**2018 Tuberculosis course**

*Institut Pasteur/Nanshan CCDC*
*October 14-26*
*Shenzhen, China*

# Phenotypic and Molecular Tests for Diagnosis and Drug Susceptibility Testing

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# Bacteriological diagnosis of TB

1. Direct smear
2. Molecular resistance detection
3. Culture
4. Phenotypic drug susceptibility testing

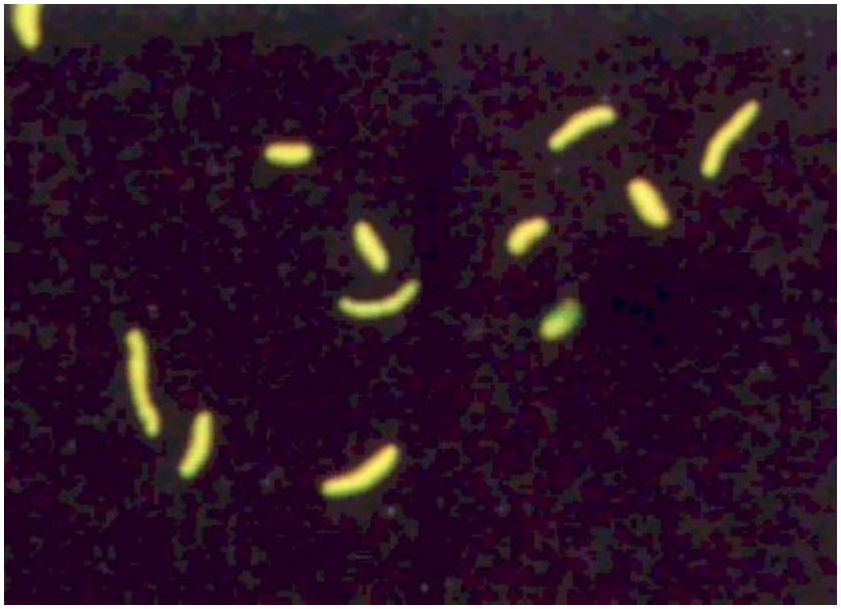
# Microscopy of stained smears

1. Auramine
2. (cold) Ziehl-Neelsen

*both based upon acido-alcohol resistance*

# Auramine staining

*Auramine - acid/alcohol – red thiazine*



## *Auramine Positive Acid Fast Bacilli*

Fluorescent microscopy

20X

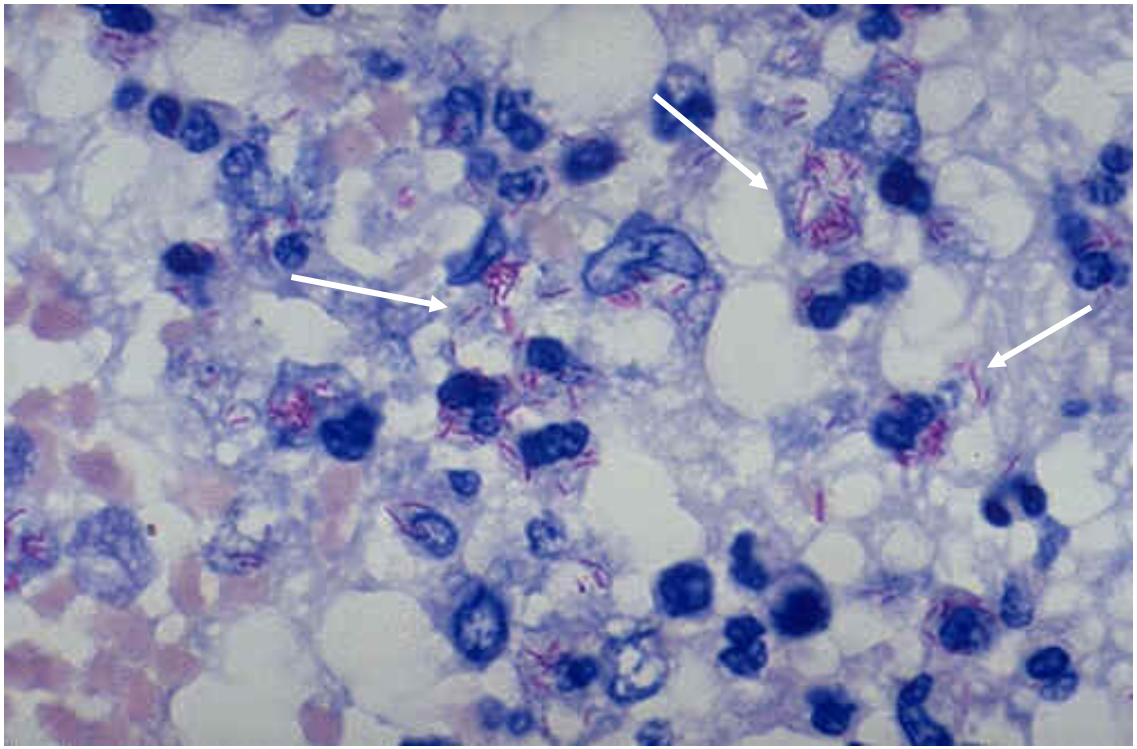
40X

- *suggests mycobacteria*
- *fast*
- *not specific → ZN*

# (cold) Ziehl-Neelsen staining

*(on the same slide)*

*Fushine - Acid/alcohol - Methylene blue*

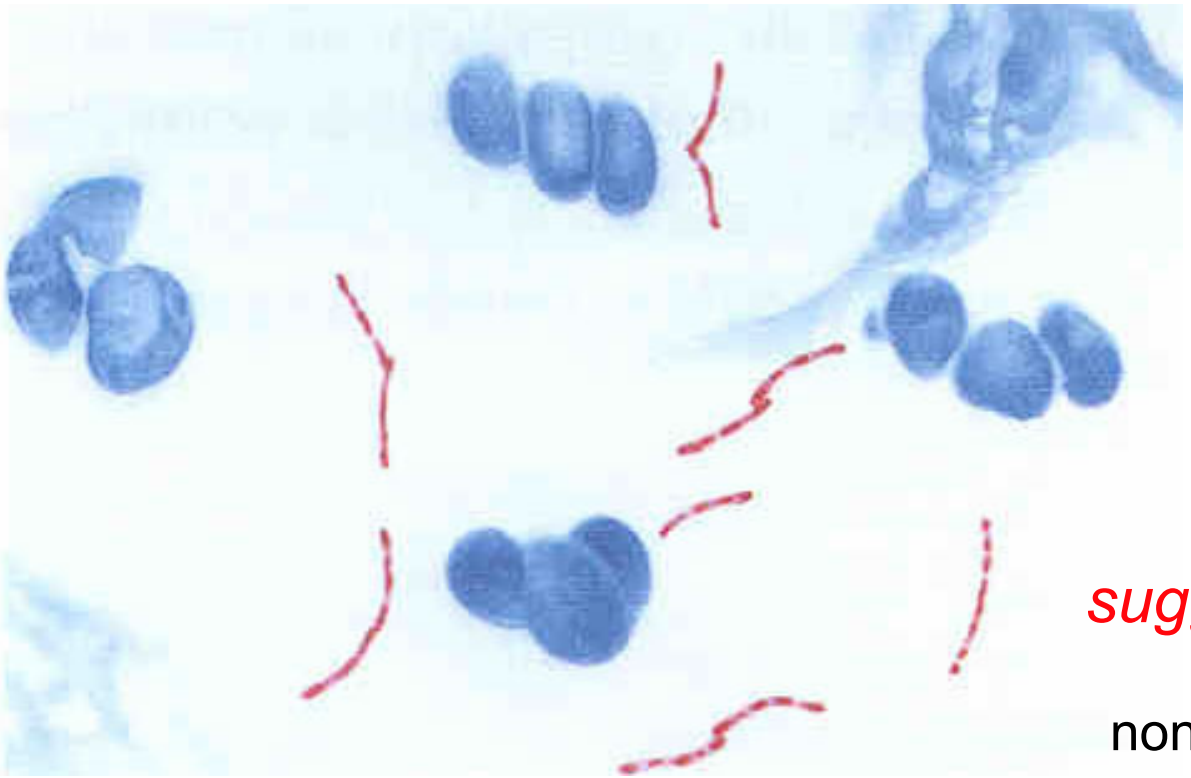


*ZN positive*
*Acid Fast Bacilli*

Optical immersion microscopy X 100

# A.F.B. positive

Ziehl-Neelsen Coloration Optical Microscope x100



*suggests tuberculosis*
but could be
non-tuberculous bacteria
actinomycetes,
rodococcus …

# Direct Smear Quantification

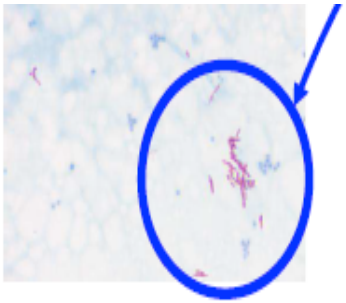
## WHO/The Union Codification

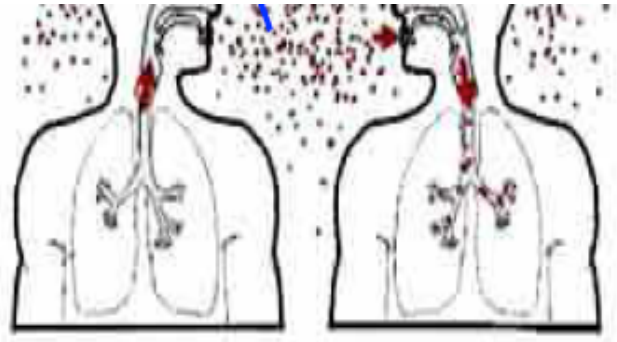
- Suspect 1-2 AFB in > 200 fields (Repeat DS)
- < 10 1 – 9 AFB in 100 fields
- + 10 – 99 AFB in 100 fields
- ++ 1 – 9 AFB / field
- +++ > 10 AFB / field

Estimation of the density

# Direct Smear

- Fast, cheap, can be repeated on 3 specimens
- Poor sensitivity: > 5 x$10^3$ bacilli / mL
- Negative AFB does not eliminate TB
- Is not specific
- Does not evaluate viability
- If pulmonary TB, patient is contagious → *notification*





# Biological Diagnosis of TB

Is it TB?

Or non tuberculous mycobacteria

→ *M tb complex detection using molecular tests*

# Biological Diagnosis of TB

Is it TB?

Is it an antibiotic resistant TB?

*Resistance is increasing*
*and*
*80% MDR cases are not diagnosed or treated*

# Drug Resistant Tuberculosis

- **MDR-TB** =

  Isoniazid-resistant and rifampicin-resistant

- **XDR-TB** = MDR

  + Fluoroquinolone-resistant
  + 1 second line injectable drug-resistant
  (amikacin, kanamycin or capreomycin)

# MDR molecular detection in clinical samples

*WHO endorsed assays*

- Rifampicin resistance *rpoB*

  Xpert® (Cepheid) MTB/RIF

  LPAs : GenoType® (Hain) MTBDR*plus*

  *95% of **RIF$^R$** are also INH$^R$ and therefore **MDR-TB***

- Isoniazid resistance *katG, inhA*

  LPAs: GenoType® (Hain) MTBDR*plus*

# Xpert® MTB/RIF (Cepheid)


Real Time PCR

*M. tuberculosis* complex DNA and

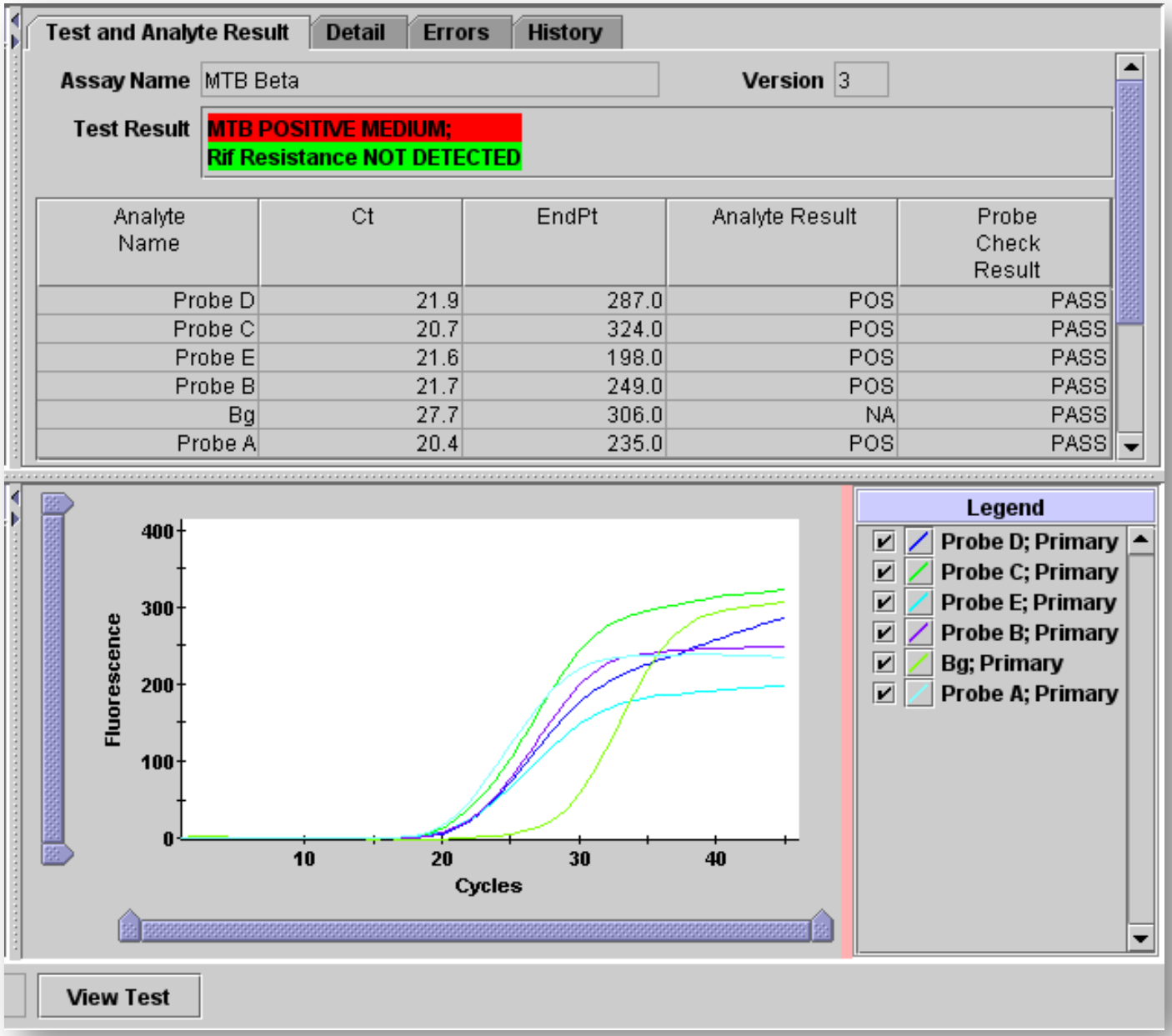
Rifampicin resistance detection

# Xpert® MTB/RIF

81 bp *rpoB* gene
Rifampicin resistance coding region


Hemi-nested PCR
5 probes bind to wt sequence
+ 1 amplification control probe

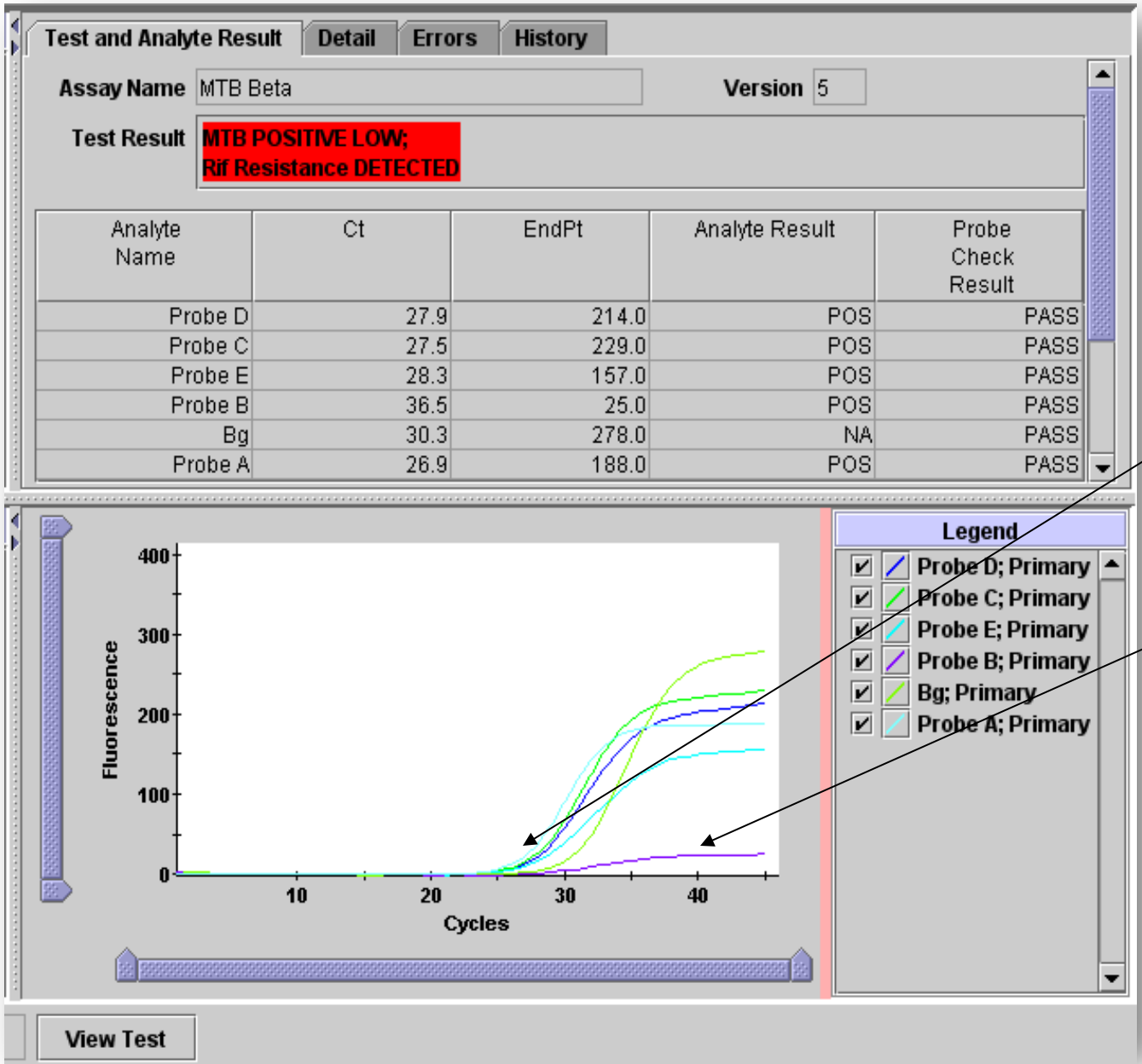
# Results window




DNA detection *M. tuberculosis* complex

No mutation associated with rifampicin resistance

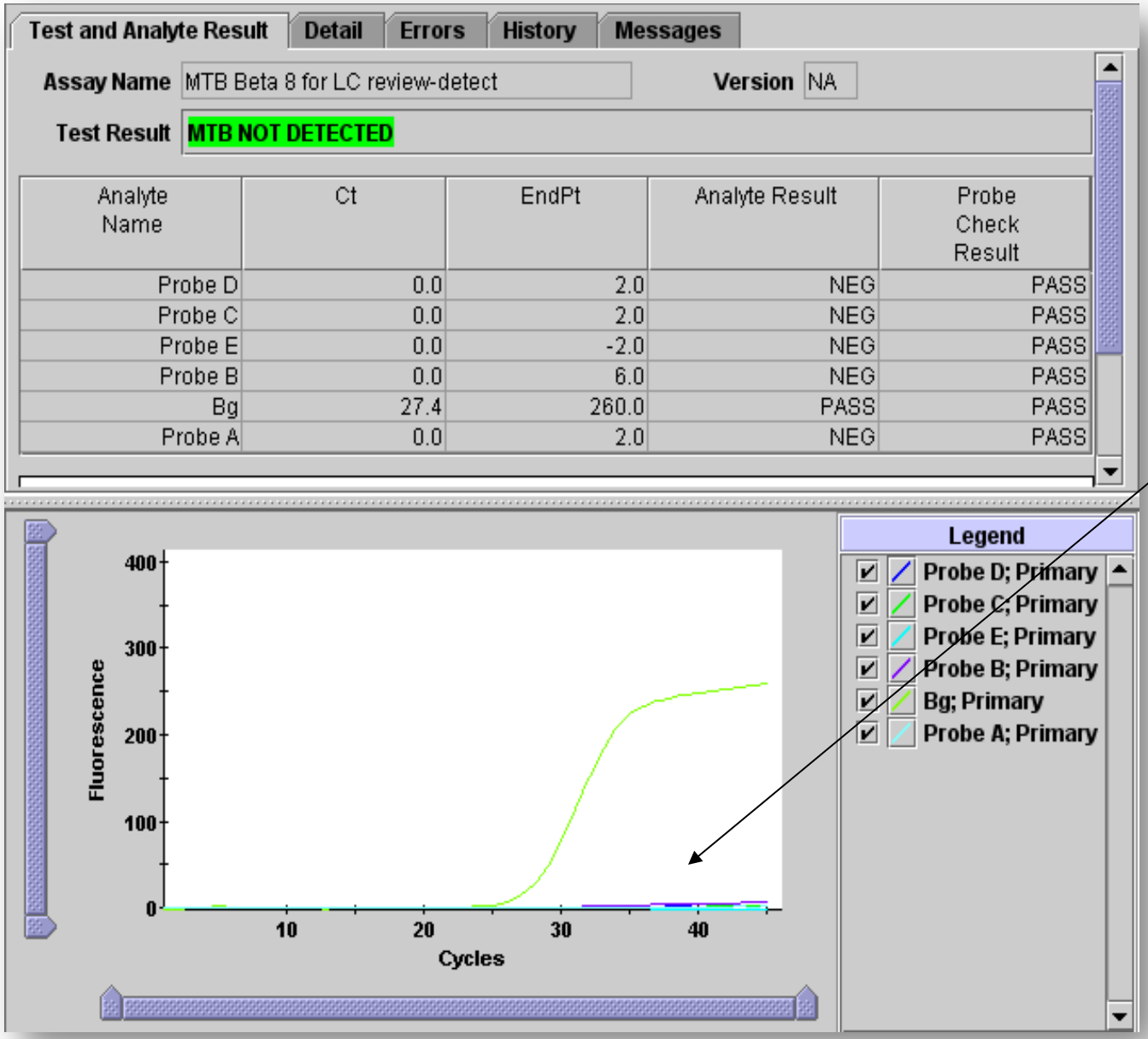
# Results window




DNA detection *M. tuberculosis* complex (small quantity)

Rifampicin Resistance associated mutation

# Results window




MTB not detected

# Xpert® MTB/RIF

*M. tuberculosis* detection in pulmonary specimens

131 bacilli per mL sputum

Sensitivity: AFB+ 98%
AFB- averaging 70%
Specificity: 98.3%

Rifampicin resistance detection

Sensitivity 96.7%
Specificity 98.6%

# GeneXpert® MTB/RIF

## Advantages

- Rapid
- Safe, easy to use closed cartridge
- Detection Mtb complex and RIF resistance
- High sensitivity/specificity

# GeneXpert® MTB/RIF

## Limitations

- Cartridge's shelf life
- Electricity, temperature, dust …

# GeneXpert® MTB/RIF

## Cepheid's OMNI

- More rugged
- Battery
- Withstand dust and heat
- Fewer training requirement

# GeneXpert® MTB/RIF

*Molecular limitations*

- Decreased capacity to detect *rpoB* C533G mutations
- Occasional false-positive RIF-resistance
  - paucibacillary samples
  - *rpoB* silent mutations (Q513Q, F514F)

# GeneXpert® MTB/RIF

## ULTRA


- Larger chamber for DNA amplification
- 2 additional targets to detect TB (IS*6110* and IS*1081*)
- Melting curve technology

→ Increased sensitivity only for bacilli detection

We expect better sensitivity for children, HIV, extra-pulmonary specimens

→ The limit could be a reduced specificity

# MDR detection

## Antibiotic Resistance sequencing

Major target genes:

*rpoB* Rifampicin

*katG* Isoniazid

*inhA* Isoniazid

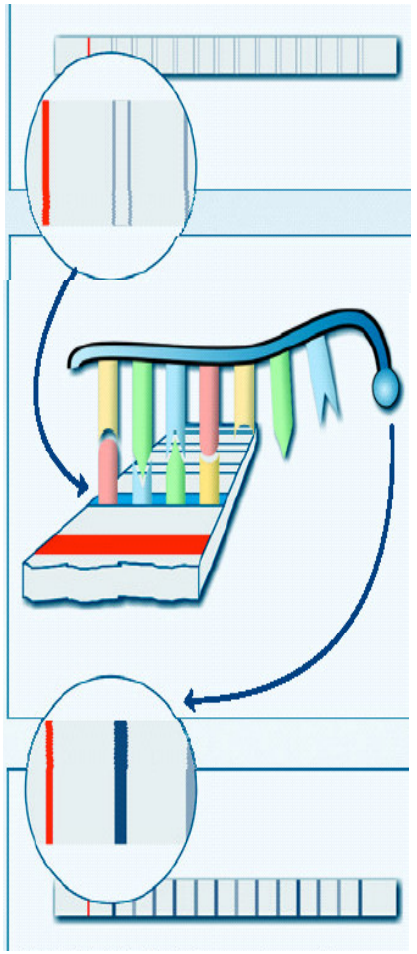
## LIPAs

GenoType® MTBDR*plus* (Hain Lifescience)

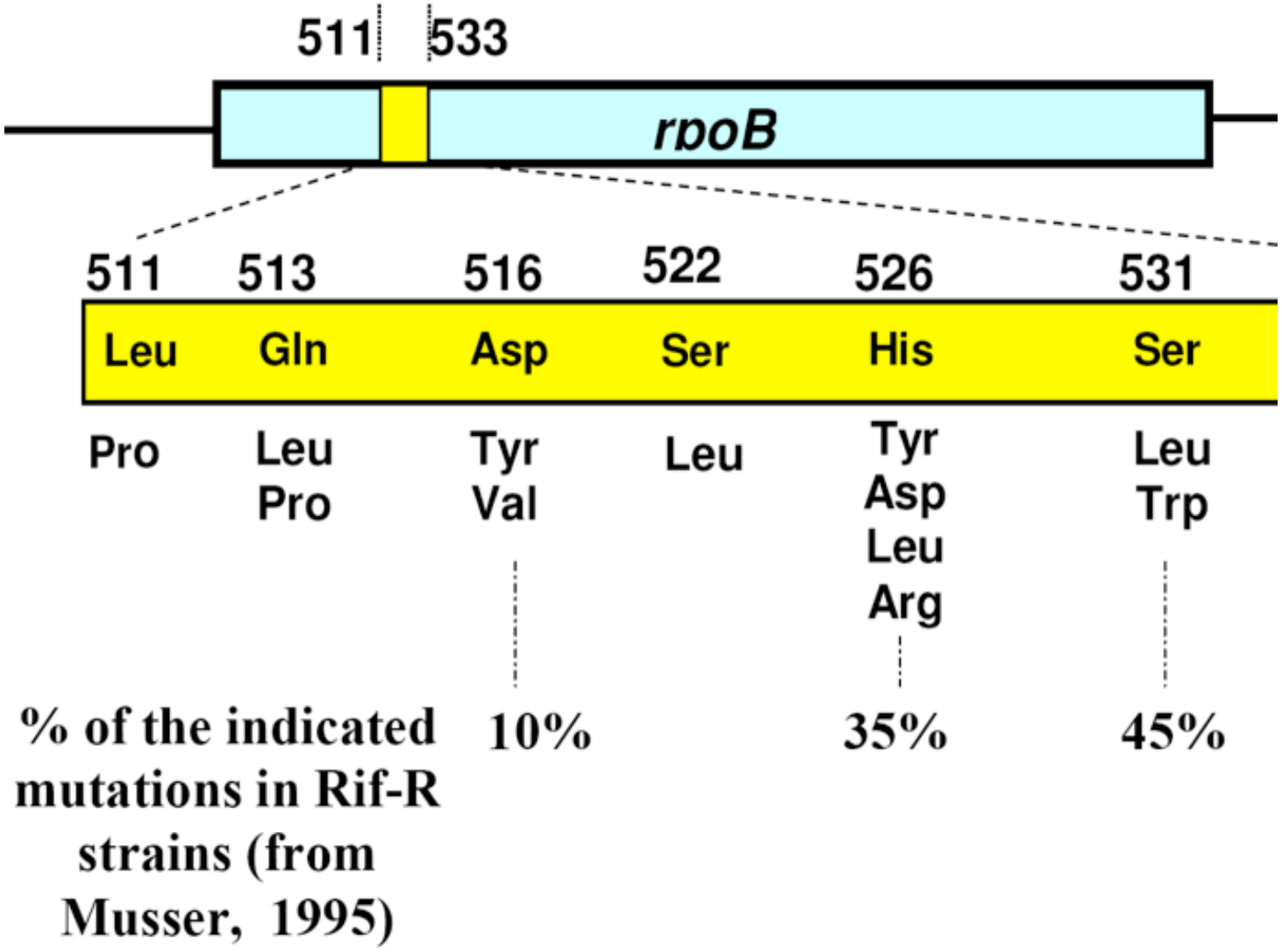
# Do the patient have MDR TB ?

## HAIN GenoType® MTBDR*plus*

Line Probe Assay

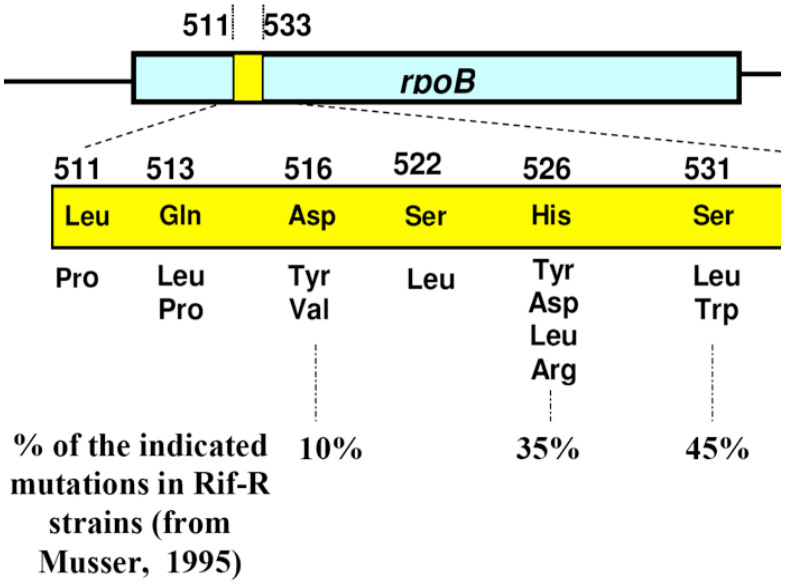



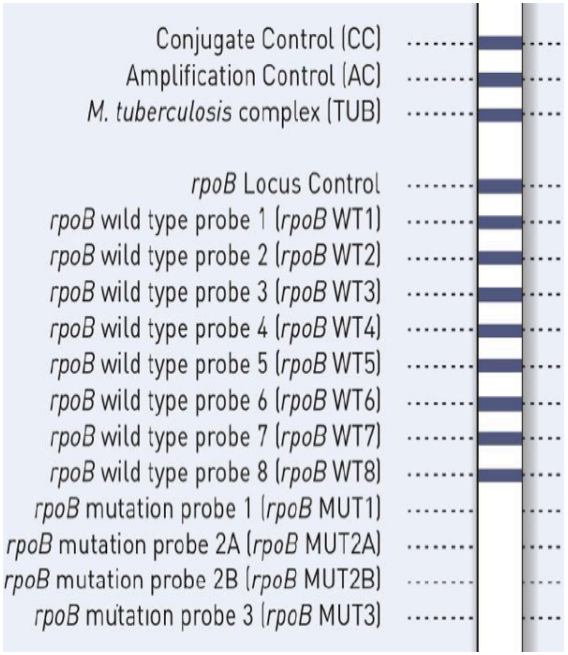
# Mutations associated with RMP resistance

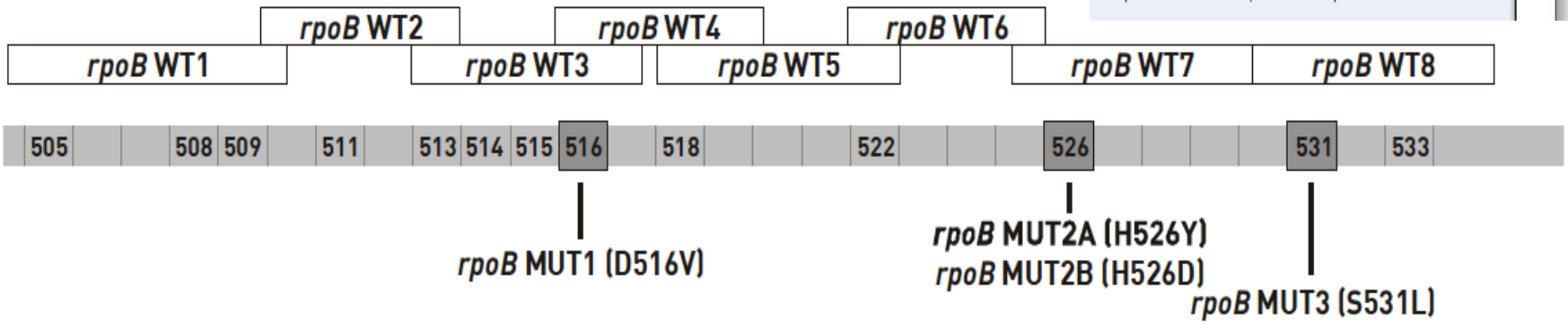


# MDR detection

## HAIN GenoType® MTBDR*plus* to **confirm RIF-R**

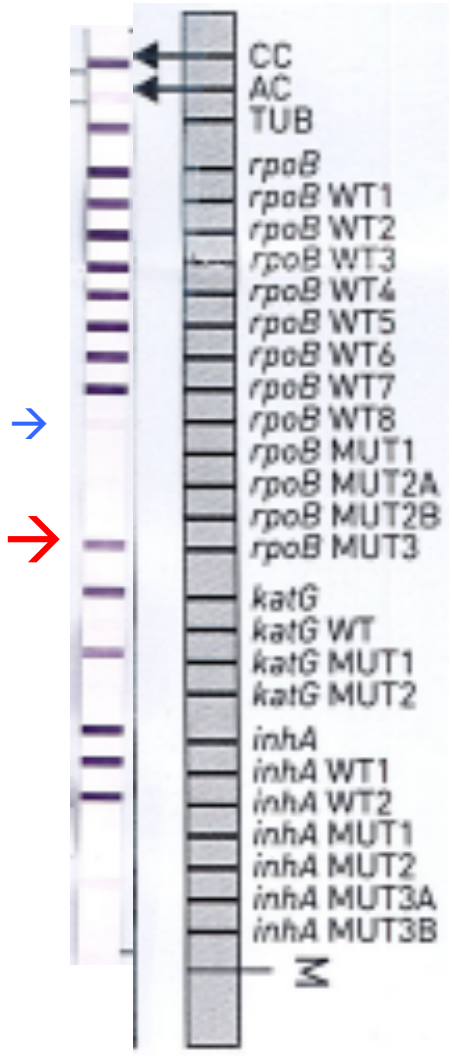


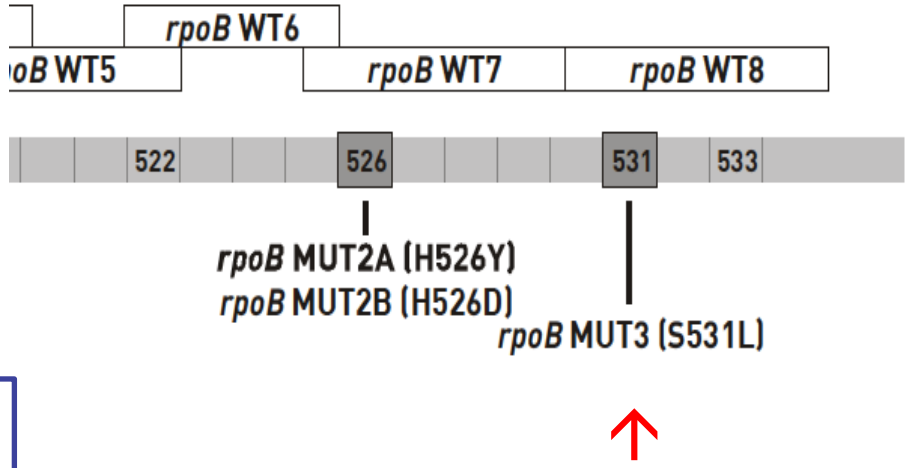



# MDR detection?

HAIN GenoType® MTBDR*plus*



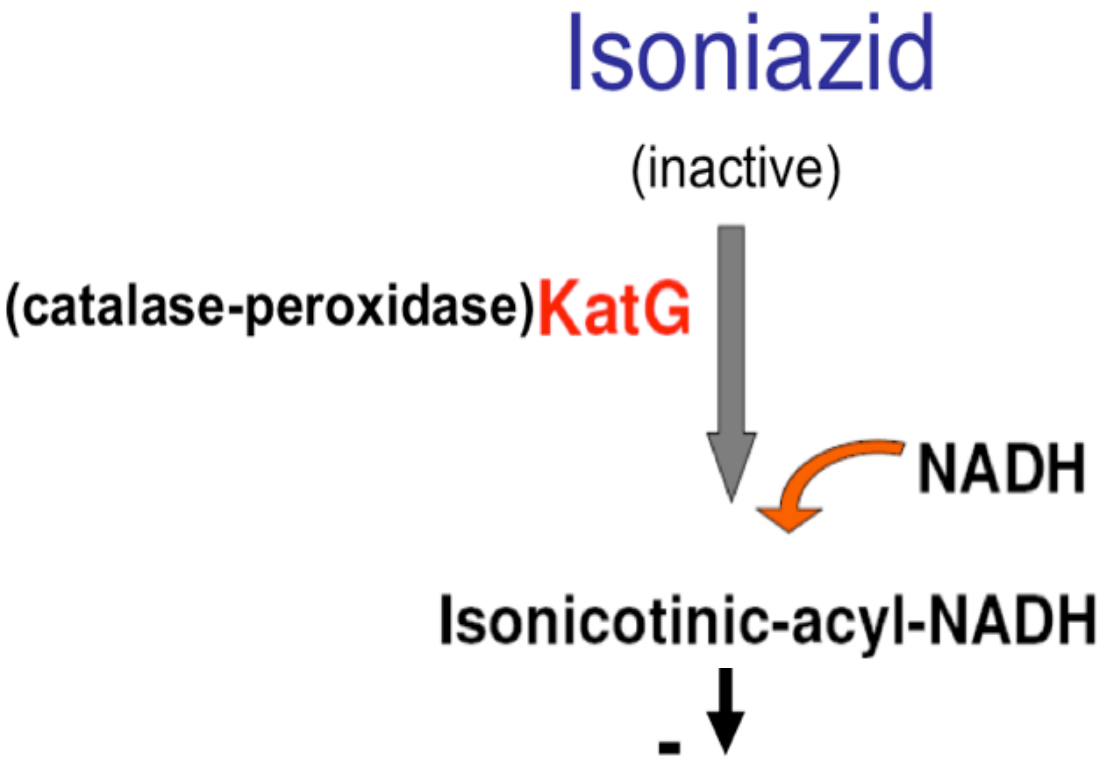




**RIF $^{R}$**

***Rifampicin resistance is confirmed***

# MDR detection

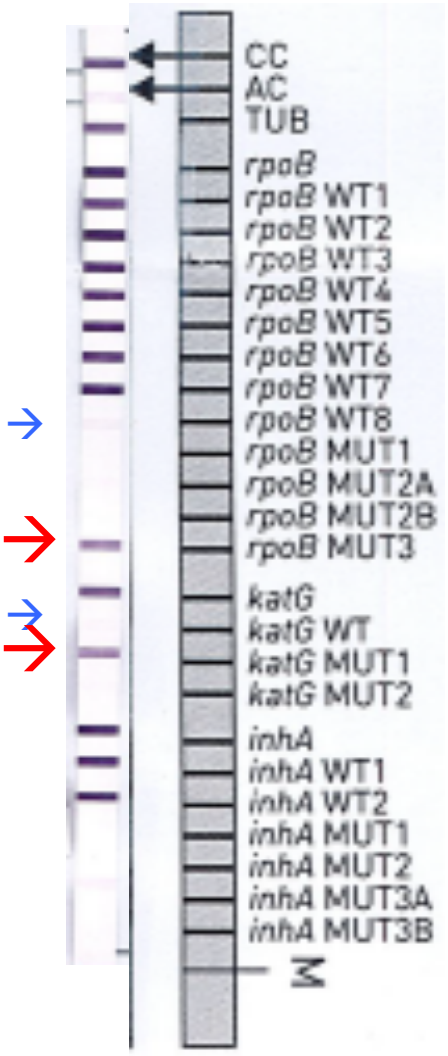
HAIN GenoType® MTBDR*plus* to **detect INH-R**




**InhA (enoyl ACP reductase)**
**(mycolic acid synthesis)**

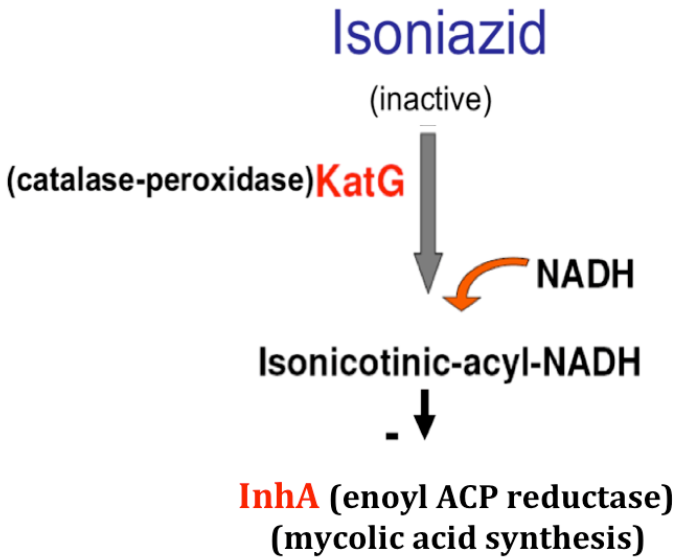
# MDR detection

## HAIN GenoType® MTBDR*plus* to detect INH-R




**RIF$^R$**

**INH$^R$**




**= MDR - TB**

# GenoType® MTBDR*plus*

## RMP and INH resistance detection

## compared to culture and clinical data

| Drug and smear status[a] | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|
| RMP | | | | |
| Pos | 98.1 | 96.0 | 98.1 | 96.0 |
| Neg | 90.7 | 96.0 | 98.0 | 82.7 |
| INH | | | | |
| Pos | 89.3 | 94.7 | 98.2 | 94.7 |
| Neg | 93.5 | 82.3 | 95.1 | 77.7 |

[a] Pos, positive; Neg, negative.

Crudu, 2012

# The patient has MDR TB

*Can we give MDR treatment ?*

*What about second line drugs susceptibility ?*

Does the patient have **X**DR TB ?

# Molecular XDR detection

Major targets genes for antibiotic resistance sequencing:

| | |
|---|---|
| *gyrA, gyrB* | Fluoroquinolones |
| *rrs, eis* | Amikacin/kanamycin/capreomycin |
| *pncA* | Pyrazinamide |

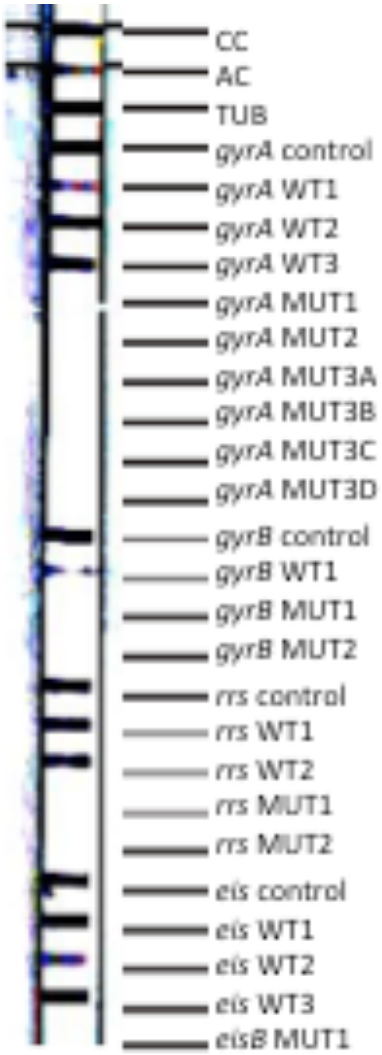
## Line Probe Assays

GenoType® MTBDR*sl* (Hain Lifescience)

# Does the patient have XDR TB ?

*MDR patient A*

HAIN GenoType® MTBDR*sl*




*gyrA* → Fluoroquinolones

***FQ$^{S}$***

*gyrB* → Fluoroquinolones

*rrs* → KAN/AMK/CAP/VIO

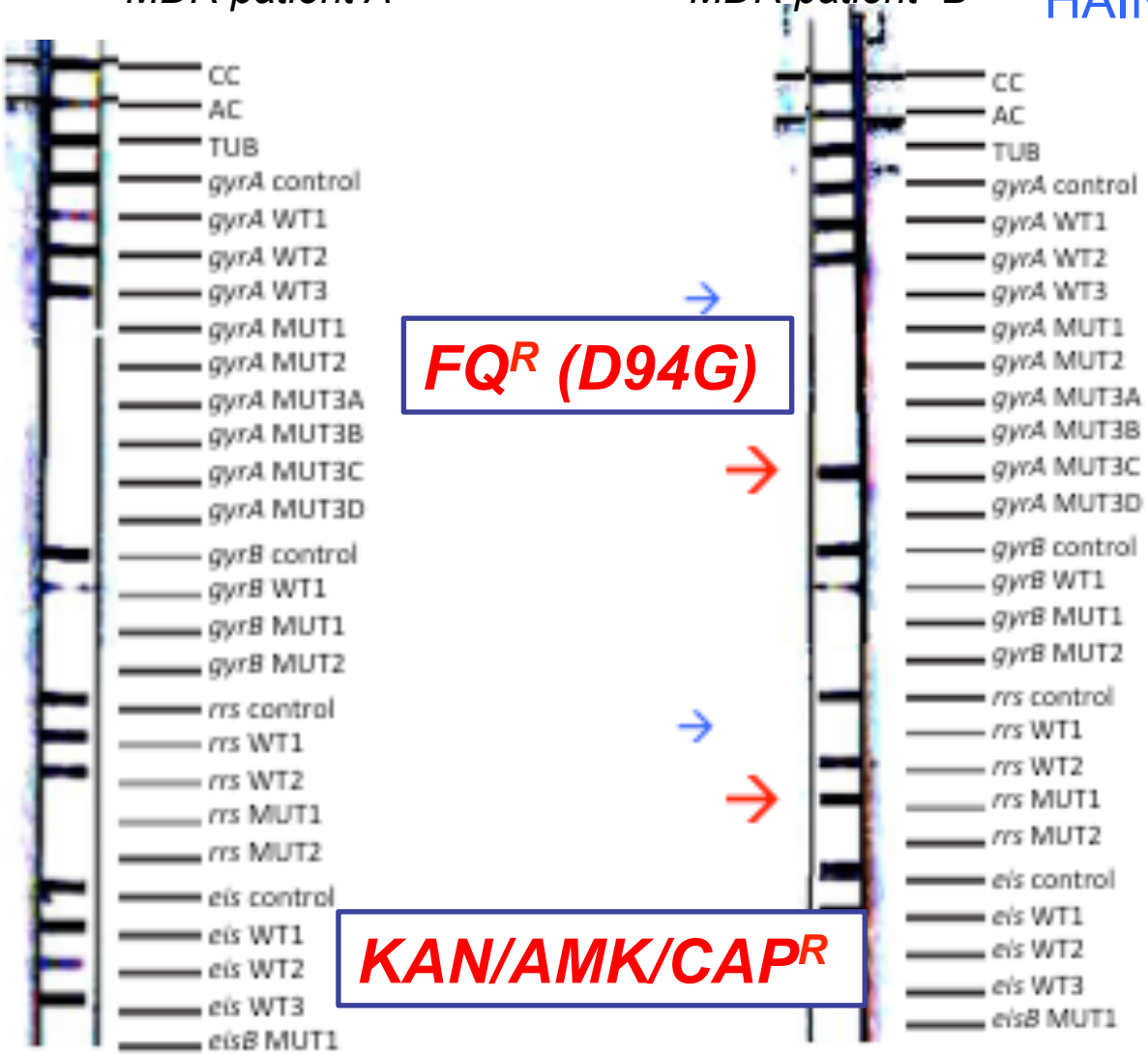
*eis* → KAN

***KAN/AMK/CAP$^{S}$***

*Patient A does not have XDR TB*

***9 months MDR treatment***

# Does patient B have XDR TB ?




*Patient B has*
***XDR** TB*

*Treatment ?*

# Next molecular diagnosis assays

## Xpert SL® XDR (Cepheid)

Isoniazid (*katG*, *inhA* promoter)
Fluoroquinolones (*gyrA, gyrB*)
Amikacin, Kanamycin (*rrs, eis* promoter)

## NGS (Illumina, Genoscreen)

# Limitations of molecular tests

- cannot distinguish live bacilli
- Extracted DNA (quality, quantity)
- Outside target zone mutations
- Mutations in sensitive isolates
- Other resistance mechanism

→ *Culture*

→ *Phenotypic drug susceptibility testing*

# Culture

- Viability of the mycobacteria
- Molecular tests
- Phenotypic drug susceptibility testing

# Culture in L3

- Negative pressure
- Double door intrance
- Safety hood
- Protective mask


*secure*
*expensive*

# Work with a *protective* mask *(FFP2)*

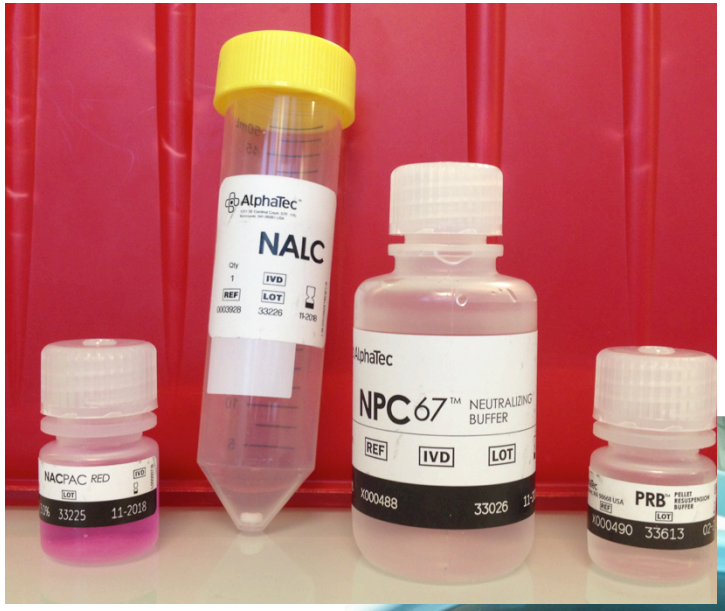
0

++

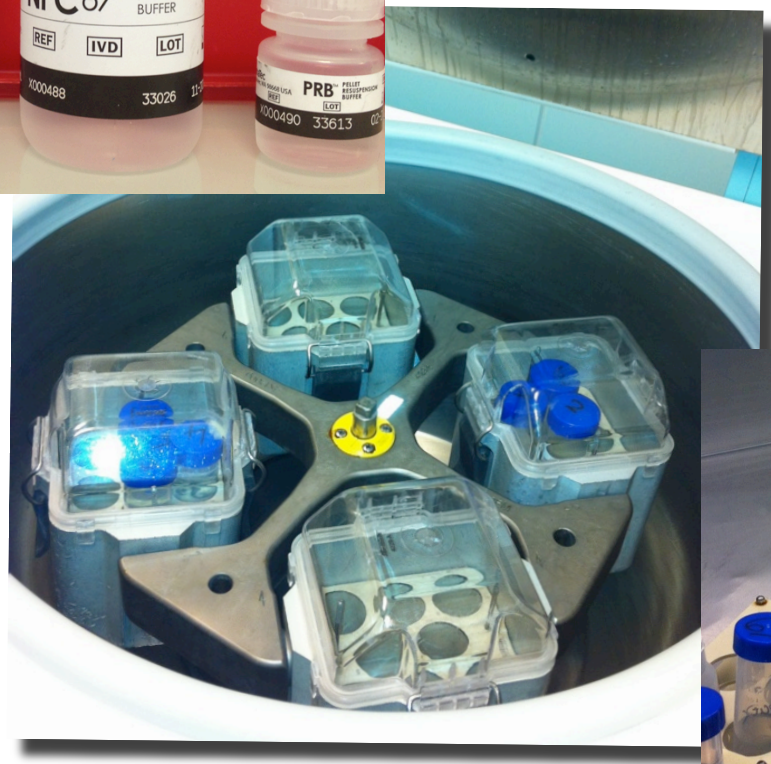
+++

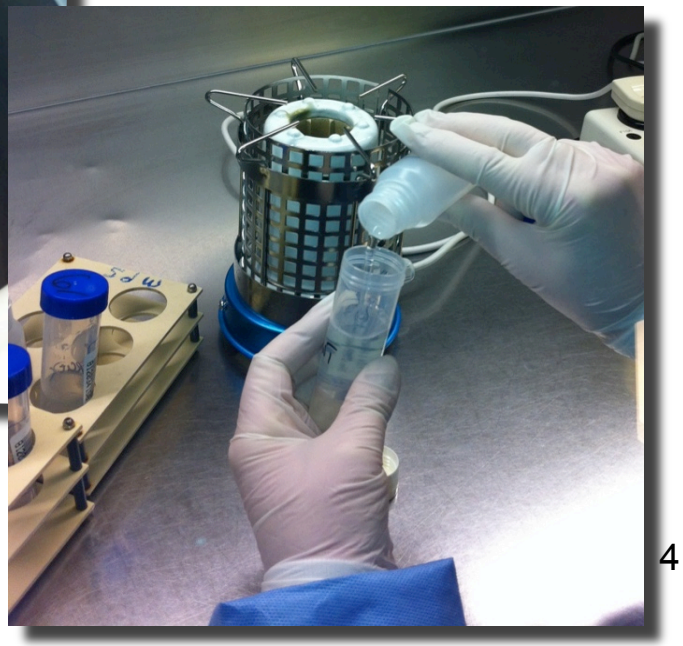
# Samples Decontamination

## Classic Petroff method



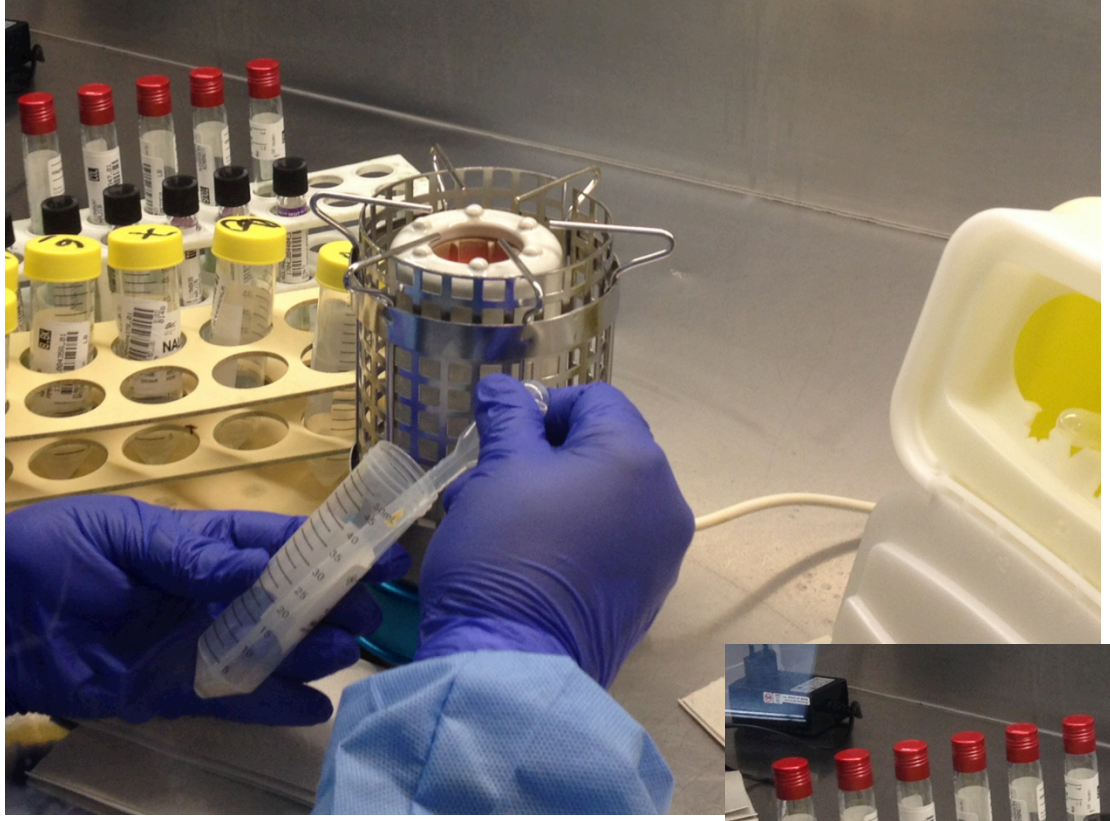

**NaOH 4% -** NALC
Centrifugation
Neutralization
Pellet resuspending buffer

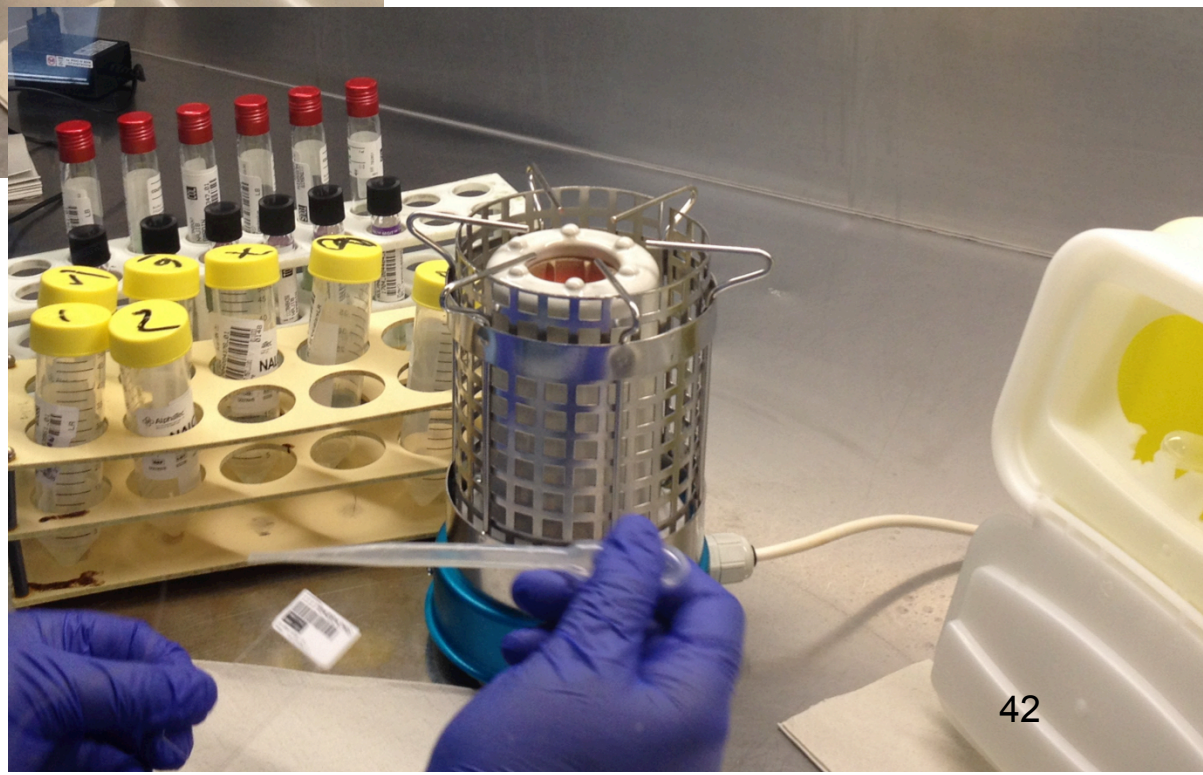




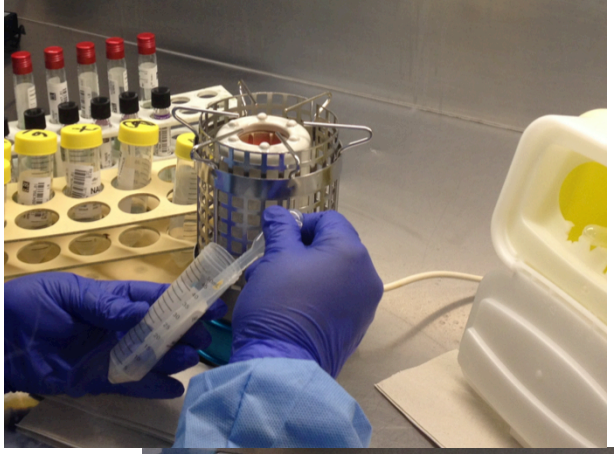
NAC-PAC®AlphaTec

# Direct smear

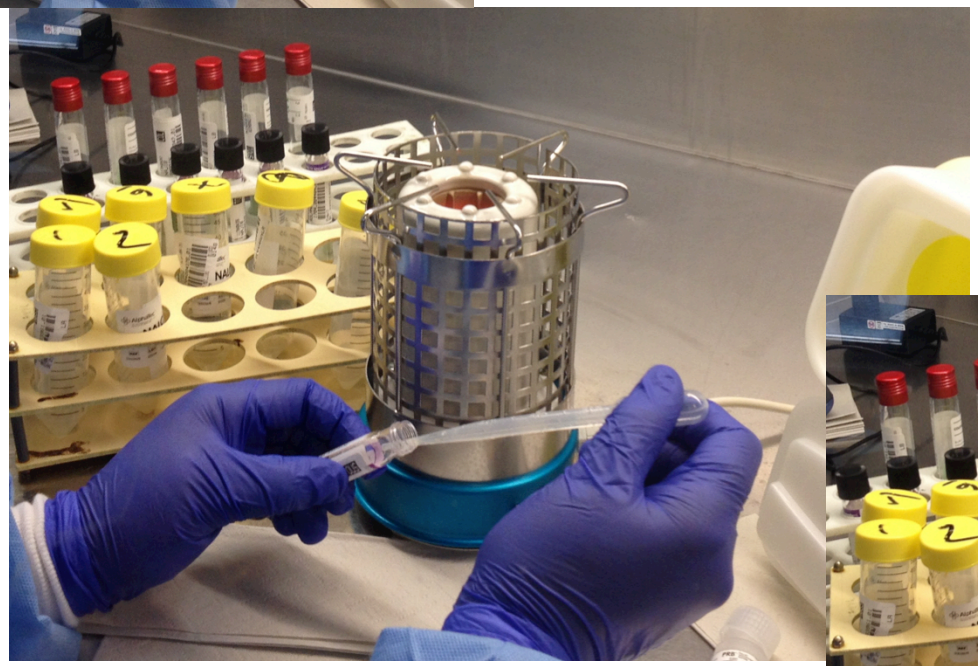




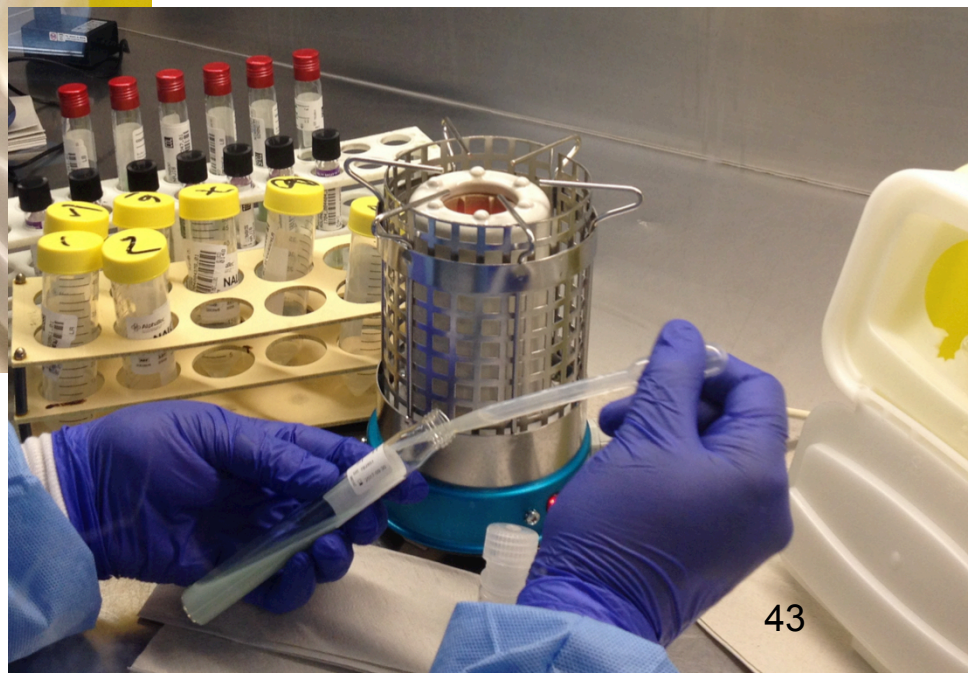
# Inoculation



**MGIT**



**LJ**



# Solid Medium Culture

- Löwenstein-Jensen or Coletsos
- Long time to positivity : 21 days
- Cultures reading 3 months

# LJ medium at 37°C

# Liquid Medium Culture

## MGIT 960

- Liquid medium 7 mL
- Nutritional Supplement
- Antibiotics

(Polymixine B, Azlocyline, Nalidixic acid, Trimetroprime, Amphothericin B)

# Liquid Medium Culture

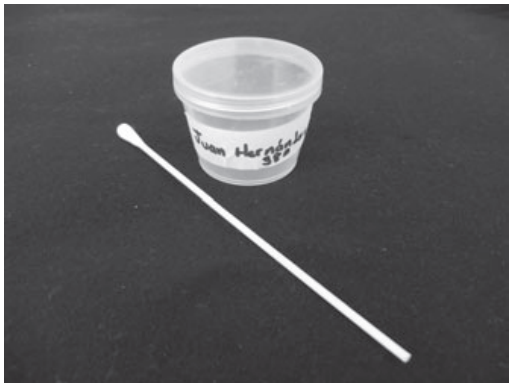
**MGIT 960**

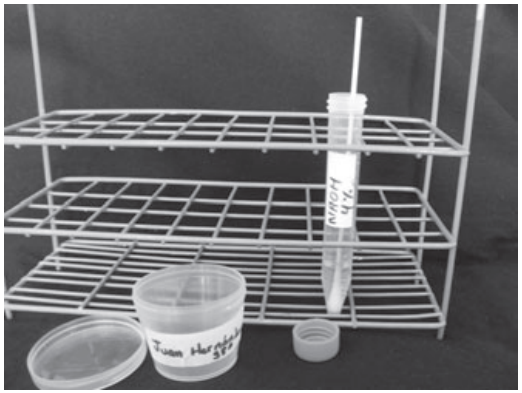
- Incubate at 37°C
- During 56 days
- Consumption of oxygen detection
- Automatic reading every hour

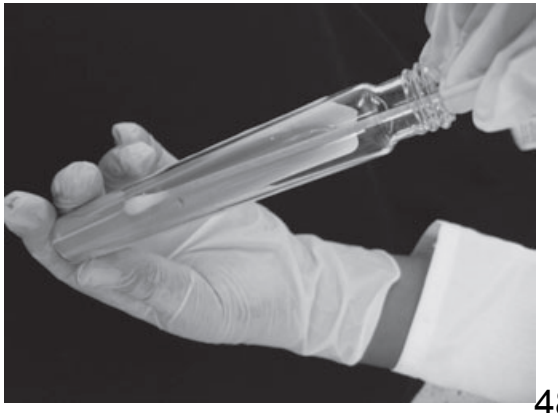
# Kudoh method

Sodium hydroxide, 4%

Ogawa modified medium pH 6.4







***No centrifugation***

# Positive culture

## Löwenstein Jensen Medium

## Ogawa (Kudoh method)

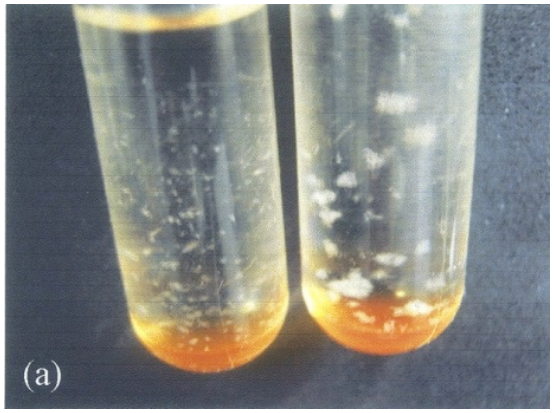
*M. tuberculosis*

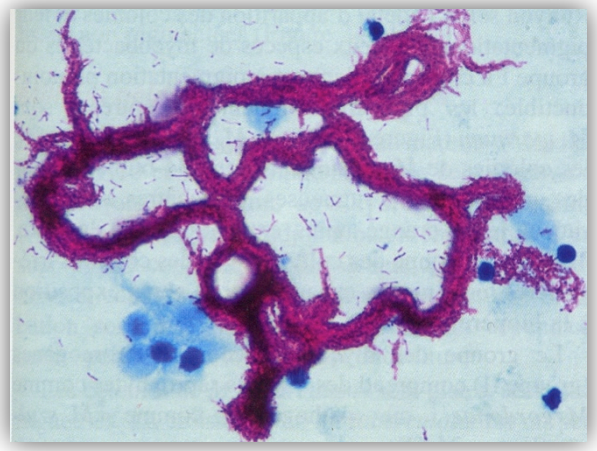
Rough colonies
**in 21 days**

# Solid Medium Culture

- Allows colony counts
- The aspect of colonies and the speed of growth gives clues as to the identification
- *Several weeks delay* …

# Liquid Medium Culture MGIT



Faster, more sensitive

BD Bactec™ MGIT™ 960

# Positive culture identification

Is it TB? TB complex detection

- Previous biochemical tests (niacine, nitrate reductase, catalase)
- Previous specific molecular probes (Geneprobe)
- Rapid Immunochromatographic Assay
  (ex.SD Bioline's TB Ag MPT64 Rapid Test)
- Molecular tests: GeneXpert® MTB/RIF or Line Probe Assays

Is it an antibiotic resistant TB? MDR/XDR detection

- Molecular tests:
  GeneXpert® MTB/RIF, Line probe assays, target genes sequencing
- Phenotypic drug susceptibility testing

# Phenotypic susceptibility testing

First, second and third line antibiotics

- Classic proportions method LJ medium
- Faster liquid medium MGIT

# Liquid Medium Phenotypic Susceptibility Testing

## BD Bactec™ MGIT™ 960

| Antibiotic | Low concentration (mg/L) | High concentration (mg/L) |
|---|---|---|
| STR | 1.0 | 4.0 |
| INH | 0.1 | 0.4 |
| RIF | 1.0 | - |
| EMB | 5.0 | 7.5 |
| PZA | 100 | |
| FQ, AMK … | | |

# Liquid Medium
# Phenotypic Susceptibility Testing

## BD Bactec™ MGIT™ 960

# Phenotypic Susceptibility Testing: Reference Proportions Method

- 1961 by Canetti, Rist and Grosset
- Numeration of the surviving colonies by comparison to the tube without antibiotics
- Sensitive strain : <1% survivor
- Reading of results starting at 21 days
- Second reading 15 days later

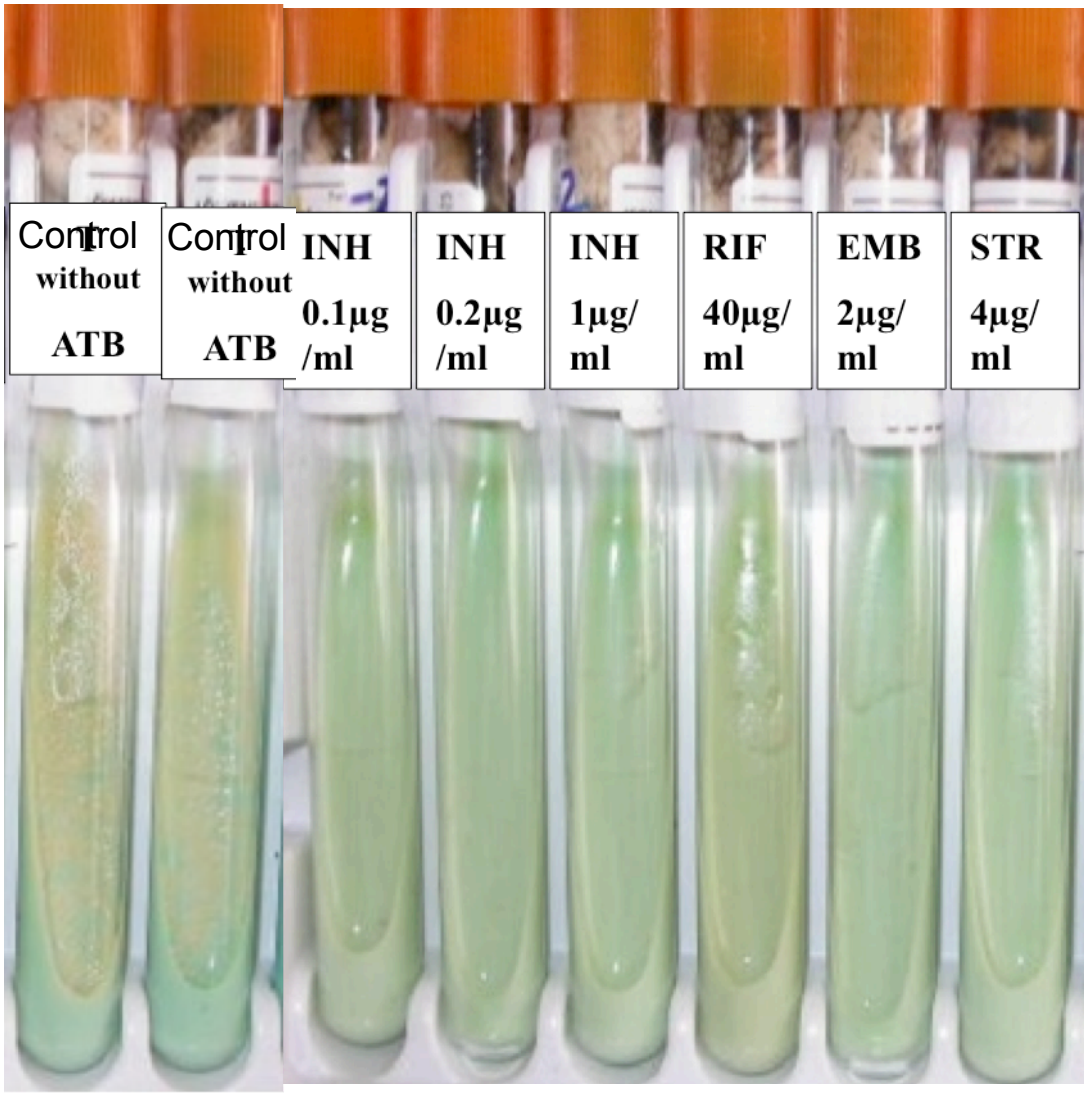
# Solid Medium
# Phenotypic Susceptibility Testing

## Proportions method

*Löwenstein-Jensen medium filled with antibiotics in various concentrations :*

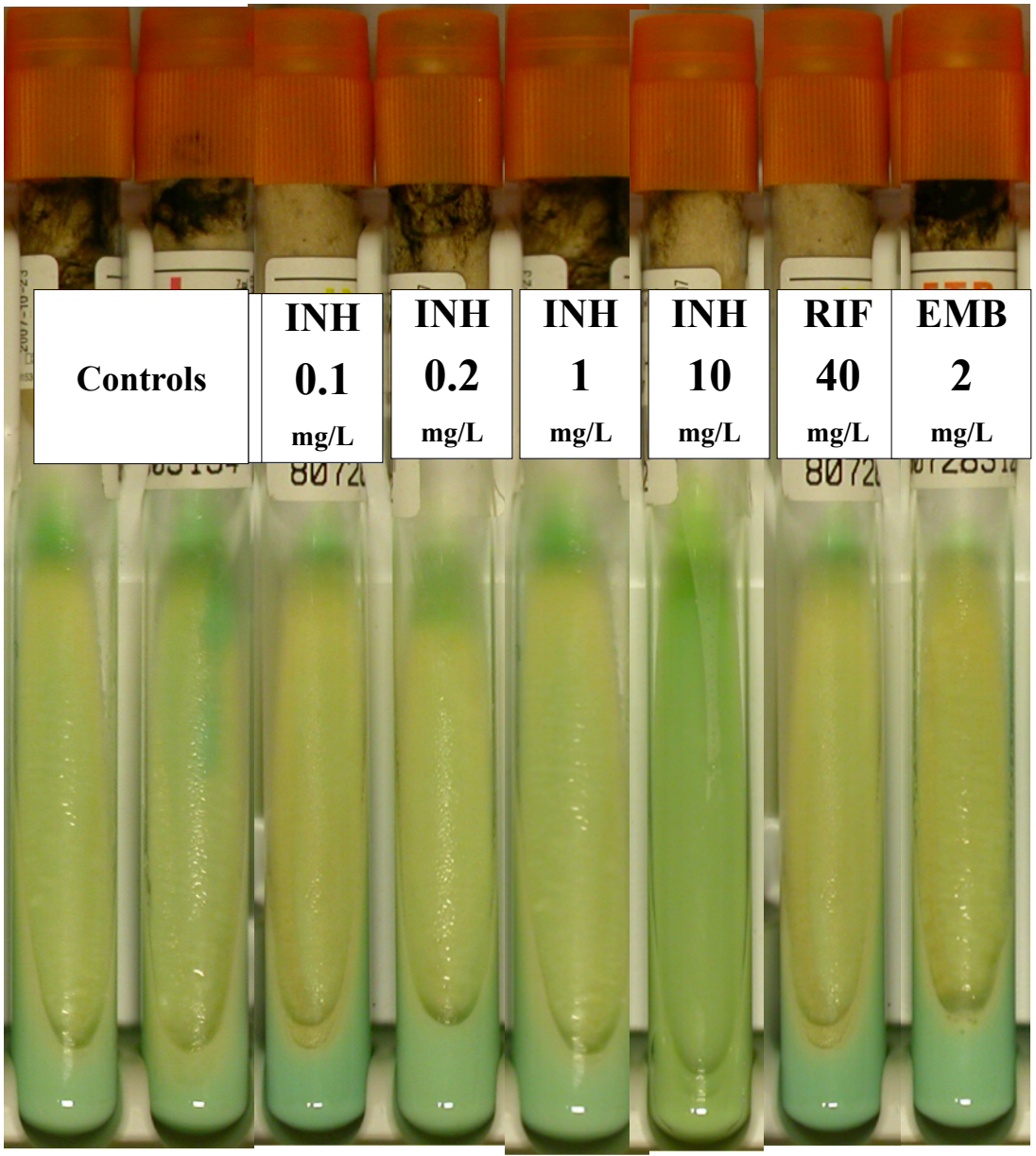
- Isoniazid (0.1, 0.2, 1 and 10 mg/L)
- Rifamycine (40 mg/L)
- Ethambutol (2 mg/L)
- Streptomycine (4 mg/L)
- Fluoroquinolones, amikacine, …

# Phenotypic Susceptibility Testing Proportions Method

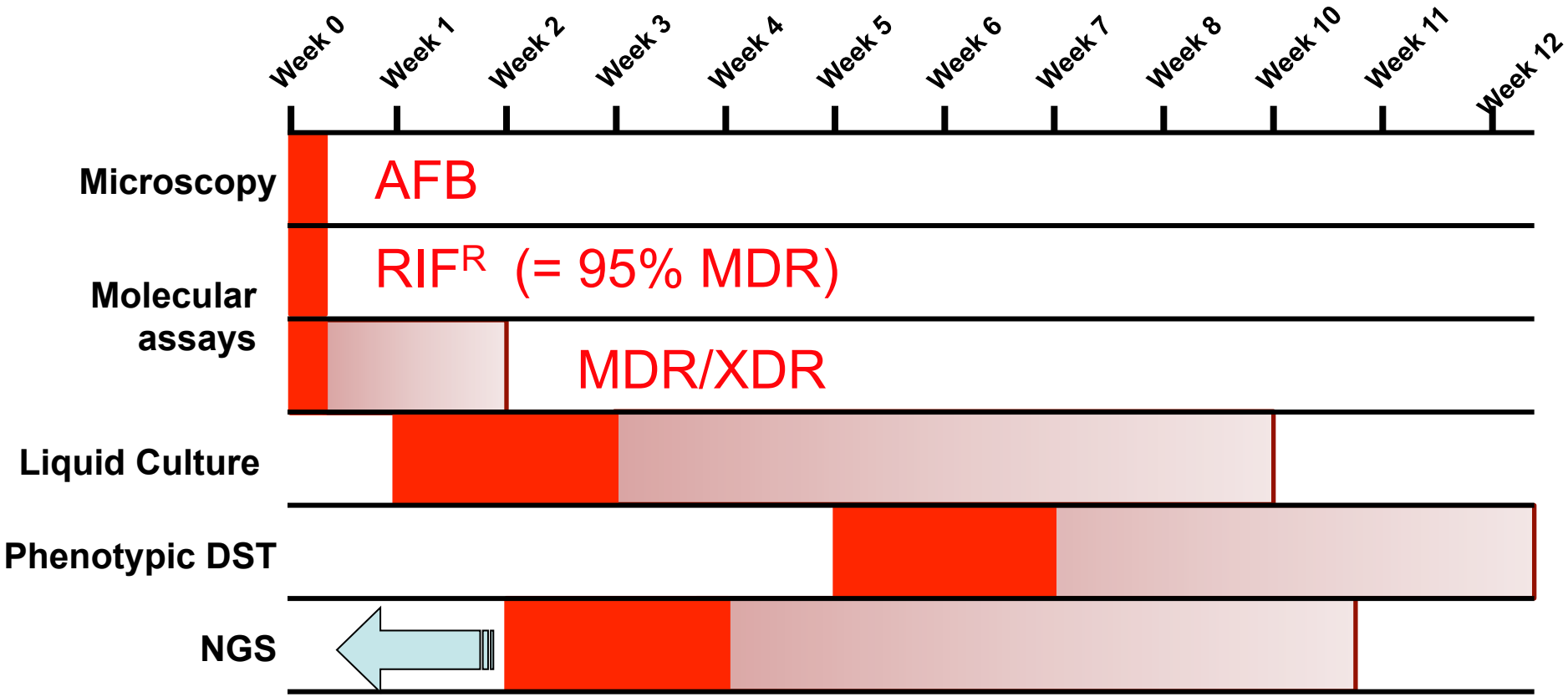



Sensitive strain

# MDR (rpoB S531L, INH$^{R}$ *katG* S315T) EMB$^{R}$



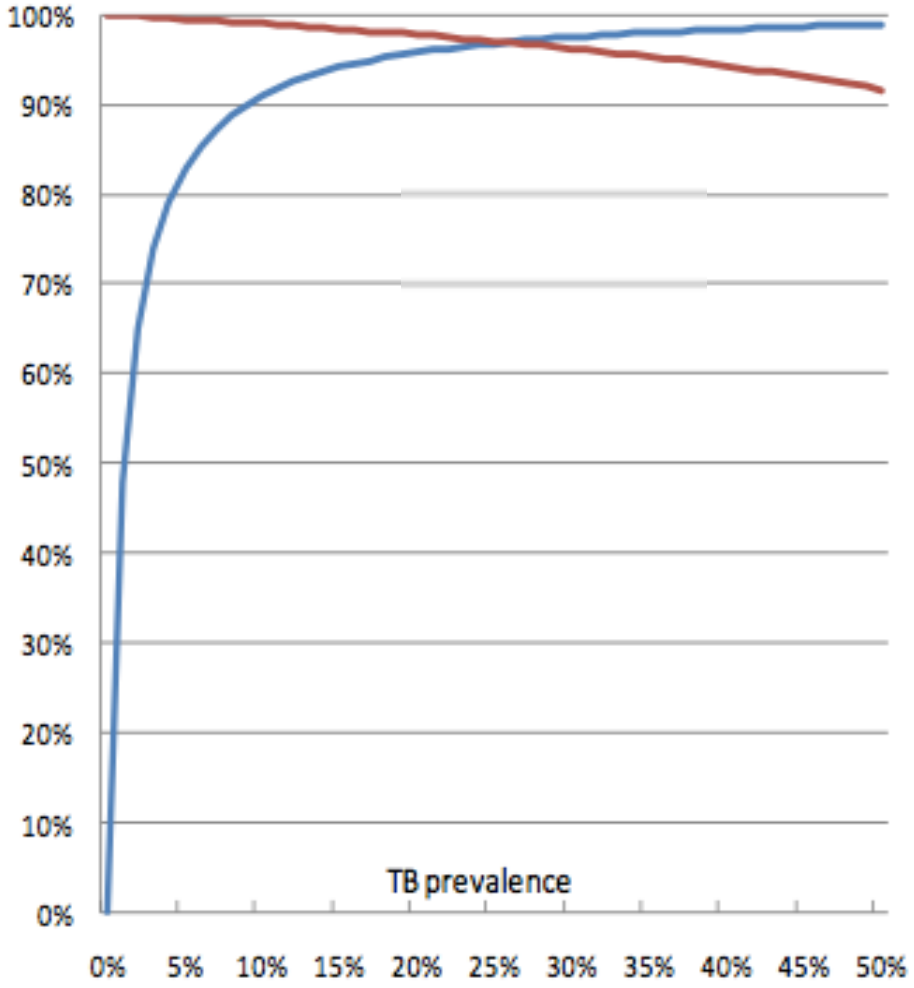
# Timelines for diagnostic testing



谢谢

*Further slides*
*are for potential questions*

# Xpert MTB/RIF



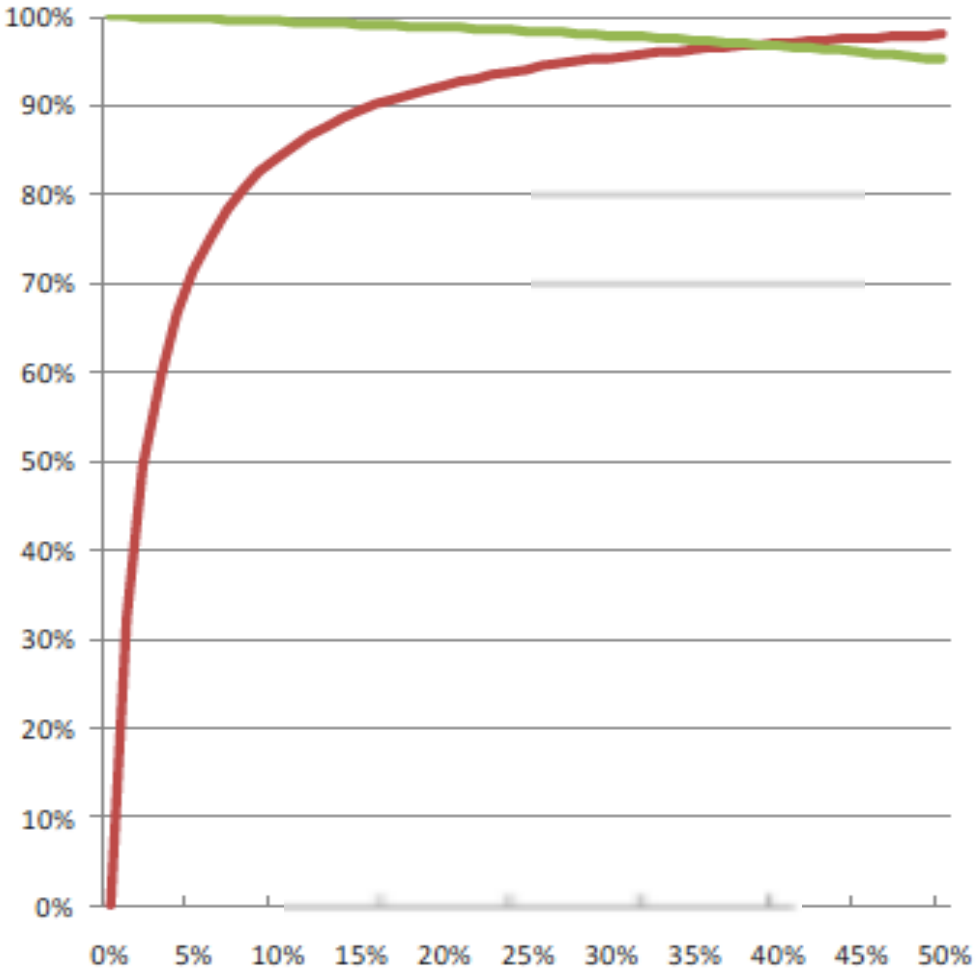

PPV

NPV

TB prevalence

In Rapid implementation of the Xpert MTB/RIF diagnostic test, OMS 2011

# Xpert MTB/RIF

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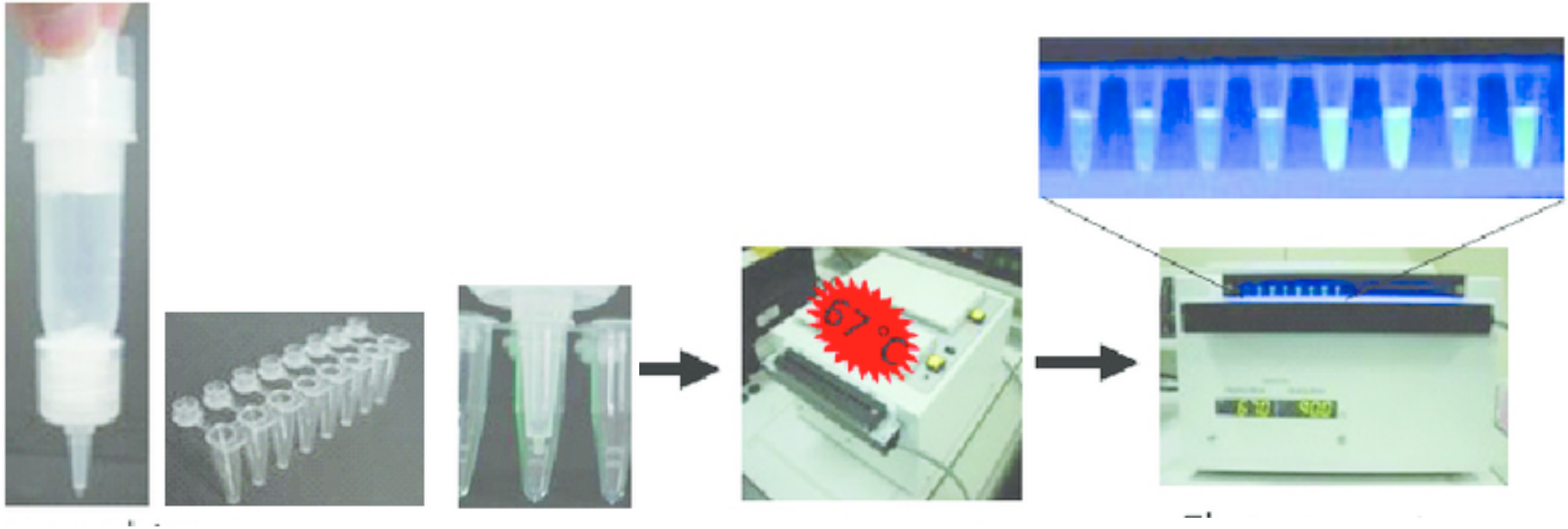
# TB LAMP test

*Loop-mediated isothermal amplification*




Prepare lysate

Dried lamp reagents

Add 30 µL mix

40 min / 67°C

Fluorescent signal detection

< 1 hour to detect MCTB (urines in HIV patients)

No sophisticated instrument

Training, electricity, temperature < 30°C