91180 Practical Week 6

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

Background COVID -19

In this prac you will:

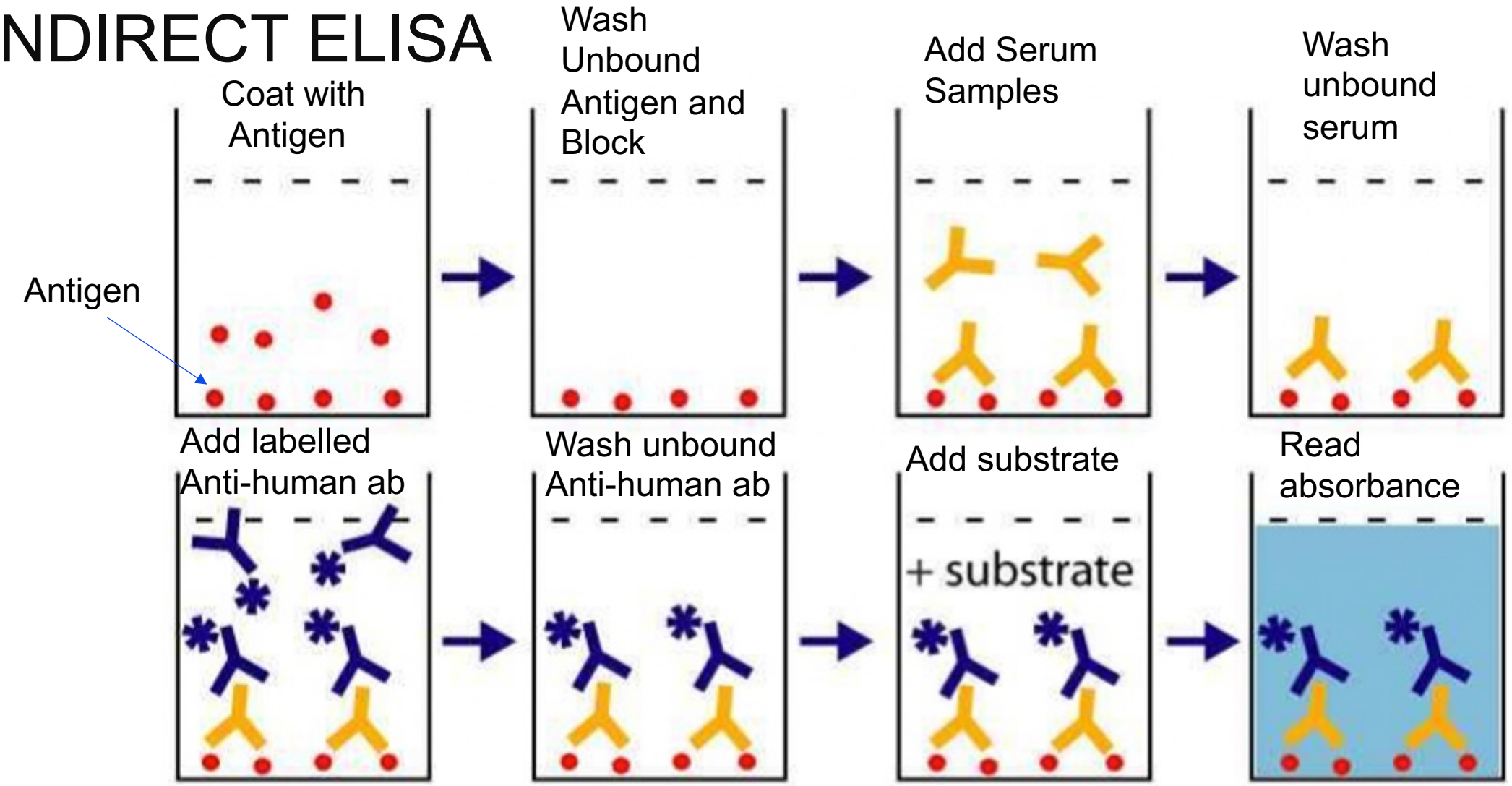
- Test for the presence of anti-SARS-CoV-2 antibodies.
- You will also attempt to isolate and identify the virus from the nasopharyngeal swab in a human cell line followed by an immunofluorescence assay.
- You will investigate whether the antibodies in the patient serum are able to neutralise the virus, using a plaque reduction assay.

Summary of experimental procedure for week 6 and 7

- In the next 2 weeks we will test 3 different immunological procedures for detecting COVID-19.
- In week 6 we will go through the Indirect ELISA.

- The images and results for the immunofluorescence and plaque forming assay are in your manual. These will be discussed in week 7.
- If you have not yet watched Matt Johansen's talk on COVID you should check this out.

INDIRECT ELISA



Indirect ELISA Video

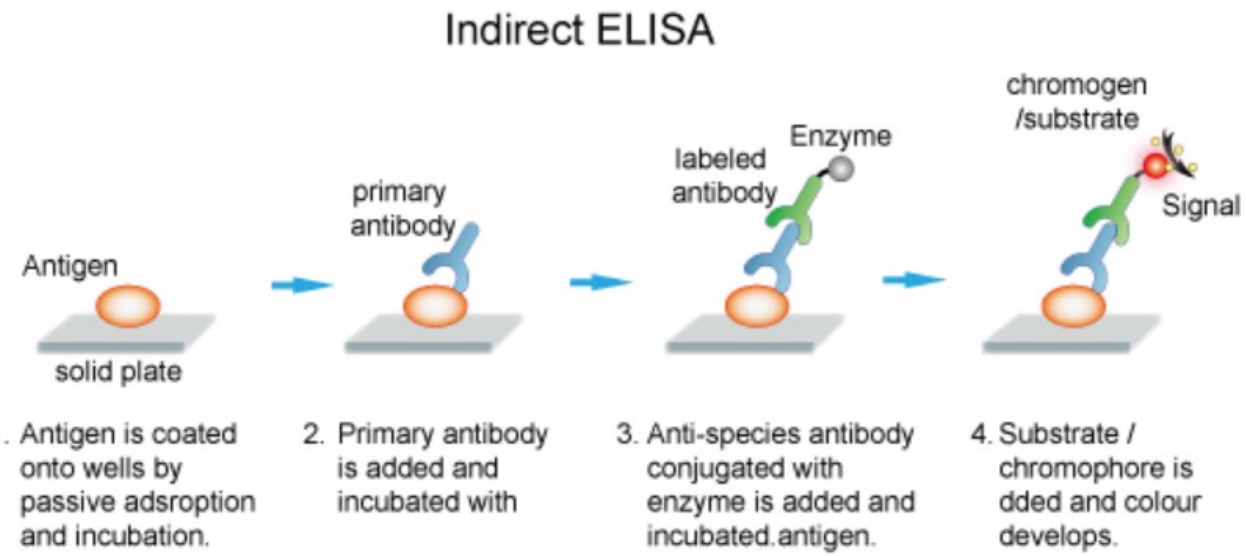
- https://www.youtube.com/watch?v=ECjGF1qMx6w (shows the steps ~ 4min)
- https://www.youtube.com/watch?v=Yfv7FtvozOg (short 90 sec Video explaining indirect ELISA)

INDIRECT ELISA

Plate Plan

3.16-fold ($10^{0.5}$-fold) dilution is called

a **half-logarithmic dilution** or **half-log**

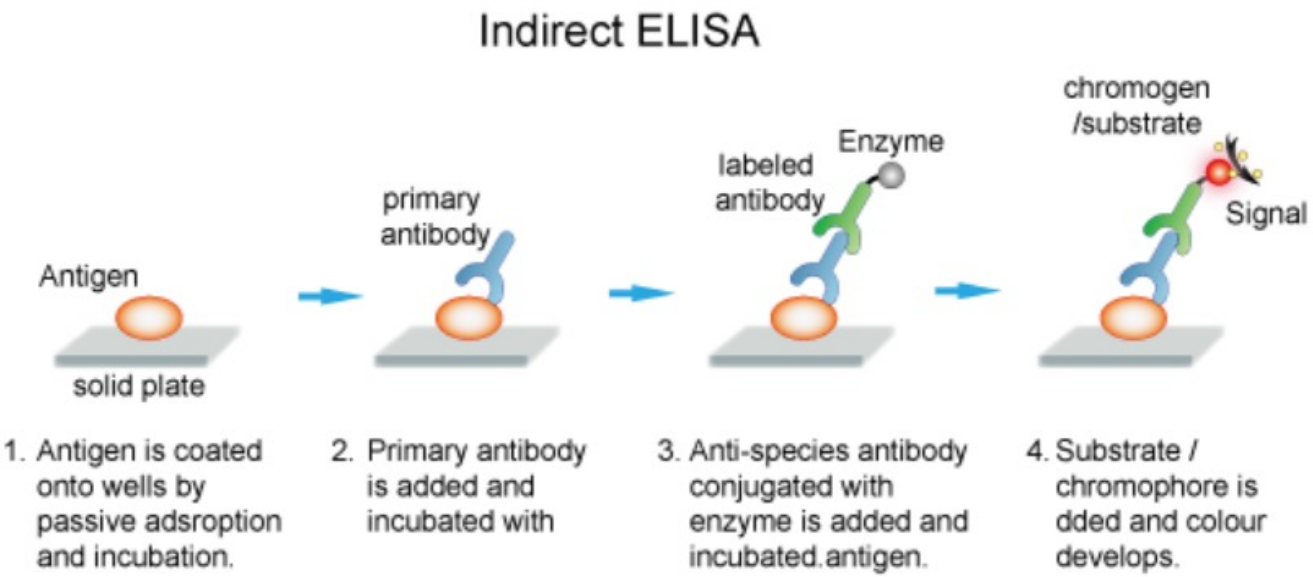


		1	2	3	4	5	6	7	8	
	A	Empty wells	Sample 1-neat	Sample 1-neat	Sample 2-neat	Sample 2-neat	Positive Serum	Positive Serum	No antigen added	
	B		3.16	3.16	3.16	3.16	1st diln	1st diln	No antigen added	
	C		10	10	10	10	2nd diln	2nd diln	No antigen added	
	D		31.6	31.6	31.6	31.6	3rd diln	3rd diln	No antigen added	
	E		100	100	100	100	4th diln	4th diln	No serum	
	F		316	316	316	316	5th diln	5th diln	No serum	
	G		1000	1000	1000	1000	6th diln	6th diln	No serum	
	H		3160	3160	3160	3160	7th diln	7th diln	No serum	

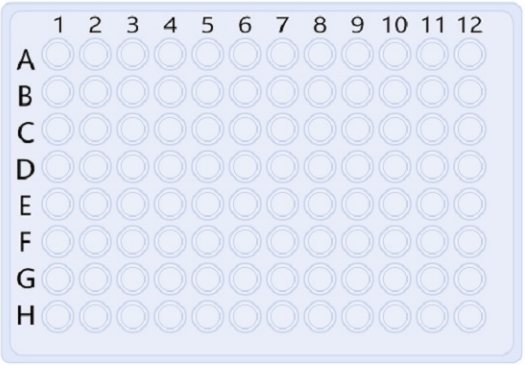
No antigen added but positive serum added in step 2

INDIRECT ELISA

Method



1: Coat wells with Antigen



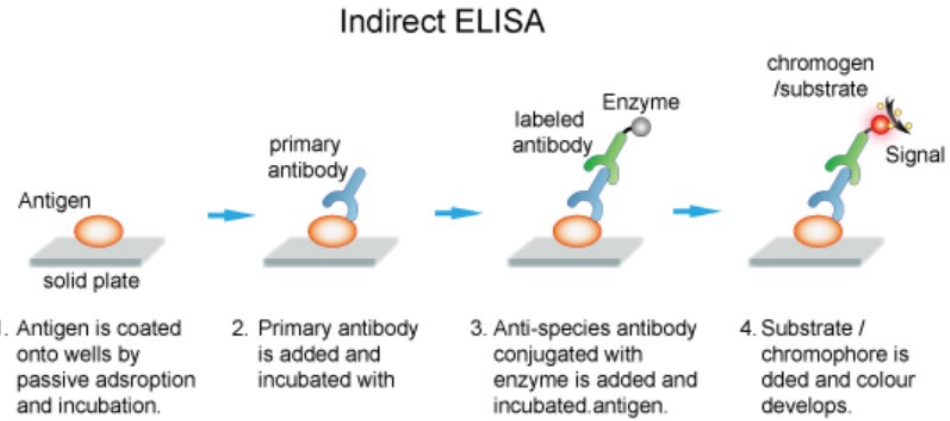
1. Add 5ug of the coronavirus S protein to 5ml of carbonate buffer.

Add 50 ul to each well of the ELISA plate -all 96 wells except

top 4- row 7, incubate 4ºC overnight.

Block Remove antigen, wash plate, add 100µl of 5% skim milk powder in phosphate buffered saline (PBS) to each well. Incubate overnight.

INDIRECT ELISA



2. Add Primary Antibody (serum samples)

Dilute serum samples using half-log dilutions of samples and controls- use a second 96 well plate.

To set up dilutions:

Add 100µl of milk/PBS to each of wells A-H of columns 1-4.

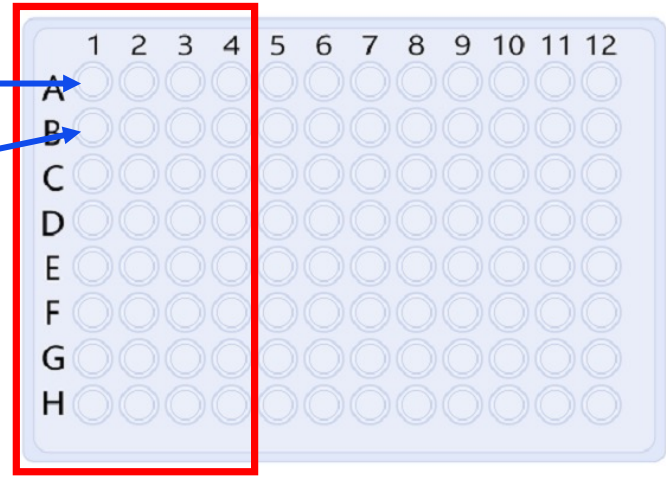
Well **A column 1** add 46.25 µl of the **sample 1** serum.

Perform 46.25µl into 100µl serial dilutions (using a new pipette tip, mix, and then remove 46.25 µl to well B,

Repeat for well C etc. to well H).

In column 2 repeat (using a clean tip) for **sample 2**.

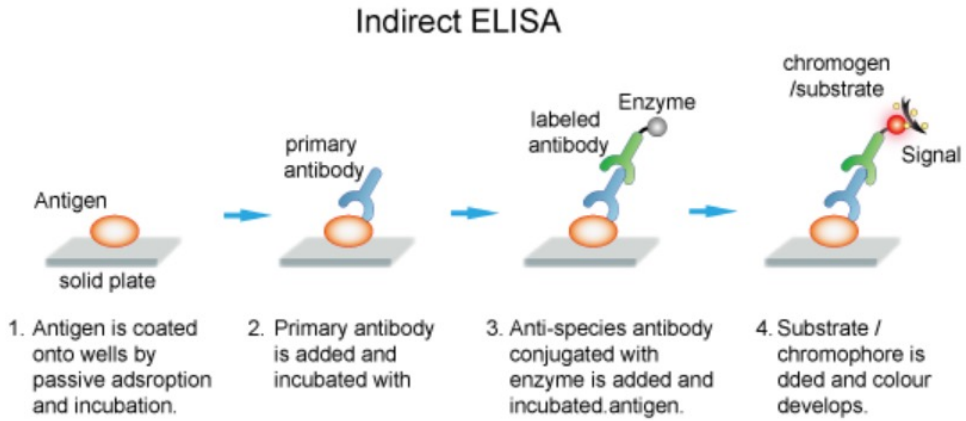
Use column 3 for the positive control serum dilutions and column 4 for the **negative control** serum dilutions.



INDIRECT ELISA

positive control serum dilutions and

negative control serum dilutions.



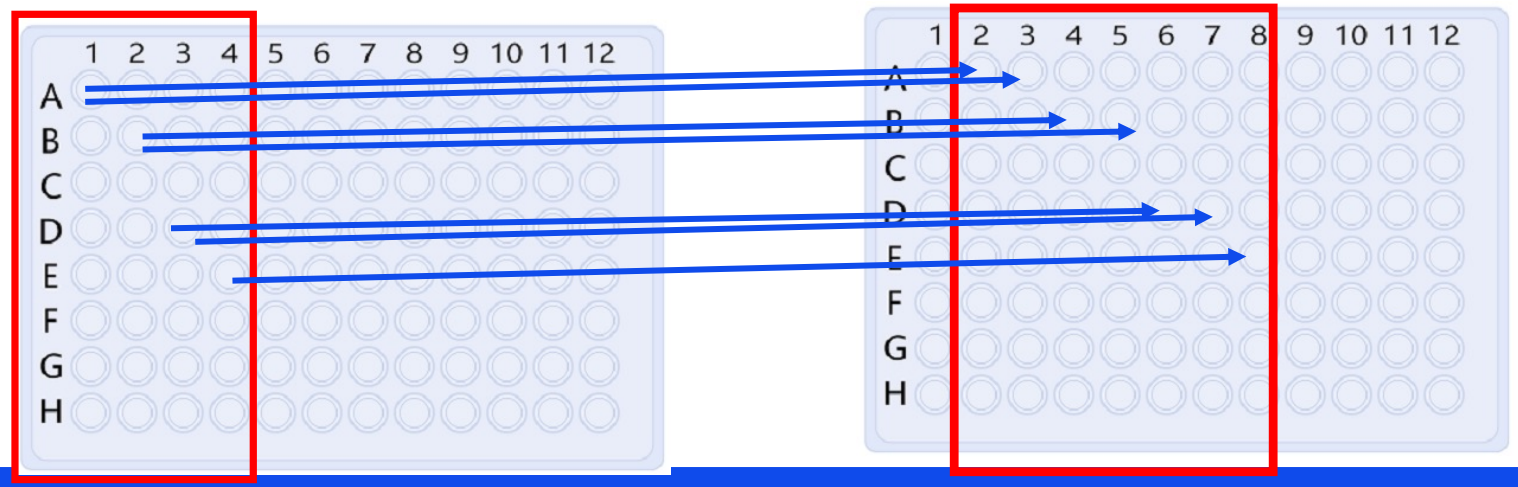
- **Negative controls** include
  o Omission of the antigen
  o Omission of the test serum or substitution with an antibody that will not bind the antigen.
- **Positive control** substitutes known positive serum for unknown serum.

INDIRECT ELISA

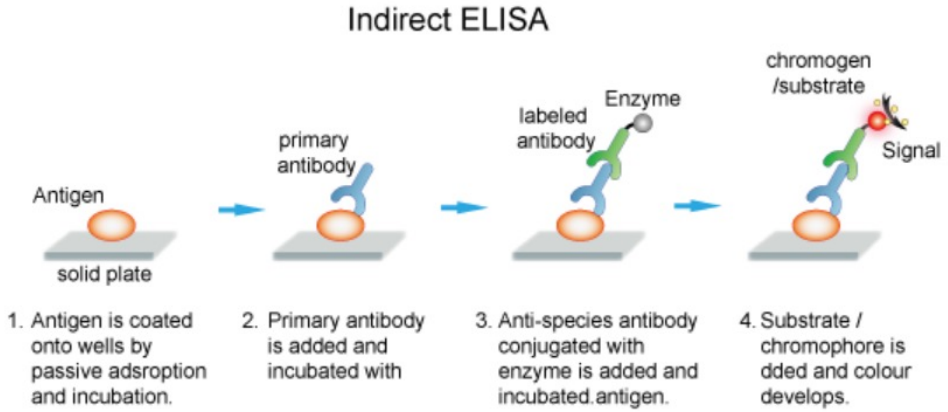
4. Empty the blocking buffer from the ELISA plate by flicking it out, and banging the plate dry over paper towels. (as per last weeks video)

5. Transfer primary Antibody (serum samples) to your coated and blocked ELISA plate, leave column 1 blank.

Add Dilutions of Sample 1 in column 1 to wells A to H of column 2 and column 3, by removing 50µl of each dilution into the appropriate well in column 2 and in column 3 to **provide duplicate column** and so on for all 4 columns- 8 columns in final plate. - Incubate the plate for 60 minutes at 37ºC.



INDIRECT ELISA



3: Add Secondary labelled Antibody

6. Remove the media, discard contents into a solution of fresh bleach, then wash each well four times with 200µl of PBS/Tween 20. Add 50µl of anti-human HRP conjugate diluted in milk/PBS to each well. - Incubate for 30 minutes at 37ºC.

7. Flick out the antiserum and wash the wells 6 times with PBS/Tween 20.

4. Add Substrate

Add 100µl of substrate (ABTS 1 mg/ml in citrate phosphate buffer pH 4 containing 0.06% v/v hydrogen peroxide) to all wells including column 1.

8. Read plates at 405nm on a microplate reader after 20 minutes incubation at room temperature.

ELISA Results

A	Empty wells	Sample 1-neat	Sample 1-neat	Sample 2-neat	Sample 2-neat	Positive Serum	Positive Serum	No antigen added
B		1st diln	1st diln	1st diln	1st diln	1st diln	1st diln	No antigen added
C		2nd diln	2nd diln	2nd diln	2nd diln	2nd diln	2nd diln	No antigen added
D		3rd diln	3rd diln	3rd diln	3rd diln	3rd diln	3rd diln	No antigen added
E		4th diln	4th diln	4th diln	4th diln	4th diln	4th diln	No serum
F		5th diln	5th diln	5th diln	5th diln	5th diln	5th diln	No serum
G		6th diln	6th diln	6th diln	6th diln	6th diln	6th diln	No serum
H		7th diln	7th diln	7th diln	7th diln	7th diln	7th diln	No serum

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.071	1.203	1.198	0. 520	0.510	1.303	1.132	0.155	0.049	0.045	0.044	0.041
B	0.103	0.820	0.881	0.379	0.415	0.682	0.606	0.123	0.058	0.052	0.063	0.046
C	0.114	0.600	0.623	0.309	0.274	0.516	0.457	0.121	0.054	0.047	0.059	0.067
D	0.093	0.429	0.402	0.237	0.205	0.422	0.361	0.109	0.046	0.056	0.050	0.051
E	0.115	0.353	0.309	0.169	0.160	0.328	0.310	0.107	0.051	0.047	0.053	0.046
F	0.109	0.283	0.287	0.141	0.140	0.234	0.207	0.129	0.046	0.044	0.048	0.055
G	0.100	0.201	0.158	0.143	0.146	0.151	0.141	0.116	0.050	0.050	0.050	0.045
H	0.109	0.120	0.126	0.124	0.122	0.120	0.111	0.119	0.039	0.053	0.053	0.043

How to Calculate Antibody Levels in Serum

- Need both negative and positive samples
- During routine sera evaluation **for research purposes**, researcher usually set a cut-off by taking into account the background levels of the assay (either without sera or with sera that is known to be negative).
- Some researchers take 2- or 3-fold the average background levels as the cut-off
- Others calculate the mean value + 3-SD for the negative wells and take this as the cut-off.
- Other use same dilution in all samples that best differentiates +ve and –ve samples
- What is important is to use a uniform criteria during a given study and report this criteria
- **For diagnostic assay**s usually have a panel of both negative and positive samples
- Use statistic methods to determine the cut-off value providing the best discrimination between them.

Calculating AB Titre

- You will need to graph your results
- Remember your serum dilutions are half log dilutions
- Calculate the antibody titre for your samples, positive and negative controls using one of the methods listed on the page before
- Create a figure of this
- Do you samples show antibody against SARS CoV-2?
- Prism has some good statistical analysis packages that you can use to compare results and to plot your graphs

Faculty of Science

Using Prism

- This link below will take you to the PRISM site and they have a series of videos about using Prism
- You are welcome to use any software you like to make graphs and do your statistical analysis, prism is an option that we use in the lab to make graphs and do simple statistical analysis like t-tests and ANOVA.

- https://www.graphpad.com/series/getting-started/

Questions

- This week we will do the indirect ELISA
- Next week we will go through the Virus Neutralisation Plague Assay and the immunofluorescence.
- The results for these are in your practical manual.