GROWTH OF ANIMAL VIRUSES IN TISSUE CULTURE

The infectivity of a virus preparation can often be measured by the ability of the virus to form a plaque in a continuous lawn or sheet of host cells. Many types of mammalian cells have been cultivated continuously in vitro for many years and these cell lines are invaluable for the growth and assay of animal viruses. One such cell line is the VERO, which was originally derived from African green monkey kidney cells. Sheets of these cells may be used to assay animal viruses in the same way as lawns of bacteria are used to assay bacteriophage (see below), but areas of clear lysis are rarely seen and instead foci of cytopathic effect (CPE) are observed. The nature of the CPE will depend upon both the virus and the host cell used, but may take the form of cell rounding, cell proliferation or cell disintegration.

1. Assay and Serological Identification of Animal Viruses

In this practical we shall assay two animal virus samples (A & B) using monolayers of VERO cells. In addition we shall attempt to identify the two viruses by using specific neutralising antisera.

Materials

- One six-well culture dish. Each well contains $10^6$ VERO cells growing as a continuous sheet on the bottom.
- Suspensions of viruses A and B.
- Antiserum to herpes simplex virus (aHSV).
- Antiserum to measles virus (aM).
- Tissue culture medium (TCM) & phosphate-buffered saline (PBS).
- Sterile pastettes.
- Safety glasses and plastic gloves.
- Six sterile incubation tubes.

Method

N.B.: As the medium used for mammalian cell culture is also an excellent growth medium for most bacteria, yeast and fungi, this experiment demands the use of good sterile technique. Please recap all bottles and tubes after use. This will be discussed by the demonstrator. Furthermore, herpes simplex virus can cause, among other problems, eye infections in humans. Safety glasses and plastic gloves must therefore be worn and there should be no contact between face and hands.

Please dispose of all liquid waste in this practical in the blue capped bottle marked "for liquid waste only" which contains bleach to make sure any infectious agents are killed. Please do not put any solid items into this bottle. Please dispose of all solid waste, including pipettes and tips, in the wide capped container marked "contaminated laboratory waste".
1. **Work in pairs.**

2. Remove 0.4 ml of virus A into each of three sterile tubes (numbers 1, 2, & 3) and 0.4 ml of virus B into a further three tubes (numbers 4, 5, & 6). These transfers must be done carefully using good sterile technique.

3. Add 0.2 ml of PBS to tubes 1 and 4; 0.2 ml of anti-HSV serum to tubes 2 and 5; and 0.2 ml of anti-measles serum to tubes 3 & 6. Mix gently and leave for 30 mins at room temperature, swirling occasionally.

4. Towards the end of this period, remove the medium gently from the edge or side of the monolayers with a pipette, taking care not to disturb the monolayer (tip - gently tilt the monolayer so the medium flows to one side of each well).

5. After the 30 min incubation at room temperature, add the contents of each tube gently to the side of the corresponding numbered well, using a **fresh** pipette for each transfer, and leave for 30 mins.

6. **Gently** add about 2 ml of TCM to the side of each well.

7. Label plates with bench number, name and experiment number. You will get the results of this experiment in Class 13 (one week's time) so will need these notes with you then in order to interpret your results.

2. **Anti-Virus Drugs**

A number of nucleoside analogues have anti-virus activity (please make sure you read the appropriate section in the accompanying Virus General Notes and bring to Class 13 so you can discuss the results of this experiment with your Demonstrator). Acyclovir (ACV) is an anti-herpes virus drug which is in clinical use. We will make an approximate comparison of the potency of this drug against the two viruses A and B, used in Experiment 1.

**Materials**
- One six-well plate containing 10^6 Vero cells per well growing as a monolayer.
- Suspensions of viruses A & B (as per experiment 1).
- Acyclovir (ACV) 5µg/ml.
- TCM (Tissue culture medium).
- Sterile pipettes.
- Sterile plastic pastettes.
- Safety glasses and plastic gloves.

**Method**

1. Remove medium from the wells.

2. Add 0.6 ml of virus A to wells 1, 2, and 3 and 0.6 ml of virus B to wells 4, 5, and 6. Leave for 30 mins, swirling occasionally.

3. Add 2 ml medium to wells 1 and 4. Add 1.8 ml medium to wells 2, 3, 5, and 6. Add 0.2 ml ACV to wells 2, 3, 5 and 6.

4. Rock gently to mix drug and medium. Label with bench number, name and experiment. You will get the results of this experiment in Class 13 (one week’s time) so will need these notes with you then in order to interpret your results.
3. Data-Handling Exercise: The Bacteriophage T4 Replication Cycle

This exercise is designed to familiarise you with the handling of experimental data, the concepts of the plaque assay and the infectious centre assay, the Poisson distribution and, of course, to complement Professor Smith's lectures on virus replication. You should start to have a look at this today and continue in the next two Virology practicals - remember to bring these notes along with you. Do make sure you discuss your answers with a demonstrator in one of the three Virology practicals.

Before attempting the calculations below, read at least the first three pages of the accompanying general notes as they contain some useful definitions. Please remember to bring this sheet and the notes to classes 12 and 13.

Background

The infectivity of a bacteriophage T4 stock is typically measured by mixing the phage with a suspension of host *Escherichia coli* bacteria (sometimes referred to as *indicator* bacteria, in sufficient number that after an overnight incubation, they will form a confluent lawn on an agar plate) in molten agar and pouring the mixture onto a nutrient agar plate. The virus adsorbs to individual bacteria and virus multiplication occurs. After 30-40 minutes, lysis of the bacterium results and about 100 new phage are released which infect neighbouring bacteria. This sequence of events is repeated until, after about 16 hours, an area of complete lysis marks the site of the original infected bacterium. The number of such plaques in the bacterial overlay indicates the number of infectious bacteriophage in the original suspension. This is usually expressed as the viral titre, which is the number of plaque-forming units (pfu) per ml.

When virus is added to susceptible host cells, the cells become infected and the virus is now intracellular. At this stage no free virus is found but the infected cells can be assayed by the same technique as above; by plating the infected cells in the presence of indicator *E. coli*, and observing plaque formation in the subsequent lawn of *E. coli*. This is called an infectious centre assay.

The Experiments

1) **Bacteriophage T4 titration.** Ten-fold serial dilutions of a bacteriophage T4 stock were prepared in phosphate-buffered saline (PBS). 0.2ml of each T4 dilution was added to tubes containing molten soft agar and indicator *E. coli* cells. The tube contents were poured onto nutrient agar plates, allowed to set and the plates incubated at 37°C overnight. The plates are shown in Figure 1.
2) **Assessment of the T4 burst size.** The T4 stock was adjusted to $10^9$ pfu/ml and 0.01 ml was used to inoculate a 10ml culture of *E. coli* in logarithmic growth phase ($10^7$ cells per ml). After five minutes, and every ten minutes thereafter up to 55 minutes, a 0.2 ml aliquot of the culture was removed. This was serially diluted in ten-fold steps in PBS and 0.2 ml aliquots of each dilution assayed for plaque formation as before, by adding the aliquot to a tube of molten soft agar containing indicator *E. coli* bacteria and pouring onto a nutrient agar plate. After an overnight incubation at 37°C, the numbers of plaques observed on each plate were counted and are shown in Table 1.

**Table 1.** Plaque numbers from the T4 burst size experiment.

<table>
<thead>
<tr>
<th>Time*</th>
<th>No. of plaques for each dilution</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>5</td>
<td>TMTC</td>
</tr>
<tr>
<td>15</td>
<td>TMTC</td>
</tr>
<tr>
<td>25</td>
<td>TMTC</td>
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<tr>
<td>45</td>
<td>TMTC</td>
</tr>
<tr>
<td>55</td>
<td>TMTC</td>
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</tbody>
</table>

TMTC = too many to count.
* Time (in minutes) following infection of bacteria with T4 (i.e.: time post-infection).

**Please answer the following questions (1 to 4).**

Space is left below each question for you to insert your calculations

1. From Figure 1, what is the titre of the original T4 stock?

2. From Table 1, what is the average T4 burst size? Suggest ways you could attempt to make the results you obtain more accurate?

3. (i) What multiplicity of infection was used in experiment 2 (which assessed the T4 burst size)? (ii) Under these circumstances, what proportion of the *E. coli* were infected? (iii) What volume of T4 stock would you use to infect 95% of the bacteria? (iv) Under the latter circumstances, what proportion of bacteria were infected by three or more phage?

You will require the formula for the Poisson distribution to answer all of question 3.

The equation for the Poisson distribution is:

\[ P_x = \frac{(m^x \cdot e^{-m})}{x!} \]

Where \( P_x \) = the probability of a target being "hit" x times

\[ m = \text{mean hits per target} = \text{multiplicity of infection (moi)}. \]

\[ e = \text{natural logarithm}. \]
4. You will note that each sample in Experiment 2 was added directly to the indicator bacteria for assay. In an additional experiment, the samples were sonicated to lyse the bacteria prior to adding to the indicator bacteria to do the assay. How do you think the results would change?

DEMONSTRATIONS: Examine the photographs displayed around the classroom.

Please disinfect your bench top area
Push back your stool beneath the bench
Wash your hands before you leave

Thank you.