COVID-19 antibodies – epidemiology

Title page

Hello, I'm David Male from the Open University

In this video I will show you how you can detect antibodies against the corona-virus SARS-Cov-2, using a technique called ELISA - an enzyme-linked immunosorbent assay. This technique is often used to quantitate antibodies in diagnostic laboratories or for research purposes. We have recreated this technique in a virtual laboratory so that you can carry out the assay yourself and test a set of serum samples from August 2021 for the presence of antibodies against the virus. At that time, the majority of the UK adult population had received a vaccination against COVID-19, and a significant number had been infected and recovered from an infection with the virus.

ELISA screen

This diagram illustrates the key steps in an ELISA. We start with a 96-well polystyrene plate that has been sensitised with antigen. In this case the antigen will be a component of the SARS-COV-2 virus, either the external spike protein or an internal antigen of the virus called the nucleocapsid.

In the first stage diluted serum is applied to the wells on the plate. If there is specific antibody in the serum, it will bind to the antigen on the plate. The bound antibody is called the primary antibody. Any unbound proteins in the serum, including non-specific antibodies are then removed in a wash step.

In the second stage a ligand is applied to the plate, which binds specifically to the primary antibody. The ligand also has a coupled enzymatic portion, which is crucial for the following step. In some cases, the ligand itself may be an antibody that binds to the primary antibody. In this case it would be called a secondary antibody. The plate is then washed again to remove any unbound ligand.

Finally, a chromogen is put into the wells. The chromogen is a colourless chemical which generates a coloured end-product when acted on by the enzyme of the ligand. The coloured end-product is detected on a plate-reader. The more primary antibody is present, the more enzyme is bound to the plate and the more coloured end-product is produced. Hence this technique can quantitate how much antibody is present in the initial serum sample.

The virtual ELISA laboratory

Now let's look at how the virtual ELISA laboratory recreates the technique. The virtual laboratory allows many options, in choosing different serum samples, and the dilution series of these samples. You can also choose the time of the incubations with the antibodies and the number and duration of the wash steps. You can select from three different ligands and there is also a choice of chromogens for the development step. Finally, the results are read on a plate-reader, and it is important to select the correct wavelength filter. The results correspond with what you would see in a real laboratory, as these experimental conditions are varied. Moreover, if you happen to choose conditions that aren't quite right then your results may not be optimal, and perhaps even provide no usable results at all. So, it's important that you note exactly what you do, at each of the steps. It is also important to use positive and negative controls in each assay.

Once you have your experiments working well, I would encourage you to modify some of the conditions and see how this affects the results. This way, you will also learn a lot about technical aspects of the assay.

The exact appearance and layout of the experiments within the on-screen experiment will depend on the web-browser you are using and the screen-size. In this introduction I am using a desktop computer and Chrome as the browser.

Timings in the virtual ELISA laboratory

Before we start, a quick word about timing. In the virtual laboratory we have telescoped time, so an incubation that would normally take 60 minutes can be done in 1 minute. The incubations are started and stopped on a timer, like this. It means that an assay which would normally take 2-3 hours can be done in a few minutes. As mentioned, ELISA is normally done using 96-well polystyrene plates, that have been sensitised with a specific antigen. They are relatively cheap, used once and then discarded. If your ELISA experiment goes wrong, you can come back to the beginning and start again. Now let's take a look at the laboratory.

Step-1

The first step is to choose serum samples. These are samples taken from individuals that may or may not contain the antibodies that you are going to test for.

You can see 8 tubes containing separated blood samples with serum at the top of the tube and red blood cells at the bottom. Each tube represents a sample from one patient. Up to 8 different serum samples can be selected, one for each row of the ELISA plate. Select a box beneath one of the sample tubes and start to type-in the identity of the sample you want to select. The program will present you with the options available and you can then click to select them. Each sample you choose will eventually be assigned to one of the rows on the 96 well plate, which you will see in the next step. In this case I am going to use a standard as the first sample in row-A– it is a positive control, and I choose a negative control as the 2nd sample which will go on row-B of the plate. Samples from patients are normally coded so that laboratory staff cannot identify an individual by name, and there can be no mistake about the exact identity of the sample

Jump to end of screen [*Samples +con -con, N9921, C4443, C5050, H1151 F1949 Z8207]*

I have now selected 6 samples from the 60 available for the remaining rows of the plate.

Step-2

Step 2 shows a 96-well plate. You will see the identities of the samples chosen in step-1 are now listed besides each row on the plate. The task in this step is to perform a serial dilution of the serum samples. Put simply, a serial dilution is a successive dilution of a starting sample. You will see why this is important as you progress through the experiment.

The serial dilution is performed by adding a known volume of serum sample to the left-most well on the plate and mixing it with a known volume of diluent. A diluent is simply a liquid medium that is compatible with the sample, which we can use to dilute the sample. Every well on the plate contains 100µl of diluent. You need to take note of this volume so that you can work out what your serial dilution will be.

To make a serial dilution a volume of each of the serum samples chosen in Step 1 is added to the left-most well on the plate - well-1, it is mixed with the diluent, and then a defined volume is transferred to well-2 and mixed. The same amount is transferred from well-2 to well-3, then from well-3 to well-4, and so on down the plate to well-12. This procedure is carried out with the multi-channel pipette illustrated. By transferring a volume of the sample from well to well, and mixing it with fresh diluent each time, each sample is successively diluted along the plate.

In the virtual laboratory, you need to decide what volume you want to transfer from well to well by changing the setting on the pipette. In this case I am going to transfer 100µl volumes down the plate. Since there is 100 µl of the diluent in each of the wells, transferring 100 µl will produce a serial dilution where the concentration of the sample decreases by half each time. In this case, well-1 is a 1/2 dilution of the serum sample. This is because 100 µl of the serum sample was added to 100 µl of the diluent. Well-2 would be a 1/4 dilution and so on. To do this I will set 100µl on the pipette and press 'Transfer', which carries out the entire dilution series for me. You can now see the dilutions are given along the top of the plate.

The pipette volume can be set at anything between 20µl and 100µl - by choosing other volumes on the multi-channel pipette you can make other serial dilutions, such as one where the concentration of the sample decreases by a third each time, giving 1/3, 1/9, 1/27 and so on. It is up to you to choose what you think is an appropriate serial dilution for the ELISA. The doubling dilution series shown here is a good starting point, but if serum samples have very high titres of the antibody under investigation, then a higher dilution series may be required.

Step-3

In an ELISA the antibody that binds to the antigen on the plate is called the primary antibody. In this step, I first have to choose the antigen-sensitised 96-well ELISA plate that I will use for the assay. The drop-down menu lists all the available options. Here, I have two options and I want to detect antibodies to spike protein of the virus. When I choose the spike-protein ELISA plate, I am essentially getting a plate where an equal amount of the antigen is bound to every well in the plate. If there are relevant antibodies in the serum samples then they will attach to the antigen on the base of the wells.

Anyone who has been vaccinated or infected with SARS cov-2 is likely to have antibodies against the spike protein, but only individuals who have been infected are exposed to the internal proteins of the virus and will therefore usually also have antibodies against the nucleocapsid.

At this stage, we have two 96-well plates. One is our antigen-sensitised plate, and the other contains the serial dilutions of our serum samples. We now transfer the contents of the serial dilution plate to the antigen-sensitised plate. This brings the antigen and any antibodies together so that they can interact.

By pressing the 'Transfer' button diluted samples are transferred across from the serial dilution 96-well plate prepared in step-2. Importantly, the contents of each well in the serial dilution 96-well plate is moved into the corresponding position on the antigen-sensitised ELISA plate. You will notice that when you press 'Transfer' the wells all turn blue to indicate that liquid has been transferred.

Now we incubate the samples on the antigen-sensitised plate, by starting the clock

[start timer]

An incubation time of 45 minutes to 1 hour is recommended. I will stop the incubation now. [stop timer]

If you incubate for a short time, the antibody has less time to bind to the antigen, and the signal at the end of the assay will be lower. If you incubate for a bit longer than 1 hour, it will not make much difference.

The incubation allows the antigen and any antibodies in the samples to interact. This is such a strong interaction that we can 'wash' the plate quite vigorously to remove any unbound antibodies and other serum proteins. In essence, washing the plate simply means removing all the liquid from each well and adding a volume of fresh washing buffer. You need to choose how many wash steps to do and for how long you leave the wash liquid to incubate during each wash. Click on the 'wash start' button to begin a wash, and press 'wash stop' to end a wash.

Three washes of 5 minutes each are the minimum recommended. If you do not wash the plate sufficiently, residual unbound antibodies in the serum samples will neutralise the detection antibody, which will be added later.

[3x 5-minute washes]

Step-4

In Step 3, the 'primary antibody', in the serum sample (if indeed it is present), became bound to the antigen. The next step is to detect any bound antibody. This is done by using an enzyme-conjugated ligand, usually another antibody that we add to all of the wells in the 96-well plate. This detection antibody is called the 'secondary antibody' because it binds to the primary antibody. The reason why we use a secondary antibody to detect the presence of the primary antibody will become clearer as you progress.

In step 4, you are presented with three vials that contain secondary antibodies. There are three major classes of antibody present in serum- immunoglobulin-M, immunoglobulin-G, and immunoglobulin-A, which are referred to as IgM, IgG, and IgA. Each of the secondary antibodies specifically detects one of the classes of serum antibodies. In this demonstration I want to detect IgG antibodies, so I select the vial Anti-human IgG which is conjugated to the enzyme horse-radish peroxidase or HPO. You should also notice that the concentration of the secondary antibody is shown on the label.

The stock of the anti-human IgG HPO conjugate has a concentration of 1.5mg/ml. This is too concentrated to use neat, so I need to prepare a dilution.

The recommended concentration for the anti-IgG antibody in this assay is 0.6µg/ml, and I need 10mls of the solution in total to add to the 96-well plate. I therefore need to add 4µl of the anti-IgG stock solution to the 10mls of diluent.

The way to calculate the required volume of stock is shown in the panel. The volume of stock is the final concentration divided by the stock concentration, multiplied by the final volume, that is 0.6µg/ml divided by 1500µg/ml multiplied by 10,000µl. If you did not follow that, just take it on trust that you need 4µl of the stock anti-human IgG.

The optimum concentration of secondary antibody depends on the reagent and batch and in a real lab each new batch of antibody would be tested. Typical optimum concentrations will be in the range 0.5µg/ml - 5µg/ml.

The pipette-volume can be set at anything between 2µl and 100µl.

If you do not use enough of the secondary, detection antibody, the signal at the end of the assay will be low. If you use too much, the background values will be high and in any case that would be wasteful of an expensive reagent.

Step-5

In step 5, the secondary (detection) antibody is added to every well on the plate.

As before, this antibody is incubated on the plate for 45-60 minutes [start timer] ….[stop timer].

This incubation allows the secondary antibody to bind to the primary antibody. If the incubation is too short, the signal at the end of the assay will be reduced.

After the incubation, the unbound secondary antibody must be removed from the plate with washes. As before, a minimum of three 5-minute washes is recommended.

[3 x 5-minute washes]

If you add an extra wash or make the washes slightly longer, it will make little difference, but you will find that 3 washes of 5 minutes are more efficient than ..say.. 1 wash of 15 minutes.

Step-6

In this step, we are going to use the enzyme activity of the horseradish peroxidase linked to the secondary antibody to develop a coloured product in each of the wells.

To do this, I will add a chromogen solution to every well on the plate. In this case there are three options. I will choose TMB which is tetra-methyl benzidine. It produces a blue-coloured end product when acted on by horseradish peroxidase. Once the reaction has started the colour develops progressively with time. At an appropriate time of your choosing, the activity of the horseradish peroxidase enzyme should be stopped by adding sulphuric acid.

You should allow enough time for the colour to develops so that you can see visible differences between successive wells. If you run the reaction for too long, eventually every well will develop colour, background values will increase.

An incubation time of 5-30 minutes is typically used for these assays. In this case, I will stop the reaction after 20 minutes by the addition of sulphuric acid.

[Start and run chromogen reaction for 20 minutes]

You will notice that the sulphuric acid not only stops the activity of the enzyme, but also turns the blue end-product of the enzyme reaction, bright yellow.

Step-7

Finally, we get to see the results of the assay by using a 96-well plate-reader that can detect the concentration of the yellow-coloured end-product in each of the wells.

Plate readers shine light at the 96-well plate and record how much light passes through each of the wells. Most plate readers can detect light of several different, defined colours, - the colour is selected by placing an optical filter in the light path.

To detect the yellow-coloured end-product a filter of 450nm is optimal, so I select that here.

[450nm filter]

If I had chosen a different chromogen in the preceding step, then I would need a different filter at this point. For example, OPD, O-phenylene diamine, produces a red end-product and the 645nm filter is appropriate.

Now we can read the absorbance values. The 96-well plate is taken into the plate reader and the absorbance read. The plate is then returned on the tray.

In the final step you can view the results, which are presented in an 8 x 12 array corresponding to their position on the ELISA plate.

The results can now be taken for analysis.

Step-6 reprise

Let's look again at the appearance of the plate. You can see that the negative control on row B has essentially no colour on any of the wells. The positive control on row-A shows a progressive decrease in colour across the plate at least up to well-8 which is a serum dilution of 1:256. The antibody titre is normally expressed as the reciprocal of the highest serum dilution that gives a detectable signal, so the positive control has a titre of 256. You can also see immediately that 4 of the samples in rows C-H are positive for IgG antibodies against spike protein and two are negative. A simple estimate by eye is very useful in health-care settings where a plate reader is not available, but we can do better than this if we have the absorbance values from the plate reader.

ELISA plate with data:

After you have read the plate using the appropriate filter, you should have a dataset that looks like this. Notice that the absorbances are set out in a 12 x 8 grid, which corresponds exactly to their position on the ELISA plate.

The samples in wells 1-12 are in doubling dilutions and the reciprocal of the dilution is now shown in the bar beneath the table.

Look first at the positive control in row-A. The most concentrated sample in well-1 has an absorbance of 1.305, and the absorbance values decrease progressively towards the most dilute sample in well-12.

If I look at the negative control row, there is a background value which is about 0.09 and the highest value, in well-B1 is 0.106. We can therefore say that an absorbance value above 0.12 is a good cut-off point for assessing whether there is a significant level of absorbance above the background.

I have now highlighted all the wells above this threshold. From this we can read off the titre in each sample, and this is now entered on the right-hand side.

It is really important to note that each assay will be slightly different, depending on the exact conditions you use. You need to work this out for yourself by looking at your own data, and it will be different on each assay. Also be aware that duplicate samples will usually give slightly different results, reflecting the variation that is seen in this type of assay.

The results will always be more accurate and reliable, if the samples and standards are done in duplicate or triplicate, and a mean value of the absorbance is used.

Conclusions:

I have now shown you how to determine the titre of IgG antibodies against SARS-Cov-2 spike protein using ELISA. You can do a lot of different analyses using this set of 60 serum samples. Here are some suggestions:

You could test a larger group of samples for IgG antibodies against spike protein, to estimate what proportion of the adult population in the UK had some immunity to SARS-Cov-2 in August 2021.

You could test the sera for antibodies against nucleocapsid antigen, to estimate what proportion of the population had had an infection with SARS-Cov-2. Both infected and vaccinated people will have antibodies to spike protein, but only people who have been infected are likely to also have antibody against nucleocapsid antigen.

Given information on the age and sex of the individuals, which is available, you could determine whether women have higher levels of antibodies than men or whether younger people have more antibody than older people.

You could also test for IgM antibodies. IgM antibodies arise early during an immune response and decline much more quickly than IgG, so comparing IgG and IgM antibodies in a single immune individual can give some indication of how long ago they were vaccinated or infected.

You could test for IgA antibodies. IgA is important in protecting mucosal surfaces of the respiratory tract. You could then see whether the titres of IgG and IgA are correlated in different individuals.

As you can see there are many investigations that could be done with this set of serum samples. I hope you have found this introduction to the ELISA technique useful and its application to investigation of COVID-19 antibodies, interesting.