Question 1.

To calculate the titre, count the plaques (figure 1) on a plate from a dilution with the highest number of countable non-overlapping plaques. In this case, the $10^{-7}$ dilution is the best. If you count the $10^{-6}$ dilution, although the plaques seem to look well separated, it is impossible to reliably count more than about 150 plaques, suggesting that actually many of the 400 or so plaques, that should be present if the ten-fold dilutions have been done correctly, have merged and are thus impossible to count. To get a higher number of countable plaques for the $10^{-6}$ dilution, you would need to plate it onto a plate with a much larger surface area, thus giving better spacing and less chance of plaques merging. Were you to count from the $10^{-8}$ dilution, you would be counting too few plaques which would give a potentially very inaccurate result.

There are about 45 plaques on the plate for the $10^{-7}$ dilution.

Titre is therefore $45 \times 10^{-7}$ in 0.2 ml of sample.

Answer - Titre is $2.25 \times 10^9$ pfu/ml

Question 2.

The burst size is the average number of pfu released per infected cell. Early on in experiment 2, 5 and 15 min post-infection, no released phage are yet present and the plaques we see are derived from infected bacteria (so-called infectious centres). At this stage, assuming all of the virus inoculum has entered the bacterial cells, the input titre of virus, or number of infected cells, equals the number of infectious centres per ml we would calculate the average titre at 5 and 15 mins post-infection, since we can be confident that at these times, the virus is still in the eclipse phase and there has been no burst yet.

Care needs to be taken as to which dilutions are to be used for your calculations. You are not given photographs of the plates, merely what another person has counted. If you think back to experiment 1 where you were given images of the resultant plates and you had to count them, you may have observed what is described above in the answer to question 1: the problem of apparently "merged plaques" if there are actually too many plaques on a plate, even if they look well separated. With this in mind, if you inspect the figures in table 1, you can see that the figures for 5 and 15 mins for the $10^{-3}$ dilution are not 10 times what the figures are for the $10^{-4}$ dilution, suggesting that the experimenter had the same difficulty counting the plaques on the less dilute plate as you may have done in experiment 1. Thus, you might conclude that the figures given for the $10^{-4}$ dilution are probably more reliable.

5min $\quad 34 \times 10^4 \times 5 = 1.7 \times 10^6$ pfu/ml
15min $\quad 28 \times 10^4 \times 5 = 1.4 \times 10^6$ pfu/ml

Average $\quad 1.55 \times 10^6$ pfu/ml

From the data, it is clear that the burst is in progress at 35 minutes (there is a clear increase in plaque numbers versus the 5, 15 and 25 minute time points). The burst has
clearly happened by the 45 and 55 minute time points. The plaques here are from progeny virus from the infected cells you have measured at 5 and 15 minutes. I would steer clear of using the 25 minute time point as it is closer to the 30 minutes when you are told burst is definitely starting. From the figures obtained, there is the suggestion that a few rogue cells may already have burst by 25 minutes, so that time point is probably best avoided. I would calculate the average titre of the time points where the burst is definitely over, say the 45 min and 55 min time points as it enables you to have some kind of duplicate count:

45min \(40 \times 10^6 \times 5 = 1.6 \times 10^8\) pfu/ml
55min \(32 \times 10^6 \times 5 = 2 \times 10^8\) pfu/ml
Average = \(1.8 \times 10^8\) pfu/ml

Answer - The burst size is the ratio of progeny phage versus the initial number of infected cells.

\[\frac{1.8 \times 10^8}{1.55 \times 10^6} = 116\ \text{average progeny phage per infected cell}\]

In order to get a more accurate result for this value, you would need to do the experiment in independent (ie separate experiments) duplicates or, better still but more work, triplicates to remove the random errors which can clearly be seen in this dataset. For example, in a perfect world, the \(10^{-5}\) samples at 45 and 55 minutes should give identical numbers. Performing triplicate titrations for each of these time points would have given you more confidence in the eventual number and even enabled you, if really enthusiastic, to carry out some form of statistical test.

**Question 3.**

(i). **What multiplicity of infection was used in experiment 2?**

Multiplicity of infection (moi) = number of pfu/number of cells

\(10^7 \text{ pfu/10ml} = 10^6 \text{pfu/ml}\)
\(10^7 \text{ cells per ml}\)

Therefore multiplicity of infection = \(10^6/10^7 = 0.1\) plaque forming units per cell (pfu/cell)

(ii). **Under these circumstances, what proportion of the E. coli were infected?**

\[P_0 = e^{-m} = e^{-0.1} = 0.905\]

\[P_{inf} = 1 - 0.905 = 0.095\]

i.e. **9.5% E. coli infected.**

(iii). **What volume of T4 stock would you use to infect 95% of the bacteria?**

Require 95% infection, therefore 5% not infected

Therefore \(P_0 = 0.05\)
Therefore \( e^{-m} = 0.05 \)
Therefore \( m = 3 \text{ pfu/cell} \)

You have a total of \( 10^8 \) cells, therefore you need \( 3 \times 10^8 \) pfu

Titre is \( 10^9 \) pfu/ml, therefore you need \( 3 \times 10^8 / 10^9 \) ml of the T4 stock = \( 0.3 \text{ ml} \)

(iv). Under the latter circumstances, what proportion of bacteria were infected by three or more phage?

At \( m = 3 \) what proportion of \( E. \text{ coli} \) are infected with 3 or more phage?

Proportion uninfected \((P_0) = 0.05\)

Proportion infected by 1 phage \((P_1) = (m^1 \times e^{-m})/1! = 3 \times 0.0498 = 0.149\)

Proportion infected by 2 phage \((P_2) = (m^2 \times 0.0498)/2! = (9 \times 0.0498)/2 = 0.224\)

\( P_0 + P_1 + P_2 = 0.05 + 0.149 + 0.224 = 0.423 \)

Therefore proportion infected by 3 or more \((P_{\geq 3}) = 1 - 0.423 = 0.577\)

i.e. \( 57.7\% \)

**Question 4.**

In the case where the cells were lysed (by sonication) before assay, you would see no plaques from the 5 and 15 minute time points (apart from a few derived from a small amount of unabsorbed inoculum) since the virus is in the **eclipse phase**, i.e.: no fully assembled viable phage are ready yet, so if you kill the cells, you get no infectious virus to assay.

At later time points, the situation is different. The burst has already occurred, so sonication should not have much effect on yields if progeny virus is all just free in the nutrient broth, as free virus is unaffected by sonication. So the simplest correct answer is that you will have the same number of plaques as you obtained if you did not sonicate the bacteria at the post-burst time points.

**Optional - Things you might wish to consider**

However, from question 3, you have shown that only 9.5% of the initial \( E. \text{ Coli} \), or \( 9.5 \times 10^6 \) cells were initially infected. This leaves \( 9.05 \times 10^7 \) uninfected cells in the culture. You might
want to consider whether the progeny phage will manage to infect an uninfected cell and initiate a new round of infection in the time available.

So, if you look at the problem in a more sophisticated manner, it is possible that the population may contain a mix of free progeny phage and cells which are undergoing a new round of infection and thus are in eclipse phase. Free phage will contribute towards a titre as above, whereas progeny phage which have already managed to infect a fresh uninfected cell will be in eclipse phase and will thus not contribute towards a titre. This is a much more complex situation and will lead to a far vaguer answer to the question.

**Experiment from Class 11 - Assay and Serological Identification of Animal Viruses**

Acyclovir resistance is most commonly caused by inactivation of the TK gene by deletion or the introduction of an inactivating mutation. It can also be caused by mutation of the DNA polymerase gene which then produces a functional enzyme which no longer recognises ACV-triphosphate. This is a so-called substrate specificity mutation.

**The data showing that Virus B is ACV resistant**

From the staining of the plates you performed earlier, you should have come to the conclusion that both viruses A and B were herpes simplex virus. However, you should have identified that Virus B was a strain that is resistant to ACV. The reason for this resistance has been investigated, and is as follows:

The thymidine kinase (TK) gene (pages 4 and 5 of the general notes) was sequenced after being PCR'd up from viral DNA. A portion of the comparative lineup of the sequence of virus A versus virus B, showing the mutation is reproduced below.

A GCCGTTCCTGGCTCCTCA\[\text{GC}\]GGGGGGAGGCTGCACATGC\[\text{GC}\]GCTGGGAGCTCACATGCCGGG

B GCCGTTCCTGGCTCCTCA

This one base pair deletion leads to a frameshift mutation. The effect of this is to put translation of the protein out of frame beyond the deleted G base. This leads to the protein sequence of TK, which should be 376 amino acids long, diverging from the correct sequence at amino acid 146, with the addition of a further 36 incorrect amino acids encoded by the new frame before the ribosome encounters a stop codon. Several essential motifs of the active site of the enzyme are between amino acids 161-193, and thus not present in the protein encoded by virus B.

A TK assay was performed on infected cell extracts for both viruses. Virus B infected cell extracts showed TK activity comparable to that seen from uninfected cell extracts; this could be either cellular TK activity or a background level at the limits of the sensitivity of the assay. The TK activity of virus B was less than 10% of the TK activity seen for infected cell extracts of virus A.

Therefore Virus B does not produce an active TK, therefore cannot phosphorylate ACV and thus does not have its DNA replication disrupted by ACV, therefore is resistant to the drug. Incidentally, *in vivo*, HSV that does not have an active TK is severely attenuated.
HAEMAGGLUTINATION-INHIBITION (HAI) TEST AS A DIAGNOSTIC TOOL

As you have seen in Class 13, you can detect the presence of specific antibodies to influenza virus by their ability to inhibit the normal haemagglutination the virus exhibits when a fixed amount of virus is incubated with a fixed amount of fowl red blood cells.

This HAI test can be used to identify strains of influenza virus which have been isolated from clinical specimens. The specimen is inoculated into fertile eggs, and a stock of the unknown virus is prepared by harvesting the allantoic fluid. Various reference sera, containing antibody against the haemagglutinin spikes of known influenza strains, are then tested for their ability to inhibit haemagglutination by the unknown virus. In the example in Figure 1, the unknown virus is clearly related to a strain of virus which first began to circulate in 1968, A/Hong Kong/68 (haemagglutinin Subtype H3).

**Antigenic Drift and Shift**

However the unknown virus is not precisely the same as that strain, since that extent of neutralisation by the anti A/Hong Kong/68 serum is not identical for both viruses. Remember, the same number of HA units of each virus were used in the assay. This difference can be attributed to “Antigenic Drift” the accumulation of point mutations in the haemagglutinin spike which slightly alter its antigenic properties from year to year.

The other type of antigenic variation observed with influenza virus, “Antigenic Shift”, refers to the appearance in the population of viruses with a haemagglutinin spike which is totally unrelated to any of the previous circulating strains. This explains why the antisera to the H1 and H2 viruses in Figure 1 do not inhibit the agglutination of the rbc by the unknown virus, although they inhibit agglutination by the virus they were raised against. Strains such as this arose in 1918 (H1 subtype), 1957 (H2 subtype) and 1968 (H3 subtype), with devastating consequences, since most of the world’s population had no protective antibody.