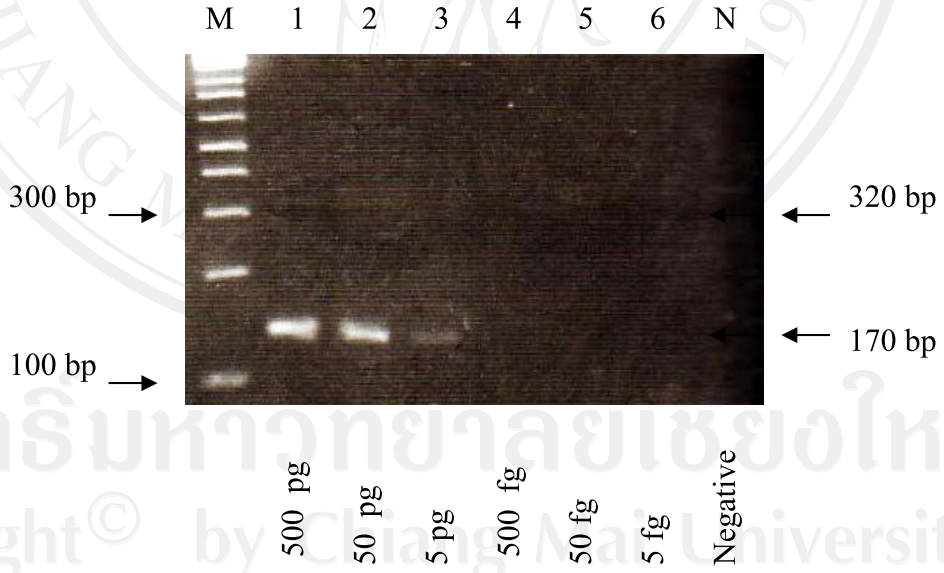

Figure27. Variation of DNA template concentration for *M. kansasii* ATCC 12478 detection by Multiplex PCR. Lane M = 1Kb plus DNA ladder; Lane N = Negative; Lane 1 = 500 pg; Lane 2 = 50 pg; Lane 3 = 5 pg; Lane 4 = 500 fg; Lane 5 = 50 fg; Lane 6 = 5 fg

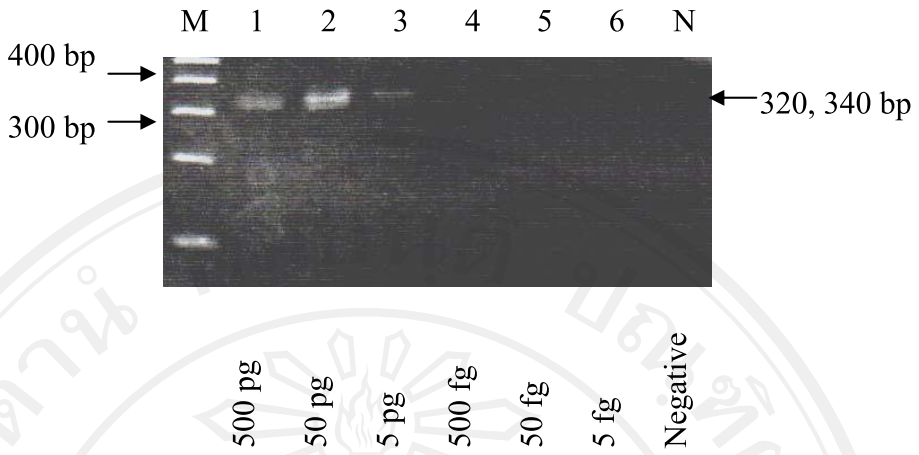

Figure28. Variation of DNA template concentration for *M. scrofulaceum* ATCC 19982 detection by Multiplex PCR. Lane M = 1Kb plus DNA ladder; Lane N = Negative; Lane 1 = 500 pg; Lane 2 = 50 pg; Lane 3 = 5 pg; Lane 4 = 500 fg; Lane 5 = 50 fg; Lane 6 = 5 fg

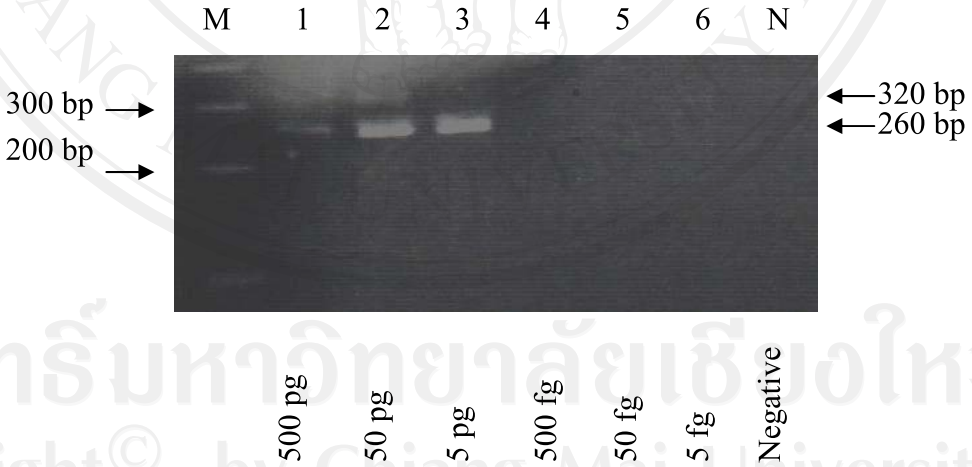

Figure29. Variation of DNA template concentration for *M. tuberculosis* ATCC 27294 detection by Multiplex PCR. Lane M = 1Kb plus DNA ladder; Lane N = Negative; Lane 1 = 500 pg; Lane 2 = 50 pg; Lane 3 = 5 pg; Lane 4 = 500 fg; Lane 5 = 50 fg; Lane 6 = 5 fg

6. Detection and identification of mycobacteria by Single-tube Multiplex PCR

All of the referent strains of *Mycobacterium* species were tested by the optimized Single-tube Multiplex PCR. The results showed the specific amplified products pattern of each species as show in **Figure 30-31**.

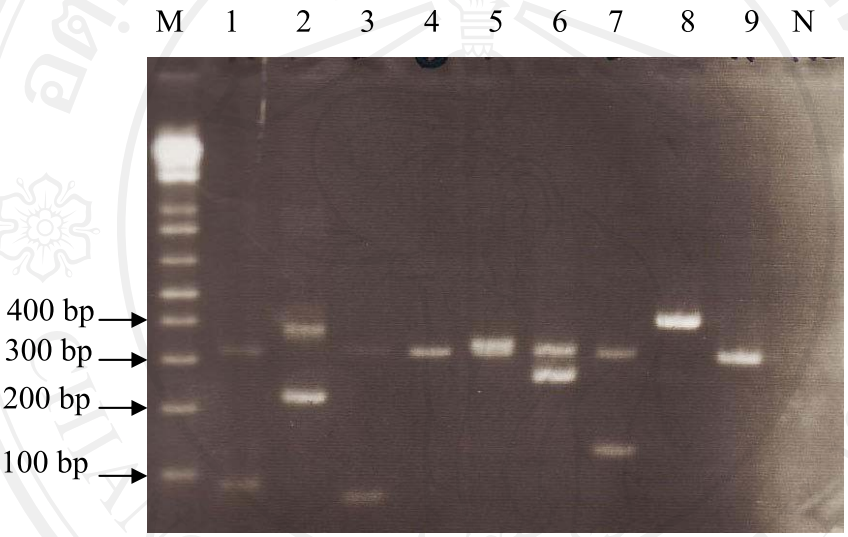

Figure30. The specific amplified products pattern of mycobacteria referent strains by optimized Single- tube Multiplex PCR. Lane M, 1 kb ladder DNA; Lane 1, *M. avium* ATCC 25291 (95/ 320 bp); Lane 2, *M. fortuitum* ATCC 6841 (210/ 370 bp); Lane 3, *M. intracellulare* ATCC 13950 (75/ 335 bp); Lane 4, *M. gordonae* ATCC 14470 (330 bp); Lane 5, *M. scrofulaceum* ATCC 19982 (340/ 320 bp); Lane 6, *M. tuberculosis* ATCC 27294 (260/ 320 bp); Lane 7, *M. kansasii* ATCC 12478 (170/ 330 bp), Lane 8, *M. phlei* ATCC 23024 (400 bp); Lane 9, *M. xenopi* ATCC 13250 (330 bp)

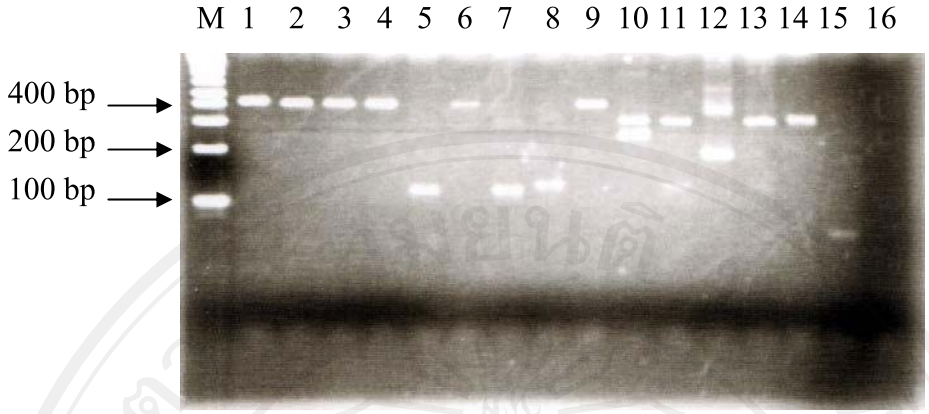

Figure31. The specific amplified products pattern of referent strain of *Mycobacterium* species and nonmycobacteria by optimized Single- tube Multiplex PCR.Lane M, 1 kb DNA ladder marker; lane 1; *M. nonchromogenicum* ATCC 19530 (400bp), lane 2, *M. neoaurum* ATCC 25795 (390 bp); lane 3, *M. terrae* ATCC 15778 (390 bp); lane4, *M. szulgai* ATCC 3201(360 bp); lane 5 and 7 *M. gordonae* TBD 014-03 (150/ 330 bp); lane 6, *M.terrae* TBD 201-02 (390 bp); lane 8 and 11, *M. kansasii* TBD 111-03 (170/ 330 bp); lane 9, *M. austroafricanum* ATCC 13250 (390 bp); lane 10, *M. bovis* BCG (260/ 320 bp); lane 12, *M. fortuitum* TBD 012-01 (210/ 370 bp); lane 13, *M. xenopi* TBD 231-02 (330 bp); lane 14, *M. scrofulaceum* TBD 192-03 (340/ 320 bp); lane 15, *M. intracellulare* ATCC 13950 (75/ 335 bp); lane 16, *Escherichia coli* ATCC 25922 (No band).

7. Comparison of the identification results by Single-tube Multiplex PCR with PCR –REA

Sixty-four clinical isolated mycobacteria were identified by Single-tube Multiplex PCR. The identification results by Single-tube Multiplex PCR were compared with PCR-REA. Six species of mycobacteria included in the identification capacity of Single-tube Multiplex PCR were all concordant identified with PCR-REA. Other species than six species capable identified by Multiplex PCR were identified as *Mycobacterium* species. One unclassified MAC and one *M. avium* complex by PCR-REA identification were identified as *M. avium* and *M. intracellulare* by Multiplex PCR. The over all results by Multiplex PCR were 98.4% concordance with PCR-REA, as shown in **Table 11.** The calculation was:

Concordance value = $\frac{63 \times 100}{64}$ = 98.4%

Table11. Comparison of 64 clinical isolated mycobacteria identification by Multiplex PCR with PCR-REA

No. of isolates	Assays		
	PCR-REA	Multiplex PCR	INNOLiPA*
1	ND	*M. avium*	*M. avium*
29	*M. avium*	*M. avium*	ND
1	Unclassified MAC	*M. avium*	ND
1	*M. avium* complex	*M. intracellularae*	ND
25	MTB complex	MTB complex	ND
1	*M. fortuitum*	*M. fortuitum*	ND
1	*M. scrofulaceum*	*M. scrofulaceum*	ND
1	*M. kansasii I*	*M. kansasii*	ND
2	*M. gordonae*	*Mycobacterium spp.*	ND
3	Rapid Mycobacteria	*Mycobacterium spp*	ND
2	ND	*Mycobacterium spp.*	*M. chelonae*

* 3 *Mycobacterium* species identified by INNOLiPA were also compared with Multiplex PCR. ND= not done

8. Comparison of the identification results by Single-tube Multiplex PCR with PNB screening test

The 25 isolates of MTB complex and 8 isolates of NTM were identified by PNB screening test in previous studies. The results showed 1 isolate out of 8 NTM was identified as MTB complex by Multiplex PCR while all 25 isolates of MTB complex were identified as MTB complex same as PNB screening test. In addition 51 nonmycobacterias which were identified with conventional biochemical test were tested by Multiplex PCR and the results of all nonmycobacteria showed negative reaction as shown in **Table 12**. The negative results for all nonmycobacteria by Multiplex PCR indicated the 100% specificity of this technique.

Table12. Comparison between Multiplex PCR and PNB screening test in detection and identification mycobacteria

Assays		Multiplex PCR			No. of isolates
		MTB complex	NTM	Nonmycobacteria	
PNB Screening test	MTB complex	25	0	0	25
	NTM	1	7	0	8
Biochemical test	Nonmycobacteria	0	0	51	51
No. of isolates		26	7	51	84