SATREPS project:

CONTROL OF TUBERCULOSIS AND GLANDERS

Activity report from the Research Institute of Tuberculosis

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Objectives

- To isolate *Mycobacterium tuberculosis* var. *bovis* from human with MGIT and Löwenstein-Jensen (L-J glycerol and pyruvate) in the national tuberculosis reference laboratory in National Centre for Communicable Disease (NCCD)
- To identify *M. tuberculosis* var. *tuberculosis* and perform drug susceptibility testing (DST) including newly introduced drugs for new WHO regimens
- To introduce next generation sequencing technology into NCCD laboratory
- To perform genotyping of *M. tuberculosis* in NCCD laboratory
- To develop standard operational procedure (SOP) to identify *M. tuberculosis* variants
- To perform risk assessment of *M. bovis* infection among Mongolian people based on the isolation from clinical specimens
- To perform TB screening with LAMP method and IGRA test to workers in slaughterhouses
- To train NCCD trainees for next generation sequencing technologies in RIT

Implementations

The Research Institute of Tuberculosis (RIT) has implemented the followings

1. Regular on-line meeting
   - Regular on-line meetings with mainly three members (Dr. Buyankhishig, Dr. Oyuntuya and Prof. Mitarai) were held since 2020. The progresses and problems of the project was discussed in the meetings.
2. Standard operational procedure development
   - Standard operational procedures development is one of the key components of this technical transfer programme. Three different types of SOPs are developed until now.
3. Overseas trainings
   - WGS practical trainings (sequencing and bioinformatics)
   - IGRA training (ELISA)
4. On-site trainings
   - WGS device installation and practices
   - IGRA test practices
5. Molecular technical development in RIT
   - Targeted NGS for Oxford Nonopore Technologies (ONT)
   - Direct sequencing technology development from clinical specimen

2.1 SOP for Löwenstein-Jensen (L-J glycerol and pyruvate)

Though *Mycobacterium tuberculosis* var. *bovis* (*M*. *bovis*) grows on normal Löwenstein-Jensen (L-J) medium with glycerol as carbon source, the use of L-J medium supplemented with pyruvate is recommended.

For the better isolation of *M*. *bovis*, L-J glycerol and pyruvate have been introduced into NCCD TB laboratory and performed since December 2021.

4. МИКОБАКТЕРИЙН ӨСГӨВӨР

4-1. ӨСГӨВӨРЛӨХ ТЭЖЭЭЛТ ОРЧИН

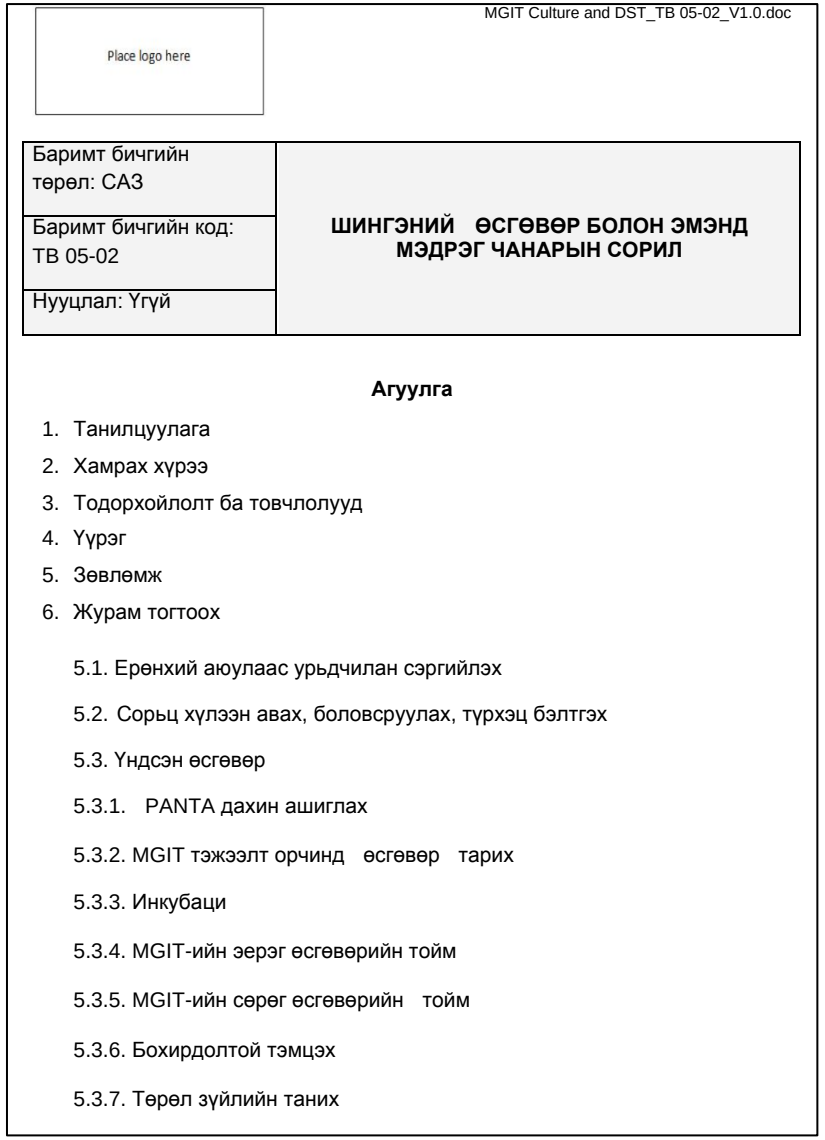
ЗАРЧИМ
Микобактери нь агаартай , уургаар баялаг орчинд (*M.leprea* аас бусад нь) ургалт өгдөг. *M.fortuitum* ба *M. chelonei* гэх мэт зарим микобактериуд нутриент агар гэх мэт уургаар баяжуулагүй тэжээлт орчинд ургадаг..
M. tuberculosis нь агаартай, уургаар баяжуулсан тэжээлт орчинд 35 – 37˚С -д удаан ургадаг, тэжээлт орчинд тарьснаас хойш 2- 3 долоон хоногт нүдэнд харагдах ургалт ажиглагдах ба нөсөө үүсгэхгүй.
M. tuberculosis нь тэжээлт орчны гадаргуугаас төвийсөн, хуурай өрөмтсөн колон өгч ургана. Энэ нь паранитробензойны хүчил (ПНБ)-д мэдрэг байх ба 25˚С хэмд ургадаггүй. Сүрьеэгийн микобактери ургуулах өндөг суурьтай болон шингэн зэрэг олон янзын тэжээлт орчингууд байдаг.

4-2. ЛЕВЕНШТЕЙН-ИЕНСЕНИЙ ТЭЖЭЭЛТ ОРЧИН (өндөг суурьтай)
Лабораторид LJ тэжээлт орчинг бэлдэнэ. Тэжээлт орчин бэлдэхдээ шинэ өндөг, хэд хэдэн давс хийж, малахит ногоон, пенициллинийгбохирдолтыг дарангуйлах зорилгоор хийдэг. Тэжээлт орчинг хоёр тюбэнд ташуу бэлдэнэ. Нэг ташуу тэжээл нь глицерол агуулсан (LJ G), нөгөө нь пируват (LJ P) агуулна. *M. tuberculosis-ын* хүний төрөл нь L-J G тэжээлд сайн ургах ба үхрийн төрөл (*M. bovis*) нь L-J P тэжээлт орчинд ургадаг.
Тавигдах шаардлага:
Өндөг
Давсны уусмал (a)Хоёр солигдолт фосфорхүчлийн кали KH2PO4
(b) Магни сульфат MgSO4
(c) Магний цитрат
(d) *L*-аспирагин
(e) Глицерол
(f) Пируваттай натри

2.2 SOP for MGIT AST for new drugs

Because the World Health Organization recommends new anti-tuberculosis treatment regimens employing new drugs for drug-resistant tuberculosis and has changed the definition of extensively-drug resistant tuberculosis in 2021, we need to introduce a new DST technology to diagnose new drug resistances.

The new method is based on MGIT automated liquid culture technology and requires to prepare drug containing medium in-house. The SOP includes procedures for levofloxacin, moxifloxacin, bedaquiline, linezolid, clofazimine and delamanid.



MGIT Culture and DST_TB 05-02_V1.0.doc

Place logo here

Баримт бичгийн төрөл: САЗ	**ШИНГЭНИЙ ӨСГӨВӨР БОЛОН ЭМЭНД МЭДРЭГ ЧАНАРЫН СОРИЛ**
Баримт бичгийн код: ТВ 05-02	
Нууцлал: Үгүй	

Агуулга

1. Танилцуулага
2. Хамрах хүрээ
3. Тодорхойлолт ба товчлолууд
4. Үүрэг
5. Зөвлөмж
6. Журам тогтоох

5.1. Ерөнхий аюулаас урьдчилан сэргийлэх

5.2. Сорьц хүлээн авах, боловсруулах, түрхэц бэлтгэх

5.3. Үндсэн өсгөвөр

5.3.1. PANTA дахин ашиглах

5.3.2. MGIT тэжээлт орчинд өсгөвөр тарих

5.3.3. Инкубаци

5.3.4. MGIT-ийн эерэг өсгөвөрийн тойм

5.3.5. MGIT-ийн сөрөг өсгөвөрийн тойм

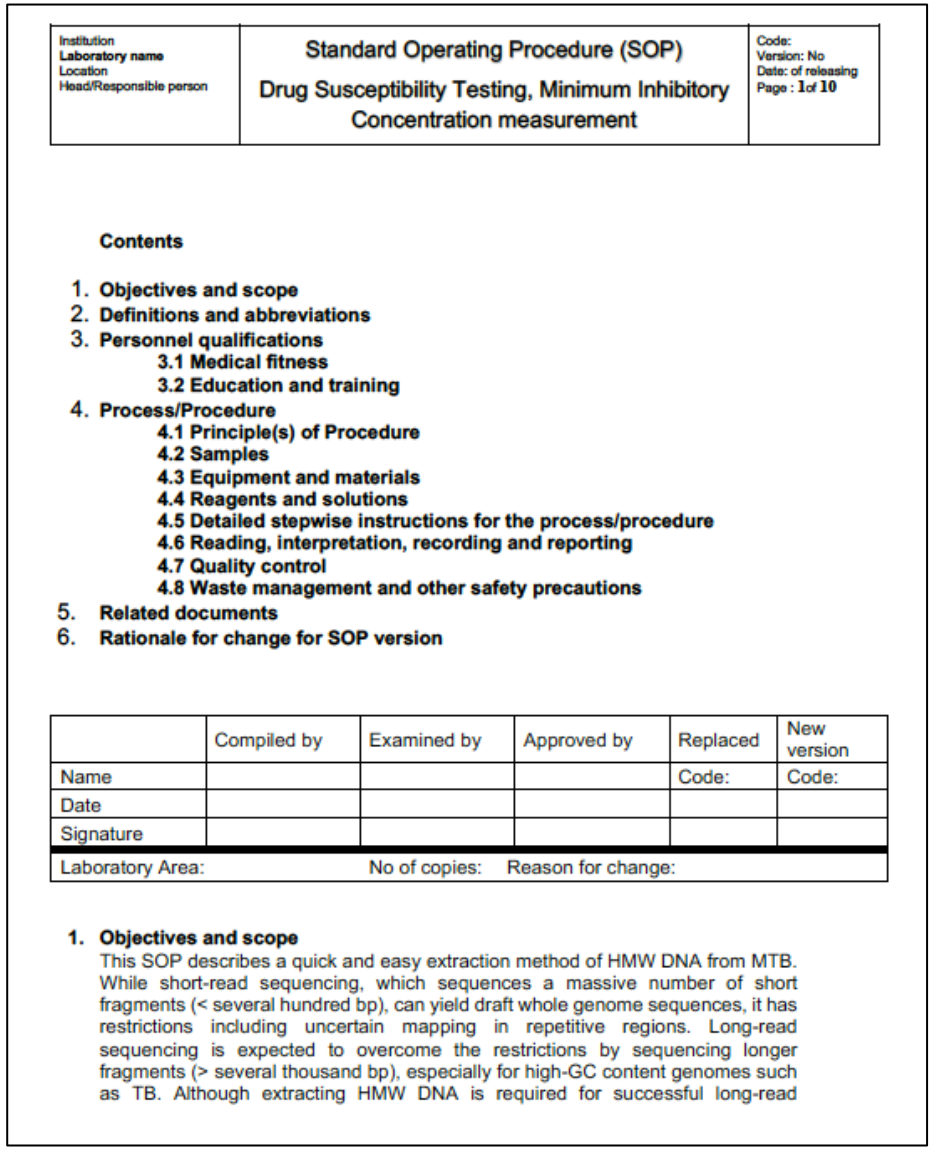
5.3.6. Бохирдолтой тэмцэх

5.3.7. Төрөл зүйлийн таних

2.3 SOP for Genome DNA extraction

This SOP describes a quick and easy extraction method of high molecular weight (HMW) DNA from MTB. While short-read sequencing, which sequences a massive number of short fragments (< several hundred bp), can yield draft whole genome sequences, it has restrictions including uncertain mapping in repetitive regions. Long-read sequencing is expected to overcome the restrictions by sequencing longer fragments (> several thousand bp), especially for high-GC content genomes such as TB.

Although extracting HMW DNA is required for successful long-read sequencing, the method is not well established in MTB. Here, the quick and easy way to extract HMW DNA from TB that is suitable for long-read sequencing is described.



Institution **Laboratory name** Location Head/Responsible person	Standard Operating Procedure (SOP) Drug Susceptibility Testing, Minimum Inhibitory Concentration measurement	Code: Version: No Date: of releasing Page : 1of 10

Contents

	Compiled by	Examined by	Approved by	Replaced	New version
Name				Code:	Code:
Date					
Signature					
Laboratory Area:		No of copies:	Reason for change:		

1. **Objectives and scope**

This SOP describes a quick and easy extraction method of HMW DNA from MTB. While short-read sequencing, which sequences a massive number of short fragments (< several hundred bp), can yield draft whole genome sequences, it has restrictions including uncertain mapping in repetitive regions. Long-read sequencing is expected to overcome the restrictions by sequencing longer fragments (> several thousand bp), especially for high-GC content genomes such as TB. Although extracting HMW DNA is required for successful long-read

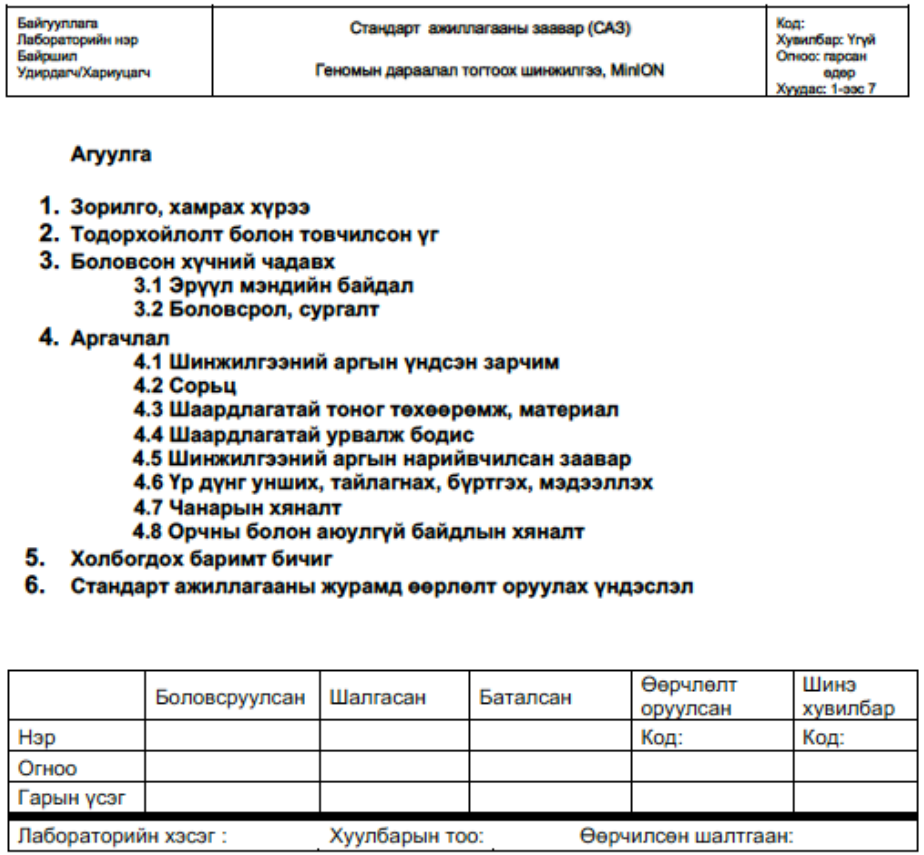
2.4 SOP for MinION sequencing

This procedure is involved in the method to perform long-read sequencing with MinION Mk1B. Most of procedure is conducted by kits and equipment sold by Oxford Nanopore Technologies.

First, HMW DNA is fragmented and barcodes are attached to the end of fragments, simultaneously.

Then, the barcoded samples are pooled, and sequencing adapters are attached to the pooled samples.

Finally, the DNA library is loaded into the flow cell and start a run. The sequence data obtained will be analysed separately.



Байгууллага Лабораторийн нэр Байршил Удирдагч/Хариуцагч	Стандарт ажиллагааны заавар (САЗ) Геномын дараалал тогтоох шинжилгээ, MinION	Код: Хувилбар: Үгүй Огноо: гарсан өдөр Хуудас: 1-ээс 7

Агуулга

	Боловсруулсан	Шалгасан	Баталсан	Өөрчлөлт оруулсан	Шинэ хувилбар
Нэр				Код:	Код:
Огноо					
Гарын үсэг					
Лабораторийн хэсэг :		Хуулбарын тоо:		Өөрчилсөн шалтгаан:	

1. **Зорилго, хамрах хүрээ**
   Энэхүү стандарт ажиллагааны заавар нь сурьеэгийн үүсгэгчээс ялган авсан өндөр молекул жинтэй ДНХ-ээс MinION Mk1B уртаар унших(long read) геном дарааллыг эхлүүлэх аргыг тайлбарласан.

2. **Тодорхойлолт болон товчилсон үг**
   өндөр молекул жинтэй ДНХ
   Энэ өндөр молекул жинтэй ДНХ-ийн хэлтэрхийн урт нь хэдэн арван кб гэсэн утгатай бөгөөд энэ нь Long-read дарааллаар цаашид дүн шинжилгээ хийхэд хангалттай юм. Өндөр молекул жинтэй ДНХ -ийн тодорхойлолт нь хэрэглээ, организм болон бусад хүчин зүйлээс хамааран хэд хэдэн кб-аас Мб хооронд хэлбэлздэг.

Implementations

The Research Institute of Tuberculosis (RIT) has implemented the followings

3 On-line training

We conducted on-line new DST training connecting NCCD TB laboratory and RIT on July 5th and 6th. This SOP describes the use of the BACTEC MGIT 960 TB System for liquid culture and drug susceptibility testing of *Mycobacterium tuberculosis*. The BACTEC MGIT 460 TB System has been found to boost culture positivity by 15–20% relative to conventional solid media and to substantially reduce the time to positivity. Liquid culture, however, is more prone to contamination. Liquid culture is now approved by WHO for use in low to middle income countries.

The training was conducted using 20 standardized *M. tuberculosis* strains which drug resistances are already known (standard results from Institute of Tropical Medicine, Antwerp, Belgium). The results are not validated as of October 19, 2022.

Implementations

The Research Institute of Tuberculosis (RIT) has implemented the followings

4 Introduction of interferon gamma release assay (IGRA)

For the screening of *M. tuberculosis* infection, IGRA is introduced into NCCD TB laboratory. QuantiFERON-TB Gold in Tube (QFT-4G, QIAGEN) is the 4th generation IGRA detecting interferon gamma release from CD4/8 cells. This is a standard method for IGRA with ELISA system, however, due to the delay of transportation of equipment necessary of ELISA to NCCD, the standard QFT system is not installed yet. After consultation with QIAGEN, the company proposed a simple semi-quantitative version of QFT (QIAReach system) which employs lateral flow immunoassay for the quantification of released IFN-gamma.

The QIAReach system has been installed into NCCD in September 2022, and validation test was performed using blood samples collected from bacteriologically confirmed TB cases, TB contacts and healthy volunteers (12 samples in total). The test was successful.

QIAreach QuantiFERON-TB

- Simple QFT-4G test with 1ml blood
- With lateral flow immunoassay
- 5-20 min to results
- Positive or negative results
- Ability to reach people in remote areas for efficient preventive TB programs with digital QIAreach technology

Laboratory procedure (QuantiFERON TB Gold Plus)

- Step1　Blood samples incubation with antigens (1-ml sample was dispensed Into each assay tube, and incubated at 37 ± 1°C for 16–24 h)
- Step2 ELISA (using standard QFT ELISA kit)

QFT-GIT

QFT-Plus

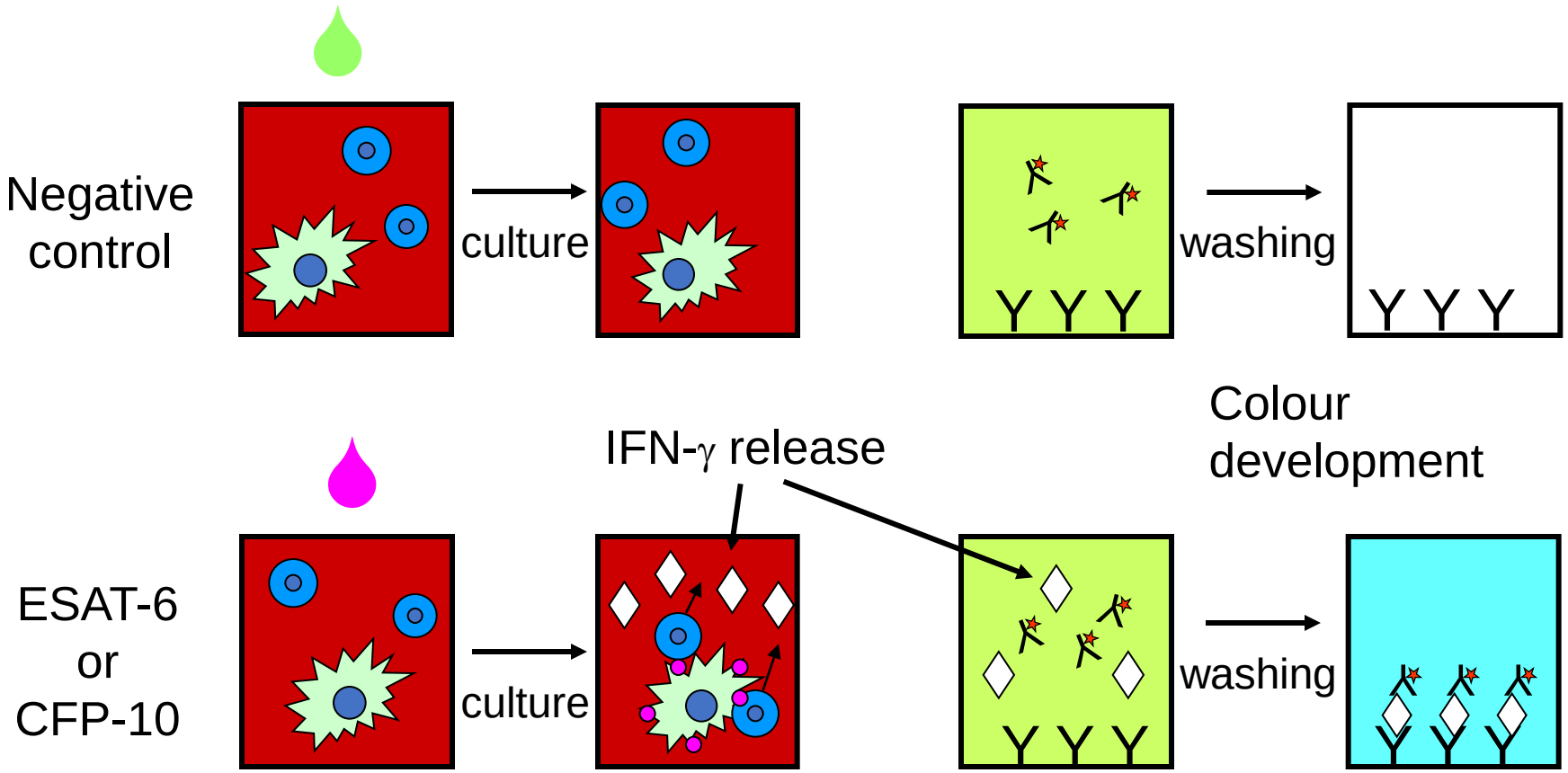
Nil (Grey Cap)	Negative control. Adjusts for background noise or non-specific IFN-γ in blood samples.
QFT-GIT Antigen Tube (Red Cap)	Contains highly specific TB antigens (ESAT-6, CFP-10, TB7.7)
Mitogen (Purple Cap)	Positive control (Detects patients immunocompromised subjects, or incorrect blood handling & incubation)
TB1 (Green Cap)	Contains long synthetic peptides from ESAT-6 and CFP-10 to stimulate $CD4^+$ T-helper lymphocytes.
TB2 (Yellow Cap)	Contains an set of short peptides targeted to stimulate $CD8^+$ cytotoxic T lymphocytes.

The IFN-γ concentration of Antigen and Mitogen are defined as follows:
Antigen (IU/mL) = IFN-γ (A) – IFN-γ (N); Mitogen (IU/mL) = IFN-γ (M) – IFN-γ (N)

QuantiFERON TB Assay

Step 1:
Stimulate lymphocytes by specific antigens

Step 2:
Evaluate IFN-gamma production by ELISA



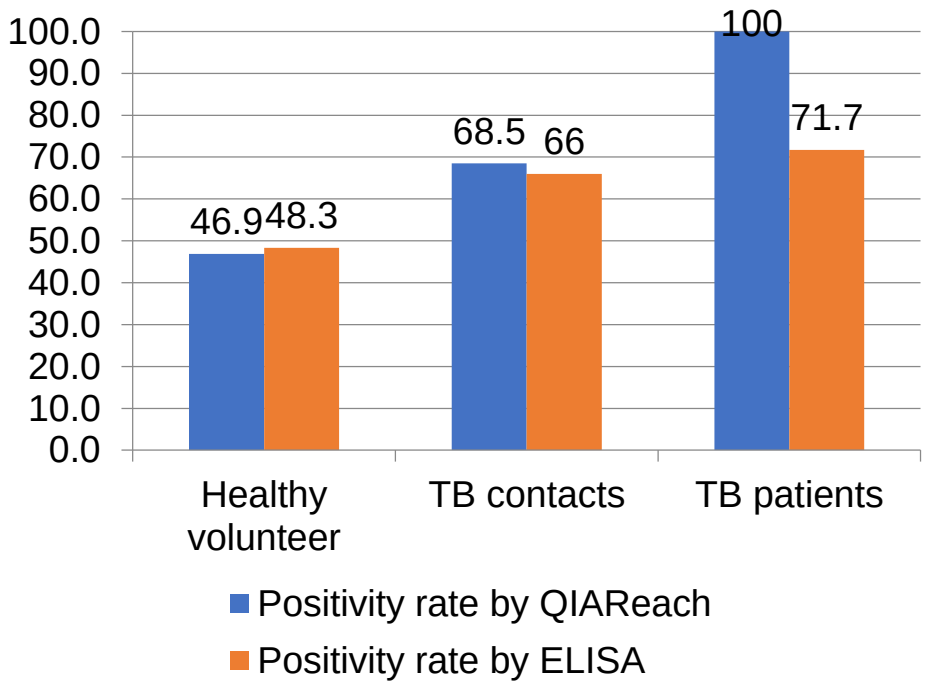
Laboratory process

- Enzyme Linked Immuno-Sorbent Assay (ELISA)
- An antibody to a target protein is immobilized on the surface of microplate wells and incubated first with the target protein and then with another target protein-specific antibody, which is labeled with an enzyme.
- After washing, the activity of the microplate well-bound enzyme is measured.
- The immobilized antibody and the enzyme-labeled antibody must recognize different epitopes of the target protein.
- Sandwich ELISA is technically demanding.

Relative evaluation of QIAReach and QFT-Plus

Implemented in Mongolia (Ulaanbaatar)
Comparison of QIAReach and QFT Gold Plus in healthy subjects, contacts, and active tuberculosis patients

Subject	Tested samples	QFT By QIAReach	Positive %	QFT by ELISA	Positive % /n
Total (sample size 600)	416 (69.3%)	363	57.3% (208/363)	190	59.5% (113/190)
Healthy volunteer	196	196	46.9% (92/196)	87	48.3% (42/87)
TB contacts	162	162	68.5 (111/163)	50	66.0% (33/50)
Tb patients	58	5	100% (5/5)	53	71.7% (38/55)



Implementations

The Research Institute of Tuberculosis (RIT) has implemented the followings

5 Overseas training

IGRA training

The IGRA training was performed using negative and positive human blood controls. The QFT Gold Plus assay kit was used. It was performed according to the manufacturer’s instruction. The most critical step was ELISA, and the quality control process was carefully implemented.

Implementations

The Research Institute of Tuberculosis (RIT) has implemented the followings

5 Overseas training

WGS sequencing and bioinformatics

The programme included DNA extraction from *M. tuberculosis* using glass-beads dispersion method followed by organic solvent purification, DNA quality and quantity measures, library preparations, and genome sequencing. The base calling is translated to sequence and analysed basically using TBProfiler solution.

Bioinformatic analysis of obtained data (fastq) was performed by using MTBSeq (public domain, github).

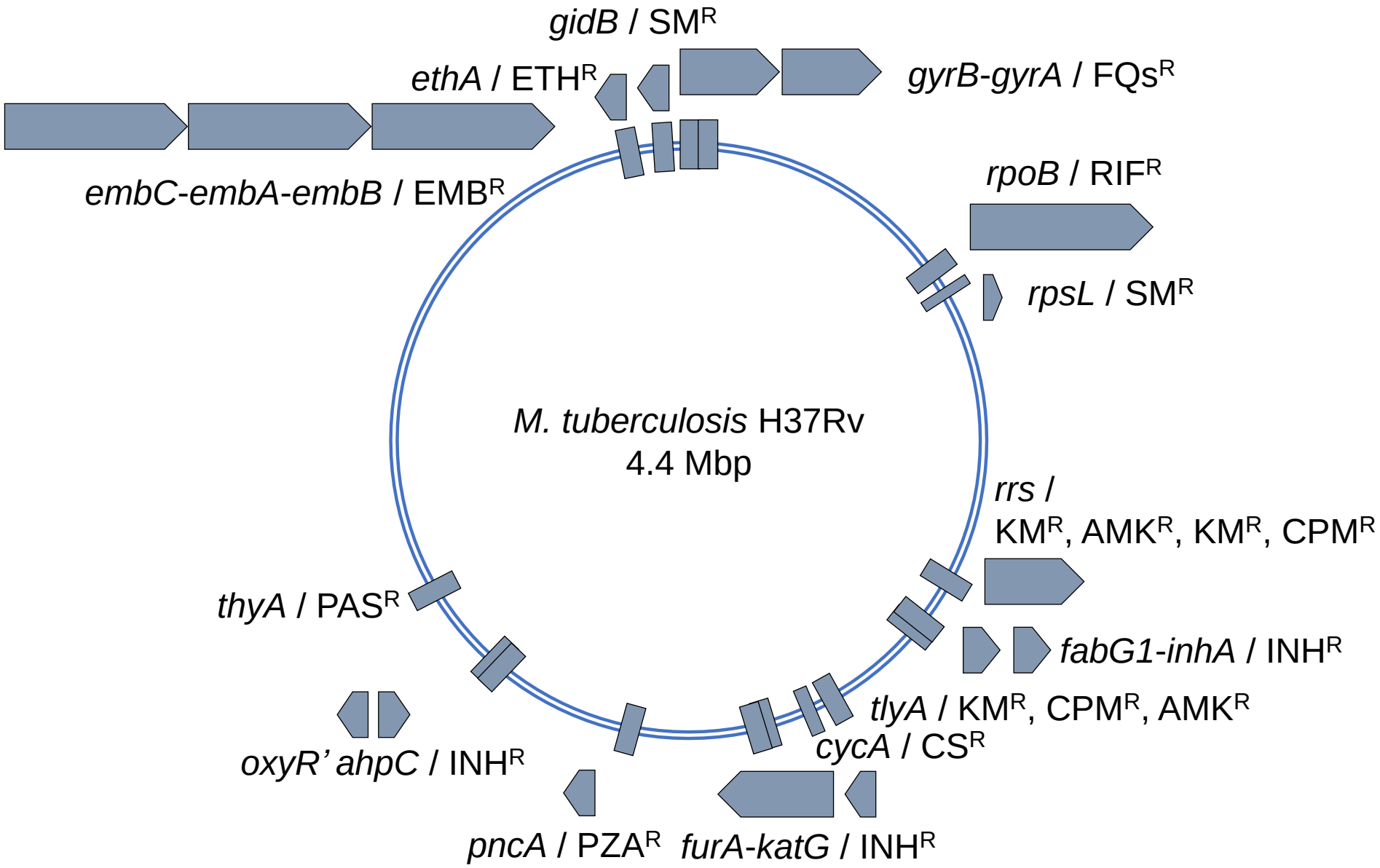
Drug resistant tuberculosis (DR-TB)

- Drug susceptibility testing (DST) is important for management of DR-TB cases.
- Current standard culture-based DST takes 1-2 months before results are available.
- Programmatic management of DR-TB (PMDT) using mWRD* is a standard strategy to efficiently manage DR-TB cases.

*mWRD: molecular World Health Organization (WHO) recommended diagnostics

Technical Development

- Targeted Next Generation Sequencing (tNGS)
  - The targeted next generation sequencing (tNGS) is a technology to amplify the target sequences which conquer the drug resistances and perform deep sequencing with amplicons. It can yield the genotypic DST results within 2–3 days and cover many drug resistant mutations and indels including newly introduced drugs.
  - Deeplex Myc-TB (Genoscreen, France) is multiplex-PCR based technology, and one of the tNGS diagnostic kits and can predict 15 drug resistances in one process.



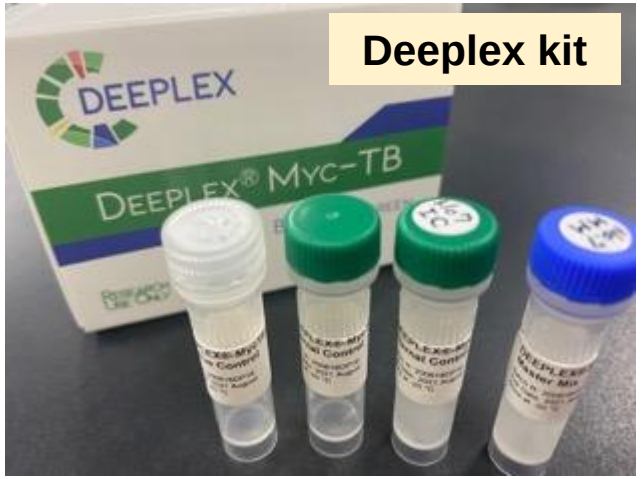
Major drug resistance genes and their locations in *M. tuberculosis* genome

Deeplex® Myc-TB (1)

DNA extraction and multiplex PCR (19 gene targets) → amplicon deep sequencing (tNGS) → SQ data analysis on Web App

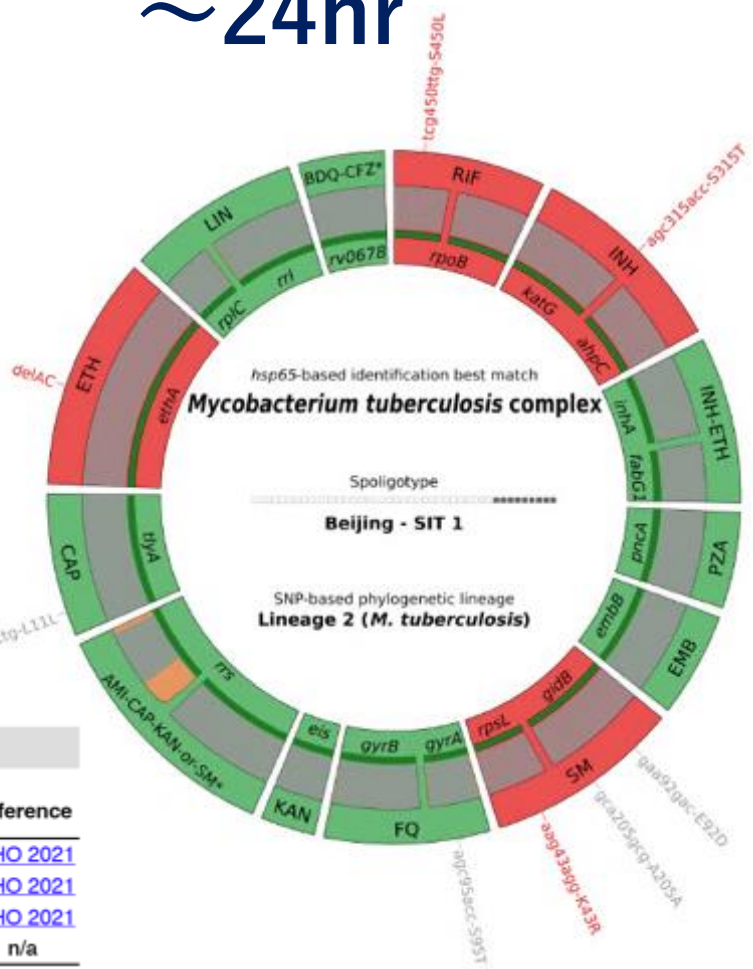
~4hr

~24hr



Deeplex kit

NGS device



DEEPLEX®-MycTB Report

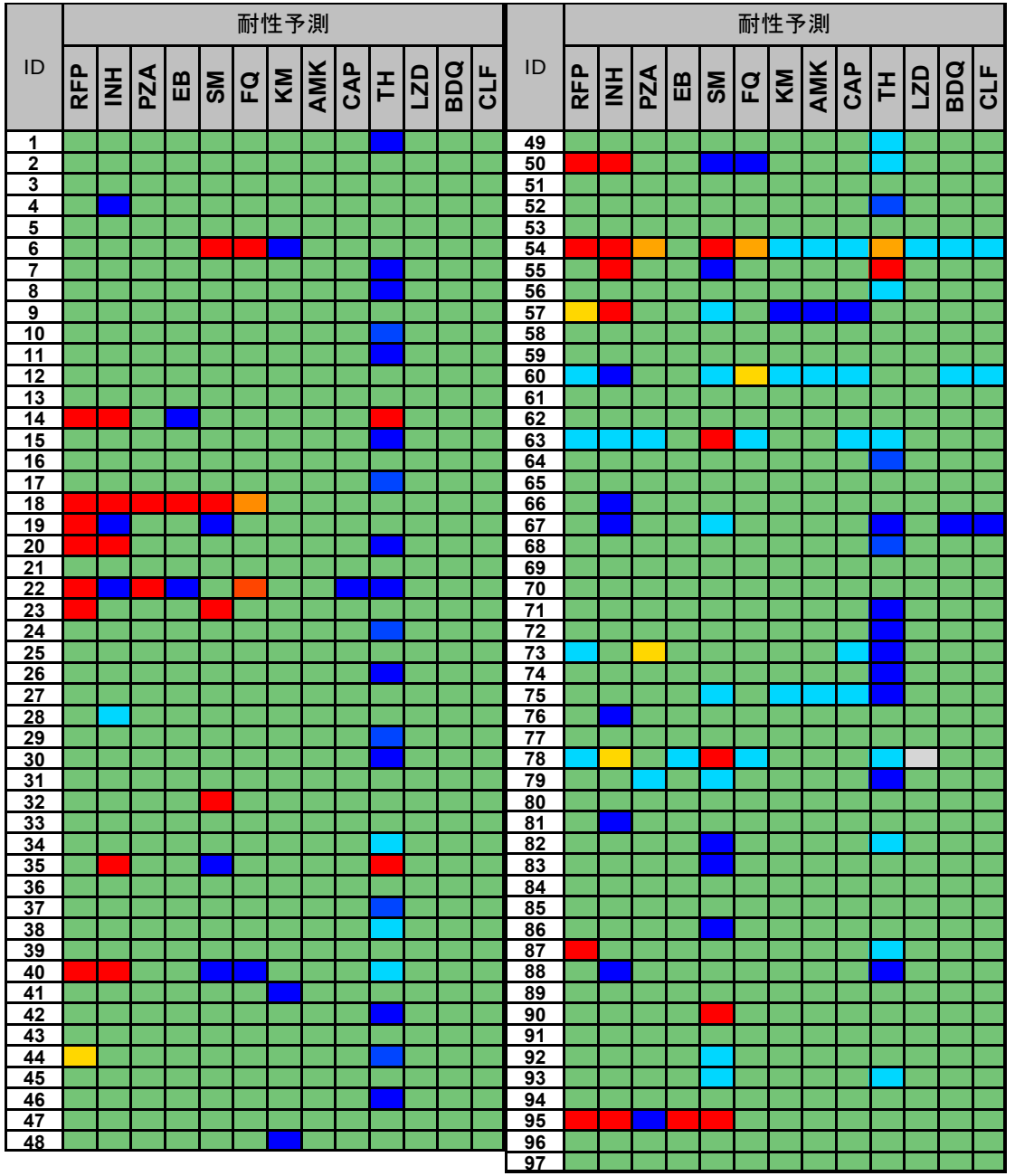
Drug resistance associated variants[3]

Gene	Genomic position	Codon change	% Variant	Dx-score	AA change	Drug*	Confidence	Resistance level	Reference
katG	2155168	agc315acc	100.000	328.50	S315T	INH	Associated with resistance	Resistant	WHO 2021
rpoB	761155	tcg450ttg	99.560	1022.75	S450L	RIF	Associated with resistance	Resistant	WHO 2021
rpsL	781687	aag43agg	99.250	427.75	K43R	STM	Associated with resistance	Resistant	WHO 2021
ethA	4326836-7	delAC	96.700	0.00	frameshift	n/a	n/a	Resistant	n/a

Results

Drug resistance prediction using Deeplex-MycTB (n=155)

- Analysis possible with smear 1+ or higher (116 samples: 74.8%)
- Actual test days; Culture + DST average 21.3 days (12.7–36.7 days)
- Resistance prediction (red); 52variants (7 drugs)
  - Consistent with conventional DST results (*rpoB*: H445L*)
    - * borderline RIF-resistance mutation
- Mixed resistance variants (orange); 2 samples
- Total of 5 types 89.2%variants (DST; FQs resistance)
  - Type 1 78% variant (DST; FQs sensitivity)
- No resistance information (blue); 63 variants (6 drugs, 30variants in ETH)
- Several %variant mixture (water/yellow)
  - 64variant (DST; sensitive)
- One case of discrepancy in phenotypic INH resistance → mutation confirmed by WGS



Whole Genome Sequencing directly from clinical specimen (sputum)

- Current WGS technology requires much amount of pure genomic DNA for sequencing.
- It will require culture process with solid medium (time consuming).
- For rapid utilisation of WGS information (DST and molecular epidemiology), direct WGS technology will be required.

Mycobacterium tuberculosis genome enrichment method from specimens

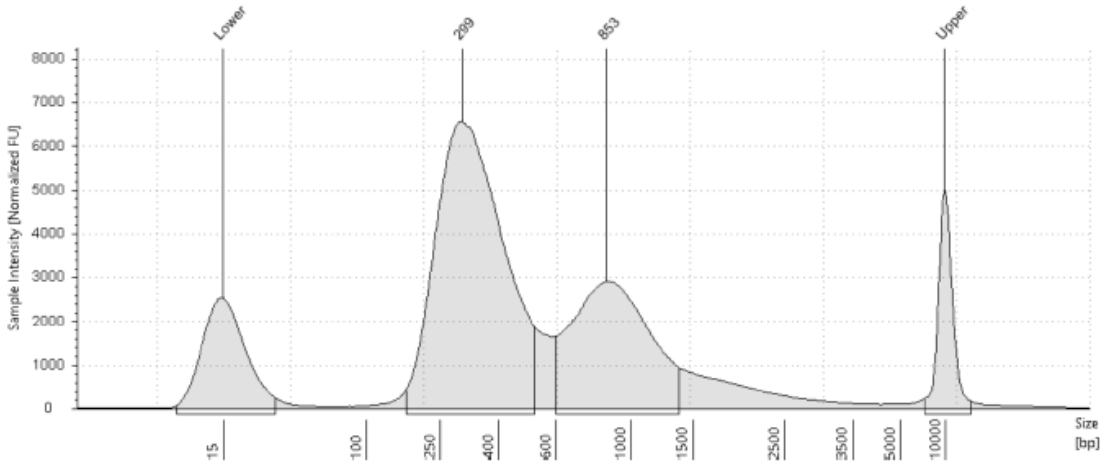
Mycobacterium tuberculosis genome enrichment using QIAseq xHYB kit (Qiagen)
A system that selectively enriches the M. tuberculosis genome by hybridizing specific regions on the genome using oligo RNA.

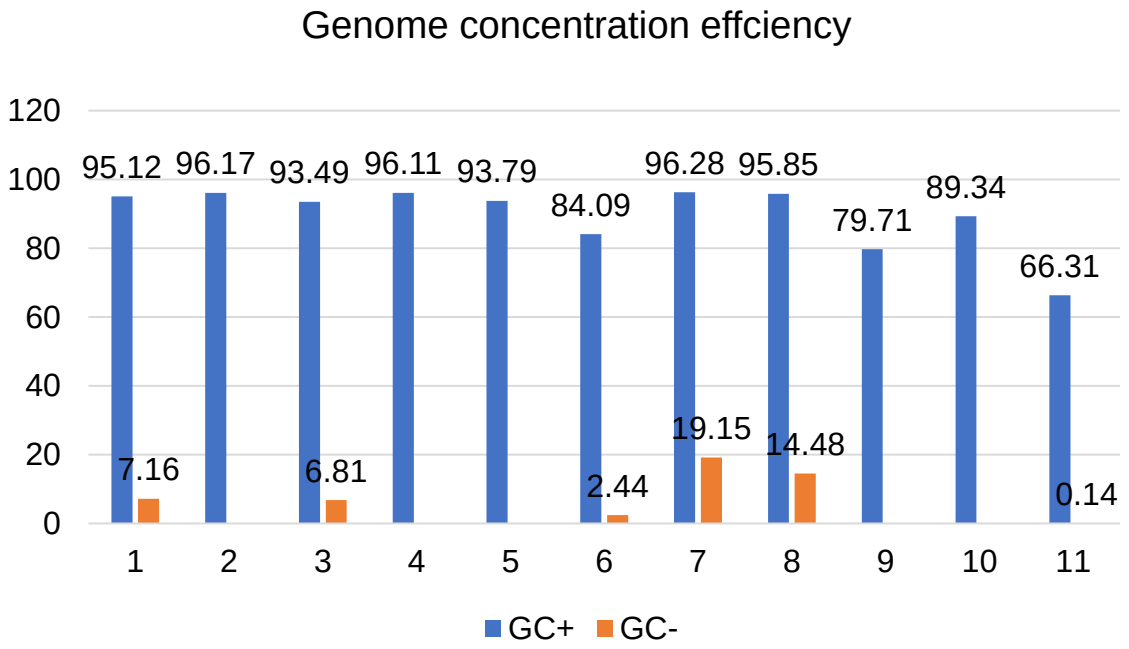
Library concentration flow

1. QIAseqFX library creation
2. Pool and hybridize multiple libraries
3. Amplify enriched Mycobacterium tuberculosis genome library → Complete SQ library

Progress

1. QIAseqFX library creation
    1. Some reagents + reaction amount used for xHYB kit
    2. PCR cycle with over amplification
    3. Need to reduce by 3–5 cycles (125–375 ng/sample)
2. PCR process after genome enrichment
    1. PCR cycle with over amplification
    2. Although reducing by 3 cycles, conditions need to be considered
3. Genome enrichment results
    1. Total number of reads with 3+ smear samples (8 samples)
    2. *Mycobacterium tuberculosis* genome ratio increased to 84.1–96.2%





Implementations

The Research Institute of Tuberculosis (RIT) has implemented the followings

6 Ethical process

The protocols for IGRA study, isolation and identification of *M. bovis* in human sample, genetic analysis (typing) of *M. tuberculosis* isolates including *M. bovis*, and genotypic DST are prepared and submitted to corresponding IRB (NCCD or Ministry of Health or both) in collaboration with NTRL/NCCD. The protocols have been approved.

Thank you.

RIT JATA
Research Institute of Tuberculosis
Japan Anti-Tuberculosis Association

Research Institute of Tuberculosis