91180 Practical Week 7

Immunological testing of potential Covid-19 samples

UTS CRICOS 00099F

Background

In this prac you will be comparing 3 methods to identify COVID-19 infection in serum samples.

For this exercise, imagine that you are a member of a PC3 laboratory here in Sydney. Using full PPE, including a respirator, you have been asked to carry out an epidemiological investigation on recent suspected cases from UTS.

Time between exposure to the virus and presentation of symptoms is typically 5 to 6 days

IgM and IgA antibodies to SARS-CoV-2 can be detected in serum at relatively low titres from around 5 days after infection

IgG antibodies can be detected after 14 days

Summary of experimental procedure for week 6 and 7

- Last week (week 6) you went through the indirect ELISA method for Identifying SARS-CoV-2 Antibody
- This week we will go through the results of the plaque forming assay and the immunofluorescence.

- The images and results for the immunofluorescence and plaque forming assay are in your manual.
- If you have not yet watched Matt Johansen's talk on COVID you should check this out.

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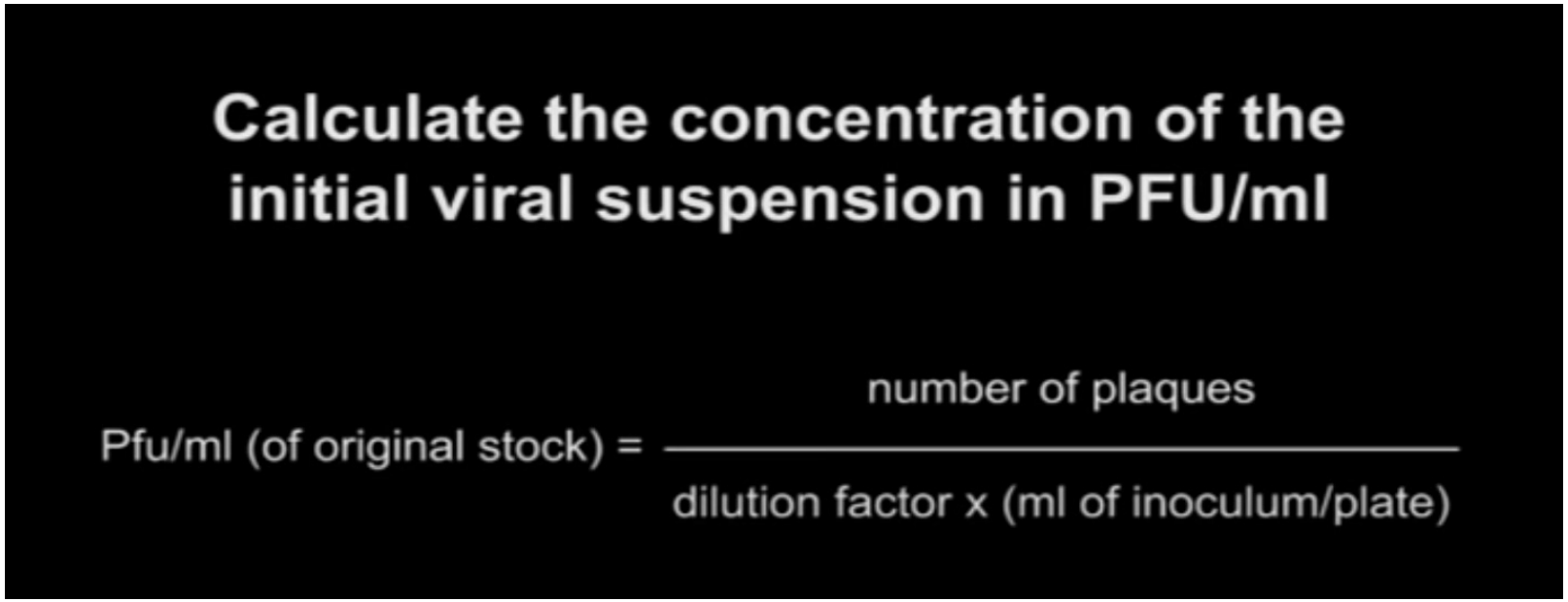
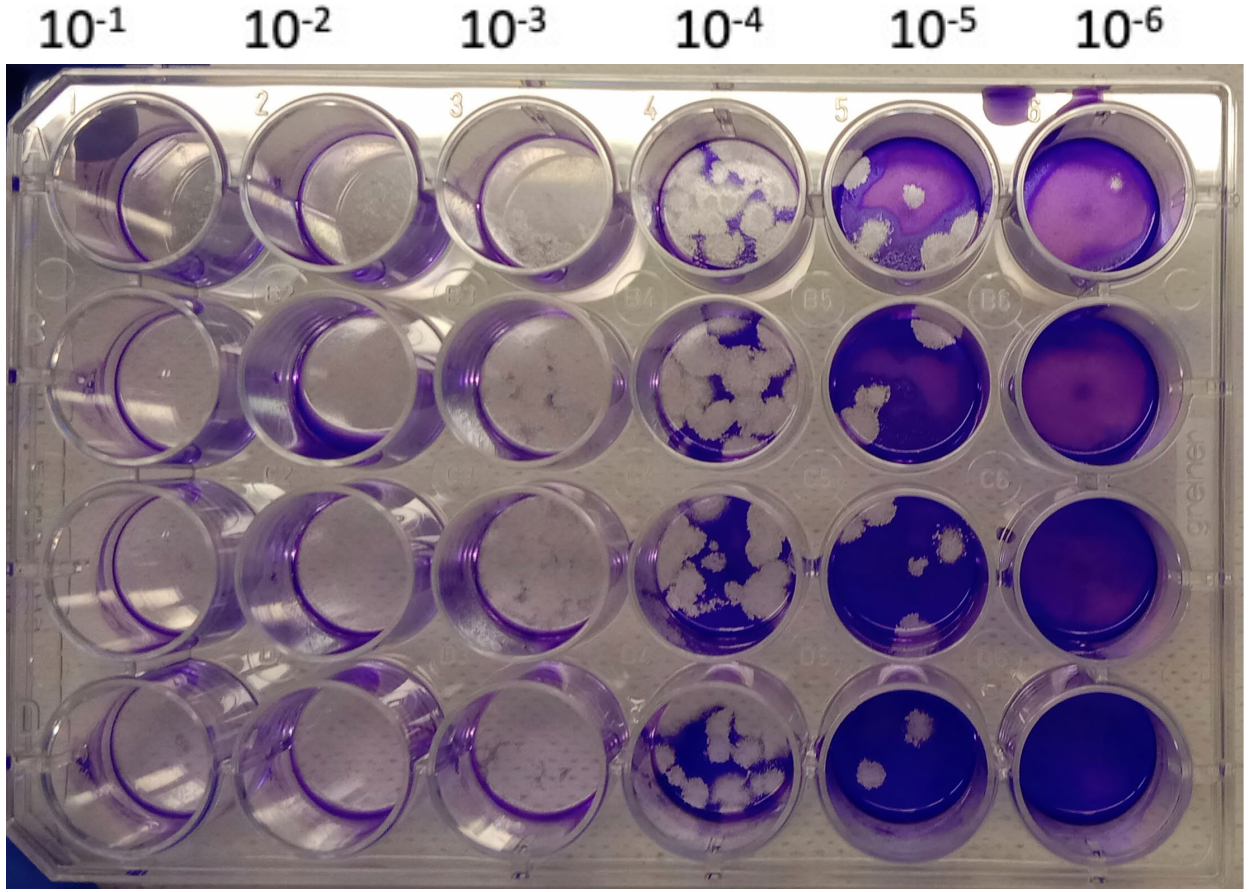
Setting up the plaque Assay

- abcam virus neutralisation assay (7min)- No voice over so could watch on double speed

https://www.youtube.com/watch?v=QThpXUHjcZM



Determining infectious viral titres



Calculating PFU/mL

0.25 mL unknown titre added to each well

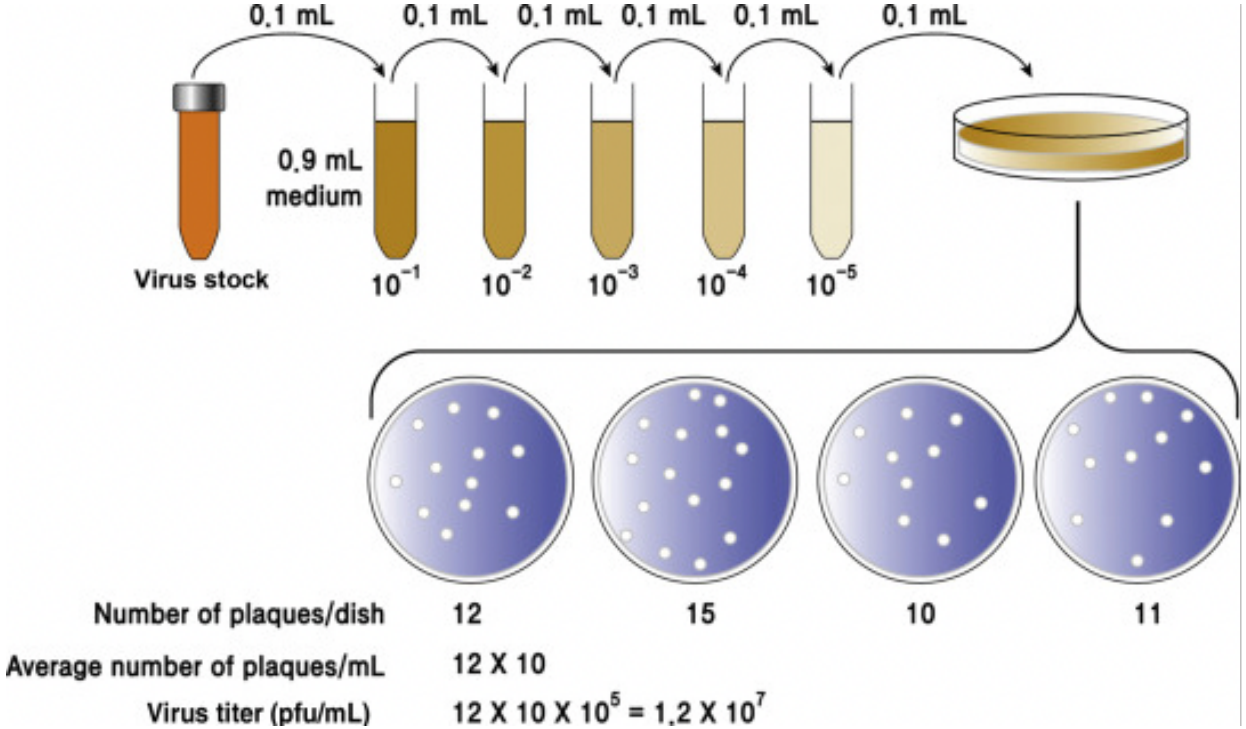
In Row 4 there are 2 plaques at 10^{-5} dilution

= 2 x 100,000 /0.25 ml = 8 x 10^5 PFU/mL

Try calculating this yourself for Rows 1, 2 and 3.

Determining infectious viral titres

- Plaque assay
- – Virus added to confluent cell monolayer
- – One infectious virus particle = 1 plaque forming unit (PFU)
- – Determines titre of viable and infectious virus



Determining infectious viral titres

Dilute out the viral samples

Serum neutralisation assay measures the ability of the antibodies in the test serum to neutralise coronavirus

1. Dilute out the serum samples from the two patients
   In disposable tubes, prepare 160µl of 1/10, 1/20 and 1/40 serum dilutions for each of the three serum samples.

2. Mix 160µl of each serum dilution with 160µl of the diluted virus preparation. As additional controls, mix 250µl of the virus with 250µl of medium for the "virus only" control, and prepare one tube with 500µl of medium for the "no virus control".

3. Vortex the tubes and incubate at 37ºC for 60 minutes.

4. After incubation, aspirate the medium from the cultured cells, and add 150µl samples from each tube to the appropriate monolayers in the 24 well plate.
   - Do not leave the monolayers without liquid for more than 30 seconds, or the cells will die.

Determining infectious viral titres

Add Virus to the cells in the 24 well plate

4- cont'd Dilutions of samples from patient 1, patient 2 and negative control sera are to be tested in duplicate. The "no virus" control and the "virus only" control samples are to be tested in triplicate. Ensure that the cell monolayers are completely covered by the inoculum.

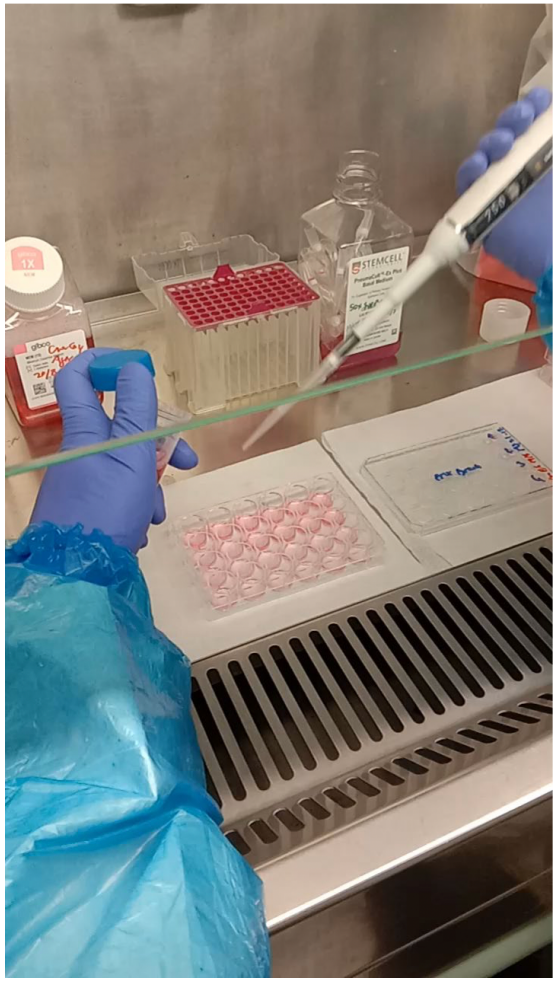
5. Incubate for 90 minutes 37ºC 5% CO2.

6. Gently remove the media from the monolayers and replace with 1ml of MEM.

7. Add 1 ml of 3% Agarose agar overlay (diluted in your MEM) – this is added to prevent spread of the virus in the medium. Virus can only infect cells directly next to each other, not all the cells.

7. Incubate the plates at 37ºC 5% CO2 for 6 to 7 days.

Determining infectious viral titres



Add agar to the plates after 1 hr

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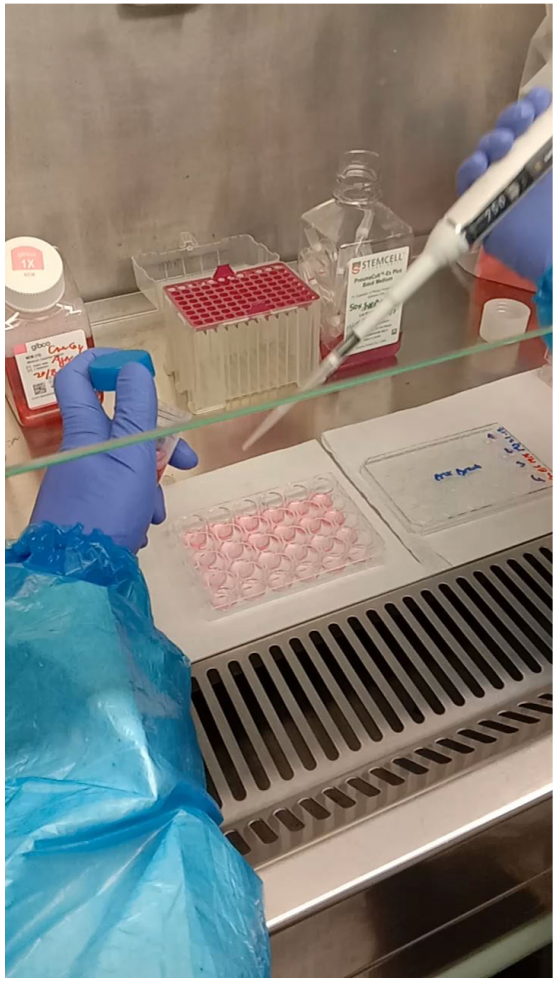
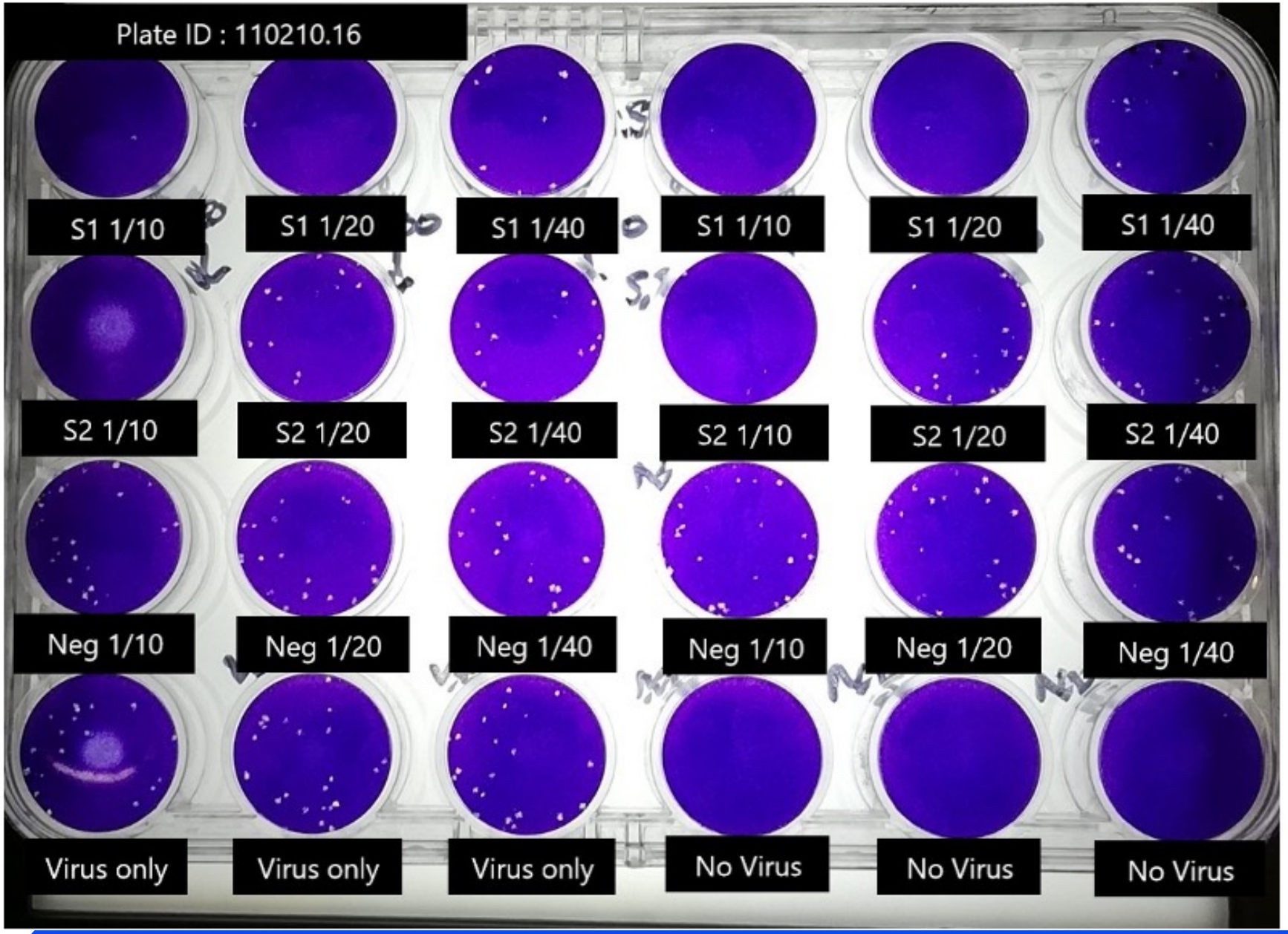


Add agar to the plates after 1 hr

Suggested layout:
Row A: Duplicates of patient 1 serum dilutions.
Row B: Duplicates of patient 2 serum dilutions.
Row C: Duplicates of the negative control serum dilutions.
Row D: Three wells of the "virus only" control, three wells of the "no virus" control.

Day 7:

8. Inspect plates for plaque formation before staining.
9. Aspirate medium from wells (this contains live virus, place in the disinfectant provided).
10. Add 200µl of 10% formalin to each well to fix the monolayers. Leave for 10 minutes
11. Aspirate the formalin and add 100µl of crystal violet solution. Leave for 20 minutes
12. Gently wash the monolayers to remove excess stain. Drain onto paper towel.



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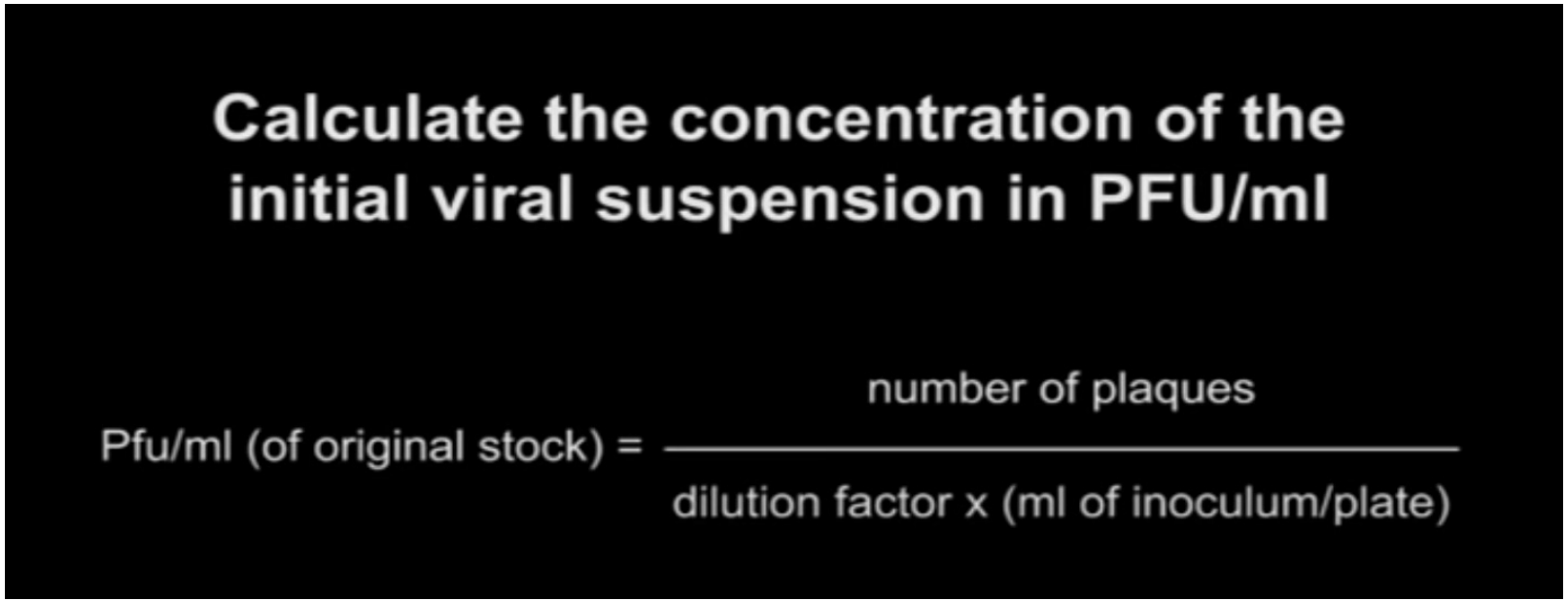
Calculate Any neutralisation in the samples by comparing virus only samples to those with diluted serum.



Immunofluorescence Assay - **Fluorescent antibody staining of virus-infected cultures**

Using Vero cells we will determine if virus can be cultured from the tissue sample collected from patient 1's nasopharyngeal swab.
Antibiotics and antifungal agents will be added to the tissue cultures to help minimise bacterial and fungal growth.
Stringent aseptic technique is still important.
Virus infection of cell monolayers can be detected by the cytopathic effects of the virus.
However, this does not tell you with any certainty what virus is present.
We will examine the Vero monolayers for coronavirus, using specific antibodies to coronavirus which binds to viral proteins.
This binding will be detected with an anti-human immunoglobulin conjugated to FITC, which fluoresces under UV light.

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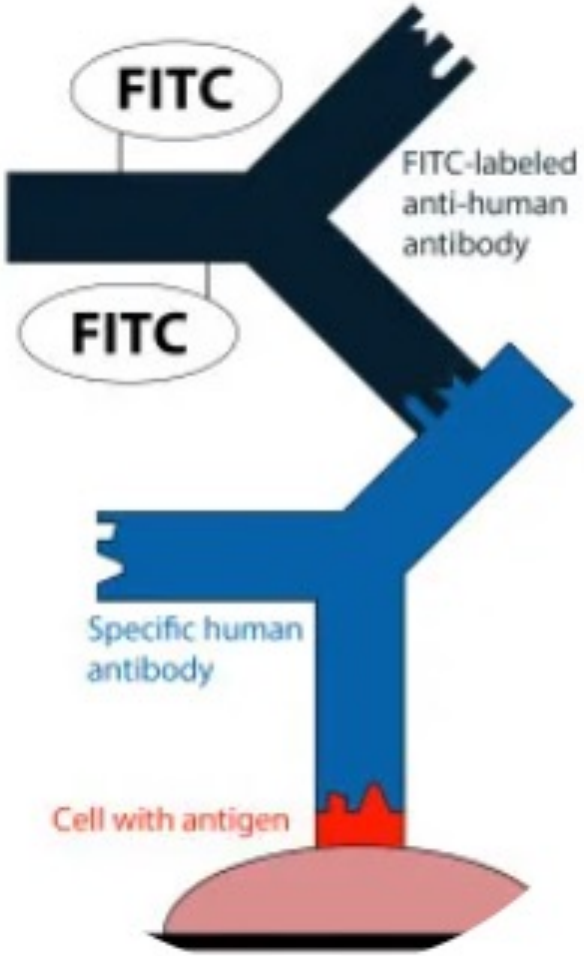
Video of the technique:

Immunofluorescence Assay - **Fluorescent antibody staining of virus-infected cultures**

Immunofluorescence methods (2-3 mins)
https://www.youtube.com/watch?v=pteO6FRWo3g



IFA for antibodies detection

Immunofluorescence Assay - Fluorescent antibody staining of virus-infected cultures

1. Aspirate the medium from the monolayers

2. Add 150µl of samples as follows:
A1 negative control (MEM), A2 swab sample, A3 positive control virus.
A4 negative control (MEM), A5 swab sample, A6 positive control virus.
Incubate the monolayers for 60 minutes 37.C 5% CO2.

3. Gently aspirate the inoculum and replace with 1ml of MEM.
Incubate for 3-4 days until the cytopathic effect can be seen in the monolayers.

4. Aspirate the MEM and add 200µl of 1:1 v/v methanol/acetone to each monolayer to fix and permeabilise the cells. Leave for 10 minutes. Aspirate the fixative.

5. Wash the monolayers 3 times with 1 ml of PBS/BSA.

Immunofluorescence Assay - Fluorescent antibody staining of virus-infected cultures

6. Add 50µl of anti-corona virus serum to each well in columns 1, 2 and 3.

7. Add 50µl of normal human serum to each well in columns 4, 5 and 6.

8. Incubate 37ºC for 30 minutes.

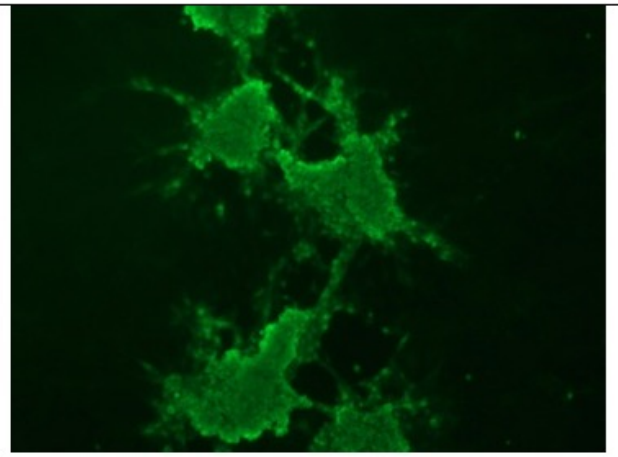
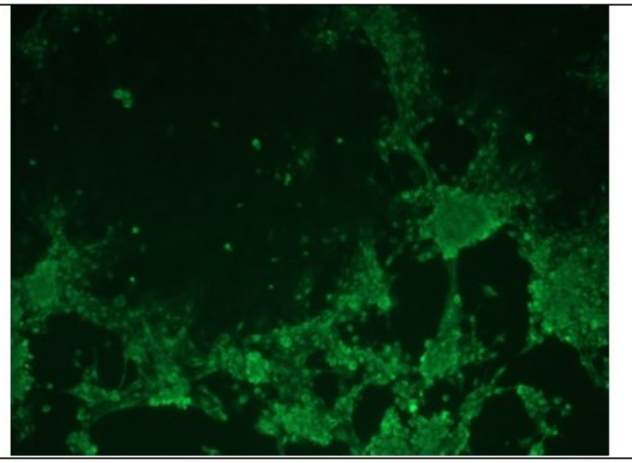
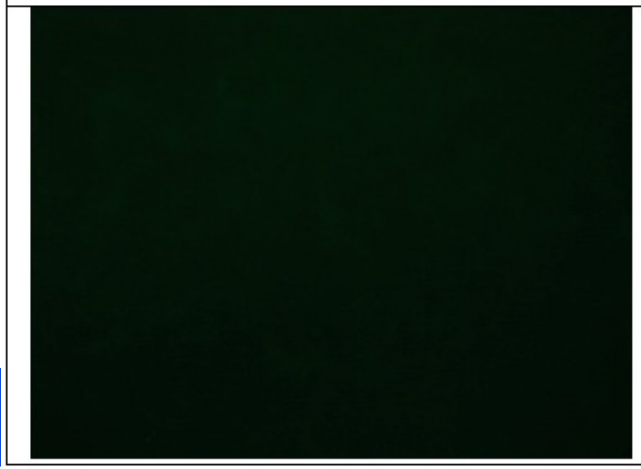
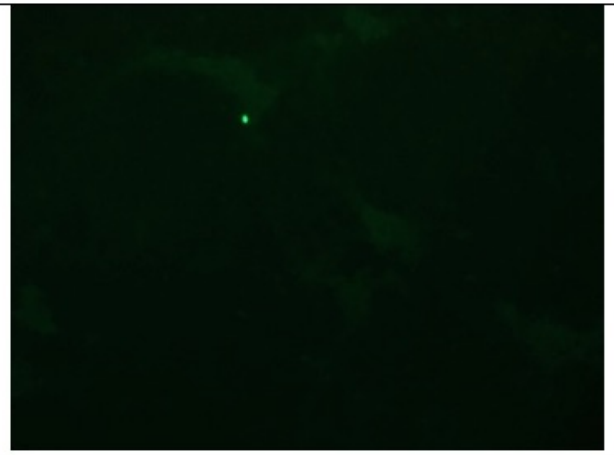
9. Aspirate the serum from the wells. Wash each well 3 times with 1 ml of PBS/BSA.

10. Add 50µl of goat-anti-human-IgG-FITC to each well. Incubate for 30 minutes at room temperature in the dark.

11. Wash the monolayers 3 times with 1 ml of PBS/BSA.

12. Aspirate and wash monolayers 3 times with 1 ml PBS/BSA. View plates using a fluorescent microscopy.

Immunofluorescence Results

(A1) Undiluted negative control with anti-Corona virus serum	(A2) Undiluted swab sample with anti-Corona virus serum	(A3) Undiluted positive control with anti-Corona virus serum
		
(A4) Undiluted negative control with negative control serum	(A5) Undiluted swab sample with negative control serum	(A6) Undiluted positive control with negative control serum.
		

Assessment: You will write this prac (week 6 and 7) up as your scientific report answering the questions below.

1. Provide a flow diagram for the method of Part A (indirect ELISA) . 1 mark

2. Document the mean absorbance for each sample and controls using data provided in Table 1- Excel file in week 6-7 module (2 marks)

Dilution	Sample 1	Sample 2	Positive control	Negative control

Assessment:

3. Plot graphs of the mean of the duplicate absorbance vs log dilution for all samples using Table 1 (4 marks)

4. Calculate the antibody titres by picking an absorbance reading in the linear part of the curve. The absorbance value you choose should be at least 0.1 units above the negative control serum absorbance at a dilution of 1/100. The antibody titre is the reciprocal of the dilution of antibody giving your chosen absorbance value. (In order to make a valid comparison, the same absorbance value must be used for all curves). (3 marks)

5. What issues could arise if we were to use the N protein as antigen in our ELISA instead of S protein? (3 marks)

Assessment:

6. Provide a flow diagram for the method of Part B (virus neutralisation). (1 mark)

7. Count plaques in all wells in Figure 1. Calculate the percentage plaque reduction at each serum dilution and estimate the titre. (3 marks)
If there are virus specific neutralising antibodies in the serum sample the number of plaques will be reduced compared to the same amount of virus incubated without serum. Antibody titre in this assay is normally reported as the reciprocal of the serum dilution giving a 50% reduction in plaque number. This may need to be calculated by interpolation. An alternative is to use the highest serum dilution giving a plaque reduction of 50% or greater.

8. Provide a flow diagram for the method of Part C (Immunofluorescence) (1 mark)

9. Briefly explain the outcome for each image (1 –2 sentences each) (6 marks)

10. Briefly describe what information is gained through the techniques of part A, B and C. How is this different to the knowledge gained from PCR? (6 marks)

Assessment:

11. Which method, if any, gives the truest measurement of anti-coronavirus antibodies? Explain (3 marks)

12. How might we determine if one of the patients is infected but not yet showing symptoms, or if they have previously been exposed to the SARS Cov2 virus? (3 marks)

13. You are preparing to present this work as a paper. Write an abstract complete with title for your paper based on this data (15 marks).