


*In The Name Of God*

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Collaborating Center for Tuberculosis
East Mediterranean Region

Mycobacteriology
Research Center

National Research Institute
Tuberculosis and Lung Disease

Collaborating Center for Tuberculosis
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# تستهای مولکولی تشخیص هویت مایکوباکتریوم

# Identification Tests: PCR, Nested-PCR & PCR-RFLP

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| | |
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**Protocol (No: 3)**

| | |
|---|---|
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**تستهای مولکولی تشخیص هویت مایکوباکتریوم**


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# تشخیص مایکوباکتریوم درنمونه های بالینی

## *Identification of Mycobacterium in Sputum Specimens*

**Step 1)** Isolated DNA is amplified using IS6110 primer

***80-85% of M.TB complex has the IS6110 in their genome. Therefore, this step is just to give an overall idea that the DNA from samples are belong to M.TB complex or not. However, as 20-15% of M. tuberculosis isolates, do not have IS6110 fragments, we follow the Nested-PCR for Hsp65 gene simultaneously. (Since the DNA is very low in clinical specimens, we perform nested-PCR).***

**Step 2)** Nested-PCR: First amplified the specific region using $TB_{15}$ and $TB_{17}$ primer.
**Step 3)** The obtained PCR-products, will re-amplify using TB11 and $TB_{12}$ primer for *hSP*65 gene.
**Step 4)** The PCR products digested with Bst*EII*, H*aeIII* restriction enzyme

***RESULTS***

***a)*** → ***Mycobacterium .tuberculosis complex***

***If with BstEII, HaeIII restriction enzyme, we obtain the M.TB complex patterns, then it is necessary to follow additional steps.***

- ***M.Bovis BCG by Multiplex PCR using primer of RD1 gene***
- ***M. tuberculosis & M.Bovis*** **Using spoligotyping**

***b)*** → ***Other than tuberculosis complex (MOTT)***

***Most of the MOTT are identified by hSP65, if not then primer for 16S-23S rRNA gene were used and the PCR-products were digested with HaeIII, CfoI, TaqI, MspI, DdeI, AvaII, HinfI restriction*** **enzyme.**

# تشخیص مایکوباکتریوم در کشت مثبت

## *Identification of Mycobacterium in Culture Positive Specimens*

**Step 1)** Isolated DNA is amplified using IS6110 primer
**Step 2)** PCR using TB11 and TB $_{12}$ primer for *hsp*65 gene
**Step 3)** The PCR products digested with *BstE*II, *Hae*III restriction enzyme

***Mycobacterium .tuberculosis complex***

***If with BstEII, HaeIII restriction enzyme, we obtain the M.TB complex patterns, then it is necessary to follow additional steps.***

***M. tuberculosis & M.Bovis Using spoligotyping***

***M.Bovis BCG by Multiplex -PCR using primer of RD1 gene***

***Other than M.TB complex***

***Most of the MOTT are identified by hsp65, if not then primer for 16S-23S rRNA gene were used and the PCR-products were digested with HaeIII, CfoI, TaqI, MspI, DdeI, AvaII, HinfI restriction enzyme***

# پرایمر های مورد نیاز
## *Primer*

**Primer name: IS6110 ($TB_1$ & $TB_2$)**
Sequence:

A: 5′-ATC CTG CGA GCG TAG GCG TCG G -3′
B: 5′-CAG GAC CAC GAT CGC TGA TCC GG -3′

**Primer name: *HSP*65 ($TB_{15}$& $TB_{17}$)**
Sequence**:**

A: 5′-CGT AYG ACG AAG AGG CCC GT-3′
B: 5′WAS GGR TCC TCS AGG ACS GC-3′

**Primer name: *HSP*65 ($TB_{11}$&$_{12}$)**
Sequence:

A: 5′-ACC AAC GAT GGT GTG TCC AT-3′
B: 5′-CTT GTC GAA CCG CAT ACC CT-3′

**Primer name: RD1**
Sequence:

ET1: 5′-AAG CGG TTG CCG CCG ACC GAC C -3′
ET2: 5′-CTG GCT ATA TTC CTG GGC CCG G -3′
ET3: 5′-GAG GCG ATC TGG CGG TTT GGG G -3′

**Primer name16s-23s rRNA (SP)**
Sequence:

A: 5′-ACC TCC TTT CTA AGG AGC ACC -3′
B: 5′-GAT GCT CGC AAC CAC TAT CCA -3′

# آنزیم های هضم کننده
# *Restriction-enzyme*

***Restriction enzyme used for PCR products using $TB_{11\text{-}12}$ primer***

1) *Bst*EII

2) *Hae*III enzyme

***Restriction enzyme used for PCR products using SP primer***

*1)* *Hae*III

2) *Cfo*I

3) *Taq*I

4) *Msp*I

5) *Dde*I

6) *Ava*II

7) *Hin*fI

# تهیه پرایمر
# *Primer Preparation*

* زمانی که می خواهید پرایمر سفارش دهید، سوالهای زیر را باید بدانید:

۱) سکانس پرایمر ۲) مقدار آن ۳) روش تخلیص آن

*When you order primer from a commercial company, you should know the following Information:

1) Sequence (5'-3') of the primer, number of nucleotides
2) The Unit size (e.g., 100 nmol)
3) Purification standard (e.g., standard desalting or gel purified)

* زمانی که پرایمر مورد نظر از شرکت دریافت شد، اطلاعات زیر همراه پرایمر می باشد:

۱) وزن مولکولی ۲) مقدار GC ۳) درجه ذوب ۴) مقدار الیگونوکلئوتید ها

*The primer will then be synthesized and sent to you in a lyophilized (freeze-dried) form, together with the following data:

1) Molecular weight
2) GC content
3) Tm (50 mM NaCl)
4) Amount of oligo (lyophilized). This could be expressed as $OD_{260}$, nM and µg

## *About primer*

مثلا پرایمر دریافتی شامل اطلاعات زیر است:

- OD260: 7.2
- Weight: 197.5 µg; 32.4 nmol
- Conc: 100 µM

**∗ Stock solution برای پرایمر با اطلاعات بالا:**

برای تهیه stock solution با غلظت 100 µM، به اندازه nmol قید شده، آب مقطر اضافه کنید.

یعنی برای پرایمر فوق مقدار ۳۲۴ میکرولیتر آب اضافه می شود که غلظت پرایمر (۱۰۰ µM/µl) به دست می آید.

برای تهیه working solution با غلظت ۱۰ µM: مقدار ۱۰ µl از محلول استوک پرایمر را با آب مقطر به حجم ۱۰۰µl می رسانیم.

غلظت پرایمر با افزودن، یک میکرولیتر از محلول 10 µM primer در واکنش۲۰ µL، برابر با 10 Pmol می باشد.

**∗ Stock solution**

Take for example a primer data sheet with the following information:

- OD260: 7.2
- Weight: 197.5 µg; 32.4 nmol
- Conc: 100 µM

To make 100 µM/µl of stock solution; Check the weight of primer, (Here is 32.4 nmol).Then, based on this weight adds the 324 µl D.W to make a stock of 100µM/µl.

To make 10 µM of working solution: Take 10 µl of stock solution and bring the volume into 100 µl.

Reaction Mixture: Adding 1 µL (of the 10 µM of working solution), to a 20 µL PCR reaction will result in a final primer concentration of 10 Pmol of primer in 20 µL volume.

## *Information on Insertion sequence (IS6110)*

**Primer name: $Tb_1$**

**Oligo sequence:** $TB_1$: 5′-ATC CTG CGA GCG TAG GCG TCG G -3′

**Delivered:** 6.4 OD**,** 185.4µg**,** 27.3 nmol **Conc:** 100.0µM

**MW:** 6792 **Tm:** 67.7, **Synthesis Scale:** 0.04 µmol **No .Bases:** 22 **GC%:** 68.2

**Dissolve in:** 273 µl .D.W

**Primer name: $Tb_2$**

**Oligo sequence:** $TB_2$: 5′-CAG GAC CAC GAT CGC TGA TCC GG -3′

**Delivered:** 6.0 OD, 171.5 µg, 24.4 nmol **Conc:** 100.0µM

**MW:** 7035 **Tm:** 67.8, **Synthesis Scale:** 0.04 µmol **No .Bases:** 23 **GC%:** 65.2

**Dissolve in:** 244 µl .D.W

# *Insertion sequence (IS6110)-PCR*

## PCR Mixture for 50 µl Reaction

dNTP: ( 0.2mM )1 µl
MgCL: (1.5 mM ) 1.5 µl
Enzyme (Taq): (1U) 0.25 µl
DMSO: (1%) 2 µl
Ung: 0.5 µl(0.5 u)

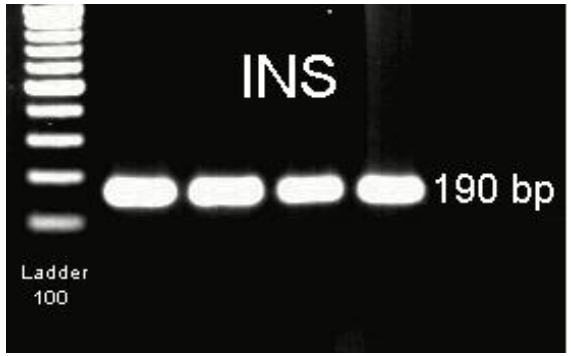
PCR Buffer: 5 µl
Primer A: (8 pmol) 0.8 µl
Primer B: (8 pmol) 0.8 µl
DNA Template: 2 µl

## PCR Cycle:

| | | | |
|---|---|---|---|
| **Initial denaturation** 10 min 95°c | | | |
| 30 Cycles | step1<br>step 2<br>step 3 | 20 Sec<br>1 min<br>20 Sec | 93˚C<br>65˚C<br>72˚C |
| **Final extension** 10 min 72˚C | | | |

## PCR Product

Primer $TB_1$ (5′- ATC CTG CGA GCG TAG GCG TCG G -3′) and $TB_2$ (5'- CAG GAC CAC GAT CGC TGA TCC GG -3′) amplified a 190-bp fragment
Photograph of PCR –Product using $TB_1$ &$TB_2$ primer



## *Information on Tb15 and $Tb_{17}$ primer*

**Primer name: $Tb_{15}$**

**Oligo sequence:** TB15: 5′-CGT AYG ACG AAG AGG CCC GT-3′

**Delivered:** 30.8 OD (849.8 µg, 138.0 nmol) **Conc:** 100.0 µM

**MW:** 6160 **Tm:** 62.4 **Synthesis Scale:** 0.04 µmol **No .Bases:** 20 **GC%:** 62.5

**Dissolve in:** 1380 µl .D.W

**Primer name: $Tb_{17}$**

**Oligo sequence:** TB17: 5′-WAS GGR TCC TCS AGG ACS GC -3′

**Delivered:** 29.5 OD (842.5µg, 137.3 nmol) **Conc.:** 100.0 µM

**MW:** 6136 **Tm:** 64.5 **Synthesis Scale:** 0.04 µmol **No .Bases:** 20 **GC%:** 67.5

**Dissolve in:** 1373 µl .D.W

## *Information on hsp65 gene ($Tb_{15}$ and $Tb_{17}$) primer*

### PCR Mixture for 50 µl Reaction

| | |
|---|---|
| dNTP: ( 0.2mM )1 µl | PCR Buffer: 5 µl |
| MgCL: (1.5 mM) 1.5 µl | Primer A: (4 pmol) 0.2 µl |
| Enzyme (tag): (1U) 0.25 µl | Primer B: (4 pmol) 0.2 µl |
| DMSO: (1%) 2.5 µl | DNA Template: 3-5 µl |

### PCR Cycle:

| | | | |
|---|---|---|---|
| **Initial denaturation** 5 min 95°c | | | |
| 30 Cycles | Step 1<br>Step 2<br>Step 3 | 20 Sec<br>1 min<br>40 Sec | 95 ˚C<br>60 ˚C<br>72 ˚C |
| **Final extension** 5 min 72˚C | | | |

### PCR Product:

Primer Tb15 (5′-CGT AYG ACG AAG AGG CCC GT-3′) and Tb17 (5′-WAS GGR TCC TCS AGG ACS GC -3′) amplified a 470bp fragment.

## *Information on $RD_1$ gene primer*

**Primer name: ET1**

**Oligo sequence:** ET1: 5′-AAG CGG TTG CCG CCG ACC GAC C -3′
**Delivered:** 6.0 OD (176.6 µg, 26.3 nmol) **Conc:** 100.0 µM**,**
**MW:** 6706 **Tm:** 69.6 **Synthesis Scale:** 0.04 µmol **No .Bases:** 22 **GC%:** 72.7%
**Dissolve in:** 263 µl .D.W

**Primer name: ET2**

**Oligo sequence:** ET2: 5′-CTG GCT ATA TTC CTG GGC CCG G -3′
**Delivered:** 6.7OD (203.2µg, 30.3 nmol) **Conc:** 100.0 µM
**MW:** 6718 **Tm:** 65.8 **Synthesis Scale:** 0.04 µmol **No .Bases:** 22 **GC%:** 63.6%
**Dissolve in:** 303µl .D.W

**Primer name: ET3**

**Oligo sequence:** ET3: 5′-GAG GCG ATC TGG CGG TTT GGG G -3′
**Delivered:** 6.3OD (180.3µg, 26.1 nmol) **Conc:** 100.0 µM
**MW:** 6903 **Tm:** 67.7 **Synthesis Scale:** 0.04 µmol **No .Bases:** 22 **GC%:** 68.2
**Dissolve in:** 261 µl .D.W

# *$RD_1$ Gene- PCR*

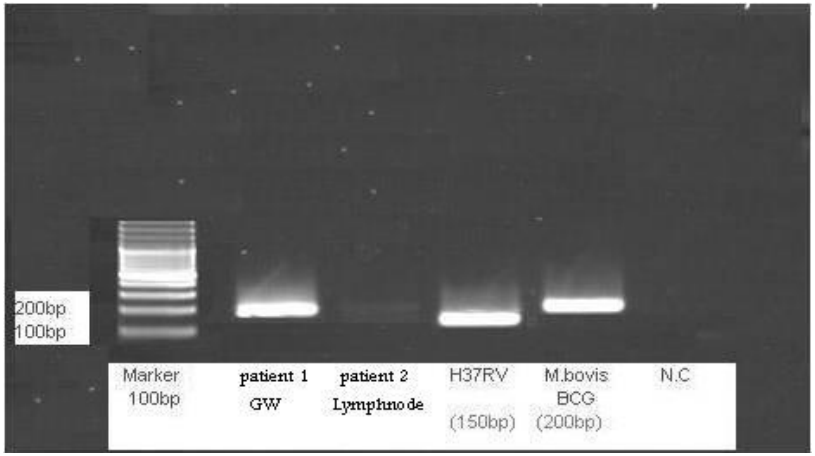
**PCR Mixture for 50 µl Reaction**

dNTP: ( 0.2mM )1 µl
MgCL: (2 mM) 2 µl
Enzyme (Taq): (1U) 0.25 µl
Primer ET3: (5 pmol) 0.5 µl
DNA Template: 5 µl
PCR Buffer: 5 µl
Primer ET1: (5 pmol) 0.5 µl
Primer ET2: (25 pmol) 2.5 µl
Total volume: 50 µl

**PCR Cycle:**

| | | | |
|---|---|---|---|
| **Initial denaturation** 3 min 95°c | | | |
| 30 Cycles | Step 1<br>Step 2 | 30 Sec<br>1 min | 94˚C<br>65˚C |
| **Final extension** 10 min 72˚C | | | |

**PCR Product:** *M.tb will give 150bp, whereas M.bovis B.C.G will give 200 bp.*



## *Information on hsp65 ($Tb_{11}$ and $Tb_{12}$) primer*

**Primer name: $Tb_{11}$**

**Oligo sequence:** Tb11: A: 5′-ACC AAC GAT GGT GTG TCC AT-3′

**Delivered:** 7.8 OD (218.4µg, 35.7 nmol) **Conc:** 100.0 µM**,**

**MW:** 6117 **Tm:** 57.3 **Synthesis Scale:** 0.04 µmol **No .Bases:** 20 **GC%:** 50.0

**Dissolve in:** 357 µl .D.W

**Primer name: $Tb_{12}$**

**Oligo sequence:** Tb12: B: 5′-CTT GTC GAA CCG CAT ACC CT-3′

**Delivered:** 7.6 OD (227.5µg, 37.8 nmol) **Conc:** 100.0 µM

**MW:** 6013 **Tm:** 59.4 **Synthesis Scale:** 0.04 µmol **No .Bases:** 20 **GC%:** 55.0

**Dissolve in:** 378 µl .D.W

# *Information on hsp65- PCR*

**PCR Mixture for 50 µl Reaction**

| | |
|---|---|
| dNTP: ( 0.2mM )1 µl | PCR Buffer: 5 µl |
| MgCL: (1.5 mM) 1.5 µl | Primer A: (8 pmol) 0.8 µl |
| Enzyme (Taq): (1U) 0.25 µl | Primer B: (8 pmol) 0.8 µl |
| DMSO: (1%) 2 µl | DNA Template: 2 µl |

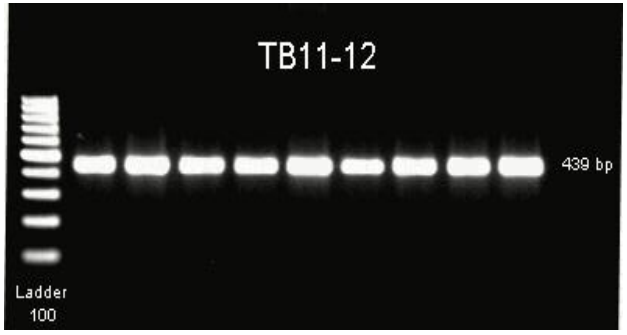
**PCR Cycle:**

| | | | |
|---|---|---|---|
| **Initial Denaturation** 4 min 95°C | | | |
| 30 Cycles | Step 1<br>Step 2<br>Step 3 | 30 Sec<br>1 min<br>40 Sec | 94 °C<br>56 °C<br>72 °C |
| **Final extension** 10 min 72°C | | | |

**PCR Product:**

Primer Tbll (5'-ACC AAC GAT GGT GTG TCC AT) and Tb12 (5'-CTT GTC GAA CCG CAT ACC CT) amplified a 439-bp fragment between positions 398 and 836 of the published gene sequence. (1)

*Photograph of PCR –Product using Tb11-12 primer*



# *HSP65 Restriction Analysis*

## Restriction analysis:

For B*stEII* digestion, 10 µl of PCR product is added directly to a mixture containing 1 µl of enzyme, 2 µl of restriction buffer (5 x buffer), and 18 µl of water, and the mixture is incubated for 1-16 h at 37°C.

For H*aeIII* digestion, 10µl of PCR product is added directly to a mixture containing 1µl of enzyme, 2µl of restriction buffer (5 x buffer), and 18 µl of water, and the mixture is incubated for 1-16 h at 37°C.

## Evaluation of restriction patterns:

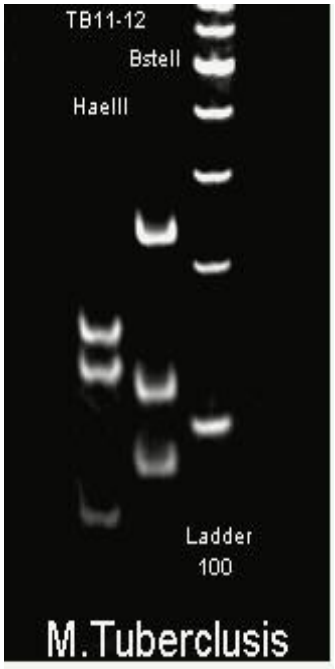
7µl of the digested PCR-product with 5µl of loading buffer were mixed and loaded onto an 8% polyacrylamid gel and visualized by UV light.

# *HSP65 Restriction Analysis*

## Evaluation of restriction patterns

PCR can be digested or not digested by B*stEII* **and** H*aeIII*.

| PCR Products Size | Restriction enzyme patterns | | Identification patterns |
|---|---|---|---|
| | **B*stEII*** | **H*aeIII*** | |
| 439 bp | 235/120/85 | 150/130/70/0 | *Mycobacterium. tuberculosis complex* |



# *Published Patterns of Mycobacterium Using BstEII and HaeIII Restriction Enzyme*

| *Bst*EII fragment length (bp)[a] | | *Hae*III fragment lengths (bp)[b] | Species[c] |
|---|---|---|---|
| No digestion | | 198 (197), 92 (87), 50 (58), 33 (40) | ***M. confluentis*** |
| | | 187 (184), 106 (107), 43 (NF), 38 (34) | ***M. gilvum*** |
| | | 177 (171), 91 (87), 74 (NF) 49 (45), 33 (36) | ***M. tusciae*** |
| | | 174 (181), 132 (126), 30 (42), 26 (36) | *M. triviale* |
| | | 146 (145), 69 (69), 58 (58), 55 (54) | ***M. brumae*** |
| | | 145 (139), 99 (98), 60 (58), 53 (51), 39 (36) | ***M. pulveris*** |
| | | 136 (135), 129 (128), 64 (65), 47 (46), 20 (35) | ***M. duvalii*** |
| | | 131 (128), 106 (103), 75 (70), 44 (42), 38 (36), 21 (22) | *M. szulgai* |
| | | 93 (87), 53 (59), 39 (36), 23 (22) | ***M. gadium*** |
| 313 ± 10 [325] | 130 ± 2 [140] | 200 (198), 60 (60), 55 (59) | *M. chelonae* subsp. *chelonae* |
| | | 178 (186), 69 (65), 58 (58) | ***M. mucogenicum*** |
| | | 163 (160), 108 (112), 106 (111) | *M. haemophilum* |
| | 117 ± 3 [125 or 120] | 204 (196), 72 (69), 60 (58), 56 (54), 43 (41) | ***M. aichiense*** |
| | | 187 (181), 132 (126), 42 (38), 36 (34) | *M. terrae* |
| | | 170 (172), 137 (139), 58 (56) | *M. neoaurum* |
| | | 167 (161), 128 (123), 59 (58), 42 (40), 38 (36) | ***M. rhodesiae*** |
| | | 151 (147), 142 (141), 60 (58), 42 (46) | ***M. diernhoferi*** |
| | | 147 (139), 84 (87), 58 (58) | ***M. chitae*** |
| | | 145 (139), 91 (94), 50 (58) | *M. nonchromogenicum* type II |
| | | 130 (127), 111 (112), 71 (70), 59 (59) | *M. gordonae* type IV[d] |
| | | 127 (127), 108 (103), 43 (42) | *M. genavense* |
| 234 ± 3 [245] | 211 ± 4 | 191 (188), 135 (126), 66 (NF) | *M. simiae* type I |
| | | 184 (179), 141 (137), 74 (72) | ***M. thermoresistibile*** |
| | | 176 (174), 141 (149), 91 (95), 50 (51) | ***M. sphagni*** |
| | | 167 (162), 96 (94), 53 (51), 46 (45), 42 (40), 37 (36) | ***M. poriferae*** |
| | | 155 (145), 136 (127), 45 (42), 39 (40) | ***M. interjectum*** |
| | | 150 (145), 127 (127), 100 (95) | *M. scrofulaceum* |
| | | 146 (145), 102 (106), 81 (78) | *M. marinum*[d] |
| | | 145 (146), 138 (140), 95 (98), 54 (52) | *M. fortuitum* subsp. *peregrinum* |
| | | 144 (139), 128 (123), 98 (101), 53 (52) | *M. porcinum* |
| | | 144 (145), 74 (69), 62 (58), 55 (52) | *M. chelonae* subsp. *abscessus*[d] |
| | | 143 (139), 86 (87), 58 (58), 40 (37) | ***M. obuense*** |
| | | 141 (140), 85 (81), 51 (54), 33 (36) | *M. phlei* |
| | | 132 (140), 104 (106), 80 (76) 41 (41) 37 (36) | ***M. branderi*** |
| | | 128 (127), 105 (103), 82 (78) | *M. kansasii* type I[d] |
| | | 127 (127), 104 (103), 51 (59) | *M. avium/paratuberculosis* |
| | | 113 (112), 104 (106) | *M. asiaticum* |
| | 131 ± 5 85 ± 3 [140/85 or 80] | 160 (161), 142 (145), 61 (59) | ***M. agri*** |
| | | 154 (154), 129 (123), 63 (58), 45 (40), 41 (36) | *M. smegmatis* |
| | | 151 (146), 99 (100), 53 (52) | ***M. alvei*** |
| | | 141 (150), 120 (124), 50 (58), 40 (43), 30 (36) | ***M. tokaiense*** |
| | | 140 (139), 130 (130), 73 (79), 43 (40) | *M. shimoidei* |
| | | 137 (139), 117 (123), 58 (59) | *M. fortuitum* subsp. *fortuitum* |
| | | 133 (126), 104 (103), 73 (69) | *M. gastri/kansasii* |
| | 117 ± 2 99 ± 4 [125 or 120/100] | 152 (145), 131 (127), 63 (57), 44 (39) | *M. intracellulare* |
| | | 147 (145), 109 (105), 81 (78) | *M. malmoense* |
| | | 145 (140), NF (106), 59 (40) 49 (36), 42 (33), 35 (23) | ***M. hiberniae*** |
| | | 130 (127), 111 (112), 43 (40), 39 (35) | *M. gordonae* type III[d] |
| | 117 ± 2 84 ± 5 [125 or 120/80] | 214 (210), 111 (115) | *M. gordonae* type II[d] |
| | | 166 (161), 103 (104), 56 (59), 44 (42), 39 (36) | *M. xenopi* |
| | | 158 (161), 111 (112), 54 (57) | *M. gordonae* type I[d] |
| | | 157 (152), 131 (127), 71 (69) | *M. tuberculosis* complex[d] |
| | | 145 (139), NF (123), 64 (58), 39 (32) | *M. senegalense* |
| | | 142 (141), 124 (123), 61 (58), 54 (52), 43 (43) | ***M. farcinogenes*** |

## *Information on 16S-23S rRNA primer*

**Primer name: SP1**

**Oligo sequence:** SP1: 5′-ACC TCC TTT CTA AGG AGC ACC-3′

**Delivered:** 13.6 OD (394µg, 62.4 nmol) **Conc** (volume 1ml) 62 pmol/µl

**MW:** 6326 g/mol **Tm:** 59.8 **Synthesis Scale:** 0.05 µmol **No .Bases:** 21 **GC%:** 52.4

**Dissolve in:** 624 µl .D.W

**Primer name: SP2**

**Oligo sequence:** SP2: 5′-GAT GCT CGC AAC CAC TAT CCA -3′

**Delivered:** 9.4 OD (265µg, 41.9 nmol) **Conc** (volume 1ml) 42 pmol/µl

**MW:** 6335g/mol **Tm:** 59.8 **Synthesis Scale:** 0.04 µmol **No .Bases:** 21 **GC%:** 52.4

**Dissolve in:** 419 µl .D.W

# *16S-23S rRNA gene - PCR*

## PCR Mixture for 50µl Reaction

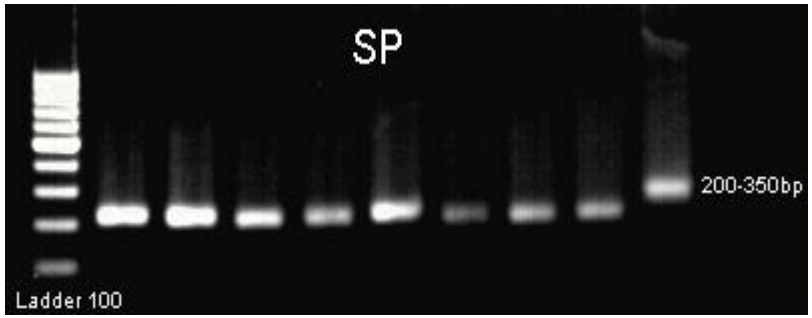
dNTP: ( 0.2mM)1 µl
Mgcl2: (1.75 mM) 1.75µl
Enzyme tag: (2.5 U) 0.25 µl
DMSO: (1%) 2 µl
Water: up to 50 µl

PCR Buffer:5 µl
Primer A :( 8 pmol) 0.8 µl
PrimerB: (8pmol) 0.8 µl
Template: 1.5 µl

## PCR Cycle

| | | | |
|---|---|---|---|
| **Initial denaturation** 4 min 95°c | | | |
| 30 Cycles | Seq1<br>Seq2<br>Seq3 | 30Sec<br>1 min<br>40 Sec | 94˚ C<br>56˚C<br>72˚C |
| **Final extension** 10 min 72˚C | | | |

**PCR Product:** *Primer $SP_1$and SP2 amplified a 200-350-bp fragment*



# *Restriction analysis of 16S-23S rRNA gene -PCR*

## Restriction analysis:

For H*ae*III digestion, 10µl (0.1-0.5µg) of PCR product is added directly to a mixture containing 1µl of enzyme, 2µl of restriction buffer (5 x buffer), and 18 µl of water, and the mixture is incubated for 16 h at 37°C.

*For Cf*oI digestion, 10µl (0.1-0.5µg) of PCR product is added directly to a mixture containing µl of enzyme, µl of restriction buffer (5 x buffer), and 18 µl of water, and the mixture is incubated for 1 h at 37°C.

For *Taq*I digestion, 10µl (0.1-0.5µg) of PCR product is added directly to a mixture containing µl of enzyme, µl of restriction buffer (5 x buffer), and 18 µl of water, and the mixture is incubated for 16.h at 65°C.

For *Msp*I digestion, 10µl (0.1-0.5µg) of PCR product is added directly to a mixture containing µl of enzyme, µl of restriction buffer (5 x buffer), and 18 µl of water, and the mixture is incubated for 16.h at 37°C.

For *Dde*I digestion, 10µl (0.1-0.5µg) of PCR product is added directly to a mixture containing µl of enzyme, µl of restriction buffer (5 x buffer), and 18 µl of water, and the mixture is incubated for 16 h at 37°C.

For *Ava*II digestion, 10µl (0.1-0.5µg) of PCR product is added directly to a mixture containing µl of enzyme, µl of restriction buffer (5 x buffer), and 16µl of water, and the mixture is incubated for 16.h at 37°C.

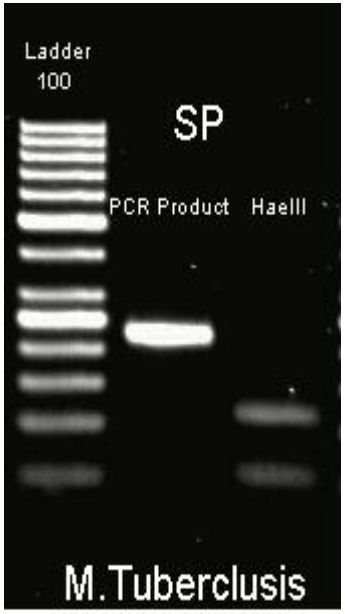
For *Hinf*I digestion, 10µl (0.1-0.5µg) of PCR product is added directly to a mixture containing µl of enzyme, µl of restriction buffer (5 x buffer), and 18 µl of water, and the mixture is incubated for 16 h at 37°C.

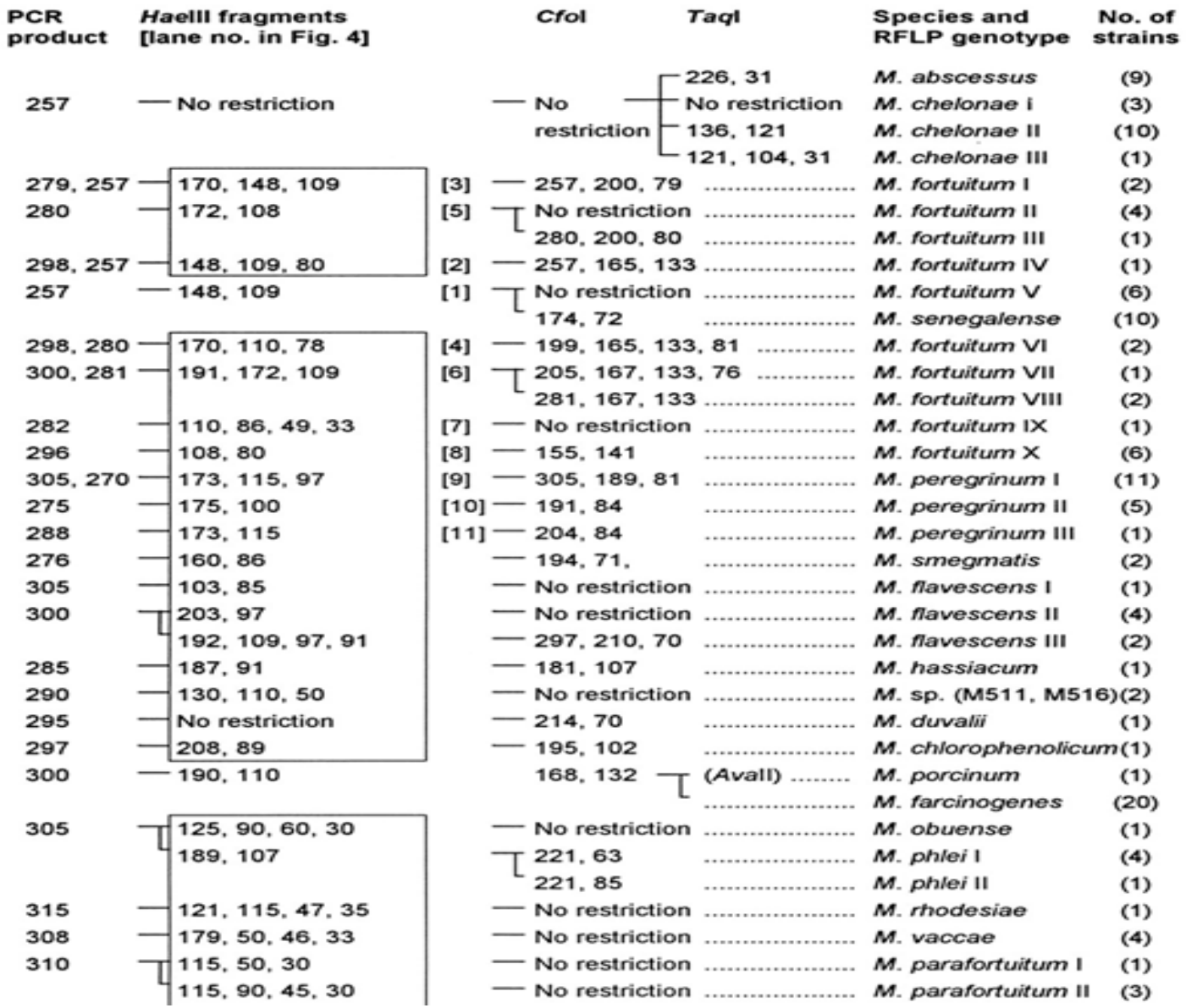
## *Restriction analysis of 16S-23S rRNA gene -PCR*

### Evaluation of restriction patterns

PCR can be digested or not digested by H*aeIII*.

| PCR Products Size | Restriction enzyme patterns<br>H*aeIII* | Identification patterns |
|---|---|---|
| 220 bp | 110/60/50 | *Mycobacterium. Tuberculosis complex* |




PCR product
HaeIII fragments [lane no. in Fig. 4]
CfoI
TaqI
Species and RFLP genotype
No. of strains
257 — No restriction
No restriction — 226, 31 — M. abscessus (9)
No restriction — No restriction — M. chelonae I (3)
No restriction — 136, 121 — M. chelonae II (10)
No restriction — 121, 104, 31 — M. chelonae III (1)
279, 257 — 170, 148, 109 [3] — 257, 200, 79 — M. fortuitum I (2)
280 — 172, 108 [5] — No restriction — M. fortuitum II (4)
280, 200, 80 — M. fortuitum III (1)
298, 257 — 148, 109, 80 [2] — 257, 165, 133 — M. fortuitum IV (1)
257 — 148, 109 [1] — No restriction — M. fortuitum V (6)
174, 72 — M. senegalense (10)
298, 280 — 170, 110, 78 [4] — 199, 165, 133, 81 — M. fortuitum VI (2)
300, 281 — 191, 172, 109 [6] — 205, 167, 133, 76 — M. fortuitum VII (1)
281, 167, 133 — M. fortuitum VIII (2)
282 — 110, 86, 49, 33 [7] — No restriction — M. fortuitum IX (1)
296 — 108, 80 [8] — 155, 141 — M. fortuitum X (6)
305, 270 — 173, 115, 97 [9] — 305, 189, 81 — M. peregrinum I (11)
275 — 175, 100 [10] — 191, 84 — M. peregrinum II (5)
288 — 173, 115 [11] — 204, 84 — M. peregrinum III (1)
276 — 160, 86 — 194, 71, — M. smegmatis (2)
305 — 103, 85 — No restriction — M. flavescens I (1)
300 — 203, 97 — No restriction — M. flavescens II (4)
192, 109, 97, 91 — 297, 210, 70 — M. flavescens III (2)
285 — 187, 91 — 181, 107 — M. hassiacum (1)
290 — 130, 110, 50 — No restriction — M. sp. (M511, M516) (2)
295 — No restriction — 214, 70 — M. duvalii (1)
297 — 208, 89 — 195, 102 — M. chlorophenolicum (1)
300 — 190, 110 — 168, 132 — (AvaII) — M. porcinum (1)
M. farcinogenes (20)
305 — 125, 90, 60, 30 — No restriction — M. obuense (1)
189, 107 — 221, 63 — M. phlei I (4)
221, 85 — M. phlei II (1)
315 — 121, 115, 47, 35 — No restriction — M. rhodesiae (1)
308 — 179, 50, 46, 33 — No restriction — M. vaccae (4)
310 — 115, 50, 30 — No restriction — M. parafortuitum I (1)
115, 90, 45, 30 — No restriction — M. parafortuitum II (3)

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