

**PLEASE READ THE GENERAL NOTES RELATING TO THIS SERIES OF PRACTICALS. THEY CONTAIN LOTS OF RELEVANT INFORMATION AND SOME EXERCISES YOU SHOULD ATTEMPT.**

**YOU SHOULD ALSO REMEMBER TO BRING THE CLASS NOTES AND THIS HANDOUT TO THE NEXT TWO CLASSES AS YOU WILL NEED THE INFORMATION IN THEM IN SUBSEQUENT SESSIONS**

### **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<sup>6</sup> VERO cells growing as a continuous sheet on the bottom.
- Suspensions of viruses A and B.
- Antisera to herpes simplex virus ( $\alpha$ HSV; one vial shared between two pairs)
- Antisera to measles virus ( $\alpha$ M; one vial shared between two pairs).
- 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.

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 from the monolayers with a pipette, taking care not to disturb the monolayer.
5. After the 30 min incubation at room temperature, add the contents of each tube to the corresponding numbered well, using a *fresh* pipette for each transfer, and leave for 30 mins.
6. Add *gently* about 2 ml of TCM to each well
7. Label plates with bench number, name and experiment number.

## 2. Anti-Virus Drugs

A number of nucleoside analogues have anti-virus activity (see Virus General Notes). 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. Suspensions of viruses A & B.  
Acyclovir (ACV) 5µg/ml. One vial shared between two pairs  
TCM (Tissue culture medium), sterile pipettes  
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.

## 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 Minson's lectures on virus replication. You should have a look at this today and discuss your answers with a demonstrator in a later Virology practical.

### 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 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.

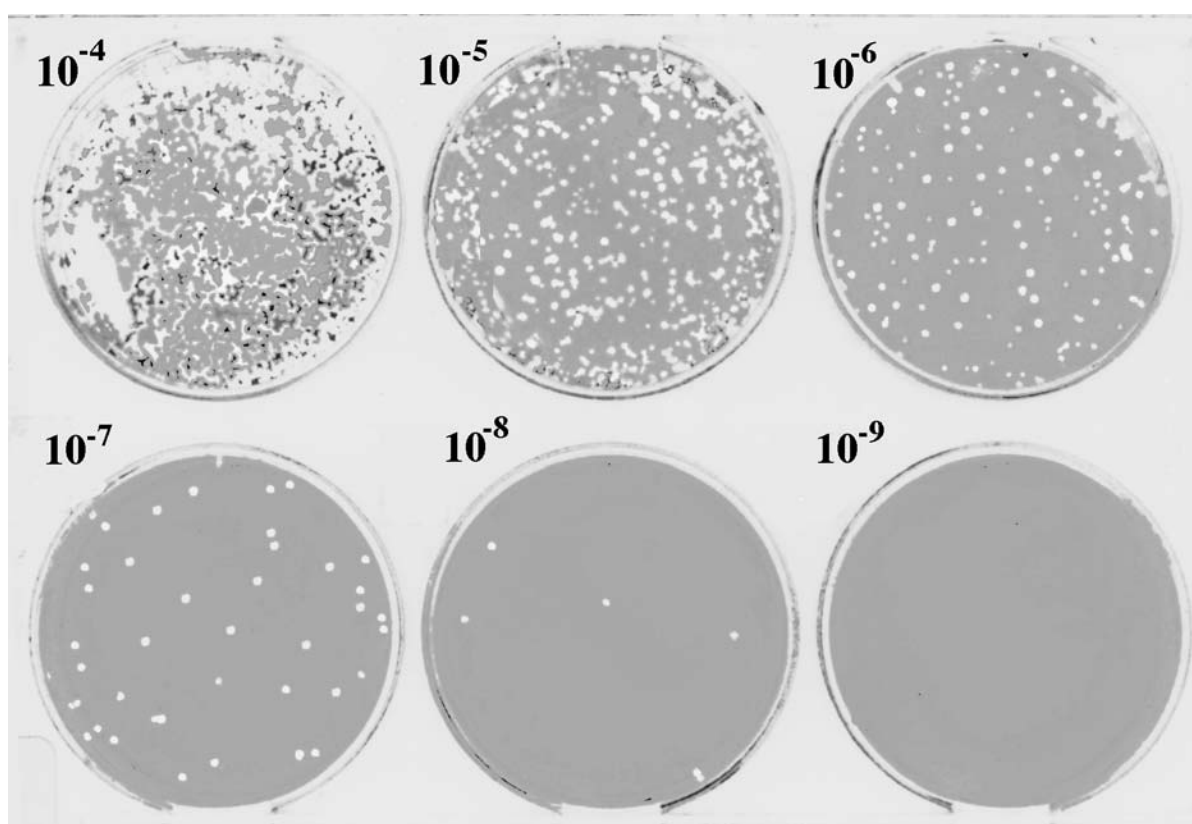
When virus is added to susceptible host cells, the cells become infected and the virus is now intracellular. At this stage, no free virus is found, but the infected cells can be assayed by the same technique as above; by plating the infected cells in the presence of indicator *E. coli*, and observing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

### The experiments

1 *Bacteriophage T4 titration.* 10-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.

Catalogue Number	Small Image	Image Map	Large Image
A_IN_VI_BT_01.jpg	<a href="#">Bacteriophage T4 titration</a>		<a href="#">Bacteriophage T4 titration</a>

**Figure 1. T4 plaque assay** (dilution indicated at top left of each plate).



2. *Assessment of the T4 burst size.* The T4 stock was adjusted to 10<sup>9</sup> pfu/ml and 0.01 ml used to inoculate a 10ml culture of *E. coli* in logarithmic growth phase (10<sup>7</sup> cells per ml). After ten minutes, and every ten minutes for one hour, a 0.2ml aliquot of the culture was removed. This was serially diluted in ten-fold steps in PBS and 0.2 ml aliquots 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.

Time*	No. of plaques for each dilution
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	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$	$10^{-8}$
10	TMTC	247	34	9	1	0	0
20	TMTC	263	28	4	0	0	0
30	TMTC	TMTC	180	25	1	0	0
40	TMTC	TMTC	TMTC	311	35	6	0
50	TMTC	TMTC	TMTC	347	32	4	0
60	TMTC	TMTC	TMTC	368	40	11	1

TMTC = too many to count.

\* Time (in minutes) following infection of bacteria with T4 (ie: time post-infection).

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

1. From Fig 1., what is the titre of the original T4 stock?
2. From Table 1, what is the average T4 burst size?
3. 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 assay. How do you think the results would change?
4. What multiplicity of infection was used in experiment 2 (which assessed the T4 burst size)? Under these circumstances, what proportion of the *E. coli* were infected? What volume of T4 stock would you use to infect 95% of the bacteria? 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 4.

The equation for the Poisson distribution is:

$$P_x = (m^x \cdot e^{-m}) / x!$$

Where  $P_x$  = the probability of a target being "hit" x times

$m$  = mean hits per target = multiplicity of infection (moi).

$e$  = natural logarithm.

**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.**

