



Training on New and rapid Tuberculosis diagnostics (first and second line Probe Assay)

Module 5: Molecular Diagnosis of
TB/MDR/XDR-
Bacteria using
TB and Drug resistant
Line probe assay (Hain
Test)

**Uganda Supranational
Reference Laboratory**

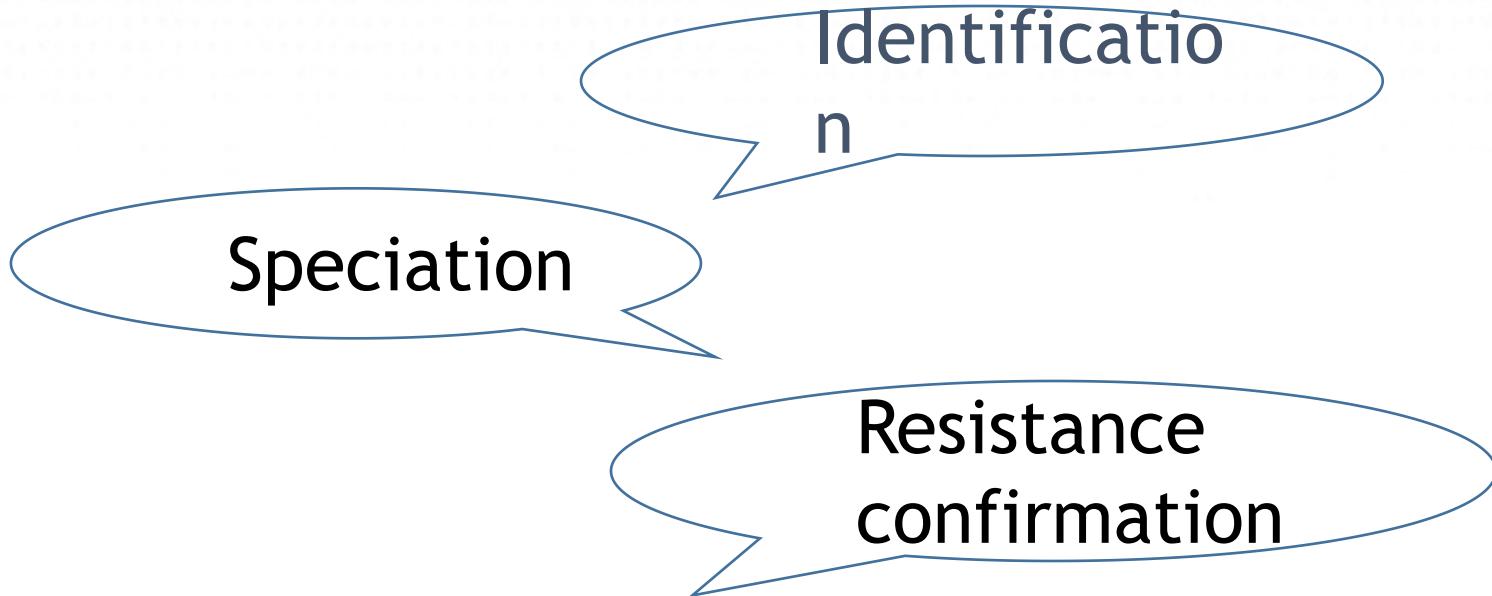
Content outline

- Introduction
- Mycobacterium Diagnosis
- Line probe assay (LPA) technology - Basic Principles
- Results interpretation and evaluation
- XDR-TB diagnosis - New assay
- Benefits of LPA
- Overview of the FluoroType system
- Additional Hain Tests



Introduction

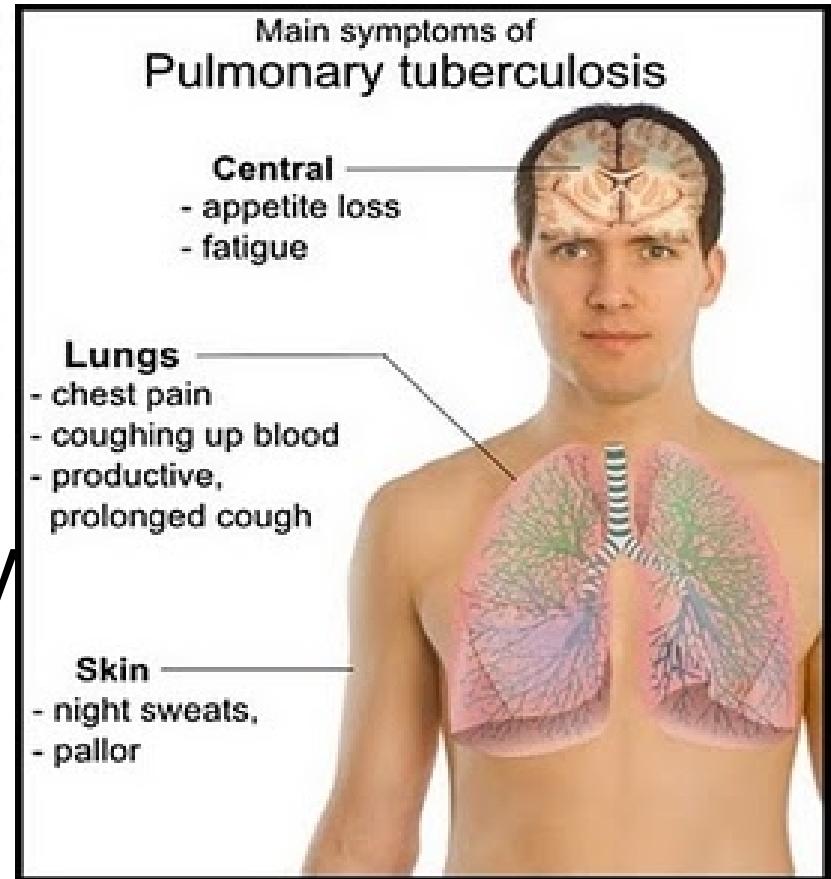
- Hain Lifescience has the world's widest range of molecular tests for Mycobacteria



Mycobacterium tuberculosis

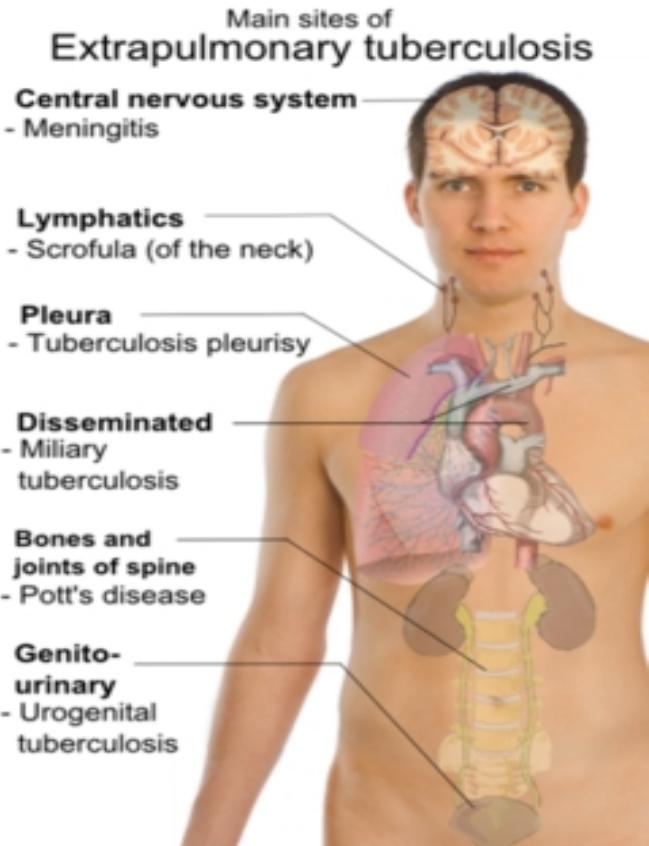
Pulmonary TB

- Most common type of TB
- Infects the respiratory system in humans



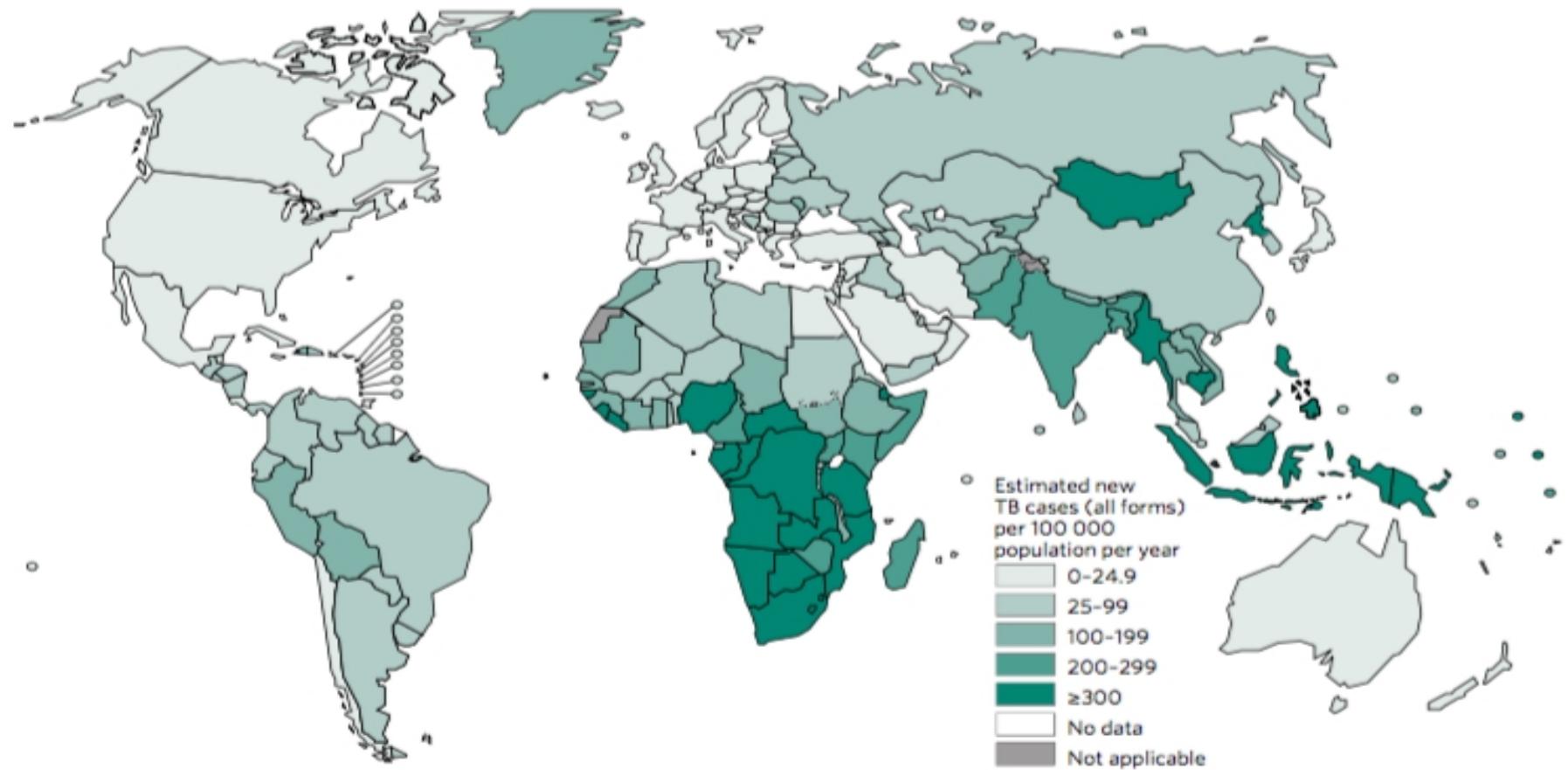
Highly infectious

- Occurs in 15 -20% of active Cases
- Most common in immuno-suppressed Individuals and children
- Major areas are, Joints, bones, Lymphatic system etc



Epidemiology of TB – WHO Report 2016

Estimated TB incidence rates, 2015



When to Suspect MDR-TB

- Retreatment patients who's sputum smear remains positive after 3 months of intensive therapy
- Treatment failure and interruption cases
- Close contacts of MDR tuberculosis cases
- Positive diagnosis with TB culture and susceptibility testing



Mycobacterium Diagnosis

- Phenotypic identification
 - Microscopy (AFB)
 - Culture
 - Liquid
 - Solid
- Genotypic identification (molecular)
 - DNA
 - RNA based assays



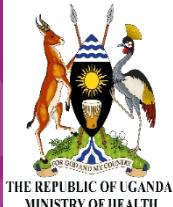
TB diagnosis using Hain test kits

- Based on DNA STRIP®Technology or DNA line probe assay (LIPA)
- Molecular genetic assay for identification of TB presence, Multi drug resistant TB (MDR-TB) and Extensively drug resistance TB (XDR-TB)
- DST- Drug susceptibility testing



ition -LPA

- Molecular test targeting nucleic acid of the Bacilli (DNA or RNA)
- DNA strip test which has a known embedded probe on to the Strip
- Simultaneous identification of tuberculosis and genetic mutations causing resistance
- Rifampin and Isoniazid - 1st line drugs



Sample Required for test

- **Pulmonary:** Respiratory (Lungs)
 - Sputum, Bronchoalveolar lavage (BAL), Tracheal aspirate, Bronchial wash samples.

Extra-pulmonary: Non respiratory

- Pericardial effusions, Pleural effusion, Cerebral Spinal Fluid (CSF) ,Gastric lavage, Pleural fluid, Lymph node aspirate, sterile biopsies etc



Qualified cases for diagnosis

- New case of TB - patient who has never been treated with Anti-TB drug or has taken drugs for less than 1 month.
- Retreatment case of TB -
 1. Treatment failure - previous treated but treatment failed
 2. Defaulted - Treatment interrupted for 2 consecutive months
 3. Relapse - completed treatment but recently diagnosed to be TB positive



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Test Principle

- GenoType®Technology and GenoQuick®Technology Platforms are based on the DNA STRIP®Technology
- Starts with nucleic acid (DNA or RNA) extraction from pulmonary or extra-pulmonary smear positive and negative patient sample and liquid or solid cultures.



Amplification by PCR, hybridization and detection

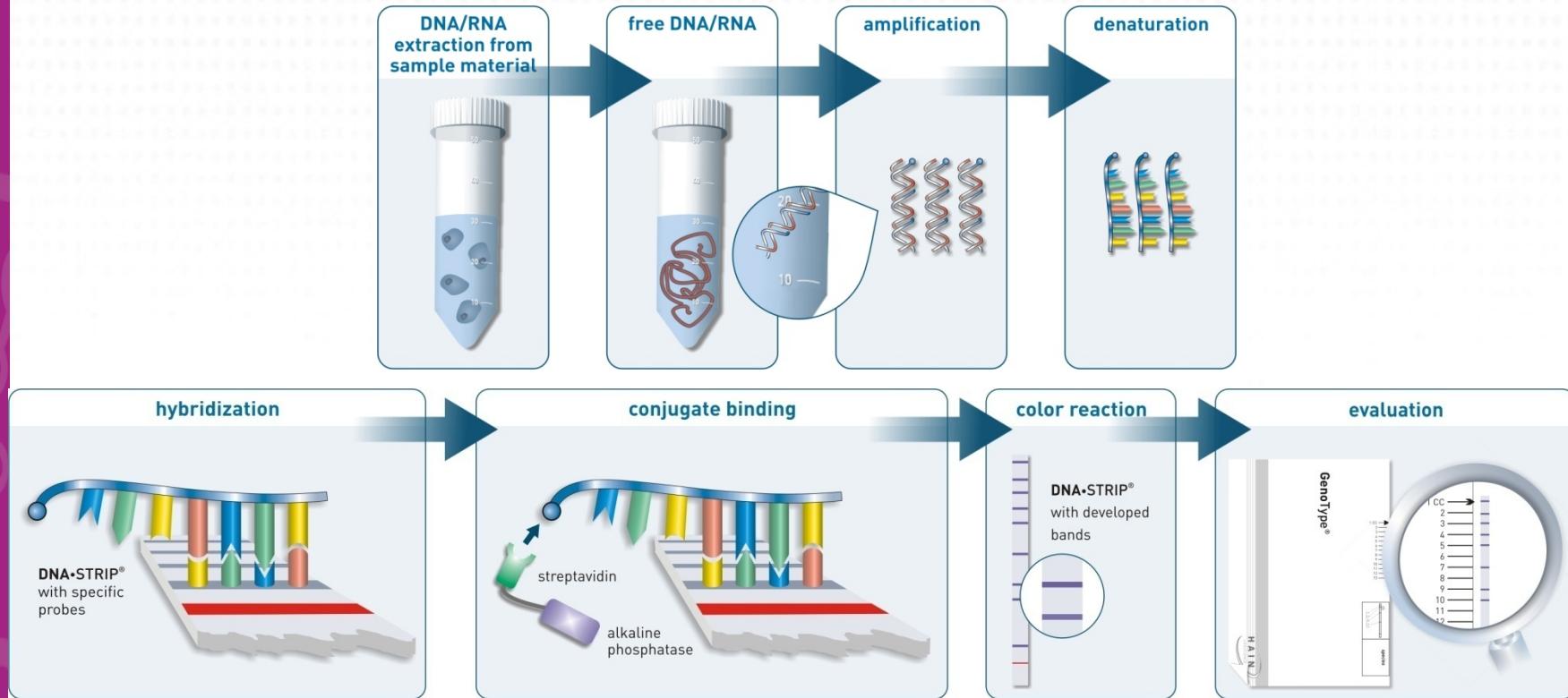
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GenoType® – DNA•STRIP® Technology



Test Principle cont..

- Amplicons specifically bind to complementary probes on the DNA STRIP®- Reverse Hybridization.
- Binding is visualized in a subsequent enzymatic colour reaction.
- As a result, a specific banding pattern develops on the strip.



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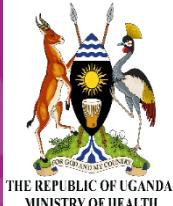
Line Probe Assays: PCR steps

- PCR reaction using cells from AFB isolate
 - Primers are labeled with biotin
 - Biotin-labeled primers are incorporated into the amplified target DNA sequences during PCR
 - More than one primer set is used in the same reaction
 - Several labeled target sequences are amplified at the same time

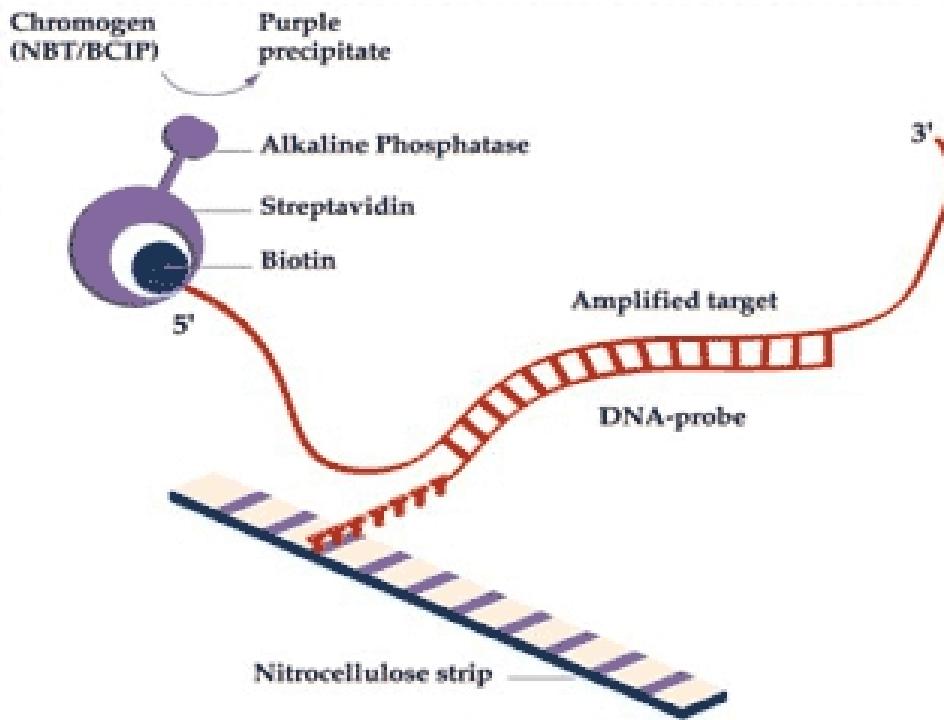


Hybridization-Detection Steps

- Reverse hybridization
 - Unlabeled probes specific for MTB complex or other Mycobacterial species are bound to strips
 - Several target sequence probes can be placed on a strip
 - Biotin-labeled target DNA binds to complementary probe on strip
- Probe-target complex is detected
 - Streptavidin-conjugated reagent detects biotin-labeled target - probe complex



Reverse Hybridization



Probe firmly attached
the membrane



Overview of Reverse Hybridization

1. Separation of amplicons into single-stranded DNA

Denaturation

2. Binding of labeled amplicons to probes on strips

Hybridization

3. Removal of amplicons that don't match exactly

Stringent Wash

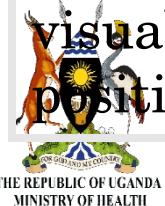
4. Binding of enzyme-conjugated protein

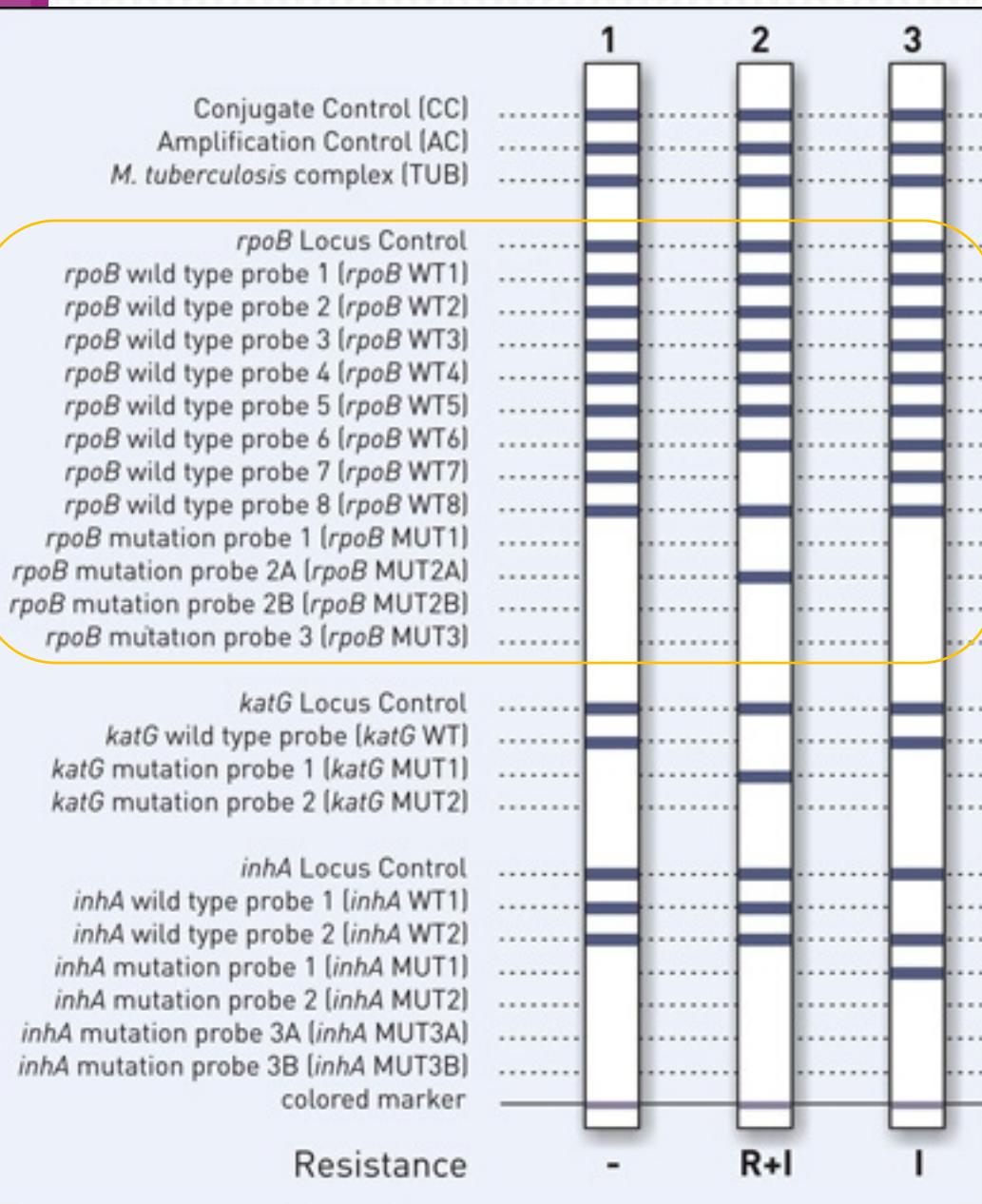
Conjugate Reaction

5. Enzymatic conversion of dye and visualization of positive bands

Substrate Reaction

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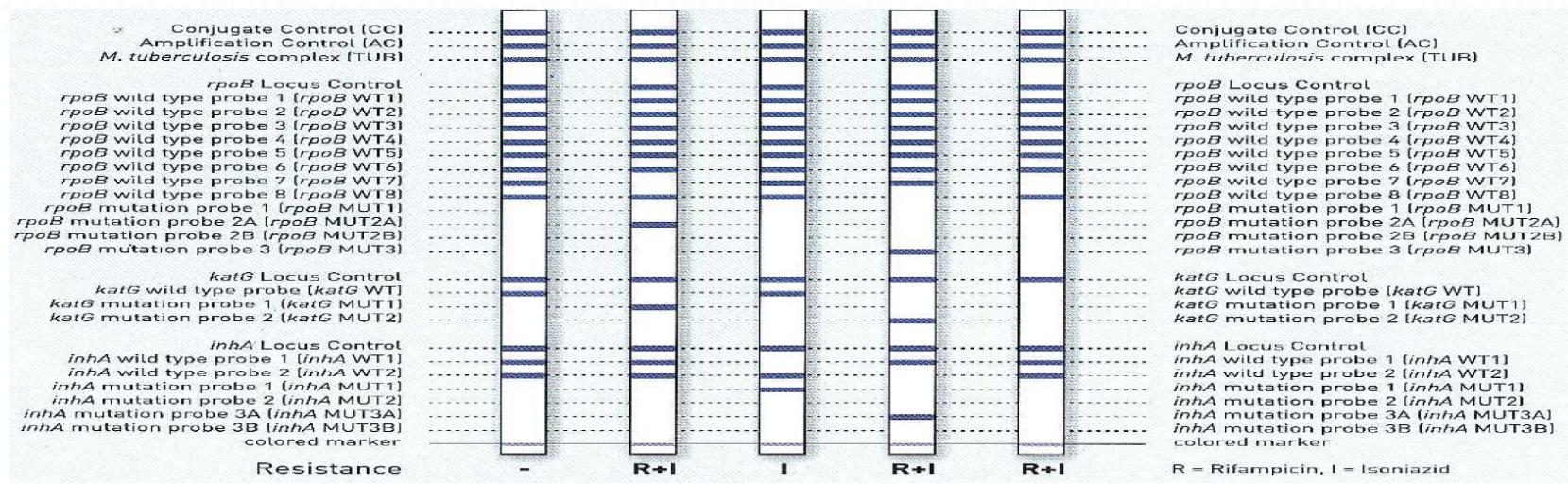


**rpoB -
RIM**

katG

INH

Interpretation of results (MTBDR plus)



Result

- The GenoType®MTBDRplus allows a safe and fast detection of the *M. tuberculosis* complex.
- Resistance to Rifampicin is by the detection of mutations in the *rpoB* gene.
- Resistance to Isoniazid is by the detection of the most common mutations in the *katG* gene and *inhA* gene.



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Comparison of GenoType MTBDRplus with Xpert MTB/RIF

- Comparison of GenoType MTBDRplus with

	GenoType MTBDRplus ver 2.0	Xpert MTB/RIF
Sensitivity (MTBC)	98.1% smear +, culture +	98.2% smear +, Culture +
	79.8% smear -, Culture +	72.5% smear -, Culture +
Specificity	99.8%	99.2%
Samples	Extra pulmonary, sputum	sputum

Valeriu Crudu et al JCM 2012; *First evaluation of an improved assay 1 for molecular genetic detection of tuberculosis as well as RMP and INH resistances*



Genotype MTBDRplus 2.0 vs MGIT & clinical TB

Crudu. JCM, 2012	Sensitivity	Specificity
MTB		
Overall	95.6%	99.2%
Smear negative	79.8%	99.2%
Rifampicin resistance		
Overall	94.3%	96.0%
Smear negative	90.7%	96.0%
Isoniazid resistance		
Overall	95.8%	88.9%
Smear negative	93.5%	82.3%

Xpert MTB/RIF

Attributes & Advantages

Simple to perform, minimal training required

Sensitivity of 93%, specificity of 98.2%

“Near-care”

Shortcomings & Disadvantages

Complex instrument (calibration, power supply)

Cost for instrument ~ USD 17,000

Detects only Rif resistance

Implementation not for high throughput labs

False positives/negatives



GenoType MTBDRplus ver 2.0. (Hain)

Attributes and advantages

- Highly sensitive and specific (98.9%, 100%)
- Detects both Rifampicin and Isoniazid
- Meant for low and high throughput labs
- Short Turn around time of 5hrs
- Affordable - ~\$10 per test

Disadvantages



Cannot be used as point of care test

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Requires biosafety facilities

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WHO Recommendation May 2016



TUBERCULOSIS DIAGNOSTICS

MOLECULAR LINE-PROBE ASSAY FOR THE DETECTION OF RESISTANCE TO SECOND-LINE ANTI-TB DRUGS (SL-LPA)

BACKGROUND

- Multidrug-resistant tuberculosis (MDR-TB) is a public health crisis and a global health security risk carrying grave consequences for those affected.
- An estimated 480 000 people developed MDR-TB in 2014 and 190 000 people died as a result of it.
- Early detection of people with MDR-TB is one of the major bottlenecks in tackling this epidemic.



BENEFITS OF THE SL-LPA



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Cont..

- In May 2016 WHO endorsed the New 2nd LPA a rapid test to detect resistance to second line drugs
- “GenoType MTBDRsl Ver 2.0”
- Detects resistance to Fluoroquinolones (Moxifloxacin, ofloxacin, levofloxacin) and aminoglycosides (KAN, AMK, CAP, VIO)
- WHO recommends all confirmed Rif resistance (RR) and MDR-TB cases to be analysed by LPA as an initial test instead of culture DST



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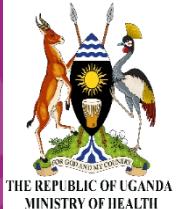
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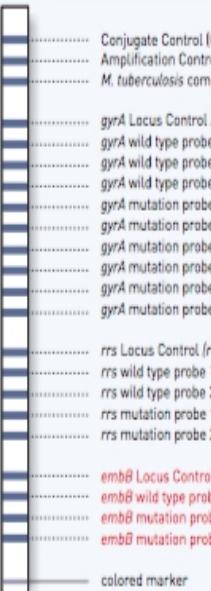
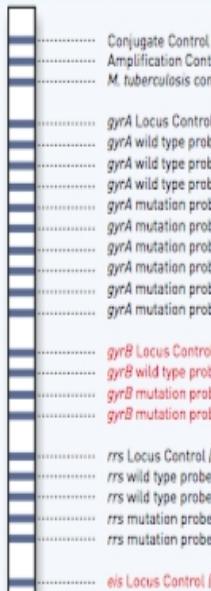
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GenoType MTBDRsI Ver 2.0 assay

- Molecular assay for simultaneous detection of TB and XDR-TB from direct sputum samples
- Sputum samples confirmed to be MDR-TB positive
- Uses the DNA Strip Technology
- Detects mycobacterium genes conferring resistance to 2nd line drugs (FQ, AG)
- An advanced test from the previous version



GenoType MTBDRsl VER 1.0		GenoType MTBDRsl VER 2.0	
			
Differences between the two versions are marked in red			
Detection of	<i>M. tuberculosis</i> complex and its resistances to fluoroquinolones, amino-glycosides/cyclic peptides and ethambutol	<i>M. tuberculosis</i> complex and its resistances to fluoroquinolones and aminoglycosides/cyclic peptides	
Sample Material	smear-positive pulmonary samples, cultivated samples	smear-positive and -negative pulmonary samples, cultivated samples	
Ready-to-use amplification mix	-	✓	
Ethambutol	Mutations in the <i>embB</i> gene that are involved in ethambutol resistance		
	✓	-	
Fluoroquinolone	Mutations in the <i>gyrB</i> gene that are involved in fluoroquinolone resistance		
	-	✓	
Kanamycin	Mutations in the <i>eis</i> gene that are involved in kanamycin resistance		
	-	✓	

Additional genes added

Additional genes added

GenoType MTBDRsI Specific genes

- gyrA gene and gyrB codes for A and B-subunit of the DNA gyrase respectively
- gyrA Its coded from codon 85 to 97
- Mutation occurs at codon 88, 90, 91, and 94
- gyrB mutation occur at codon 538, 540
- Fluoroquinolones (ofloxacin and moxifloxacin) Inhibits DNA gyrase which allows ease of the DNA to be unwound during replication.



GenoType MTBDRsl ver 2.0. Specific genes

- rrs gene encodes for cell wall development
- rrs gene is 300bp
- Its coded at codon 1401 to 1484
- 67.4% kanamycin resistance (*Yasuhiko Suzuki et al 1998; Journal of Clinical Microbiology. P 1220-1225*)
- Mutation occur at 1401, 1402, and 1484



Cont...

- Eis gene encodes for low level Kanamycin
- Mutation occur at codon, 2, 10,12, 14, 37



Benefits of assay

- Highly Sensitive and specific: Test can be performed from +ve and -ve sputum smear samples
- Efficient diagnosis: Patients previously tested and are MDR-TB positive don't need to give out another sputum sample
- Rapid XDR-TB results generated within 4 hours
- Improved version contains extra genes (*gyrB* gene) in fluoroquinolones. This reduces negative/positives cases

Cont..

- Enables early patient isolation and therapy initiation preventing spread of the strain
- Laboratory procedure remains the same



FluoroType – New Assays

FluoroType®



DNA-Extraction with
GenoXtract

Amplification and
Detection with FluoroCycler®

Result

Semi-quantitative real-time PCR

Based on Hybeacon technology

3 hours turn-around time

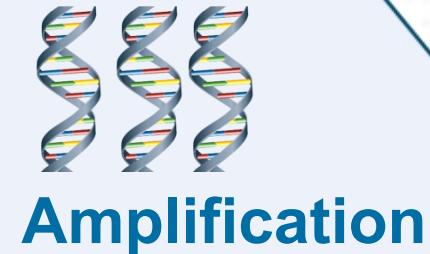


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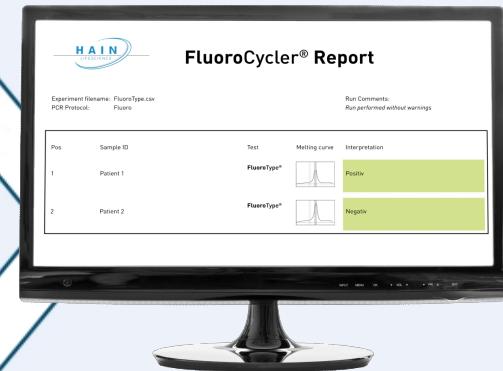
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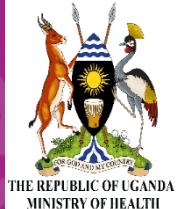
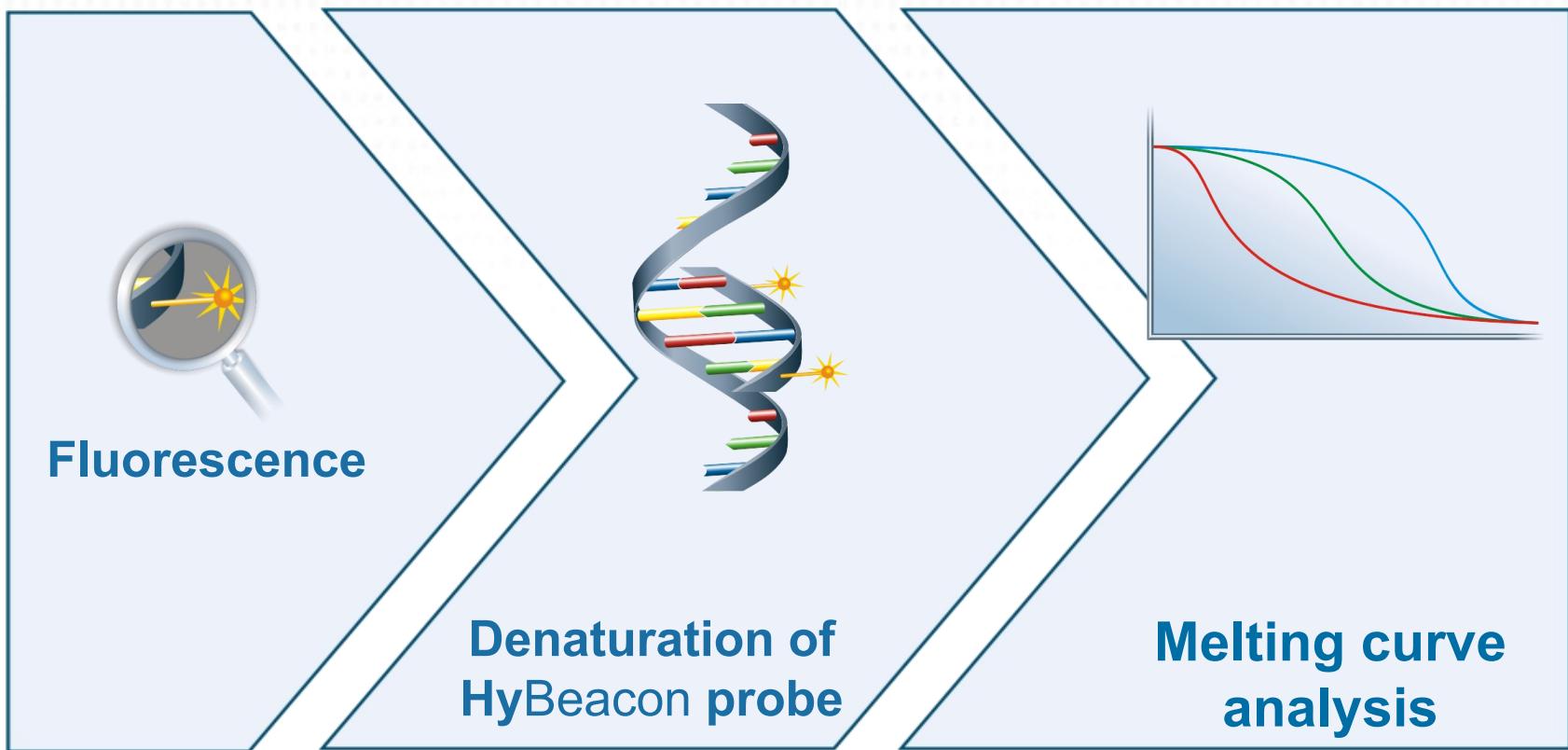
Detection with
melting curve
analysis



FluoroCycler®

Result

What happens in FluoroCycler®?



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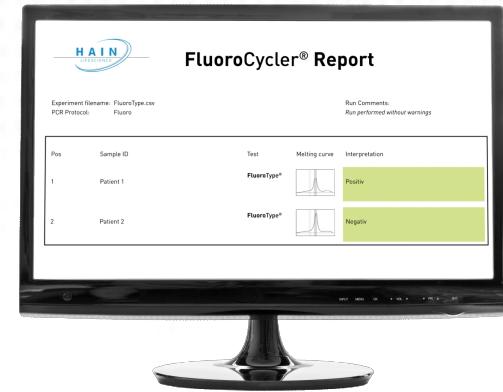
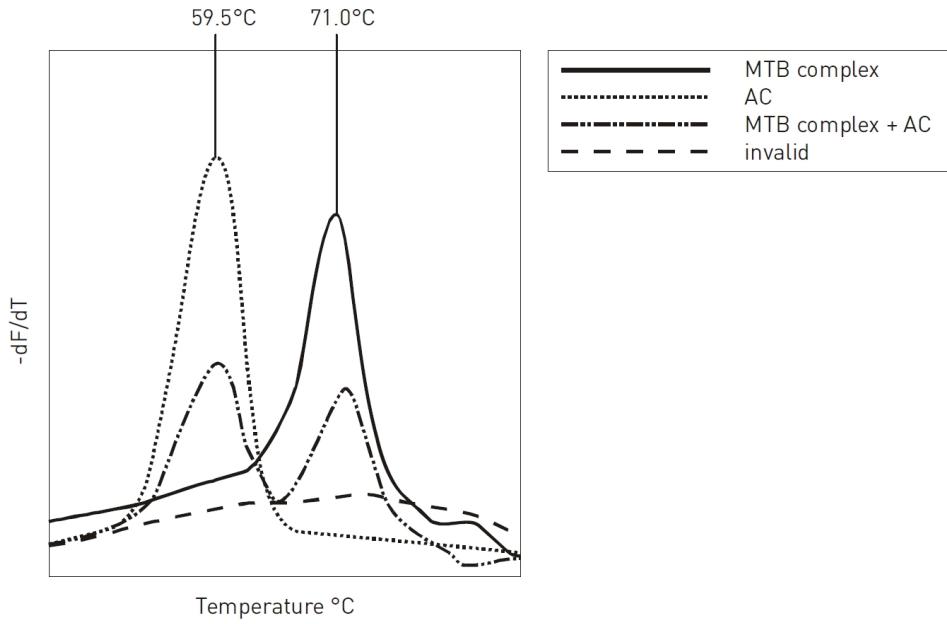
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What happens in FluoroCycler®?

- Melting curve



Results after
approx. 3
hours

Additional Hain Microbiology Assays

- GenoQuick® MRSA GenoQuick® Bordetella
- GenoType Cdiff GenoType® MRSA Direct
- GenoType® MRSA
- GenoType® Staphylococcus
- GenoType® HelicoDR
- GenoType® BC gram-positive GenoType® BC gram-negative
- GenoType® EHEC
- GenoType® Enterococcus
- micro-Ident® (Dental)



Why Molecular (PCR) test system?

1. Molecular genetic detection methods in TB diagnostics is now the test for precise results
2. They have the capability to reduce bio-hazard and increase speed, sensitivity and specificity
3. Genetic based Drug Sensitivity Testing enables rapid diagnosis and timely start in correct treatment
4. This could lead to more individualized therapies



References

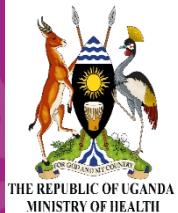
- GLI TB training package
<http://www.stoptb.org/wg/gli/trainingpackages.asp>
- www.hain-lifesciences.com



Acknowledgments



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