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APPENDIXES

APPENDIX A: Media Preparation

Bacterial Media

1. LB medium (Luria-Bertani medium)

Per liter:

bacto-tryptone	10 gm
bacto-yeast extract	5 gm
sodium chloride	5 gm

Adjust pH to 7.0. Sterilize by autoclaving at 121 °C for 15 min.

2. SOB medium

Per liter:

bacto-tryptone	20 gm
bacto-yeast extract	5 gm
sodium chloride	0.5 gm

Add 10 ml of 250mM potassium chloride and adjust pH to 7.0. For plating, 20 gm agar is added. Sterilize by autoclaving per usuasl protocol. Just before use, add 5 ml of sterile solution of 2M magnesium chloride.

3. SOC medium

SOC medium is identical to SOB medium except that it contains 20mM glucose. Just before use, add 20 ml of sterile 1M solution of glucose.

Cell Culture Media

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1. Tryptose phosphate broth

Per liter:

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bacto-tryptose	20 gm
bacto-dextrose	2 gm
sodium chloride	5 gm
disodium phosphate	2.5 gm

Sterilize in autoclave. Store at -20 °C until use.

2. Growth medium for propagation of BHK-21 cells

Glasgow minimum essential medium (GMEM) as modified by MacPherson and Stocker (1962) supplemented with:

tryptose phosphate broth	10 %

fetal bovine serum 10 %
MEM non-essential amino acid solution 1 %
penicillin 100 IU/ml
streptomycin 100 ug/ml

Mix above ingredients in sterile double distilled water.

Adjust the pH to 7.4-7.6 with 1N HCl. Store at 4 °C and warm up to 37 °C before use.

3. Maintenance medium for propagation of rabies virus

Maintenance medium is identical to growth medium except that it contains 2% fetal bovine serum.

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APPENDIX B: General Techniques Employed in Cell Culture

1. Trypsinization of monolayer Culture.

Cells are most commonly removed from the culture substrate by treatment with trypsin or trypsin-EDTA. If trypsin is being solubilized or diluted from a concentrated solution, it is important to use a buffered salt solution that contains no Ca^{++} or Mg^{++}. Adjust pH of trypsin solution to 7.4-7.6.

1. Remove medium from culture vessel and wash the monolayer with PBS to remove all traces of serum.
2. Dispend the prewarmed trypsin-EDTA solution into the culture vessel to completely cover the monolayer of cells for approximately 2 min.
3. Remove the trypsin-EDTA solution and place culture vessel into 37 °C incubator untill cells detach from the surface. Progress can be checked by examination with a stereomicroscope.
4. Cells can be resuspended in complete medium by gentle pipetting up and down to disperse the clamps. Further dilution can be made for subculturing or cell count.

2. Titration of virus suspension

Rabies virus can be quantitated by a rapid tissue culture test (Wiktor, 1973). In microtiter plate, BHK-21 cells are added to a series of viral diluent and monolayers are allowed to form. After 14 h incubation, the cell monolayer is examined by FA staining for the presence of rabies virus infectivity. The 50% end point dilutions are calculated according to Reed & Muench method (Lorenz and Bogel, 1973).

1. Suspend the viral seed into a ten-fold serial dilution with maintenance medium (see Appendix C).
2. Load 50μl of virus suspension into 96-well microtiter plate.
3. Trypsinize the BHK cells of 48 h old and disperse in maintenance medium.
4. Add 50μl of BHK cell suspension containing $1.67X10^6$ cells to all viral dilutions and mix well by pipetting.
5. Place the microplate in the CO_2 chamber for 14 h.
6. Remove the cultural fluid, rinse once with phosphate-buffered saline (PBS), fix in cold 80% acetone for 3 min and air dry.
7. Add 50μl of FITC-labelled antinucleocapsid conjugate (1:500 dilution in PBS) to each wells.

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8. Return the microplate to the CO_2 chamber. Incubate for 1/2 h.
9. Drain off and rinse once with PBS, and then air dry.
10. Examine under Epifluorescent microscope using ten low-power (400X) microscopic fields for 8 fields/well.
11. Calculate the 50% endpoint dilutions $TCID_{50}$ titres by Reed & Meunch method.

The 'starting point dilution' is the dilution showing a infection next below 50%. The formula given below is used to determine the difference between the logarithm of the starting point dilution and the logarithm of the 50% endpoint dilution ('difference of logarithms'). When the infection decreases with increasing dilution, the 50% end point dilution will be lower than the starting point dilution. The 'difference of logarithm' is therefore to be substracted from the logarithm of the reciprocal of starting point dilution.

For example, titration of virus suspension give the following results:

Virus dilution	*No. of field observation*		*Cumulative total*		*Percentage infection*
	-ve fields	*+ve fields*	*-ve fields*	*+ve fields*	
10^{-5}	0	8	0	14	14/14 = 100
10^{-6}	3	5	3	6	6/9 = 67
10^{-7}	7	1	10	1	1/11 = 9

Total are accumulated from 10^{-5} to 10^{-7} for negative numbers of field observation from 10^{-7} to 10^{-5} for positive numbers of field observation.

In this example the dilution factor is 10 and the starting point dilution showing a infection next below 50% is 10^{-7}.

Calculate the 'difference of logarithms' from the formula:

$$\frac{50\%\text{-(infectivity next below 50\%)}}{\text{(infectivity next above 50\%-infectivity next below 50\%)}} \times \text{logarithm of dilution factor}$$

Hence, 'difference of logarithms' $= \frac{50-9}{67-9} \times 1$

$= \frac{41}{58} \times 1$

$= 0.71$

Since the 50% end point dilution is lower than the starting point dilution and is calculated by substracting the 'difference of logarithm' as follows:

log (reciprocal of 50% end point dilution) = log (reciprocal of starting point dilution)-'difference of logarithms'
= log 10^{-7}- 0.71
= 7- 0.71
= 6.29

Hence,
log (50% end point dilution) = -6.29
and 50% end point dilution ($TCID_{50}$ titre) = $10^{-6.29}$

3.Cell Counts

Hemocytometer technique is one of serveral methods recommended for the determination of cell numbers. The technique is rapid and simple without the use of sophisticated instrument. Cells can be counted under standard light microscope.

1. Prepare a cell suspension in PBS.
2. With a cover-slip in place, use a pastuer pipet to transfer a small amount of cell suspension to chamber of the hemocytometer. Touch the edge of the cover-slip with care and allow the chamber to be filled by capillary action.
3. Count all the cell in the 1 mm center square and four 1 mm corner squares.

Each square of the hemocytometer, with cove-slip in place, represents a total volume of 10^{-4} ml. The cell concentration per ml can be determined using the following calculations:
cells per ml = average count per square X dilution factor X 10^4

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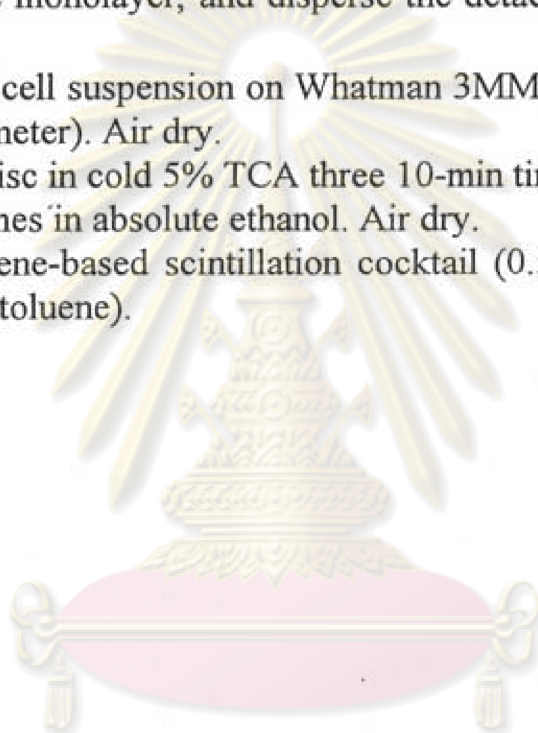
Appendix C: Other Laboratory Techniques

1. Measurment of RNA Synthesis

Synthesis of RNA was determined by measuring the incorporation of ^{3}H-uridine into trichloroacetic acid-precipitable material as described previously by Tobey and Campbell (1965).

1. Incubate the 100 μCi of ^{3}H-uridine (43 Ci/mmole) with 5 X 10^{6} monolayered cells at 37 °C in CO_2 chamber for 30 min.
2. Drain off and wash twice with cold PBS.
3. Trypsinize the monolayer, and disperse the detached single cells in 500 μl of PBS.
4. Dot 100 μl of cell suspension on Whatman 3MM chromatography paper disc (2.5 cm diameter). Air dry.
5. Immerse the disc in cold 5% TCA three 10-min time.
6. Wash three times in absolute ethanol. Air dry.
7. Count in toluene-based scintillation cocktail (0.5% [w/v] PPO, 0.03% [w/v] POPOP in toluene).



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BIOGRAPHY

Mr. Jaturaporn Pornsilapatip was borned on 12 July, 1959 in Bangkok. He graduated from Faculty of Sciences and Arts, Kasetsart University in 1981 with the Bachelor of Science (Microbiology). After that he had enrolled in Chulalongkorn Graduate School and recieved his Master of Science (Industrial microbiology) in 1985.

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