THE THIRD JOINT COORDINATING COMMITTEE MEETING FOR THE SATREPS PROJECT

SATREPS project:

CONTROL OF TUBERCULOSIS AND GLANDERS

Activity report from the Research Institute of Tuberculosis

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Department of Mycobacterium Reference and Research,

the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association

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

Inputs

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. Technical transfer to NCCD
   - NGS capacity (GridION, Oxford Nanopore Technologies)
   - IGRA (ELISA)
   - Drug susceptibility testings (*in-house* MGIT, MIC)
4. Overseas trainings
   - IGRA
   - Genome sequencing and *in silico* analysis
5. Diagnostics development (RIT)
   - Metagenomic NGS

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.

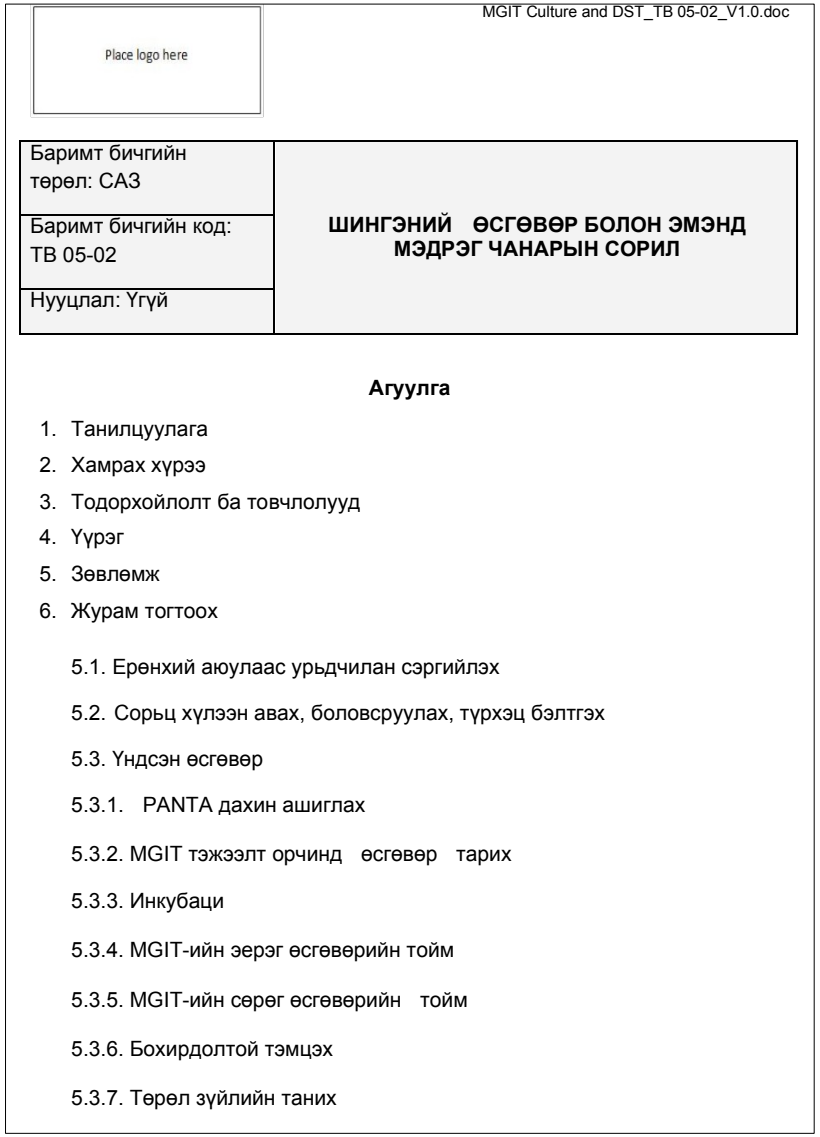
4-2. ЛЕВЕНШТЕЙН-ИЕНСЕНИЙ ТЭЖЭЭЛТ ОРЧИН (өндөг суурьтай)
Лабораторид LJ тэжээлт орчинг бэлдэнэ. Тэжээлт орчин бэлдэхдээ шинэ өндөг, хэд хэдэн давс хийж, малахит ногоон, пенициллинийгбохирдолтыг дарангуйлах зорилгоор хийдэг. Тэжээлт орчинг хоёр тюбэнд ташуу бэлдэнэ. Нэг ташуу тэжээл нь глицерол агуулсан (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.

Minimum Inhibitory Concentration by broth microdilution method is installed, including new drugs to evaluate drug resistance mutation/indels.



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

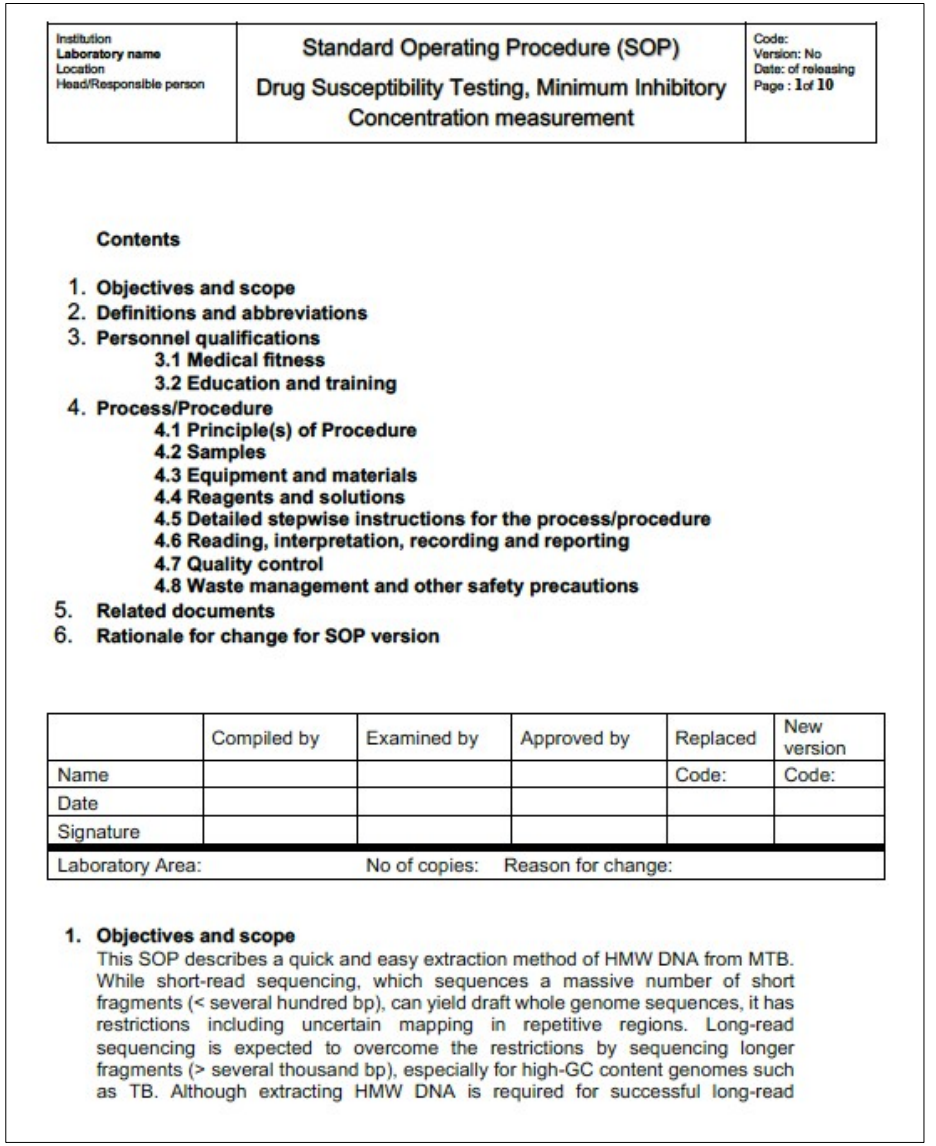
5.3.6. Бохирдолтой тэмцэх

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

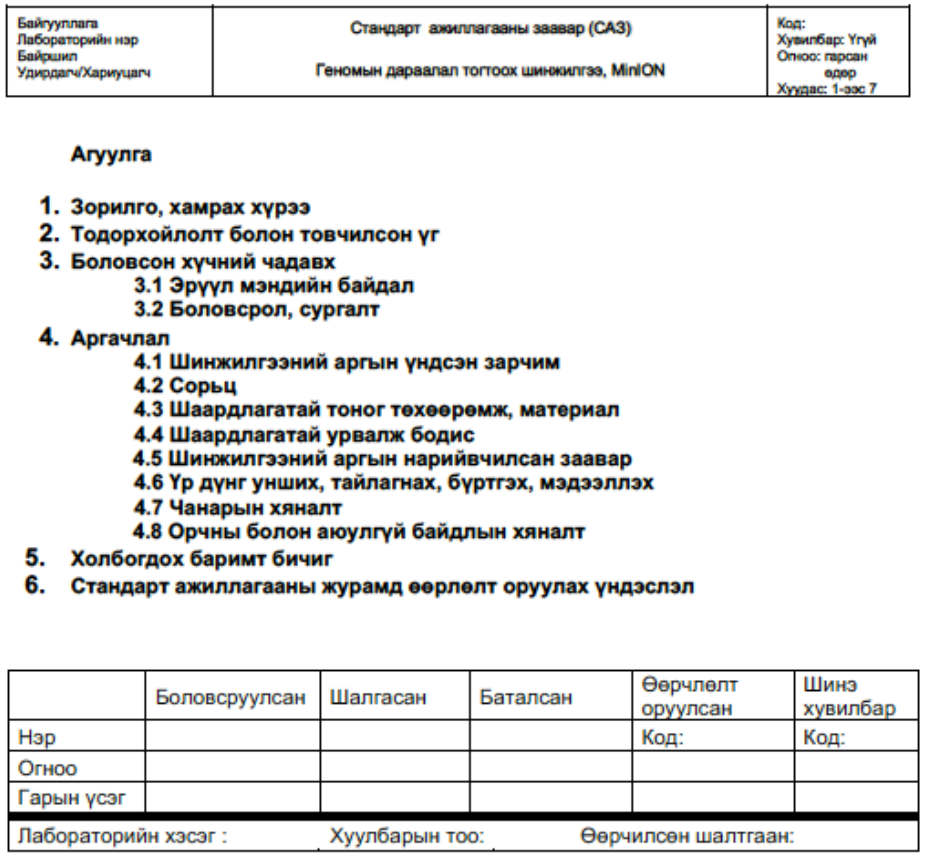
2.4.1 SOP for MinION sequencing

This procedure is involved in the method to perform long-read sequencing with MinION Mk1B and GridION. 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:	

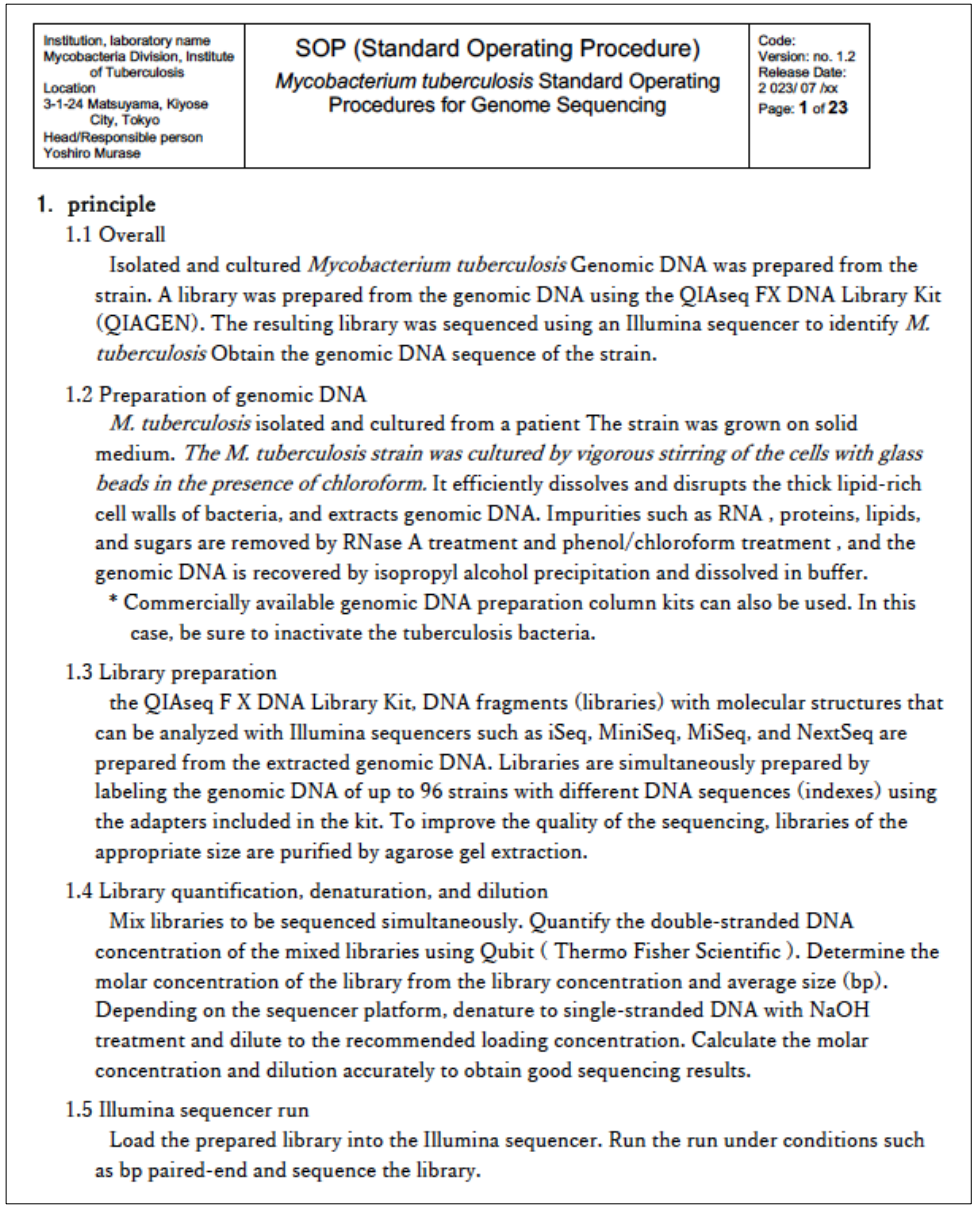
1. **Зорилго, хамрах хүрээ**
   Энэхүү стандарт ажиллагааны заавар нь сурьеэгийн үүсгэгчээс ялган авсан өндөр молекул жинтэй ДНХ-ээс MinION Mk1B уртаар унших(long read) геном дарааллыг эхлүүлэх аргыг тайлбарласан.

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.

2.4.2 SOP for Illumina sequencing

Isolated and cultured *Mycobacterium tuberculosis* Genomic DNA was prepared from the strain. A library was prepared from the genomic DNA using the QIAseq FX DNA Library Kit (QIAGEN). The resulting library was sequenced using an Illumina sequencer to identify *M. tuberculosis* Obtain the genomic DNA sequence of the strain.

Libraries are simultaneously prepared by labeling the genomic DNA of up to 96 strains with different DNA sequences (indexes) using the adapters included in the kit. To improve the quality of the sequencing, libraries of the appropriate size are purified by agarose gel extraction.



Institution, laboratory name Mycobacteria Division, Institute of Tuberculosis Location 3-1-24 Matsuyama, Kiyose City, Tokyo Head/Responsible person Yoshiro Murase	SOP (Standard Operating Procedure) *Mycobacterium tuberculosis* Standard Operating Procedures for Genome Sequencing	Code: Version: no. 1.2 Release Date: 2 023/ 07 /xx Page: **1** of **23**

1. principle

1.1 Overall

Isolated and cultured *Mycobacterium tuberculosis* Genomic DNA was prepared from the strain. A library was prepared from the genomic DNA using the QIAseq FX DNA Library Kit (QIAGEN). The resulting library was sequenced using an Illumina sequencer to identify *M. tuberculosis* Obtain the genomic DNA sequence of the strain.

1.2 Preparation of genomic DNA

M. tuberculosis isolated and cultured from a patient The strain was grown on solid medium. *The M. tuberculosis strain was cultured by vigorous stirring of the cells with glass beads in the presence of chloroform.* It efficiently dissolves and disrupts the thick lipid-rich cell walls of bacteria, and extracts genomic DNA. Impurities such as RNA , proteins, lipids, and sugars are removed by RNase A treatment and phenol/chloroform treatment , and the genomic DNA is recovered by isopropyl alcohol precipitation and dissolved in buffer.

* Commercially available genomic DNA preparation column kits can also be used. In this case, be sure to inactivate the tuberculosis bacteria.

1.3 Library preparation

the QIAseq F X DNA Library Kit, DNA fragments (libraries) with molecular structures that can be analyzed with Illumina sequencers such as iSeq, MiniSeq, MiSeq, and NextSeq are prepared from the extracted genomic DNA. Libraries are simultaneously prepared by labeling the genomic DNA of up to 96 strains with different DNA sequences (indexes) using the adapters included in the kit. To improve the quality of the sequencing, libraries of the appropriate size are purified by agarose gel extraction.

1.4 Library quantification, denaturation, and dilution

Mix libraries to be sequenced simultaneously. Quantify the double-stranded DNA concentration of the mixed libraries using Qubit (Thermo Fisher Scientific). Determine the molar concentration of the library from the library concentration and average size (bp). Depending on the sequencer platform, denature to single-stranded DNA with NaOH treatment and dilute to the recommended loading concentration. Calculate the molar concentration and dilution accurately to obtain good sequencing results.

1.5 Illumina sequencer run

Load the prepared library into the Illumina sequencer. Run the run under conditions such as bp paired-end and sequence the library.

Inputs

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

3　On-line training (during COVD-19 pandemic)

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.

Inputs

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

4　Installation and implementation of interferon gamma release assay (IGRA)

For the screening of *M. tuberculosis* infection, IGRA is introduced into NCCD TB laboratory.

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.

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. It was installed into NCCD in 2023.

Screening of TB patients, TB contacts and healthy volunteers are conducted. High IGRA positive results indicated the active tuberculosis transmission in the community.

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

QuantiFERON-TB Gold Plus

Since the T1 tube induces IFN-γ from CD4 positive cells and the T2 tube induces IFN-γ from CD4+CD8 positive cells, four reaction tubes will be used in addition to the positive and negative controls.

- The criteria for diagnosis have also changed. Previously, IFN-γ concentrations between 0.1 IU/ml and less than 0.35 IU/ml were considered "indeterminate," but this category has been abolished and the cutoff level is now 0.35 IU/ml, with only positive or negative results being determined.

<table>
<tr><th>Nil (IU/ml)</th><th>TB1 value (IU/ml)</th><th>TB2 value (IU/ml)</th><th>Mitogen (IU/ml)</th><th>Result</th><th>Interpretation</th></tr>
<tr><td rowspan="4">≤8.0</td><td>≥0.35 and ≥25% of the Nil value</td><td>Any</td><td rowspan="2">Any</td><td rowspan="2">Positive</td><td rowspan="2">Suspected tuberculosis infection</td></tr>
<tr><td>Any</td><td>≥0.35 and ≥25% of the Nil value</td></tr>
<tr><td colspan="2" rowspan="2"><0.35 or
≥0.35 and less than 25% of the Nil value</td><td>≥0.5</td><td>Negative</td><td>No tuberculosis infection</td></tr>
<tr><td><0.5</td><td rowspan="2">Indeterminate</td><td rowspan="2">It is not possible to determine whether or not someone is infected with tuberculosis</td></tr>
<tr><td>>8.0</td><td colspan="2">Any</td><td></td></tr>
</table>

Inputs

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

5 Overseas trainings

A total of 9 trainees from NCCD received training at RIT (2022–2024)

- Basic GLPs for *M*. *tuberculosis* in BSL3 laboratory
- DNA preparation for genome sequencing
- Genome sequencing technologies by illumina and nonopore platforms
- Genome data analysis *in silico*
- Interferon gamma release assay (ELISA)
- Broth microdilution method for Minimum Inhibitory Concentration

Inputs

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

6 Genome sequencing of *M*. *tuberculosis* isolates from patients

- For the detection/identification of *M*. *bovis* in human isolates.
- A retrospective study using stored *M*. *tuberculosis* (complex) isolates in NCCD.
- A total of 2,000 isolates for genome sequencing.
  - 1,000 in RIT and another 1,000 in NCCD

Illumina sequencing results (RIT)

Species/Lineage analysis (N=1,001)

Lineage	Number	Appendix
La1	1	*M. Bovis* BCG
lineage2	819	
lineage2;lineage4	13	
lineage3	1	
lineage4	166	
M.canetti;lineage2;lineage6	1	*M. africanum*?

Drug resistance analysis (gDST) (N=1,003)

Drug resistance category	**Number**	Proportion (%)
Susceptible	504	50.2
HR-TB	135	13.5
RR-TB	10	0.01
MDR-TB	260	25.9
Pre-XDR-TB	37	0.04
XDR-TB	3	0.003
Other	54	0.05

XDR-TB

sample	Main lineage	Sub lineage	rifampicin	isoniazid	ethambutol	pyrazinamide	moxifloxacin	levofloxacin	bedaquiline	delamanid	pretomanid	linezolid	streptomycin	clofazimine	ethionamide
607	lineage2	lineage2.2.1	rpoB p.Asp435Tyr (1.00), rpoB p.Ser450Leu (1.00)	inhA c.-777C>T (1.00), inhA p.Ser94Ala (1.00)	embB p.Met306Val (1.00)	pncA p.Asp12Glu (1.00)	gyrA p.Asp94Gly (1.00)	gyrA p.Asp94Gly (1.00)	mmpR5 c.141_142dupTC (0.86), mmpR5 c.198dupG (0.12)	ddn c.172dupC (0.24), fbiC c.1617_1618dupCG (0.11), fgd1 c.273dupC (0.15)	ddn c.172dupC (0.24), fbiC c.1617_1618dupCG (0.11), fgd1 c.273dupC (0.15)	-	rpsL p.Lys43Arg (1.00)	mmpR5 c.141_142dupTC (0.86), mmpR5 c.198dupG (0.12)	inhA c.-777C>T (1.00), inhA p.Ser94Ala (1.00)
1005	lineage2	lineage2.2.1	rpoB p.Asp435Tyr (1.00), rpoB p.Ser450Leu (1.00)	inhA c.-777C>T (1.00), inhA p.Ser94Ala (1.00)	embB p.Met306Val (1.00)	pncA p.Asp12Glu (1.00)	gyrA p.Ala90Val (1.00), gyrB p.Ile486Leu (1.00)	gyrA p.Ala90Val (1.00), gyrB p.Ile486Leu (1.00)	mmpR5 c.198dupG (0.89)	-	-	-	rpsL p.Lys43Arg (1.00)	mmpR5 c.198dupG (0.89)	inhA c.-777C>T (1.00), inhA p.Ser94Ala (1.00)
864	lineage2	lineage2.2.1	rpoB p.Asp435Tyr (1.00), rpoB p.Ser450Leu (1.00)	inhA c.-777C>T (1.00), inhA p.Ser94Ala (1.00)	embB p.Met306Val (1.00)	pncA p.Asp12Glu (1.00)	gyrA p.Asp94Gly (1.00)	gyrA p.Asp94Gly (1.00)	-	-	-	rplC p.Cys154Arg (0.39)	rpsL p.Lys43Arg (1.00)	-	inhA c.-777C>T (1.00), inhA p.Ser94Ala (1.00)

Inputs

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

7 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.

Shotgun metagenome sequencing

- DNA extraction: MAGicBead™ cfDNA Isolation Kit
- Library preparation: QIAseq FX Library Library preparation
- Sequencing: NextSeq 500/550 High-Output v2.5 Kit (300 cycles)
- Data analysis: The obtained fastq read data was analysed using CLC genomics workbench (Qiagen, CA, US). A curated database (CNBI) for detecting *M. tuberculosis* was used for metagenomic analysis after filtering out the sequences of human origin (hg38).

Results of shotgun metagenome

- All samples tested are MTB fragment positive.
- Controls: Positive (+), Negative (-)
- ADA: 29.1±26.6 (0.92 – 120.85)
- MTB fragments: 87.1±300.5 (7 – 2278)
- Coverage: 0.0025±0.0092 (9.50E-5 – 6.9E-2)

- There should be a threshold for MTB fragment number?

Acknowledgements

We extend our sincere gratitude to

❖The SATREPS project titled “Control of Tuberculosis and Glanders”

Sincerely thankful for their invaluable financial support

❖The Japanese International Cooperation Agency (JICA)

❖The Japanese Agency for Health Research and Development (AMED)

❖Global Fund (GF) TB,HIV project in Mongolia

Special sincerely thankful for their kind cooperation

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❖The Institute of Veterinary Medicine (IVM)

❖The Ministry of Health, Mongolia

❖The Hokkaido University, School of Veterinary Medicine (HU, FVM)

❖The Hokkaido University Center for Zoology (HU, RCZC)

❖The Tuberculosis Research Institute (RIT)

Thank you.

RIT JATA
Research Institute of Tuberculosis
Japan Anti-Tuberculosis Association

Research Institute of Tuberculosis