結核菌鑑定與非結核分枝桿菌鑑定
-分子診斷法

高雄醫學大學附設中和紀念醫院檢驗醫學部
楊淵傑 總級醫檢師
Molecular Diagnosis

- Rapid
  - Do not have to wait for cell growth to a certain number
- More sensitive than traditional methods
  - Theoretically 1~1000 cells can be detected
- With great potential for large scale screening or even identification
What to consider before developing new molecular identification assays?

**Selection of Target Sites for Identification**

- Sequence conservation and stability
- multi-copy?
- DNA or RNA?
- Target size?
Molecular Diagnosis of *Mycobacterium*

- **Detection** of *M. tuberculosis complex* from specimens
- **Identification** of *Mycobacterium species* from colonies
Specific sequences for molecular diagnosis of Mycobacterium

- IS6110 (TBC)
- \textit{rpoB}: Differentiation of species in MTBC.
- 36-bp direct repeat sequence (DP) (TBC)
- 16S-23S rRNA intergenic spacer (ITS): Identification of MTBC and NTM
- 65 kDa heat shock protein (HSP) antigen (all mycobacterium)
DNA extraction

- Bacterial suspension
  - Lysis bf (SDS)
  - GUSCN (Guanidin thiocyanate)
  - 80°C, 5 min
- Ammonium acetate
- Protein precipitation
  - 100% Ethanol or Isopropanol
  - -70°C or -20°C
  - DNA precipitation
    - 70% Ethanol
    - de-salt
    - dry
    - DNA resuspend
Molecular diagnosis of clinical microbiology

Technique used:

- PCR with specific primers
- RFLP (restriction fragment length polymorphism)
- Hybridization
- DNA array
PCR: Polymerase Chain Reaction

30 - 40 cycles of 3 steps:

**Step 1: Denaturation**
1 minute 94 °C

**Step 2: Annealing**
45 seconds 54 °C
forward and reverse primers !!!

**Step 3: Extension**
2 minutes 72 °C
only dNTP's

(Andy Vierstraete 1999)
聚合酵素連鎖反應（Polymerase chain reaction），是一種快速的DNA複製方法
**TB-PCR (in house)**

- Detection of TB directly from clinical samples

```
specimens
    decontamination & digestion
    DNA extraction & purification
    PCR amplification of IS6110 DNA fragment(542bp)
    Agarose gel electrophoresis

No product
    (Negative)
```

```
Confirm PCR product with Dral
    317+225bp DNA fragments
    M.tuberculosis complex
```
PCR productions from the IS6110 of M. tuberculosis (542 bp)

- **M**: marker
- **Lane 1~8**: product of IS6110 (542 bp)
- **N**: negative control
- **P**: positive control

M. tuberculosis complex cut with Dral
PRA; PCR-Restriction enzyme Analysis

Genomic DNA

PCR products

65KD HSP DNA

Electrophoresis
Recognition site of Restriction Endonuclease

**Dra I**

```
TTT
AAA
AAA
```

**BstE II**

```
G|G_T_N_A_C_C
C_C_A_N_T_G
```

**Hae III**

```
G|G_C_C
C_C|G_G
```
Mycobacteria

DNA extraction & purification

PCR amplification of 439bp DNA fragment encode a 65KD protein

Restriction analysis (*Bst*EII, *Hae*III)

Agarose gel electrophoresis

Compare with restriction map

Bst E II

No Digestion

180/140 bp ______ M. triviale
175/80 bp ______ M. vaccae
140/105 bp ______ M. szulgai
140 bp ______ M. flavescens I
210 bp ______ M. chelonae ss. chelonae
175/125 bp ______ M. haemophilum
190/140 bp ______ M. terrae
140/115/70 bp ______ M. gordonae IV
140/105 bp ______ M. genavense
200/135 bp ______ M. simiae
160/115/80 bp ______ M. marinum
160/60 bp ______ M. chelonae ss. abscessus
155/150/100 bp ______ M. fortuitum ss. peregrinum
155/135/95 bp ______ M. scrofulaceum

170/110 bp ______ M. asiaticum
175/80 bp ______ M. aurum
160/130 bp ______ M. smegmatis
150/110/70 bp ______ M. shimoidei
140/105/70 bp ______ M. gastri / M. kansasii
155/140/60 bp ______ M. intracellulare/ MAI st. 18
155/110/70 bp ______ M. malmbense
140/120 bp ______ M. gordonae III
235/115 bp ______ M. gordonae II
170/115 bp ______ M. gordonae I
170/105 bp ______ M. xenopi
160/140/70 bp ______ M. tuberculosis complex
160 bp ______ M. nonchromogenicum
155/135 bp ______ M. fortuitum ss. fortuitum
150/35 bp ______ M. fortuitum ss. 3rd variant

Hae III

125 bp

140 bp

220 bp

140/115 bp ______ M. gordonae V
140/105/70 bp ______ M. kansasii
140/105 bp ______ M. avium
115/110 bp ______ M. asiaticum
175/80 bp ______ M. aurum
160/130 bp ______ M. smegmatis
150/110/70 bp ______ M. shimoidei
140/105/70 bp ______ M. gastri / M. kansasii
155/140/60 bp ______ M. intracellulare/ MAI st. 18
155/110/70 bp ______ M. malmbense
140/120 bp ______ M. gordonae III
235/115 bp ______ M. gordonae II
170/115 bp ______ M. gordonae I
170/105 bp ______ M. xenopi
160/140/70 bp ______ M. tuberculosis complex
160 bp ______ M. nonchromogenicum
155/135 bp ______ M. fortuitum ss. fortuitum
150/35 bp ______ M. fortuitum ss. 3rd variant
IDENTIFICATION OF MYCOBACTERIA

Copyright © 1999 Hospices cantonaux

last update: 15-September-2007

http://app.chuv.ch/prasite/index.html
QUERY PRA DATABASE (Simplified Query Form)

Choose one of the patterns proposed in the list for each enzyme

BstEII fragments

HaeIII fragments

Submit

To enter your own values of the length of the digested fragments press "Form"
## QUERY PRA DATABASE RESULTS

<table>
<thead>
<tr>
<th>ID</th>
<th>Species</th>
<th>BstE II</th>
<th>Hae III</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>Mycobacterium gordonae type 1</td>
<td>235/120/85</td>
<td>160/115/60</td>
<td>10</td>
</tr>
<tr>
<td>163</td>
<td>Mycobacterium tuberculosis complex type 1</td>
<td>235/120/85</td>
<td>150/130/70</td>
<td>15</td>
</tr>
<tr>
<td>48</td>
<td>Mycobacterium fortuitum type 1</td>
<td>235/120/85</td>
<td>145/120/60</td>
<td>16</td>
</tr>
<tr>
<td>51</td>
<td>Mycobacterium fortuitum s. acetamidolyticum type 1</td>
<td>235/120/85</td>
<td>145/120/60</td>
<td>16</td>
</tr>
<tr>
<td>152</td>
<td>Mycobacterium smegmatis type 1</td>
<td>235/130/85</td>
<td>145/125/60</td>
<td>18</td>
</tr>
<tr>
<td>100</td>
<td>Mycobacterium mageritense type 1</td>
<td>235/130/85</td>
<td>145/125/60</td>
<td>18</td>
</tr>
<tr>
<td>66</td>
<td>Mycobacterium gordonae type 9</td>
<td>235/120/100</td>
<td>160/115/60</td>
<td>18</td>
</tr>
<tr>
<td>58</td>
<td>Mycobacterium goodii type 1</td>
<td>235/130/85</td>
<td>145/125/60</td>
<td>18</td>
</tr>
<tr>
<td>44</td>
<td>Mycobacterium farcinogenes type 1</td>
<td>235/120/85</td>
<td>140/125/60</td>
<td>20</td>
</tr>
<tr>
<td>113</td>
<td>Mycobacterium neworleanense type 1</td>
<td>235/120/85</td>
<td>140/125/60</td>
<td>20</td>
</tr>
</tbody>
</table>
PCR productions from the TB 11,12 of *Mycobacterium* (439 bp)

M: marker
Lane 1~8: product of TB 11,12 (440 bp)
N: negative control
P: positive control
### PCR-restriction fragments analysis for Mycobacteria identification


<table>
<thead>
<tr>
<th>Organism</th>
<th>Mycobacterium abscessus type 2</th>
<th>Mycobacterium fortuitum type 2</th>
<th>Mycobacterium gordonae type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BstEII pattern</td>
<td>235 / 210 / 0</td>
<td>235 / 120 / 85</td>
<td>235 / 120 / 100</td>
</tr>
<tr>
<td>HaeIII pattern</td>
<td>200 / 70 / 60 / 50</td>
<td>140 / 120 / 60 / 55</td>
<td>130 / 115 / 0 / 0</td>
</tr>
<tr>
<td>Phenotype</td>
<td>rapidly growing non-pigmented</td>
<td>rapidly growing non-pigmented</td>
<td>slowly growing scotochromogen</td>
</tr>
</tbody>
</table>
Real Time PCR ---- TaqMan

Polymerization

Strand displacement

Cleavage

Completion of polymerization

NFO = Nonfluorescent quencher
MGB = Minor groove binder
R = Reporter
P = Hot-start DNA polymerase
<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation Maxima (nm)</th>
<th>Emission Maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>495</td>
<td>520</td>
</tr>
<tr>
<td>SGI</td>
<td>497</td>
<td>522</td>
</tr>
<tr>
<td>TET</td>
<td>521</td>
<td>540</td>
</tr>
<tr>
<td>VIC</td>
<td>530</td>
<td>552</td>
</tr>
<tr>
<td>JOE</td>
<td>526</td>
<td>552</td>
</tr>
<tr>
<td>HEX</td>
<td>540</td>
<td>557</td>
</tr>
<tr>
<td>CY3</td>
<td>552</td>
<td>570</td>
</tr>
<tr>
<td>TAMRA</td>
<td>552</td>
<td>578</td>
</tr>
<tr>
<td>ROX</td>
<td>585</td>
<td>605</td>
</tr>
<tr>
<td>Texas Red</td>
<td>595</td>
<td>615</td>
</tr>
<tr>
<td>CY5</td>
<td>643</td>
<td>667</td>
</tr>
</tbody>
</table>
Real-Time PCR
Real Time PCR
PCR & hybridization

- PCR
- Genomic DNA
- PCR products
- Electrophoresis
- Southern transfer (*Probe)
- Autoradiography
DNA loading

Labeled Size marker

Electrophoresis

Gel

Blotting

Paper towels

sponge

Gel

Salt solution

Nitrocellulose membrane

DNA transferred

Probing (hybridization)

Probe hybridized To target DNA

Autoradiogram
The Underlying Principle of DNA array

- Base-pairing
  - A:T
  - G:C
- Hybridization
  Complementary binding of DNA

Probe

Target
Internal transcribed spacer (ITS)

- Located between the rRNA genes
- Non-transcribed but essential for rRNA maturation
- Highly conservative in a species
- Highly variable between species
- High copy number: 50–150 copies/genome

Prokaryotic

Prokaryotic DNA is typically shown as a single circular chromosome, which is easier to illustrate with a single line. The rRNA genes (16S, 23S) are located on this chromosome, with the ITS region separating them. The ITS region is non-transcribed but essential for rRNA maturation.

Eukaryotic

Eukaryotic DNA is typically shown as multiple linear chromosomes. The rRNA genes (18S, 5.8S, 28S) are located on these chromosomes, with the ITS regions (ITS1, ITS2) separating them. The ITS regions are non-transcribed but essential for rRNA maturation.
Hybridization procedures

- Probes fixed on nylon membrane
- Amplicon labeled with digoxygenin
- DNA chip
- Anti-Dig antibody labeled with alkaline phosphatase
- Color development
- Hybridization

Migyp5b  PC
### Layout of oligonucleotide probes on the array

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>MTBC1</td>
<td>MTBC3</td>
<td>MTBC4</td>
<td>M</td>
</tr>
<tr>
<td>B</td>
<td>Mtub2</td>
<td>MbovG3R</td>
<td>Mbov2</td>
<td>Mcap2</td>
<td>Mmic5R</td>
<td>Mcan5</td>
<td>M</td>
</tr>
<tr>
<td>C</td>
<td>MtubW2</td>
<td>MbovGW3R</td>
<td>MbovW2</td>
<td>McapW2</td>
<td>MmicW5R</td>
<td>McanW5</td>
<td>M</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>E</td>
<td>Mavi2-4</td>
<td>Mint3-2</td>
<td>Mabs4-4</td>
<td>Mche1</td>
<td>Mfor5-1L</td>
<td>Mma/ul4-2</td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>Msme2R</td>
<td>Mter3</td>
<td>Mper5</td>
<td>Mszu4</td>
<td>Mkan3-1</td>
<td>Mnon3</td>
<td>M</td>
</tr>
<tr>
<td>G</td>
<td>Mvac1-1</td>
<td>Mgas5</td>
<td>Mscr5-2</td>
<td>Mgor4</td>
<td>Mxen4-1</td>
<td>PC (PAN-03)</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>
Hybridization patterns of NTM

- *M. abscessus* (CCUG 20993T)
- *M. avium* (BCRC 15441T)
- *M. chelonae* (CCUG 35749)
- *M. fortuitum* (BCRC 15320)
- *M. gastri* (CCUG 29062T)
- *M. gordonae* (CCUG 21801T)
- *M. intracellulare* (CCUG 28005T)
- *M. kansasii* (CCUG 27785)
- *M. lentiflavum* (CCUG 47901)
- *M. marinum* (ATCC 25039)
# Commercial Method for Direct Detection of *Mycobacterium tuberculosis* Complex in Clinical Samples

<table>
<thead>
<tr>
<th>Method</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real-time PCR</td>
<td>COBAS® TaqMan® MTB Test</td>
</tr>
<tr>
<td></td>
<td>Xpert® MTB/RIF test</td>
</tr>
<tr>
<td>Isothermal amplification</td>
<td>GEN-PROBE AMPLIFIED MTD</td>
</tr>
<tr>
<td></td>
<td>Loopamp MTBC Detection Kit</td>
</tr>
<tr>
<td></td>
<td>ProbeTec DTB</td>
</tr>
<tr>
<td>Reverse hybridization</td>
<td>GenoType® Mycobacteria Direct</td>
</tr>
<tr>
<td></td>
<td>DR. MTBC Screen™ IVD Kit</td>
</tr>
</tbody>
</table>
DR. MTBC Screen™ Chip Kit
Reverse hybridization

- Base-pairing
  A=T
  G=C
- Hybridization
  Complementary binding of DNA
操作流程

1. 倍性PCR
2. 杂交
3. Strept-AP
4. NBT/BCIP
整個流程約需8小時。
結果判讀

結核分枝桿菌群檢驗晶片探針示意圖

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>B1,B3</td>
<td>Hybridization positive control</td>
<td></td>
</tr>
<tr>
<td>A2,C2</td>
<td>MTB complex specific probe</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>PCR positive control</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>Negative Control</td>
<td></td>
</tr>
</tbody>
</table>

檢驗結果及解釋：

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>檢體中有結核分枝桿菌群之病原菌存在</td>
<td>檢體中無結核分枝桿菌群之病原菌存在</td>
<td></td>
</tr>
</tbody>
</table>
效能

Table 8. DR. MTBC test 與 TB culture 的比對結果

<table>
<thead>
<tr>
<th></th>
<th>TB Culture</th>
<th>DR. MTBC test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Kappa (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>北榮</td>
<td>Positive</td>
<td>14 (3.3%)</td>
<td>3 (0.7%)</td>
<td>17 (4.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>11 (2.6%)</td>
<td>395 (93.4%)</td>
<td>406 (96.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>25 (5.9%)</td>
<td>398 (94.1%)</td>
<td>423(100.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.0325*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>北榮</td>
<td>Positive</td>
<td>26 (7.1%)</td>
<td>1 (0.3%)</td>
<td>27 (7.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>21 (5.7%)</td>
<td>318 (86.9%)</td>
<td>339 (92.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>47 (12.8%)</td>
<td>319 (87.2%)</td>
<td>366(100.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>&lt; 0.0001*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>高榮</td>
<td>Positive</td>
<td>135 (22.8%)</td>
<td>11 (1.9%)</td>
<td>146 (24.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>24 (4.1%)</td>
<td>421 (71.2%)</td>
<td>445 (75.3%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>159 (26.9%)</td>
<td>432 (73.1%)</td>
<td>591 (100.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.0280*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data from pakage insert
# Sensitivity & Specificity

<table>
<thead>
<tr>
<th>DR. MTBC</th>
<th>No. of specimens</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTB (n=190)</td>
<td>Neg. (n=1190)</td>
<td>(%)(%)</td>
</tr>
<tr>
<td>Positive (231)</td>
<td>175</td>
<td>56</td>
<td>92.1</td>
</tr>
<tr>
<td>Negative (1149)</td>
<td>15</td>
<td>1134</td>
<td></td>
</tr>
</tbody>
</table>

The kappa coefficient was 0.801 (95% C.I. 0.757 ~ 0.846; P<0.0001).
待測的痰液檢體需以痰液消化&去污染劑處理後才能開始使用本產品進行檢測。

由於檢體核酸已不具傳染性，故檢體核酸之增幅放大、雜交、洗滌及呈色、結果判讀步驟皆於一般合格實驗室操作即可，但所有廢液/物仍須嚴格收集滅菌始能丟棄。

本產品能偵測到 *M. tuberculosis complex*，包括：*M. tuberculosis*，*M. bovis*，*M. bovis BCG*，*M. africanum*，*M. microti* 和 *M. canetti*，但是並無法鑑別上述六種分枝桿菌。

晶片呈色後，Hybridization Positive Control 及 PCR Positive Control必須有訊號，才為成功之測試。
COBAS® TaqMan® MTB Test
**TaqMan Probe Assay Principle**

**Hydrolysis Probe Technology**

Probe Intact:Reporter (R) Fluorescence Emission Suppressed by Quencher (Q)

Probe Cleavage by 5’-Nuclease Activity of Enzyme

Probe Cleaved: Fluorescence Emission Detected

R = Reporter      Q = Quencher
COBAS® TaqMan® 48 Analyzer

Test Workflow

Sample Preparation

Add Master Mix and Sample to K-tube/K-tray

Select Test/Create Order

Load Analyzer
Start amplification/detection

Data Analysis

AL3.2 or higher
This test is for use only with sputum or BAL that has been liquefied, decontaminated and concentrated using NALC–NaOH methods.
The Quantitation Standard

- Demonstrates sample Prep and PCR amplification has occurred in each sample: Quantification Reference

- **Benefit**
  - allows more accurate quantitation and controls for sample inhibition
  - no external standard curves required
  - Increases throughput
  - Provides internal control
Roche AmpliLink Software

User-friendly control samples and results
Selective Amplification Using AmpErase® Uracil N-glycosylase (UNG)

- Specifically detects and destroys previously amplified DNA without harming target.

![Diagram showing the process of selective amplification using AmpErase enzyme](image)
Test Performance

The overall sensitivity, specificity, positive predictive value, and negative predictive value for the Cobas TaqMan MTB test were 91.5%, 98.7%, 91.5%, and 98.7%, respectively.


Analytical Sensitivity of COBAs TaqMan MTB.

<table>
<thead>
<tr>
<th>Member</th>
<th>Nominal Input CFU/PCR</th>
<th># Invalid</th>
<th># Valid</th>
<th># Pos</th>
<th># Trials</th>
<th>% Hits</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>0</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td>100.0%</td>
<td>94.6 - 100%</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td>100.0%</td>
<td>94.6 - 100%</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td>100.0%</td>
<td>94.6 - 100%</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0</td>
<td>54</td>
<td>51</td>
<td>54</td>
<td>94.4%</td>
<td>84.6 - 98.8%</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>0</td>
<td>54</td>
<td>46</td>
<td>54</td>
<td>85.2%</td>
<td>72.9 - 93.4%</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>0</td>
<td>54</td>
<td>33</td>
<td>54</td>
<td>61.1%</td>
<td>46.9 - 74.0%</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>54</td>
<td>0</td>
<td>54</td>
<td>0.0%</td>
<td>0.0 - 5.4%</td>
</tr>
</tbody>
</table>

95% LOD by PROBIT = 0.46 CFU/PCR

Data from package insert
Performance Comparison of Roche MTB Test System

**COBAS TaqMan vs COBAS Amplicor**

- **NALC-NaOH decontamination of clinical specimens**
  - ~ 45 min

- **Instrument Setup & Mastermix**
  - 15 min
  - 10 min
  - 45 min
  - 15 min

- **COBAS TaqMan**
  - ~ 150 min

- **COBAS Amplicor**
  - ~ 250 min

**Up to 44 specimens**
- **Up to 20 specimens**

**Next day results**
操作之注意事項：

- 只可接受的呼吸道檢體，如咳出以及誘導產生的痰液等。並使用NALC–NaOH方法將這檢體液化、除污及濃縮。
- 已除污的檢體於−20°C下可保存最多2週。已處理的檢體於−70°C下可保存最多6個月,或於2–8°C下可保存最多4天。
- 如果檢體製備、擴增和偵測兩者是在分開不同的工作天進行,則試劑製備與擴增和偵測要在同一天進行。
- RW、RL、RN、MTB MMX、MYCO Mg2+、MYCO IC、MTB (+) C和MYCO(−) C均含有Sodium azide。Sodium azide可以與鉛及銅管反應並形成高爆炸性的金屬疊氮化物。當含有疊氮化鈉的溶液倒於實驗室水槽內丟棄時,要用大量的水沖洗排水管,以防止azide堆積在水槽管道中。
- 在檢體和品管液製備時必需使用螺旋蓋管，藉以防止濺潑及檢體的潛在交叉污染。不要使用snap cap tubes。
- COBAS TaqMan MTB Master Mix以及加了工作MMX的已處理檢體和品管液都是光敏感的。這些試劑均要避光。
Xpert® MTB/RIF test

We are saving lives with fast, simple MTB/RIF testing that gives results in minutes, not weeks.

In here is a more hopeful world.

Cepheid. Cares About Tuberculosis
Supporting Access to Xpert® MTB/RIF in High Burden Developing Countries
Xpert MTB/RIF Overview

- Semi-Quantitative, Multiplex, Hemi-nested real-time PCR assay
  - Sputum sample
  - Concentrated sediments prepared from induced or expectorated sputa
  - Smear positive or smear negative samples
- Uses 3 specific primers and 5 unique Molecular Beacon® probes
  - To differentiate between the conserved wild-type sequence and mutations in the 81 bp core region that are associated with RIF resistance
- Sample Processing Control (SPC)
  - non-infectious sample preparation control spore
在待測DNA片段中，設計高專一性probe，在probe 5’及3’上分別標記Reporter dye與Quancher dye；完整probe在游離狀態下，R的能量會被Q吸收，偵測器無法測得螢光。當進行PCR時，Probe黏合倒待測模板上，此時具有5’exonuclease的Taq polymerase會將probe水解，使得R游離，R與Q分開後，R被激發的能量才以螢光形式釋放出來。
檢體處理流程

Procedure – Expectorated Sputum Samples

1. Add 2:1 Sample Reagent to sample (minimum sample 1ml)
2. Shake then stand 10 minutes
3. Shake then stand further 5 minutes
4. Transfer 2ml to cartridge
5. Begin Test...

Procedure – Sputum Sediment

1. Inoculate media and prepare smear from deposit
2. Add 1.5ml Sample Reagent to 0.5ml resuspended deposit
3. Shake then stand 10 min.
4. Shake then stand further 5 minutes
5. Transfer the 2ml to cartridge
6. Begin Test...
操作流程

1. Sputum liquefaction and inactivation with 2:1 sample reagent
2. Transfer of 2 ml material into test cartridge
3. Cartridge inserted into MTB-RIF test platform (end of hands-on work)
4. Sample automatically filtered and washed
5. Ultrasonic lysis of filter-captured organisms to release DNA
6. DNA molecules mixed with dry PCR reagents
7. Seminested real-time amplification and detection in integrated reaction tube
8. Printable test result

Time to result, 1 hour 45 minutes
Figure 6. MTB DETECTED MEDIUM; Rif Resistance DETECTED (Privileged User View)
結果判讀

- 以5段Probe針對wild type rpoB gene進行測定，任2段(含2段)陽性則MTB(+)；在MTB(+)前提下，當任一段Neg(表示mutate)，則RIF(R)。
- 當5段都陰性或只有1段陽性，則MTB(-)，不會有RIF報告。
- 當檢體(+)SPC(-)為可接受，因為PCR反應有確實進行；當檢體(-)SPC(-)，結果會“INVALID”。
- 當Ct>40則反應為陰性。
- 具有可半定量偵測的特性

<table>
<thead>
<tr>
<th>MTB result</th>
<th>Ct range</th>
<th>CFU/ ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>&lt;16</td>
<td>(10^7)</td>
</tr>
<tr>
<td>Medium</td>
<td>16–22</td>
<td>(10^6)</td>
</tr>
<tr>
<td>Low</td>
<td>22–28</td>
<td>(10^4–10^5)</td>
</tr>
<tr>
<td>Very low</td>
<td>&gt;28</td>
<td>(10^2–10^3)</td>
</tr>
</tbody>
</table>
Sensitivity and Specificity (原廠)

<table>
<thead>
<tr>
<th></th>
<th>Smear negative</th>
<th>Smear Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture Positive</td>
<td>Culture Negative</td>
</tr>
<tr>
<td>Xpert Positive</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>Xpert Negative</td>
<td>7</td>
<td>289</td>
</tr>
</tbody>
</table>

- Sensitivity in smear negative, culture positive (S-C+) was 90.9% (70/77)
- Sensitivity in smear positive, culture positive (S+C+). was 100% (275/275)
- Specificity of the assay was 98.3%
- Sensitivity observed for Rifampicin resistance was 96.7%
- Specificity observed for Rifampicin resistance was 98.6%
Catharina C. Boehme: Rapid Molecular Detection of Tuberculosis and Rifampin Resistance, *NEJM* 2010

Results – Detection of *Mycobacterium tuberculosis* from 3 Samples Per Patient

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All Culture Positive</td>
<td>Smear and Culture Positive</td>
</tr>
<tr>
<td>Overall</td>
<td><strong>97.6%</strong></td>
<td><strong>99.8%</strong></td>
</tr>
<tr>
<td>Lima</td>
<td>99.1%</td>
<td>100%</td>
</tr>
<tr>
<td>Baku</td>
<td>96.6%</td>
<td>100%</td>
</tr>
<tr>
<td>Cape Town</td>
<td>95.9%</td>
<td>99.0%</td>
</tr>
<tr>
<td>Durban</td>
<td>95.6%</td>
<td>100%</td>
</tr>
<tr>
<td>Mumbai</td>
<td><strong>98.4%</strong></td>
<td>100%</td>
</tr>
</tbody>
</table>
# Results – Detection of Rifampicin Resistance

<table>
<thead>
<tr>
<th>Location</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>99.1%</td>
<td>100%</td>
</tr>
<tr>
<td>Lima</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Baku</td>
<td>98.1%</td>
<td>100%</td>
</tr>
<tr>
<td>Cape Town</td>
<td>93.8%</td>
<td>100%</td>
</tr>
<tr>
<td>Durban</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Mumbai</td>
<td>99.2%</td>
<td>100%</td>
</tr>
</tbody>
</table>

(After discrepant result resolution by sequencing)

Mycobacterium tuberculosis complex DNA in sputum samples or concentrated sediments prepared from induced or expectorated sputa that are either acid-fast bacilli (AFB) smear positive or negative.

The performance of Xpert MTB/RIF Assay for the detection of MTB complex has not been demonstrated from non-respiratory specimens, such as blood, CSF, stool or urine.

When processing more than one sample at a time, open only one cartridge, add the Sample Reagent-treated and close the cartridge before adding Sample Reagent-treated sample to the next cartridge.

Do not use a cartridge that has been dropped or shaken after you have added the treated sample.

Mutations or polymorphisms in primer or probe binding regions may affect detection of new or unknown MDR-MTB or RIF-resistant strains resulting in a false negative result.
Gen–Probe

Amplified MTD® Test

MTD : Mycobacterium Tuberculosis Direct
Amplified molecular diagnostic assay detects *M. Tuberculosis* directly from clinical specimens in less than 2hrs30

- Two enzymes, Two primers system
- Uses TMA technique
- Transcription of RNA *amplicon* from ribosomal targets
- Amplicon is detected by DNA probes in HPA
- Single tube format
- The first FDA-approved direct test for helping to diagnose smear-positive and negative specimens.
Sample preparation:

- Prepare and sonicate specimen

Amplification: TMA

- Add to tube: amplification reagent, oil prepared specimen
- Add enzyme reagent
- Denature at 95°C, 15 mins
- Anneal 42°C, 5 mins
- Amplify 42°C, 30 mins

Detection: HPA

- Add Probe Reagent
- Hybridization 60°C, 15 mins
- Selection 60°C, 15 mins
- Read on luminometer
Hybridization Protection Assay (H.P.A) in Solution Assays

- Hybridization
  - Acridinium ester-labeled DNA probe specifically detects RNA amplicon

- Selection
  - Separation of hybridized from unhybridized probes by chemical hydrolysis in solution phase (no washing step)

- Detection
  - Chemiluminescence
Detection of amplicon with DNA probes and the Hybridization Protection Assay (HPA) technique. (A) Acridinium ester (AE)-labeled DNA probes are added and allowed to hybridize to specific target sequences within the amplicon produced in the TMA reaction. (B) Separation of hybridized from unhybridized probes is done by the addition of selection reagent which hydrolyzes the AE on the unhybridized probes. No light is emitted in the luminometer from the unhybridized probes. (C) The AE on the hybridized probes is protected within the double helix and is not hydrolyzed by the selection reagent. Light is emitted and detected by the luminometer.
Sensitivity, Specificity and Predictive Values after Resolution of Discrepant Results


(N=823) | Sensitivity | Specificity | PPV | NPV |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear-Positive respiratory</td>
<td>100%</td>
<td>99.7%</td>
<td>99.5%</td>
<td>100%</td>
</tr>
<tr>
<td>Smear-Negative respiratory</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Smear-Positive nonrespiratory</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Smear-Negative nonrespiratory</td>
<td>100%</td>
<td>98.9%</td>
<td>90%</td>
<td>100%</td>
</tr>
<tr>
<td>All</td>
<td>100%</td>
<td>99.6%</td>
<td>97.4%</td>
<td>100%</td>
</tr>
</tbody>
</table>
The amplified MTD Test is the first FDA approved rapid TB test able to detect *M. Tuberculosis* directly in clinical samples.

- Single tube format (no transfer or wash step)
- Isothermal reaction
- Consistent clinical performance in laboratories throughout the world
- Definitive, accurate diagnostic result in less than 2 hrs 30
Summary

- **DR. MTBC Screen™ IVD Kit**
  優點: 國內廠商具價格優勢，較無缺貨疑慮。
  缺點: 操作過程較為繁複。

- **COBAS® TaqMan® MTB Test**
  優點: 利用Uracil N–glycosylase的設計，可避免DNA汙染。
  缺點: 每次操作須帶陽性及陰性對照組，若每批檢體量不大，則會使成本大幅增加。

- **Xpert® MTB/RIF test**
  優點: 操作簡便，不需整批上機，屬POCT (Point of care test)。
  缺點: 試劑成本過高，Cartridge的設計可能會增加感染性廢棄物。

- **GEN-PROBE AMPLIFIED MTD test**
  優點: 以RNA為偵測標的，陽性可反映菌之活性。
  缺點: 試劑成本過高，每次操作須帶陽性及陰性對照組，若每批檢體量不大，則會使成本大幅增加，目前未取得衛署許可。
Commercial Method for Identification of MTBC and NTM in culture samples
DR. TBDR/NTM IVD Kit for Direct Detection of *Mycobacterium tuberculosis* Isolates, Including Rifampin-Resistant Isolates, and NTM

- designed to target MTBC, rifampin-resistant *M. tuberculosis*, and 15 species of NTM,
The accuracy rate of this kit for identification of *Mycobacterium* species was 95.5% (105/110).

Journal of Clinical Microbiology. 2012, 50(10) : 3398–3401
Molecular Identification of M. tuberculosis and NTM (line probe assay)

• **GenoType® MTBC**
  - 23S rRNA+gyrB sequence polymorphisms+RD1 deletion for identification of M. bovis BCG
  - Identification of BCG strain and its *subspecies*

• **GenoType® Mycobacterium (CM/AS)**
  - 23S rRNA gene
  - M. tuberculosis complex and 14 most common NTM species (CM) and 16 additional NTM species (AS)

• **INNO-LiPA MYCOBACTERIA v2**
  - 16S–23S rRNA gene spacers
  - 16 different mycobacterial species
By using DNA strip technology (Line Probe Assay) to detect TB
Method: GenoType® – DNA•STRIP® Technology

FIG. 1. GenoType MTBDRplus work flow
Hain Lifescience. Genotype® MTBDRplus product insert. Version 1
GenoType® MTBC

Can be performed from solid or liquid culture material.

- DNA· Strip® Assay for differentiation of Mycobacterium tuberculosis complex
  - M. tuberculosis
  - M. africanum
  - M. bovis ssp. bovis
  - M. bovis ssp. caprae
  - BCG
  - M. microti

- Based on the detection of SNPs in the gyrB gene and on the deletion of the RD1 region in BCG
GenoType® MTBC

Conjugate Control (CC)
A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.

Universal Control (UC)
This zone detects, as known, all known mycobacteria and members of the group of gram-positive bacteria with a high G+C content. If this zone and the Conjugate Control zone stain positive but the remaining banding pattern can not be assigned to a specific mycobacterium, additional methods have to be applied to identify the respective bacterial species.

MTBC
This zone hybridizes, as known, with amplicons generated from all members of the Mycobacterium tuberculosis complex.

Other bands
Specific probes, for evaluation see interpretation chart.
### GenoType® MTBC - Typical Results

<table>
<thead>
<tr>
<th>M.tub</th>
<th>M.microti</th>
<th>M.afr</th>
<th>M.bovis ssp. bobis</th>
<th>BCG</th>
<th>M.bovis ssp. caprae</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Strips" /></td>
<td><img src="image2.png" alt="Strips" /></td>
<td><img src="image3.png" alt="Strips" /></td>
<td><img src="image4.png" alt="Strips" /></td>
<td><img src="image5.png" alt="Strips" /></td>
<td><img src="image6.png" alt="Strips" /></td>
</tr>
</tbody>
</table>

---

*Note: Images of strips are placeholders for actual results.*
GenoType® Mycobacterium (CM/AS)

- Genotype® Mycobacterium CM (common species)
  - permits the simultaneous molecular genetic identification of the M. tuberculosis complex
  - 24 of the most common NTM species
- Genotype® Mycobacterium AS (additional species)
  - permits the simultaneous molecular genetic identification of 19 additional NTM species

- Identification and differentiation of \textit{M. tuberculosis} complex and 40 most common NTM
- **Starting material**: liquid and/or solid culture samples
Genotype® Mycobacterium CM&AS

1. Species may possibly be further differentiated with the Genotype® Mycobacterium AS
2. For further differentiation use the Genotype® Mycobacterium AS
3. For further differentiation use the Genotype® MTBC
INNO-LiPA MYCOBACTERIA v2
INNO-LiPA MYCOBACTERIA v2 is a line probe assay for the simultaneous detection and identification of the genus *Mycobacterium* and 16 different mycobacterial species. The test is based on the nucleotide differences in the 16S-23S rRNA spacer region and can be performed starting from either liquid or solid culture.

The following *Mycobacterium* species can be detected simultaneously: *M. tuberculosis* complex, *M. kansasii*, *M. xenopi*, *M. gordonae*, *M. genavense*, *M. simiae*, *M. marinum* and *M. ulcerans*, *M. celatum*, MAIS, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. malmoense*, *M. haemophilum*, *M. chelonae* complex, *M. fortuitum* complex, and *M. smegmatis*. 
Line probe assay

Chromogen (NBT/BCIP)

Alkaline phosphatase

Streptavidin

Biotin

Biotin-labeled single-stranded amplified target

DNA probe

Nitrocellulose strip

Color reaction
Features & Benefits

- The 16S - 23S rRNA spacer region brings the precision of molecular testing to species differentiation
- Mixed populations easily identified
- Discriminates subgroups within *M. kansasii* and *M. chelonae*
- Quick visual interpretation
- Applicable on early liquid culture - no need for colony morphology
- Quick screening of major species
- Fully automated processing of the strips possible using Auto-LiPA 48
### Mycobacterial genes with mutations associated with antimicrobial resistance

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Species</th>
<th>Gene</th>
<th>% Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP</td>
<td>M. tuberculosis, M. avium, M. africanum, M. leprae</td>
<td><em>rpoB</em></td>
<td>&gt;96</td>
</tr>
<tr>
<td>INH</td>
<td>M. tuberculosis</td>
<td><em>katG</em></td>
<td>90</td>
</tr>
<tr>
<td>INH-ethionamide</td>
<td>M. tuberculosis</td>
<td><em>inhA locus</em></td>
<td>90</td>
</tr>
<tr>
<td>INH</td>
<td>M. tuberculosis, M. leprae</td>
<td><em>ahpC</em></td>
<td>90</td>
</tr>
<tr>
<td>INH</td>
<td>M. tuberculosis</td>
<td><em>kasA</em></td>
<td>90</td>
</tr>
<tr>
<td>EMB</td>
<td>M. tuberculosis</td>
<td><em>embB</em></td>
<td>47-65</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>M. tuberculosis, M. smegmatis</td>
<td><em>rpsL</em></td>
<td>70</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>M. tuberculosis</td>
<td><em>rrs</em></td>
<td>70</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>M. tuberculosis</td>
<td><em>pncA</em></td>
<td>72-97</td>
</tr>
</tbody>
</table>
Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis

Updated Recommendation:

NAA testing should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB for whom a diagnosis of TB is being considered but has not yet been established, and for whom the test result would alter case management or TB control activities.
Thanks for attention