Report of Attachment Program
Advanced Diagnostics of Plant Viruses

at
Laboratory of Tropical Plant Protection
Tokyo University of Agriculture (Tokyo NODAI), Japan
October 26 – December 25, 2015

By

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(Viet Nam)

Organized by:

Tokyo University of Agriculture
(Tokyo NODAI), Japan

In Collaboration with:

bionet aseanet

ASEAN Network on Taxonomy

2016

REPORT OF ATTACHMENT PROGRAM IN JAPAN

FROM October 27th to December 24th 2015

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Country: Vietnam

1. Background information

The ASEAN Plant Health Cooperation Network (APHCN) – ASEANET Project ***"Taxonomic capacity building to support market access for agricultural trade in the ASEAN region"***, funded by the Japan ASEAN Integration Fund (JAIF) has been implemented for 2 (two) years starting from 15th May 2015 covering several activities related to training and attachment program.

Due to the first workshop *"**Training Workshop on Plant Viruses**"*, which was successfully held from 17-28 August 2015 at UPLB, Philippines with nine ASEAN countries including Brunei Darussalam, Indonesia, Cambodia, Laos, Malaysia, Myanmar, Philippine, Thailand, and Vietnam. Under this ASEAN-endorsed Project, three outstanding participants from the training workshop are to be selected for a two-month attachment program "***Training Workshop on Advanced Diagnostics on Plant Viruses"*** at the Laboratory of Tropical Plant Protection of the Tokyo University of Agriculture (TUA), Japan, for further training under supervision of Prof. Keiko Natsuaki (Vice President of TUA) and Dr. Marita S. Pinili (Institute of Plant Breeding, University of the Philippines Los Banos).

2. Objectives of the attachment program

- To provide diagnostic skills for identification of plant virus diseases based on typical symptoms.
- To study the methods for detection and identification of plant viruses by serological and molecular techniques.
- To study the transmission of plant viruses from the sources to target host.
- To apply the suitable techniques for plant viruses disease management in our country.

3. Daily program

Date	Activity	Venue	Resource Person(s)
Monday Oct. 26th	Arriving at Tokyo University of Agriculture (TUA) and visit Laboratory of Tropical Plant Protection (HOGOKEN Lab.)	Tokyo University of Agriculture (TUA) and HOGOKEN Lab.	Mr. Takeda
Tuesday Oct. 27th	Visiting the campus and making phosphate buffer for mechanical inoculation.	HOGOKEN Lab.	Mr. Chung; Ms Kudo and Mr. Ikeda
Wednesday Oct. 28th	Detecting virus on bamboo by ELISA method	HOGOKEN Lab.	Ms. Hiraiwa and Ms Matsumoto
Thursday Oct. 29th	- Inoculating *potyvirus* to passionfruit seedlings by mechanical method and sawing the index plants. - Visiting the NODAI Biorium	HOGOKEN Lab.	Prof. Keiko Natsuaki and Mr. Ikeda
Friday Oct. 30th	- Sampling the samples with virus-like symptom. - Wrapping up of the first week.	HOGOKEN Lab.	Ms. Hiraiwa and Ms Takada
Saturday Oct. 31st	Attending the TUA's festival and the dinner party at the HOGOKEN Lab.		
Sunday Nov. 1st	Day off		
Monday Nov. 2nd	Preparing the sample conservation buffers	HOGOKEN Lab.	Ms. Hiraiwa and Mr. Ikeda
Tuesday Nov. 3rd	Day off – national holiday		
Wednesday Nov. 4th	Detecting virus on passionfruit by ELISA method	HOGOKEN Lab.	Mr. Ikeda
Thursday Nov. 5th	Detecting virus on passionfruit by ELISA method (continued)	HOGOKEN Lab.	Ms. Takada
Friday Nov. 6th	- Using the information network regarding plant virus identification (DDBJ/NCBI).	HOGOKEN Lab.	Prof. Keiko Natsuaki

Date	Activity	Venue	Resource Person(s)
	- Wrapping up of the second week.		
Saturday Nov. 7th	Attending the ISSAAS conference.	NODAI Academia Center	
Sunday Nov. 8th	Attending the ISSAAS conference.	NODAI Academia Center	
Monday Nov. 9th	Attending the ISSAAS excursion.	Kawaguchiko Lake; Fuji mountain	
Tuesday Nov. 10th	Day off – catch up holiday for Nov. 8th		
Wednesday Nov. 11th	- DNA extraction from *Banana bunchy top virus* (BBTV)-infected banana samples. - Running PCR reaction and preparing the agarose gel.	HOGOKEN Lab.	Dr. Marita S. Pinili
Thursday Nov. 12th	- Short presentation on plant parasitic nematodes. - Gel electrophoresis. - Extraction and detection of BBTV from fresh and preserved banana and abaca samples.	HOGOKEN Lab.	Dr. Marita S. Pinili
Friday Nov. 13th	Post-laboratory discussion	HOGOKEN Lab.	Dr. Marita S. Pinili
Saturday Nov. 14th	Day off		
Sunday Nov. 15th	Day off		
Monday Nov. 16th	- Conducting gel electrophoresis of BBTV PCR products. - Impregnation and extraction of virus nucleic acid from FTA plant card.	HOGOKEN Lab.	Dr. Marita S. Pinili
Tuesday Nov. 17th	- Performing PCR assay of DNA from FTA plant card.	HOGOKEN Lab.	Dr. Marita S. Pinili

Date	Activity	Venue	Resource Person(s)
	- Carrying out gel electrophoresis and gel cut. - Purification of DNA product.		
Wednesday Nov. 18th	- Extraction of BBTV from banana aphids (*Pentalonia nigronervosa)* as a vector of virus. - Conducting the PCR assay. - Ligation of purified DNA using pGEM vector.	HOGOKEN Lab.	Dr. Marita S. Pinili
Thursday Nov. 19th	- Extraction of BBTV from aphid impregnated on FTA plant card. - Conducting the PCR assay and gel electrophoresis. - Transformation of ligated plasmid.	HOGOKEN Lab.	Dr. Marita S. Pinili
Friday Nov. 20th	- Checking the colonies of transformation step. -Post-laboratory discussion	HOGOKEN Lab.	Dr. Marita S. Pinili
Saturday Nov. 21st	Day off		
Sunday Nov. 22nd	Day off		
Monday Nov. 23rd	- Picking the transformed colonies. - Culturing the *E.coli* colonies to LB medium.	HOGOKEN Lab.	Dr. Marita S. Pinili
Tuesday Nov. 24th	- Performing miniprep and insert check.	HOGOKEN Lab.	Dr. Marita S. Pinili
Wednesday Nov. 25th	- Conducting the precipitation step. - Preparing for DNA sequencing.	HOGOKEN Lab.	Dr. Marita S. Pinili
Thursday Nov. 26th	- Attending the lecture on Phylogenetic tree and	HOGOKEN Lab.	Dr. Noriko Furuya (from DNA Data Bank

Date	Activity	Venue	Resource Person(s)
	constructing phylogenetic tree by MEGA software.		of Japan – DDBJ).
Friday Nov. 27th	-Post-laboratory discussion	HOGOKEN Lab.	Dr. Marita S. Pinili
Saturday Nov. 28th	Day off		
Sunday Nov. 29th	Day off		
Monday Nov. 30th	- Discussion about the plan schedule of Yokohama Trip and short visit to Utsunomiya University. - Attend Halal Seminar by Nodai cooperate with Putra Malaysia University	HOGOKEN Lab. NODAI Academia Center	Prof. Keiko Natsuaki
Tuesday Dec. 1st	Preparation of LB medium for culturing bacteria	HOGOKEN Lab.	Ms. Takada
Wednesday Dec. 2nd	Transferring the banana aphids from healthy plant to BBTV-infected plant for acquisition of virus for transmission experiment.	HOGOKEN Lab.	Ms. Takada
Thursday Dec. 3rd	Visiting to Utsunomiya University to learn the method for dsRNA extraction.	Laboratory of Plant Pathology	Dr. Tomohide Natsuaki and his students
Friday Dec. 4th	Continue dsRNA extraction.	Laboratory of Plant Pathology	Dr. Tomohide Natsuaki and his students
Saturday Dec. 5th	Day off		
Sunday Dec. 6th	Day off		
Monday Dec. 7th	- Conducting RNA extraction for potyvirus from passion fruit (inoculated plant) using phenol chloroform method.	HOGOKEN Lab.	Ms. Minho

Date	Activity	Venue	Resource Person(s)
	- Performing the cDNA synthesis assay.		
Tuesday Dec. 8th	Carrying out the PCR assay and gel electrophoresis.	HOGOKEN Lab.	Mr. Ikeda
Wednesday Dec. 9th	Gel checking	HOGOKEN Lab.	Ms. Matsumoto
Thursday Dec. 10th	- Re-conducting the PCR assay because of not good result. - Preparing the LB medium	HOGOKEN Lab.	Ms. Takada
Friday Dec. 11th	- Study visit to Yokohama Plant Protection Station and Research Center.		Prof. Keiko Natsuaki
Saturday Dec. 12th	Day off		
Sunday Dec. 13th	Day off		
Monday Dec. 14th	Performing the gel electrophoresis and DNA purification.	HOGOKEN Lab.	
Tuesday Dec. 15th	- Performing the DNA ligation. - Detection of protein using SDS-PAGE and western blot	HOGOKEN Lab.	
Wednesday Dec. 16th	Performing the DNA transformation.	HOGOKEN Lab.	
Thursday Dec. 17th	Conducting dsRNA purification.	HOGOKEN Lab.	
Friday Dec. 18th	Attending the doctoral thesis defense of Ms. Ayaka Uke	HOGOKEN Lab.	
Saturday Dec. 19th	Day off		
Sunday Dec. 20th	Day off		
Monday Dec. 21st	- Performing the final presentation for attachment project.	HOGOKEN Lab.	

Date	Activity	Venue	Resource Person(s)
	- Conducting the DNA ligation step.		
Tuesday Dec. 22nd	- Conducting the DNA transformation step. - Visiting Bio-molecular laboratory to gain the information about illumina's sequencing technology		
Wednesday Dec. 23rd	Culturing *E.coli* in TB medium	HOGOKEN Lab.	
Thursday Dec. 24th	- Conducting the miniprep step. - Moving to Narita view Hotel	HOGOKEN Lab.	
Friday Dec. 25th	- Coming back to Vietnam		

4. Activities

4.1. Laboratory studies:

After two months in Tokyo University of Agriculture (TUA), I have gained a lot of valuable knowledge related to detection and identification of plant virus diseases.

4.1.1. Observation of symptoms of samples affected by plant viruses

Fig 1. Observation and samples collecting with virus infection symptoms

Fig. 2: Symptoms of virus infection on Bamboo (*Pleioblastus chino*); Passion fruit (*Passiflora edulis*); Taro (*Colocasia esculenta*) and Banana (*Musa* sp.)

4.1.2. Preparation of several buffers for diagnosis of plant viruses

Fig. 3: Making buffers for ELISA technique

4.1.3. Detection of plant viruses using ELISA method

During the program, ELISA method was used for detection of potyvirus on Bamboo (*Pleioblastus chino*), Passion fruit (*Passiflora edulis*) and Taro (*Colocasia esculenta*).

Fig. 4: Detection of potyvirus from selected crops using for ELISA technique

4.1.4. Mechanical inoculation of potyvirus onto Passion fruit seedlings

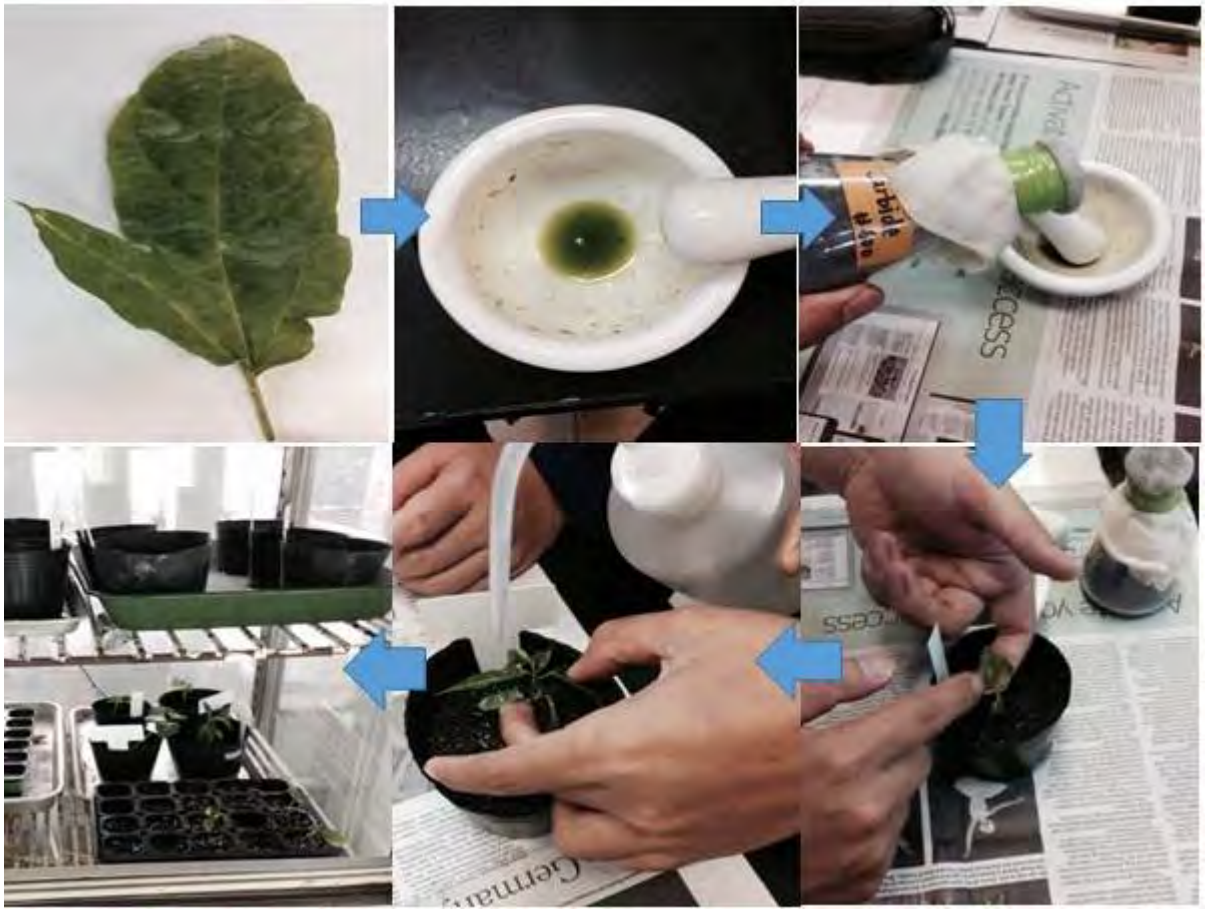


Fig. 5: The process of mechanical inoculation of potyvirus onto Passionfruit seedlings

4.1.5. Detection of Banana Bunchy Top Virus (BBTV) on Banana and Abaca samples using PCR technique

Fig. 6: Preparation for leaf extraction from selelcted samples

Fig. 7: Performance of PCR assay

Fig. 8: Performance of gel electrophoresis

Fig. 9: Gel staining with EtBr and viewing of DNA band under UV transilluminator

4.1.6. Detection of plant viruses using Electron Microscope

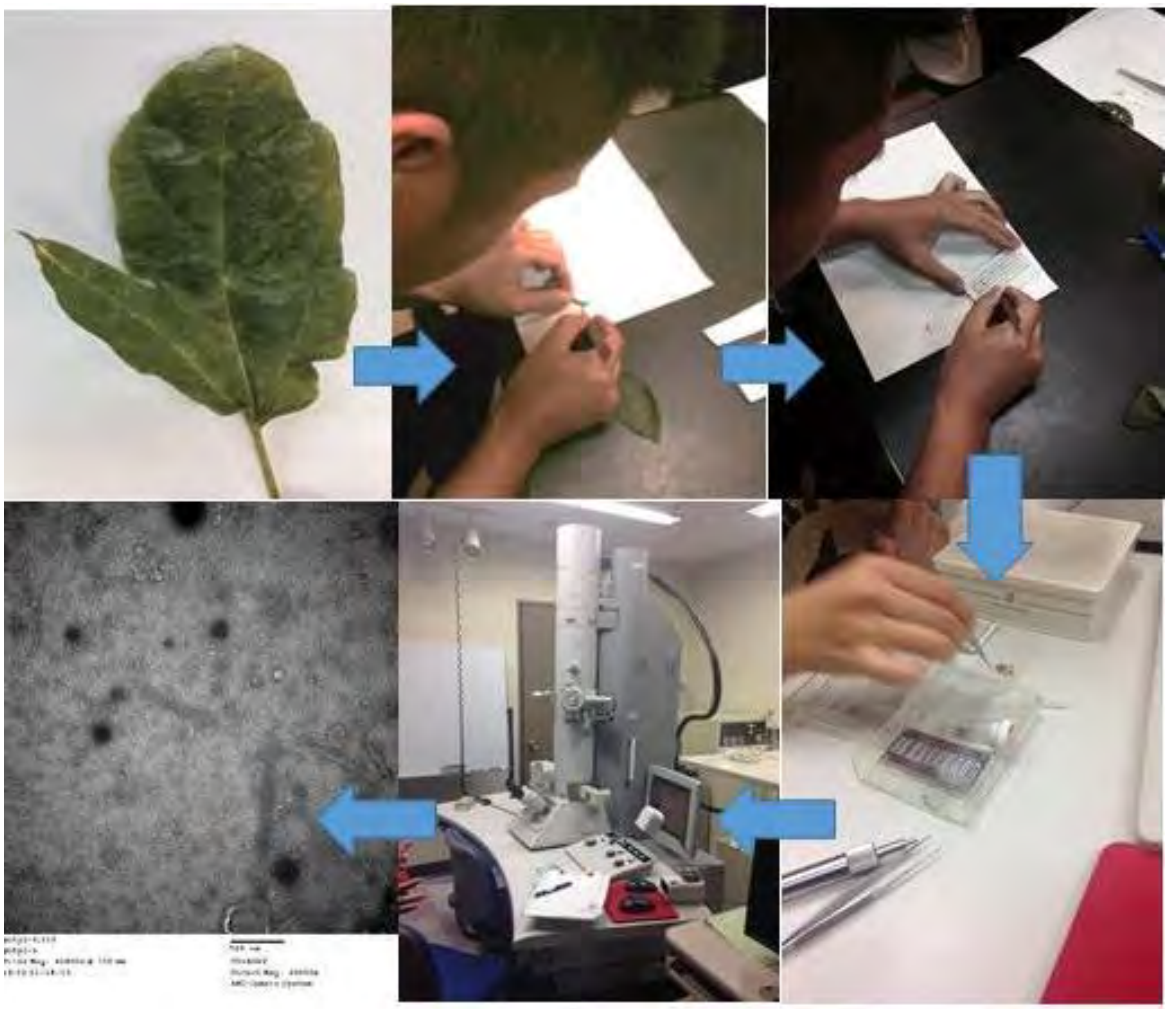


Fig. 10: The process of detection of plant viruses using Electron Microscope

4.1.7. Preservation of virus-infected samples and aphids on FTA plant card

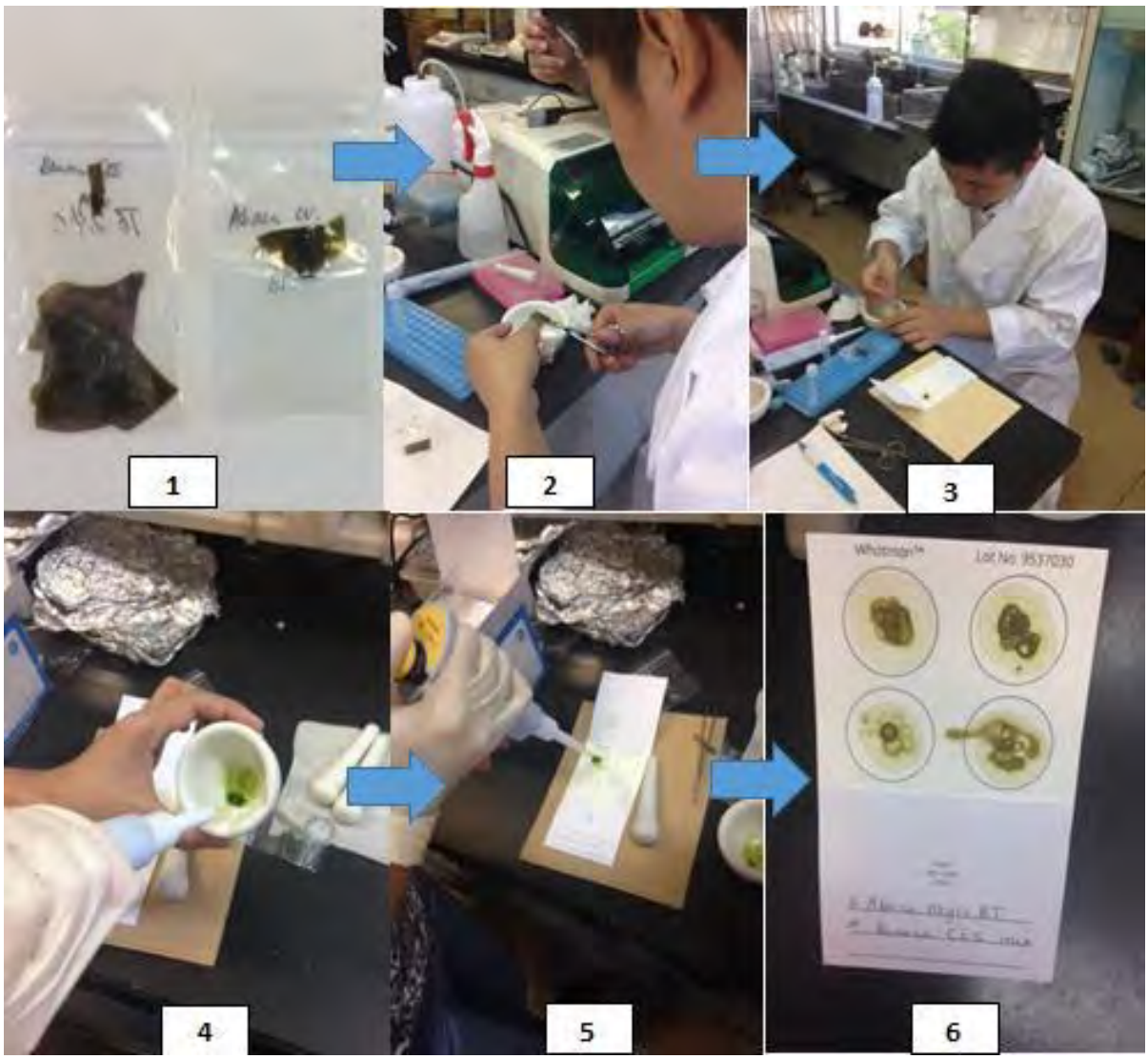


Fig. 11: Preservation of virus-infected leaf on FTA plant card

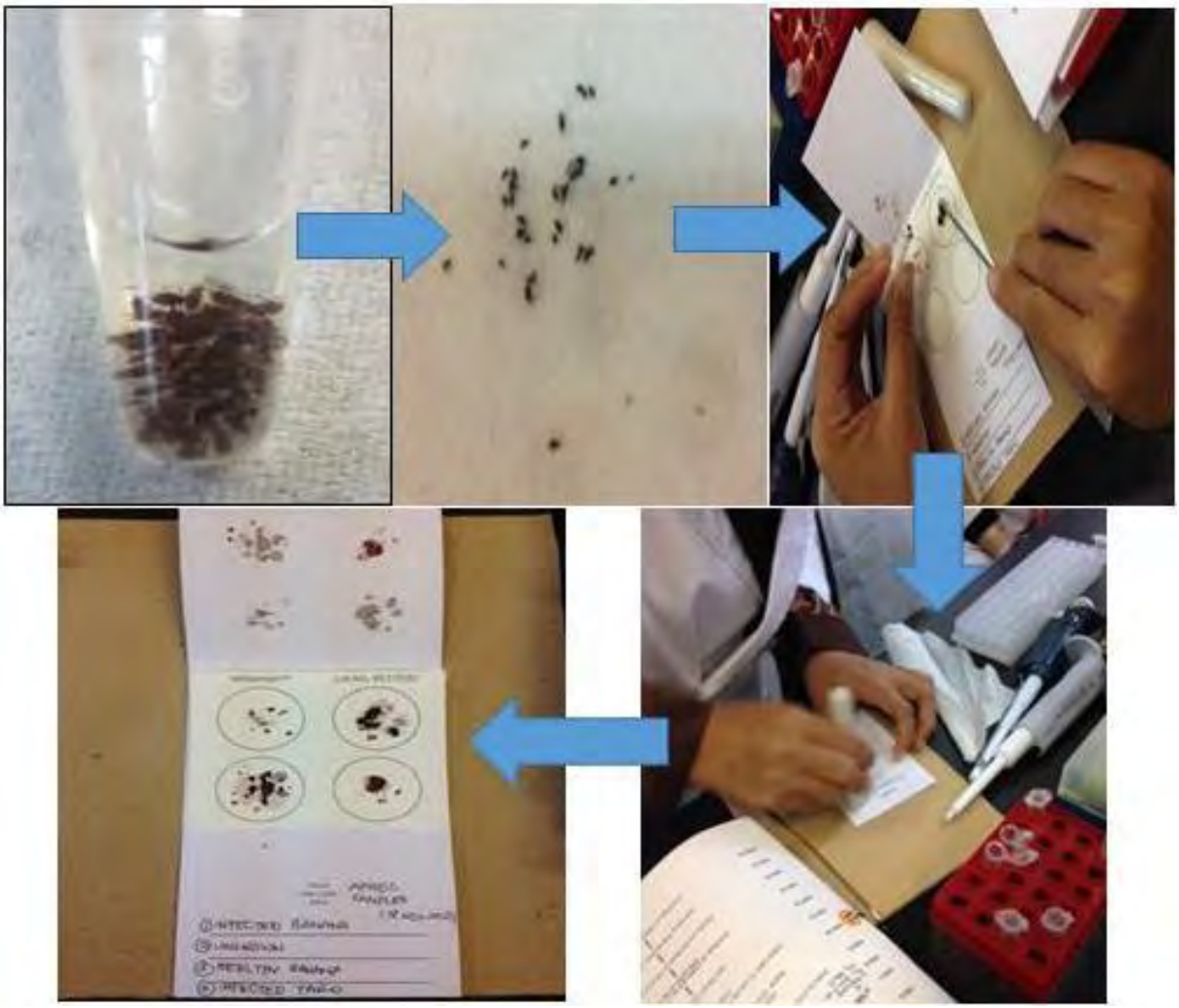


Fig. 12: Preservation of aphids (vector of plant viruses) on FTA plant card

4.1.8. Detection of plant viruses from samples impregnated on FTA plant card

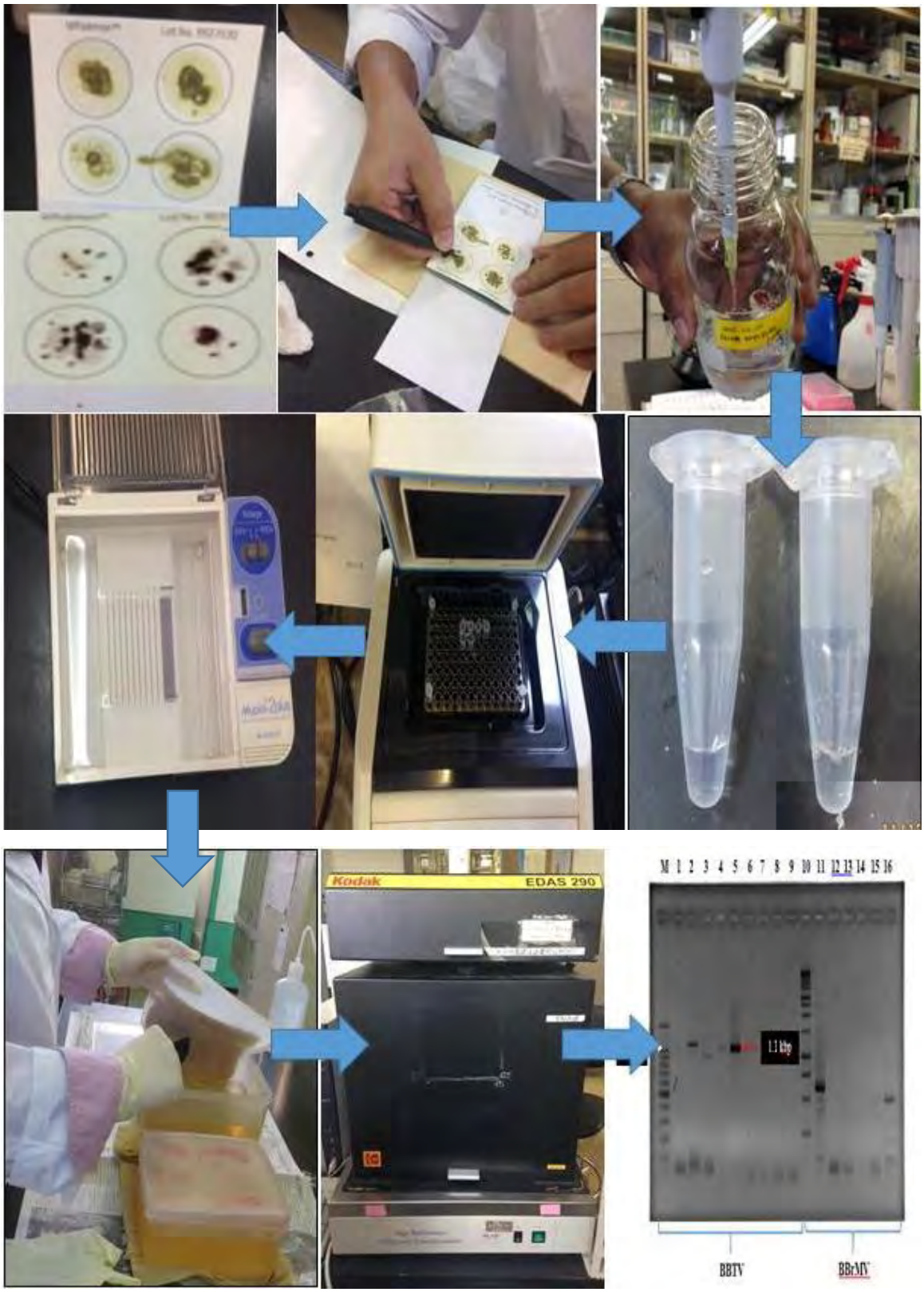


Fig. 13: Detection of plant viruses from samples impregnated on FTA plant card

4.1.9. Method for DNA purification

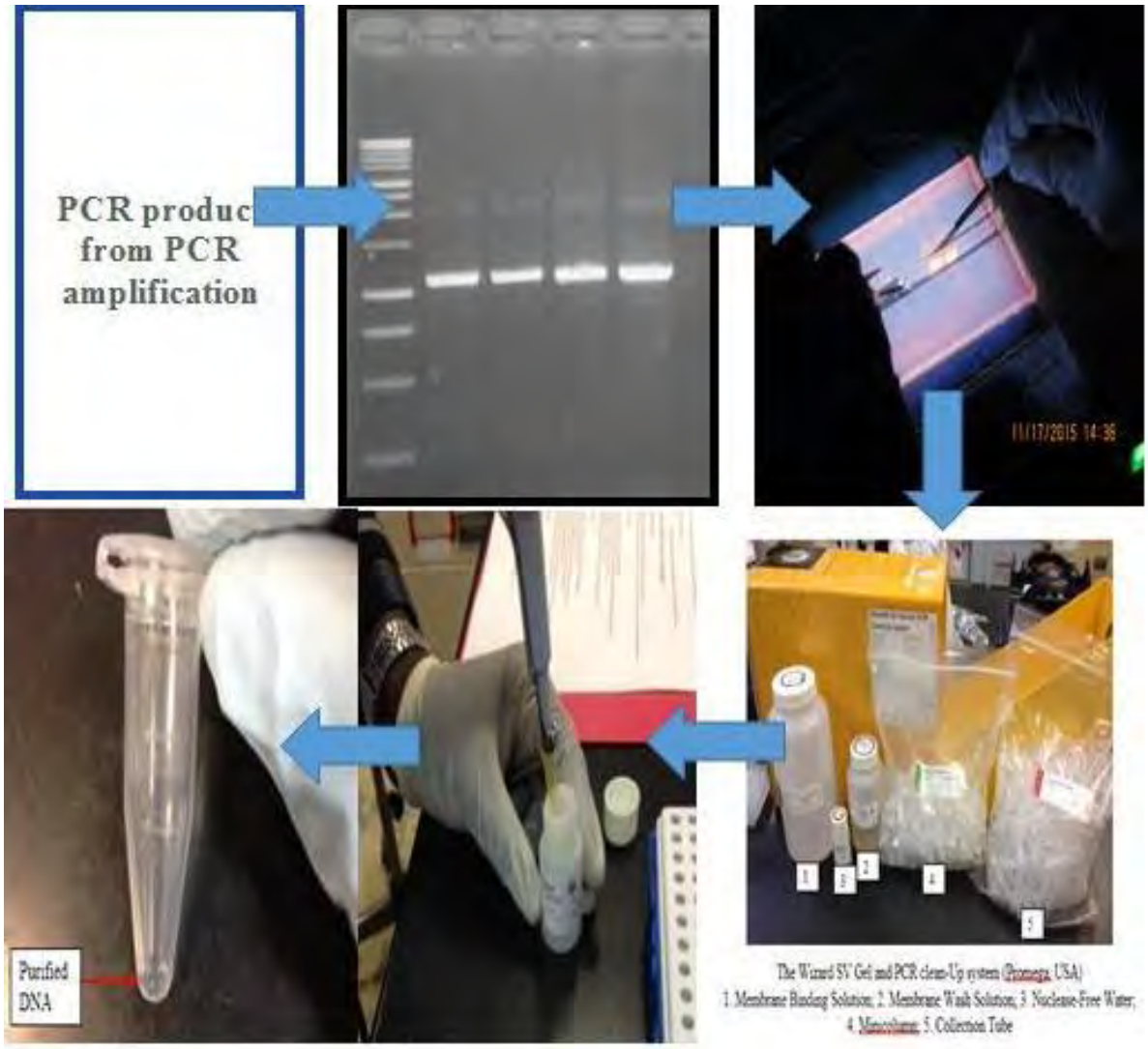


Fig. 14: DNA purification from gel agarose

4.1.10. Method for cloning

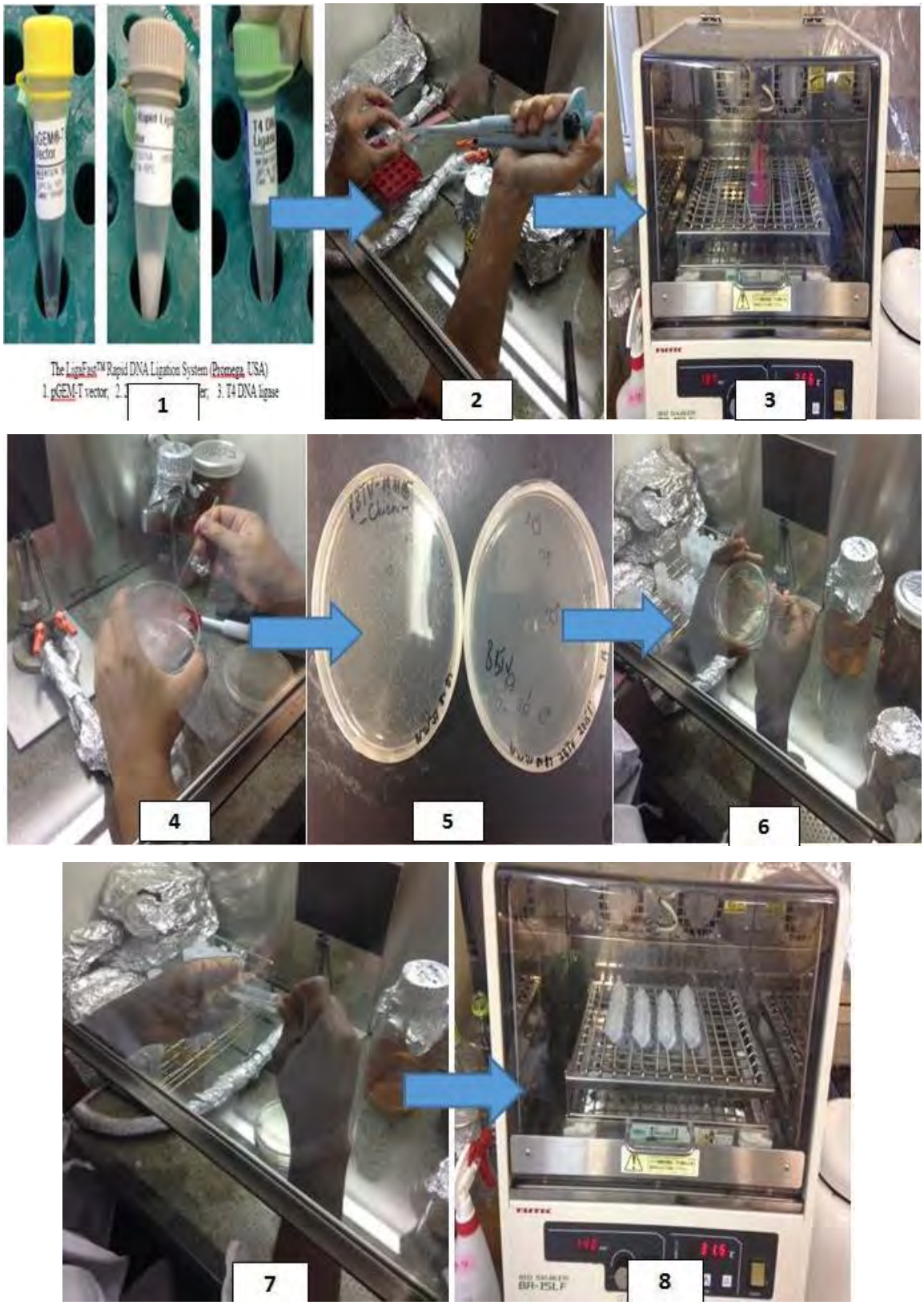
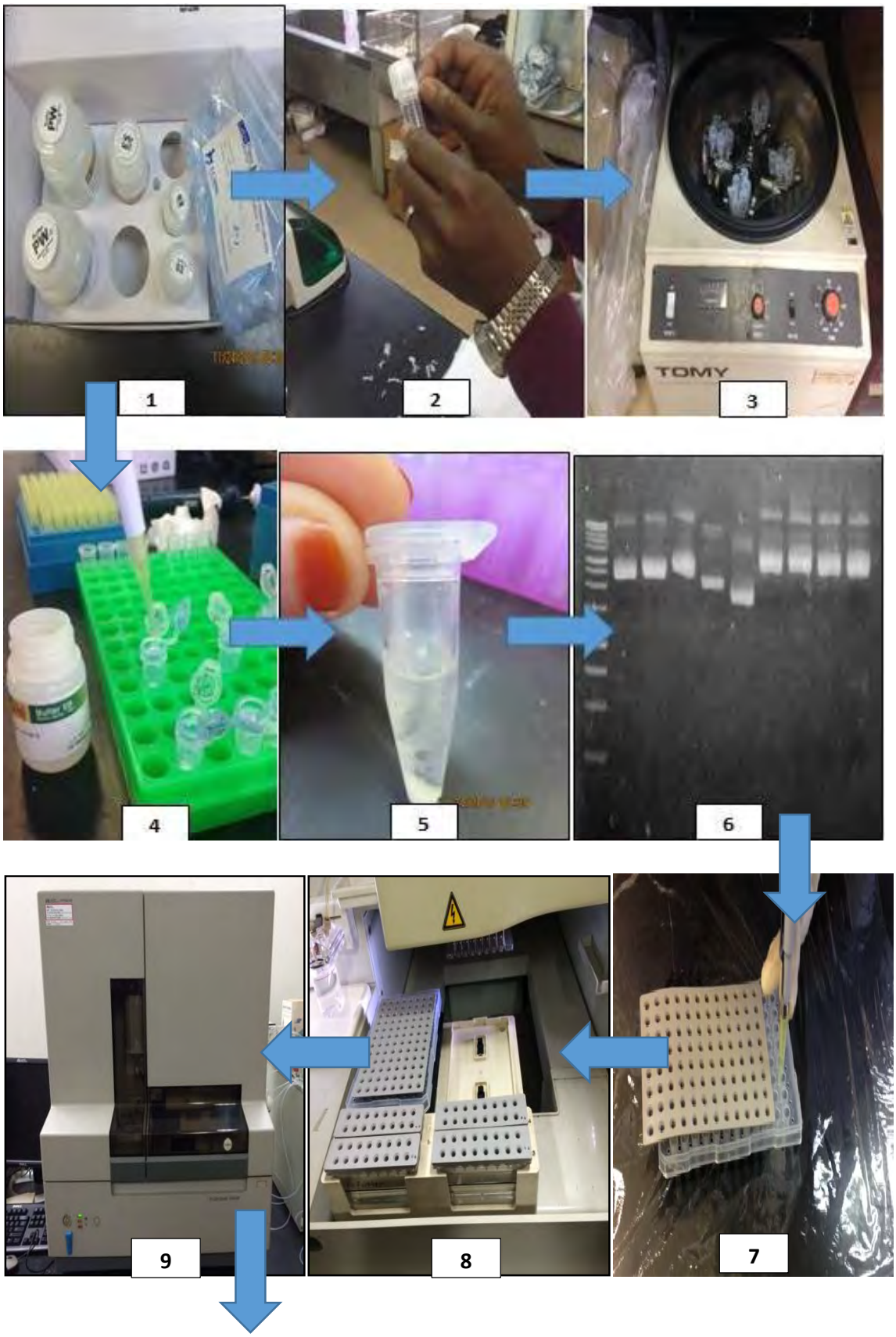


Fig. 15: Cloning of pGEM-inserted colonies

4.1.11. Method for DNA Sequencing



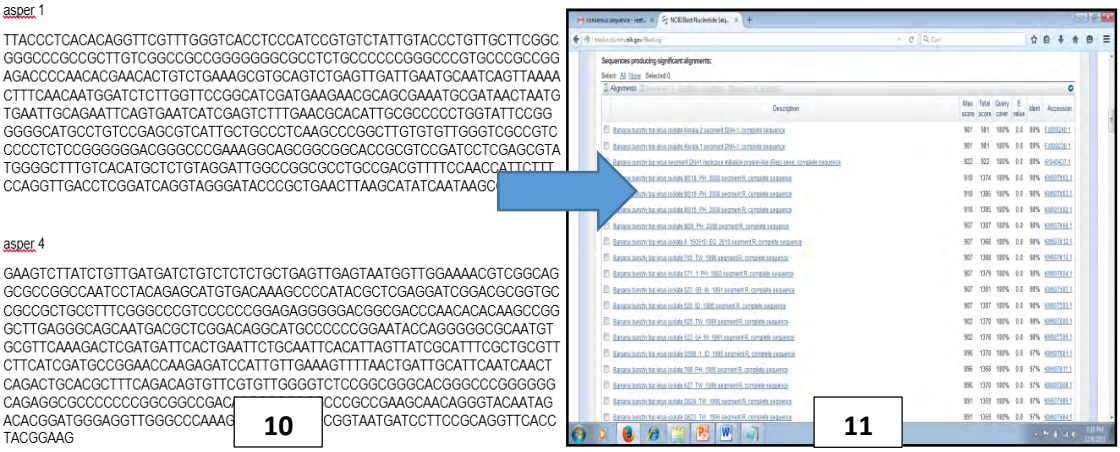


Fig. 16: DNA sequencing of virus isolated from virus infected plant

4.1.12. Construction of phylogenetic tree using MEGA software

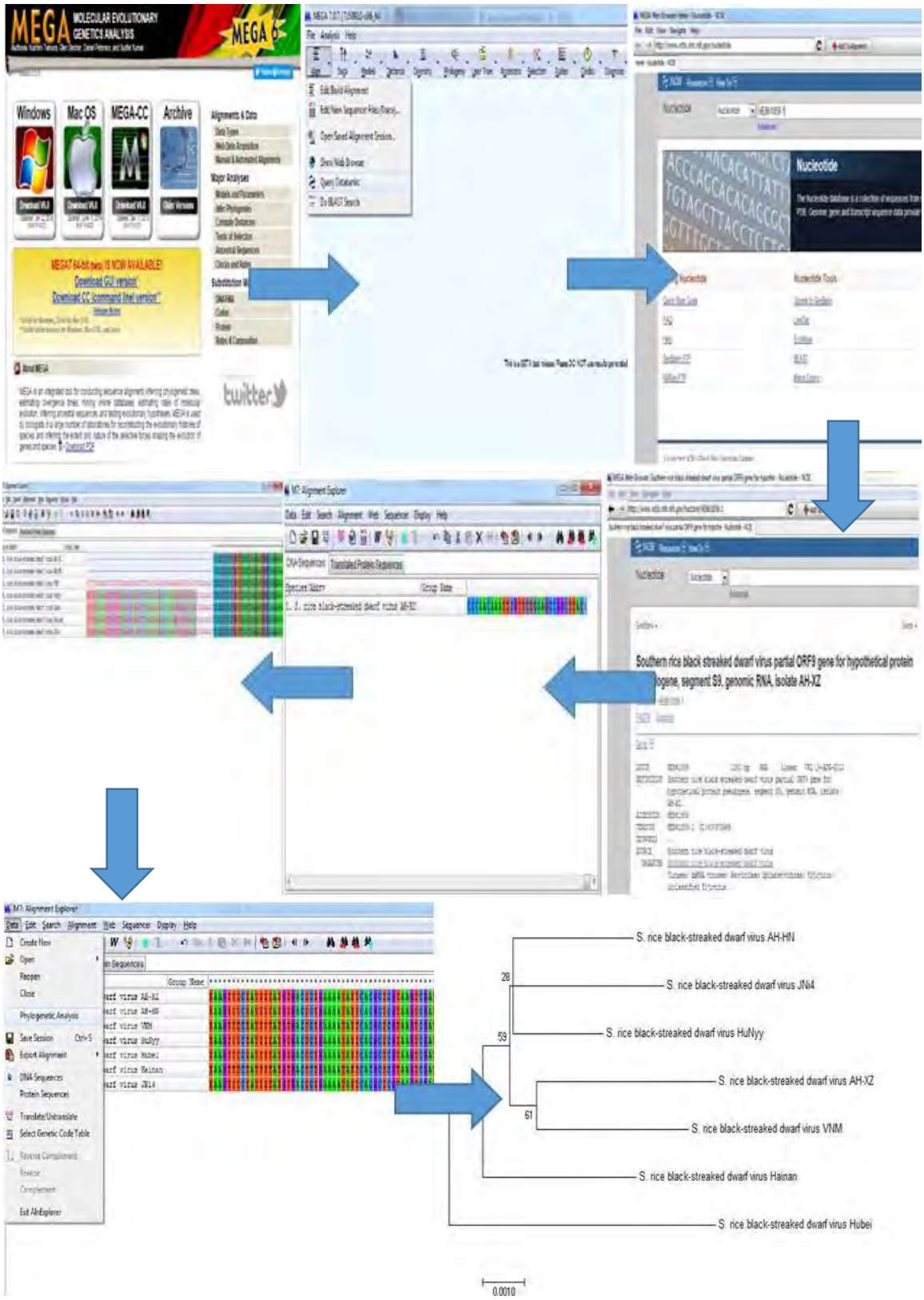


Fig. 17: Construction of phylogenetic tree using MEGA software

4.1.13. Extraction of double-stranded RNA(dsRNA) of plant to detect CMV

Fig. 18: Extraction of dsRNA plant to detect CMV

4.1.14. RNA extraction of plant viruses using phenol-chloroform method

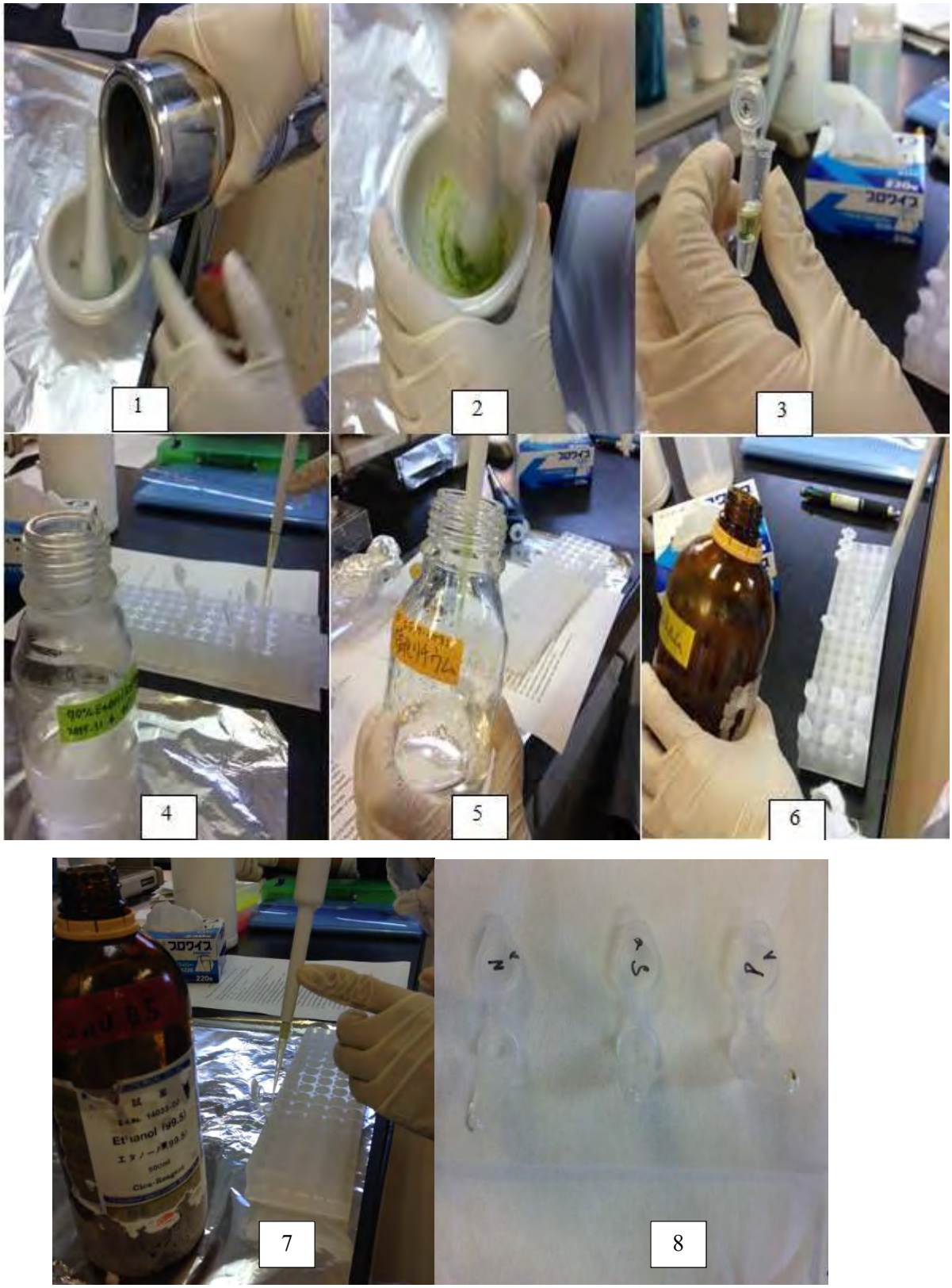


Fig. 19: Extraction of RNA of plant viruses using phenol-chloroform method

4.1.15. Detection of protein present using SDS PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) & Western Blot

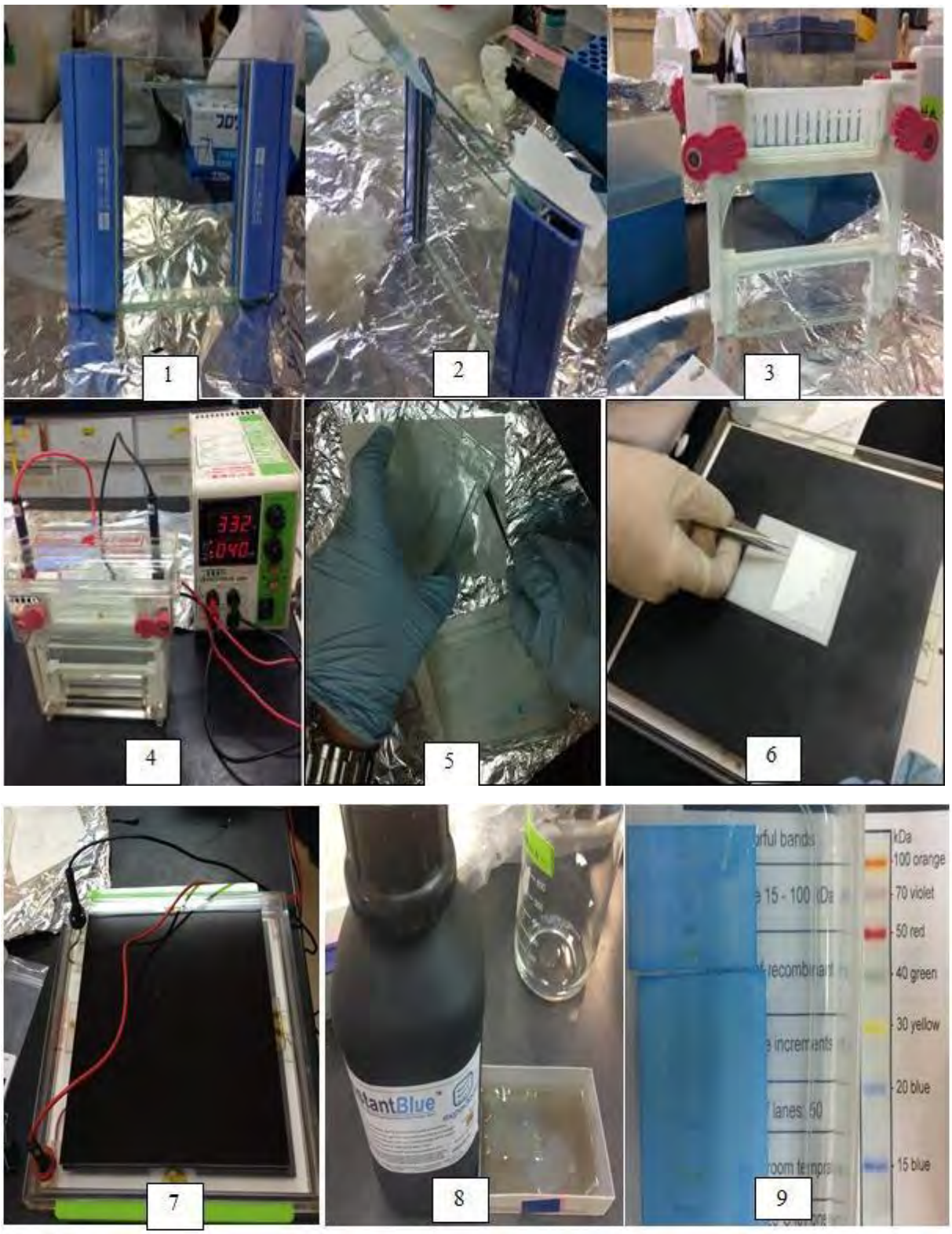


Fig. 20: Pictures show SDS PAGE method and Western Blot for detection of protein from bovine serum albumin (BSA)

Fig 21: Attending the lecture on plant parasitic nematode as vector of plant viruses by Dr. Marita S. Pinili

Fig 22: Attending the lecture on DNA sequencing analysis and phylogenetic tree by Dr. Noriko Furuya from DNA Data Bank of Japan (DDBJ)

Fig 23: Studying on the method for dsRNA extraction from plant viruses by Dr. Tomohide Natsuaki – Vice President of Utsunomiya University

4.2. Study visit to Yokohama Plant Protection Station

- Having an overview on the plant quarantine system in Japan.
- Understanding the function and operation of each division under plant quarantine system of Japan.
- Visiting to the plant quarantine facilities of Research Center of Yokohama Plant Protection Station.
- Discussing and learning on the experiences in plant quarantine field from plant quarantine officers.

Fig 24. Attending the lecture on plant quarantine system in Japan

Fig 25. Introduction of facilities and on-going activities at the Research Center

Fig 26. Visiting to the exhibition room of Yokohama Plant Quarantine Station

Fig 27. Group photo in front of Yokohama Plant Quarantine Station

4.3. *Attending the 2015 ISSAAS (International Society for Southeast Asian Agricultural Sciences) International Congress held at Tokyo University of Agriculture (Setagaya Campus)*

- Having an opportunity to gain knowledge through keynote lectures, plenary lectures, scientific presentations and parties.
- Making friend with researchers from various countries and build up network with them for possible future collaboration.

Fig 28. Memorial pictures with researchers met in ISSAAS 2015

Fig 29. Attending several activities during ISSAAS conference 2015

4.4. *Other activities*

4.4.1. *Participating in the celebration of The 124th Anniversary of Tokyo NODAI*

- Having wonderful experiences during the event, including Japanese tea ceremony, Japanese agricultural product exhibition, Japanese traditional sports, etc.
- Enjoying special foods prepared by foreign students studied in Tokyo NODAI.

Fig 30. Attending cultural activities during the *124th anniversary of Tokyo NODAI*

4.4.2. *Visiting to NODAI Food and Agriculture Museum*

- Understanding the foundation, history and development of Tokyo University of Agriculture (Tokyo NODAI).
- Interesting information on specimens of chicken variety collected over the world.
- Obtaining knowledge on Japanese culture; agriculture and method for making Sake.

Fig 31. Photos in *NODAI Food and Agriculture Museum*

4.4.3. *Sightseeing to some famous places*

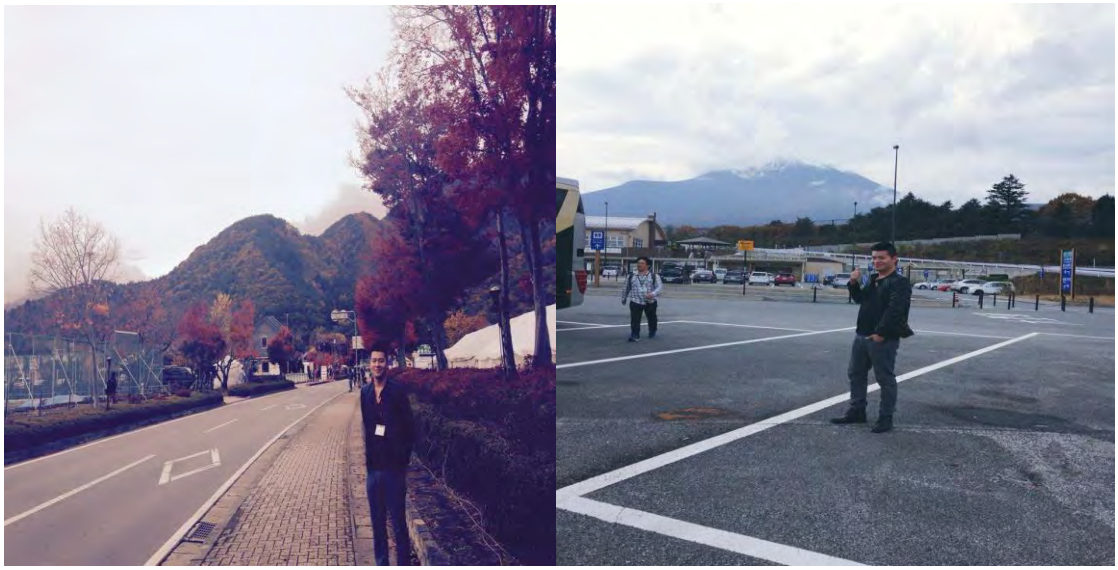
Meiji temple

NHK studio park

Asakusa temple

Ueno Zoo

Nikko – the world heritage



Fuji Mountain – the symbol of Japan

4.5. *What have impressed me during the two months program in Tokyo NODAI*

- The environment in Japan is always clean and neat.
- The great metro systems in Tokyoare convenient and eco-friendly.
- The Japanese are very polite, hospitable and friendly.
- Japanese students in HOGOKEN Laboratory in particular are well-knowledge in their major, laborious, helpful and friendly.

5. Summary and Recommendation

5.1. Summary

The two-month attachment program "***Training Workshop on Advanced Diagnostics on Plant Viruses"*** has been implemented in Tokyo University of Agriculture (TUA), Japan from October 27th to December 24th 2015 with three participants from Malaysia, Indonesia and Vietnam respectively. The program was under supervision by Prof. Keiko Natsuaki and Dr. Marita S. Pinili. Practical trainings have been carried out at the Laboratory of Tropical Plant Protection (HOGOKEN Lab.), Department of International Agricultural Development, TUA. During the program, I was able to learn on the tecniques and gained knowledge especially on diagnostic of plant viruses. In addition, the two days visit and hands-on work on the dsRNA extraction method at Utsunomiya University was a good exposure to the advanced technology in diagnostic of viruses. This laboratory activity was under supervision of Dr. Tomohide Natsuaki. Moreover, we were able to attend three expertise lectures on "Attenuated plant viruses" by Prof. Keiko Natsuaki; "Plant parasitic nematodes" by Dr. Marita S. Pinili and "Phylogenetic tree" by Dr. Noriko Furuya. Besides experiments at the laboratory, we were given opportunity to participate at the 2015 International Congress of International Society for Southeast Asian Agricultural Sciences (ISSAAS). In addition, the study visit to Yokohama Plant Protection Station was a good opportunity for the ASEAN participants to understand the plant quarantine system in Japan. Before the end of attachment program, a final meeting was held for the ASEAN participants to perform their final reports on the program.

In summary, I was able to improve our diagnostic skills in plant viruses and quarantine test procedures starting from symptoms observation until the identification of the plant viruses for agriculture products trade and applying the correct management techniques in order to control the spread of plant virus infections within the country.

5.2. Recommendations

The attachment program is great opportunity for myself and the other ASEAN participants to learn the advanced techniques on diagnostic of plant virus diseases from Japan – one of the top world countries in agricultural science. This program is excellent and I do not have any comments. Besides knowledge gained, I was able to see the interesting Japanese cultures and met with excellent people throughout the program. . I will try my best to apply the knowledge and experiences gained from the program in my work and share with my co-workers. I hope there are more projects collaboration between the ASEAN Plant Health Cooperation Network (APHCN) – ASEANET and Japan government for ASEAN participants in the future.

6. Acknowledgement

I would like to express my deepest appreciation to Japan-ASEAN Integrated Fund (JAIF) and the ASEAN Plant Health Cooperation Network (APHCN) of ASEANET to organize the training program for ASEAN countries' participants.

Special thanks to Dr. Lum Keng Yeang and Dr. Soetikno Sastroutomo who given me opportunity to participate in this project and helping me on the preparation for the attachment program in Japan.

In addition, I would like to express my sincere gratitude to Professor Keiko Natsuaki of Tokyo University of Agriculture (Tokyo NODAI) for her guidance, encouragement and experience sharing throughout the attachment program. Thank you for taking care our needs and accommodate us during our stay in Japan.

My sincere thanks to Dr. Tomohide Natsuaki, Dr. Marita S. Pinili and Dr. Noriko Furuya for the great lectures and practical instructions.

To all my friends from HOGOKEN Laboratory, thank you for your help and support during the experiments despite their busy schedule in studying. Last but

not least, my friends from Malaysia and Indonesia, thank you for making the training program exciting and full of fun.

LIST OF ANNEXES

Report 1. Detection of unknown virus on bamboo plant by Indirect-ELISA

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Oct. 28th, 2015

2. Material

- Indirect ELISA Kit (SRA 27200/0500) for detection of *Potyvirus* provided by Agdia company.

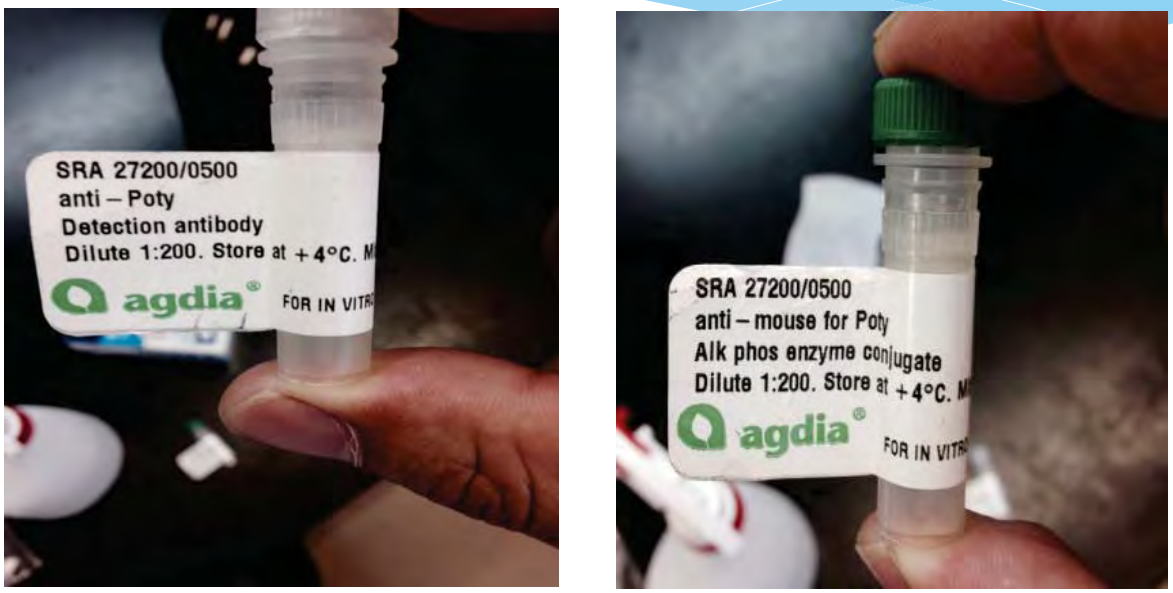


Fig. 1: Indirect ELISA kit (SRA 27200/0500) for detection of *Potyvirus* (Agdia, USA).

3. Samples

Bamboo (*Pleioblastus chino*) collected in Main gate of Tokyo NODAI on Oct. 28th

Fig. 2: sample 1 with symptoms: mosaic & local chlorotic spots

Fig. 3: sample 2 with symptoms: mosaic, long yellow stripe, necrosis on leaves

4. Procedure

– ***Step 1: Extraction of sample***

- Weigh 0.1 g sample (bamboo leaf) and transfer to the mortar. Add the nitrogen with liquid form.

- Add 1.0 ml sample extraction buffer (1X) into the mortar and grind.

- Centrifuge the plant sap at 15.000 rpm within 5 minutes.

- Load 200 µl plant sap per well of the ELISA plate.

- Incubate at Room temperature for 1 hour under dark condition.

4. Procedure (continued)

– ***Step 2: To bind detection antibody***

- After incubation, remove the plant sap from the wells by ELISA-washing machine.

- Gently tap the ELISA plate on paper tissue to make ELISA wells dry totally but not too long time.

- Add 200 µl Poty-specific antibody with a dilution of 1:200 in ECI buffer (1X) to each well.

- Incubate at room temperature for 2 hours under dark condition.

4. Procedure (continued)

– ***Step 3: To bind Enzyme-linked antibody (secondary antibody)***

- Wash the ELISA plate

- Tap the ELISA plate to remove excess washing buffer

- Add 200 µl Enzyme-linked antibody with a dilution of 1:200 in 1X ECI buffer to each well.

- Incubate at room temperature for 1 hour under dark condition.

4. Procedure (continued)

– ***Step 4: To add PNP tablet and result reading***

- Dilute 1 PNP tablet (0.5 mg) with 5 ml PNP buffer.

- Mix thoroughly.

- Add 100 µl this solution to each well.

- Incubate at room temperature under dark condition.

- Read the absorbance of ELISA plate using ELISA Reader (Microplate Reader/ Bio-RAD) after 15, 30, 45 and 60 minutes of incubation.

5. Result

Sample	Absorbance value of ELISA plate*				Conclusion
	15 minutes	30 minutes	45 minutes	60 minutes	
Negative control	0.064	0.068	0.073	0.079	
Positive control	0.161	0.261	0.370	0.468	
Buffer	0.065	0.066	0.067	0.070	
Sample 1	1.711	3.206	(very high)	(very high)	+
Sample 1	0.125	0.182	0.250	0.313	+

**** Average value of two wells***

6. Discussion

- Two bamboo samples collected in Tokyo University of Agriculture get positive with *Potyvirus*.
- Sample 1 has the highest absorbance value indicated that the concentration of virus in this sample is very high.

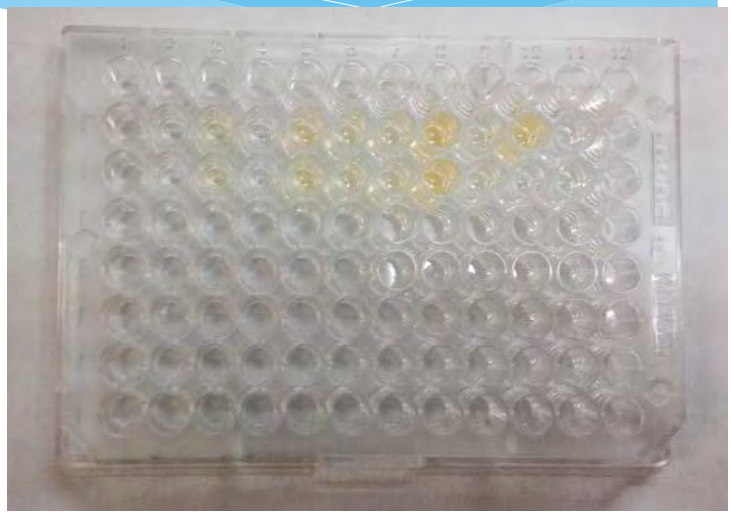


Fig. 4: result of Indirect ELISA reaction for detection of virus on bamboo. Two bamboo samples also get positive with *potyvirus*.


THANK YOU FOR YOUR ATTENTION
Terraced Field in Sapa !
Viet Nam

Report 2. Method for inoculation of unidentified passionfruit virus to passionfruit seedlings

1. PLACE AND TIME

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Oct. 29th, 2015

2. MATERIAL

- Mortars and pestles kept in freezing container of the fridge.
- Phosphate buffer 0.1 M (pH = 7.0)
- Carborundum powder.

3. SAMPLES

- Leaf of passion fruit plant suspected to be infected by a *Potyvirus*
- Healthy passion fruit seedling

Fig. 1: *potyvirus* infected leaf of passionfruit with symptom: mosaic, leaf deformation, leaf crinkle, chlorotic spots and yellow vein.

4. PROCEDURE

- Step 1:

Grind well *potyvirus* infected leaf in 0.1 M Phosphate buffer (10v/w) using mortar and pestle.

4. PROCEDURE (CONTINUED)

- Step 2:

Add carborundum powder into sap juice.

4. PROCEDURE (CONTINUED)

- Step 3:

Rub sap with carborundum powder on passionfruit seedling tenderly.

4. PROCEDURE (CONTINUED)

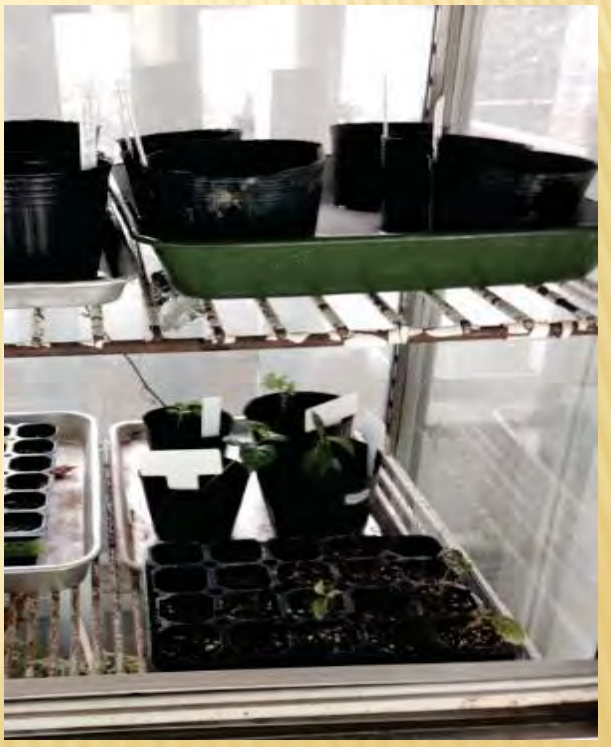
- Step 4:

After 3~6 min., wash away extra plant sap and carborundum from inoculated leaves by water very gently.

4. PROCEDURE (CONTINUED)

- Step 5:

Label and transfer the inoculated seedling to anti-insect chamber and observe symptom after latent period.



5. THE INFORMATION ABOUT VIRUS DISEASES ON PASSION FRUIT (*PASSIFLORA* SPP.) IN THE WORLD

Table 1. List of passion fruit viruses reported in the world

No	Name of virus	Genus of virus	Country of origin	Presence in Vietnam	Main host plants	Manners of transmission
1	Bean yellow mosaic virus	Potyvirus	Croatia	No	Groundnut, pea, chickpea, soybean, lupin, common bean…	- Aphids (more than 20 species): *Myzus persicae*, *Aphis fabae*…
2	Citrus leprosis virus	Cilevirus	Brazil	No	Citrus spp.	- Mites: *Brevipalpus phoenicis*
3	Cowpea aphid-borne mosaic virus	Potyvirus	Brazil	No	Cowpea, pea, soybean, passion fruit, groundnut...	- Aphids: *Myzus persicae* - Mechanical inoculation - Seeds
4	Cucumber mosaic virus	Cucumovirus	Australia	Yes	Chili, chickpea, cucumber, carrot, soybean, tobacco, passion fruit, tomato, potato…	- Aphids (more than 60 species): *Myzus persicae*, *Aphis fabae*, *Aphis craccivora*… -Mechanical inoculation - Seeds (19 plant species)

Table 1. List of passion fruit viruses reported in the world

No	Name of virus	Genus of virus	Country of origin	Presence in Vietnam	Main host plants	Manners of transmission
5	East Asian Passiflora Virus	Potyvirus	Japan	No	Passion fruit	- Aphids: Aphis gossypii, Hyperomyzus lactucae, Myzus persicae
6	Giant granadilla malformation virus	Begomovirus	Colombia	No	Passion fruit	-Whitefly: *Bemisia tabaci*
7	Jatropha mosaic virus	Begomovirus	Puerto Rico	No	Passion fruit	-Whitefly: *Bemisia tabaci*
8	Maracuja mosaic virus	Tobramovirus	India	No	Passion fruit	-Mechanical inoculation - Contact between plants
9	Passiflora latent virus	Carlavirus	Germany	No	Passion fruit	-Mechanical inoculation
10	Passiflora ringspot virus	Potyvirus	Ivory Coast	No	Passion fruit	- Aphids: *Aphis gossypii*, *A spiraecola* -Mechanical inoculation
11	Passiflora virus Y	Potyvirus	Australia	No	Passion fruit	- Aphids: *Aphis gossypii* -Mechanical inoculation
12	Passion flower little leaf mosaic virus	Begomovirus	Brazil	No	Passion fruit	-Whitefly: *Bemisia tabaci* -Mechanical inoculation
13	Passion fruit crinkle virus	Potyvirus	Taiwan	No	Passion fruit, soybean	- Aphids: *Myzus persicae* - Mechanical inoculation

Table 1. List of passion fruit viruses reported in the world

No	Name of virus	Genus of virus	Country of origin	Presence in Vietnam	Main host plants	Manners of transmission
14	Passion fruit green spot virus	Cilevirus	Brazil	No	Passion fruit	-Mites: Brevipalpus phoenicis
15	Passion fruit vein clearing virus	unassigned genus	Brazil	No	Passion fruit	- Unknown
16	Passion fruit yellow mosaic virus	Tymovirus	Brazil	No	Passion fruit	-Beetle: Diabrotica speciosa -Mechanical inoculation
17	Passionfruit mottle virus	Potyvirus	Taiwan	No	Passion fruit	- Aphids: Myzus persicae - Mechanical inoculation
18	Passionfruit woodiness virus	Potyvirus	Australia	No	Passion fruit	- Aphids: Aphis gossypii, Myzus persicae - Mechanical inoculation
19	Purple granadilla mosaic virus	unclassified	Brazil	No	Passion fruit	-Beetle: Diabrotica speciosa -Mechanical inoculation
20	Soybean mosaic virus	Potyvirus	Colombia	No	Soybean, passion fruit	- Aphids (more than 16 species): Myzus persicae, Aphis fabae… -Mechanical inoculation - Seeds (30% or higher) - Pollen


THANK YOU FOR YOUR ATTENTION
Terraced Field in Saga
Viet Nam

Annex 3

Report 3. How to make the PTA solution and detect viruses by electron microscope

1. Place and time

- Place: HOGOKEN Lab.
- Time: Nov. 2nd, 2015

2.1. How to make the PTA (Phosphatungstic acid) solution

**** Materials***

- Phosphatungstic acid
- Purified water
- Potassium hydroxide (KOH)

**** Procedure***

- Weight 2 g Phosphatungstic acid
- Dissolve 2 g Phosphatungstic acid in 100 ml purified water
- Adjust pH = 7.0 by Potassium hydroxide (KOH)
- Store at 2-8°C

2.2. How to bind the plant sap on to a carbon-coated collodion membrane covered copper grid (for EMS observation)

*** Materials**

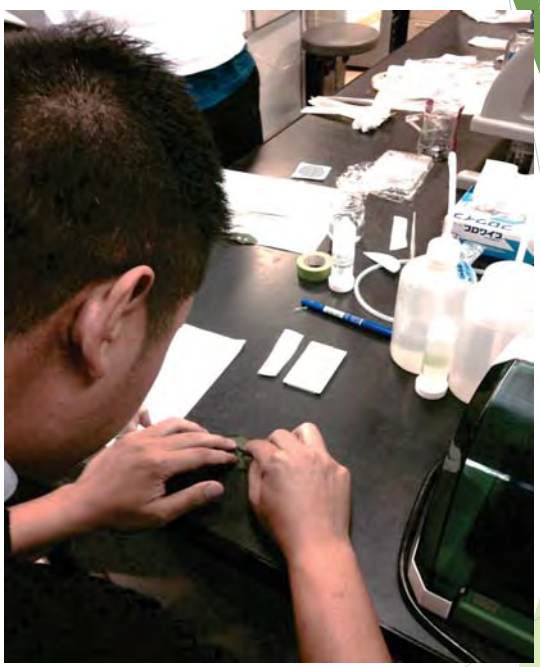
- 2% PTA solution
- Blades
- Virus-infected leaf
- Glass slide
- Carbon-coated collodion membrane covered copper grid
- Laboratory forceps
- Laboratory pipette
- Paper tissue

Produce

Step 1:

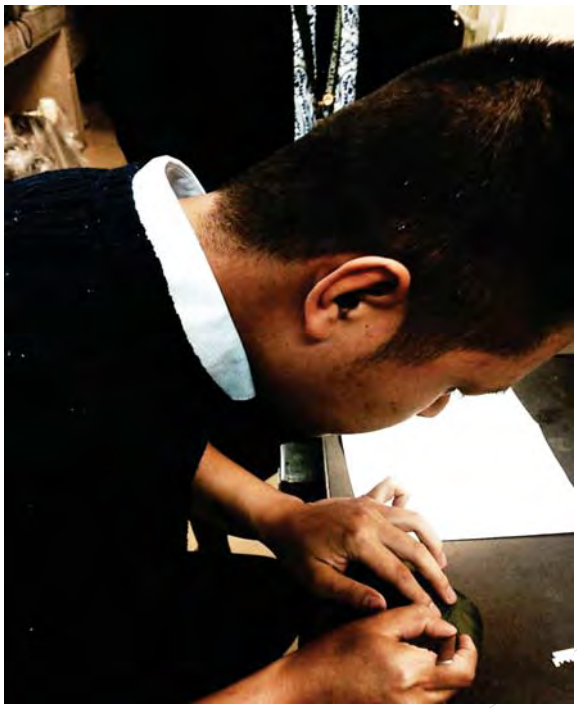
- Use blade to take a small piece of leaf (sample) from the virus-infected leaf.

*Note: prefer taking the vein of virus-infected leaf where the concentration of virus is highest



Step 2:

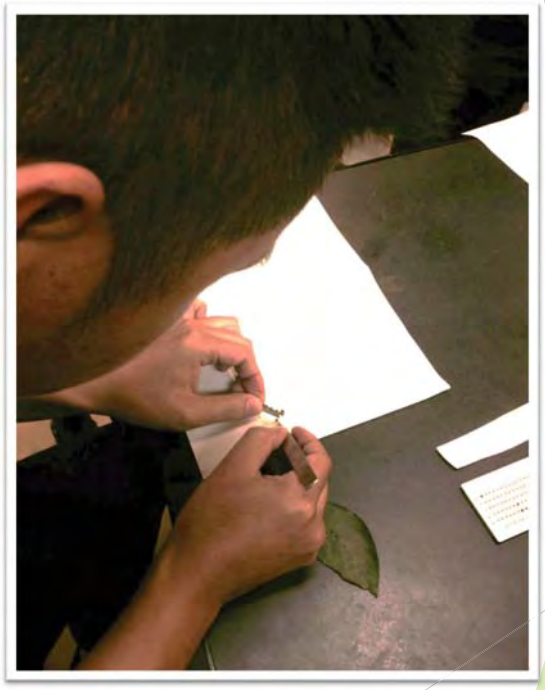
- Use the laboratory forceps to transfer sample to glass slide tenderly



Step 3:

- Use laboratory pipette to drop a small amount of PTA solution on to glass slide.

- Use the blade to crush the sample with PTA solution to make the plant sap (*this activity should be done within 10-15 seconds*).



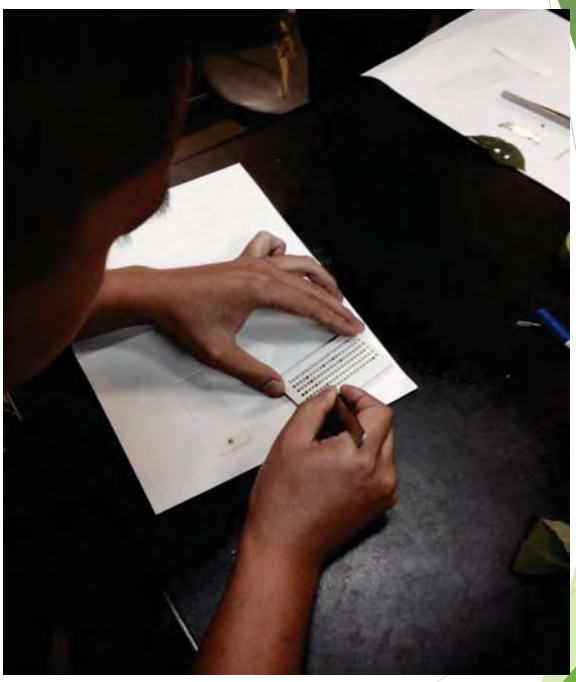
Step 4:

- Pick up the Carbon-coated collodion membrane with laboratory forceps and gently dip it in plant sap.

- Exclude the excess of plant sap by paper tissue

Step 5:

- Transfer carefully the membrane dipped plant sap to Grid storage box and prepare for EMS observation



Annex 4

Name: **Tran Van Chien**

Country: **Vietnam**

REPORT ON INTRODUCTION ABOUT PLANT VACCINE OF *PEPPER MILD MOTTLE VIRUS*

1. Place and time

Place: HOGOKEN meeting room

Time: Nov. 2nd, 2015

2. Materials

The video on introduction about plant vaccine of *Pepper mild mottle virus* (PMMoV)

3. Contents

- Introduction about the production of bell pepper (*Capsicum annuum*) in Japan.

- Introduction about *Pepper mild mottle virus* (PMMoV) and its damage on bell pepper in Japan.

- How to produce the PMMoV-vaccinated seedlings

- Yield comparison between healthy, PMMoV-infested and PMMoV-vaccinated plants after harvesting.

- Vitamim C comparison between fruits collected from healthy and PMMoV-vaccinated plants

4. Discussion about the difficulty in applying vaccinated plants in our countries

***In Vietnam:** In my opinion, the most important difficulty in applying plant vaccines in Vietnam is economic aspect. Vietnam now is the developing country, so the famers are still poor. Their optimum management method for plant pests and diseases in general, and virus diseases in particular is chemical control. Therefore, it's very hard to persuade the famers to use the vaccinated plants instead of chemical. In addition, the habit of cultivation in Vietnam is different from Japan. Almost Vietnamese famers sow the plants on the fields (not in the green house), so not only

the virus diseases can attack the plants but also many pathogenic agents (fungi, bacteria, nematodes…) as well as other pests. Consequently, it's difficult to use the vaccinated plants in Vietnam. However, I hope in the near future, when the Vietnamese famers have more money and more knowledge, they can use the vaccinated plants in their cultivation as one of advanced management controls.

*** In other countries (Indonesia, Malaysia and Uganda):** The participants from these countries also said that they have had the same difficulty with Vietnam. However, Mr. Patrick believed that it would be possible if applying the vaccinated plants in vegetable cultivation in Uganda.

Report 5. Detection of Potyvirus on Passionfruit plant by Indirect ELISA

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 4th, 2015

2. Material

- Indirect ELISA Kit (SRA 27200/0500) for detection of *Potyvirus* provided by Agdia company.

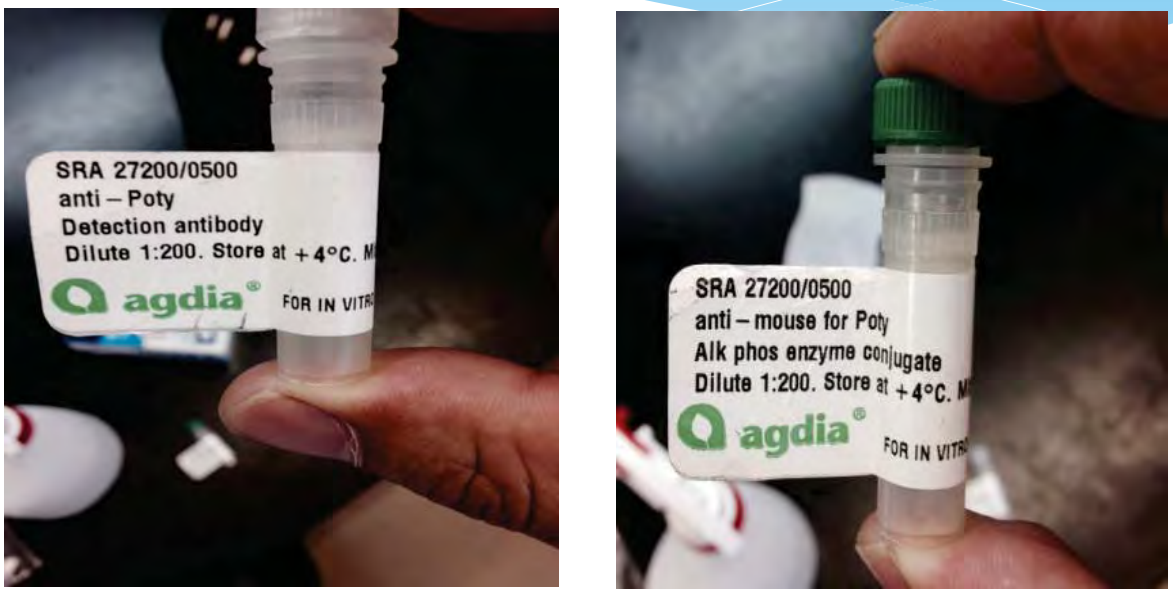


Fig. 1: Indirect ELISA kit (SRA 27200/0500) for detection of *Potyvirus* (Agdia, USA).

3. Samples

Potyvirus-inoculated Passionfruit seedling (date of inoculation: Oct. 29th, 2015)

Fig. 2: *Potyvirus*-inoculated Passionfruit seedling without typical symptoms.

4. Procedure

– ***Step 1: Extraction of sample***

- Weigh 0.1 g sample (bamboo leaf) and transfer to the mortar. Add the nitrogen with liquid form.

- Add 1.0 ml sample extraction buffer (1X) into the mortar and grind.

- Centrifuge the plant sap at 15.000 rpm within 5 minutes.

- Load 200 µl plant sap per well of the ELISA plate.

- Incubate at Room temperature for 1 hour under dark condition.

4. Procedure (continued)

– ***Step 2: To bind detection antibody***

- After incubation, remove the plant sap from the wells by ELISA-washing machine.

- Gently tap the ELISA plate on paper tissue to make ELISA wells dry totally but not too long time.

- Add 200 µl Poty-specific antibody with a dilution of 1:200 in ECI buffer (1X) to each well.

- Incubate at room temperature for 2 hours under dark condition.

4. Procedure (continued)

– ***Step 3: To bind Enzyme-linked antibody (secondary antibody)***

- Wash the ELISA plate

- Tap the ELISA plate to remove excess washing buffer

- Add 200 µl Enzyme-linked antibody with a dilution of 1:200 in 1X ECI buffer to each well.

- Incubate at room temperature for 1 hour under dark condition.

4. Procedure (continued)

– ***Step 4: To add PNP tablet and result reading***

- Dilute 1 PNP tablet (0.5 mg) with 5 ml PNP buffer.

- Mix thoroughly.

- Add 100 µl this solution to each well.

- Incubate at room temperature under dark condition.

- Read the absorbance of ELISA plate using ELISA Reader (Microplate Reader/ Bio-RAD) after 15, 30, 45 and 60 minutes of incubation.

5. Result

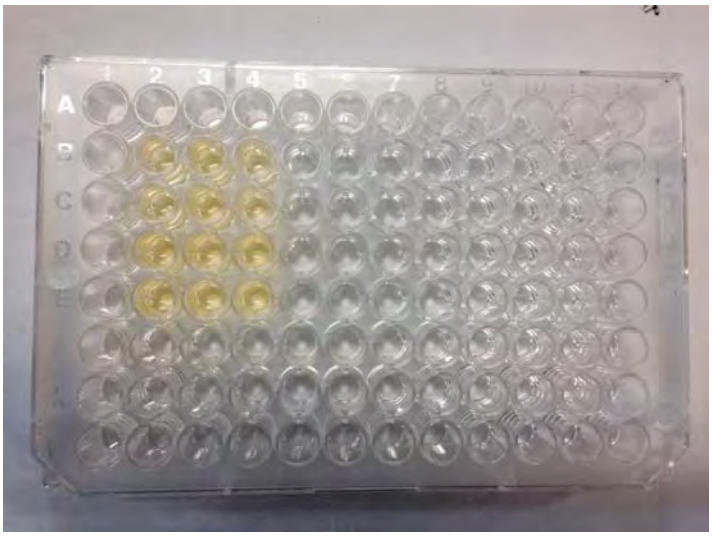
Sample	Absorbance value of ELISA plate*				Conclusion
	15 minutes	30 minutes	45 minutes	60 minutes	
Negative control	0.071	0.073	0.076	0.079	
Positive control	0.073	0.083	0.096	0.116	
Buffer	0.071	0.072	0.073	0.080	
Sample 1	0.062	0.099	0.125	0.165	+

**** Average value of two wells***

6. Discussion

- All the ELISA wells (includes Negative control and Buffer) turned yellow color

- This result may be explained that: the unspecific binding was occurred in Step 2 or the contamination was happened during conduct the In-ELISA process.

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Terraced Field in Sapa !
Viet Nam

Annex 6

Report 6. Detection of *Potyvirus* on Taro plant by Indirect ELISA

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 5th, 2015

2. Material

Indirect ELISA Kit (SRA 27200/0500) for detection of *Potyvirus* provided by Agdia company.

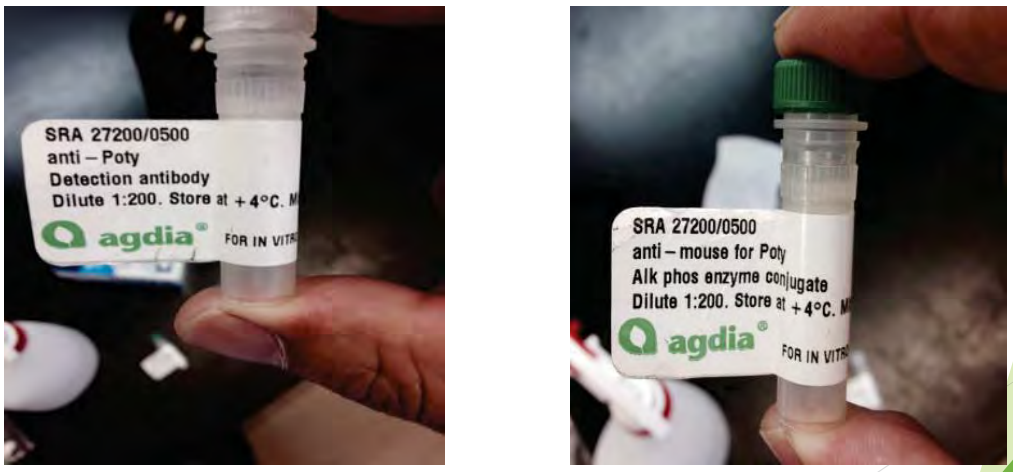


Fig. 1: Indirect ELISA kit (SRA 27200/0500) for detection of *Potyvirus* (Agdia, USA).

3. Samples

Fig. 2: Taro leaf with symptoms: yellow mottle and necrosis on the margin of leaf (sample 1)

Fig. 3: Taro leaf with symptoms: yellow mottle and necrosis on the margin of leaf (sample 2)

Fig. 4: yellow mottle on the margin of leaf, mosaic and leaf malformation (sample 3)

4. Procedure

- ***Step 1: Extraction of sample***

- Weigh 0.1 g sample (bamboo leaf) and transfer to the mortar. Add the nitrogen with liquid form.

- Add 1.0 ml sample extraction buffer (1X) into the mortar and grind.

- Centrifuge the plant sap at 15.000 rpm within 5 minutes.

- Load 200 µl plant sap per well of the ELISA plate.

- Incubate at Room temperature for 1 hour under dark condition.

4. Procedure (continued)

- ***Step 2: To bind detection antibody***

- After incubation, remove the plant sap from the wells by ELISA-washing machine.

- Gently tap the ELISA plate on paper tissue to make ELISA wells dry totally but not too long time.

- Add 200 µl Poty-specific antibody with a dilution of 1:200 in ECI buffer (1X) to each well.

- Incubate at room temperature for 2 hours under dark condition.

4. Procedure (continued)

- ***Step 3: To bind Enzyme-linked antibody (secondary antibody)***

- Wash the ELISA plate

- Tap the ELISA plate to remove excess washing buffer

- Add 200 µl Enzyme-linked antibody with a dilution of 1:200 in 1X ECI buffer to each well.

- Incubate at room temperature for 1 hour under dark condition.

4. Procedure (continued)

- ***Step 4: To add PNP tablet and result reading***

- Dilute 1 PNP tablet (0.5 mg) with 5 ml PNP buffer.

- Mix thoroughly.

- Add 100 µl this solution to each well.

- Incubate at room temperature under dark condition.

- Read the absorbance of ELISA plate using ELISA Reader (Microplate Reader/ Bio-RAD) after 15, 30, 45 and 60 minutes of incubation.

5. Result

Sample	Absorbance value of ELISA plate*				Conclusion
	15 minutes	30 minutes	45 minutes	60 minutes	
Negative control	-	-	-	-	
Positive control	-	-	-	-	
Buffer	-	-	-	-	
Sample 1	-	-	-	-	
Sample 2	-	-	-	-	
Sample 3	-	-	-	-	

6. Discussion

- All the ELISA wells (includes Negative control and Buffer) turned yellow color.
- This result may be explained that: the unspecific binding was occurred in Step 2 or the contamination was happened during conduct the In-ELISA process.

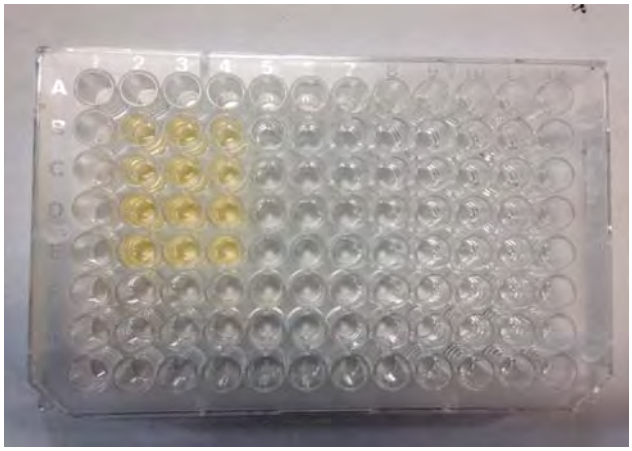
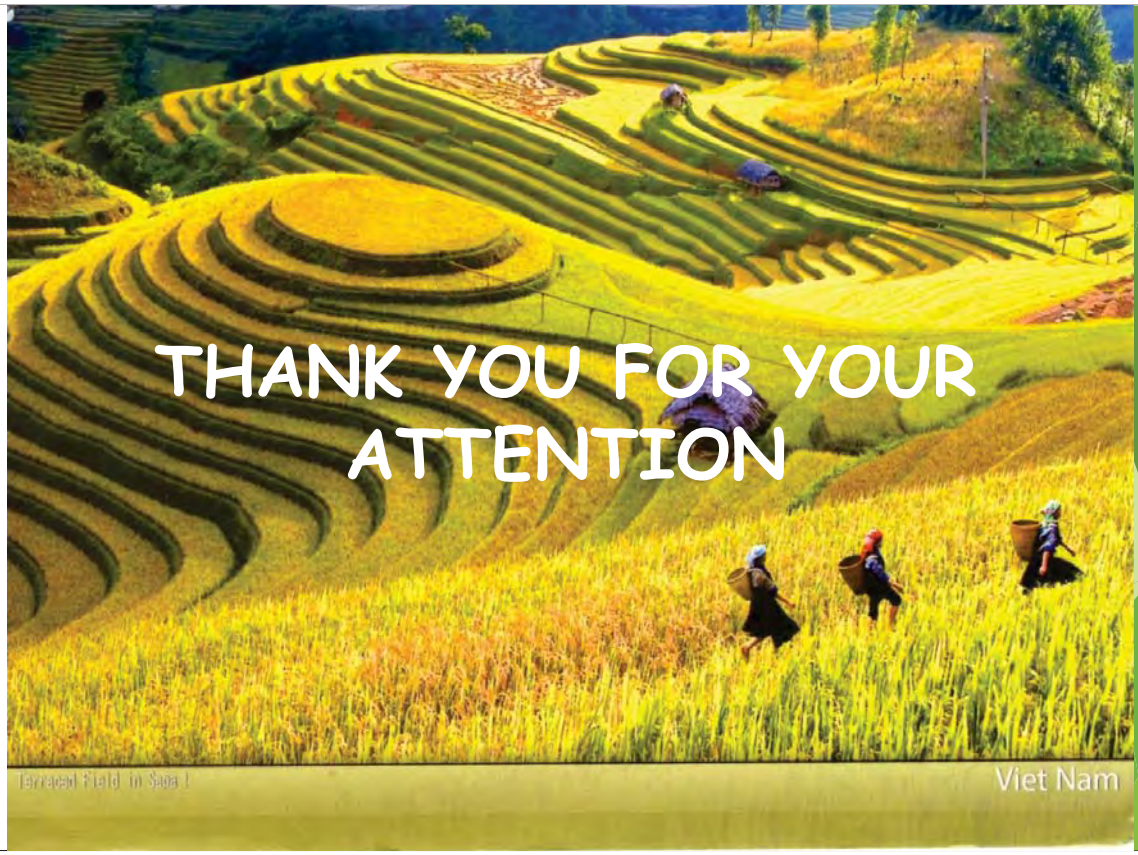


Fig. 5: result of Indirect ELISA reaction for detection of virus on taro plant. All the ELISA wells (includes Negative control and Buffer) turned yellow color.


THANK YOU FOR YOUR ATTENTION
Viet Nam

Annex 7

Report 7: Detection of Banana Bunchy Top Virus (BBTV) on Banana by PCR

1. PLACE AND TIME

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 11th - 13th, 2015

2. MATERIALS

- DNA extraction Kit PHYTOPURE (RNP-8511) (GE healthcare, USA).
- Specific PCR primers for BBTV (D11 – forward/ D12 – reverse) (Karan et al., 1994).

Fig 1: DNA extraction Kit PHYTOPURE (RNP-8511) (GE healthcare, USA).

3. SAMPLES

Fig. 2. The BBTV inoculated- plant without symptoms

Fig. 3. Healthy plant

3. SAMPLES

Fig. 4. Banana plant used to rear banana aphid (*Pentalonia nigronervosa*)

Fig. 5. BBTV-inoculated banana plant with typical symptoms: stunt, bunchy top, yellow (chlorotic/ necrotic) leaf margins

4. PROTOCOLS FOR DNA EXTRACTION

- Step 1:

- Weigh 0.1 g sample and pulverize in liquid Nitrogen quickly using a mortar and pestle.

- Transfer the plant sap powder into new 1.5 ml tube.

4. PROTOCOLS FOR DNA EXTRACTION

- Step 2:

- Add 300 µl plant DNA extraction Reagent 1 into tube with plant sap powder, turn the tube upside down or gently shake.

4. PROTOCOLS FOR DNA EXTRACTION

- Step 3:

- Add 100 µl plant DNA extraction Reagent 2 into tube with plant sap powder and Reagent 1, turn the tube upside down or gently shake.

4. Protocols for DNA extraction

- Step 4:

- Vortex the tube for few second and heat for 10 minutes at 65°C (Dry Thermo Unit).

- Put the tube in cold box for 20 minutes

4. Protocols for DNA extraction

- Step 5:

- Add 250 µl Chloroform into tube and gently shake.

- Then, add 50 µl Resin into tube (note: pre-vortex the resin before using to avoid sedimentation)

4. Protocols for DNA extraction

- Step 6:

- Shake tube for 10 minutes in sharking machine (Bio Shaker BR-15LF/ TAITEC) at 25.5°C (note: check carefully the tube cap to ensure that it has been closed tightly).

4. Protocols for DNA extraction

- Step 7:

- Centrifuge at 2500 rpm for 10 minutes at room temperature (KUBOTA 3300). Repeat centrifugation if plant debris are not completely settled.

- Collect the supernatant (about 250 µl) and transfer into new 1.5 ml tube (avoid the plant tissue debris).

4. PROTOCOLS FOR DNA EXTRACTION

- Step 8:

- Add 250 µl 2-propanol and shake gently (note: the volume of 2-propanol must be equal with collected supernatant in step 11).

- Centrifuge at 15,000 rpm for 5 minutes at room temperature. The DNA pellet will be precipitated at the bottom of tube.

4. PROTOCOLS FOR DNA EXTRACTION

- Step 9:

- Pipette out the liquid carefully and ddd 100 µl 70% ethanol and gently wash the tube.

- Centrifuge at 15,000 rpm for 2 minutes at room temperature, then discard carefully as much ethanol as possible

4. Protocols for DNA extraction

- Step 10:

- Air-dry for 2-3 minutes at room temperature

- Add 100 µl 1X TE buffer and break the DNA pellet by touching with a pipette tip.

5. Protocols for PCR assay

- Step 1: Prepare the cocktail mixture; calculate the required amount as follow:

	For detection	For sequencing
q.s.	17.4 µl	34.8 µl
10x Ex Taq Buffer	2.5 µl	5.0 µl
dNTP mixture	2.0 µl	4.0 µl
Forward primer (25 pmol)	0.25 µl	0.5 µl
Reverse primer (25 pmol)	0.25 µl	0.5 µl
TaKaRa Ex Taq (5 units/µl)	0.1 µl	0.2 µl
Template DNA	2.5 µl	5.0 µl
Total	25 µl	50 µl

5. PROTOCOLS FOR PCR ASSAY

- Step 2:

- Transfer 22.5 µl the cocktail mixture into PCR tube, then add 2.5 µl of extracted DNA.

- Flash PCR tube for few seconds (must not have the bubbles).

- Conduct PCR assay by PCR machine (DNA Engine/ BioRAD) with above given PCR conditions.

.

5. PROTOCOLS FOR PCR ASSAY

- Step 2:

- Transfer 22.5 µl the cocktail mixture into PCR tube, then add 2.5 µl of extracted DNA.

- Flash PCR tube for few seconds (must not have the bubbles).

5. Protocols for PCR assay

- Step 3: Conduct PCR assay by PCR machine (DNA Engine/ BioRAD) with above given PCR conditions.

6. Protocols for *Gel electrophoresis*

- Prepare 2% agarose gel in 1X TAE buffer.
- Completely dissolve the agarose in the buffer using a microwave.
- Let the solution to slightly cool down (~ 5 minutes).
- Pour the solution slowly into the casting tray with the comb in place. Avoid forming any bubbles.
- Let the agarose get to solidify (~30 – 45 minutes) then carefully remove the comb.
- Place the solidified agarose gel into the electrophoresis unit. Fill in with 1X TAE buffer until the gel is fully submerged.

6. PROTOCOLS FOR *GEL ELECTROPHORESIS (CONTINUED)*

- Put 2 µl blue juice in a piece of parafilm.
- Mix 13 µl PCR product (for 6 band-comb) into blue juice by carefully pipetting the solution in and out of the tip. Avoid forming any bubbles. (For 8 bands (small comb) use 8 µl PCR product with 2 µl blue juice).
- Load the sample mixture into gel starting with 100 bp ladder/marker (for BBTV) at 15 µl; followed by the negative control.
- Run samples for 25 – 30 minutes using electrophoresis machine (Mupid-2plus/ Advance).
- After running, stain the gel by submerging it into Ethidium bromide solution for 5 minutes.
- De-stain the gel in distilled water for 1 – 2 minutes.
- View DNA band under UV illumination and take photo using EDAS 290 (Kodak, Japan).

7. RESULT

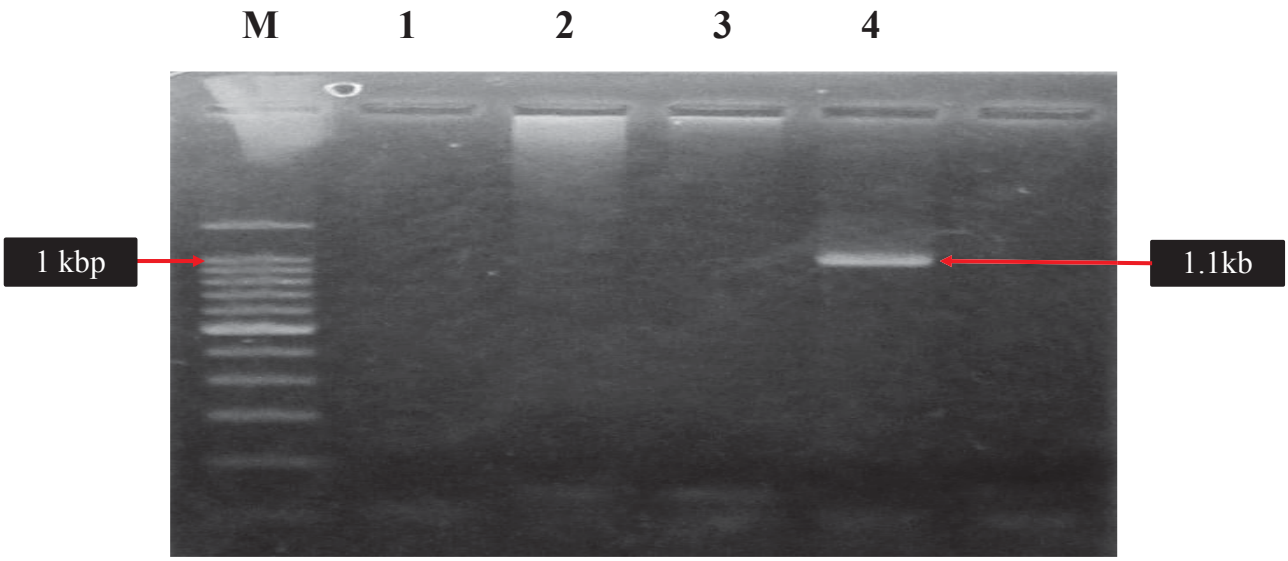


Fig 4. PCR assay of *Banana bunchy top virus* (BBTV) collected from HOGOKEN Lab. with D11/D12 primers. The PCR band with the size of ~1.1kb (red arrow) was amplified from Sample 4 (lane 4). No band was amplified from Sample 1-3 (lanes 1-3). The 100bp DNA ladder (Promega, USA) was included as marker.

8. DISCUSSION

- The BBTV-inoculated banana plant (sample 4) shows symptoms: stunt, bunchy top, yellow (chlorotic/ necrotic) leaf margins (Fig. 5) resulted positive to BBTV presence. Sample 2 (BBTV-inoculated banana plant without symptoms – Fig. 2) as well as other samples gave negative to BBTV.

THANK YOU FOR YOUR ATTENTION

Report 8. Detection of Banana Bunchy Top Virus (BBTV) on Abaca (*Musa textilis*) by PCR

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 12th - 16th, 2015

2. Material

- DNA extraction Kit PHYTOPURE (RNP-8511) (GE healthcare, USA).
- Specific PCR primers for BBTV (D11 – forward/ D12 – reverse) (Karan et al., 1994).

Fig 1: DNA extraction Kit PHYTOPURE (RNP-8511) (GE healthcare, USA).

3. Samples

Fig. 2: the samples of Abaca (*Musa textilis*) collected in the Philippines in 2011 and was kept at (-) 30°C in HOGOKEN Lab. Sample 1: Positive control for BBTV; Sample 2: was named "Abaca forestry"

4. Procedure for DNA extraction, PCR assay and gel electrophoresis

- Procedure for DNA extraction, PCR assay and gel electrophoresis was described in previous report (see report no. 7)

5. Result

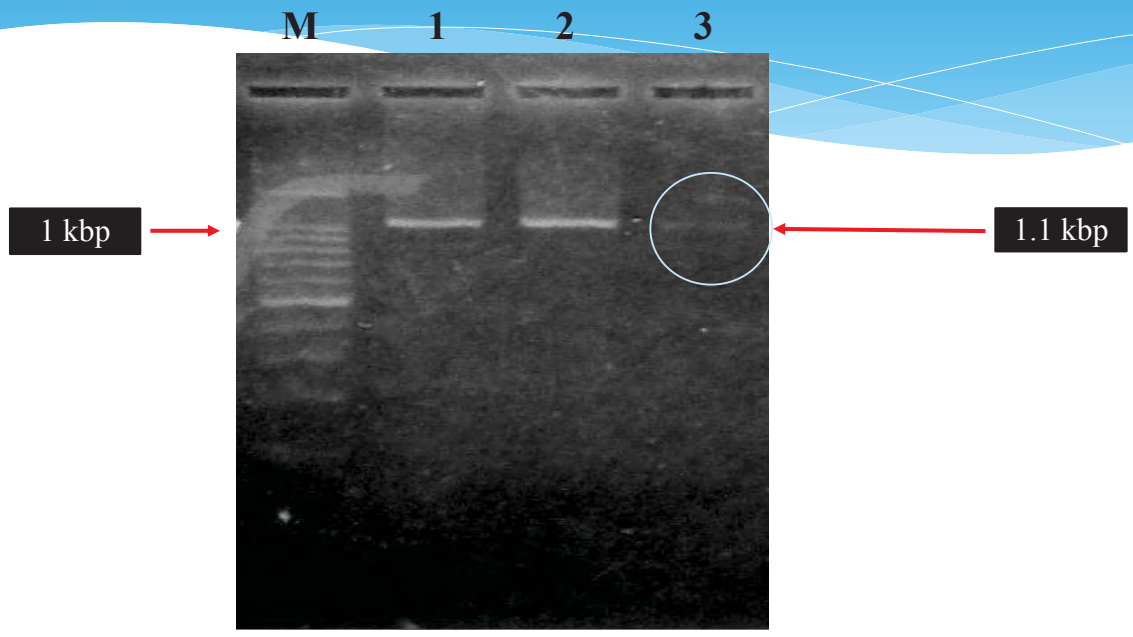


Fig 3. PCR assay of *Banana bunchy top virus* (BBTV) on Abaca (*Musa textilis*) collected from the Philippines with D11/D12 primers. The PCR band with the size of ~1.1kb (red arrows) was amplified from Positive controls (lanes 1-2). The PCR band in was amplified from Sample 2 (lane 3) was unclear (in circle). The 100bp DNA ladder (Promega, USA) was included as marker.

6. Discussion

- The sample 2 named "Abaca forestry" (Fig. 2) resulted positive to BBTV presence. However, the PCR band was unclear. This result may be caused due to reduction of concentration of virus during storage period.

Annex 9

Name: **Tran Van Chien**

Country: **Vietnam**

REPORT ON LECTURE ABOUT PLANT PARASITIC NEMATODES AS THE VECTOR FOR PLANT VIRUSES

1. Lecturer

Dr. Marita S. Pinili, from IPB, University of the Philippines Los Banos

2. Place and time

Place: HOGOKEN meeting room, Tokyo University of Agriculture (Tokyo NODAI).

Time: Nov. 12th, 2015

3. Contents

3.1. Introduction about plant nematodes

3.1.1. General morphological characteristics

Nematodes are worm shape (vermiform), unsegmented, pseudoceolomate, multicellular, triploblastic, and bilaterally simetrical. Nematodes were once classified with a very large and heterogeneous cluster of animals grouped together on the basis of their overall worm-like appearance, simple structure of internal body set, and well-defined head.

3.1.2. Biological characteristic

Life cycle of nematode parasitic plants are: egg, juvenile, and adult. Egg hatched as a juvenile. There are four juvenile stadia, separated another by molt. Juvenile is the most damaged stadia of nematode in plant. Major type nematodes feeding strategies/parasitic habit nematode are:

1. Ectoparasites in which the nematode remains outside of the plant and uses its stylet to feed from the cells of the plant roots

2. Semi-endoparasites nematodes are able to partially penetrate the plant and feed at some point in their life cycle
3. Migratory parasites nematodes can spend much of their time migrating through root tissues destructively feeding on plant cells
4. Sedentary endoparasites. The most damaging nematodes in the world have a sedentary endoparasitic. The two main nematodes in this group are the cyst nematodes (*Heterodera* and *Globodera*) and the root-knot nematodes (*Meloidogyne*)
5. Stem and bulb nematodes (*Ditylenchus* spp.) are, as their name suggests, nematodes that attack the upper and lower parts of plants
6. Seed gall nematodes (*Anguina* spp.) . These nematodes migrate as J2s in water films to the leaves of plants where they feed as ectoparasites at the tips, causing distortion of the leaves.
7. Foliar nematodes are in the genus *Aphelenchoides*. The adult nematodes migrate in water films on the stems to the leaves of their host plant and penetrate the leaves through natural openings (stomata)

3.1.3. Damages caused by plant nematodes

There are two type of nematodes which are free-living nematodes and plant parasitic nematodes. Roles nematodes in disease development there are as a pathogen, incitant, and vector of virus. Nematodes pathogen can cause disease even if the absence of other organism. Nematodes can attack healthy plants tissues that creates infection courts for other organism and causes minimal damage. Nematodes as a vector can carries other pathogen in host tissues but it is not further involved in disease development.

3.2. Plant parasitic nematodes as the important vectors of plant viruses

3.2.1. Defined genera of plant parasitic nematodes as the vector of plant viruses.

Genera of nematodes that carries plant viruses are Triplonchida and Dorylaimida. Triplonchida transmit *Tobravirus* (*Trichodorus* sp., *Paratrichodorus* sp.), while Dorylaimida can transmits genus of *Nepovirus* (*Xiphinema* sp, *Longidorus* sp., and *Paralongidorus* sp.).

Tobravirus derived from *Tobacco Rattle Virus* and have straight tubular particle. All *Tobravirus* species are ssRNA positive-strand (+ss RNA) viruses. Some other important *Tobravirus* are *Pea early-browning virus* (PEBV) and *Pepper ringspot virus* (PepRSV). While *Nepovirus* derived from *Nematode Polyhedral Virus* which have isometric particle of 28 nm diameter, and have bipartite genome with two functional RNA molecules. They are ssRNA positive-strand (+ss RNA) viruses. Some other important *Nepovirus* are *Tobacco ringspot virus* (TRSV); *Grapevine fanleaf virus* (GFLV) and *Beet ringspot virus* (BRSV).

3.2.2. Manner of transmission of plant viruses by plant parasitic nematodes.

Plant viruses are transmitted by plant parasitic nematodes following non-persistent manner. Virus cannot replicate in nematodes body, not passed transovarily through nematode eggs, and is eliminated from nematode body when juvenile's molt.

- Ingestion : the intake of virus particles during feeding
- Acquisition: act of ingesting virus particles
- Absorption: the active of process by which virus particles adhere to specific sites of retention in the feeding apparatus

- Retention : the period during which specifically absorbed attached of the site retention in the feeding apparatus
- Release: the dissociation of the virus particles from the specific site of retention in the feeding apparatus
- Transfer: The placement of virus particles in plant cell

3.4. Method for conducting the experiments on virus-transmission by plant parasitic nematodes and detection of virus in nematodes.

- Extraction nematode from rhizospere of infected plant
- Add nematode to healthy bait plant grown in sterilized soil
- Transmission confirmed by manifestation of similar symptom

Detection and identification of virus can be done by serology methods (ELISA, ISEM) or molecular method (molecular hybridization technique, PCR, and RT-PCR).

Report 10. Method for storage of virus-infected samples by FTA plant card

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 16th, 2015

2. Materials

- *FTA plant card*

Fig. 1: The FTA plant cards can be purchased from Whatman company (GE healthcare Life Science). This plant cards are easy to be used and stored in Laboratory.

3. Samples

Fig. 2: The BBTV-infected samples were collected in the Philippines in 2011 and stored at (-) 30°C in HOGOKEN Lab.

4. Produce

Step 1:

- Use scissor to cut sample into small pieces and transfer to the mortar.

Step 2:

- Add 200 µl sterilized water into mortar and grind the sample by pestle.

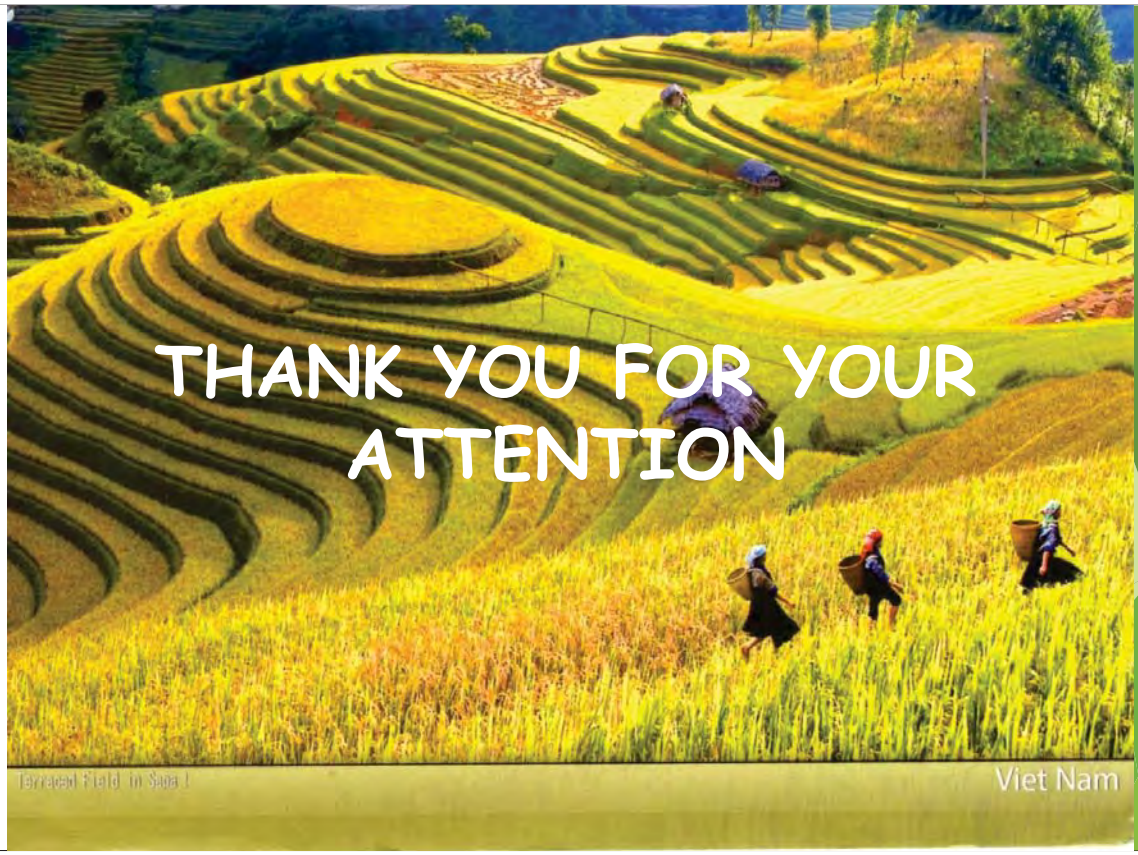
Step 3:

- Use the pipet to transfer the plant sap into FTA plant card (**note:** do not pipet the plant sap out of the round cycle of plant card to avoid the contamination with other samples).

Step 4:

- Let FTA plant card dry under room temperature within 1 hour.

- Put the FTA plant card with plant sap in plastic bag with silicagen and keep in cool dry place. The plant sample will be stored by FTA plant card more than 1 year.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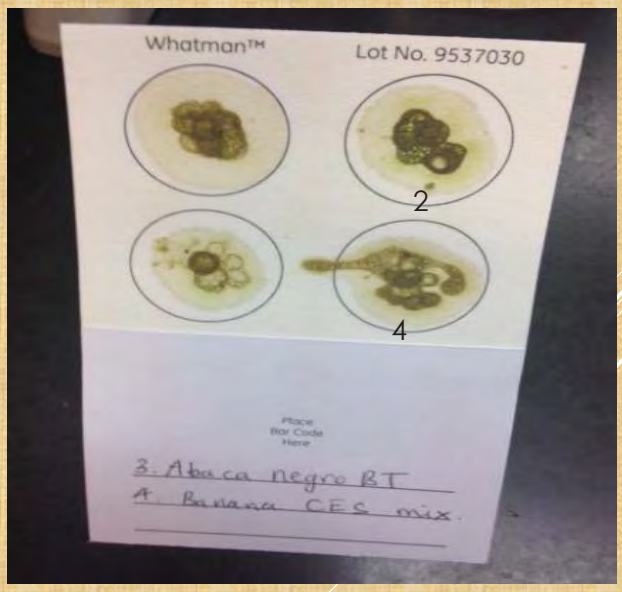
Report 11. Detection of Banana Bunchy Top Virus (BBTV) and Banana Bract Mosaic Virus (BBrMV) from FTA plant card.

1. PLACE AND TIME

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 16th - 17th, 2015

2. SAMPLES AND MATERIALS

- *2.1. Samples*
- - FTA plant card with banana and abaca samples which are infected by BBTV and BBrMV. These samples were collected in the Philippines in 2011 by Dr. Marita Pinili (IPB, University of the Philipoines Los Banos) and stored at (-) 30°C in HOGOKEN Lab.



Sample 2. Abaca Forestry isolate

Sample 4. Banana CES mixed infection

2. SAMPLES AND MATERIALS

- *2.2. Materials*

- FTA plant Kit (GE healthcare Life Science, UK)

(1) FTA membrane puncher; (2) Cutting mat; (3) FTA purification reagent

3. PROTOCOLS

3.1. Extraction of total plant DNA from FTA plant card

- Step 1:

- Use puncher to take 8 pieces of sample on FTA plant card and transfer into 1.5 ml eppendorf tube (note: should not take much plant tissue debris).

- Step 2:

- Add 100 µl processing buffer, then add 1 µl RNAse inhibitor into 1.5 ml tube.

- Then, incubate on ice with mixing every 5 minutes interval for 30 minutes.

▸ Step 3:

- Transfer the supernatant into new 1.5 ml tube and add 10 µl 3M Sodium acetate (pH = 5.2) and 10 µl of cold 2-propanol.

- Incubate at (-) 80°C (Panasonic, Japan) for 30 minutes.

▸ Step 4:

- After finishing the incubation, centrifuge the tube at 15,000 rpm (KUBOTA 3300) for 10 minutes.

- Step 5:

- Discard supernatant and wash total plant DNA pellet with 500 µl 75% Ethanol.

- Centrifuge the tube at 15,000 rpm (KUBOTA 3300) for 02 minutes.

- Step 6:

- Remove the Ethanol by pipet, then dry tube at room temperature for 2-3 minutes.

- Dissolve total plant DNA pellet in 30 µl DEPC-treated water and store at (-) 30°C for next using.

3.2. Synthesis of cDNA (for BBrMV)

- Prepare the cocktail mixture in PCR tube; calculate the required amount as follow:

5X RT buffer	4.0 µl
dNTP mixture (10 mM)	2.0 µl
Reverse primer of BBrMV	1.0 µl
RNAse inhibitor (10U/ µl)	1.0 µl
ReverTra Ace™	1.0 µl
Total RNA	11 µl
Total	**20 µl**

3.2. Synthesis of cDNA (for BBrMV)

- Conduct synthesis of cDNA by PCR machine (DNA Engine/ BioRAD) with PCR conditions below:

Temperature (°C)	Time (min.)
42	20
99	5
4	endless

3.3. PCR assay and Gel electrophoresis

- Conduct PRA assay with PCR conditions as follow:
- For BBTV

Temperature (°C)	Time (min.)	Cycles
94	4	
94	1	29
61	1	
72	2	
72	10	

- 2% gel agarose was used for gel electrophoresis of PCR product.

3.3. PCR assay and Gel electrophoresis

- For BBrMV

Temperature (°C)	Time (min.)	Cycles
94	1	
94	0.5	32
61	1	
72	1	
72	3	

- 2% gel agarose was used for gel electrophoresis of PCR product.

4. RESULT

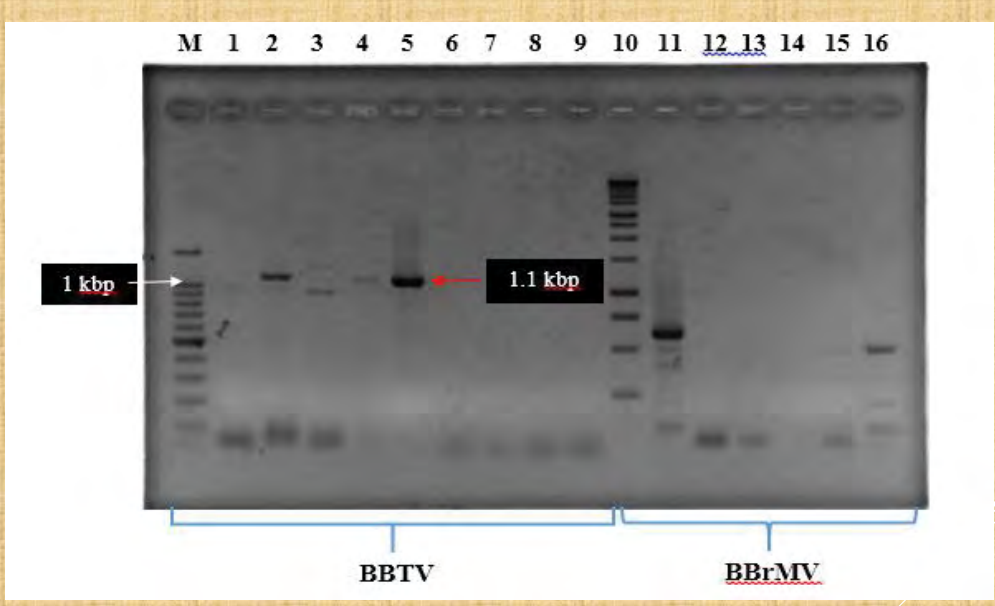


Fig 1. PCR assays on frozen samples collected in the Philippines in 2011 (Abaca Forestry isolate – lane 2; 13 and Banana CES mixed infection – lane 4; 14) with D11/D12 primer pairs for *Banana Bunchy Top Virus* (BBTV) and Bract 1/ Bract 2 for *Banana Bract Mosaic Virus* (BBrMV). For BBTV, the PCR band with approx. size of ~ 1.1kb (red arrow) was amplified from both samples (lane 2; 4). For BBrMV, no band was amplified from these samples (lane 12-13). The 100bp DNA and 1kb DNA ladder (Promega, USA) was included as marker.

5. DISCUSSION

- Both samples (Abaca Forestry isolate and Banana CES mixed infection) resulted to positive with BBTV, whereas got negative with BBrMV.
- These samples which has been kept at (-) 30°C for over 4 years still have a good concentration of virus for PCR assay. It showed that making plant samples in freezing condition at (-) 30°C or below is one of the best ways to store virus-infected samples.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Report 12. Detection of Banana Bunchy Top Virus (BBTV) from Aphid

1. PLACE AND TIME

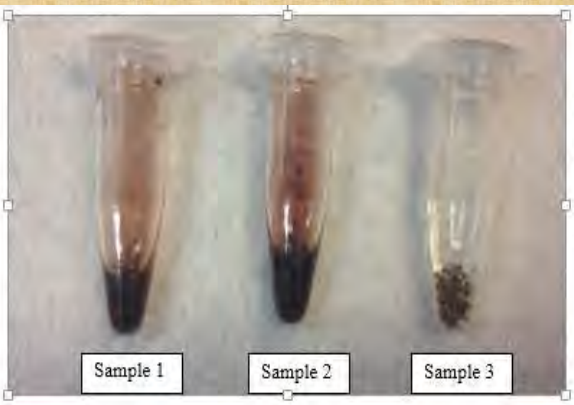
Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).

Time: Nov. 18th – 19th, 2015

2. SAMPLES AND MATERIALS

2.1. Samples

- The banana aphid (*Pentalonia nigronervosa*) samples were collected from banana and taro plant in HOGOKEN Lab. 1 week ago and kept in 1.5 ml tube with 70% Ethanol.



Sample 1. The aphids were collected from BBTV-infected banana plant.

Sample 2. The aphids were collected from BBTV-inoculated banana plant but not shown symptoms.

Sample 3. The aphids were collected from BBTV-inoculated taro plant but not shown symptoms.

2. SAMPLES AND MATERIALS

2.2. Materials

- DNA extraction Kit (GE healthcare Life Science, UK)

3. PROTOCOLS

3.1. Extraction of total DNA from banana aphid

1. Immobilize 15-20 aphids by 70% Ethanol.
2. Remove the Ethanol and dry the aphid by tissue/ filter paper at room temperature.
3. Transfer the aphids into cold mortar and grind in liquid nitrogen.
4. Collect the sample quickly and place in 1.5 ml tube.
5. Perform DNA extraction following the procedure of Nucleon Phytopure DNA Extraction Kit (GE healthcare Life Science, UK).

3.2. PCR assay and Gel electrophoresis

Conduct PRA assay with PCR conditions as follow:

Temperature (°C)	Time (min.)	Cycles
94	4	
94	1	29
61	1	
72	2	
72	10	

- 2% gel agarose was used for gel electrophoresis of PCR product.

4. RESULT

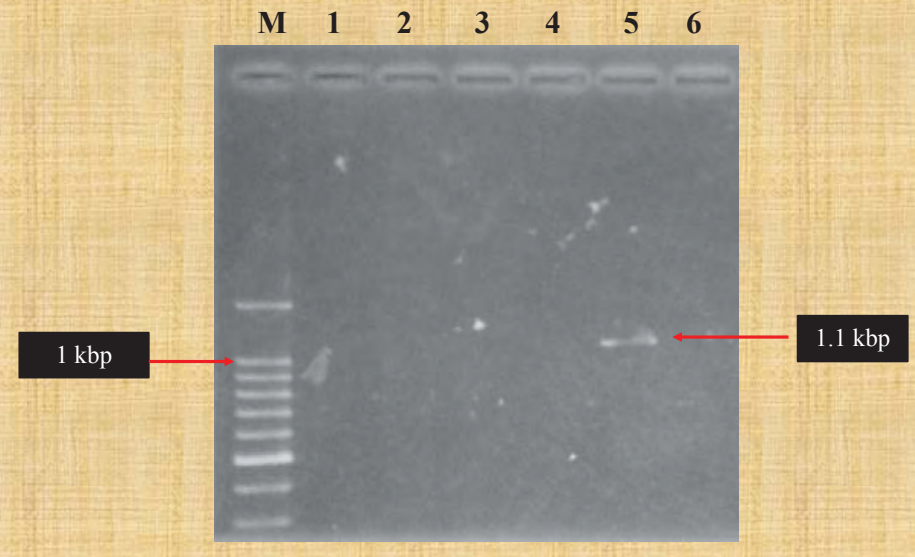


Fig 1. PCR assay of *Banana bunchy top virus* (BBTV) on banana aphid with D11/D12 primers. The PCR band with the size of ~1.1kb (red arrow) was amplified from Sample 1 (lane 5). No band was amplified from Sample 2 and 3 (lanes 3; 4; 6, respectively). Lane 2 was negative control. No band in lane 1 (replication test of sample 1). The 100bp DNA ladder (Promega, USA) was included as marker.

5. DISCUSSION

The aphid sample collected BBTV infected banana plant (sample 1) resulted positive to BBTV presence. Sample 2 (aphids on BBTV-inoculated banana plant) and Sample 3 (aphids on BBTV-inoculated taro plant) gave negative to BBTV. There was no band in lane 1 (replication test of sample 1). This reason might be occurred because of problem in DNA extraction step. There was no total DNA of sample 1 obtained after this step.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Report 13. Detection of BBTV from aphid-impregnated FTA plant card.

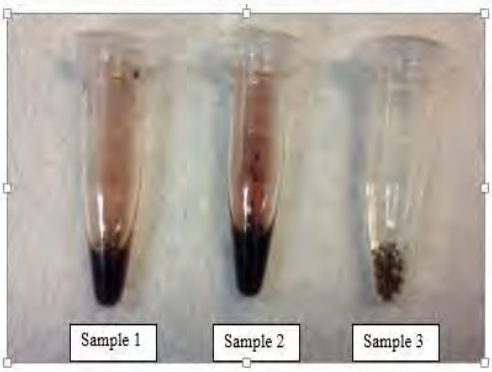
1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 18th - 19th, 2015

2. Samples and materials

- *2.1. Samples*

- The banana aphid (*Pentalonia nigronervosa*) samples were collected from banana and taro plant in HOGOKEN Lab. 1 week ago and kept in 1.5 ml tube with 70% Ethanol.



Sample 1. The aphids were collected from BBTV-infected banana plant.

Sample 2. The aphids were collected from BBTV-inoculated banana plant but not shown symptoms.

Sample 3. The aphids were collected from BBTV-inoculated taro plant but not shown symptoms.

2. Samples and materials

- *2.2. Materials*

- FTA plant card (GE healthcare Life Science, UK)

2. Samples and materials

- *2.2. Materials*

- FTA plant Kit (GE healthcare Life Science, UK)

(1) FTA membrane puncher; (2) Cutting mat;

(3) FTA purification reagent

3. Protocols

3.1. Impregnating of aphid's nucleic acid into FTA plant card

- Step 1:

- Immobilize 15-20 aphids by 70% Ethanol.

- Remove the Ethanol and dry the aphid by tissue/ filter paper at room temperature.

- Step 2:

- Place the aphid in membrane of FTA plant card.

- Step 3:

- Fold the cover of FTA plant card, then macerate the aphid by pestle till the nucleic acid impregnate into the card.

3.2. Extraction of DNA of Aphid from FTA plant card

1. Use the puncher to take 1-2 disks from the FTA plant card, then put into PCR tube.
2. Add 200 µl 90% Ethanol into the PCR tube and incubate for 5 minutes at room temperature.
3. Remove the Ethanol and add again 200 µl 90% Ethanol and incubate for 30 minutes at room temperature.
4. Add 200 µl FTA purification reagent and incubate for 5 minutes at room temperature.
5. Remove the reagent and repeat step 4 two times more.
6. Add 200 µl 1X TE buffer (pH = 8.0) and incubate for 5 minutes at room temperature.
7. Repeat step 6.
8. Remove the liquid and dry the disks for 1 – 2 hours at room temperature.
9. Use these disks for PCR assay or store at (-) 30°C for next using.

3.3. PCR assay and Gel electrophoresis

- Conduct PRA assay with PCR conditions as follow:

Temperature (°C)	Time (min.)	Cycles
94	4	
94	1	29
61	1	
72	2	
72	10	

- 2% gel agarose was used for gel electrophoresis of PCR product.

4. Result

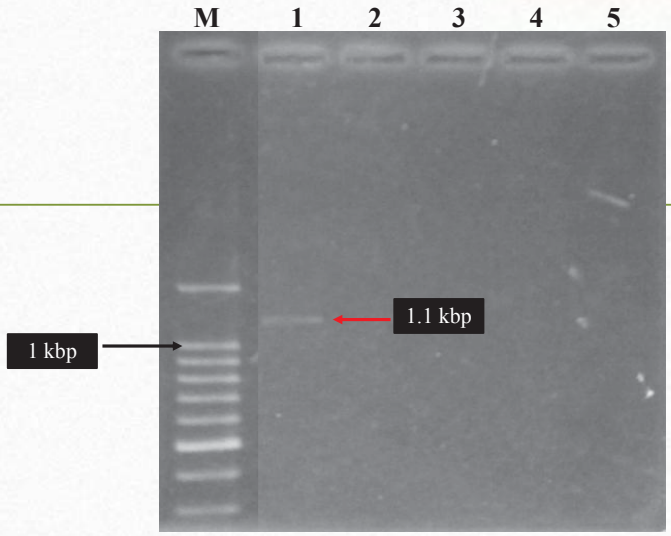


Fig 1. PCR assay of *Banana bunchy top virus* (BBTV) on banana aphid impregnated into FTA plant card with D11/D12 primers. The PCR band with the size of ~1.1kb (red arrow) was amplified from Sample 1 (lane 1). No band was amplified from Sample 2 and 3 (lanes 2-3). Lane 4 was negative control. The 100bp DNA ladder (Promega, USA) was included as marker.

5. Discussion

- The aphid sample collected BBTV infected banana plant (sample 1) resulted positive to BBTV presence. Sample 2 (aphids on BBTV-inoculated banana plant) and Sample 3 (aphids on BBTV-inoculated taro plant) gave negative to BBTV.
- The PCR assay for detecting BBTV on banana aphid can be performed with aphid-impregnated FTA plant card.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Report 14. Method for developing phylogenetic tree using MEGA software

1. Lecturer

- Dr. Noriko Furuya

From DNA Data Bank of Japan (DDBJ).

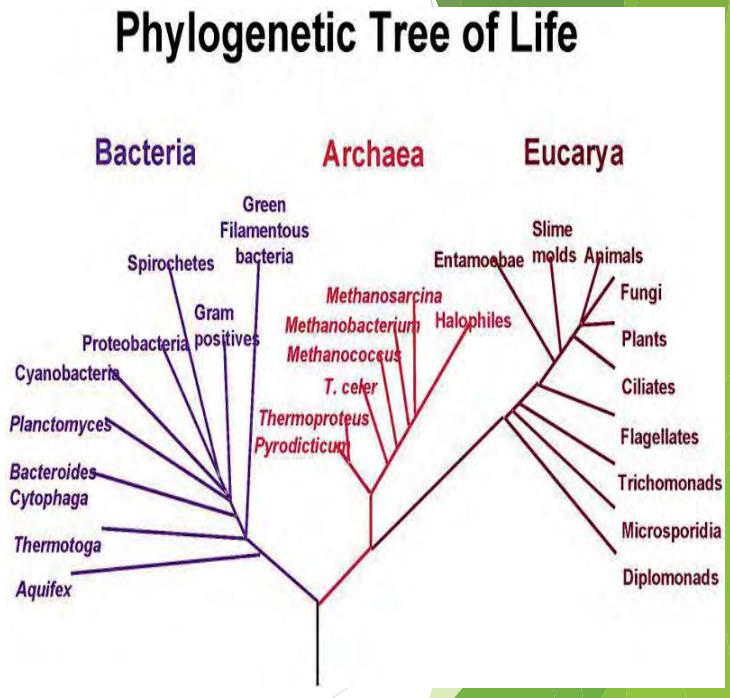
2. Place and time

- Place: HOGOKEN meeting room
- Time: Nov. 26th, 2015

3. Contents

3.1. Definition of Phylogenetic tree

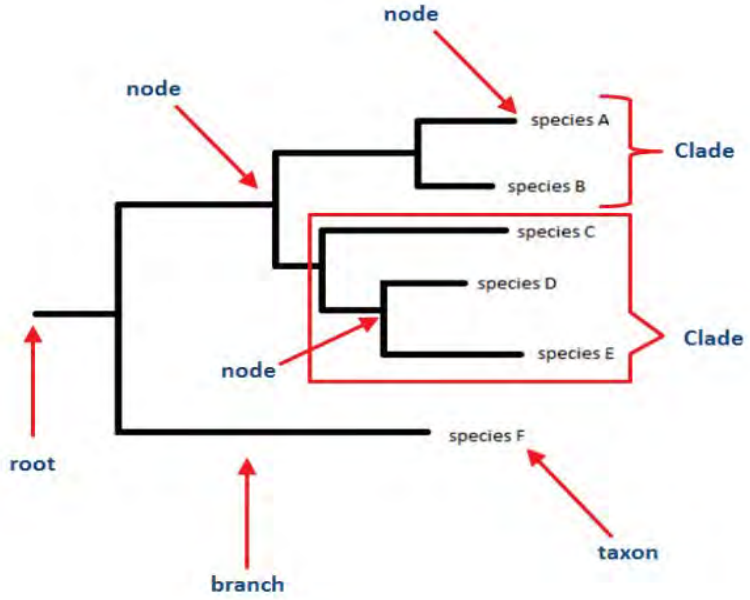
- A branching diagram showing the inferred evolutionary relationships among various biological species based upon similarities and differences in their physical or genetic characteristics.



Source:
http://www.dnabaser.com/articles/phylogenetic-tree/

3.2. Main parts of a Phylogenetic tree

- Root
- Branches
- Nodes
- Leaves (taxon)



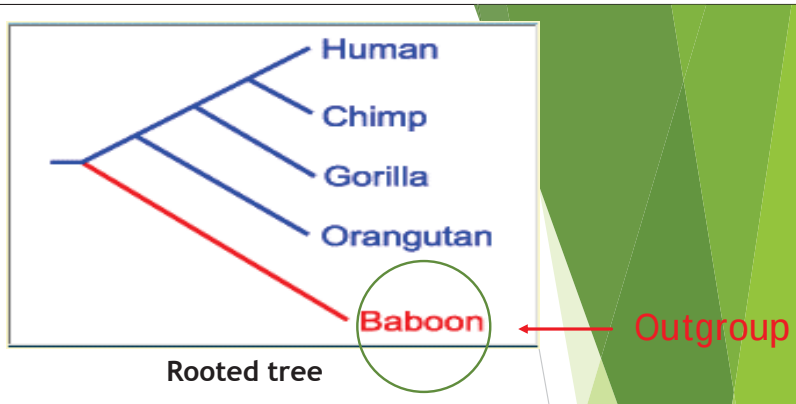
*Source:
http://www.cs.us.es/~fran/students/julian/phylogenetics/phylogenetics.html

3.3. Rooted tree

- A directed tree with an unique node corresponding to the most recent common ancestor of all the entries at the leaves of the tree.

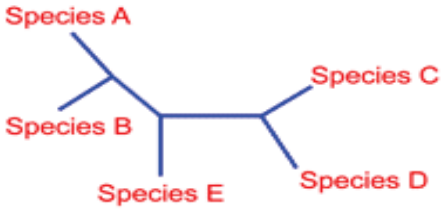
- Outgroup is the entry that is not included in the target group at present.

- If there is the outgroup entry, it is able to transform an unrooted tree to the rooted tree.



Rooted tree

Unrooted Tree with Unscaled Branches

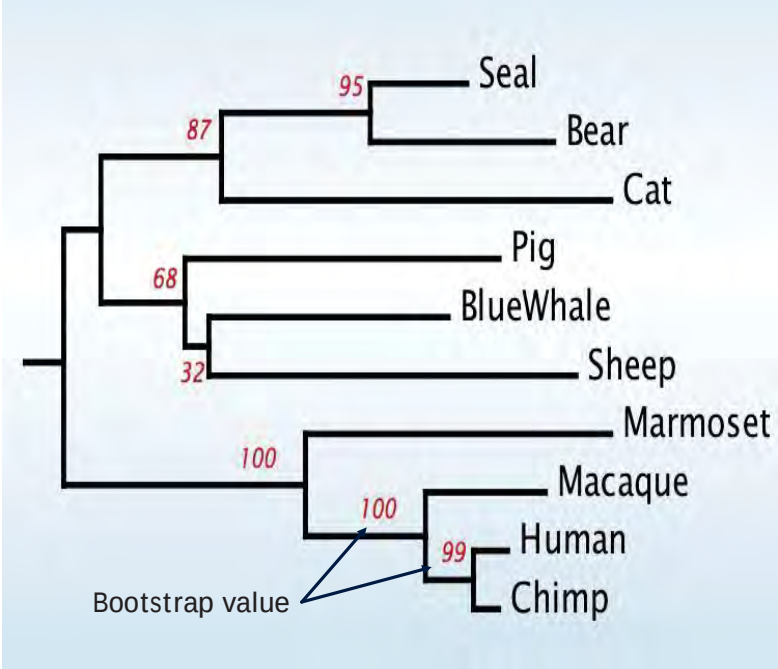


Unrooted tree

*Source:
http://www.ncbi.nlm.nih.gov/Class/NAWBIS/Modules/Phylogenetics/phylo9.html

3.4. Bootstrap method

Bootstrapping is a resampling analysis that involves taking columns of characters out of your analysis, rebuilding the tree, and testing if the same nodes are recovered. This is done through many (100 or 1000, quite often) iterations.



*Source:
http://cabbagesofdoom.blogspot.jp/2013/04/how-to-read-phylogenetic-tree.html

3.5. MEGA software

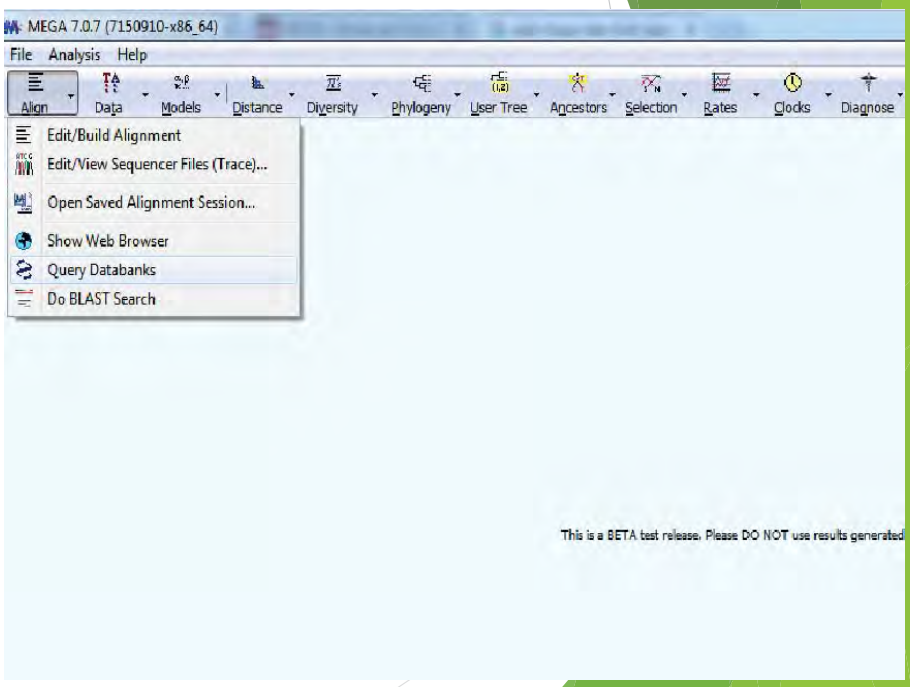
- MEGA is an integrated tool for conducting sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses.
- MEGA is used by biologists in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of the selective forces shaping the evolution of genes and species

Source: http://www.megasoftware.net/

3.6. Method for constructing a phylogenetic tree using MEGA software

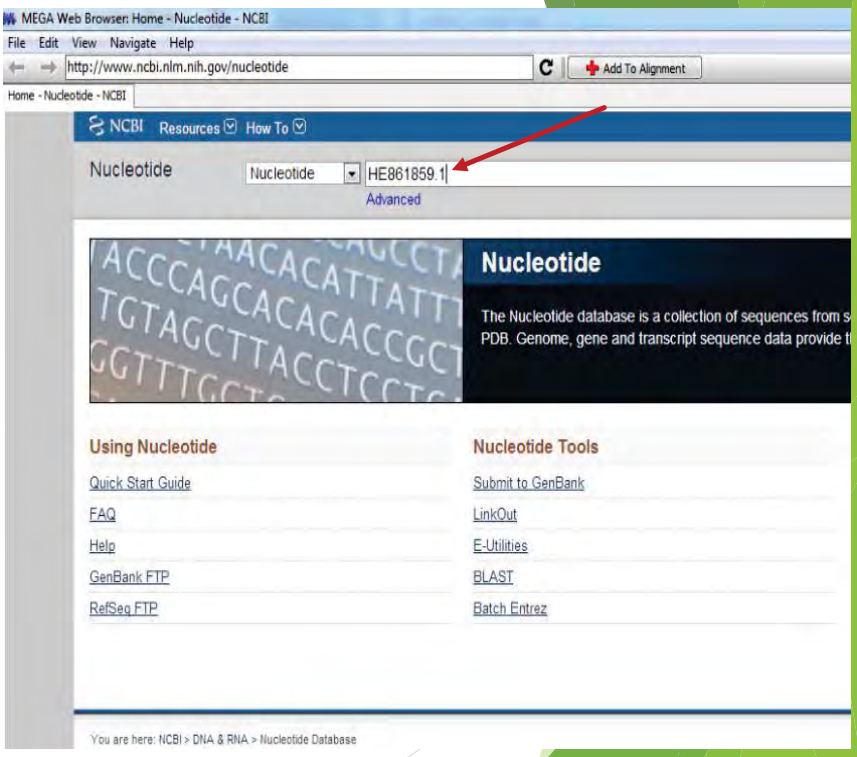
▶ Step 1.

- Open MEGA software
- Click “Align” and choose “Query Databanks”



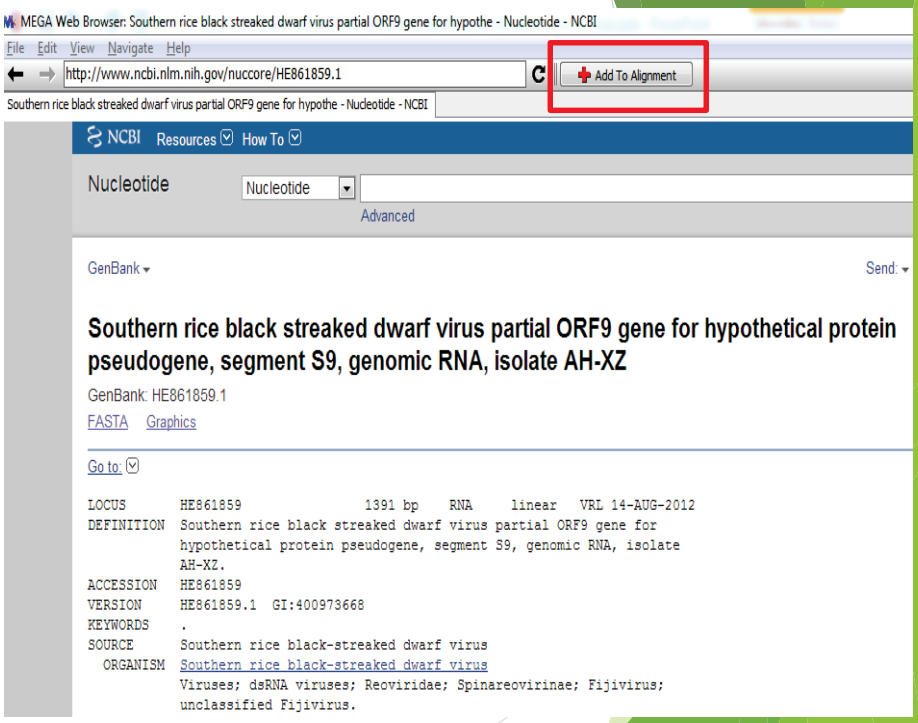
➢Step 2.

- Type the accession of target nucleotide sequence.



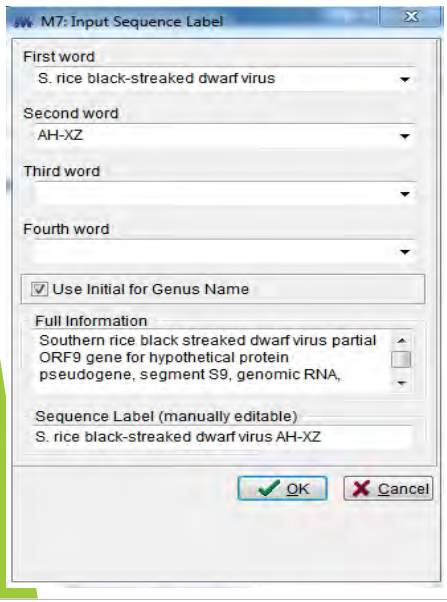
▶Step 3.

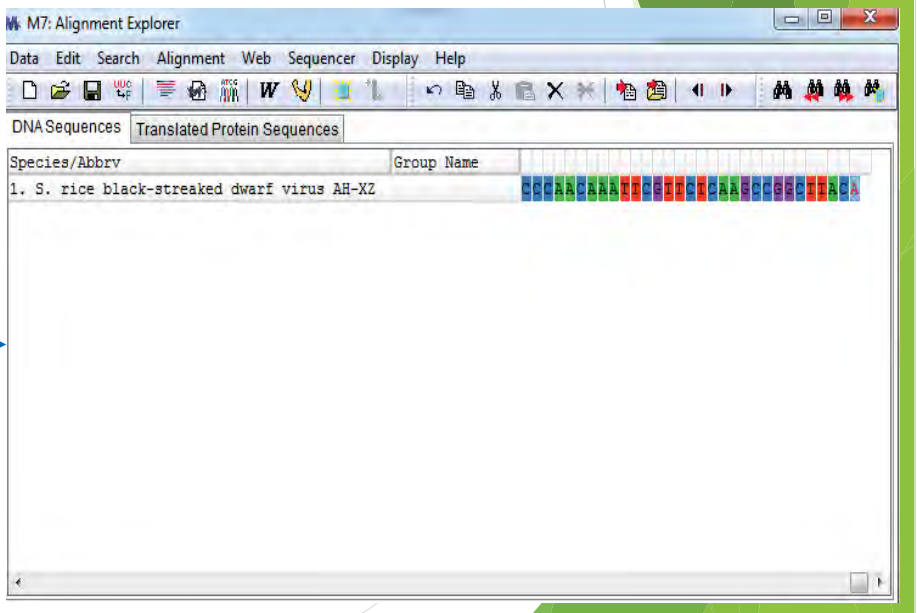
- Click “Add to Alignment”



▶Step 4.

- Finish the sequence label.
- Then, click “OK”.

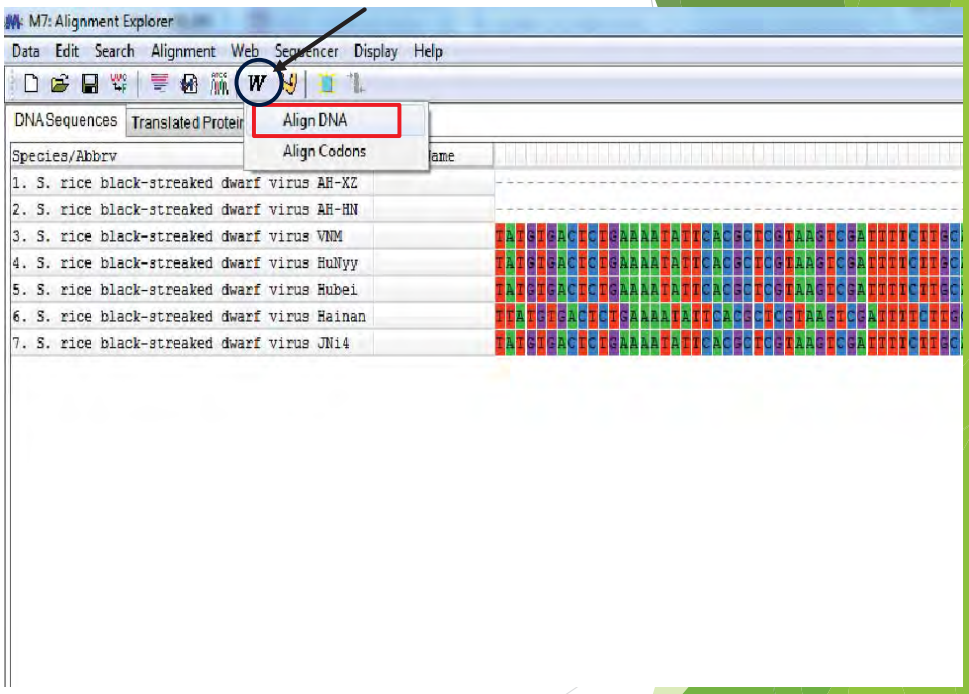




▶ Step 5.

- Repeat 4 previous steps for each nucleotide sequence chosen to construct a phylogenetic tree.

- Afterwards, click icon "W" on the window of MEGA software. Press "Ctrl" + "A" to choose all nucleotide sequences and click "Align DNA"



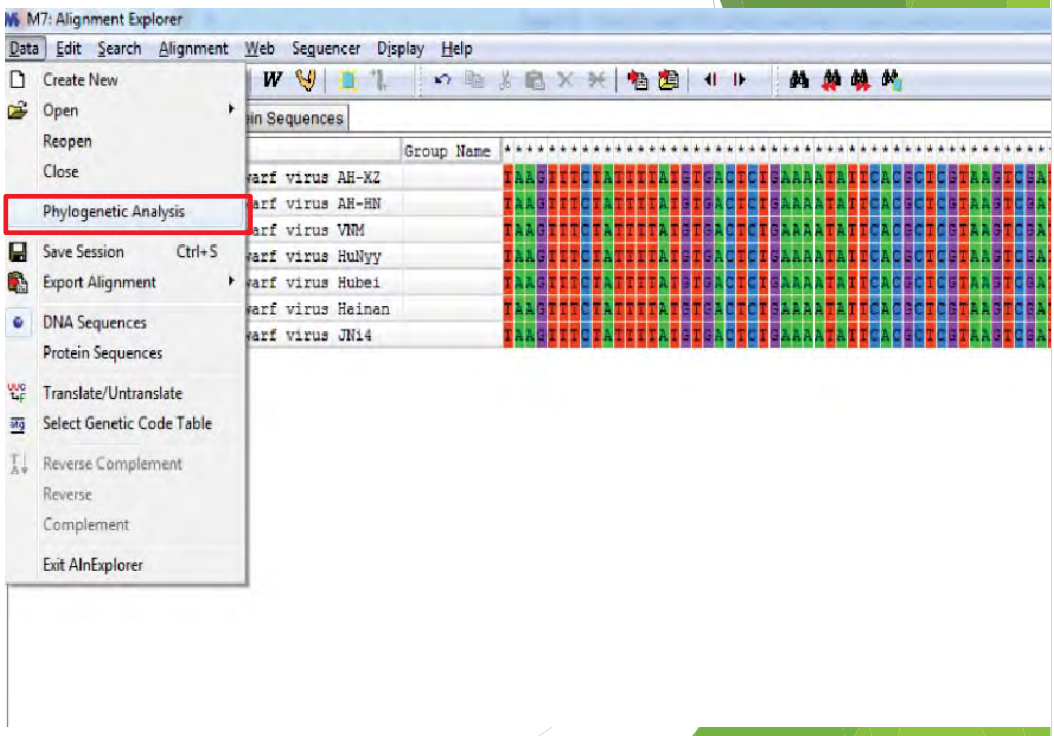
▶ Step 6.

- Trim and align by hand all the sequences to get nice result of nucleotide sequence alignment.

Align by hand

▶ Step 7.

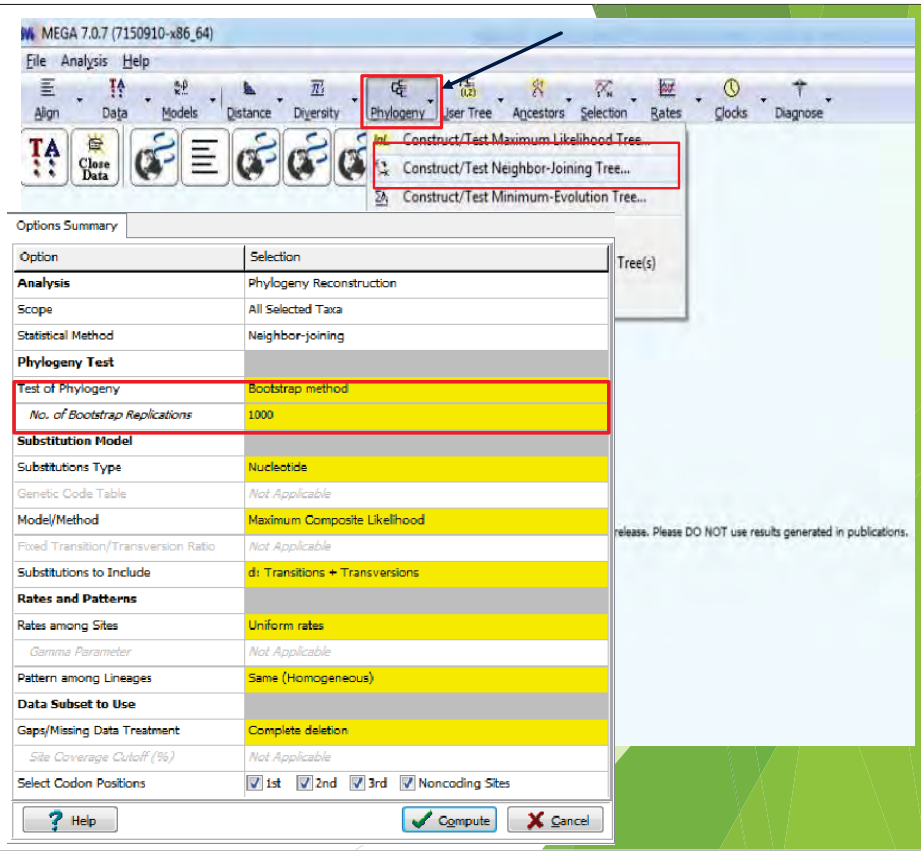
- Click “Data” and choose “Phylogenetic analysis”



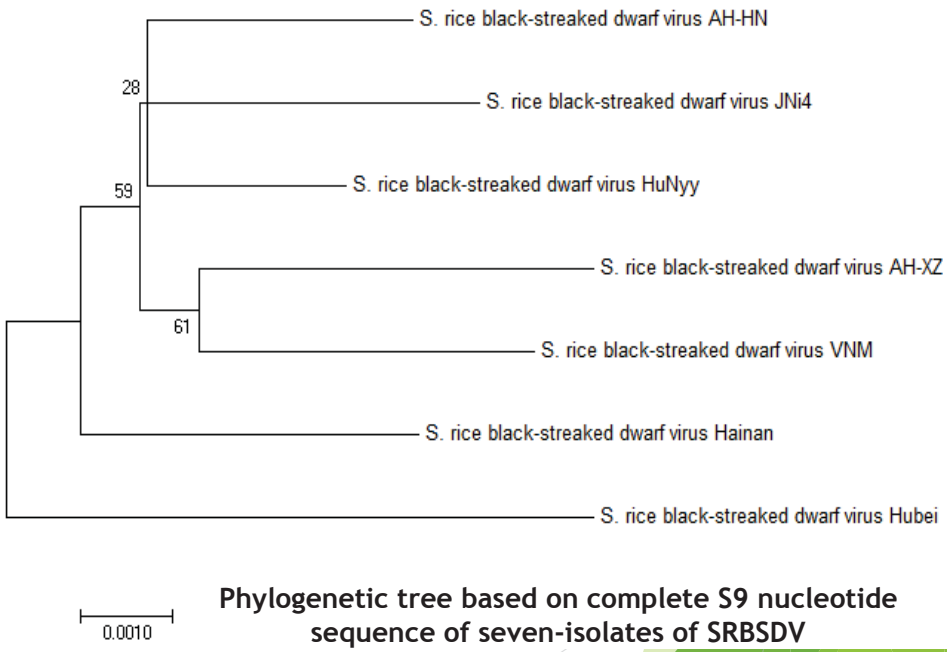
▶ Step 8.

- On the main window MEGA software, click “Phylogeny” and choose “”Construct/Test Neighbor-Joining Tree”.

- In option “Test of Phylogeny”, click “Bootstrap method” with “Bootstrap replication of 1000”. Click “compute”.



The phylogenetic tree will appear with bootstrap value.



Phylogenetic tree based on complete S9 nucleotide sequence of seven-isolates of SRBSDV

Report 15. Method for purification, ligation, transformation, cloning and sequencing of DNA of plant viruses.

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 18th - 27th, 2015

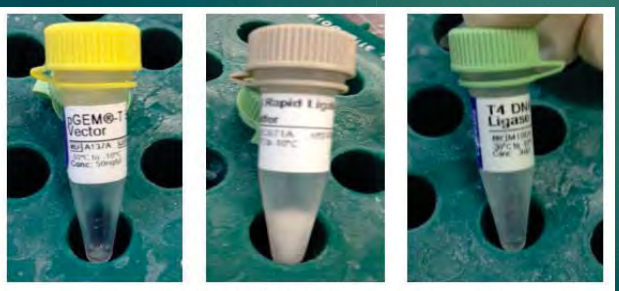
2. Samples and materials

- *2.1. For gel extraction*

- Wizard SV Gel and PCR clean-Up system (Promega, USA).

- *2.2. For Ligation*

- LigaFast™ Rapid DNA Ligation System (Promega, USA).



The LigaFast™ Rapid DNA Ligation System (Promega, USA)
1. pGEM-T vector; 2. 2X rapid ligation buffer; 3. T4 DNA ligase

The Wizard SV Gel and PCR clean-Up system (Promega, USA)
1. Membrane Binding Solution; 2. Membrane Wash Solution; 3. Nuclease-Free Water; 4. Minicolumn; 5. Collection Tube

2. Samples and materials

- *2.3. For transformation*

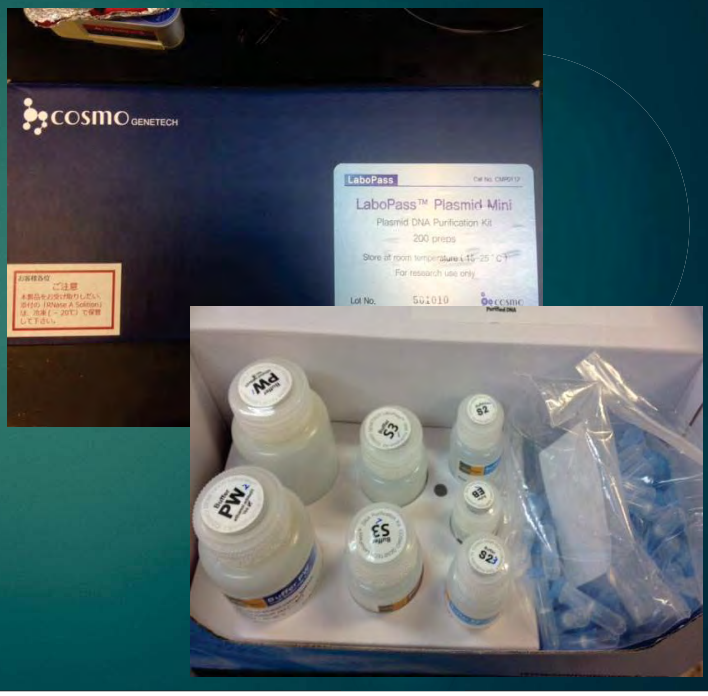
- pGEM-T vector-inserted DNA

- DNA fragments of *Banana Bunchy Top Virus* (BBTV) have been inserted into pGEM-T vector by T4 ligase.

- *E. coli* plasmid

- *2.4. For sequencing*

- LaboPass Plasmid Mini Kit/ Plasmid DNA purification Kit (COSMO GENETECH).



3. Protocols

3.1. Gel extraction

- Step 1: Gel electrophoresis

- Separate the DNA amplified products on a 2% agarose gel (if the size of expected DNA is smaller than 1.5kbp; on the other hand, it is recommended to used 1% agarose gel with 1kbp DNA ladder if the expected DNA size is larger than 1.5 kbp) in 1X TAE buffer and stain in Ethidium bromide solution.

- Load 10 µl 100bp DNA ladder and 12 µl solution (10 µl PCR product + 2 µl blue juice (loading dye)) into each well.

- Run the gel with 100 Voltage for 25-30 minutes.

- Prepare a clean 1.5 ml tube and get its weight.

- View the DNA band under UV light, use the blade to cut the gel and place in the pre-weighted 1.5 ml tube.

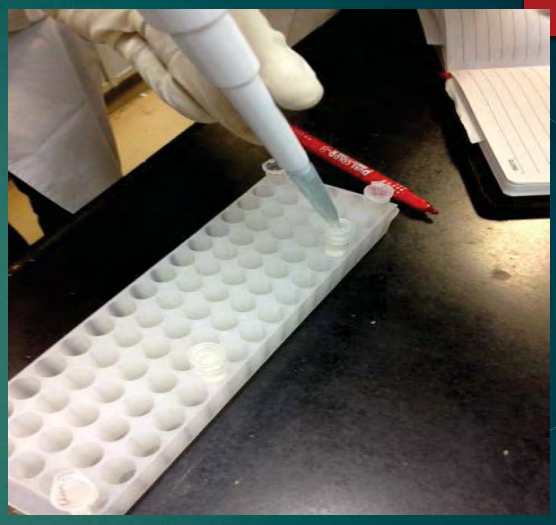
- Weight the tube again. *Weight of the gel = weight of the tube with gel - weight of the gel*

3.1. Gel extraction

- Step 2: Dissolving the gel

- Add 10 µl Membrane Binding Solution per 10 mg of gel slice.

- Vortex and incubate at 55°C for 10-15 minutes until gel slice is completely dissolved.



3.1. Gel extraction

- Step 3: Binding DNA

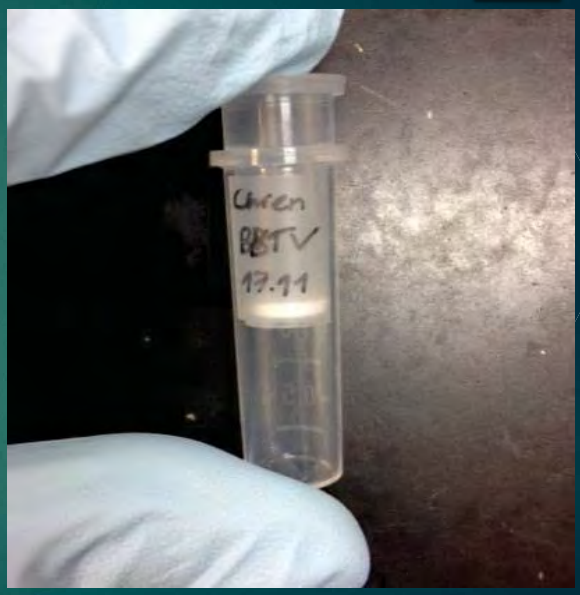
- Add 700 µl Membrane Wash Solution (Ethanol added).

- Transfer all dissolved gel solution into the minicolumn assembly.

- Incubate at room temperature for 1 minute.

- Centrifuge the minicolumn assembly at 16,000 rpm (KUBOTA 3300) for 1 minute.

- Discard the solution and re-insert minicolumn into collection tube.



3.1. Gel extraction

- Step 4: Washing

- Insert Minicolumn into Collection tube.

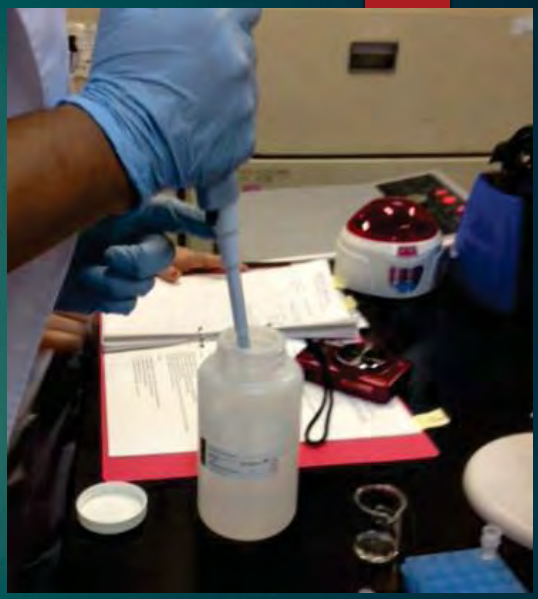
- Centrifuge the minicolumn assembly at 16,000 rpm (KUBOTA 3300) for 1 minute.

- Discard the solution and re-insert minicolumn into collection tube.

- Repeat step 1 with 500 µl Membrane Wash Solution.

- Centrifuge the minicolumn assembly at 16,000 rpm (KUBOTA 3300) for 5 minute.

- Empty the collection tube and re-centrifuge the minicolumn assembly for 1 minute with opening the microcentrifuge lid to alow evaporation of any residual ethanol.



3.1. Gel extraction

- Step 5: Elution

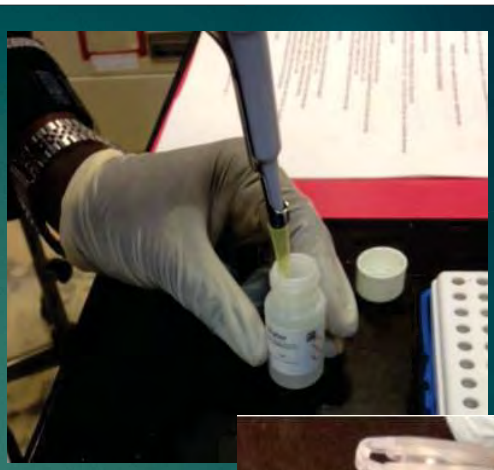
- Carefully transfer minucolumn to new 1.5 ml tube.

- Add 30 µl Nuclease-Free Water to the minicolumn.

- Incubate at room temperature for 1 minute.

- Centrifuge at 16,000 rpm (KUBOTA 3300) for 1 minute.

- Discard the minicolumn and store DNA at 4°C or -20°C.



3.2. Ligation

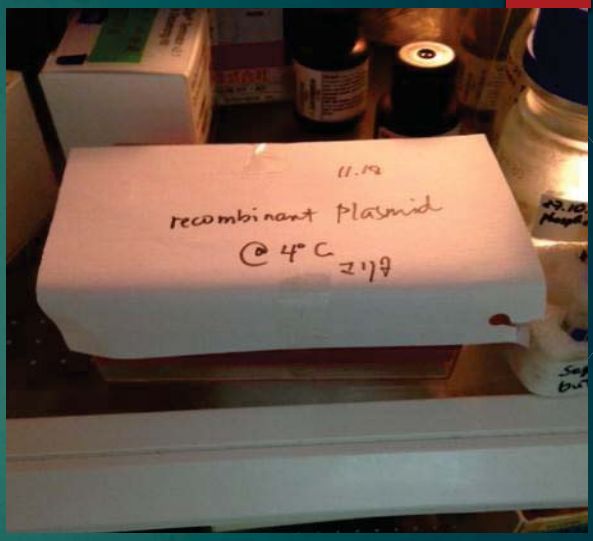
- Step 1: Add the cocktail mixture into 1.5 ml tube following:

2X Rapid ligation buffer	5 µl
pGEM-T vector	1 µl
T4 DNA ligase	1 µl
Purified DNA	3 µl
Total	**10 µl**

3.2. Ligation

- Step 2: Mix well with pipette and incubate over night at 4°C (Sharp SJ56S).

- After ligation, the insert DNA is will be transformed into bacterial cells for propagation for sequencing



3.3. Transformation of inserted DNA into E. coli plasmid

- Step 1.

- Leave the competent *E. coli* (105 µl/ tube) in ice box for 1.0 – 1.5 hours. Using pipette to mix gently the competent *E. coli* to be totally dissolved.

3.3. Transformation of inserted DNA into E. coli plasmid

- Step 2.

- Add 50 µl competent *E. coli* to inserted DNA and incubate in ice box for 30 minutes.

- Put the tube including competent *E. coli* and inserted DNA in heat block at 42°C (Dry Thermo Unit) for 45 seconds and immediately transfer back to ice box for 2 minutes.

- Step 3.

- Under laminar flow (CLEAN BENCH/ Hitachi, Japan), add 1 ml S.O.C medium into the tube and wrap the lid of tube with parafilm.

- Step 4.

- Shake the tube horizontally by shaker (Bio Shaker BR-15LF/ TAITEC) for 45 minutes at 37°C.

- Step 6.

- After shaking, centrifuge with 15,000 rpm (KUBOTA 3300) for 2 minutes to get pellet of *E. coli*.

▶ Step 6.

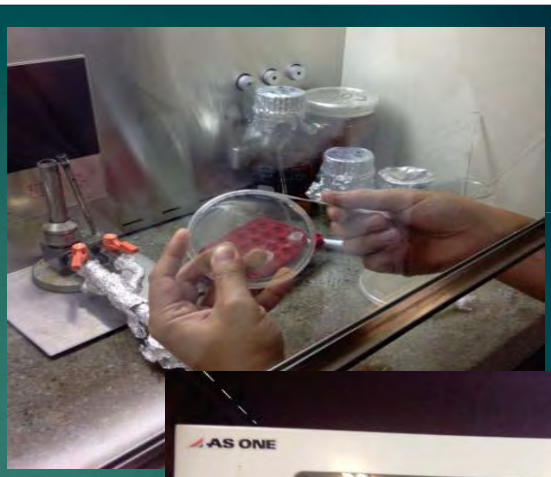
- Use the pipette to remove the S.O.C medium and just remain about 200 µl in tube.

▶ Step 7.

- In the laminar flow, drop the solution in tube onto the petri with LB medium and spread well with sterilized triangle rod until the solution completely dry.

- Step 8.

- Wrap the petri with parafilm and incubate at 37°C overnight (AS ONE) and transfer to 4°C for 1-2 days (Sharp SJ56S).



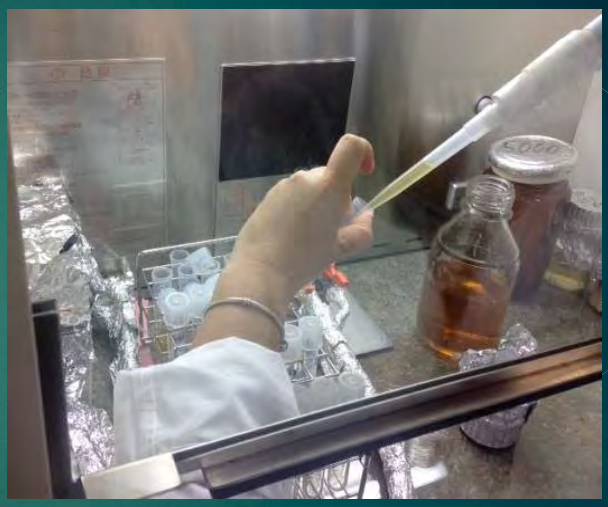
3.4. Cloning recombinant plasmid in TB medium

- Step 1.

- Select 05 white colonies of recombinant plasmid from the petri and 01 blue colony as negative control.

- Step 2.

- Use pipette to transfer 4 ml TB medium into one 15 ml falcon tube.

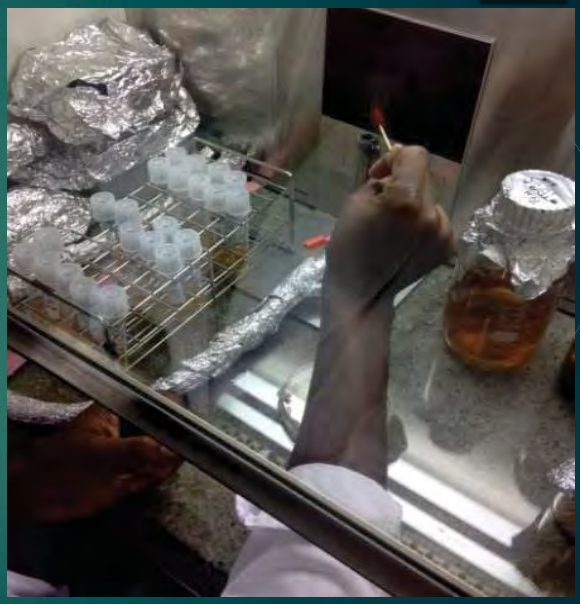


- Step 3.

- Use sterile toothpick to take a single colony and then transfer into falcon tube.

- Step 4.

- Flame the used toothpick afterwards to avoid the contamination.



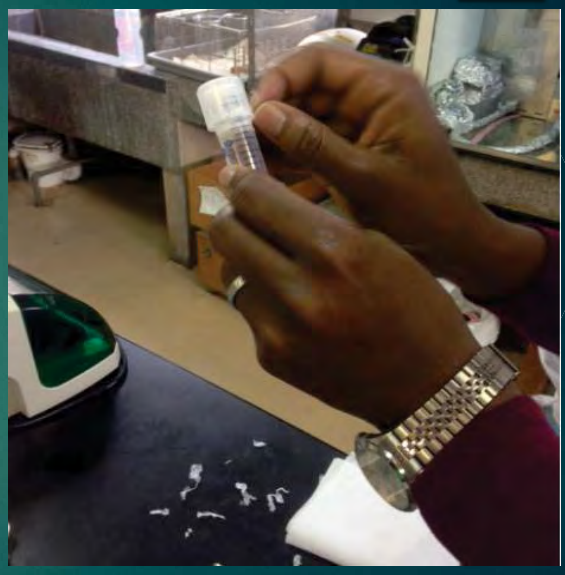
- Step 5.

- Cover the lid of falcon tube with parafilm, then shake (Bio Shaker BR-15LF/ TAITEC) overnight at 37°C.

3.5. Miniprep

- Step 1.

- After shaking the recombinant plasmid overnight, take the falcon tube from shaking machine and remove the parafilm from lid of tube.



- Step 2.

- Centrifuge the falcon tube at 3,500 rpm (TOMY LC-100 Low speed centrifuge) for 7 minutes.

- Step 2.

- Pour off the TB medium and keep the pellet.

- Step 3.

- Add 250 µl S1 buffer with RNAse added before using (do not let the tip touch the tube).

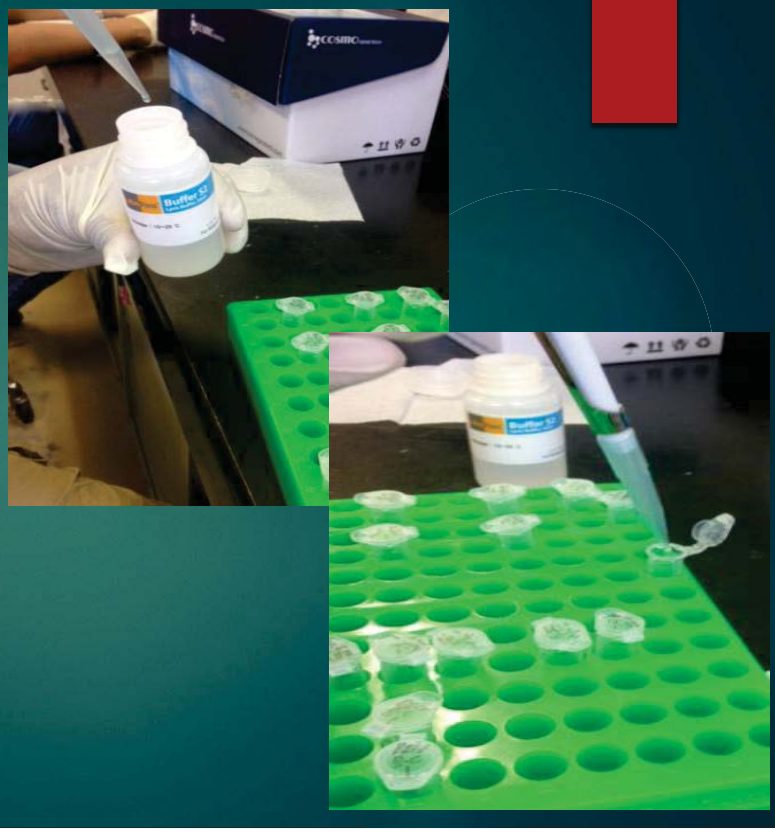
- Re-suspend the pellet and vortex the falcon tube for 30 seconds and transfer all the suspension into new 1.5 ml tube.

.

▶ Step 4.

- Add 250 µl S2 buffer (S2 buffer must be shake before using) and afterwards invert the tubes 3 – 4 times (do not vortex the tubes).

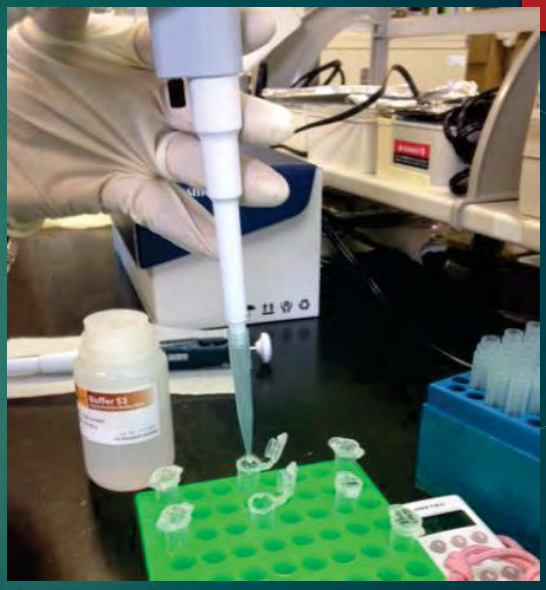
- Incubate the tubes at room temperature for 5 minutes.



▶ Step 5.

- Add 350 µl S3 buffer into the tubes (S3 buffer must be shake before using) and then invert the tube 3 – 4 times.

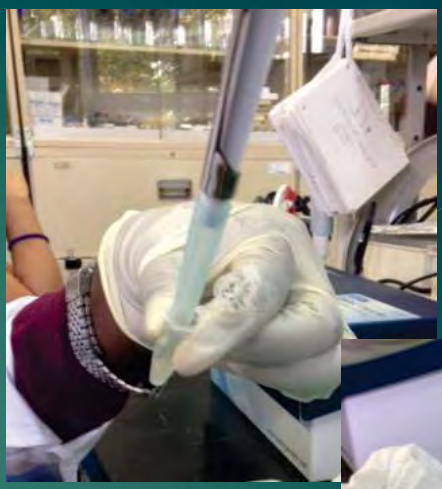
- Centrifuge the tubes for 10 minutes at 14,000 rpm (KUBOTA 3300).

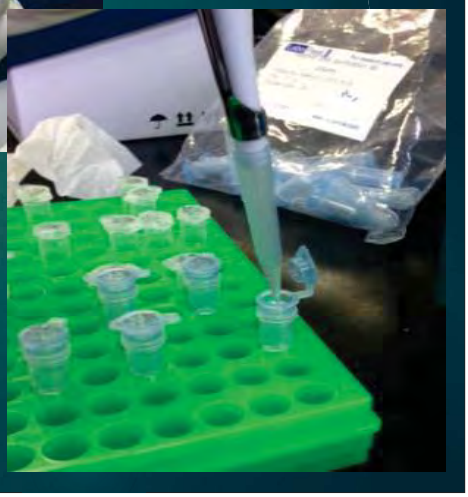


▶ Step 6.

- Use the pipette to transfer the supernatant (clear solution) into the spin column. The plasmid will be trapped in the filter.

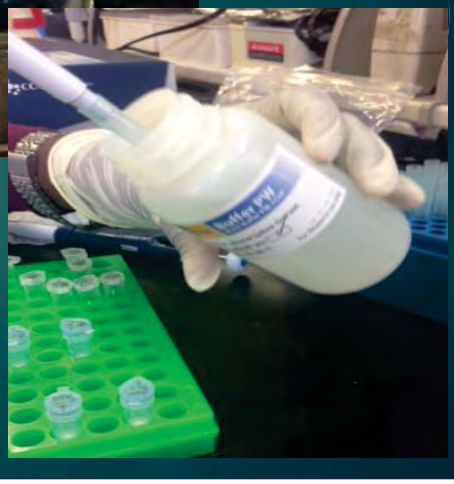
- Centrifuge the tubes for 10 minutes at 14,000 rpm (KUBOTA 3300).





▶ Step 7.

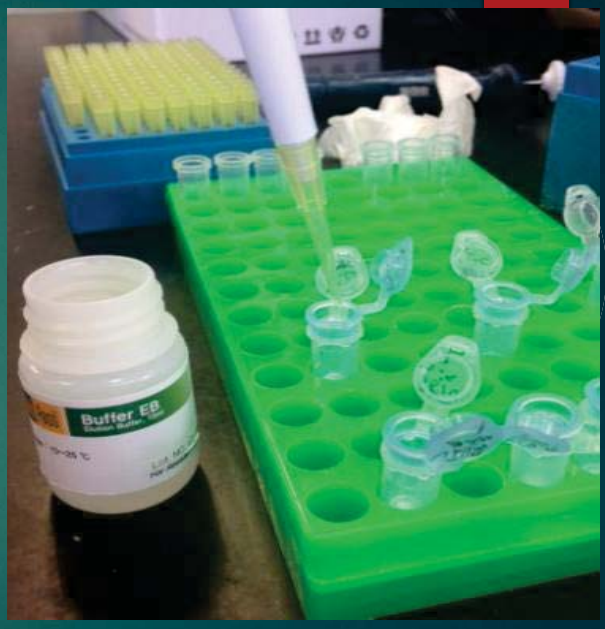
- Pour off the liquid, afterwards adding 750 µl PW buffer and centrifuge the tubes for 1 minutes at 14,000 rpm (KUBOTA 3300) (PW buffer will further wash the filter to purify the plasmid).



▶ Step 8.

- Pour off the liquid again and centrifuge the tubes for 1 minutes at 14,000 rpm (KUBOTA 3300). Afterward, transfer the spin column into new 1.5 ml tube.

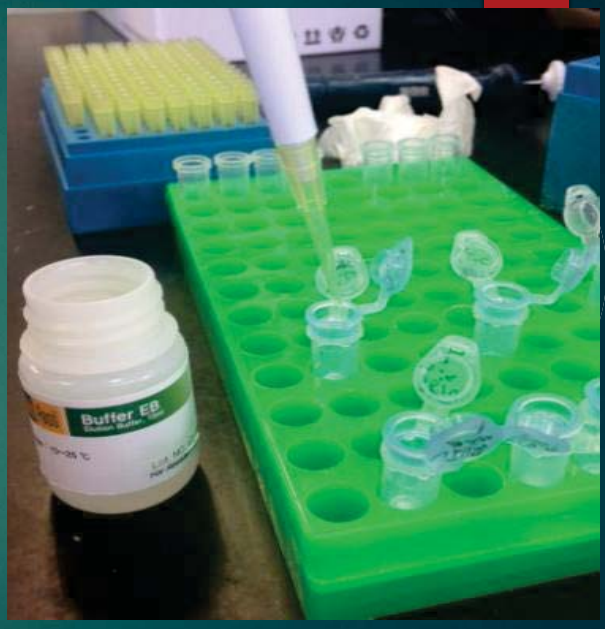
- Add 50 µl EB buffer into the center of the filter and keep at room temperature for 1 minute (EB buffer will dissolve the plasmid from the filter).



▶ Step 9.

- Centrifuge the tubes for 1 minutes at 14,000 rpm (KUBOTA 3300) and keep the plasmid.

- The collected plasmid is used in the next steps for the sequencing or kept at 4°C.



3.6. Insert check

- Prepare 1.5% agarose gel.
- Load 4µl 1.5 kb DNA ladder (Promega, USA) as a marker onto the first well of 1.5% prepared agarose gel.
- Mix 2µl loading buffer (6X load dye) and 2µl DNA plasmid of blue colony and load onto the agarose gel as a negative control.
- Mix 2µl loading buffer and 2µl DNA plasmid of each samples and load onto agarose gel.
- Run samples for 25 – 30 minutes using electrophoresis machine (Mupid-2plus/ Advance).
- After running, stain the gel by submerging it into Ethidium bromide solution for 3 minutes.
- De-stain the gel in distilled water for 1 minute.
- View DNA plasmid band under UV illumination and take photo using EDAS 290 (Kodak, Japan).

3.7. Cycle Sequence

- Step 1:

- Prepare the cocktail mixture; calculate the required amount as follow:

q.s. (double distilled water)	2.0 µl
Sequence buffer (Big Dye Terminator 5X sequencing buffer)	1.0 µl
*Primer (sp6/ T7)	0.5 µl
Premix	2.0 µl
Inserted plasmid DNA (PCR product)	4.5 µl
Total	**10 µl**

***note:** 1 sample of successfully inserted DNA will be conducted cycle sequence with 2 reactions separately (one with sp6 primer and one with T7 primer).

- Step 2:

- Conduct Cycle sequence by PCR machine (DNA Engine/ BioRAD) with the following PCR conditions:

Temperature	Time	Cycles
96°C	10 seconds	24
50°C	5 seconds	
60°C	4 mintes	
4°C	endless	

3.8. Precipitation

- Step 1:

- After thermal cycle, prepare the cocktail mixture in 1.5 ml tube as below:

Cycle sequencing product	10.0 µl
3M Acetic acid	1.0 µl
99.5% Ethanol	30.0 µl
Total	41 µl

3.8. Precipitation

- Mix and centrifuge the cocktail mixture briefly (KUBOTA 3300).
- Put the 1.5 ml tube in ice box for 10 minutes.
- Centrifuge at 20°C for 20 minutes at 14.000 rpm (HITACHI, Japan).
- Discard the supernatant and keep the pellet.
- Add 100 µl 99.5% ethanol (not cold) into the tube, afterwards centrifuging for 5 minutes at 14.000 rpm (KUBOTA 3300).
- Discard the supernatant and keep the pellet.
- Add 100 µl 99.5% ethanol (kept in -30°C) into the tube, then roll (horizontal) the tube for 20 – 30 seconds.
- Centrifuge the tube for 5 minutes at 14.000 rpm (KUBOTA 3300).
- Discard the supernatant and dry pellet for 2 minutes at room temperature with lip of tube is opened.
- Put the tube in heat block (Dry Thermo Unit) at 95°C for 2 minutes with lip of tube is opened.
- Take the tube from heat block and put it again in ice box for 5 minutes with lip of tube is closed.
- Keep the pellet at 4°C (Sharp SJ56S).

3.9. DNA analysis using Automate Sequencer

- Step 1.

- Dissolve the pellet with 20 µl Hi-Di formamide.

- Transfer the solution into the 96-Well Reaction Plate carefully.

- Step 2.

- Check all wells to ensure that there are no bubbles inside the wells.

- Cover the plate by Plate Septa 96-Well.

- Keep the plate 4°C (Sharp SJ56S) for sequencing using sequencer machine.

Report 16. Method for extraction of dsRNA from double-stranded RNA plant viruses

1. Lecturer

Ph.D. **Tomohide Natsuaki**

Vice-President for International Exchange

Vice-Dean for Research & Professor

Utsunomiya University

2. Place and time

- Place: Laboratory of Plant Pathology, Faculty of Agriculture, Utsunomiya University
- Time: Dec. 3rd – 4th, 2015

3. Samples and dsRNA target

- **3.1. Sample:**

  Japanese snake gourd (*Trichosanthes pilosa*) infected by *Cucumber mosaic virus* (CMV) is stored at - 80°C.

- **3.2. dsRNA target**

  dsRNA from *Cucumber mosaic virus* (CMV)

4. Protocols

- Step 1.

- Weigh 05 grams of sample and grind well using cold mortal & pestle

4. Protocols

- Step 2.

- Add 10 ml 2X STE buffer and 50 µl 2-mercaptoethanol into mortal.

- Mix with sample, then transfer the plant sap into the homogenizer tube.

4. Protocols

- Step 3.

- Keep the mortal with remain of plant sap, add 2.5 ml phenol; 2.5 ml chloroform and 0.1 ml iso amyl alcohol into the mortal, then mix well.

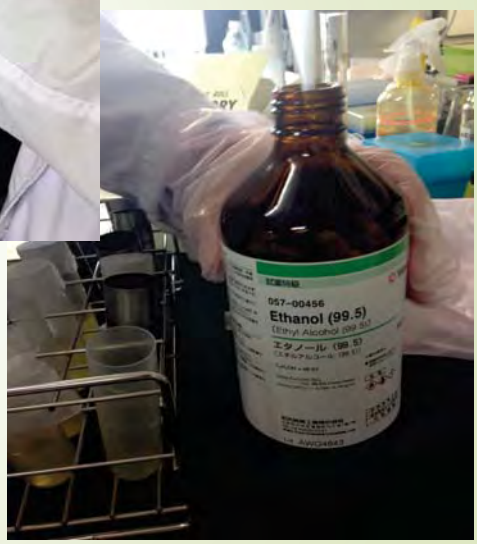
- Transfer the solution into the same homogenizer tube (step 2) and homogenize at 5000 rpm for 5 minutes (Ace Homogenizer, Nihonseiki Kaisha ltd.).

4. Protocols

- Step 4.

- After finishing the homogenizing step, centrifuge the solution at 10,000 rpm for 10 minutes.

- Use pipette to transfer 10 ml supernatant into new falcon tube. Then add amount of 99.5% ethanol following the specific formula:



$$\left[\frac{\text{Volume of supernatant}}{0.85}\right] - \text{Vol. of supernatant} = \text{Vol. of added 99.5\% ethanol}$$

4. Protocols

- Step 5.

- Incubate the solution at 4°C for 60 minutes, then centrifuge at 10,000 rpm for 10 minutes.

- Use pipette to transfer all the supernatant into new glass tube. Afterwards, add 1 g fibrous cellulose powder (Whatman) into glass tube.

- Vortex the mixture for 15 minutes with 30 seconds interval.

4. Protocols

- Step 6.

- Cut the tissue paper to make a filter and push it inside the chromatography tube.

- Hang up and transfer the mixture into the chromatography tube.

- Place the glass under chromatography tube.

4. Protocols

- Step 7.

- Transfer 100 ml 1X STE buffer and 15% ethanol into conical flask.

- Hang it up over the chromatography tube. Use the specific pipe to connect conical flask and chromatography tube.

- Let the sample precipitate totally, put again the filtered solution into the chromatography tube. The total DNA will be kept in cellulose powder.

4. Protocols

- Step 8.

- Weigh 0.8 g fibrous cellulose powder (Whatman) and put it into new glass tube. Afterwards, place the glass tube under the chromatography tube.

- Add 20 ml 1X STE buffer into the chromatography tube and let the solution precipitate totally.

4. Protocols

- Step 9.

- Add 3.6 ml 99.5% ethanol into the new cellulose solution and Vortex for 15 minutes with 30 seconds interval.

- Put the mixture into the chromatography tube and let the solution precipitate totally.

- Add 8 ml 1X STE buffer into the chromatography tube and let it filter down into a new falcon tube. Repeat the same process for another falcon tube. (Total volume of filtered solution is 16 ml (2 falcon tube)).

4. Protocols

- Step 10.

- Add 2µl DNase and ½ of small spoon of $MgCl_2$ $.6H_2O$ into each falcon tube.

- Cover the tube by parafilm and incubate in warm water (30°C) for 30 minutes to eliminate DNA.

4. Protocols

- Step 11.

- Add 8 ml 99.5% ethanol into each falcon tube with DNA eliminated solution.

- Store at -20°C overnight or -80°C for 1 hour.

4. Protocols

- Step 12.

- Centrifuge the sample in cold temperature (0°C) at 10,000 rpm for 15 minutes.

- Discard supernatant and keep the pellet.

- Prepare the RNA-dissolving solution by mixing 100 µl 10X loading dye and 500 µl RNase free water

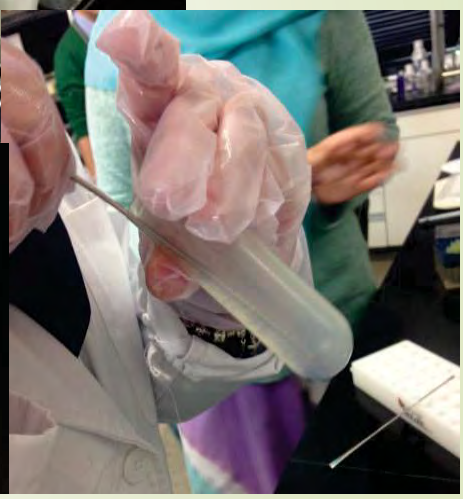
- Put the falcon tube upside down inside the dryer for 5 minutes to remove ethanol totally.

4. Protocols

- Step 13.

- Use pipette to drop 50 µl RNA-dissolving solution into each tube and mix well by laboratory spatula to dissolve RNA.

- Spin the tube for few seconds and transfer all the sample into 1 PCR tube.



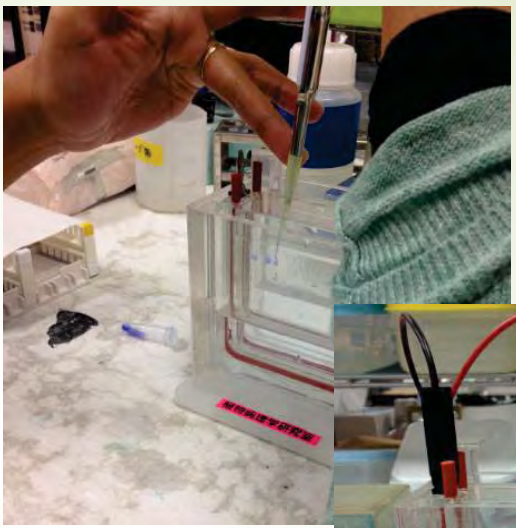
4. Protocols

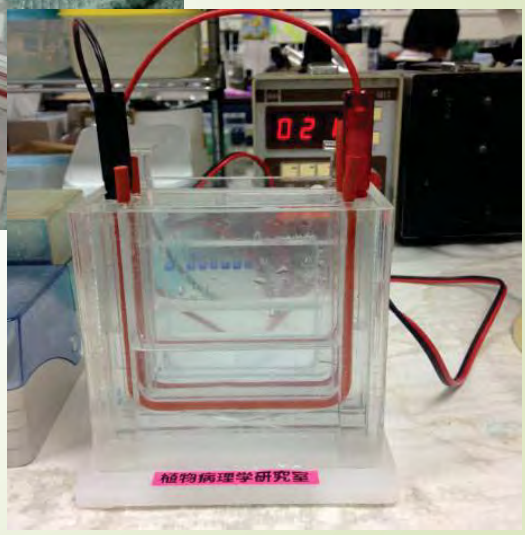
- Step 14.

- Pour 5% polyacrymide gel into the gel case and place under the light to fasten the solidification of gel.

- Put 1X TBE buffer inside gel case. Make sure that middle part of gel case is filled up by 1X TBE buffer.

- Load 10 µl 1kb DNA ladder as a marker and 6 µl sample into the gel. Conduct gel electrophoresis at 20 mA for 50 minutes.



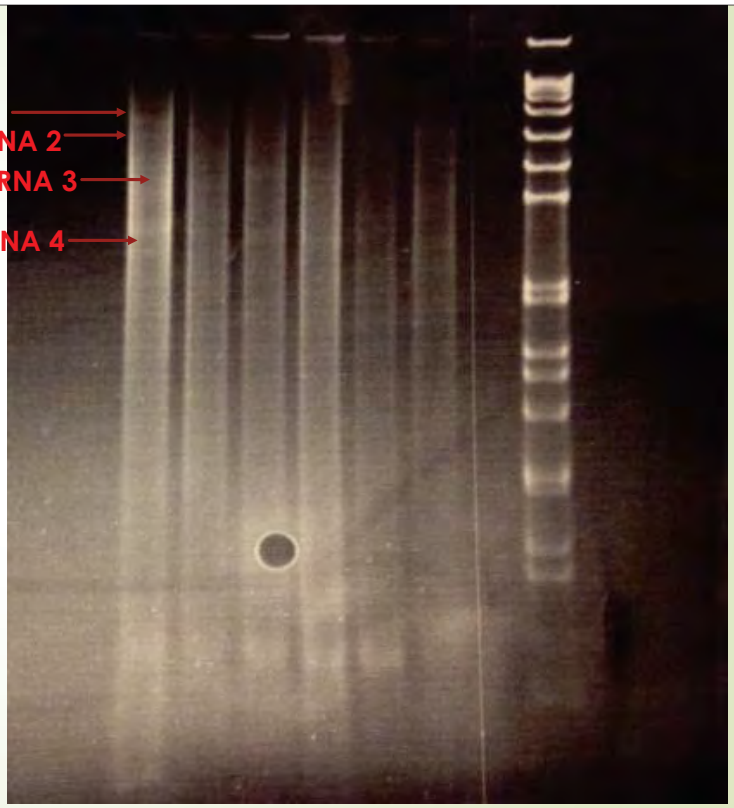


4. Protocols

- Step 15.

- After finishing gel electrophoresis, stain gel with Ethidium bromide for 1 minute and wash with dH_2O for 30 seconds.

- View the result by UV transilluminator.

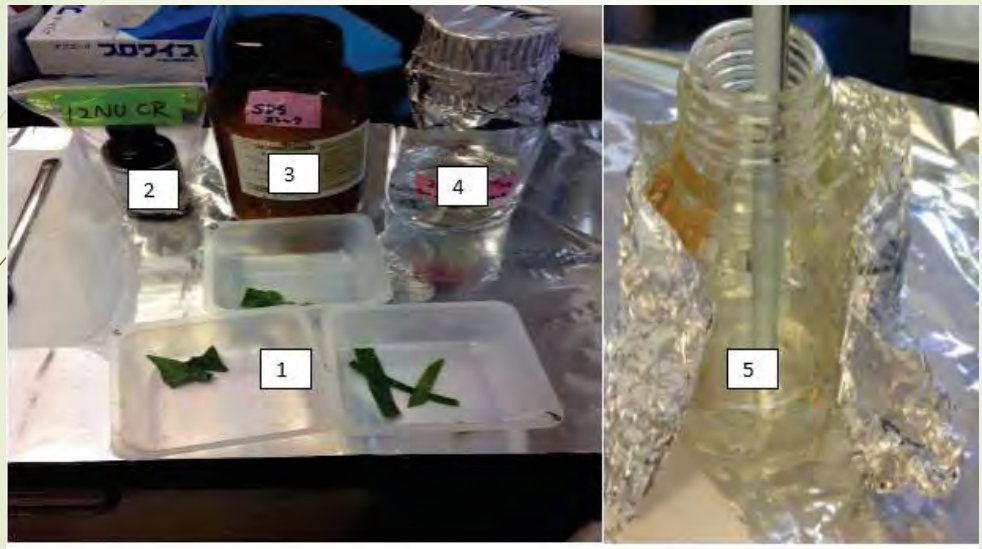


Report 17. Method for extraction of RNA of plant viruses using phenol-chloroform solution

1. Place and time

- Place: Laboratory of Plant Pathology, Faculty of Agriculture, Utsunomiya University
- Time: Dec. 7th, 2015

2. Samples and materials



1. Samples (potyvirus-inoculated passionfruit plant); 2. 2-mercaptoethanol; 3. SDS; 4. 2X STE buffer; 5. Phenol-Chloroform solution

3. Protocols

- Step 1.

- Make the extraction buffer:

2X STE buffer ..... 1 ml

SDS.....0.01 g

2-mercaptoethanol.....10µl

Mix well by vortex.
This volume is used for extraction of 1 sample.

3. Protocols

- Step 2.

- Weigh 0.1g sample and put it into the cold mortar and pestle.

- Add liquid nitrogen into mortar and grind well.

3. Protocols

- Step 3.

- Add 800-1000 µl extraction buffer into mortar and re-grind well.

3. Protocols

- Step 4.

- Transfer all the plant sap into 1.5 ml microcentrifuge tube and centrifuge in cold condition (4°C) at 15,000 rpm for 5 minutes.

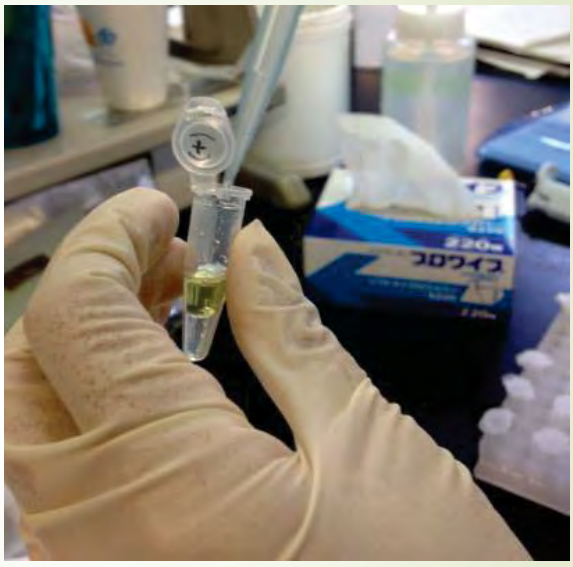
- After centrifuging, transfer 600 µl of supernatant into new 1.5 ml microcentrifuge tube.

3. Protocols

- Step 5.

- Add Phenol-Chloroform solution with equal volume of supernatant (600 µl).

- Vortex for few seconds and centrifuge in cold condition (4°C) at 15,000 rpm for 5 minutes.



3. Protocols

- Step 6.

- Repeat step 5 with 500 µl of supernatant and Phenol-Chloroform instead.

- Take 400 µl of supernatant and transfer it into new 1.5 ml microcentrifuge tube.

3. Protocols

- Step 7.

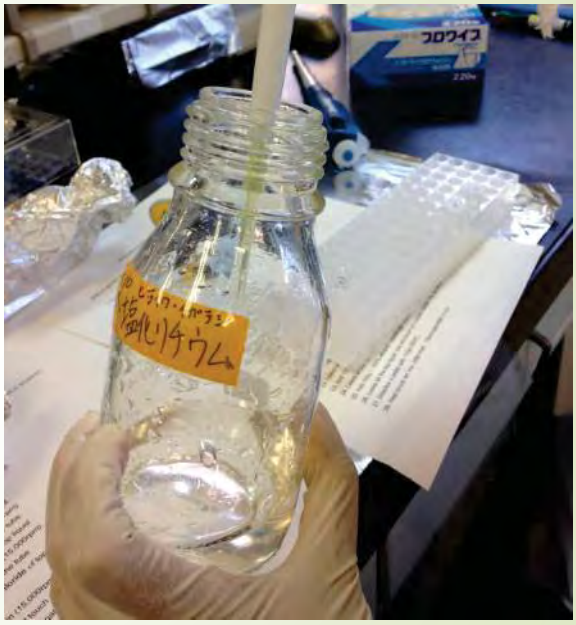
- Add 400 µl of Chloroform and centrifuge in cold condition (4°C) at 15,000 rpm for 5 minutes.

3. Protocols

- Step 8.

- Repeat step 7 with the volume of supernatant and chloroform added are 300 µl.

- Transfer 150 µl of supernatant into new 1.5 ml microcentrifuge tube and add 50 µl 8M lithium chloride (LiCl) (with 1:3 ratio (v/v)), vortex for few seconds and incubate in ice box for 1 hour.



3. Protocols

- Step 9.

- Centrifuge in cold condition (4°C) at 15,000 rpm for 20 minutes.
- Discard the supernatant and add 150 µl 70% ethanol.

3. Protocols

- Step 10.

- Centrifuge in cold condition (4°C) at 15,000 rpm for 5 minutes.

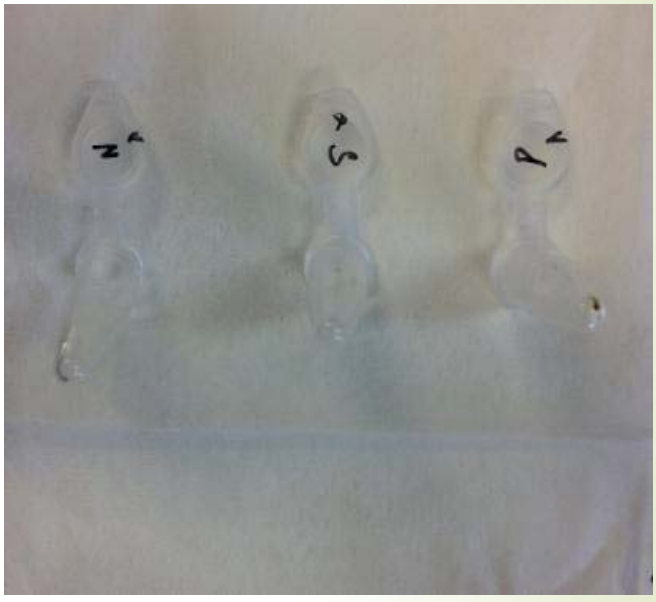
- Repeat step 14 and 15 with using 100% ethanol instead of 70% ethanol.

3. Protocols

- Step 11.

- Discard the supernatant and dry the pellet at room temperature for 10 minutes.

- Dissolve the pellet with 110 µl DEPC water.



4. Protocols

- Step 12.

- Put the tube with RNA in heat block at 65°C (Dry Thermo Unit) for 10 minutes, afterwards transfer to ice box for 10 minutes.

- Use the RNA for cDNA synthesis and PCR reaction, unless store RNA at -80°C.

Report 18. Method for purification of dsRNA of plant viruses from RNA-dissolving solution

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Dec. 14th, 2015

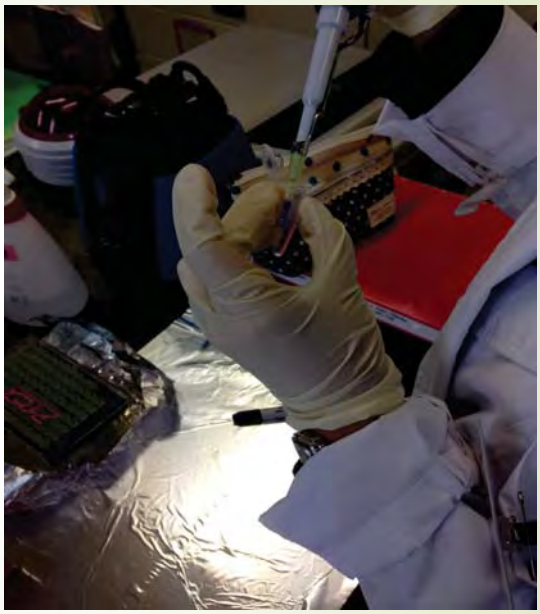
2. Samples and materials

- The dsRNA of *Cucumber mosaic virus* (CMV) was dissolved in RNA-dissolving solution (loading dye and RNase free water).

3. Protocols

- Step 1.

- Transfer all the dsRNA solution into new 1.5 ml tube.



3. Protocols

- Step 2.

- Add 200 µl 1X TE buffer and 100 µl cold chloroform in 1.5 ml tube.

3. Protocols

- Step 3.

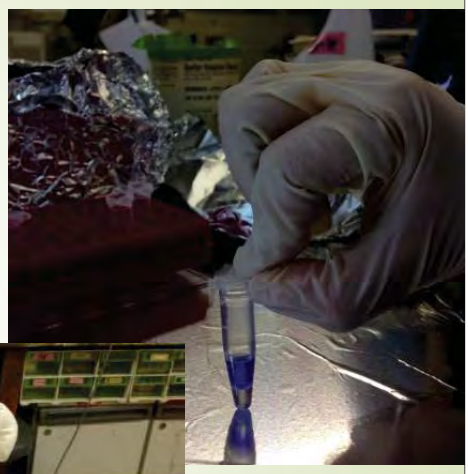
- Vortex the tube for few seconds and centrifuge at 15,000 rpm for 5 minutes (CF15RN, Hitachi, Japan).

3. Protocols

- Step 4.

- Transfer 200 µl of supernatant to new 1.5 ml tube.

- Add cold 99.5% ethanol with twice amount of supernatant.



3. Protocols

- Step 5.

- Vortex for 1 minute and incubate at -80°C for 1 hour.

3. Protocols

- Step 6.

- Discard the supernatant using pipette and centrifuge at 15,000 rpm for 5 minutes.

- Discard the remaining supernatant and dissolve the dsRNA pellet in 20 µl 1X TE buffer.

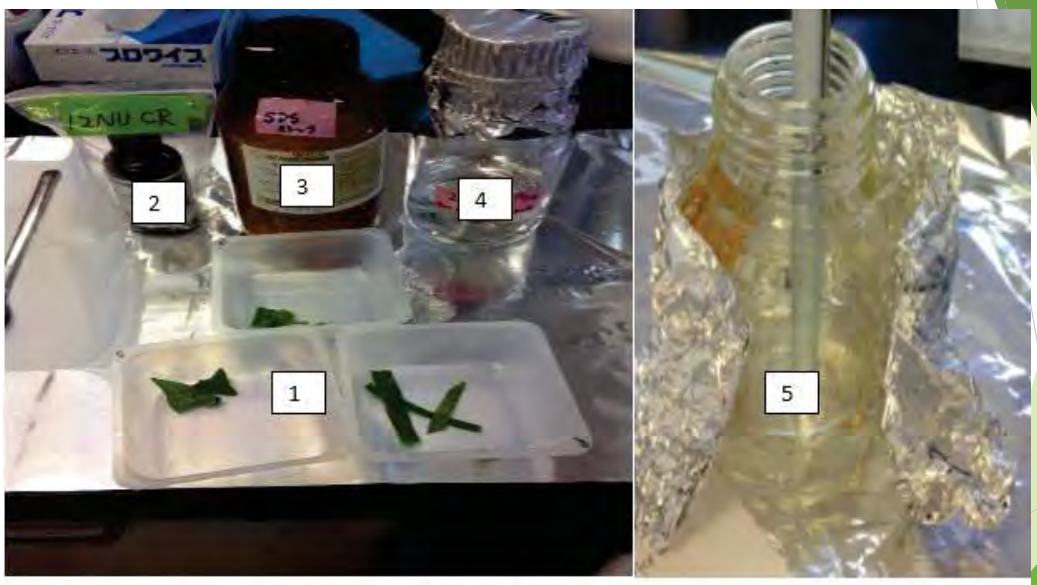
- Keep the dsRNA at -80°C for the next using.

Report 19. Detection of unknown-*potyvirus* on passionfruit plant

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Dec. 7th-18th,2015

2. Materials and samples



1. Samples (potyvirus-inoculated passionfruit plant); 2. 2-mercaptoethanol; 3. SDS; 4. 2X STE buffer; 5. Phenol-Chloroform solution

3. Procedure

- RNA extraction
- cDNA synthesis, PCR assay and gel electrophoresis
- Gel extraction
- Ligation, transformation and cloning
- Miniprep, cycle sequence and DNA analysis

3.1. RNA extraction

- Conduct RNA extraction using phenol-chloroform method.
- The protocol of RNA extraction was described in previous report.

3.2. cDNA synthesis, PCR assay and gel electrophoresis

3.2.1. cDNA synthesis

- Prepare the cocktail mixture with required amount as follow:

5X RT buffer	4.0 µl
dNTP mixture (10 mM)	2.0 µl
Reverse primer of potyvirus universal primers	1.0 µl
RNAse inhibitor (10U/ µl)	1.0 µl
ReverTra Ace™	1.0 µl
Total RNA	11 µl
Total	20 µl

3.2. cDNA synthesis, PCR assay and gel electrophoresis (continued)

3.2.1. cDNA synthesis

- Carry out the cDNA synthesis with PCR conditions below:

Temperature (˚C)	Time (min.)
42	20
99	5
4	endless

3.2. cDNA synthesis, PCR assay and gel electrophoresis (continued)

3.2.2. PCR assay

- PCR assay was performed using *potyvirus* universal primer pairs. The expected band size will be 1.7 kbp.
- PCR conditions for *potyvirus* have been described as follow:

Temperature (˚C)	Time (min.)	Cycles
94	5	
94	0.5	34
47	1	
72	2	
72	10	

3.2. cDNA synthesis, PCR assay and gel electrophoresis (continued)

3.2.3. Gel electrophoresis

- Gel electrophoresis was conducted using 1.5% agarose gel.
- The protocol of gel electrophoresis performance was presented in previous report (see report 7)

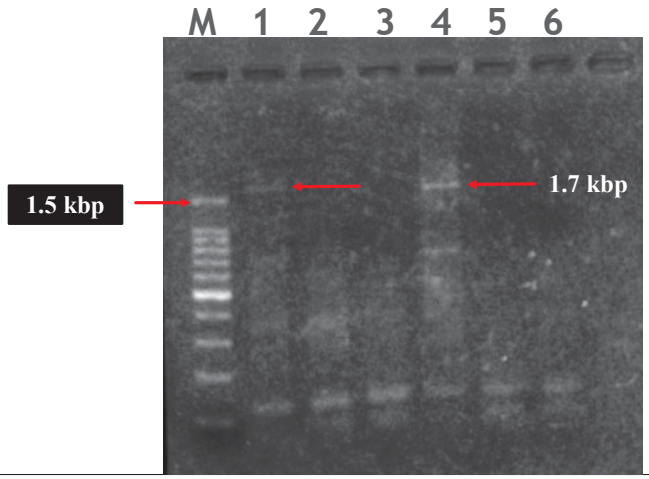


Fig 1. PCR assay of unknown *Potyvirus* inoculated passionfruit plant collected from HOGOKEN Lab. with *potyvirus* universal primers. The PCR band with the size of ~1.7 kbp (red arrows) was amplified from positive control (lane 1 & 4). No band was amplified from *Potyvirus* inoculated plant (lanes 2 & 5) and negative control (lanes 3 & 3). The 100bp DNA ladder (Promega, USA) was included as marker.

3.3. Gel extraction

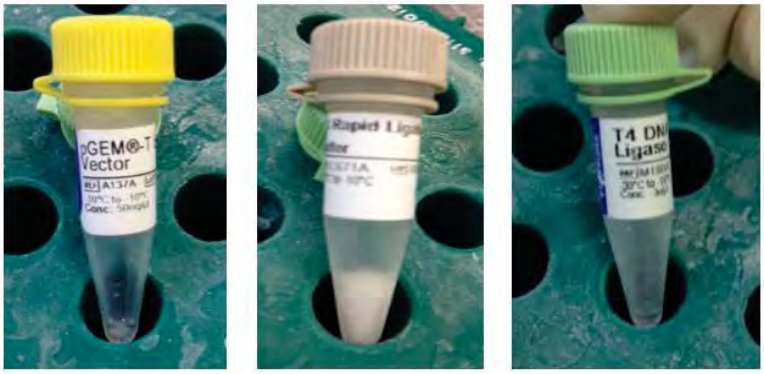
- Gel extraction was performed using Wizard SV Gel and PCR clean-Up system (Promega, USA).
- Procedure of gel extraction was presented in previous report (see report 13)

The Wizard SV Gel and PCR clean-Up system (Promega, USA)
1. Membrane Binding Solution; 2. Membrane Wash Solution; 3. Nuclease-Free Water; 4. Minicolumn; 5. Collection Tube

3.4. Ligation, transformation and cloning

3.4.1. Ligation

- Ligation was carried out using LigaFast™ Rapid DNA Ligation System (Promega, USA).
- Procedure of ligation was presented in previous report (see report 13)



The LigaFast™ Rapid DNA Ligation System (Promega, USA)
1. pGEM-T vector; 2. 2X rapid ligation buffer; 3. T4 DNA ligase

3.4. Ligation, transformation and cloning (continued)

3.4.2. Transformation

- The purpose of this step is to transfer the unknown *potyvirus* inserted pGEM vector into *E. coli* plasmid.
- The procedure of transformation was described in previous report (see report 14).

3.4. Ligation, transformation and cloning (continued)

3.4.3. Cloning of recombinant plasmid

- Cloning of recombinant plasmid was conducted on TB medium
- The procedure of cloning was described in previous report (see report 14).

Annex 20

Name: **Tran Van Chien**

Country: **Vietnam**

REPORT ON VISIT STUDY TO YOKOHAMA PLANT PROTECTION STATION

1. Place and time

Place: Yokohama plant protection station.

Time: Dec. 11th, 2015

2. Activities in Yokohama plant protection station

2.1. Having a presentation on Plant Quarantine System in Japan

Japan's Plant Quarantine is an organization under the Ministry of Agricultural, Forestry and Fisheries (MAFF). In line with global market, the varieties and quantities of crops to be imported to Japan will increase and causing greater risk of plant pests and diseases introduction. The objective of quarantine is to prevent the introduction and spread of pests in all areas of Japan.

Fig. 1: Presentation on Plant Quarantine System in Japan

Plant protection have function to protect Japan's agriculture resources. Japan plant quarantine stations implement quarantine procedures that target both domestic and overseas products. Such quarantine procedure include import quarantine to prevent the introduction of overseas plant pests, export quarantine in response request from other countries and domestic quarantine to control pests in Japan.

Japan's plant quarantine system consist of:

1. International Plant Quarantine
    a. Import Quarantine (Import plant inspection, post entry quarantine, and pre-shipment quarantine)
    b. Export Quarantine (export plant inspection and field inspection of export plant)
2. Domestic Plant Quarantine
    a. Quarantine of Domestic Seed and Seedlings
    b. Eradication/control Program for Designated Pests
    c. Monitoring Survey for Newly Invasive Pests
    d. Emergency Action

The major plant pests and diseases requiring precaution for Japan are Mediterranean Fruitfly (*Ceratitis capitata*), Fire Blight disease (*Erwinia amylovora*), Coddling Moth (*Cydia pomonella*), Tobacco Blue Mold (*Peronospora tabacina*), and others.

2.2. Visit the Yokohama plant protection station's exhibition

After the presentation, we had an opportunity to see the Yokohama plant quarantine station's exhibition to more understand about the foundation, history and development of Plant Quarantine System in Japan as well as Yokohama plant protection station.

Fig. 2: Visiting the Yokohama plant protection station's exhibition

2.3. Visiting the plant quarantine facilities of Research Center, Yokohama plant protection station.

Beside Plant Quarantine Station, we also visited Center Research where support the system of plant quarantine in technical aspect. Center Research have some Section such as:

1. Disinfestation Technology section to develop new technology about quarantine treatment
2. Entomology and Nematology Section: a laboratory that had work on developing new method about insect and nematode identification
3. Plant Pathology Section: a laboratory that had work on developing new method about identification of plant pathogen
4. Pest Risk Assessment Section: Work on assessment of quarantine pest
   Pest risk analysis (PRA) evaluates scientific evidence to determine whether an organism is a pest. If so, the analysis evaluates the probability of introduction and spread of the pest and the magnitude of potential economic consequences in a defined area, using biological or other scientific and economic evidence.
5. Pest Risk Management Section: Work on risk management of quarantine pest including recommendation of quarantine treatment based on result of pest risk assessment. If the risk is deemed unacceptable, the analysis may continue by suggesting management options that can reduce the risk to an acceptable level. Subsequently, pest risk management options may be used to establish phytosanitary regulations.
6. Living Modified Organism Section: Work on living modified organism research based on Cartagena Protocol and Biosafety to the Convention on Biological Diversity. This section seeks to protect biological diversity from

the potential risks posed by genetically modified organisms resulting from modern biotechnology

7. Pest Identification Section: work on identify quarantine pest including plant pest and pathogen

Fig. 3: Visiting the plant quarantine facilities of Research Center, Yokohama plant protection station

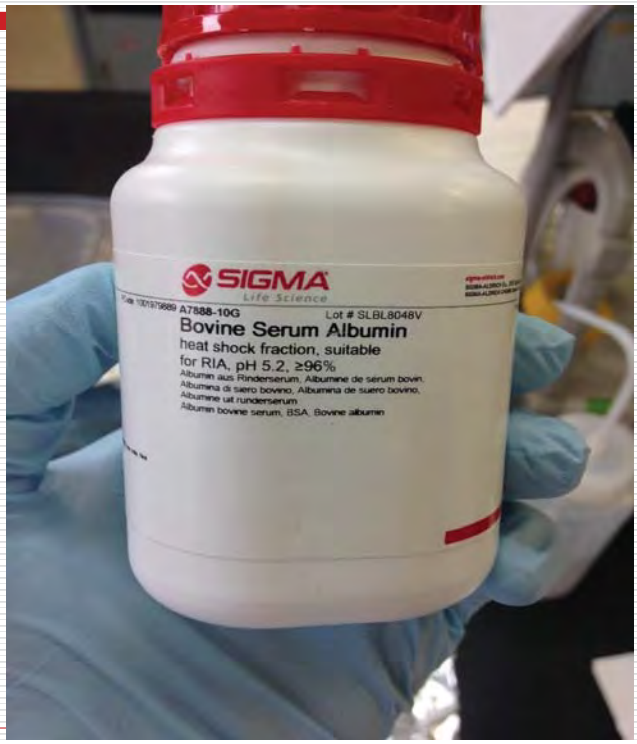
Report 21. Method for SDS PAGE (Sodium Dodecyl Sulfate PolyAcrylamide) electrophoresis

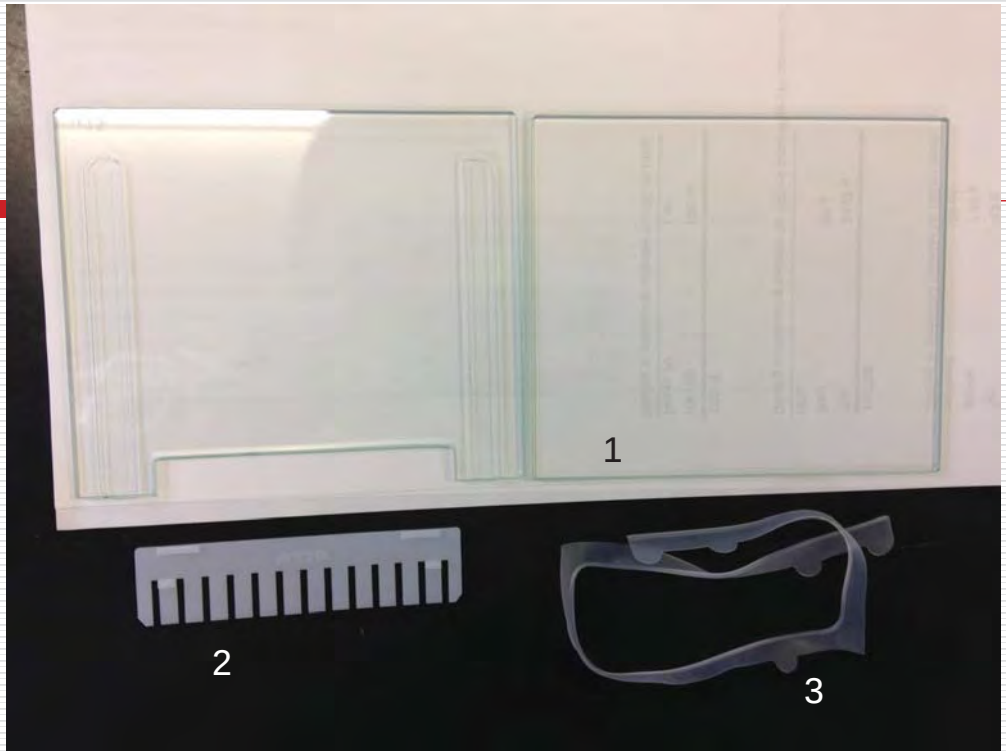
1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Dec. 15th, 2015

2. Samples and materials

- The protein used to conduct the experiment was Bovine Serum Albumin (BSA) (Sigma, USA).

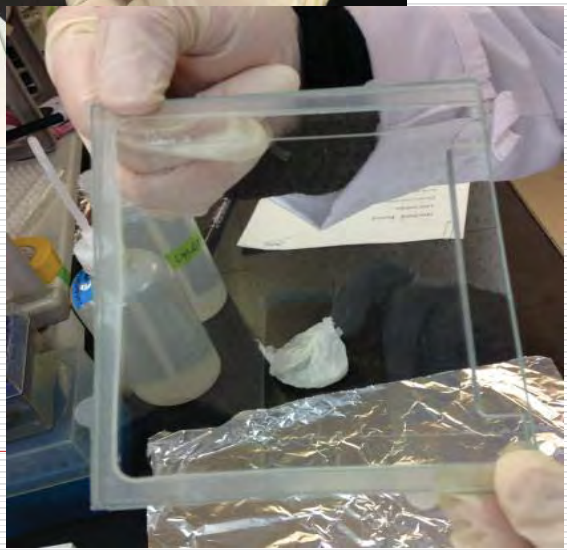




1: Glass plates; 2: Comb; 3: Spacer

3. Protocols

3.1. Preparation of SDS PAGE gel



- Step 1.

- Wipe glass plates, spacer and comb by 70% ethanol.

- Assemble the glass plates with spacers.

3.1. Preparation of SDS PAGE gel

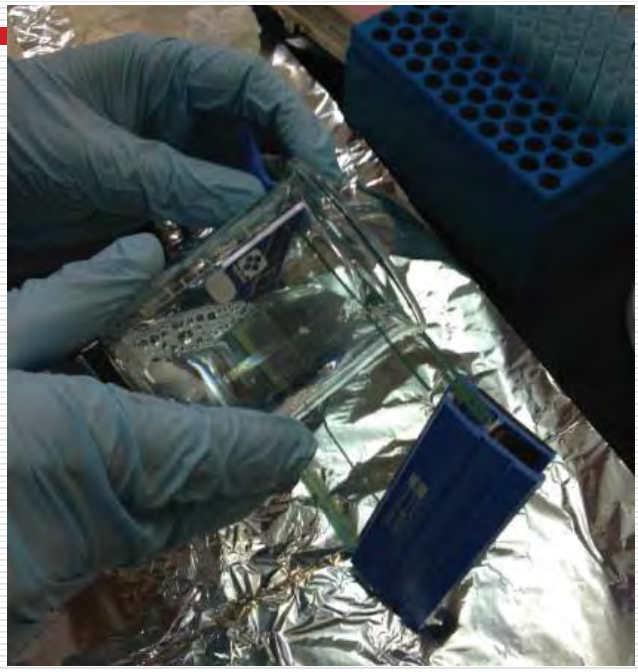
- Step 2.

- Secure gel glass assembly with bull clips on each side.

3.1. Preparation of SDS PAGE gel

- Step 3.

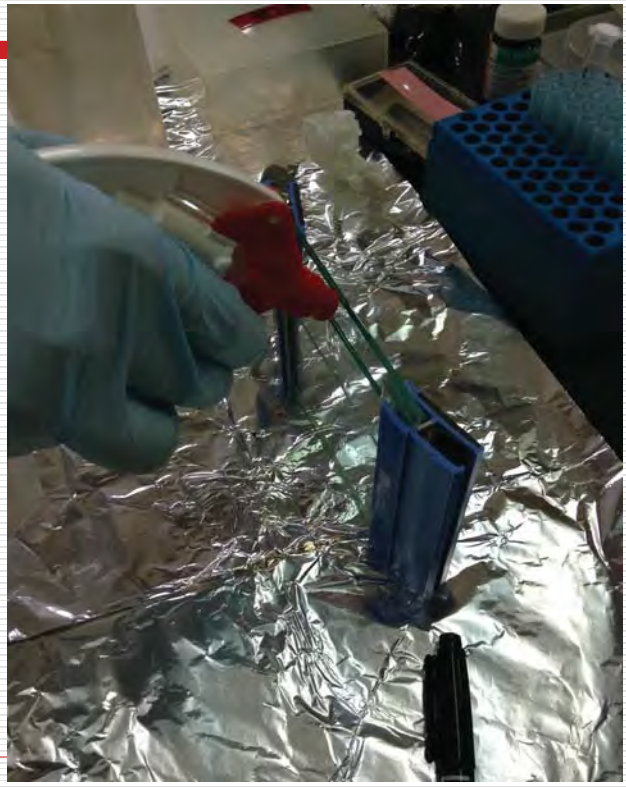
- Pour the separating gel into space of gel plate assembly up to 2/3 of height of gel plate assembly.



3.1. Preparation of SDS PAGE gel

- Step 4.

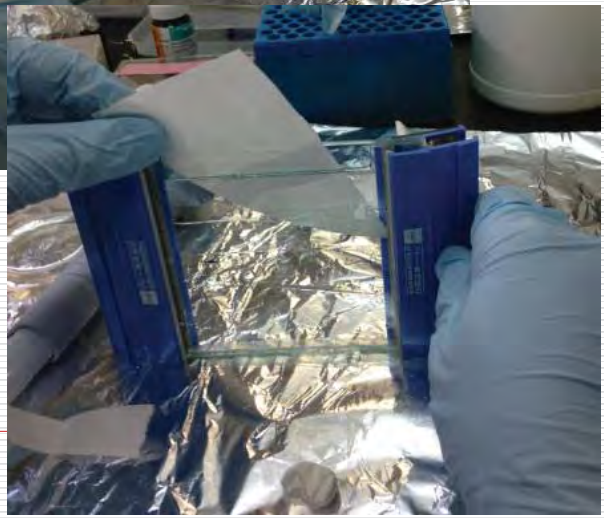
- Pour little amount of 70% ethanol into the space between two glasses to eliminate totally bubbles .



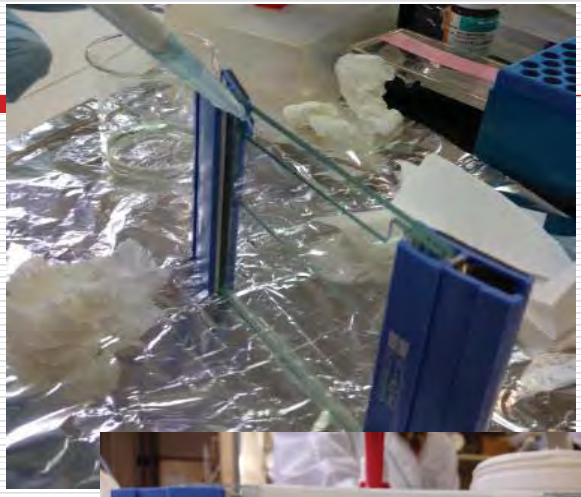
3.1. Preparation of SDS PAGE gel

- Step 5.

- After the gel get solidification, remove the ethanol and wash the surface of gel by water and filter paper. Do not scratch gel's surface.



3.1. Preparation of SDS PAGE gel

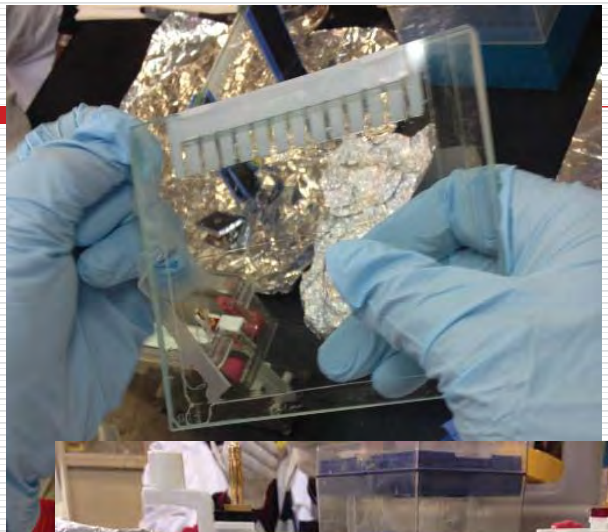


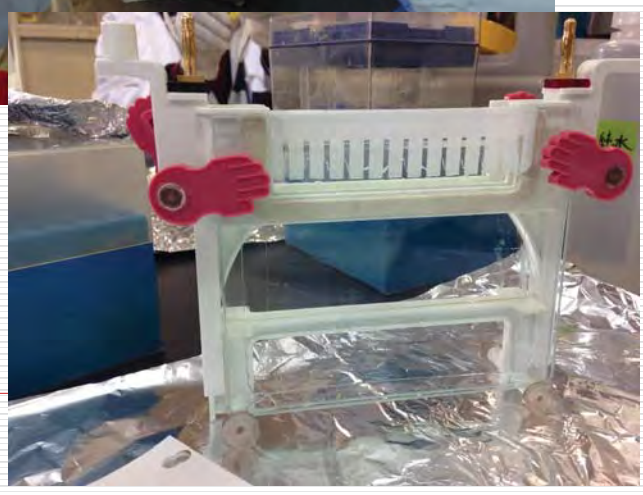
- Step 6.

- After removing the ethanol, use pipette to pour the staking gel onto the separating gel. Do not make bubbles.

- Finally, insert the comb into the space.

3.2. SDS PAGE gel electrophoresis





- Step 1.

- Remove the spacer from gel plate assembly.

- Put gel plate assembly into electrophoresis tank.

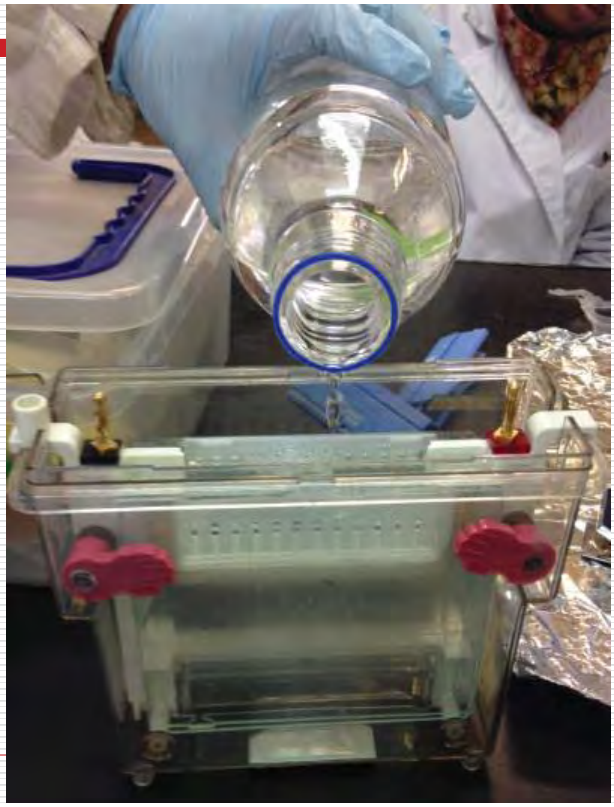
3.2. SDS PAGE gel electrophoresis

- Step 2.

- Pour 1X electrophoresis buffer into electrophoresis tank. The top of the gel is completely submerged in the buffer.

- For two side spaces of gel plates, pour the buffer up to ½ of the height of gel plates.

- Remove the bubbles inside the tank using syringe.



3.2. SDS PAGE gel electrophoresis

- Step 3.

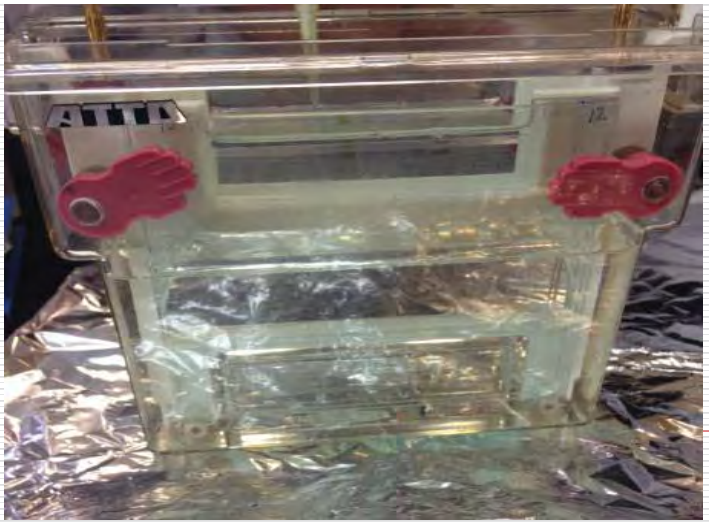
- Carefully remove the comb from the gel plates. Adjust the shape of wells using syringe.

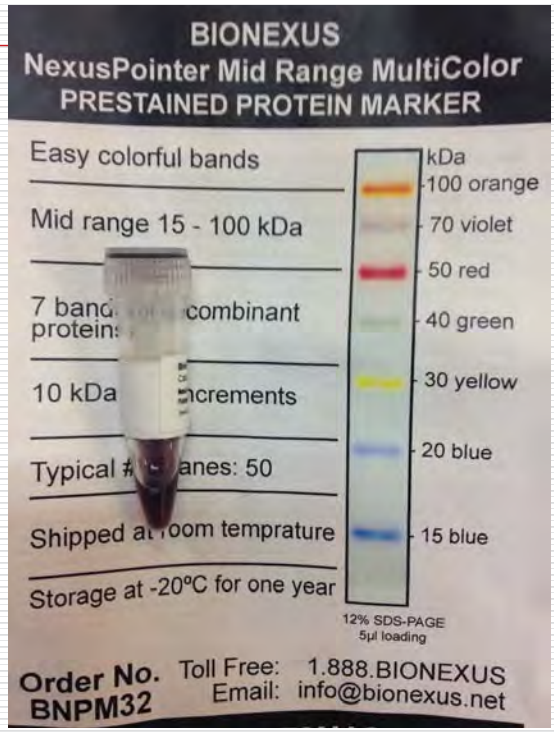
- Mix the same volume of the sample and sample buffer. Afterward, heat the mixture at 100°C for 5 minutes.

3.2. SDS PAGE gel electrophoresis

- Step 4.

- Load the marker (Bionexus, USA) and heated samples into the wells.



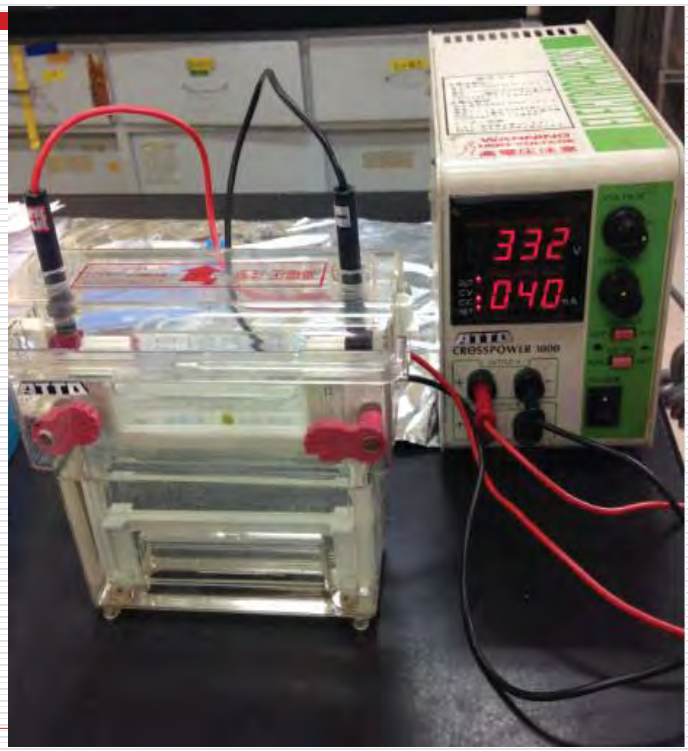


3.2. SDS PAGE gel electrophoresis

- Step 5.

- Run the electrophoresis initially at (20 x number of gel plate) mA. After the protein bands pass through the stacking gel, increase the electricity to (40 x number of gel plate) mA.

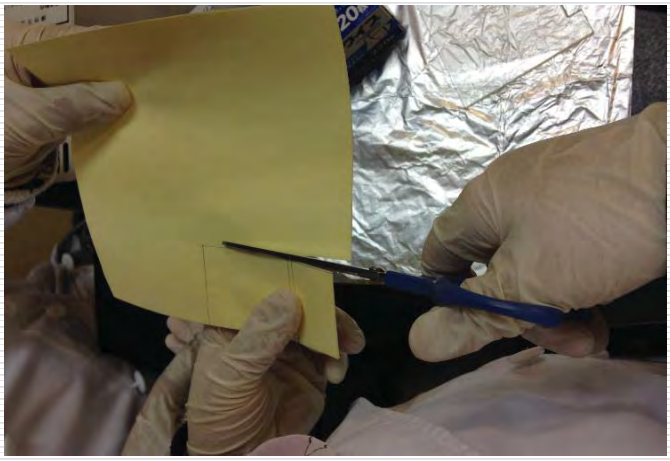
- The electrophoresis will be finished when dye front is near the bottom of the gel (above 5 mm).



3.3. Protein transfer

- Step 1.

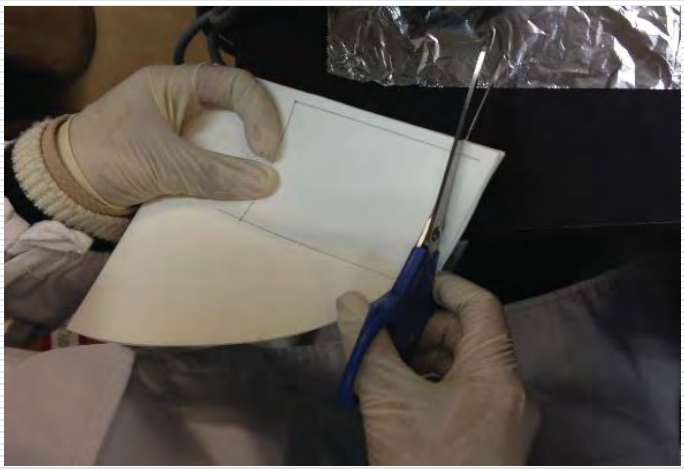
- Cut the Nitrocellulose Membranes (BIO-RAD, Germany) to fit with the size of gel.



3.3. Protein transfer

- Step 2.

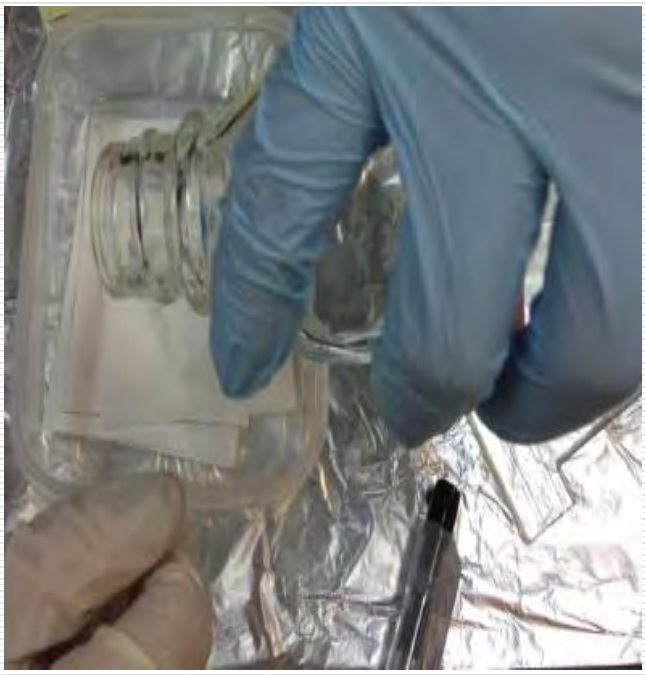
- Prepare the filter papers having the length and width are 1 cm larger than membrane.



3.3. Protein transfer

- Step 3.

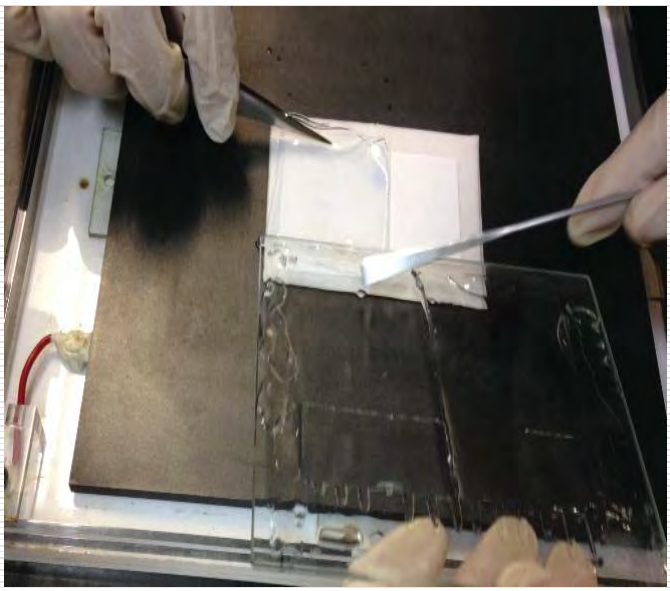
- Make the filter paper wet by soaking in transfer buffer.



3.3. Protein transfer

- Step 4.

- Put the membrane, gel and filter papers into the transfer device in order: three filter papers at the bottom, gel, membrane and three filter papers at the top.

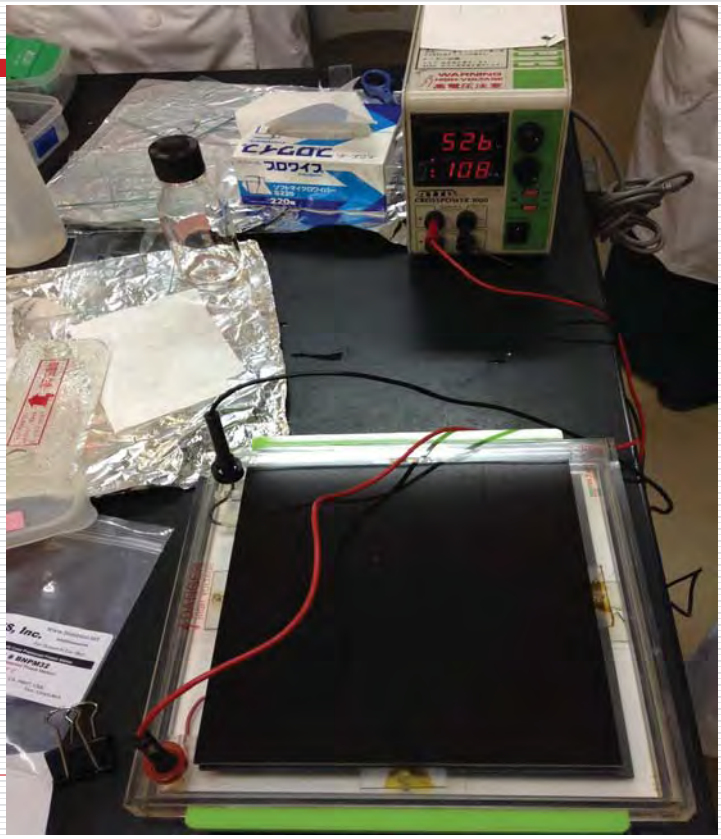


3.3. Protein transfer

- Step 5.

- Conduct the transferring process by using the electricity at 40V, 20W, (2 x membrane area) mA for 1 hour.

- For example: if the area of membrane is 20 cm^2, the mA of electricity will be set at (2 x 20) = 40 mA.



3.4. Detection of protein on membrane

- Step 1.

- After 1 hour, soak membrane in staining solution for protein gel (Instant Blue, Expedeon, UK) for 2 minutes.

3.4. Detection of protein on membrane

- Step 2.

- Shake the membrane with staining solution for 5 minutes using shaker.

3.4. Detection of protein on membrane

- Step 3.

- Pour off the staining solution.

- Submerge membrane in distilled water added a small amount of 70% ethanol.

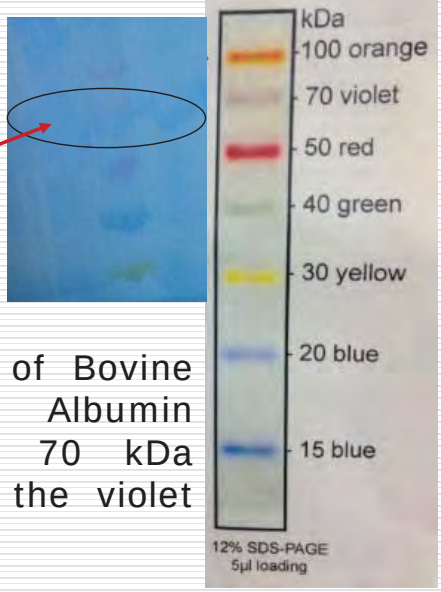
- Shake for 3 minutes using shaker.

3.4. Detection of protein on membrane

- Step 4.

- Pour off solution.

- Observe the protein band on the membrane by naked eyes.



The size of Bovine Serum Albumin (BSA) is 70 kDa revealing the violet color

THANK YOU FOR YOUR ATTENTION