Project: Animal tuberculosis

Subprojects JFY2020:

1. Development of Loop mediated isothermal amplification (LAMP) method for the detection of bovine tuberculosis

2. Grasping the prevalence of animal tuberculosis and strengthening the basis for quarantine measures

Principal Investigator:
Dr. Yasuhiko Suzuki
Director & Professor
Hokkaido University International Institute for Zoonosis Control

Co-principal Investigator:
Dr. Chie Nakajima
Professor
Hokkaido University International Institute for Zoonosis Control

Project: Animal tuberculosis

1. Development of Loop mediated isothermal amplification (LAMP) method for the detection of bovine tuberculosis

2. Grasping the prevalence of animal tuberculosis and strengthening the basis for quarantine measures

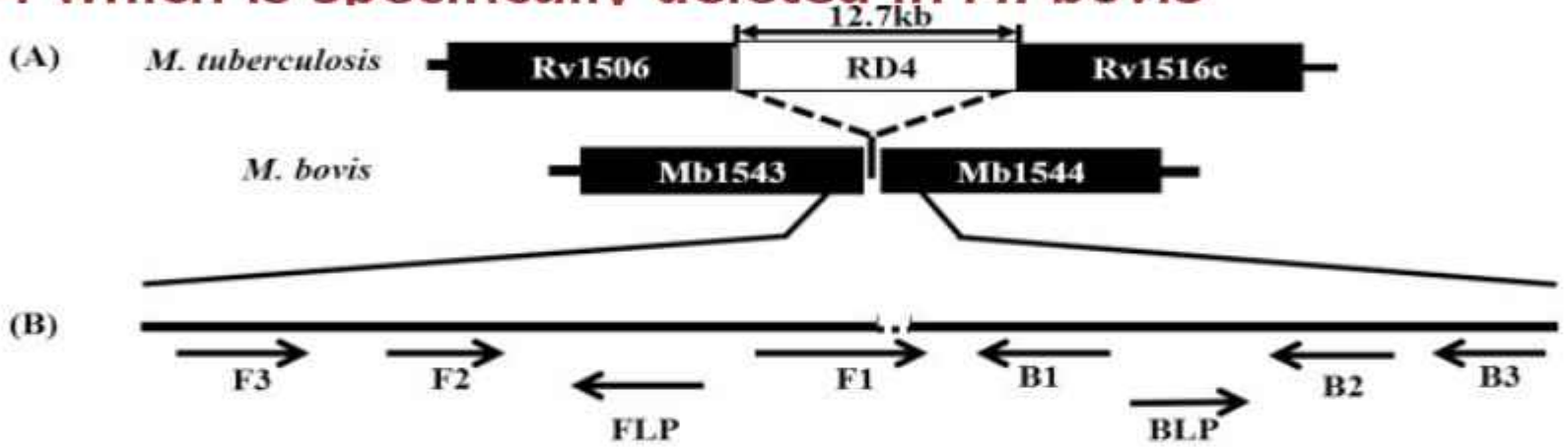
Principal Investigator:
Dr. Yasuhiko Suzuki
Director & Professor
Hokkaido University International Institute for Zoonosis Control

Co-principal Investigator:
Dr. Chie Nakajima
Professor
Hokkaido University International Institute for Zoonosis Control

Subproject 1:Development of LAMP method method for the detection of bovine tuberculosis

Primers used for LAMP

→ Primer sets were designed targeting Region of Difference 4 which is specifically deleted in *M. bovis*



gccgctcccaaaaattaccaatggtttggtcatgacgccttcctaaccagaattgtgaattcatac

F3 F2 *Eco*RI

aagccgtagtcgtgcagaagcgcaacactcttggagt.....ggcctacaacggcg

FLP F1

ctctccgcggcgcgggcgtaccggatatcttagctggtcaatagccatttttcagcaatttctcag

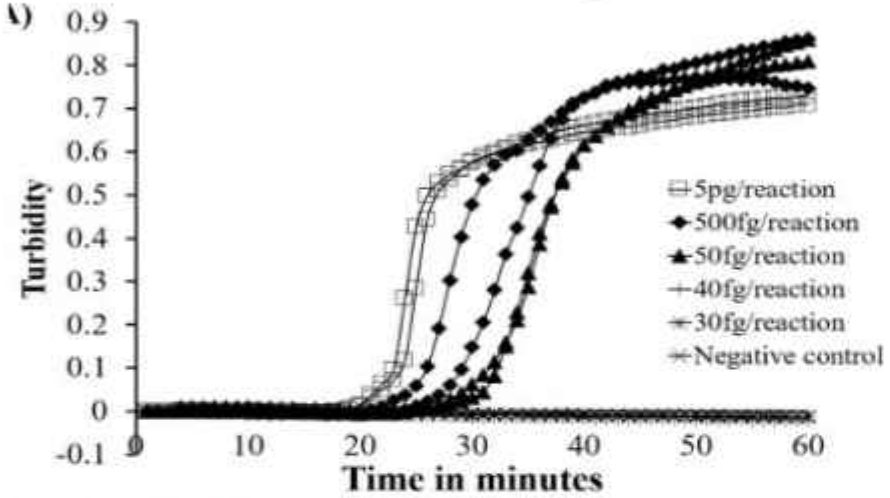
B1 BLP

taacgctacggggcgcgccgtgccgtagtagcgtccccactga

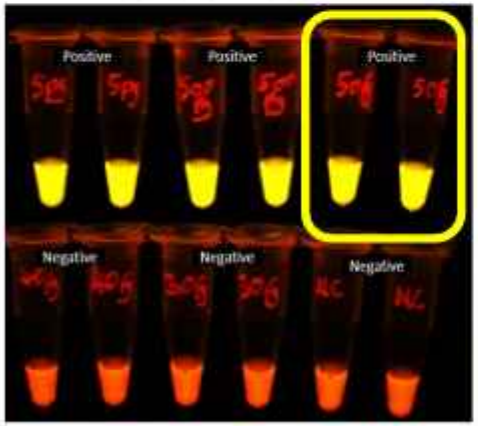
B2 B3

Subproject 1:Development of LAMP method method for the detection of bovine tuberculosis

A. Trend of turbidity



B Results



Under LED lamp

Under day light

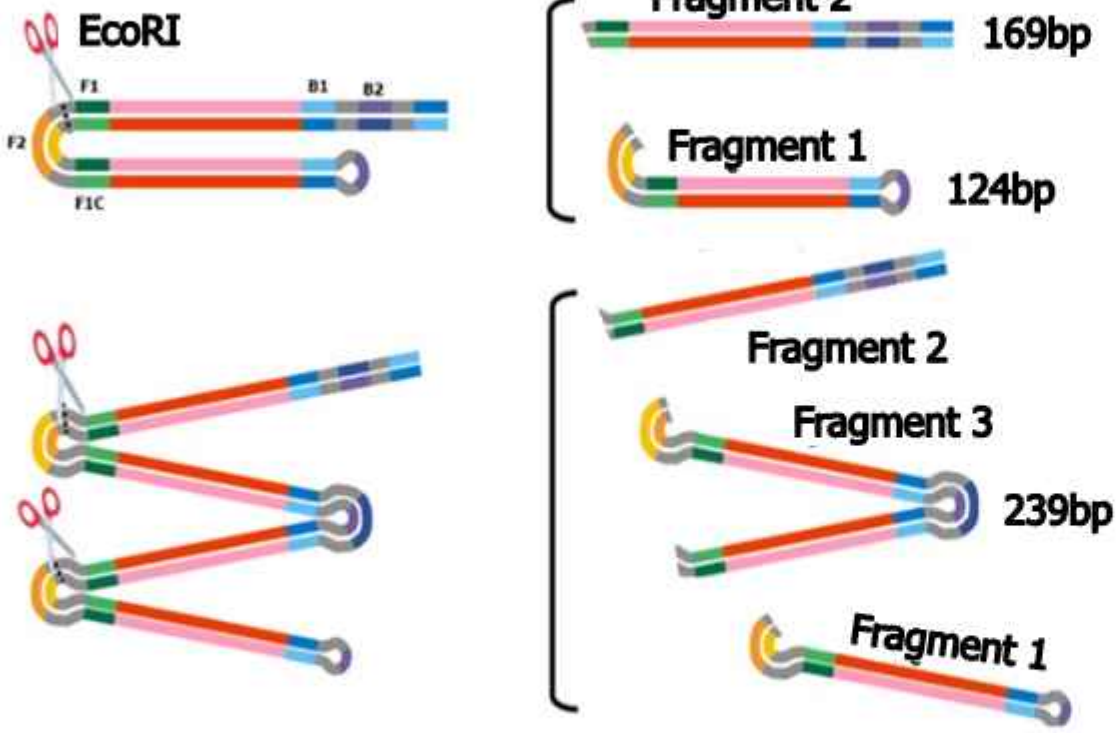
C. PCR

Lane M, 50bp DNA marker; lanes 1 – 9, *M. bovis* BCG Tokyo 172 genomic DNA 500pg, 50pg, 20pg, 5pg, 500fg, 50fg, 40fg, 30fg, 20fg/reaction; lane 10, Negative Control; lane 11, *M. tuberculosis* H37Rv. (Bakshi et al., 2005)

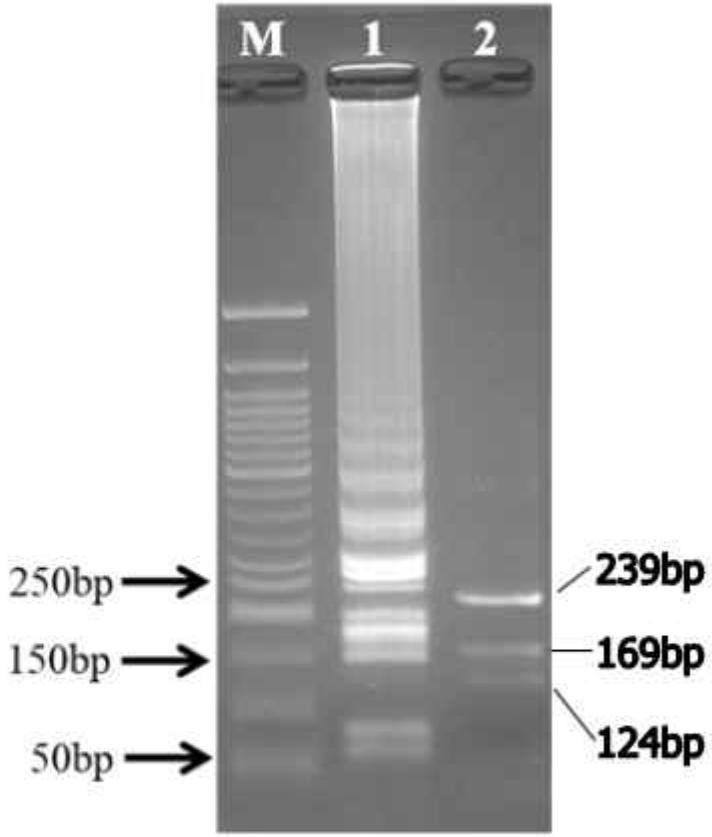
→ This method can detect up to 50 fg of DNA (equivalent to 10 *M. bovis*), which is about 100 times more sensitive than the conventional method (PCR).

Subproject 1:Development of LAMP method method for the detection of bovine tuberculosis

A. *Eco R*I digestion



B Agarose gel



→ Restriction enzyme Eco RI cleavage of the LAMP product showed a fragment of the predicted size, confirming the specific amplification.

Subproject 1:Development of LAMP method method for the detection of bovine tuberculosis

Specificity of developed LAMP method

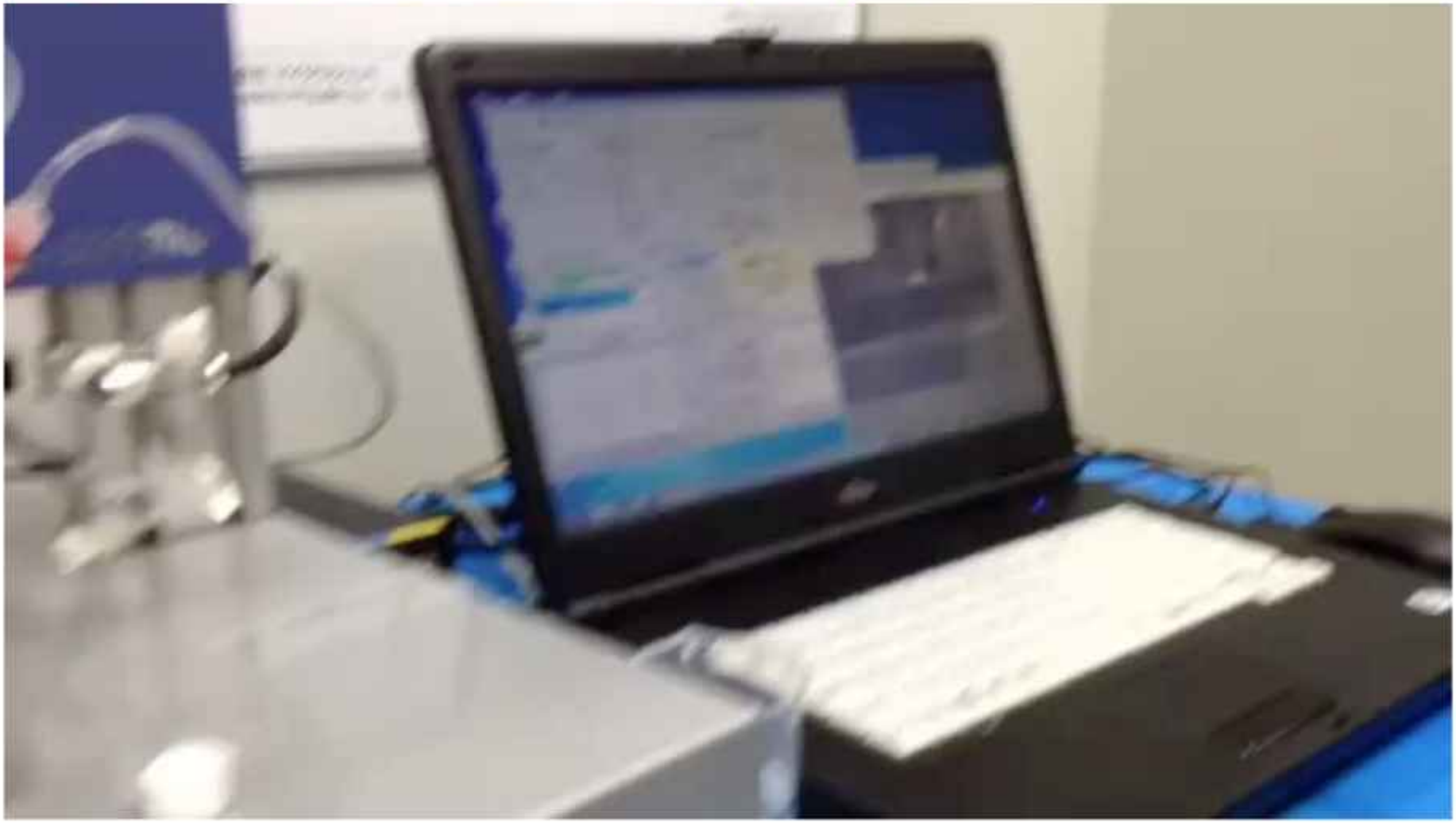
No. of samples	Established LAMP	Reference method[a]
M. bovis **isolates (65)**	**65**	**65**
M. tuberculosis **isolates (40)**	**0**	**40**
MTC species (7)	**0**	**7**
NTM strains (22)	**0**	**0**
Other bacteria (5)	**0**	**0**
Total (139)		

[a]MTBC discriminatory multiplex PCR

→High specificity to detect *M. bovis* was confirmed.

Subproject 1: Development of dry form LAMP method for the easy use at the fields

Drying and kit production using inkjet printers



Subproject 1: Development of dry form LAMP method for the easy use at the fields

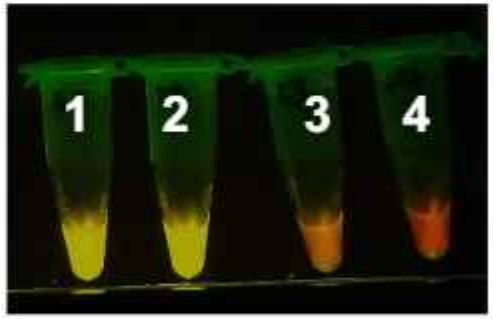
Drying and kit production using inkjet printers（2）

Subproject 1: Development of dry form LAMP method for the easy use at the fields

Hand made

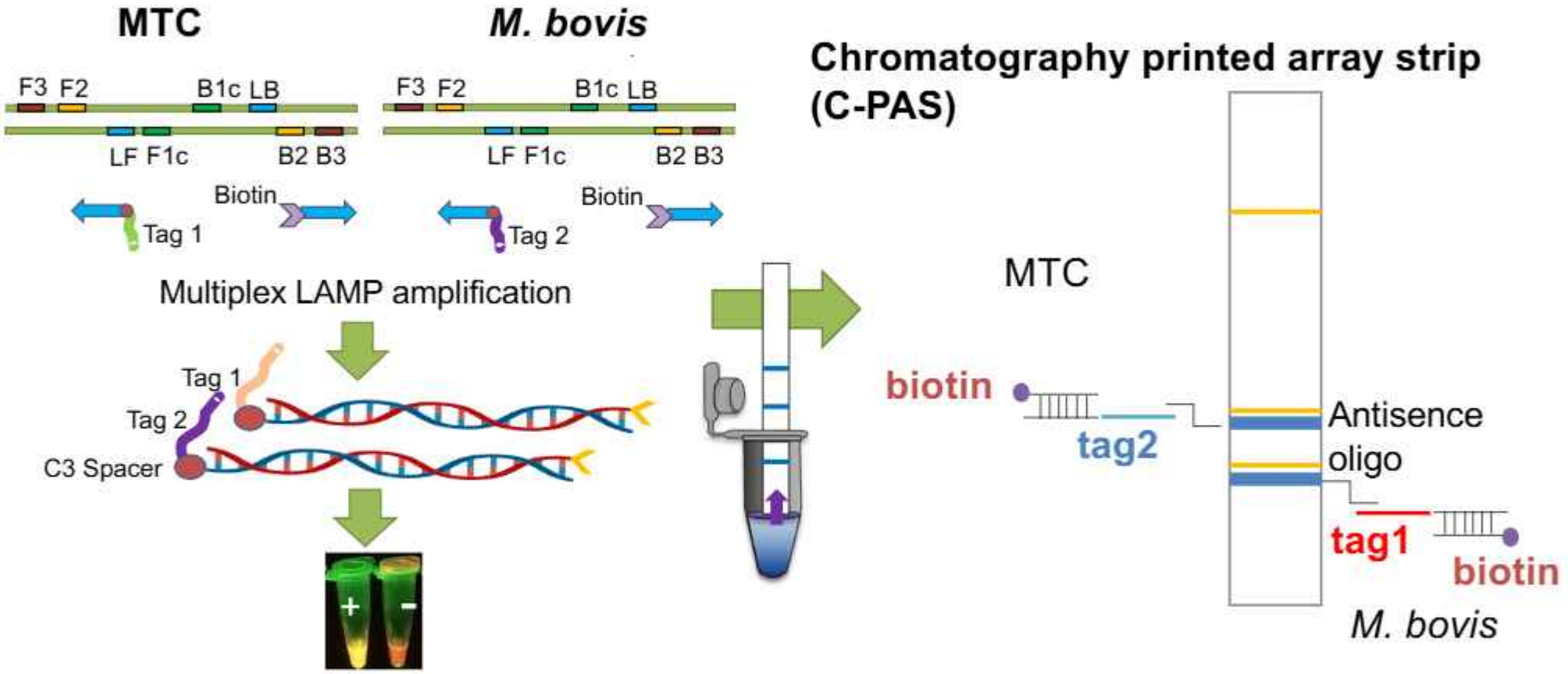
Automated

→ Automated production of dried genetic diagnostic kits for bovine tuberculosis diagnosis using the LAMP method with an ink-jet printer is now possible.



1, 2: Pos、3, 4: Neg

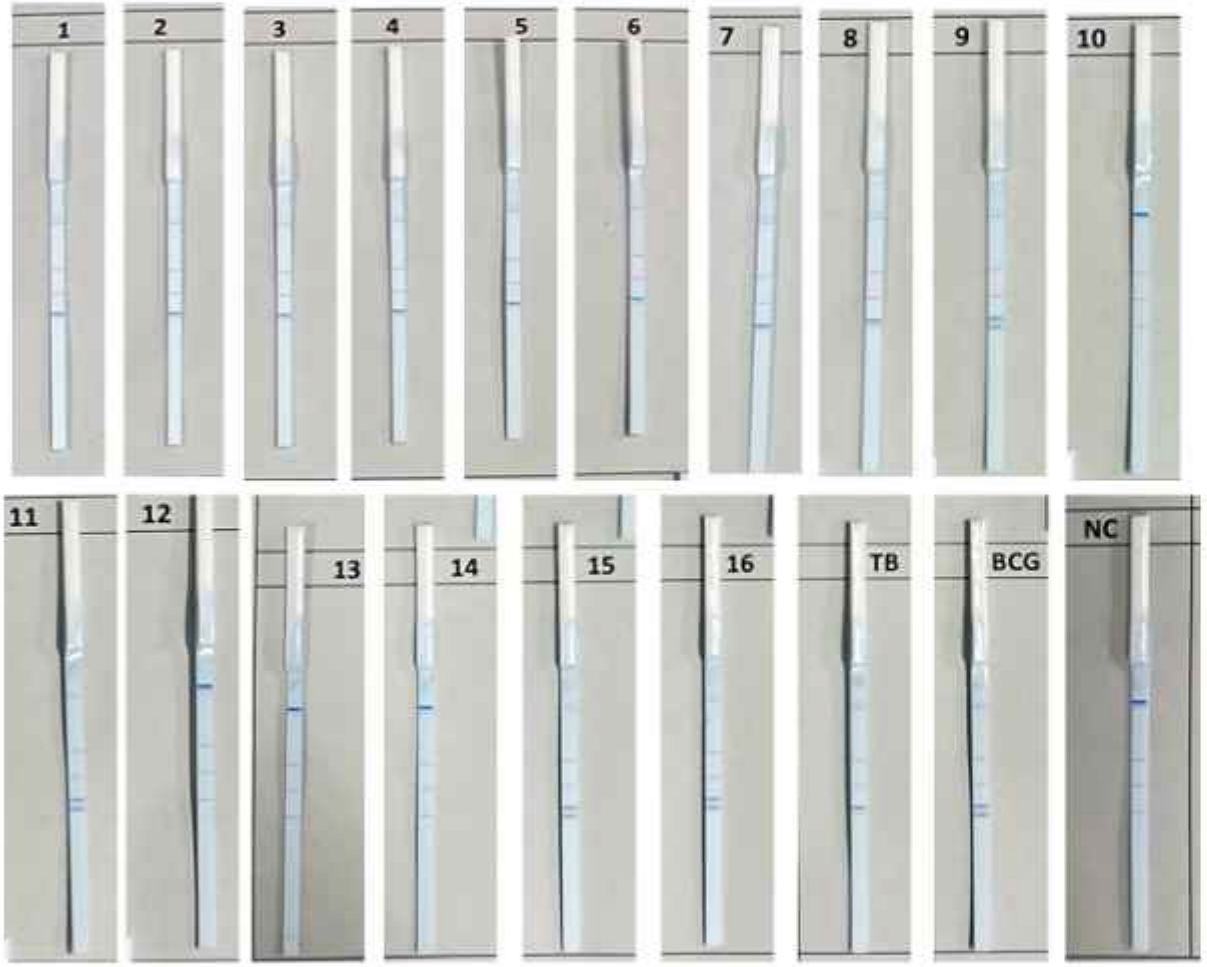
Subproject 1: Development of dry form LAMP method for the easy use at the fields

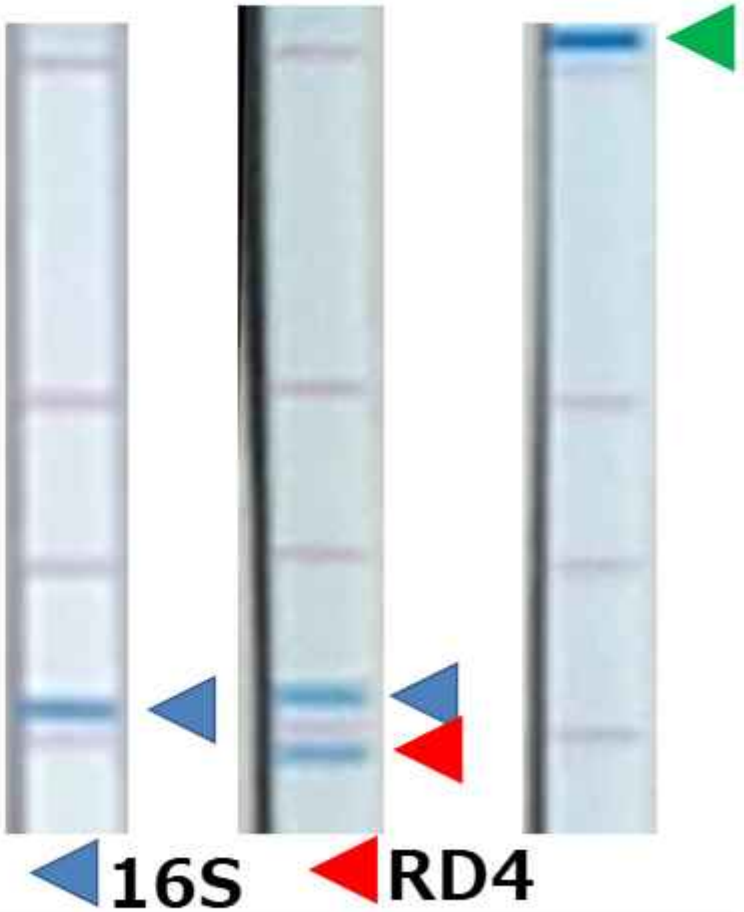


- **Multiplex amplicons indistinguishable by fluorescence**
- **Simultaneous identification of multiplexed amplicons**
- Simple with limited materials

Subproject 1: Development of dry form LAMP method for the easy use at the fields

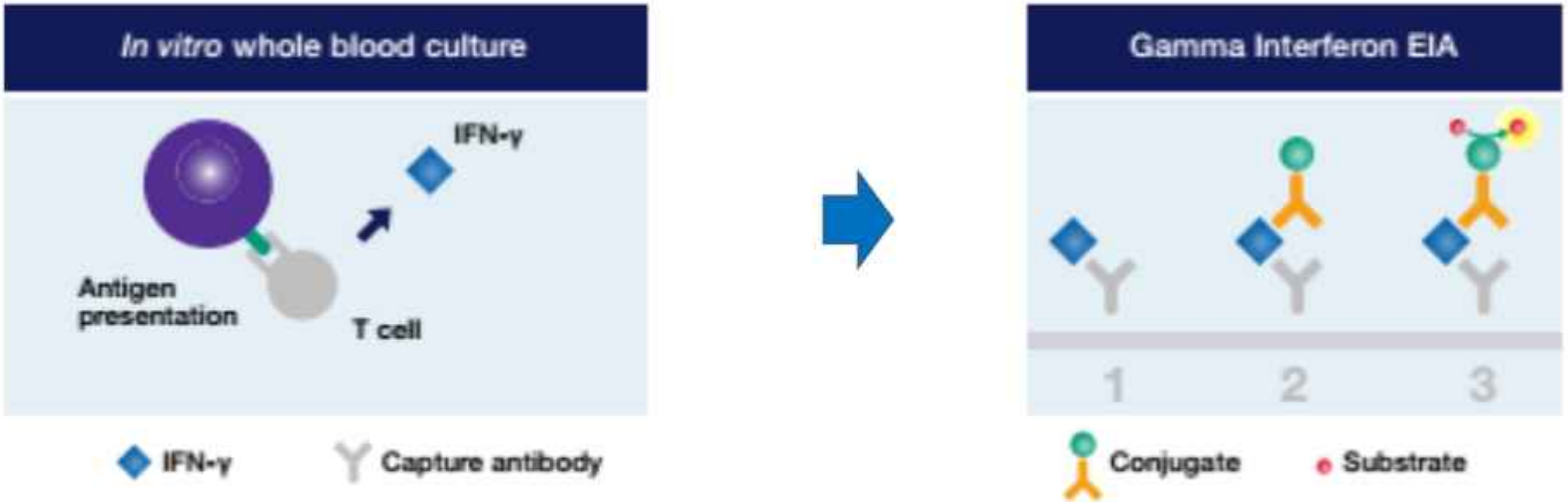
Differentiation of M. bovis by LAMP-DNA chromatography





Subproject 3:Development of immunological methods for bovine tuberculosis diagnosis

Principle of Interferon γ Release assay（IGRA）



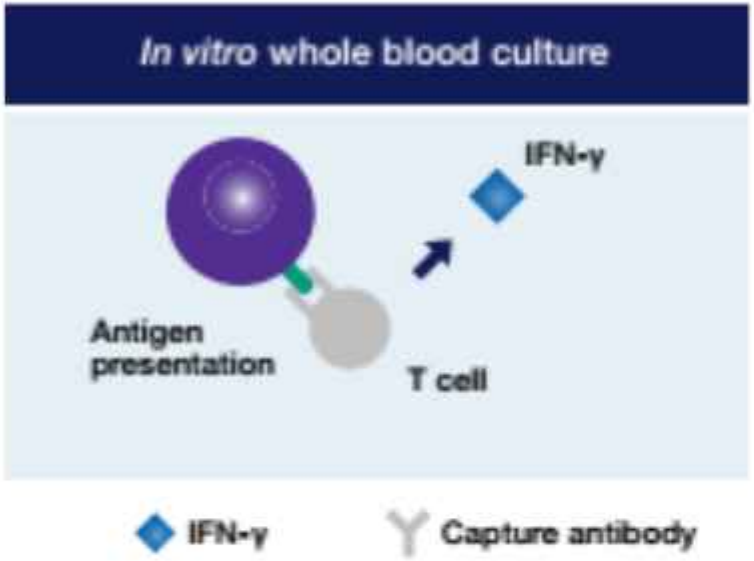
PRIONICS

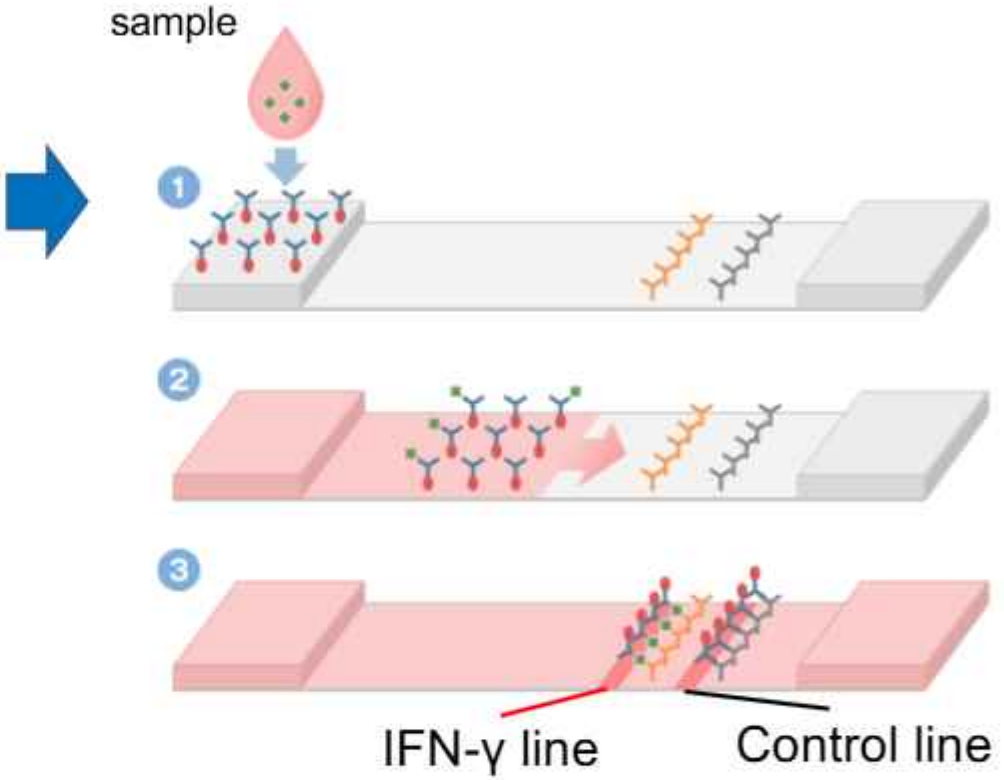
→ A commercial product is available, however, it is expensive and complicated to operate.

→ Development of an low cost and simple IGRA that can be used in Mongolia is necessary.

Subproject 3:Development of immunological methods for bovine tuberculosis diagnosis

Objectives:



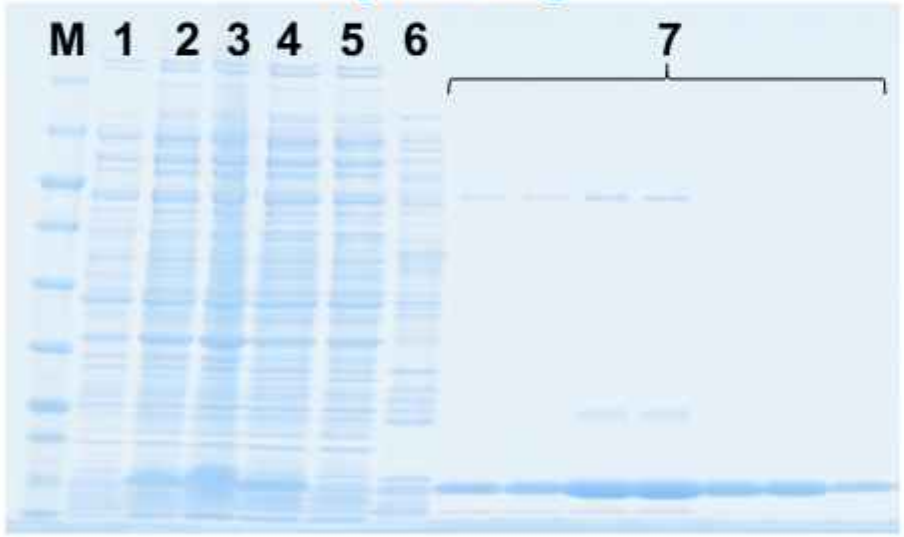


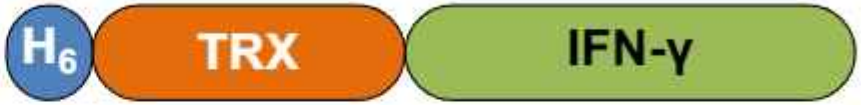
→ **Development of semi-quantitative IFN-γ immunochromatography**

Subproject 3:Development of immunological methods for bovine tuberculosis diagnosis

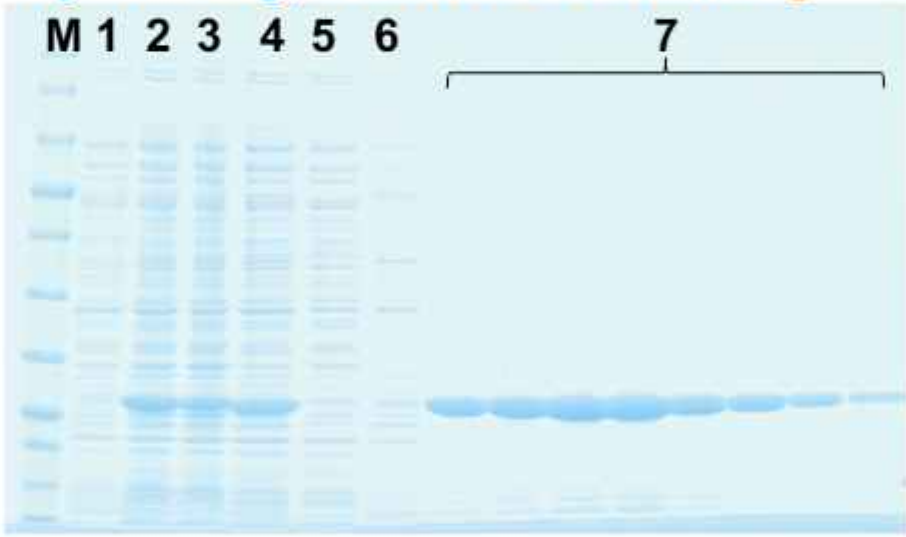
Expression and purification of recombinant bovine IFN-γ using *E. coli* expression system

H_6:His-tag





H_6: His-tag, TRX:thioredoxin-tag

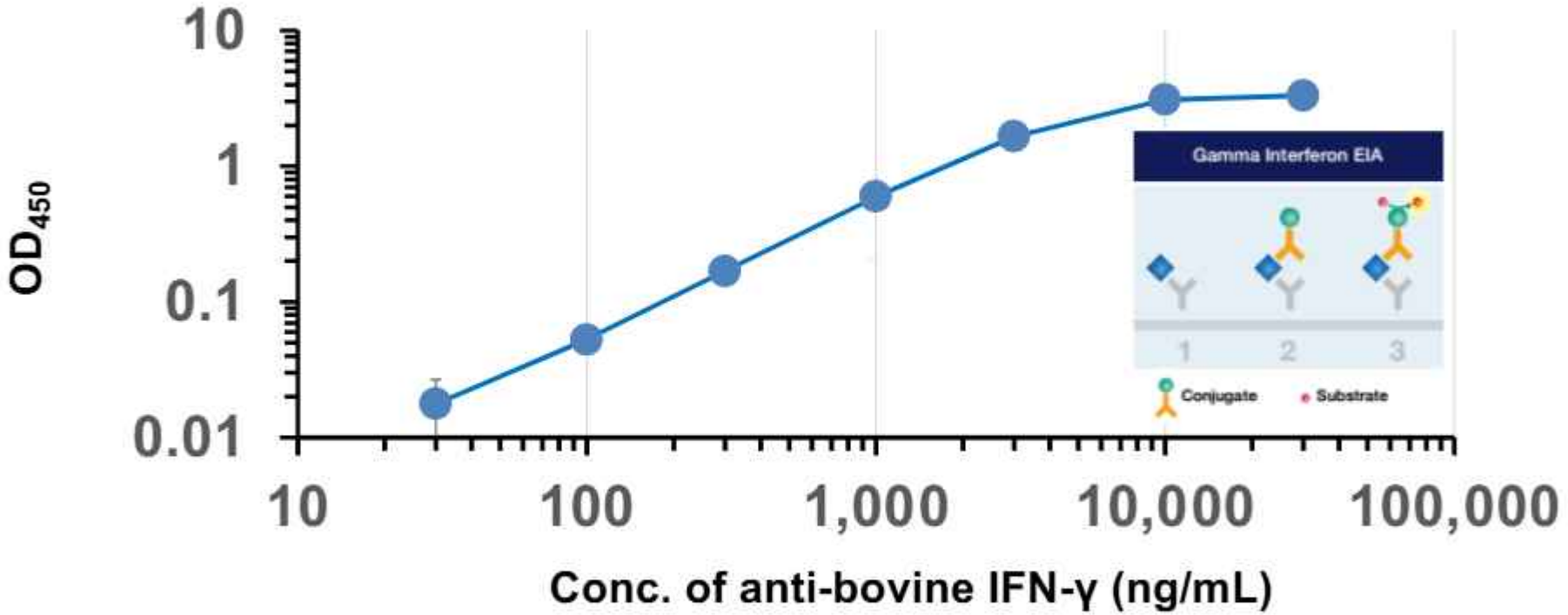


M:MW marker , 1:IPTG(-) , 2 :IPTG(+) , 3: sup after sonication and centrifugation, 5:pellet after sonication and centrifugation, 6:Ni-column pass, 7:imidasol eluates

→ We succeeded mass production and purification of two types of recombinant bovine IFN-γ with different tags. Preparation of antiserum is in progress.

Subproject 3:Development of immunological methods for bovine tuberculosis diagnosis

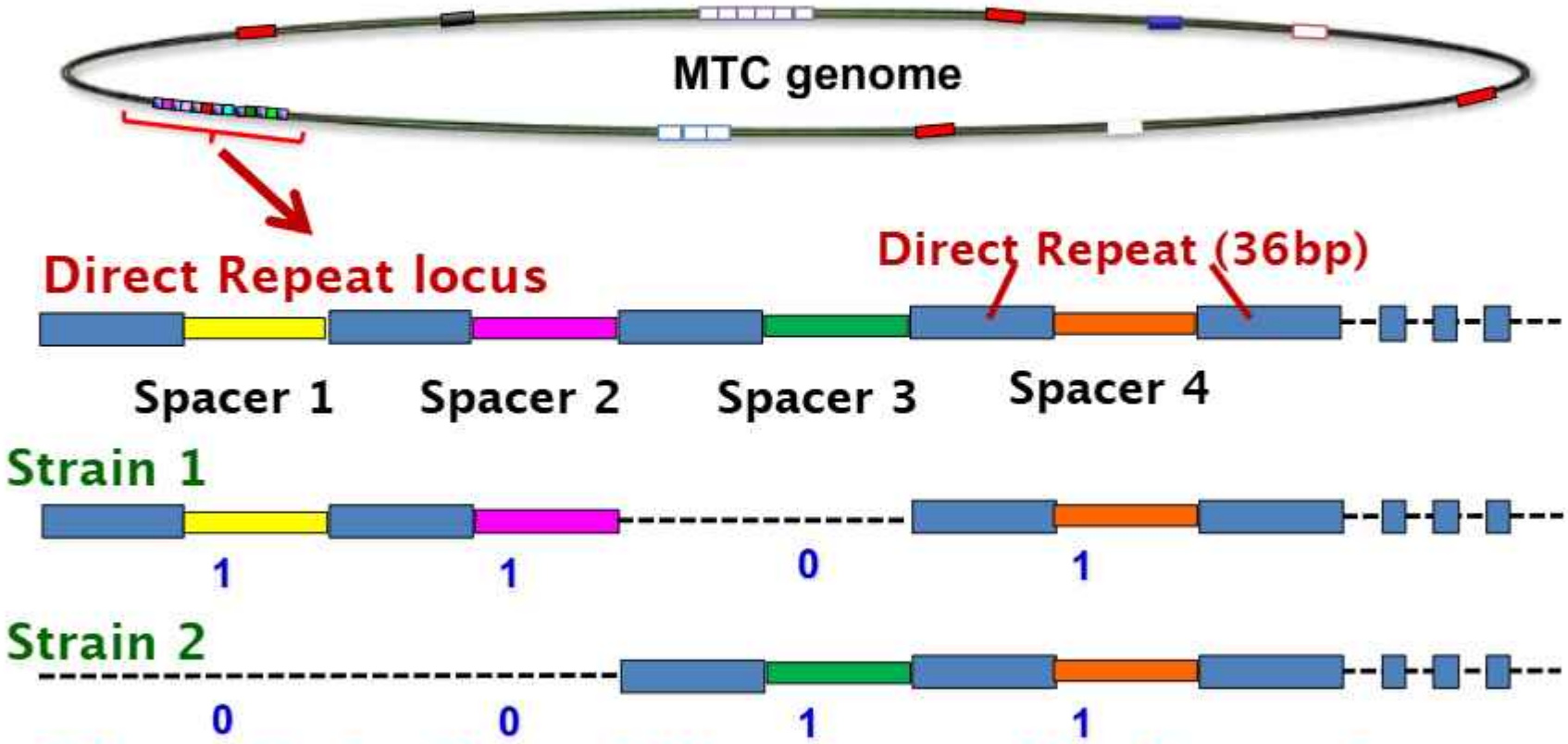
Trial to detect bovine IFN-γ using sandwich ELISA



→ We succeeded to detect IFN-γ as low as 100 ng/mL by utilizing anti-bovine IFN-γ antibody affinity purified from sera of rabbit immunized by recombinant His-tagged anti-bovine-IFN-γ. Recombinant goat, sheep, yak, camel and horse IFN-γ have successfully been produced and purified.

Subproject 3:Development of immunological methods for bovine tuberculosis diagnosis

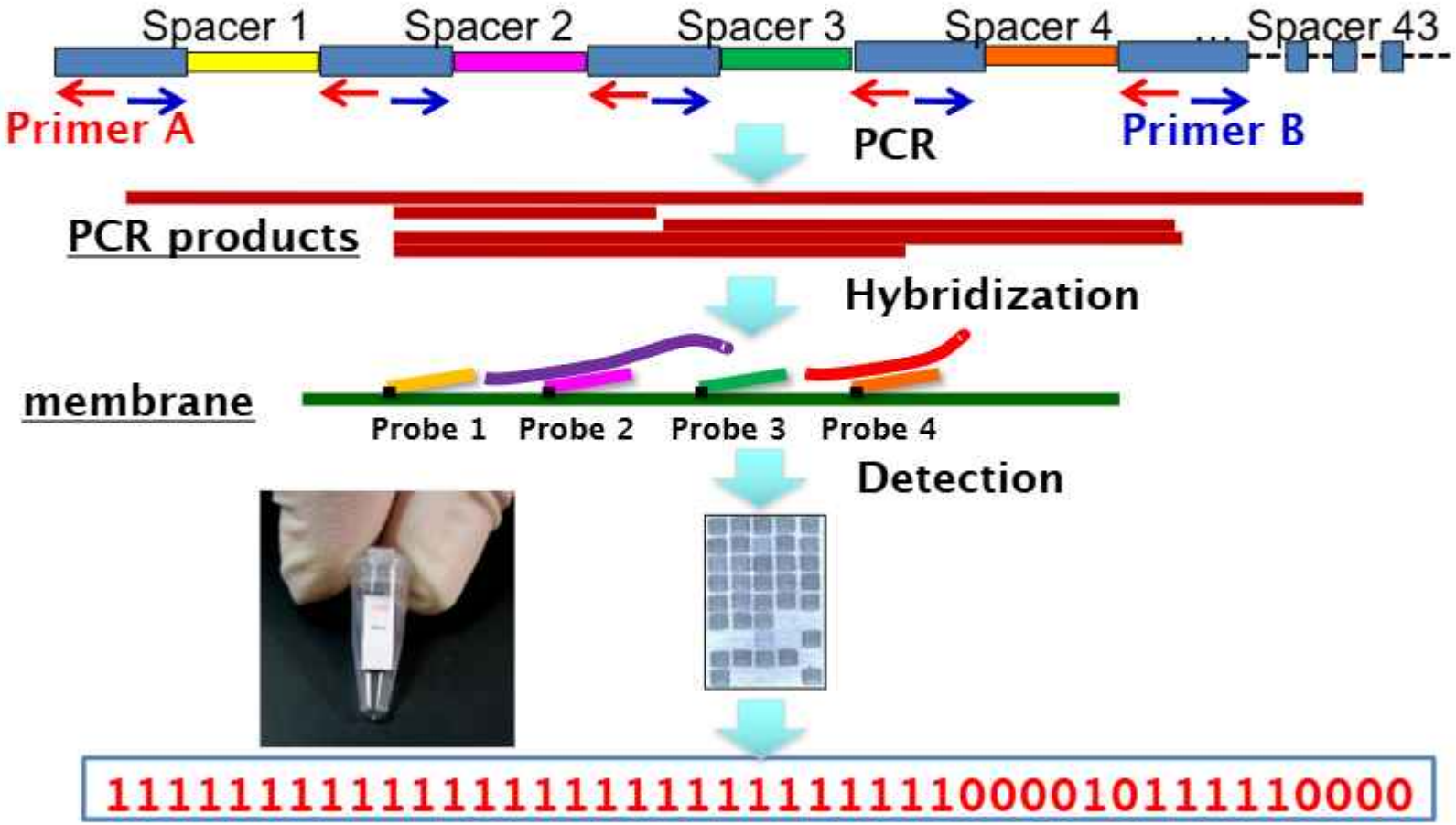
Introduction of simple and low cost genotyping method of *M. tuberculosis* complex



Characterization of direct repeat loci can give digitalized genotype

Subproject 3:Development of immunological methods for bovine tuberculosis diagnosis

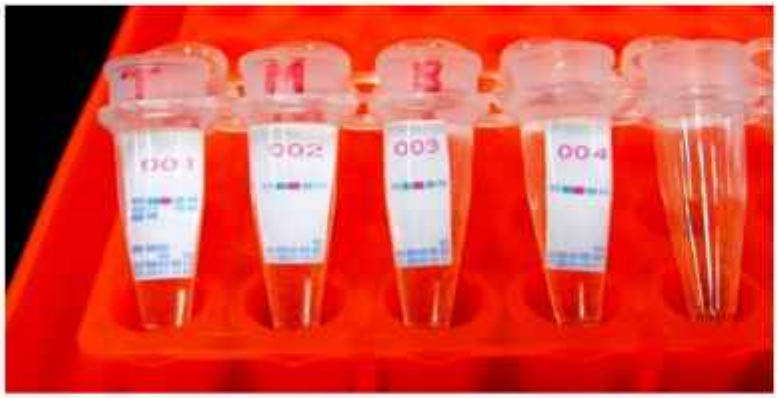
Methodology



Subproject 3:Development of immunological methods for bovine tuberculosis diagnosis

Easy use of In-PCR-tube SpoligoArray

Dr. Khin Saw Aye from Myanmar

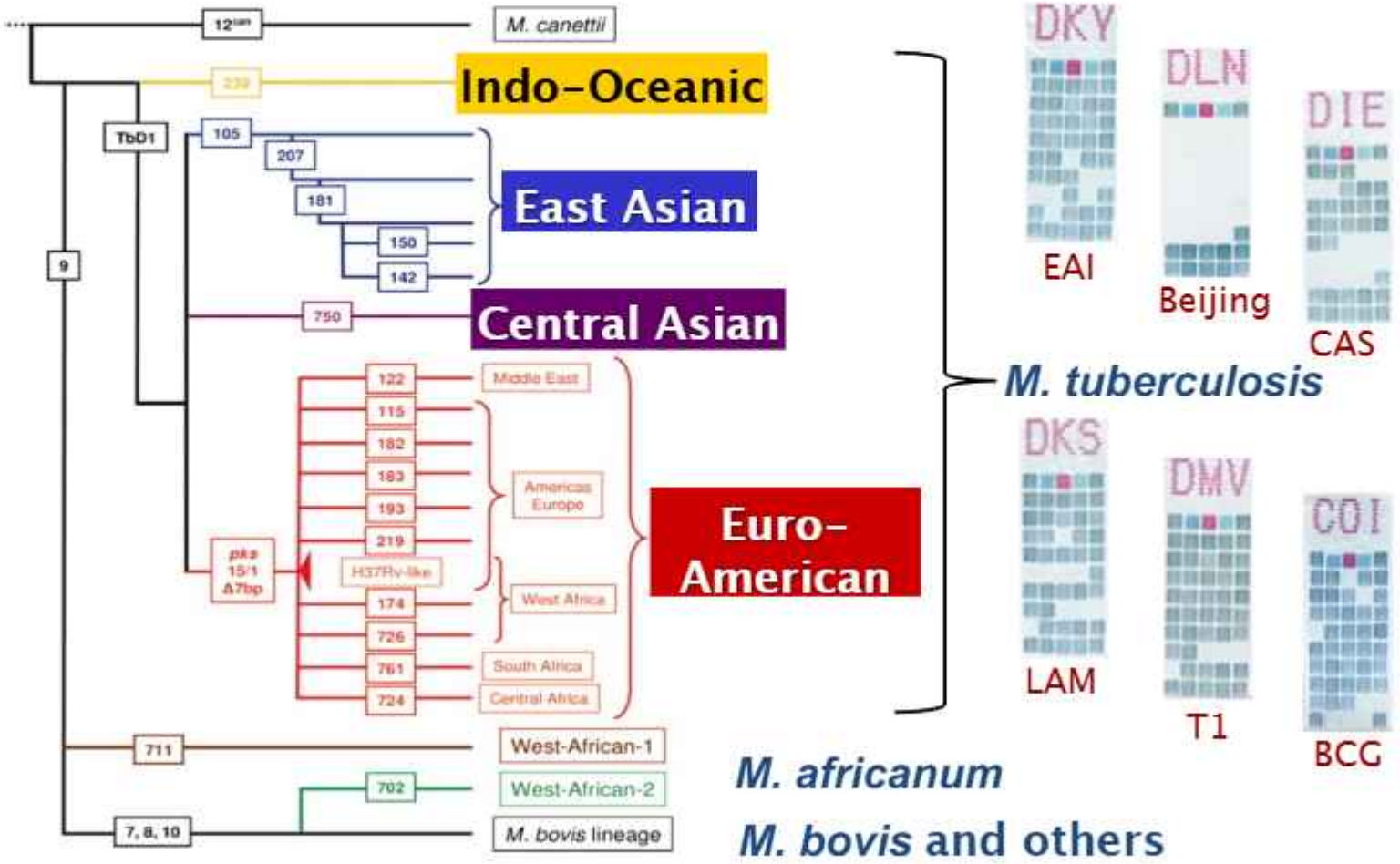


PCR tube = small volume → lower reagent amount and cost

Easy temperature control → accurate results, rapid reaction

Subproject 3:Development of immunological methods for bovine tuberculosis diagnosis

Phylogenetic tree of MTC and SpoligoArray results



Plan of Operation

Output 1: The function of laboratory diagnosis for zoonotic diseases is enhanced in Mongolia through the development of LAMP / immunochromatography-based novel rapid diagnostic methods (kits) for detecting **Mycobacterium** bovis and Burkholderia mallei as well as updating existing disease diagnostic systems.

Inputs	Year	2020				2021				2022				2023				2024				2025				Remarks	
	Month	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec		
1.1.1. To develop a LAMP-based method for detecting *M. bovis*-specific genetic region at the Hokkaido University (including drying of reagents).	Plan																							RCZC	IVM
	Activity																								
1.1.2. To make the *M. bovis* gene detection method into a "*kit*" using an ink-jet printer In Hokkaido University (trial production of a rapid diagnostic test kit).	Plan																							RCZC	IVM
	Activity																								
1.1.2.2 To make the M. bovis gene detection method using lateral Flow method	Added																								
	Activity																								
1.1.3. To evaluate the sensitivity and specificity of the developed kit(s) with biological specimens of *M. bovis*-infected patients and animals in Mongolia.	Plan																							RIT RCZC→IIZC	NCDC IVM
	Activity																								
1.1.4. To prepare Standard Operating Procedure(s) (SOPs) for the genetic diagnosis of *M. bovis* infections in humans at NCCD following the improvements are made as appropriate based on the aforementioned evaluation results.	Plan																							RIT RCZC→IIZC	NCCD
	Activity																								
1.1.5. To prepare SOPs for the genetic diagnosis of *M. bovis* infections in livestock at IVM following the improvements are made as appropriate based on the aforementioned evaluation results.	Plan																							RCZC→IIZC	IVM
	Activity																								

Plan of Operation

Output 3: The epidemics of tuberculosis and glanders as zoonotic diseases in livestock are evaluated using seroepidemiological and molecular epidemiological / seroepidemiological techniques, respectively.

Inputs	Year	2020				2021				2022				2023				2024				2025				Remarks	
	Month	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec		
3.1.1. To isolate tuberculosis complex using the L-J medium in IVM, from the tubercle-suspected granulomas samples collected from cattle and sheep at slaughterhouses and meat markets in the project target areas.	Plan																							RCZC→HZC	IVM
	Activity																								
3.1.2. To estimate the prevalence of *M. bovis* in the tuberculosis complex isolated from cattle and sheep by determining the presence of *M. bovis* in the colonies grown on L-J medium using the genetic detection method developed in the Activity 1.1.	Plan																							RCZC→HZC	IVM
	Activity																								
3.1.3. To investigate the endemic status of *M. bovis* infection including subclinical infection by performing the Interferon-Gamma Release Assay (IGRA) on herds in which *M. bovis*-detected cattle were kept.	Plan																							RCZC→HZC	IVM
	Activity																								
3.1.3.1 To To establish in house IGRA.	Added																							RCZC→HZC	
	Activity																								
3.1.4. To assess the transmission and distribution of *M. bovis* in animals (amongst cattle, between cattle and sheep, and amongst sheep) by performing the comprehensive gene screening using a next-generation sequencer (e.g., MinION) on the colonies grown on the L-J media for *M. bovis* as well as in consideration of the IGRA results of endemic status in herds.	Plan																							RCZC→HZC	IVM
	Actual																								
3.1.4.1 Introduction of simple and low-cost genotyping method of M. tuberculosis complex	Added																							RCZC→HZC	
	Activity																								
3.1.5. To develop draft revision 3 of the current guideline 3 for the diagnosis of livestock infectious diseases, the program for the control of livestock infectious diseases and/or equivalent documents on the basis of the prevalence of *M. bovis* infections in livestock as well as the results of epidemiological evaluations of its transmission and dissemination, with consultation from relevant authorities such as the Ministry of Food, Agriculture and Light Industry (MOFALI) and the General Authority for Veterinary Services (GAVS).	Plan																							RCZC→HZC	MOFALI GAVS IVM
	Actual																								
3.1.6. To conduct specific consultations with the authorities concerned such as MOFALI and GAVS for the revision of the guidelines, programs and/or equivalent document(s) on the basis of the epidemiological evidences.	Plan																							RCZC→HZC	MOFALI GAVS IVM
	Actual																								
3-2. To evaluate the *M. bovis* contamination status in milk, which are sold in markets such as milk stands, by testing them with the gene detection method developed in the Activity 1.1.	Plan																							RCZC→HZC	IVM
	Actual																								