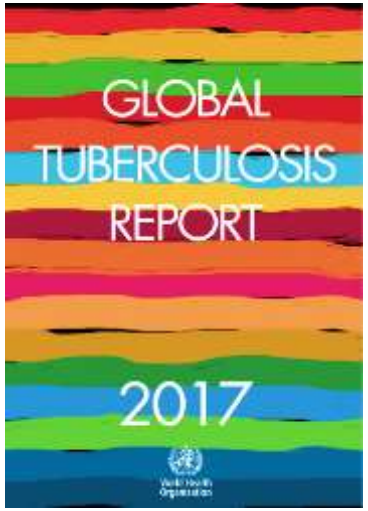
Molecular diagnosis of drug resistant tuberculosis

1-2-2019



The global data

	Estimated incidence, 2016	Estimated number of deaths, 2016
All forms of TB	10.4 million (8.8–12.2 million)	1.3 million* (1.2–1.4 million)
HIV-associated TB	1.0 million (0.9–1.2 million)	374,000 (325,000–427,000)
Multidrug- / rifampicin-resistant TB (MDR/RR-TB)	600,000 (540,000–660,000)	240,000 (140,000–340,000)

Source: WHO Global Tuberculosis Report 2017

* Excluding deaths attributed to HIV/TB

Indian data

Drug-resistant TB care, 2016

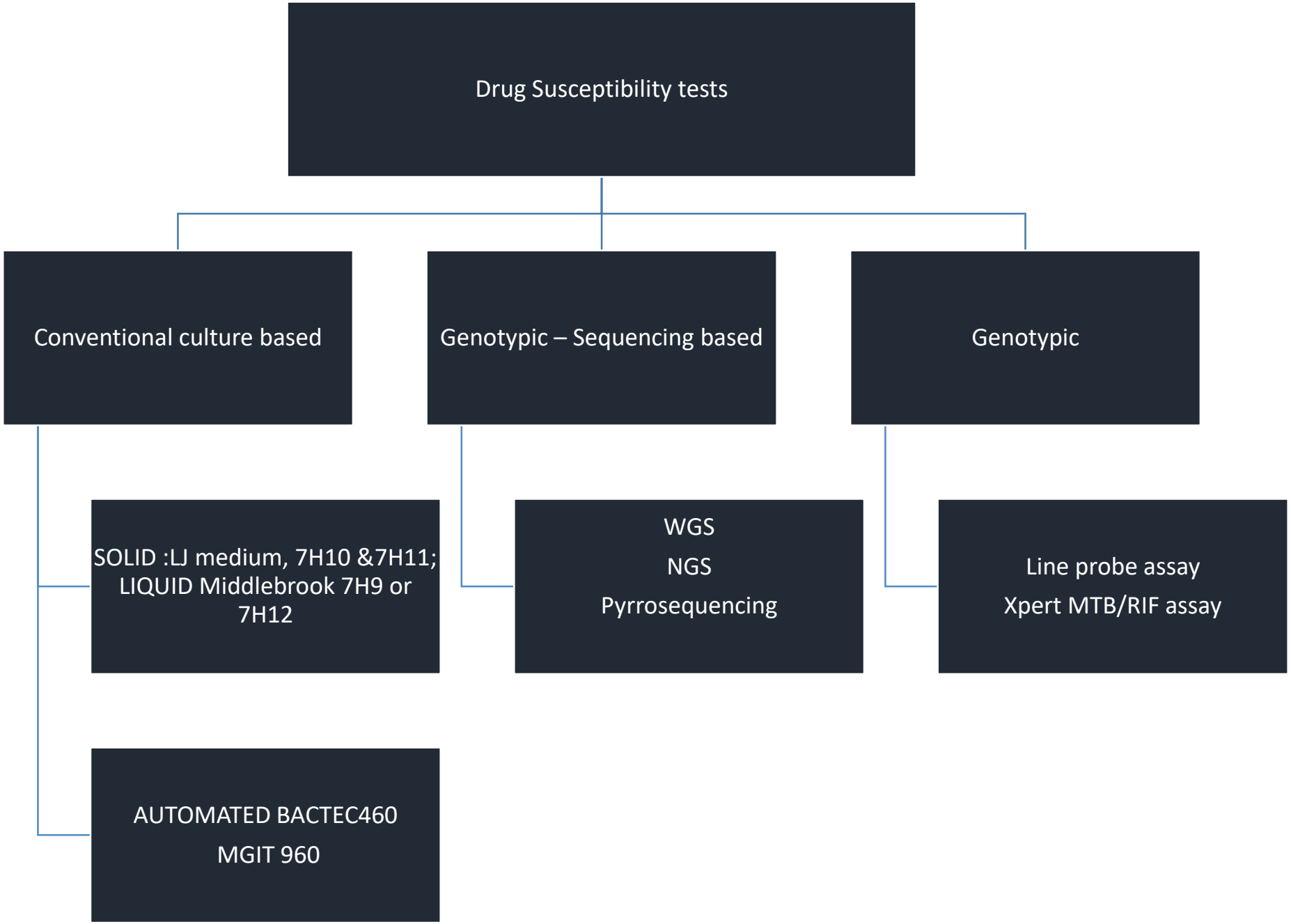
	New cases	Previously treated cases	Total number[c]
Estimated MDR/RR-TB cases among notified pulmonary TB cases			84 000 (72 000–95 000)
Estimated % of TB cases with MDR/RR-TB	2.8% (2–3.5)	12% (10–13)	
% notified tested for rifampicin resistance	20%	67%	580 438
MDR/RR-TB cases tested for resistance to second-line drugs			22 492
Laboratory-confirmed cases			MDR/RR-TB: 37 258, XDR-TB: 2 464
Patients started on treatment[d]			MDR/RR-TB: 32 914, XDR-TB: 2 475

WHO Global tuberculosis report 2017

The MDR TB menace

- Globally 4.1% of new cases and 19% of previously treated cases with MDR/RR-TB
- 240000 people died from MDR/RR-TB in 2016
- Only 41% of bacteriologically-confirmed and previously-treated TB patients were tested for rifampicin resistance, and only 39% of the notified DR-TB patients had additional DST results for the fluoroquinolones and second-line agents
- Only 22% of the estimated incident DR-TB cases were started on TB treatment
- Rapid DR-TB diagnostic assays, including technologies that provide a comprehensive picture of the drug resistance profile of a TB clinical sample, are needed to fill these gaps

WHO Global tuberculosis report 2017


Drug Susceptibility tests
Conventional culture based
Genotypic – Sequencing based
Genotypic
SOLID :LJ medium, 7H10 &7H11; LIQUID Middlebrook 7H9 or 7H12
AUTOMATED BACTEC460
MGIT 960
WGS
NGS
Pyrrosequencing
Line probe assay
Xpert MTB/RIF assay

Phenotypic methods of DST

- Culture based
- Longer turn around time
- Higher biosafety level (BSL III)
- Phenotypic testing often lacks accuracy and reproducibility - for drugs such as pyrazinamide

Types of molecular tests available

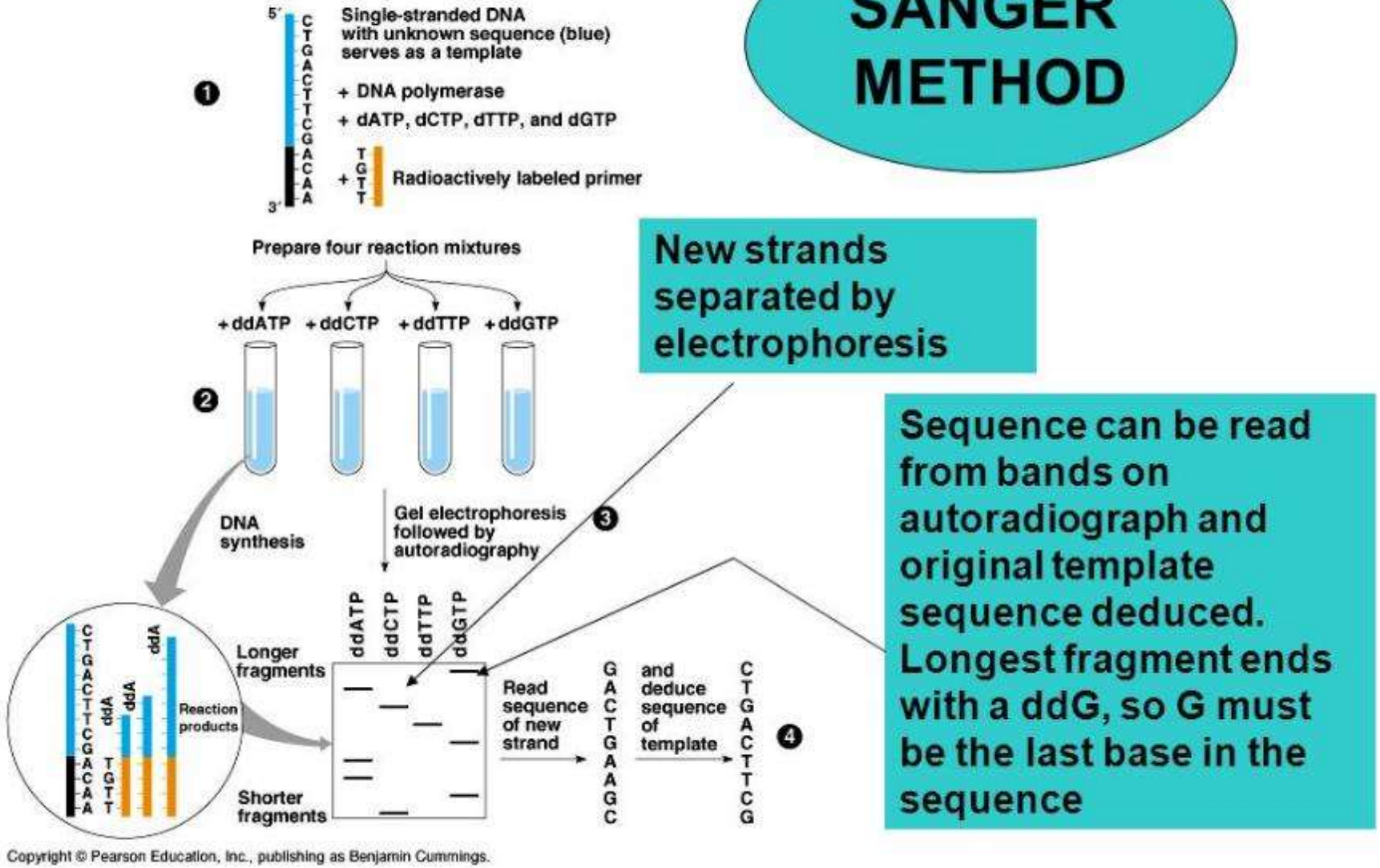
- Non sequencing based
  - XpertMTB/RIF
  - Xpert MTB/RIF Ultra
  - GeneXpert Omni
  - TrueNat MTB
  - LPA
- Sequencing based
  - Sanger sequencing
  - Next generation sequencing
  - Pyrosequencing
  - Melting curve analysis

Sequence based techniques

Sanger sequencing

- Dideoxynucleotide method or chain termination method
- Technique for DNA sequencing based upon the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication
- The DNA polymerisation is carried out in 4 separate chambers with each chamber containing one dideoxy-nucleotide
- The polymerisation or addition of nucleotides is terminated when the specific dideoxynucleotide gets added to the sequence
- DNA fragments of variable length are obtained
- The DNA fragments are then separated on agarose gel by electrophoresis and based on different lengths
- Bands of variable length are obtained from each chamber and that enables sequence determination

SANGER METHOD



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New strands separated by electrophoresis

Sequence can be read from bands on autoradiograph and original template sequence deduced. Longest fragment ends with a ddG, so G must be the last base in the sequence

Sanger sequencing

- Limitations
  - Low throughput
  - Labour intensive
  - Can sequence only smaller genes/ small segments of DNA
  - Limited set of resistance mutations can be studied at a time unlike NGS/WGS where entire genome

Next generation sequencing

- A high-throughput, massively parallel sequencing method used to determine the nucleotide sequence of a whole genome (whole genome sequencing (WGS) or part of a genome (targeted NGS) in a single biochemical reaction volume
- NGS is performed by non-Sanger-based sequencing technologies that are capable of sequencing multiple DNA fragments in parallel, which are then pieced together and mapped to a reference genome using bioinformatics analyses

NGS

- NGS assays can provide detailed sequence information for multiple gene regions or whole genomes of interest
- DNA is first extracted from clinical samples or cultured isolates
- DNA goes through enzymatic processing
- Multiple fragments of DNA are sequenced in parallel
- Bioinformatics analyses are used to map the individual reads to the reference genome
- Each DNA base is sequenced multiple times, this assessment provides high depth to deliver accurate data and insight into occurrence of genetic polymorphisms

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Advantages of NGS/WGS

- Confirm the presence or absence of indels and assess the occurrence of SNP and other sequence-level data that may evade detection by other molecular assays
- Since drug resistance in TB are attributed to point mutations NGS/WGS will have diagnostic implication
- Heteroresistance or a mix of multiple genetic populations in a clinical sample
- Assessment of genetic information for additional organisms that may be present in a clinical sample

NGS

- WGS can provide the near complete genome of *Mycobacterium tuberculosis* in a sample while targeted NGS can generate MTB sequence data at specific genetic loci of interest
- Targeted NGS and WGS both rely upon the same basic NGS workflow and both applications may be run on the same NGS instrument
- Targeted NGS offers great promise for rapid diagnosis of MDR TB

WGS

- WGS is generally performed only on strains grown in culture due to the need for a relatively high quantity of good quality DNA to generate full WGS data for a given sample
- Direct sputum samples are associated with contamination from human DNA and nasopharyngeal flora
- Targeted NGS – from sputum or culture isolates

Same-Day Diagnostic and Surveillance Data for Tuberculosis via Whole-Genome Sequencing of Direct Respiratory Samples

40 smear positive samples subjected to WGS and compared with 28 MGIT positive isolates

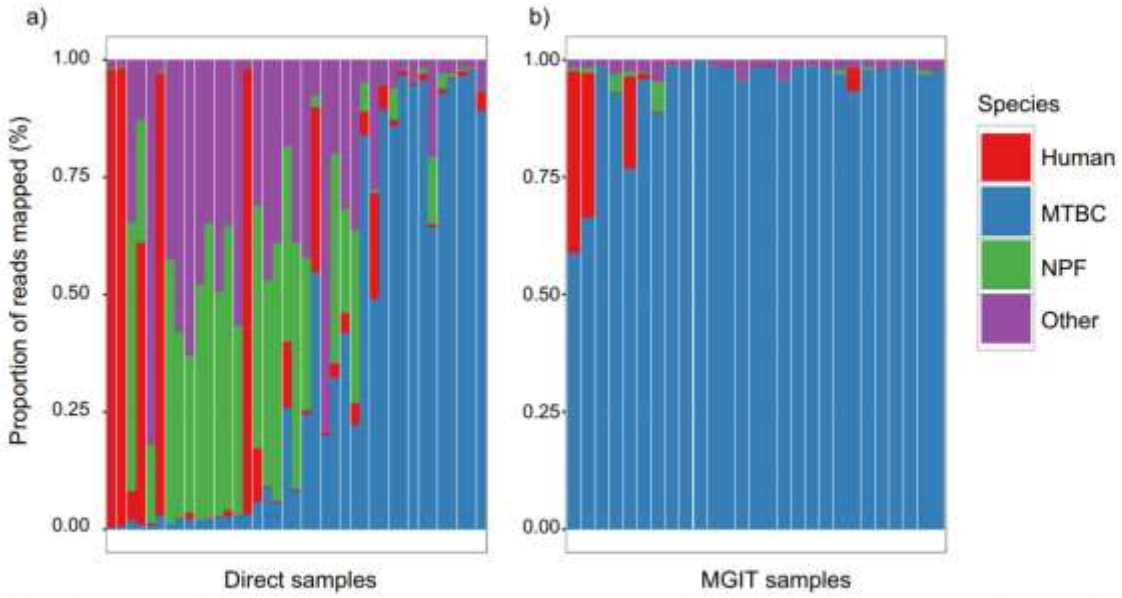


FIG 2 Proportions of reads assigned to various species categories in each sample sorted by increasing total counts of *M. tuberculosis* complex (MTBC) reads. (a) Direct samples show that removal of human DNA (red) has been broadly successful, but removal of NPF (green) and other bacteria (purple) had more variable success. (b) MGIT samples show much more uniform dominance of *M. tuberculosis* reads, as expected after 2 weeks of culture designed to favor mycobacterial growth.

Votintseva *et al.* J Clin Microbiol. 2017 May;55(5):1285-1298

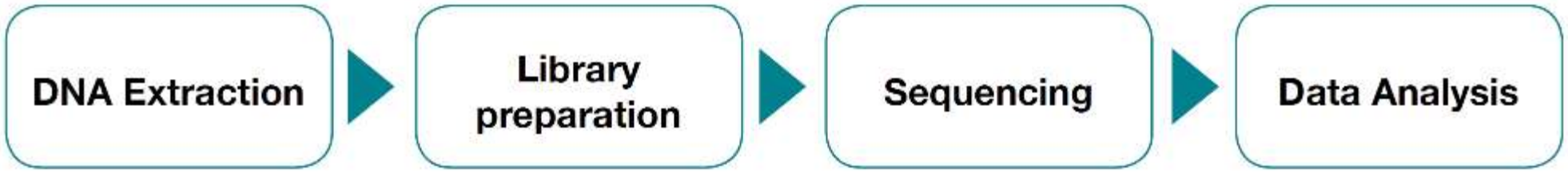
Same-Day Diagnostic and Surveillance Data for Tuberculosis via Whole-Genome Sequencing of Direct Respiratory Samples

- 70% (30/39) of the direct samples contained <10% human reads
- 46% (18/39) contained <10% NPF and other bacterial reads
- 26% (10/39) contained >40% reads from nonmycobacterial, non-NPF, bacteria
- 21/39 samples have >12× depth and recover >90% of the genome and 14/39 samples have <3× depth and recover <12% of the genome
- Antibiotic resistance prediction
  - 92 (96%) of 96 predictions for the first-line antibiotics were concordant with reference laboratory DST in those with depth >3X

Votintseva *et al.* J Clin Microbiol. 2017 May;55(5):1285-1298

NGS workflow

- DNA extraction from a TB sample
- Library preparation
- Sequencing
- Data analysis



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DNA extraction

- Thermal methods (thermolysis)
- Organic/enzymatic-based methods (e.g. phenol-chloroform proteinase K)
- Mechanical-based methods (e.g. sonication, bead beating, magnetic beads)
- Chemical-based methods (e.g. centrimonium bromide, sodium chloride)
- Following extraction – quantification using spectrophotometer and size and integrity assessed via agarose electrophoresis or microfluidic instruments

DNA library preparation

- DNA library, or a collection of specifically fragmented DNA to which oligonucleotide adaptors have been attached, must be prepared and quantified for both targeted NGS and WGS applications
- DNA library kits chosen for NGS or WGS
- General steps of library preparation include
  - DNA fragmentation
  - End-repair
  - Phosphorylation of the 5’ prime ends A-tailing of the 3’ ends
  - Ligation to sequencing adapters
  - PCR
  - library purification and quantification

Table 1: Examples of commercially available library preparation kits for different NGS instruments

Library preparation kit	System compatibility	NGS application
Nextera XT	All Illumina	WGS or Targeted
Nextera DNA Flex	All Illumina	WGS or Targeted
AmpliSeq	All Illumina	Targeted
Ion Xpress Plus Fragment	PGM and S5 Ion Torrent	WGS or Targeted
MuSeek	PGM and Proton	WGS or Targeted
Rapid Sequencing Kit	All ONT devices	WGS or Targeted
Ligation Sequencing Kit 1D	All ONT devices	WGS or Targeted
Low Input by PCR Sequencing	All ONT devices	WGS or Targeted
$1D^2$ Sequencing	All ONT devices	WGS or Targeted

Next-generation sequencing: Platforms

Platform	Manufacturer	Principle	Time	Cost	Strengths	Weakness
MiSeq™	Illumina	Sequencing: PCR	4-55 hours	100,000 USD	Long readlngth	Long run time
Ion Personal Genome Machine™	Thermofisher Scientific	Sequencing PCR	3-10 hours	80,000 USD	Short run time	Low readlength
MinION	ONT	Real time sequencing and data analysis	30min-48 hours	1000 USD	Short run time, portable Real time data analysis	High error rate

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Table 3: Comparison of commercially available NGS instruments

Sequencing platform	Sequencing kit	Number of samples per run	Sequencing cost* per sample (USD)	Typical run output	Sequencing run time	Type of analysis possible
Illumina MiSeq	MiSeq v2 (2x250bp)	54	$22.41	7.5-8.5 GB	39 hrs	SNP and INDEL analysis
Ion Torrent	Ion 318 Chip v2 (400 base)	13	$48.75	1.2-2 GB	7.3 hrs	SNP and INDEL analysis
Oxford Nanopore	Ligation Sequencing kit 1D	22 54	$42.86 $16.67	2-3 GB 4-8 GB	6 hrs 48 hrs	Genomic rearrangement, SNP, and INDEL analysis

Two run times are possible with the Oxford Nanopore system. Qiagen was not included in the above table due to a lack of publicly available information regarding the assay. All comparisons assume WGS is performed on MTB and average 30x coverage.
* Price for sequencing only, not including library preparation price, and not including bulk price discounts

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Data analysis

- Bioinformatics software platforms are used to analyse the sequencing data
- Relational Sequencing TB Data Platform (ReSeqTB) established to collect global TB patient data from multiple private and public databases and identify correlations between MTB mutations and clinically relevant resistance with the ultimate goal of guiding sequencing data interpretation for personalized TB patient care
- Four web servers (CASTB, PhyResSE, TBProfiler, and GenTB) and two software solutions (KvarQ and Mykrobe Predictor TB) developed for facilitated interpretation of TB drug resistance from genomic data
- Analytical tools developed based on the current knowledge of the molecular basis of TB drug resistance, and results will likely only improve with time given additional knowledge of TB drug resistance mechanisms and associations with phenotypic drug resistance

Prediction of resistance to different Anti tubercular drugs based on sequencing data

- WHO collaboration with FIND
- Systematic review to establish correlation between mutations detected by sequencing mycobacterial genome or specific genes with the corresponding phenotypic data
- Sequencing data and culture sensitivity, MIC data was collected
- Collated data used to calculate the frequency of each mutation in resistant and susceptible MTBC isolates
- Level of resistance conferred by mutations based on the MIC ranges found in the strains with resistance was studied and grading of mutations were done based on OR

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Table 1: Overview of confidence levels for grading mutations associated with phenotypic resistance

	LR* and OR	
	p-value	**value**
High confidence for association with resistance Strong association of the mutation with phenotypic drug resistance; sufficient evidence that the mutation confers or is strongly associated with drug resistance	< 0.05	>10
Moderate confidence for association with resistance Moderate association of the mutation with phenotypic drug resistance; additional data desirable for improved evidence that the mutation confers or is strongly associated with drug resistance	< 0.05	5 < ... ≤ 10
Minimal confidence for association with resistance Weak association of the mutation with phenotypic drug resistance; inconclusive evidence that the mutation confers or is strongly associated with drug resistance. Substantial additional data required	< 0.05	1 < ... ≤ 5
No association with resistance No evidence of association between the mutation and drug resistance	< 0.05	< 1
Indeterminate No statistically significant threshold reached; additional data required	≥ 0.05	

Only those confidence levels established based upon combined liquid and solid media data for each resistance mutation were included in this analysis.

Isoniazid resistance

- Mutations
  - *KatG* – catalase peroxidase INH activation
  - *fabG1/inhA or mabA/inhA* (*inhA* – mycolic acid synthesis)
  - *OxyR-ahpC*
  - *furA* (ferric acid upregulator)
  - *ndh* (NADH dehydrogenase)
  - *nat* (arylamine acetyl transferase – inactivates INH)
  - *MshA* – glycosyl transferase (mycolic acid synthesis)

Isoniazid resistance

- Mutations associated with high level of phenotypic resistance with high confidence
  - katG S315I
  - KatG S315T
  - KatG S315N
- Sensitivity and specificity of NGS technique in predicting phenotypic resistance 84% ((95% CI 82.9-84.3%) and 98% (95% CI 96.8-98.4%) respectively

Rifampicin resistance

- >95% of the rifampicin resistance localised to the 81 bp region of *rpo*B gene
- This region is called the RRDR
- Mutations impede binding of drug to ß subunit of DNA dependent RNA polymerase
- Sensitivty and specificity of NGS in predicting phenotypic rifampicin resistance is 96% (95% CI 95.2-96.1%) and 99% (95% CI 98.1-98.8%) respectively

Fluoroquinolone resistance

- FQs inhibit MTB DNA gyrase
- DNA gyrase 2 subunits coded by gyrA and gyrB
- Most resistance in QRDR codons 74-113 and 500-538 in gyrA and B respectively
- Majority in codon 88,90,91 and 94
- Sensitivity and specificity of predicting phenotypic FQ resistance based on the sequencing is 94% (95% CI 91.5-95.5%) and 100% (95% CI 99.2-100.0%) respectively

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Pyrazinamide resistance

- Culture-based PZA phenotypic DST difficult to perform and produces unreliable results
- Bactec MGIT 960 liquid culture method is the only WHO-recommended method for PZA susceptibility testing
- No other rapid molecular test (like Xpert LPA) for detecting PZA
- *pncA* gene and promoter, which lead to a reduction or loss of pyrazinamidase activity – most PZA resistance
- Unlike RIF and INH no hotspot or single resistance determining region
- Mutations scattered throughout the *pncA* gene and promoter – Hence sequencing techniques important

Pyrazinamide resistance

- Sensitivity and specificity for PZA resistance detection were 83% (95% CI 81.7-84.5%) and 94% (95% CI 92.9-94.7%) respectively
- The reduced sensitivity of sequencing of the pncA gene for the detection of PZA resistance compared with phenotypic DST is likely due to the limited reproducibility of the phenotypic test

SLID resistance

- AMK KAN – inhibit 16S rRNA in MTB 30S ribosome
- CAP interferes with translation inhibiting phenylalanine synthesis
- Primary gene regions conferring resistance
  - Mutations in *rrs* gene – all three injectables
  - *tlyA* gene mutations – CAP
  - *eis* promoter mutations KAN resistance

SLID resistance

SLID	Mutations	Sensitivity (95% CI)	Specificity (95% CI)
AMK	*rrs*	79% (75.8-81.5%)	100% (99.7-100.0%)
KAN	*rrs* *eis* promoter	68% (65.3-71.2%)	100% (99.8-100.0%)
CAP	*rrs* *tlyA*	71% (68.3-74.2%)	100% (99.8-100.0%)

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Accuracy of sequencing in a multi-country, population-based study for determining drug resistance in *Mycobacterium tuberculosis* complex

- Population-based surveys conducted in 7 countries
- Azerbaijan Bangladesh Belarus Pakistan Philippines South Africa and Ukraine between 2010 and 2014
- Sites were selected in each country either by cluster sampling or by including all diagnostic facilities in the country
- Both newly diagnosed and previously treated sputum samples included
- Phenotypic DST based on standard critical concentrations after LJ or MGIT culture
- WGS/NGS
- INH RIF PZA AMK KAN CAP OFX

Accuracy of sequencing in a multi-country, population-based study for determining drug resistance in *Mycobacterium tuberculosis* complex

- Results
  - 7,094 patients
  - Pooled sensitivity values for genetic sequencing among all TB cases were 91% (95% CI: 87-94%) for *rpoB*
  - 86% (95%CI: 74-93%) or *katG, inhA* and *fabG* promoter combined
  - 85% (95%CI: 77-91%) for *gyrA* and *gyrB* combined (OFX)
  - The sensitivity or *pncA* when compared with MGIT 960 testing was 54% (95%CI: 39-68%)

Prediction of Susceptibility to First-Line Tuberculosis Drugs by DNA Sequencing

- 23 collections of *M. tuberculosis* complex isolates from 16 countries analyzed
- Sequencing - Illumina platforms
- Data analysis - Public Health England bioinformatics
- Drug susceptibility - MGIT 960, LJ medium, microscopic observation drug-susceptibility with method specific critical concentrations
- Genotypic predictions were based on mutations in or upstream from genes associated with resistance to isoniazid (*ahpC*, *inhA*, *fabG1*, and *katG*), rifampin (*rpoB*), ethambutol (*embA*, *embB*, and *embC*), and pyrazinamide (*pncA*)

N Engl J Med 2018;379:1403-15

Prediction of Susceptibility to First-Line Tuberculosis Drugs by DNA Sequencing

- Isolates containing resistance mutations were predicted to be phenotypically resistant, whereas isolates containing only wild-type sequence, phylogenetic mutations, or mutations that were considered to be consistent with susceptibility were predicted to be susceptible
- Using phenotypic results as the standard – sensitivity and specificity for the correct assignment of susceptibility or resistance calculated

N Engl J Med 2018;379:1403-15

Prediction of Susceptibility to First-Line Tuberculosis Drugs by DNA Sequencing

Drugs	Sensitivity	Specificity
INH	97.1%	99.0%,
RIF	97.5%	98.8%,
PZA	94.6%,	93.6%,
EMB	91.3%	96.8%

NPV (Percentage of genotypic predictions of susceptibility that were correct) > 98.5% all drugs

Limitations

- Discrepancies not addressed - 322 resistant phenotypes that had been predicted to be susceptible and 822 susceptible phenotypes that had been predicted to be resistant
- Phenotypic error, resistant minority bacterial populations that went undetected by sequencing, mechanisms of resistance unknown to us, or laboratory labeling error
- Lack of clinical outcome data– success or failure of treatment in 10000 patients not taken into account

Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: a retrospective cohort study

Timothy M Walker, Thomas A Kohl*, Shaheed V Omar*, Jessica Hedge*, Carlos Del Ojo Elias, Phelim Bradley, Zamin Iqbal, Silke Feuerriegel, Katherine E Niehaus, Daniel J Wilson, David A Clifton, Georgia Kapatai, Camilla L C Ip, Rory Bowden, Francis A Drobniewski, Caroline Allix-Béguec, Cyril Gaudin, Julian Parkhill, Roland Diel, Philip Supply, Derrick W Crook, E Grace Smith, A Sarah Walker, Nazir Ismail†, Stefan Niemann†, Tim E A Peto†, and the Modernizing Medical Microbiology (MMM) Informatics Group‡*

Summary

Background Diagnosing drug-resistance remains an obstacle to the elimination of tuberculosis. Phenotypic drug-susceptibility testing is slow and expensive, and commercial genotypic assays screen only common resistance-determining mutations. We used whole-genome sequencing to characterise common and rare mutations predicting drug resistance, or consistency with susceptibility, for all first-line and second-line drugs for tuberculosis.

Methods Between Sept 1, 2010, and Dec 1, 2013, we sequenced a training set of 2099 *Mycobacterium tuberculosis* genomes. For 23 candidate genes identified from the drug-resistance scientific literature, we algorithmically characterised genetic mutations as not conferring resistance (benign), resistance determinants, or uncharacterised. We then assessed the ability of these characterisations to predict phenotypic drug-susceptibility testing for an independent validation set of 1552 genomes. We sought mutations under similar selection pressure to those characterised as resistance determinants outside candidate genes to account for residual phenotypic resistance.

Findings We characterised 120 training-set mutations as resistance determining, and 772 as benign. With these mutations, we could predict 89·2% of the validation-set phenotypes with a mean 92·3% sensitivity (95% CI 90·7–93·7) and 98·4% specificity (98·1–98·7). 10·8% of validation-set phenotypes could not be predicted because uncharacterised mutations were present. With an in-silico comparison, characterised resistance determinants had higher sensitivity than the mutations from three line-probe assays (85·1% *vs* 81·6%). No additional resistance determinants were identified among mutations under selection pressure in non-candidate genes.

Interpretation A broad catalogue of genetic mutations enable data from whole-genome sequencing to be used clinically to predict drug resistance, drug susceptibility, or to identify drug phenotypes that cannot yet be genetically predicted. This approach could be integrated into routine diagnostic workflows, phasing out phenotypic drug-susceptibility testing while reporting drug resistance early.

Lancet Infect Dis 2015; 15: 1193–1202

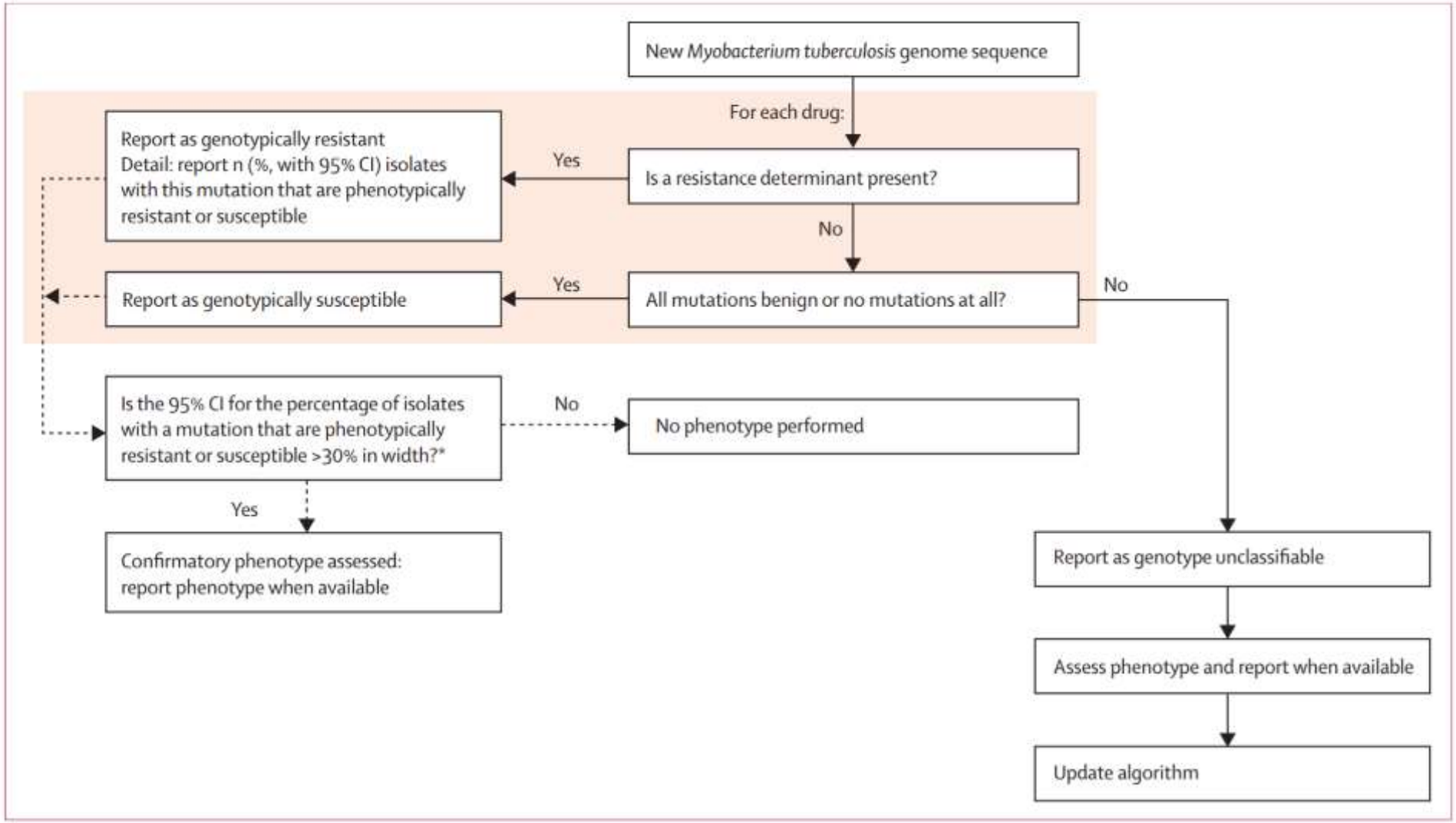


***Figure 5*: Proposed workflow for transition towards whole-genome sequencing-based drug-susceptibility testing**

*The 30% CI width suggested is arbitrary, and represents how the precise proportion of isolates with a mutation is probably less relevant than understanding whether this proportion is very high, moderate, or low. However, the precise width could be determined by what is regarded as an acceptable degree of clinical risk, and could also vary by the estimate of proportion resistant. For example, with a targeting width of less than 30%, ten phenotypically resistant isolates of ten isolates with a mutation (100%) has a lower 97·5% CI of 69%, so mutations that are uniformly resistant would need to be phenotyped 11 times before confirmatory phenotyping would stop. For a mutation associated with resistance in 50% of isolates, phenotyping would need to happen 48 times, and for a mutation associated with resistance in either 25% or 75% isolates, 36 times

Lancet Infect Dis 2015; 15: 1193–1202

Reporting of NGS/WGS

- Sequencing type (WGS or targeted NGS)
- Sequencing platform utilized
- Bioinformatics software/pipeline and version used for analysis
- Drugs of interest included in resistance profile Known drug resistance-conferring target gene(s) and respective loci IDs
- Mutations detected
- % of resistance alleles at loci of interest (to identify mixed infections when present)
- Coding effect (synonymous or nonsynonymous)
- Amino acid changes (when non-synonymous)
- Phenotypic resistance prediction
- Phylogenetic lineage/sub-lineage/local-strain information
- Detected genomic clustering (if any)
- Comments (for disputed or uncertain
- mutations)

Figure 2: Example of a standardized clinical WGS report for MTB*

Barcode XXXXXXXXXXXX

MYCOBACTERIUM TUBERCULOSIS SEQUENCING REPORT

Sample Details

Patient Name	JOHN DOE	Patient ID	12345678910
Birth Date	2000-JAN-01	Location	SOMEPLACE
Sample Type	SPUTUM	Sample Collection Date	2016-DEC-25
Sample Source	PULMONARY	Sequenced From	CULTURED ISOLATE (LJ)
Sample ID	A12345678	Sample Received Date/Time	2017-JAN-02, 12:22
Laboratory Technician	TECHNICIAN NAME	Report Date/Time	2017-JAN-05, 11:45
Requested By	REQUESTER NAME	Requester Contact	REQUESTER@EMAIL.COM

Assay Details

Sequencer	ILLUMINA HISEQ 2500	Method	WHOLE GENOME SEQUENCING
Pipeline	RESEQTBV.3.2C (https://platform.reseqtb.org)	Reference	H37RV (NC_000962.3)

Final Result

The sample was positive for ***Mycobacterium tuberculosis.***
It is **resistant to isoniazid, rifampin, capreomycin, kanamycin, ofloxacin, and moxifloxacin.**

Lineage

Mycobacterium tuberculosis, lineage 2.2.1 **(East-Asian Beijing)**.

Drug Susceptibility

Resistance is reported when a high likelihood resistance-conferring mutation is detected in loci of interest.[1] **No mutation detected does not exclude the possibility of resistance.**

☐ No mutations detected
☐ Multi-drug resistance predicted
☑ **Extensive drug resistance predicted**

	Interpretation	Drug	Gene Target (Mutation, Allele %)	Comments
First Line	Resistant	Isoniazid	*katG* (Ser315Thr, 100%)	
		Rifampin	*rpoB* (Ser450Leu, 100%)	Rifabutin resistance likely
	Sensitive	Ethambutol		No mutation detected
		Pyrazinamide		***Expert consultation advised***
Second Line	Resistant	Capreomycin	*rrs* (C1402T, nucleotide 100%)	
		Kanamycin	*rrs* (C1402T, nucleotide 100%)	
		Moxifloxacin	*gyrA* (Ala90Val, 14%)	At least low-level resistance predicted
		Ofloxacin	*gyrA* (Ala90Val, 14%)	
	Sensitive	Amikacin		No mutation detected
		Ethionamide		No mutation detected
		Streptomycin		No mutation detected

Disclaimer

[1]Loci of interest derived from ReSeqTB Data Platform and from Miotto P, et al. Eur Respir J. 2017 PMID: 29284687
Low frequency hetero-resistance below the limit of detection by sequencing may affect typing results. The interpretation provided is based on the current understanding of genotype-phenotype relationships. All results reference the *M. tuberculosis* mutation numbering system which differs from the *E. coli* numbering system.

Authorized By

Name	AUTHORIZER NAME	Position	LAB SUPERVISOR
Signature		Date	2017-JAN-05
Reporting Laboratory	LAB NAME	LAB ADDRESS	LAB PHONE NUMBER

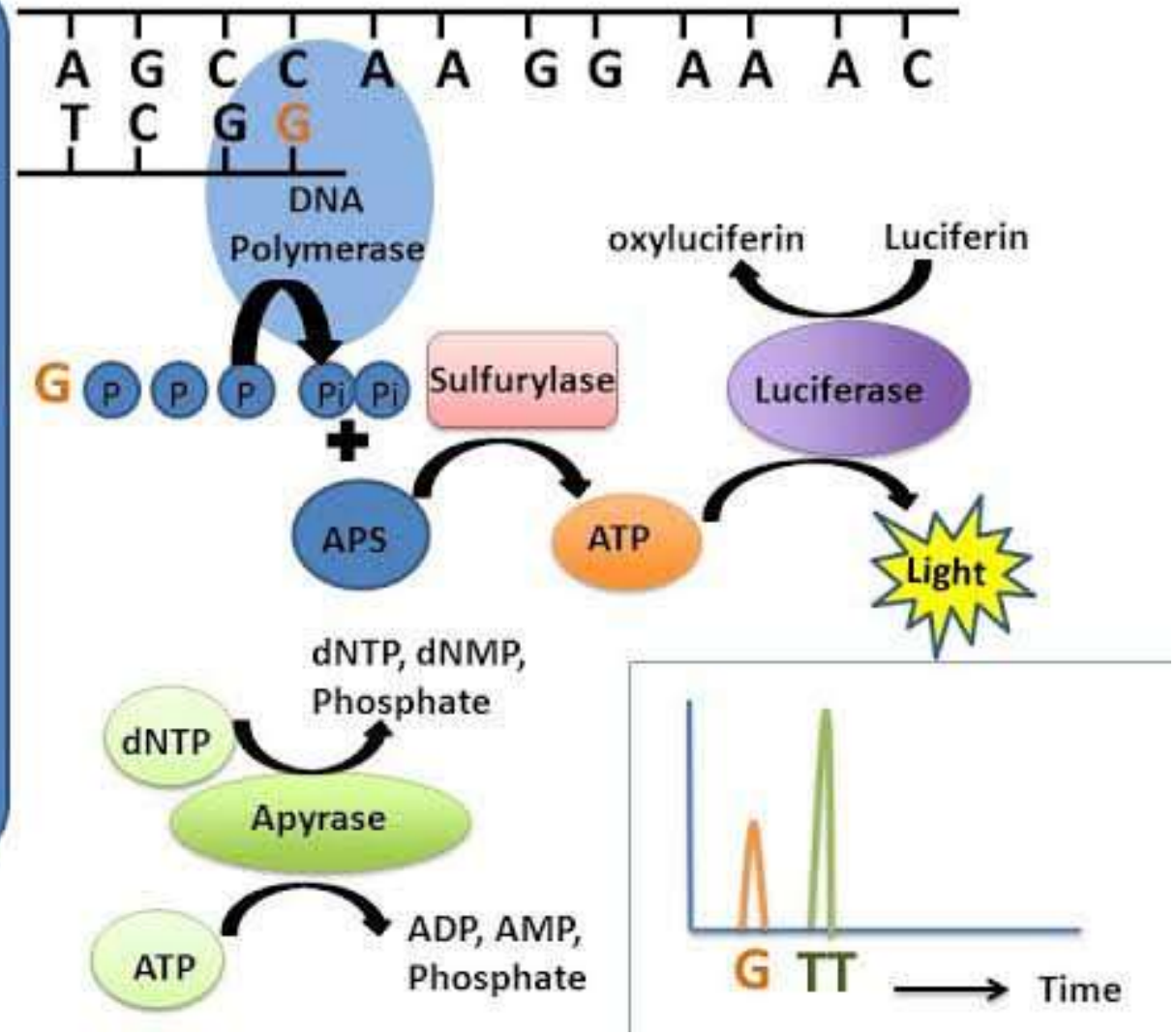
*For demonstration purposes, only.

Pyrosequencing

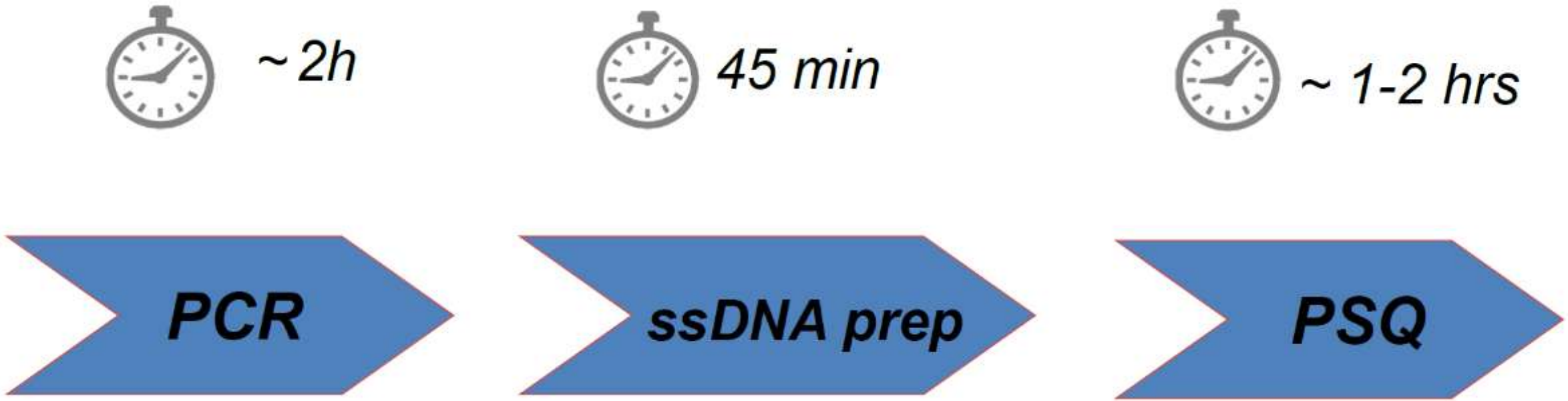
- During DNA strand elongation → addition of nucleotides accompanied by release of pyrophosphates
- Pyrophosphate → ATP synthesis → Luciferin oxidation → Release of light which can be detected as peak
- The nature of the peak determines the nucleic acid sequence

Pyrosequencing

DNA template
DNA polymerase
Primer
dNTP
ATP sulfurylase
Luciferase
Luciferin
Apyrase
APS
(Adenosine 5' phosphosulfate)



Pyrosequencing workflow



Pyrosequencing in DR-TB

- PSQ is a robust, rapid, and high-throughput diagnostic sequencing technique
- Capable of simultaneously detecting M. tuberculosis and the primary canonical mutations conferring phenotypic resistance to INH, RIF, MOX/OFX, AMK, KAN, and CAP
- Highly adaptable allows for the detection of novel mutations within the targeted gene regions through the utilization of specific deoxy nucleoside triphosphate dispensation orders
- Provision of detailed sequence information, allowing users to interpret sequence results based on current and evolving knowledge about phenotypic expression

TABLE 2 Diagnostic performance of PSQ compared to MGIT 960 DST as the reference standard

Drug (*n*)	Phenotypic DST results (no. of isolates)		Accuracy analysis[c]		
	Resistant	Susceptible	% sensitivity	% specificity	% concordance
INH (187)					
Mutations detected	153	1[a]	94 (89-97)	96 (80-99)	94
No mutations	10	23			
RIF (181[b])					
Mutations detected	146	0	96 (92-98)	100 (88-100)	97
No mutations	6	29			
OFX (187)					
Mutations detected	115	0	93 (87-96)	100 (94-100)	95
No mutations	9	63			
MOX (187)					
Mutations detected	114	1	93 (86-96)	98 (92-100)	95
No mutations	9	63			
AMK (187)					
Mutations detected	79	0	84 (75-90)	100 (96-100)	92
No mutations	15	93			
CAP (187)					
Mutations detected	76	3	88 (80-94)	97 (92-99)	93
No mutations	10	98			
KAN (187)					
Mutations detected	79	0	68 (59-76)	100 (95-100)	80
No mutations	37	71			

[a] An isolate with an *inhA* −15T mutation tested as INH susceptible.
[b] For RIF the sensitivity and specificity was calculated after excluding the six isolates with SNPs known to confer only low-level resistance.
[c] The 95% CI is indicated in parentheses where applicable.

Evaluation of pyrosequencing for detecting extensively drug-resistant Mycobacterium tuberculosis among clinical isolates from four high-burden countries. Antimicrob Agents Chemother 59:414 – 420

J Med Microbiol. 2018 Sep;67(9):1212-1216. doi: 10.1099/jmm.0.000669. Epub 2018 Jul 20.

Pyrosequencing: a rapid and effective sequencing method to diagnose drug-resistant tuberculosis.

Govindaswamy A[1], Sakthi D[2], Pai R[2], Jeyaseelan L[3], Michael JS[1].

Author information

Abstract

PURPOSE: This study was undertaken to evaluate the efficiency of the pyrosequencing (PSQ) assay for the rapid detection of resistance to rifampicin (RIF), fluoroquinolones (FQs) and second-line injectables (SLIs) such as capreomycin (CAP) and kanamycin (KAN) in Mycobacterium tuberculosis (Mtb) clinical isolates.

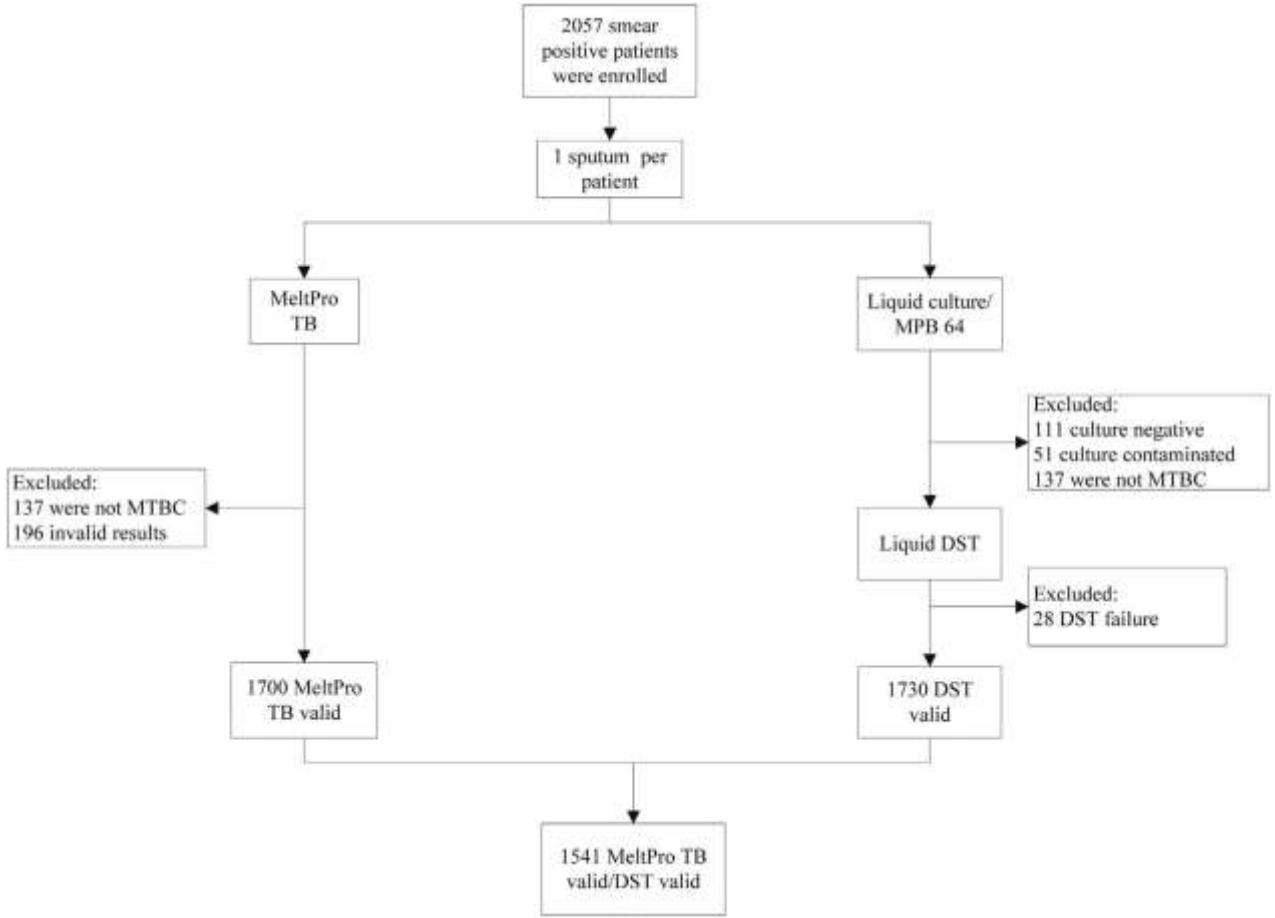
METHODOLOGY: Pyrosequencing is a simple and accurate short read DNA sequencing method for genome analysis. DNA extraction from Mtb clinical isolates was performed using Tris-HCl buffer and chloroform. The rpoB (RIF), gyrA (FQs) and rrs (aminoglycosides) genes were amplified, followed by sequencing using the PyroMark Q24 ID system. The PSQ results were compared with the results from the conventional drug susceptibility testing performed in the laboratory.

RESULTS: The sensitivity of the PSQ assay for the detection of resistance to RIF, FQ, CAP and KAN was 100%, 100%, 40% and 50%, respectively. The specificity of the PSQ assay was 100%.

CONCLUSION: The PSQ assay is a rapid and effective method for detecting drug resistance mutations from Mtb clinical isolates in a short period of time.

Rapid diagnosis of MDR and XDR tuberculosis with the MeltPro TB assay in China

- Principle – based on different melting temperature of DNA with SNPs



Pang *et al* Sci Rep. 2016 May 6;6:25330

Rapid diagnosis of MDR and XDR tuberculosis with the MeltPro TB assay in China

- Sensitivity of the MeltPro TB assay was 94.2% for detectin resistance to rifampicin and 84.9% for detecting resistance to isoniazid
- For second-line drugs, the assay showed a sensitivity of 83.3% for ofloxacin resistance, 75.0% for amikacin resistance, and 63.5% for kanamycin resistance
- MeltPro TB assay demonstrated good performance for the detection of MDR and XDR-TB, with a sensitivity of 86.7% and 71.4%, respectively

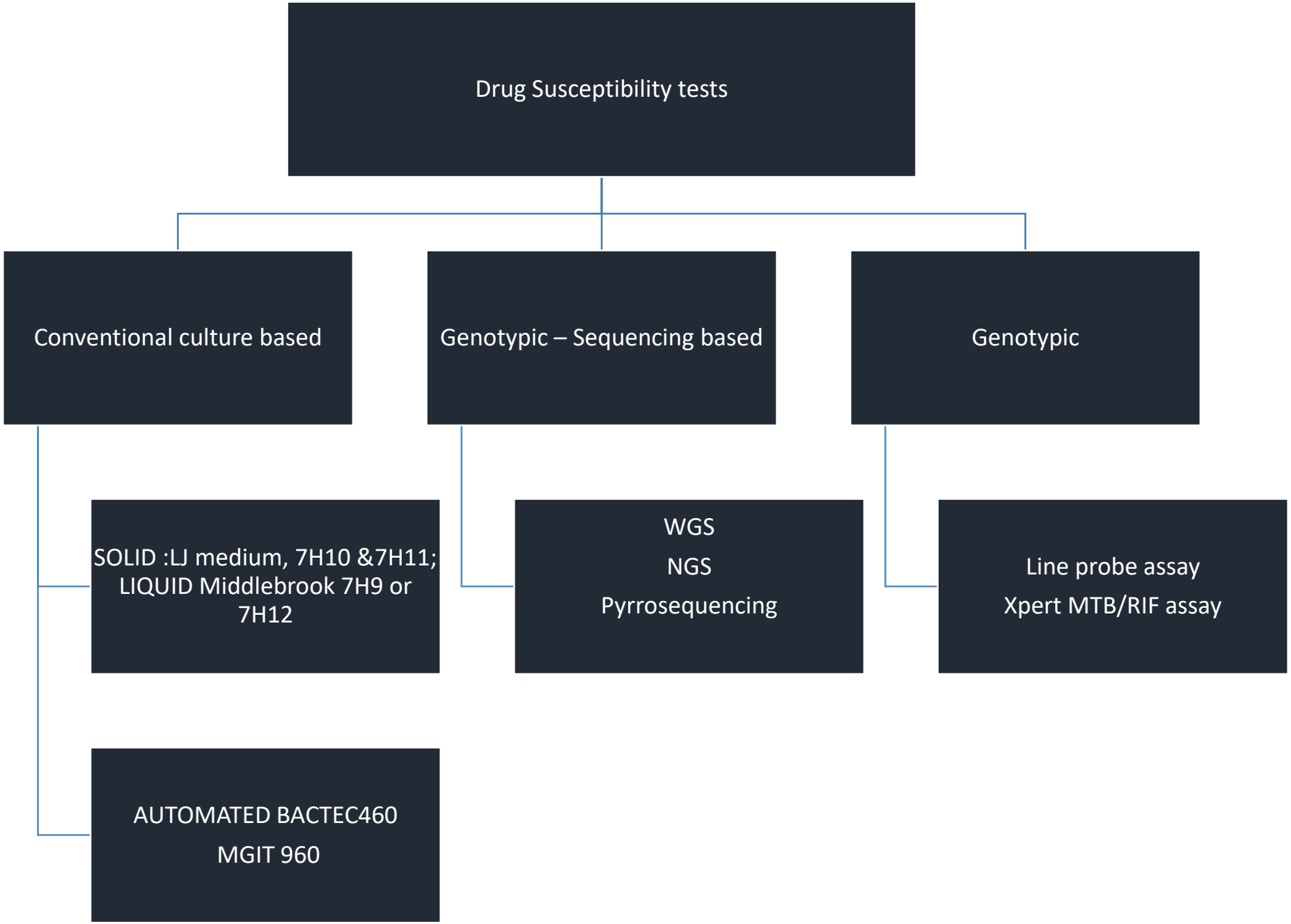
Pang *et al* Sci Rep. 2016 May 6;6:25330

Rapid diagnosis of MDR and XDR tuberculosis with the MeltPro TB assay in China

Diagnostic tools	Reference	Target sequence(s)	Performance[a]				Turnaround time (hours)	Need for special equipment	Cost per kit (USD)[b]
			RIF		INH				
			Sensitivity (%)	Specificity(%)	Sensitivity(%)	Specificity(%)			
GeneXpert	35	RIF: *rpoB*	95	98	NA	NA	2.5	Yes	45
Genechip	8	RIF: *rpoB*; INH: *katG*, *inhA* promoter	87.6	98.0	80.3	95.8	6	Yes	30
GenoType MTBDR V1.0	36	RIF: *rpoB*; INH: *katG*, *inhA* promoter	97.1	97.1	94.4	96.4	6	Yes	30
GenoType MTBDR V2.0	36	RIF: *rpoB*; INH: *katG*, *inhA* promoter	98.2	97.8	95.4	98.9	6	Yes	NA
MeltPro	Data from this study	RIF: *rpoB*; INH: *katG*, *inhA*, *oxyR-ahpC intergenic region*, *inhA* promoter	94.2	97.5	84.9	98.0	3.5	No	25

Table 9. Comparison of the performance and cost of various molecular diagnostic tools. [a]RIF: rifampicin; INH: isonizid; NA: not available. [b]Costs are calculated according to the market price for clinical practice in China.

Pang *et al* Sci Rep. 2016 May 6;6:25330


Drug Susceptibility tests
Conventional culture based
Genotypic – Sequencing based
Genotypic
SOLID :LJ medium, 7H10 &7H11; LIQUID Middlebrook 7H9 or 7H12
AUTOMATED BACTEC460
MGIT 960
WGS
NGS
Pyrrosequencing
Line probe assay
Xpert MTB/RIF assay

Xpert MTB/RIF

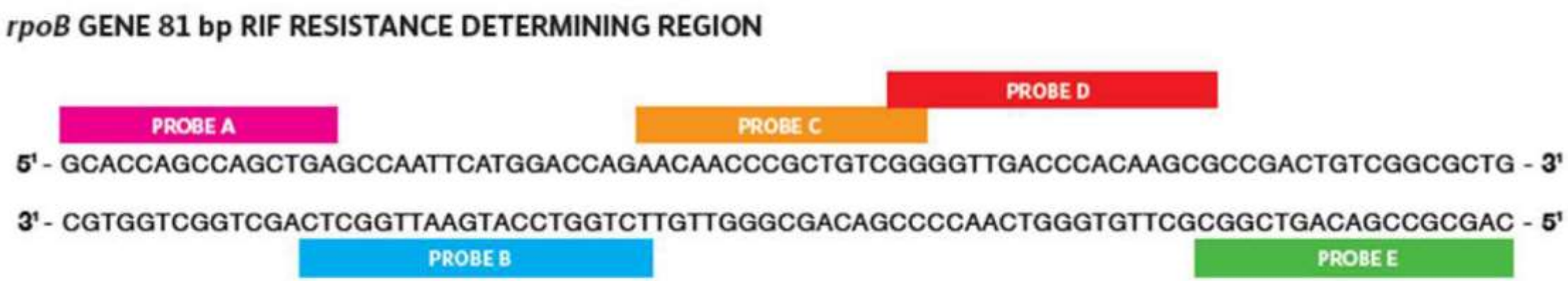
- NAAT to diagnose and detect rifampicin resistance
- Xpert uses a hemi-nested PCR to amplify the rifampin resistance-determining region (RRDR) of the *M. tuberculosis rpoB* gene
- Purifies and concentrates MTB from sputum samples isolates genomic material from the captured bacteria by sonication and subsequently amplifies the genomic DNA by PCR

WHO Policy: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children

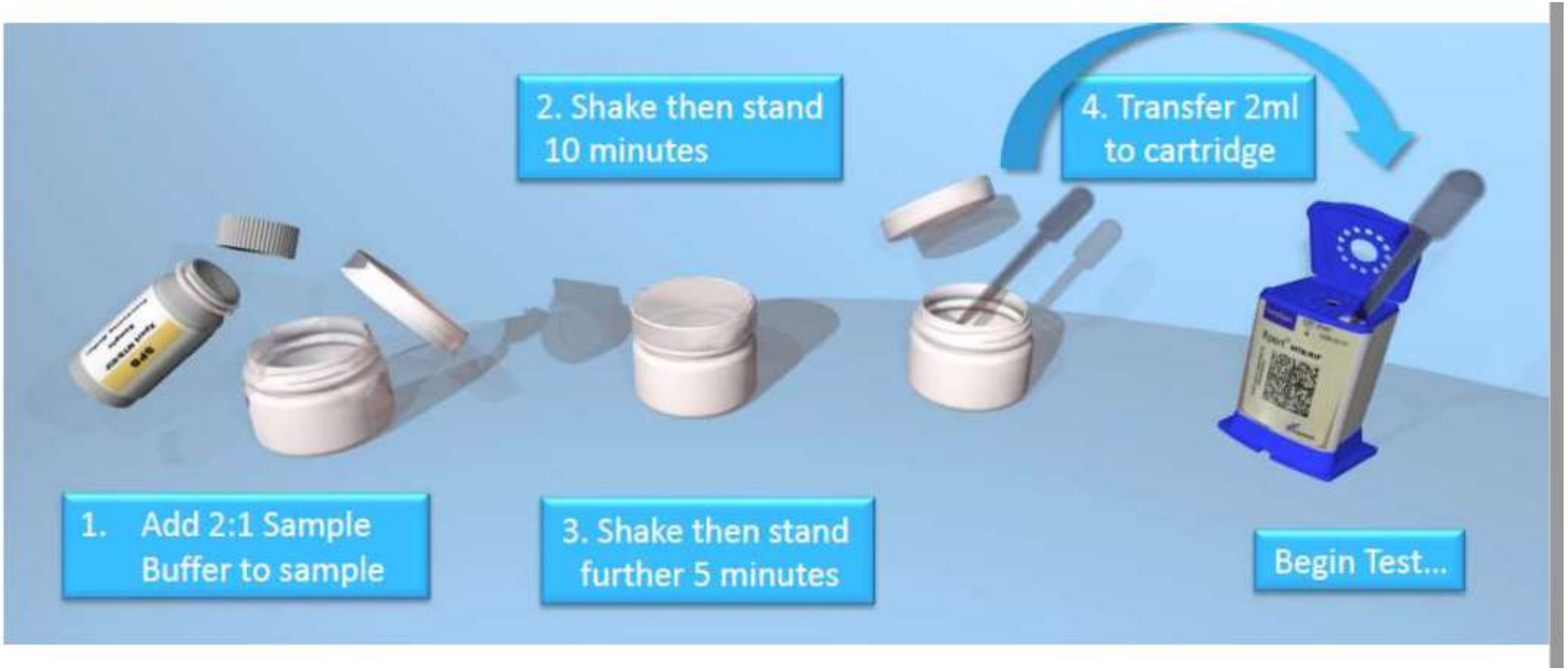
Nested PCR

- Used to increase the specificity of DNA amplification
- Two sets of primers are used in two successive reactions
- In the first PCR, one pair of primers is used to generate DNA products, which will be the target for the second reaction
- Using one (hemi-nesting) or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity

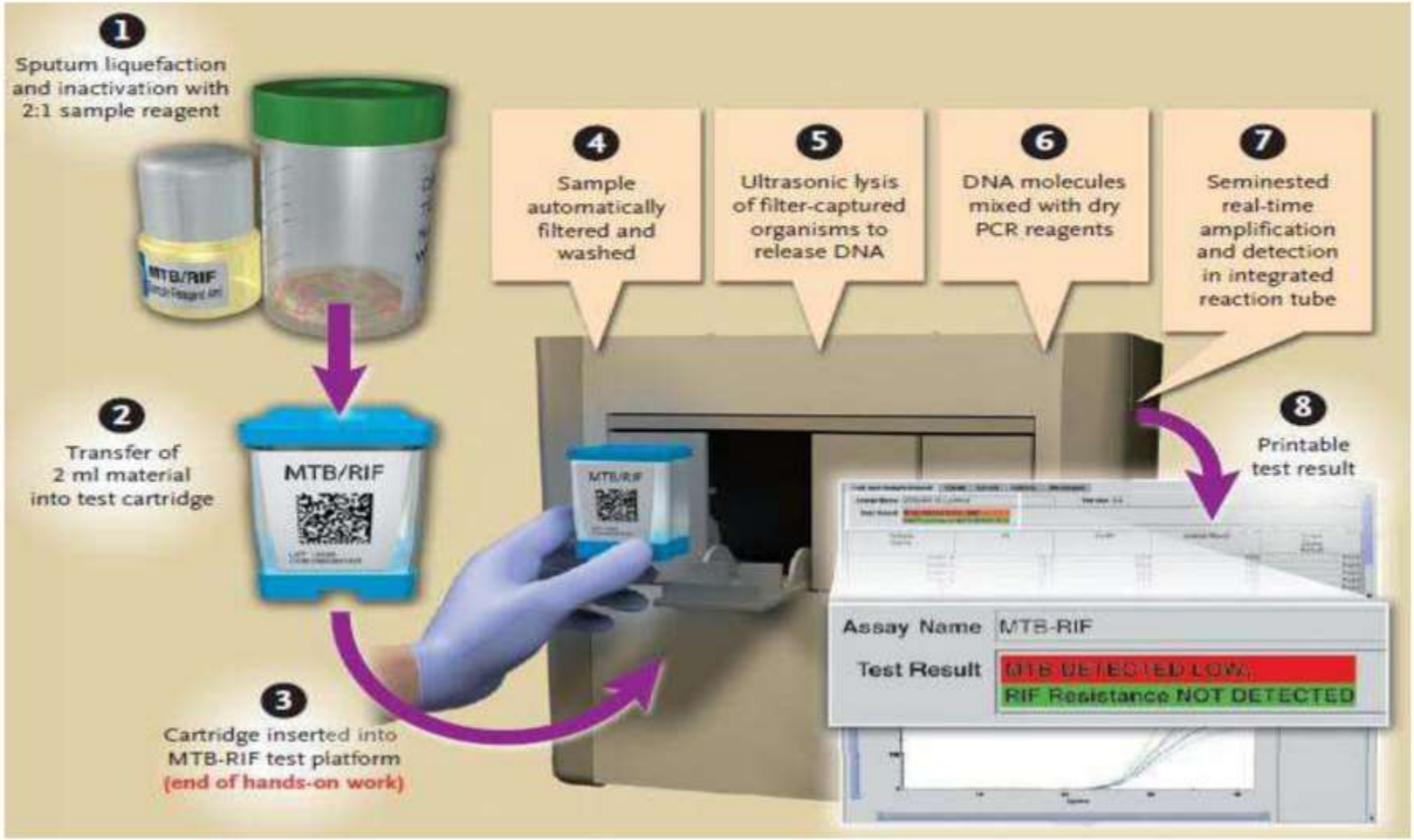
Xpert MTB/RIF



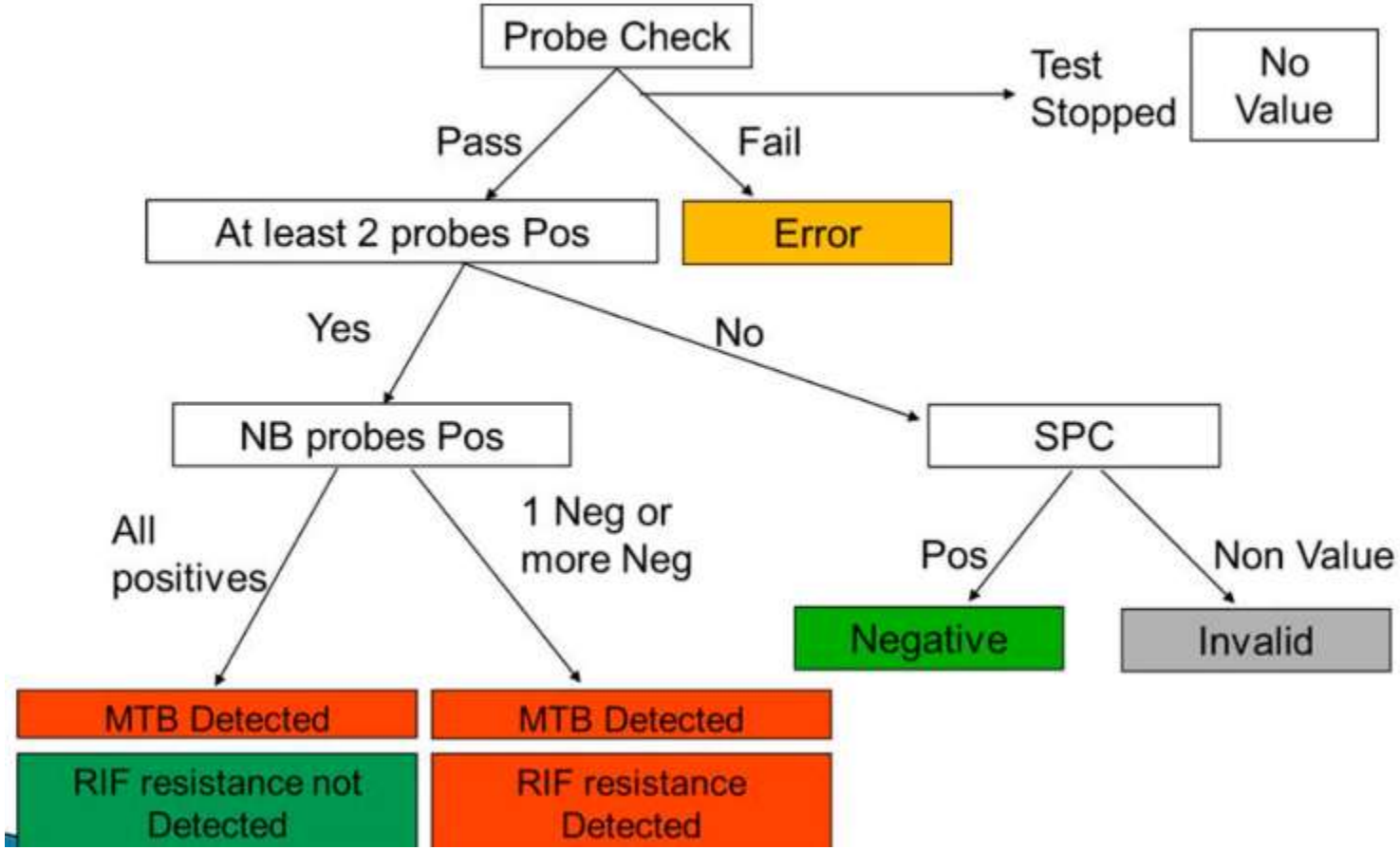
Processing sputum for XpertMTB/RIF



Procedure: Xpert MTB/RIF

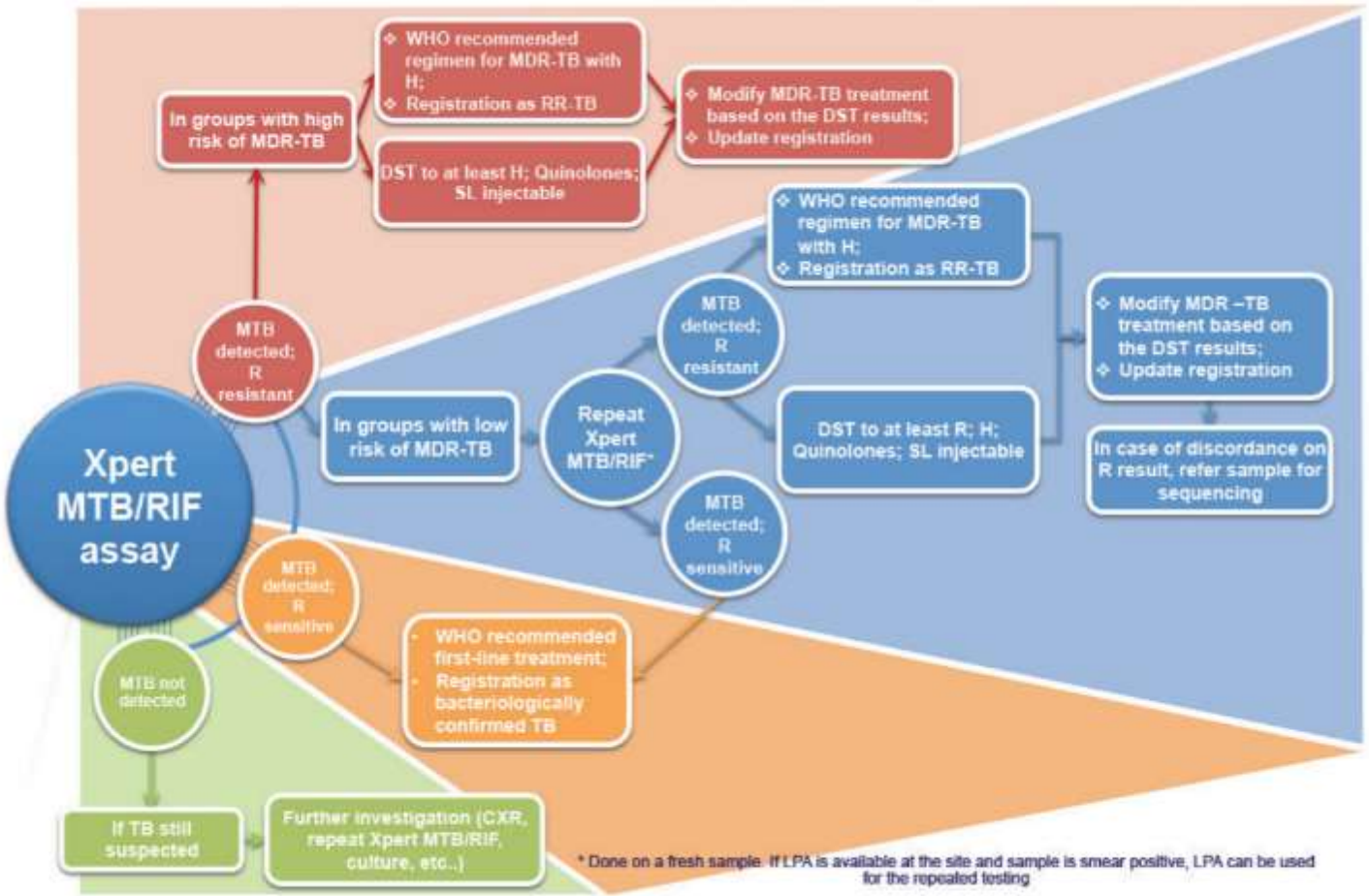


Interpreting result



Interpreting result: Xpert MTB/RIF

Figure 1. Interpreting results from Xpert MTB/RIF tests



CXR [chest X-ray], DST [drug-susceptibility testing], H [isoniazid], LPA [line probe assay], MDR-TB [multidrug-resistant TB], MTB [Mycobacterium tuberculosis], R [rifampicin], RR-TB [rifampicin-resistant TB]

Considerations for MTB/RIF Assay

Advantages

- Self-contained, single-use, semi-quantitative
- disposable cartridges
- Cross-contamination among samples is reduced hands on time is minimal
- Limited biosafety concerns
- Results within 2 hours of specimen collection

Disadvantages

- Specific mutations are not provided
- Silent mutations could lead to false-resistance
- Potential low positive predictive value in low prevalence populations
- Confirmatory testing needed
- Heteroresistance may be missed

Using Xpert MTB/RIF to diagnose pulmonary TB and detect RR

	Sensitivity %	Specificity %
Smear-positive culture-positive TB	98%	>99%
Smear-Negative culture-positive TB	68%	99%
Initial diagnostic test replacing smear microscopy	88%	99%
RR Detection	95%	98%

WHO Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children

Using Xpert MTB/RIF to diagnose extra pulmonary TB

Table 1. Meta-analysis of the sensitivity and specificity of Xpert MTB/RIF in diagnosing extrapulmonary TB and rifampicin resistance in adults and children compared against culture as a reference standard as well as against a composite reference standard, by type of extrapulmonary specimen

Specimen type	Comparison (No. of studies, No. of samples)	Median (%) pooled sensitivity (pooled 95% CrI)	Median (%) pooled specificity (pooled 95% CrI)
Lymph node tissue and aspirate	Xpert MTB/RIF compared against culture (14 studies, 849 samples)	84.9 (72–92)	92.5 (80–97)
	Xpert MTB/RIF compared against a composite reference standard (5 studies, 1 unpublished)	83.7 (74–90)	99.2 (88–100)
Cerebrospinal fluid	Xpert MTB/RIF compared against culture (16 studies, 709 samples)	79.5 (62–90)	98.6 (96–100)
	Xpert MTB/RIF compared against a composite reference standard (6 studies, 512 samples)	55.5 (51–81)	98.8 (95–100)
Pleural fluid	Xpert MTB/RIF compared against culture (17 studies, 1385 samples)	43.7 (25–65)	98.1 (95–99)
	Xpert MTB/RIF compared against a composite reference standard (7 studies, 698 samples)	17 (8–34)	99.9 (94–100)
Gastric lavage and aspirate	Xpert MTB/RIF compared against culture (12 studies, 1258 samples)	83.8 (66–93)	98.1 (92–100)
Other tissue samples	Xpert MTB/RIF compared against culture (12 studies, 699 samples)	81.2 (68–90)	98.1 (87–100)

CrI, credible interval; the CrI is the Bayesian equivalent of the confidence interval.

Xpert MTB/RIF Ultra

- Uses the same platform as Xpert MTB/RIF

	Xpert MTB/RIF	XPERT MTB/RIF Ultra
Amplification for TB detection	Single target : rpoB core region	Multi-copy target : rpoB core region Insertion sequence : IS6110, IS1081
Resistance detection	5 probes bind to rpoB gene	4 probes bind to rpoB gene
PCR reaction	25ul	50ul
Assay TAT	112 min	65-87min
LOD	131 cfu/ml	16cfu/ml

WHO Meeting Report of a Technical Expert Consultation: Non-inferiority analysis of Xpert MTB/RIF Ultra compared to Xpert MTB/RIF

Performance of Ultra

	Tuberculosis detection*					Detection of rifampicin resistance†	
	Sensitivity: all culture-positive (95% CI; n/N)	Sensitivity: smear-negative (95% CI; n/N)	Sensitivity: HIV-negative (95% CI; n/N)‡	Sensitivity: HIV-positive (95% CI; n/N)‡	Specificity (95% CI; n/N)	Sensitivity (95% CI; n/N)	Specificity (95% CI; n/N)
Xpert	83% (79 to 86; 383/462)	46% (37 to 55; 63/137)§	90% (84 to 94; 143/159)	77% (68 to 84; 88/155)	98% (97 to 99; 960/977)	95% (91 to 98; 167/175)	98% (96 to 99; 369/376)
Xpert Ultra	88% (85 to 91; 408/462)	63% (54 to 71; 86/137)§	91% (86 to 95; 145/159)	90% (83 to 95; 103/115)	96% (94 to 97; 934/977)	95% (91 to 98; 166/175)	98% (97 to 99; 370/376)
Difference (Xpert Ultra minus Xpert)	5·4% (3·3 to 8·0; 25/162)	17% (10 to 24; 23/137)	1·3% (−1·8 to 4·9; 2/159)	13% (6·4 to 21; 15/115)	−2·7% (−3·9 to −1·7; 36/977)	−0·6% (−3·2 to 1·6; 1/175)	0·3% (−0·7 to 1·5; 1/376)
Non-inferiority margin	Not predefined	−7%	Not predefined	Not predefined	Not predefined	−3%	−3%

Results are based on initial testing of the first sample with Xpert MTB/RIF and Xpert MTB/RIF Ultra (Xpert Ultra) assays. Uninterpretable results (contaminated cultures or non-determinate Xpert or Ultra results) were excluded from the analysis. Culture contamination averaged 4·3–7·8%, depending on sample and culture type. Non-determinate results (invalid, error, no result) are reported in the main text. Sensitivities of Xpert and Xpert Ultra for detection of smear-positive tuberculosis (n=323) were 99% (95% CI 97–100) and 99% (97–100). *Accuracy for tuberculosis detection was estimated in study participants in the case detection group. Patients with unknown HIV-infection status are excluded from analyses stratified by HIV status but included in all other analyses. †Accuracy for detection of rifampicin resistance was estimated in all study participants with available drug susceptibility test results and valid rifampicin resistance results for both Xpert and Xpert Ultra. ‡Data on HIV-infection status were not available for 188 culture-positive and 336 culture-negative study participants. Sensitivity of Xpert and Xpert Ultra in study participants with missing HIV status was 81% and 85%, respectively. Note that the estimate for pooled sensitivity of Xpert Ultra irrespective of HIV status does not fall between the estimates for HIV-infected and HIV-uninfected individuals. §Accuracy estimates are based on the reference standard as defined in the Methods section (using four cultures to define tuberculosis); using a less stringent reference standard with only one liquid and one solid culture (both from sputum sample 2), which is similar to the reference standard used in 21 of 22 studies included in the most recent Cochrane systematic review of the Xpert assay,[4] resulted in Xpert sensitivity for smear-negative tuberculosis of 73% (Cochrane review pooled estimate 67%) and Xpert Ultra sensitivity of 84% (appendix p 5).

Table 2: **Comparative accuracy for detection of tuberculosis and rifampicin resistance**

Lancet Infect Dis 2018; 18: 76–84

WHO meeting report of a technical expert consultation: Non-inferiority analysis of Xpert MTB/RIF Ultra vs Xpert MTB/RIF

- 1,520 persons with signs and symptoms of TB were enrolled

- Overall, sensitivity of the Ultra was 5% higher than that of Xpert MTB/RIF (95%CI +2.7, +7.8), but Specificity was 3.2% lower (95%CI -2.1, -4.7)

- Sensitivity increases were highest among smear-negative culture- positive patients and among HIV infected patients (61.3% vs 44.5% and 87.5% vs 61.3% respectively)
- CSF (95% vs 60%); Gastric aspirates (71% vs 50%)

Xpert MTB/RIF Ultra

- Ultra has higher sensitivity in smear negative patients, HIV patients, EPTB and CSF specimens
- Specificity lower than Xpert MTB/RIF
- Rifampicin resistance - Ultra has both high sensitivity and specificity
- Better differentiation of resistance causing mutations from silent ones
- Lower specificity - limitations of the reference standard (culture) used to compare the two versions of the molecular assays Ultra may be more prone to detecting small numbers of non-replicating or non-viable bacilli present in patients with a recent history of TB treatment, reducing the specificity of the assay

WHO Meeting Report of a Technical Expert Consultation: Non-inferiority analysis of Xpert MTB/RIF Ultra compared to Xpert MTB/RIF

Line probe assay

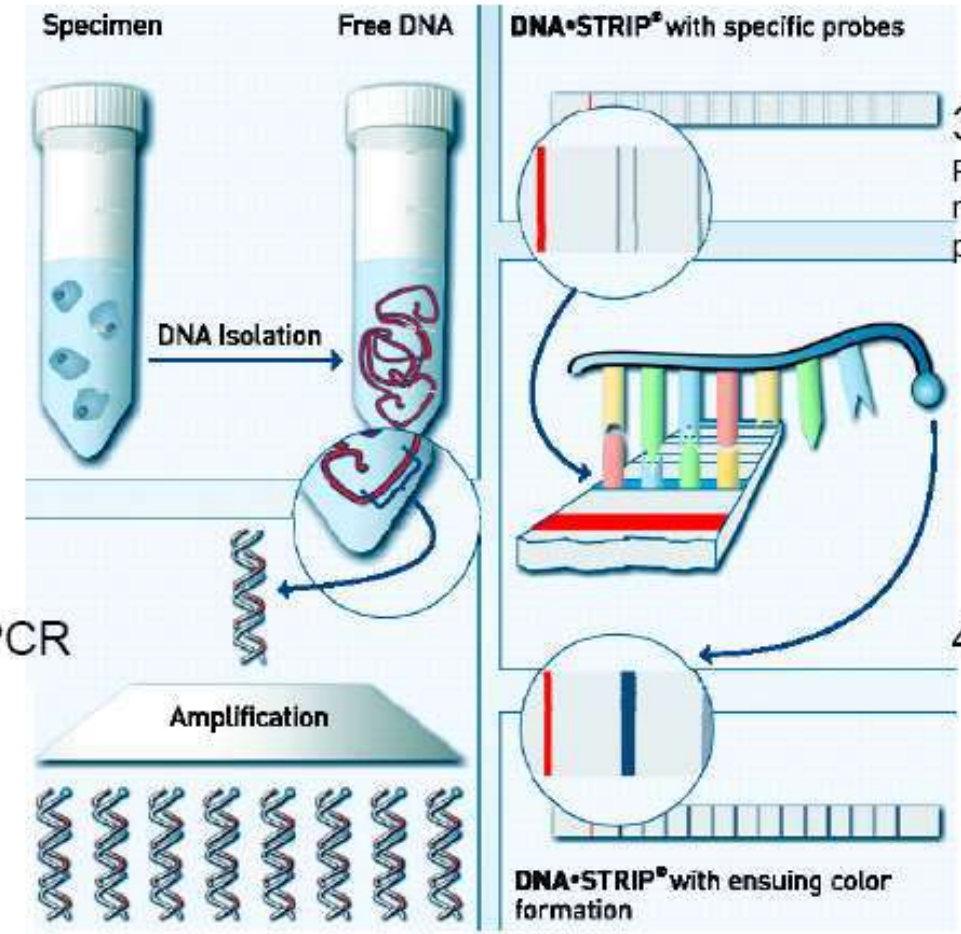
- LPA technology involves the following steps:
  - DNA is extracted from M. tuberculosis isolates (indirect testing) or directly from clinical specimens (direct testing)
  - PCR amplification of the resistance-determining region of the gene under question is performed using biotinylated primers
  - Following amplification, labeled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip
  - Captured labeled hybrids are detected by colorimetric development, enabling detection of the presence of M. tuberculosis complex, as well as the presence of wild-type and mutation probes for resistance

Line probe assay

- If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe
- Mutations are detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations
- The post hybridization reaction leads to the development of coloured bands on the strip at the site of probe binding

GenoType® MTBDR*plus* test procedure

1) DNA extraction



2) Amplification by PCR

3) Hybridization

Reverse hybridization of amplified nucleic acids to specific DNA probes bound on strips

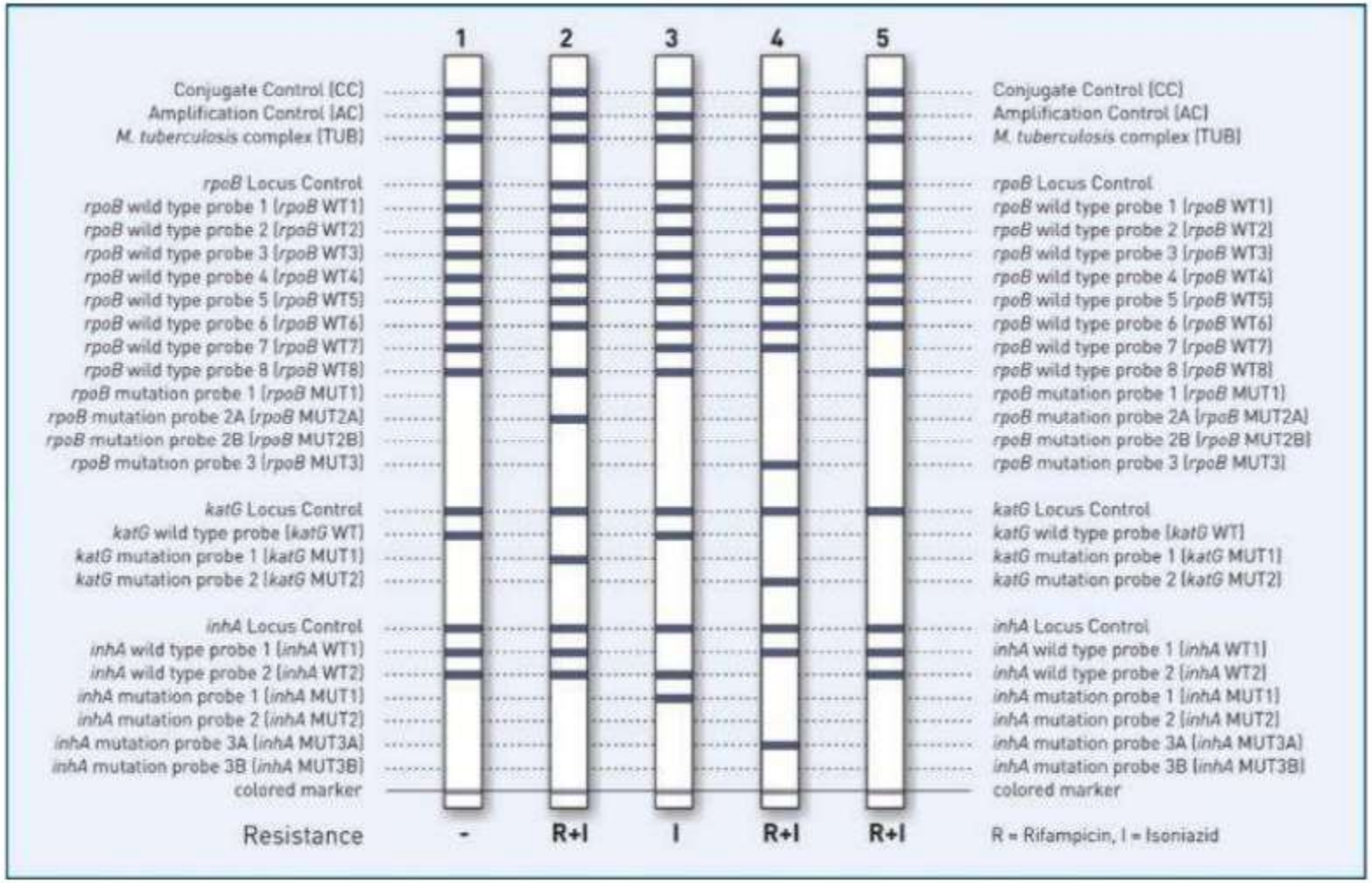
4) Evaluation

HAIN LIFESCIENCE

Available LPA platforms

	Mutations detected	Drug
INNO-LiPA Rif.TB	rpoB	Rif
Genotype MTBDR	rpoB, KatG	Rif INH
Genotype MTBDRplus	rpoB, KatG inhA	Rif INH INH
Genotype MTBDRsl v1	rrs gyrA EMB	SLID FQ Ethambutol
Genotype MTBDRsl v2	rrs, eis gyrA, gyrB	SLID FQ

FL LPA result strip



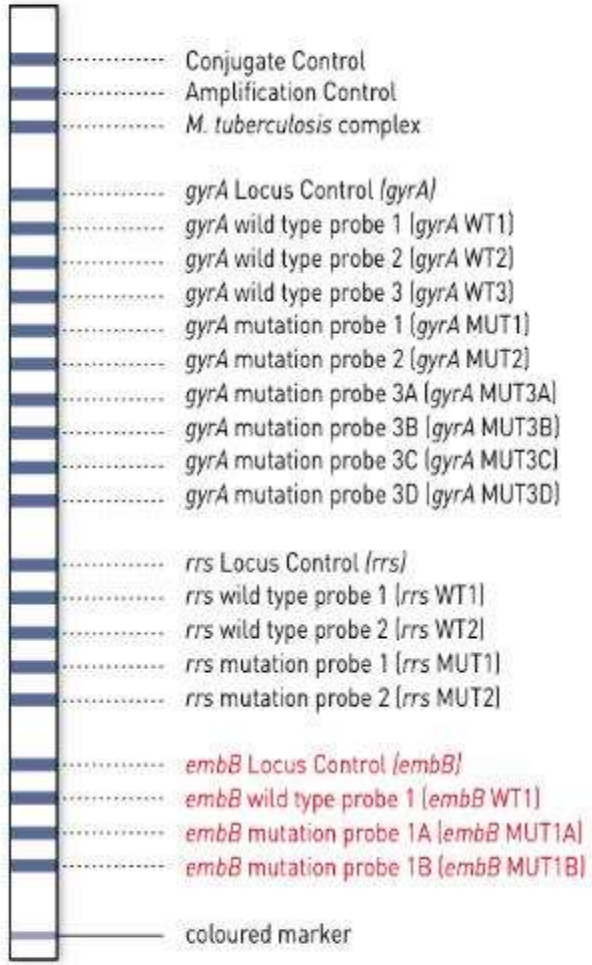
Performance of FL LPA (Version 1 & 2)

8		Sensitivity	Specificity
RR (direct)	Hain Ver 1 Hain Ver 2	97.1% 98.2%	97.1% 97.8
RR (indirect)	Hain Ver 1 Hain Ver 2	91.3% 91.3%	97.1% 97.1%
INH R (direct)	Hain Ver 1 Hain Ver 2	94.4% 96.4%	97.1% 97.1%
INH R (indirect)	Hain Ver 1 Hain Ver 2	89.4% 89.4%	98.9% 98.9%

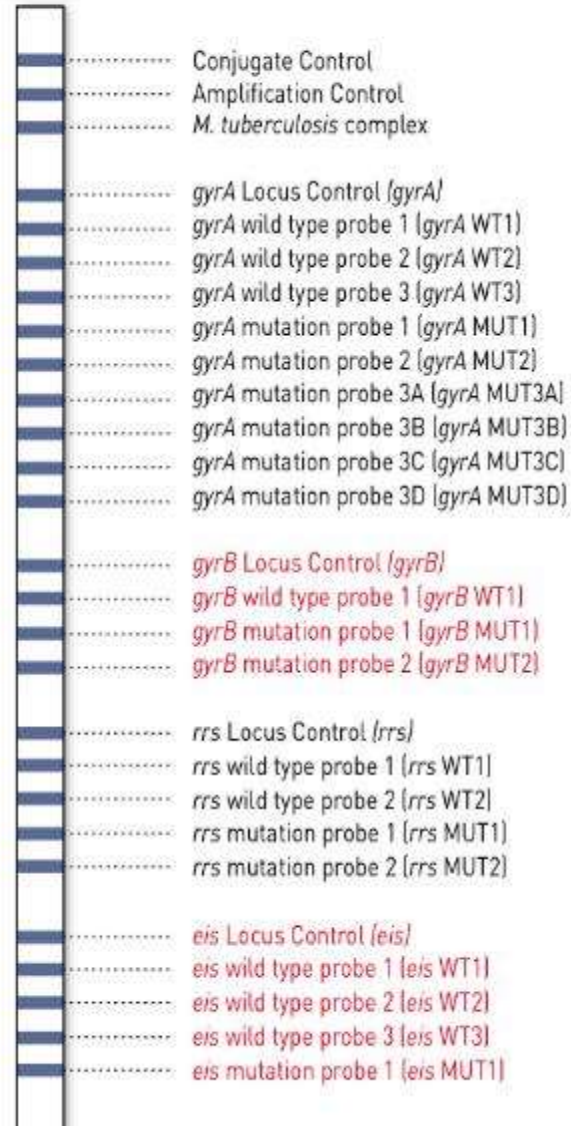
Second line LPA

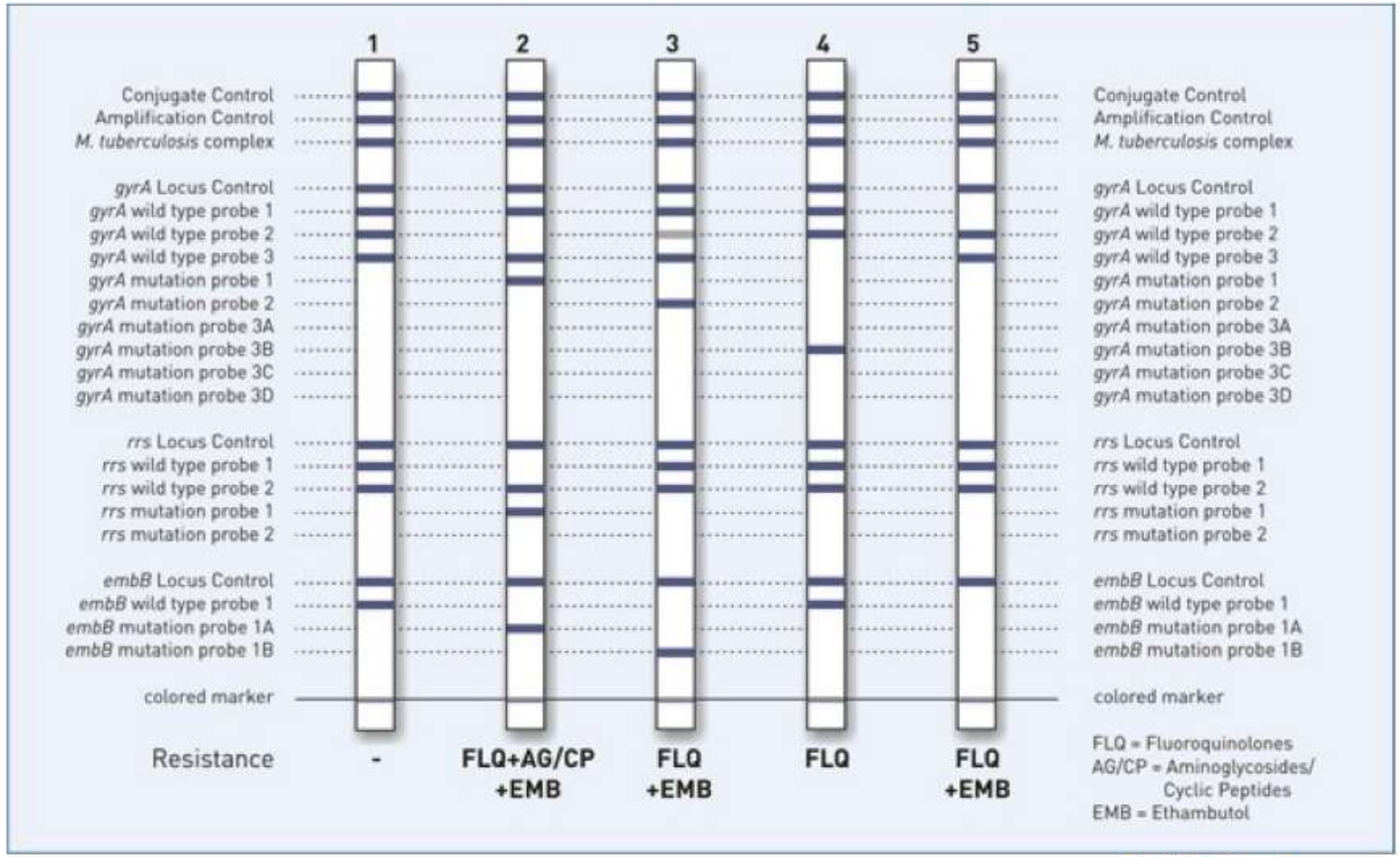
	Genotype MTBDRsl ver 1.0	Genotype MTBDRsl ver 2.0
Detection of	MTBC, resistance to FQ, Aminoglycosides and **ETHAMBUTOL**	MTBC, resistance to FQ, Aminoglycosides
Sample material	Smear **+** pulmonary and cultivated samples	Smear **+ and -** pulmonary and cultivated samples
Ethambutol resistance	Detects **embB** gene mutations	-
FQ resistance	gyrA	Detects gyrA and **gyrB** gene mutations
Kanamycin resistance	rrs	Detects rrs and **eis** gene mutation

GenoType **MTBDRsl** VER 1.0



GenoType **MTBDRsl** VER 2.0





FIND
FOUNDATION FOR INNOVATIVE NEW DIAGNOSTICS

Performance of MTBDRsl version 1 (smear +ve)

Pooled sensitivity (95% CI)	Pooled specificity (95% CI)	Pooled sensitivity (95% CI)	Pooled specificity (95% CI)
Fluoroquinolones, indirect testing (19 studies, 2 223 participants)		**Fluoroquinolones, direct testing** (9 studies, 1 771 participants)	
85.6% (79.2 to 90.4)	98.5% (95.7 to 99.5)	86.2% (74.6 to 93.0)	98.6% (96.9 to 99.4)
Second-line injectable drugs, indirect testing (16 studies, 1 921 participants)		**Second-line injectable drugs, direct testing** (8 studies, 1 639 participants)	
76.5% (63.3 to 86.0)	99.1% (97.3 to 99.7)	87.0% (38.1 to 98.6)	99.5% (93.6 to 100.0)
XDR-TB, indirect testing (8 studies, 880 participants)		**XDR-TB, direct testing** (6 studies, 1 420 participants)	
70.9% (42.9 to 88.8)	98.8% (96.1 to 99.6)	69.4% (38.8 to 89.0)	99.4% (95.0 to 99.3)

Performance of MTBDRsl version 2.0

Direct testing		sensitivity	specificity
FQ resistance	Smear +	100%	100%
	Smear -	100%	90%
SLID resistance	Smear+	62%	91%
	smear -	83%	78%
XDR-TB	Smear+	100%	100%
	Smear -	100%	90%

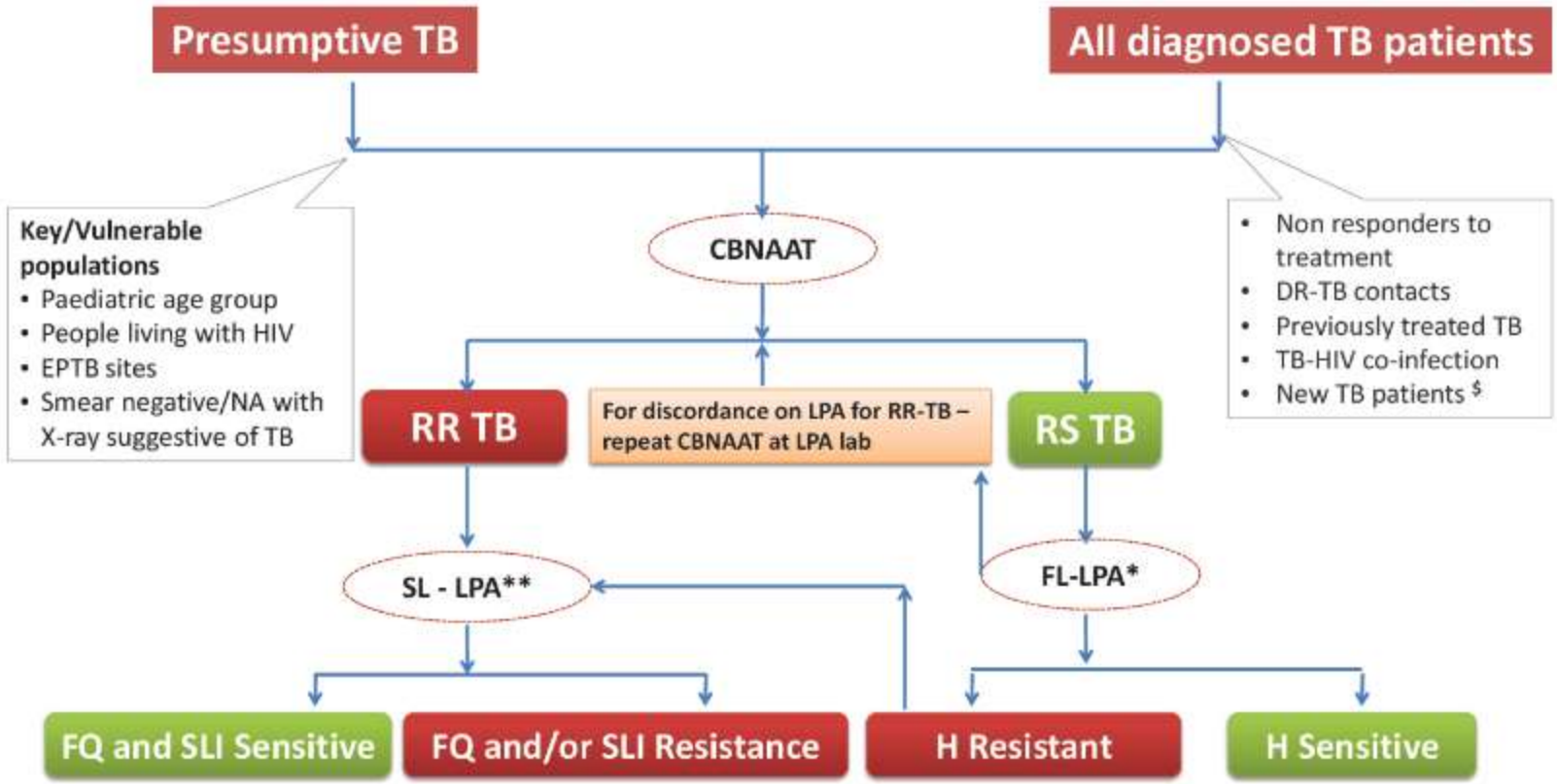
Indirect testing	sensitivity	specificity
FQ resistance	84-100%	99-100%
SLID resistance	72-89%	90-99%
XDR-TB	75-80%	91-100%

The use of molecular LPAs for the detection of resistance to second line ATT drugs: WHO policy guidance 2016

Line probe assays

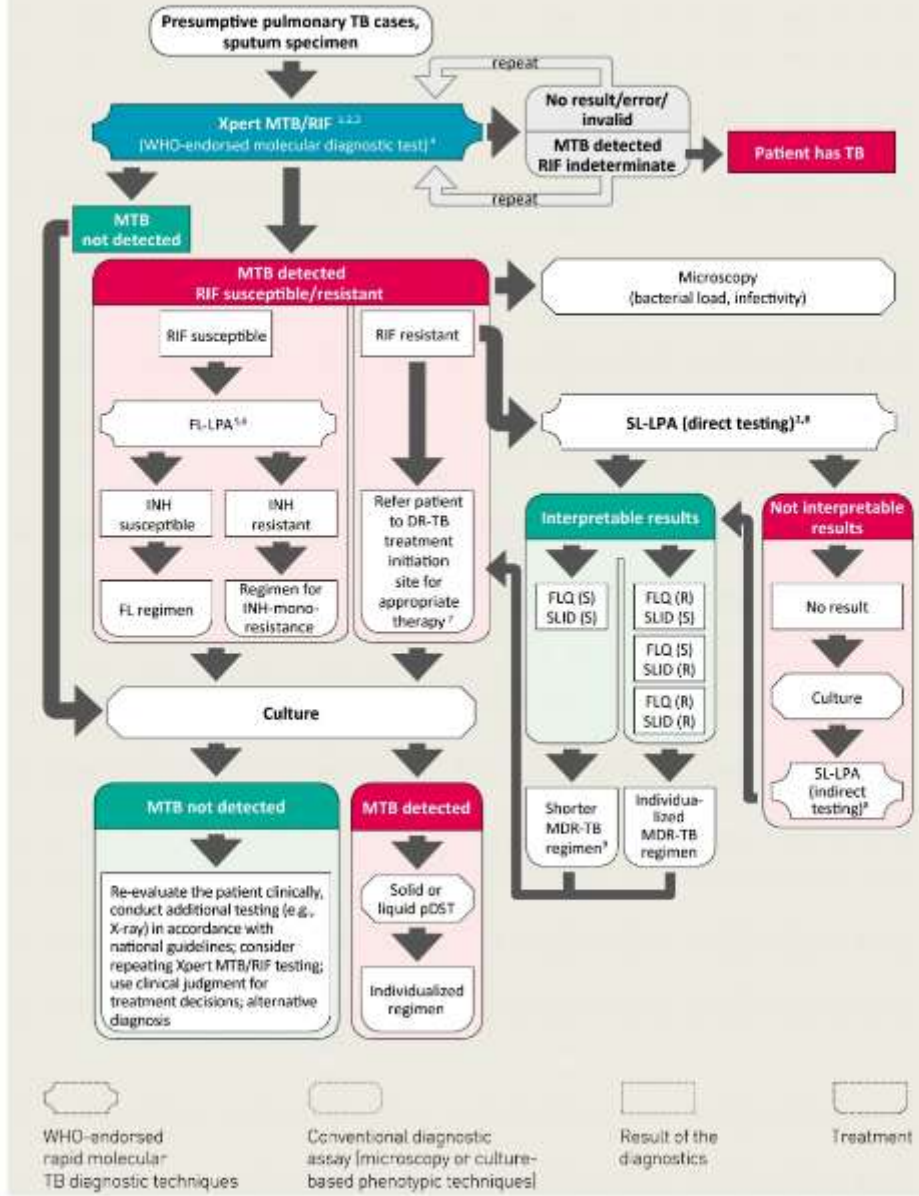
- Merits :
  - Lower contamination rates than culture
  - Detects resistance genes for Rifampicin INH FQ SLID
  - Results available in 48-72 hours
- Demerits :
  - Requires skilled man power
  - Specialized equipment
  - Dedicated space to avoid cross-contamination between specimens
  - Manual processing of specimen
  - Complexity & no. of steps preclude use in peripheral settings
  - Do not perform well on pauci-bacillary specimen indeterminate results with smear negative

Figure 5.2 DR-TB Diagnostic Algorithm



*Offer molecular testing for H mono/poly resistance to TB patients prioritized by risk as per the available lab capacity
**LC DST (Mfx 2.0, Km, Cm, Lzd) will be done only for patients with any resistance on baseline SL-LPA. DST to Z, Cfz, Bdq & Dlm would be considered for policy in future, whenever available, standardized & WHO endorsed.
$ States to advance in phased manner as per PMDT Scale up plan for universal DST based on lab capacity and policy on use of diagnostics

Fig. 1. Algorithm for the initial laboratory diagnosis of individuals with symptoms consistent with pulmonary TB



Where do sequencing techniques fit in?

- To be set up at reference laboratories
- Surveillance – NDRS – utilised culture and phenotypic DST
- Discordant cases, SL LPA/ FL LPA inconclusive
- Patients not responsive despite phenotypic or genotypic drug susceptibility confirmed
- Ultra – trace calls in whom MDR/XDR status cannot confirmed despite repeat testing

Take home message(s)

- Conventional DST (phenotypic) are imperfect gold standards
- The number of MDR TB cases diagnosed and the number started on appropriate therapy are alarmingly discordant, cause of concern may lead to health care exigency if not addressed
- Molecular methods that are economical, have rapid turn around times, and that can provide complete information about the mutations causing phenotypic resistance are required to bridge the gap and personalise therapy
- Gene sequencing can now characterize profiles of susceptibility to anti tuberculosis drugs with great degree of accuracy sufficient for clinical use but cost and lack of technical expertise limits use especially in low-middle income countries
- Xpert Ultra is more sensitive less specific for diagnosis equal to Xpert for RR
- LPA is only feasible rapid test in armamentarium for rapid diagnosis of XDR but needs fine tuning for smear negative specimens

Test for MDR TB diagnosis	Sensitivity RIF	Specificity RIF	Sensitivity INH	Specificity INH	Turn around time	Cost	Comments
Phenotypic DST	-	-	-	-	80d	5-10 USD	Comparison
Xpert/MTB RIF	95%	98%	-	-	2.5 hours	40USD	Diagnosis and RR status in 2 hrs
Xpert Ultra	95%	98%	-	-	1.5 hours	40USD	High sensitivity low specificity
LPA FL	Direct 97% Indirect 91%	Direct 97% Indirect 97%	Direct 94% Indirect 89%	Direct 95% Indirect 98%	6 hours	30USD	Only on sputum + or culture positive isolates Poor - paucibacillary
Sequencing	97.5%	98.8%	97.1%	99%	12-55 hours	150 USD	All mutations benign or resistance causing mutations picked up
Pyrosequencing	96%	100%	94%	96%	6 hours	80-100USD	
Meltpro assay	94.2%	97.5%	84.9%	98%	3.5 hours	25 USD	Cheap rapid high sen and specificity