Report of Students Exchange Program

at Research Institute for Microbial Disease (RIMD), Osaka University

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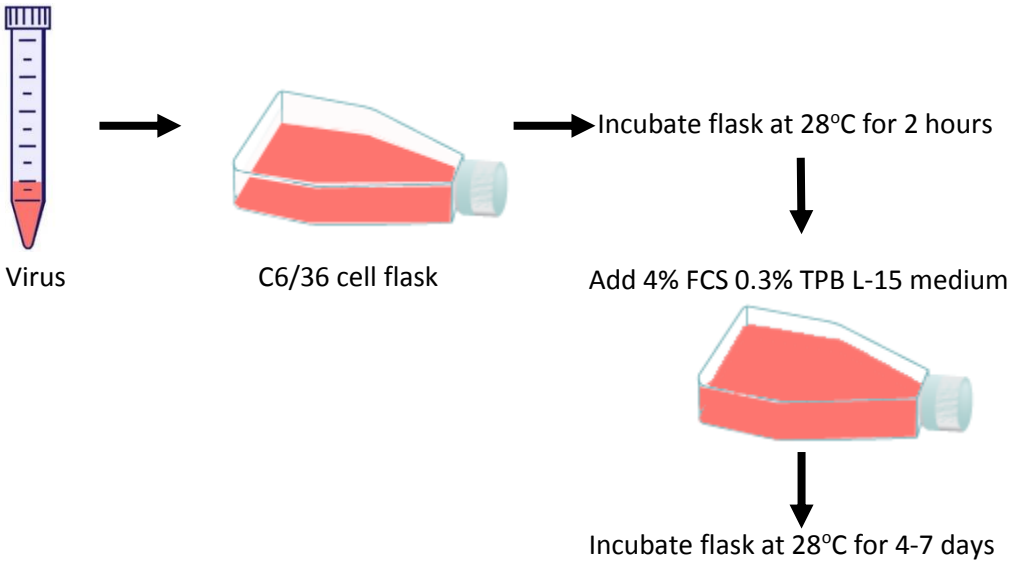
I am Khwanchit Boonha, third year student in master course at Faculty of Tropical Medicine, Mahidol University, Thailand. I am very glad to have a very good opportunity to participate in student exchange program. This is good chance for me to learn many techniques in Microbiology filed that include cell culture, virus propagation, virus neutralization assay, antibody-dependent enhancement (ADE) assay and real time PCR for dengue virus research. This all knowledge I am very thankful to SA Associate Professor Masahiro Sasaki for teaching all technique and practice.

Laboratory practice:

1. **Dengue virus propagation**

My work is propagate a dengue virus type 2 stain in C6/36 cell and will use this propagated virus in future work. Briefly, C6/36 cells were growth in T75 cm^2 flask with 10% Fetal Calf Serum (FCS) 0.3% Tryptose Phophate Broth (TPB) Leibovitz's L-15 Medium and after cell confluent (monolayer), virus was prepared with FCS (-) L-15 medium (serum free) and added to C6/36 cell surface. After 2 hours incubation, Virus flask was added 4% FCS 0.3% TPB L-15 medium and incubated at 28°C for 4-7 days (until the day when virus kill around 30-34% of cell). Virus was collected and kept at -80°C.

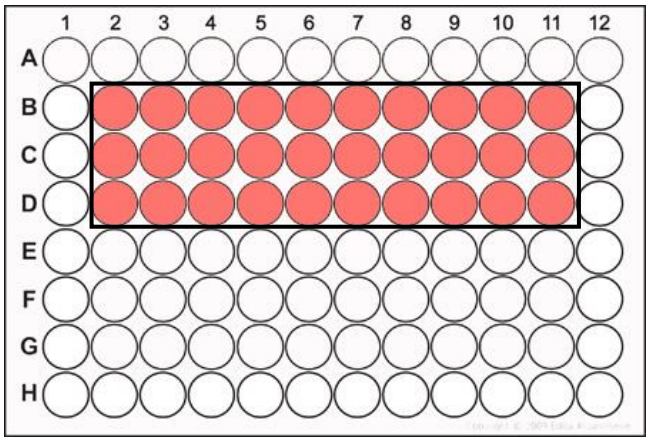


2. Virus titration

Briefly step for demonstrate a titer of propagated virus follow; make a 10-fold serial dilution of virus using FCS (-) Minimum Essential Medium Eagle (MEM). After that, a diluted virus was added to confluent vero cells plate for triplicate per dilution and incubated at 37^{o}C for 1 hour. After incubation, all well was overlaid with 100 µl 1% CMC 3% FCS MEM medium (2% final FCS concentration) and incubated the virus plate at 37^{o}C with 5% CO_2 for 48 hours. After that, all well was washed with PBS for 5 times and added 4% formaldehyde-PBS in each well for 15 minutes and follow with 0.1% Triton-X in PBS and incubate for exactly 5 minutes. Then, plate was washed again and added 1st antibody; dengue virus antibody and incubate at 37^{o}C for 1 hour. After washing 3 times, each well was added 2nd antibody; Alexa Flour® 488 goat anti-human IgG (H+L) and incubate for 1 hour at 37^{o}C. After that, each well was wash 3 times with PBS, counted colony number and measure a virus titer.

3. Virus Neutralization

Virus titration and neutralization test are my main work. Briefly of neutralization assay, first, Prepare 2-fold serial dilution of antibody in PBS (sterile) start at 25 µg/µl and keep on ice or 4^{o}C until use and then prepare 100 FFU/25 µl virus dilution using FCS (-) MEM. After that, add virus solution in each antibody dilution and incubate for 1 hour at 37^{o}C with 5% CO_2. After incubation, wash confluent vero cells plate with PBS for once and then add virus and antibody solution in each well for triplicate (middle area, figure below).



Next, incubate plate at 37^{o}C with 5% CO_2 for 1 hour and then overlay the well with 1% CMC with 3% FCS MEM (final concentration at 2% FCS). Incubate plate for 48 hours at 37^{o}C with 5% CO_2. After that, wash with PBS for 5 times and follow with add 50 µl of 4% formaldehyde-PBS in each well for 15 minute. Aspirate formaldehyde solution and add 0.1% Triton-X in PBS for 5 minutes. Then, wash each well with PBS, 3 times and add 50 µl of 1st antibody; anti-dengue, incubate at 37^{o}C for 1 hour. After wash with PBS for 3 times, add 50

µl of 2nd antibody; Alexa Flour® 488 goat anti-human IgG (H+L) and incubate for 1 hour at 37°C. Wash with PBS 3 times again and Count the colony number.

4. Antibody-dependent enhancement (ADE) assay

Start with 10-fold dilution of antibody in PBS stat at 100ng/µl and prepare virus (MOI 0.5) in FCS (-) RPMI. Then, add virus to diluted antibody tube and incubate at 37°C for 30 minutes. After incubation, prepare 12 x 10^5 cells/ml of K562 cells and add cells in each tube. Incubate tube again for 1 hour at 37°C and then add 2% FCS RPMI in 48-well before add antibody, virus and cells solution to each well. Then, incubate plate for 3 days at 37°C with 5% CO_2. Three days later, pipet all cells and solution in each well to 1.5 ml tube for RNA extraction using Trizol. After collection of cells and supernatant, spin down and discard the supernatant. Tapping cells and add 300 µl of Trizol, vortex and incubate at room temperature for 5 minutes. Next, Add 200 µl Chloroform, vortex and incubate at room temperature for 2-3 minutes. Centrifuge the solution tube at 12,000g for 20 minutes, 4°C. Slowly shift only clear solution to new 1.5 ml tube and avoid to pipet a red solution of trizol. After that, add 200 µl of isopropanol and mix by slowly inverting and incubate at room temperature for 10 minutes. Centrifuge again the solution tube at 12,000g for 20 minutes, 4°C. And then discard supernatant. Follow with add 500 µl 75% Ethanol for 5 minutes. Next, Centrifuge at 7,500g for 10 minutes, observe a pellet and discard supernatant. Air dries of pellet until white to clear jelly and then elute RNA with 20 µl Rnase free water.

5. Hybridoma Preparation for mouse MAb

1. Isolation splenocyte

Briefly step, Collect a spleen from mice and place on 10cm^2 dish with DMEM medium and then, isolate splenocyte from spleen by make a hole and press. Filtration the splenocyte and wash with FCS (-) DMEM medium and centrifuge 1500g for 5 minutes, twice.

2. PAI cells

Collect all PAI cell to 50 ml tube and centrifuge 1000 rpm for 5 minutes, remove all supernatant and add 10 ml fo FCS (-) DMEM to wash the cells and then centrifuge 1000g for 5 minutes (twice). Finally, count PAI cells to prepare 6 x 10^6 PAI cells (for 1 spleen).

3. Fusion

Add PAI cells to the splenocyte tube and mix. Then, Centrifuge cells solution tube at 1400 rpm for 5 minutes. After that, aspirate all supernatant and tap the cells. Add PEG 1500 slowly with stirring and rolling the cells tube for 1 minute. Continue stir for 1 minute and add 10 ml of FCS (-) DMEM slowly with stirring and rolling the cells tube for 2 minutes. Next, add 10 ml of 15% FCS DMEM and then centrifuge at 800g for 5 minutes. Remove solution and add 10 ml 15% FCS DMEM and centrifuge again. Then, aspirate all supernatant and add 10 ml HAT medium, gently mix. Add 5ml fusion solution to 45 ml HAT medium and add 200 µl of solution per well in 96-well plate. Incubate plate at 37°C with 5% CO_2 and change medium every 3 days until cloning

There are my work that practiced in Japan including cell culture of vero cell, THP-1, C6/36 and K562 cells and making a stock of all material. And I got some experiment to perform on virus neutralization test by demonstrate a different effect between aspirate and non-aspirate step before overlay with CMC medium. And the result is not much different between this two.

For my future, I can use the knowledge that I learn in this project for my work and may teach and help someone else in the same and develop more technique skill.

Finally, I would like to deeply thank for this program for accept me and give me a great opportunity. And thank you all member in RIMD laboratory especially, Professor Kazuyoshi Ikuta and SA Associate Professor Masahiro Sasaki to teach me many knowledge and give me a good experience and I will apply this knowledge for my work in future.