PRAC: ELISA
VENUE: FNB2A 53 PCs and FNB2B 69 PCs
Date: Monday 7 May
Time: 14:00-17:00

We will be sitting 2 students per computer

** You are welcome to do this prac ahead of time and just hand it in on Monday at 2pm at the prac venue.

Measuring immune responses

What to measure?

- Types and amount of pathogen present
  - E.g. HIV viral load or HBV surface antigen

- Types and amounts of immune cells present
  - E.g. how many CD4+ T cells present?
  - E.g. what % of all leukocytes are granulocytes?

- How well an immune cell is functioning
  - E.g. test proliferation, cytotoxicity , specificity

- Types and amount of substances produced by immune cells
  - E.g. antibodies against specific pathogen – how much made, what type?
  - E.g. how much of a particular cytokine was produced

What to measure?

- Types and amount of pathogen present
  - E.g. HIV viral load or HBV surface antigen

e.g. PCR for HiV DNA

- Types and amounts of immune cells present
  - E.g. how many CD4+ T cells present?
  - E.g. what % of all leukocytes are granulocytes?

e.g. flow cytometry

- How well an immune cell is functioning
  - E.g. test proliferation, cytotoxicity , specificity

Complex assays

- Types and amount of substances produced by immune cells
  - E.g. antibodies against specific pathogen – how much made, what type?
  - E.g. how much of a particular cytokine was produced

e.g. ELISA

What to measure?

- Types and amount of substances produced by immune cells
  - E.g. antibodies against specific pathogen – how much made, what type?
  - E.g. how much of a particular cytokine was produced

i.e we are trying to

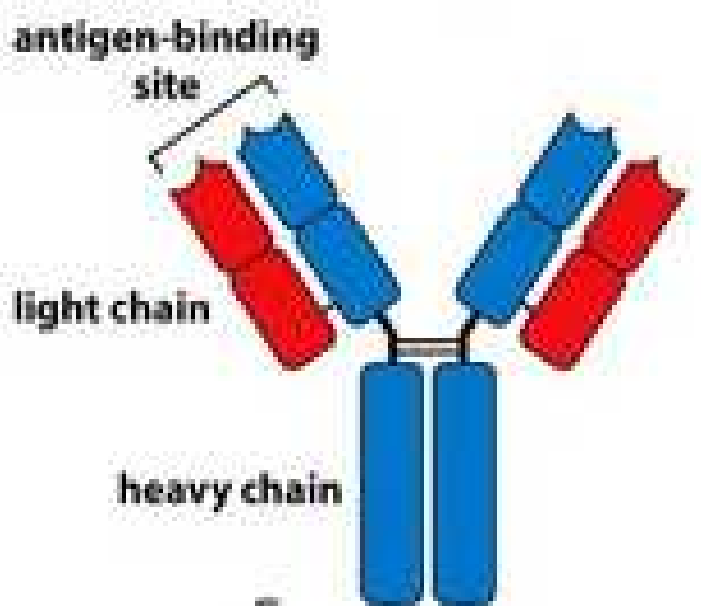
1. detect (presence/ absence)
2. quantify (how much)

a SPECIFIC PROTEIN

How?
ANTIBODIES BIND TO PARTICULAR TARGETS WITH HIGH SPECIFICITY….

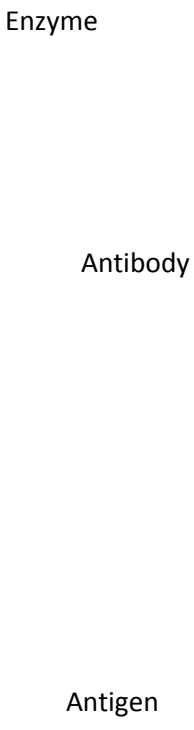
Antibodies

- **=immunoglobulins**
- are proteins secreted by B cells into the plasma
- High antigen specificity i.e. only recognise a particular piece of a particular pathogen - can bind to that piece of pathogen
- Can specifically bind to other substances e.g other proteins, toxins
- Dimers: each part has a light chain and a heavy chain
- the ends of the chains are different in every antibody – this is where the antigen binds
- Your body can produce ~ 10^{12} different types of antibodies



IMMUNOASSAYS

- Biochemical test
- measures the presence/concentration of a specific macromolecule in a solution – this is known as the analyte
- Technique uses an analyte-specific antibody
- Binding of analyte to antibody must result in a measurable signal.
- Often the antibody is linked to a labelling substance that will produce a detectable signal
- Many types of labels:
  - emit radiation
  - Enzymes that produce a color change in a solution
  - fluoresce under light
  - emit light.



LABEL: something detectable attached to antibody, allows us to measure the binding of antibody to analyte

ANTIBODY: binds specifically to the analyte

ANALYTE: what we are trying to detect or quantify

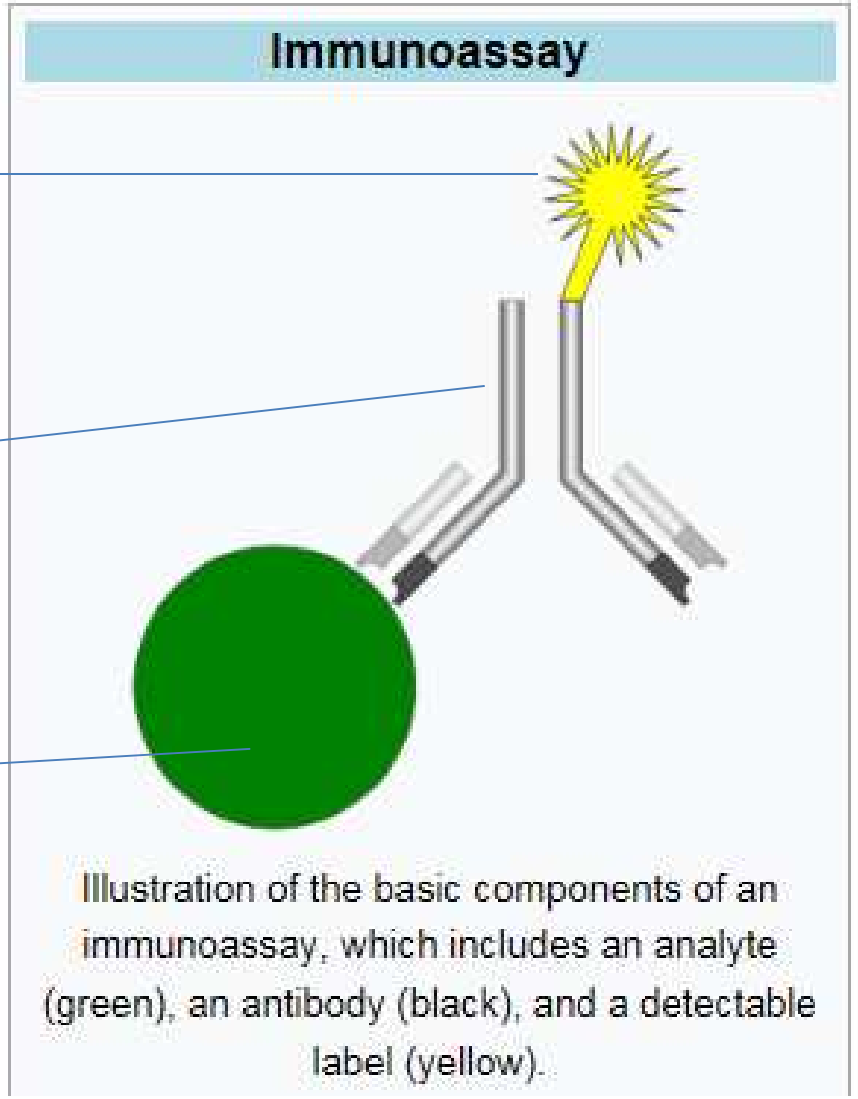
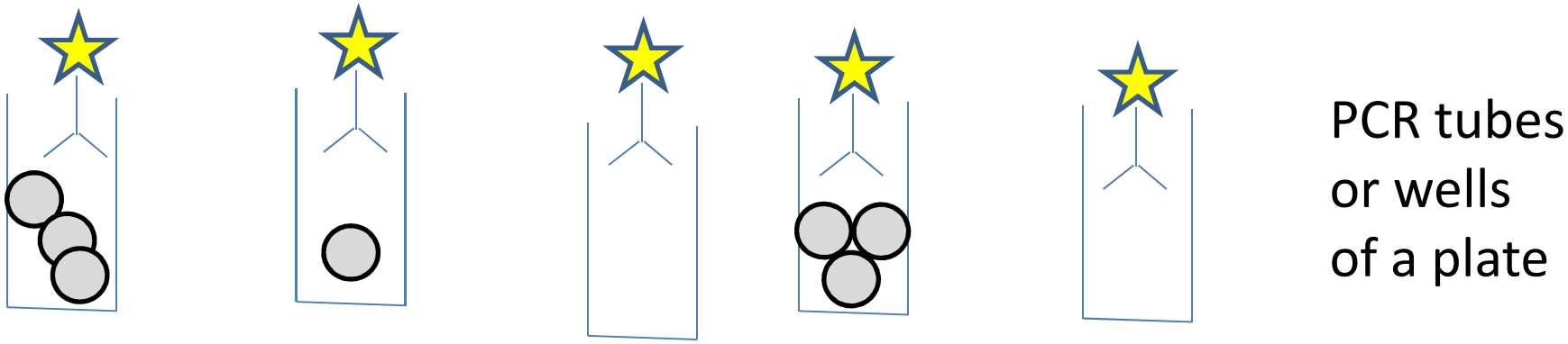


Illustration of the basic components of an immunoassay, which includes an analyte (green), an antibody (black), and a detectable label (yellow).

Example of a simple immunoassay

1. Add test samples and controls to tubes
2. Add analyte-specific, labelled antibody
3. Wash off any unbound antibody



Test Sample 1

test sample 2

test sample 3

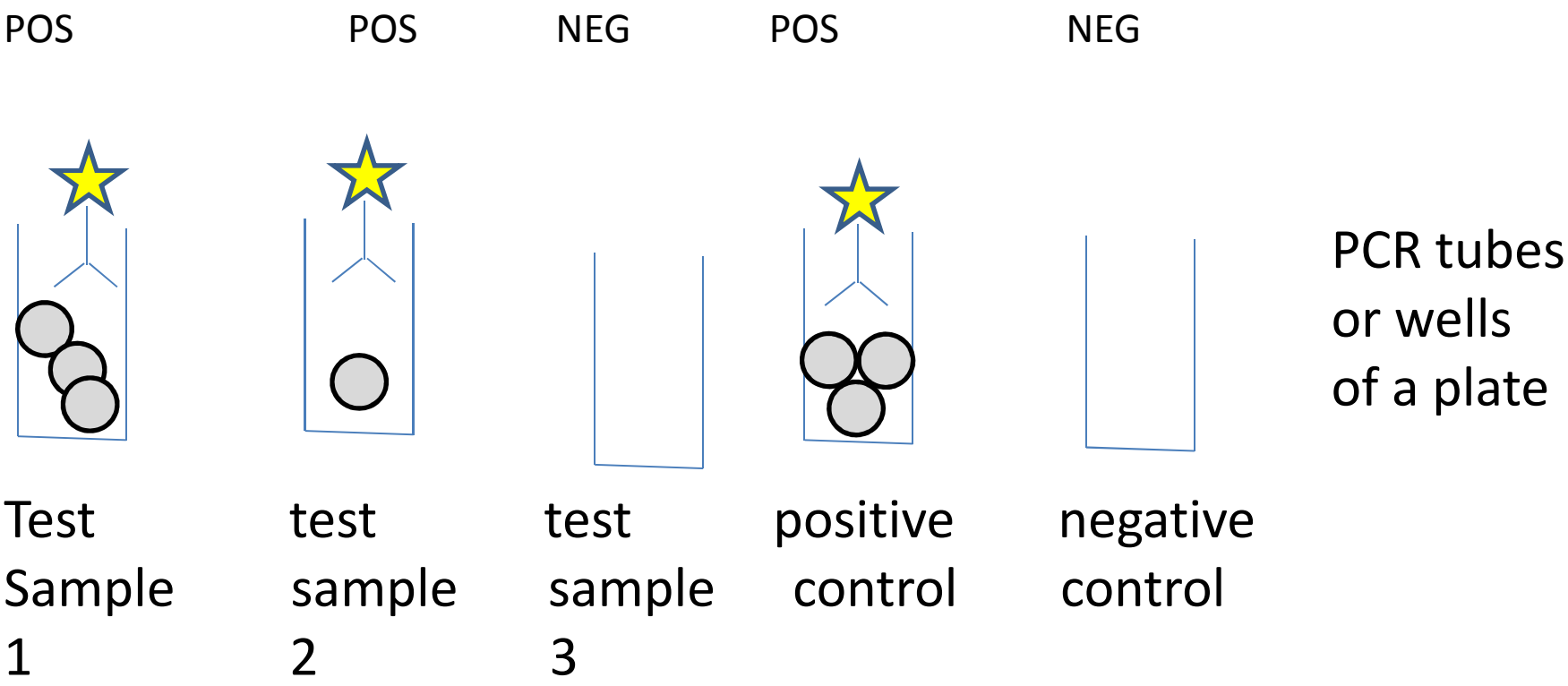
positive control

negative control

PCR tubes or wells of a plate

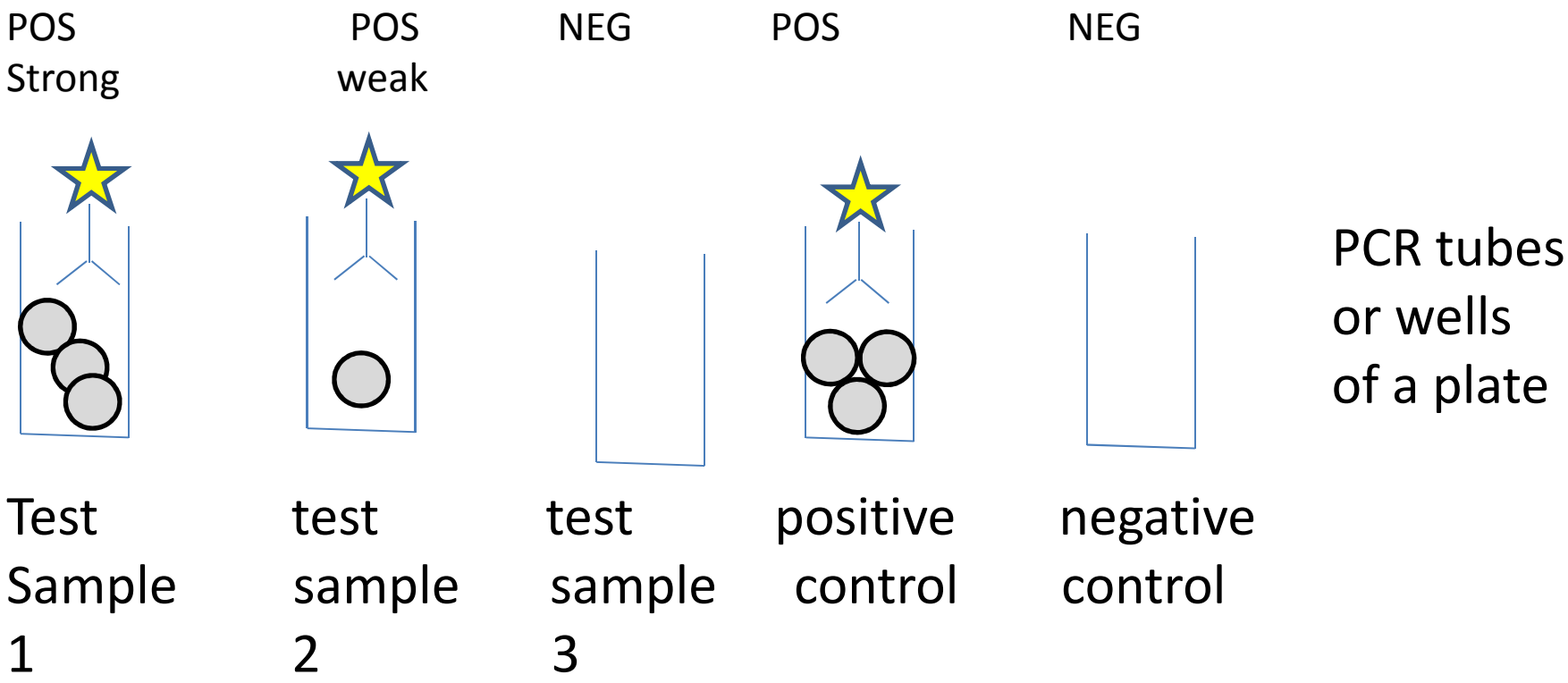
Example of a simple immunoassay

1. Add test samples and controls to tubes
2. Add analyte-specific, labelled antibody
3. Wash off any unbound antibody
4. Read results (e.g. look for light, fluorescence)



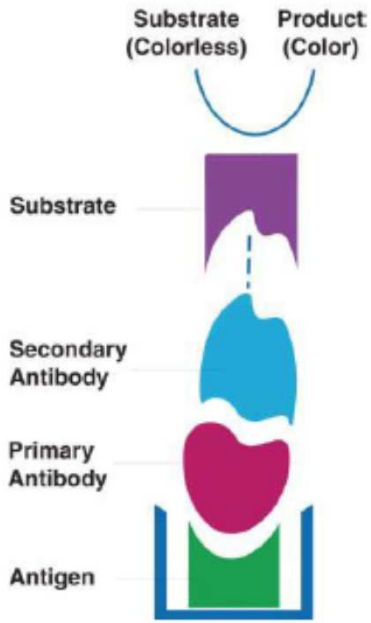
Example of a simple immunoassay

1. Add test samples and controls to tubes
2. Add analyte-specific, labelled antibody
3. Wash off any unbound antibody
4. Read results (e.g. look for light, fluorescence or colour)



ELISA

=enzyme-linked immunosorbent assay



Other types of labels:

Are often enzymes that can drive a colour change in the solution

The most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP)

In the presence of hydrogen peroxide (H2O2), HRP oxidises colourless substrate 3,3',5,5'-tetramethylbenzidene (TMB) to form a blue colour

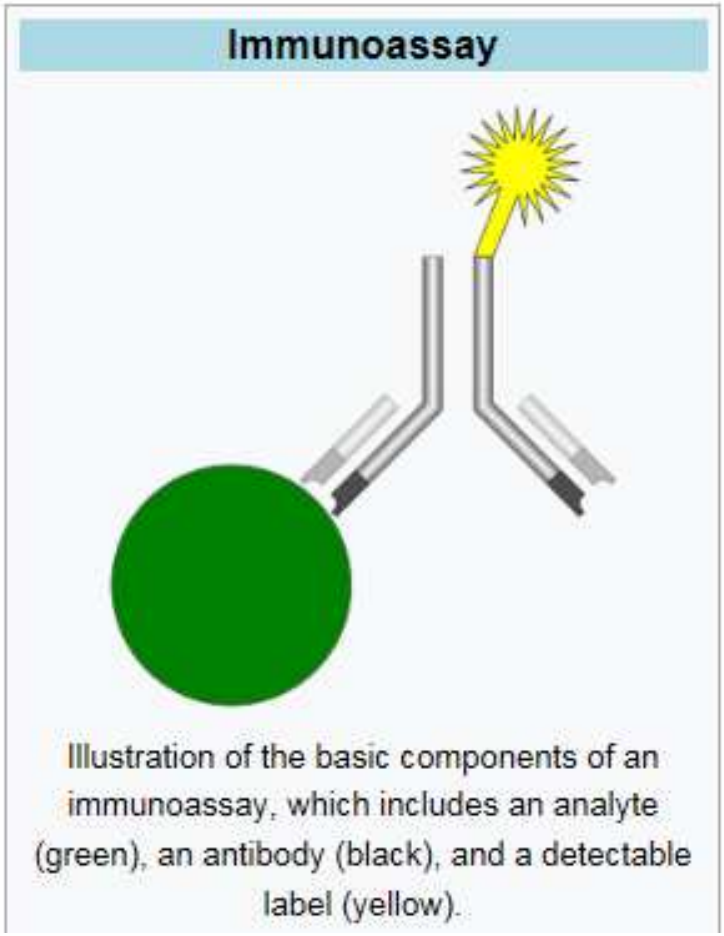


Illustration of the basic components of an immunoassay, which includes an analyte (green), an antibody (black), and a detectable label (yellow).

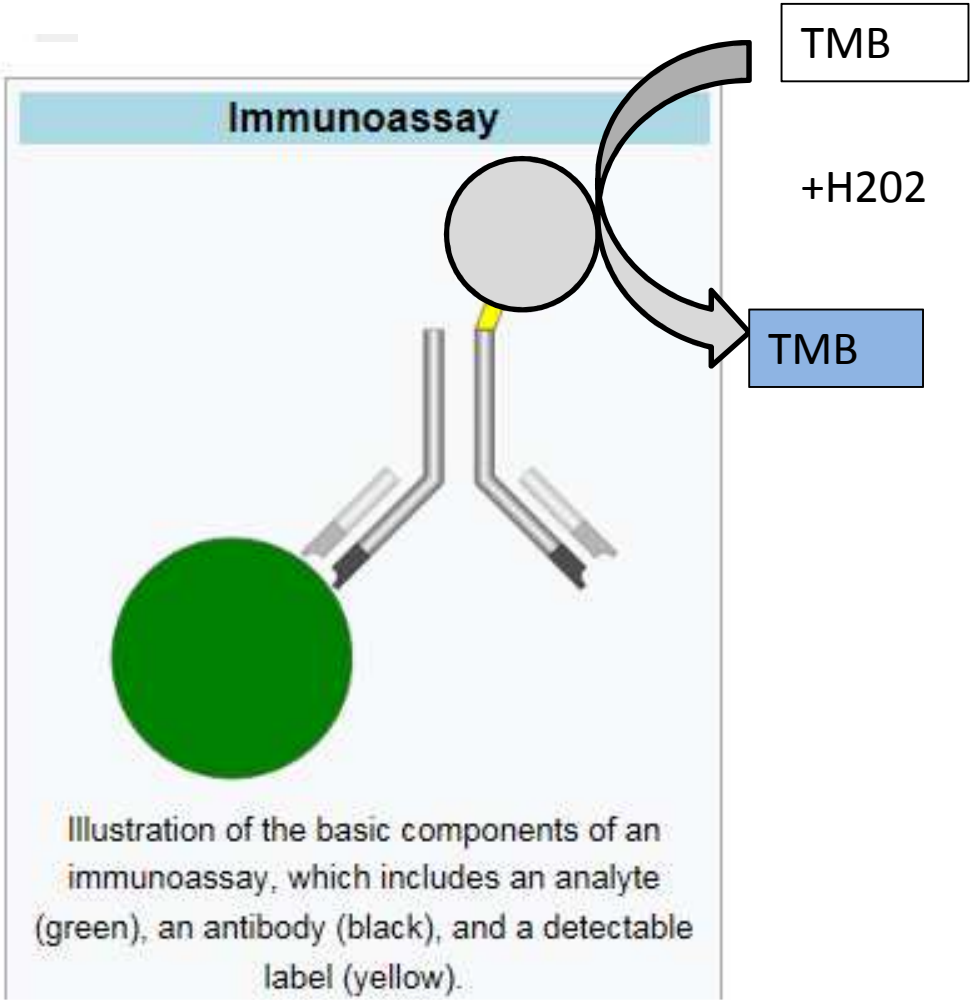
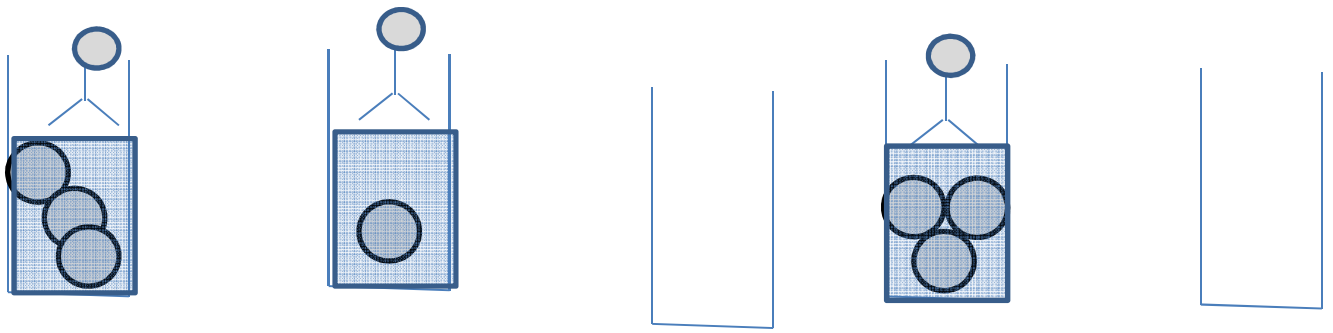


Illustration of the basic components of an immunoassay, which includes an analyte (green), an antibody (black), and a detectable label (yellow).

Example of a simple immunoassay

1. Add test samples and controls to tubes; add TMB (colourless)
2. Add analyte-specific, labelled antibody (has HRP enzyme)
3. Wash off any unbound antibody; add H2O2
4. Read results (e.g. look for TMB = blue colour)

POS Strong	POS weak	NEG	POS	NEG



Test Sample 1	test sample 2	test sample 3	positive control	negative control

PCR tubes or wells of a plate

ELISA = Enzyme-linked immunosorbent assay

- Also called solid-phase enzyme immunoassay (EIA)
- Can be used to detect various proteins such as antibodies, antigens, cytokines , hormones
- Sample types to be tested = serum, plasma, urine etc

- Qualitative – is antigen/antibody present (Y/N)
- Quantitative– amount of antigen / antibody present
- Units of measurement: numerical concentration e.g. pg/ul

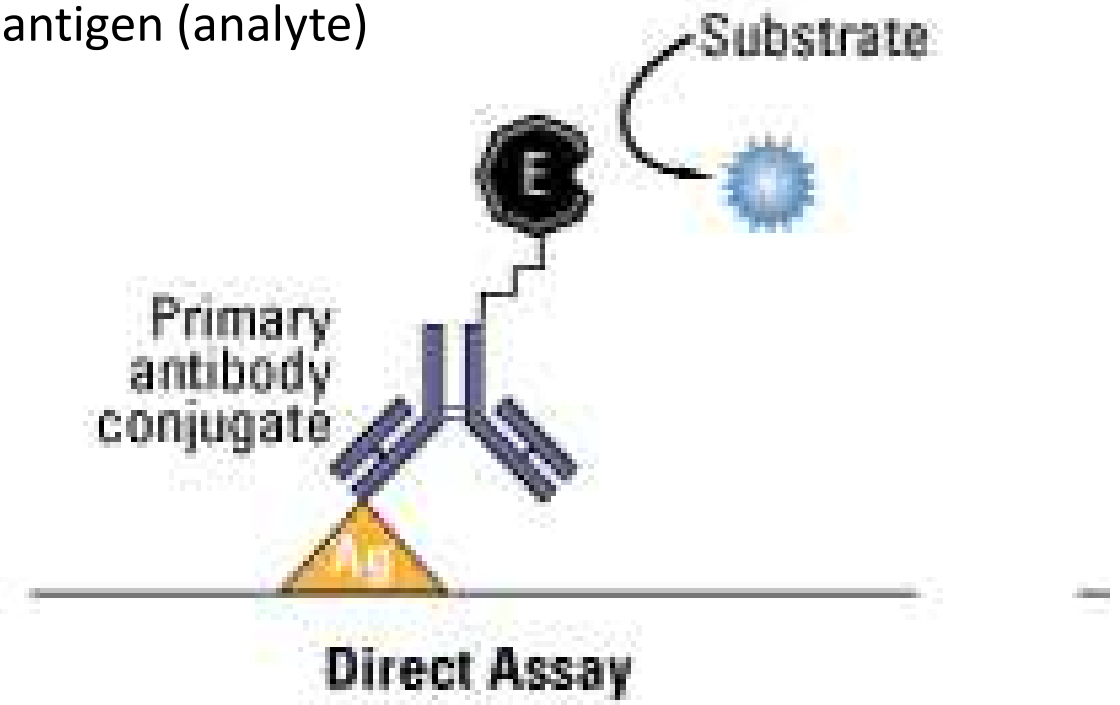
- highly specific antibody-analyte interaction
- Label to detect binding

- 3 types
- 1. direct ELISA
- 2. indirect ELISA
- 3. sandwich ELISA

Direct ELISA for antigen detection

Uses ONE labelled antibody

1. Add sample to the plate – may or may not contain antigen (analyte) of interest
2. Add specific antibody with label e.g. enzyme for colour change
3. If antigen is present in sample: highly specific antibody-antigen binding
4. Wash off unbound antibody
5. Add substrate; enzyme drives substrate colour change
6. Colour indicates presence of antigen (analyte)

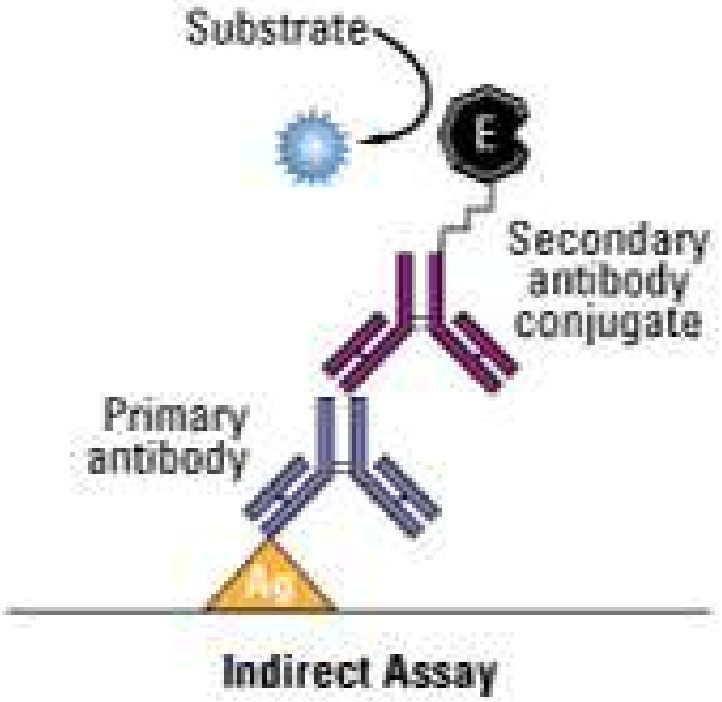


Indirect ELISA for antigen detection

Uses one unlabelled antibody and one labelled antibody

1. Add sample to the plate – may or may not contain antigen of interest
2. Add specific primary antibody; this antibody does NOT have any label attached to it
3. Add secondary antibody: the secondary antibody has the detectable label attached to it; the secondary antibody specifically binds to the primary antibody

4. if antigen is present in the sample: high specific antibody-antigen interaction
5. Wash off unbound antibody
6. Add substrate; enzyme drives substrate colour change
7. Colour indicates presence of antigen



Primary (1°) antibodies: The antibodies that recognize and bind to the antigen in an immunoassay are primary antibodies.

e.g. human antibodies against HIV antigen

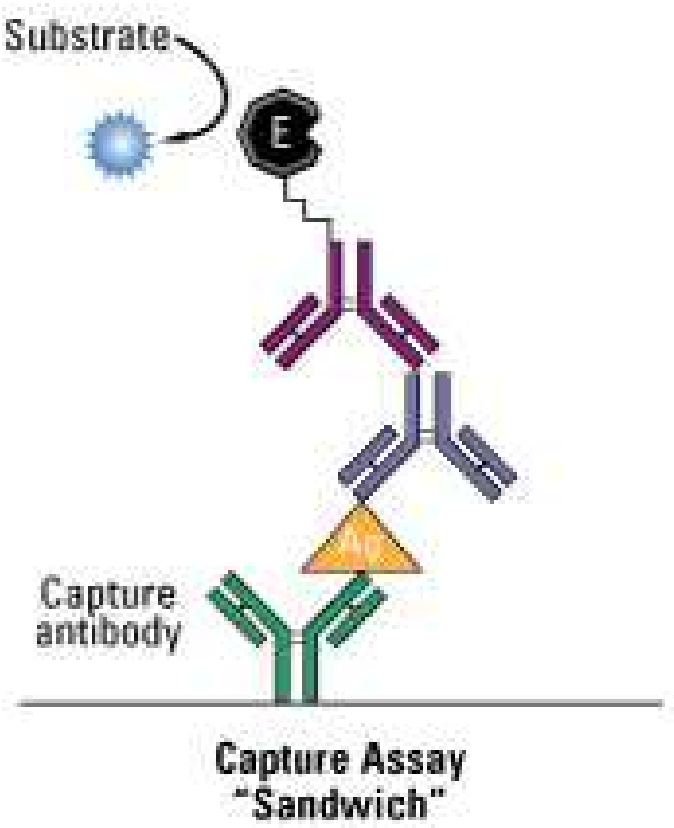
Secondary (2°) antibodies: Secondary antibodies recognize and bind to primary antibodies. They are made in animals of a different species than that used to make the primary antibody.

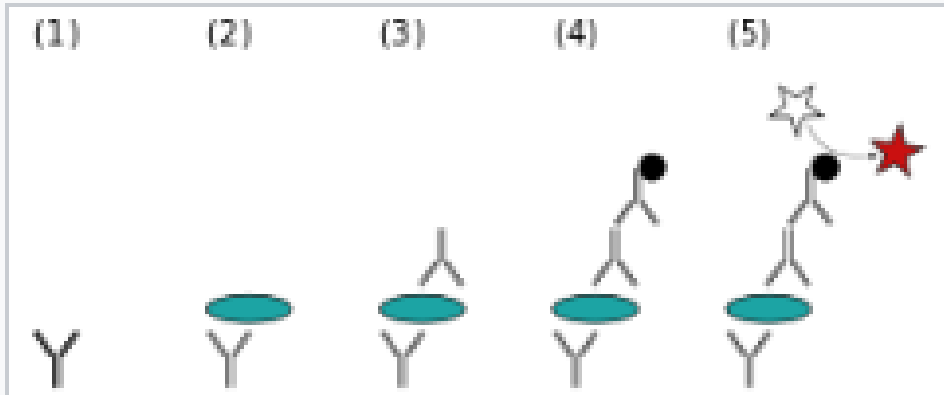
e.g. anti-human antibodies produced in rabbits

Sandwich ELISA for antigen detection

Uses one capture antibody, one unlabelled detection antibody and one labelled detection antibody

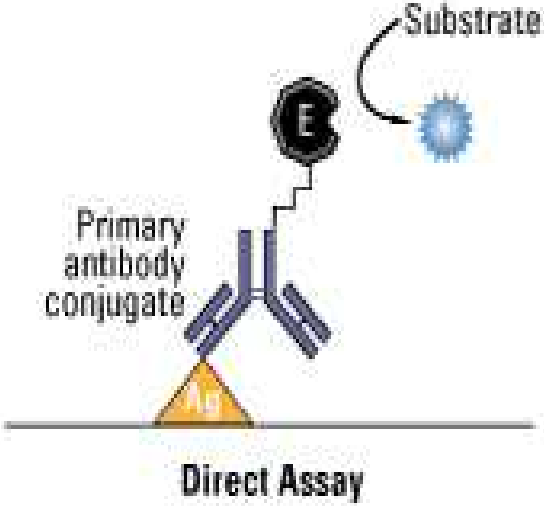
1. Add capture antibody to plate
2. Add sample to the plate – may or ma not contain antigen of interest - antigen binds to capture antibody
3. Add specific antibody-enzyme conjugate
4. if antigen is present in the sample: highly specific antibody-antigen interaction
5. Wash off unbound antibody
6. Add substrate; enzyme drives substrate colour change
7. Colour indicates presence of antigen



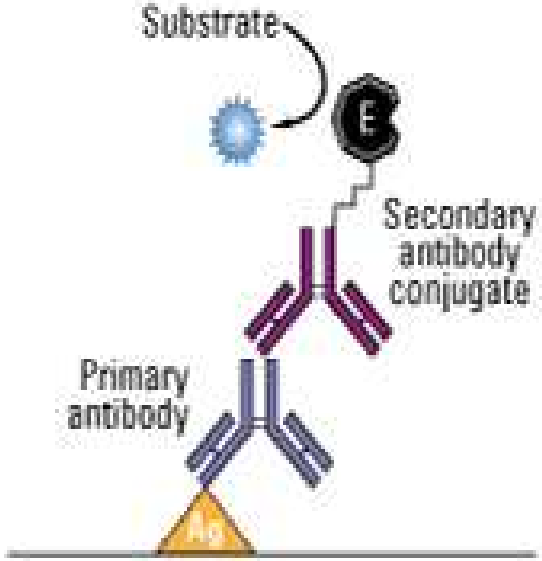


A sandwich ELISA. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.

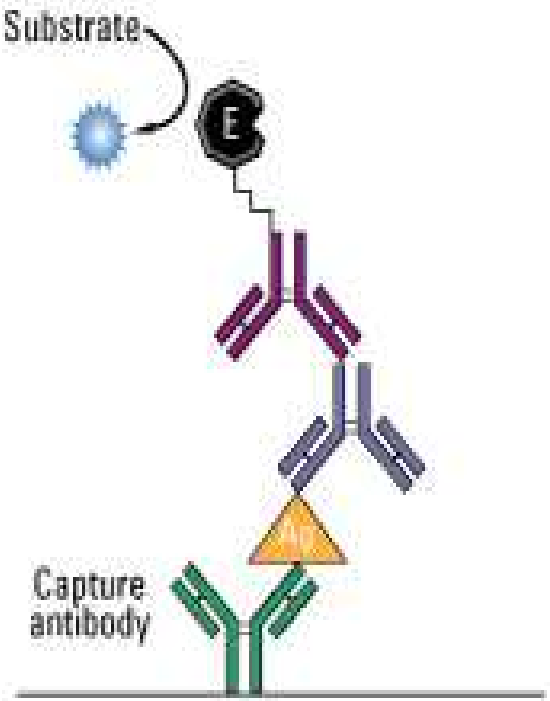
3 ELISA methods for antigen detection



Direct Assay



Indirect Assay

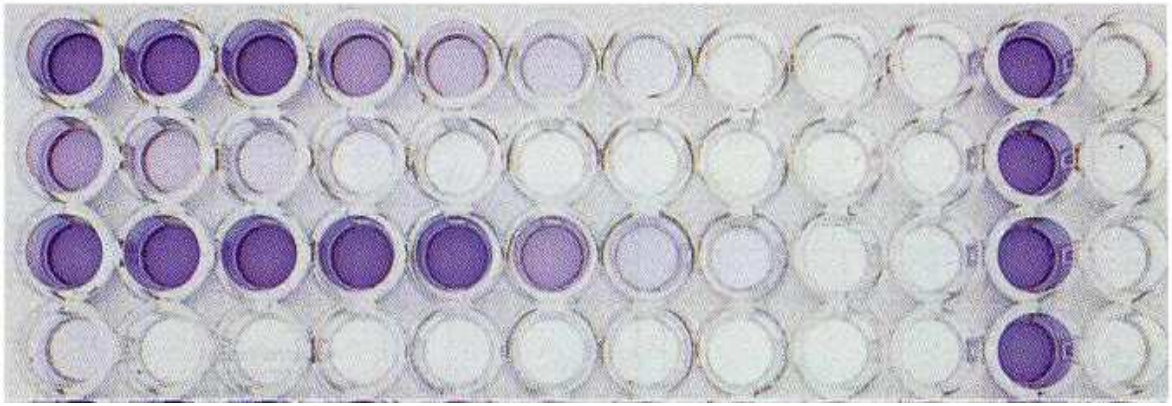


Capture Assay "Sandwich"

Microplate readers

- quantitate the absorbance of light by the colored substrate in each well of a microplate.
- They use the negative control wells to set a baseline and then read the absorbance of each well at a specified wavelength.
- For example, the peak absorbance for TMB is at 655 nm.
- Quantitative ELISA controls include a dilution series of known concentrations that is used to create a standard curve. This standard curve allows the concentration of antigen in a sample to be quantitated

- Assay measured as: colour detection units (a number)
- Strength of signal allows both qualitative (antigen or antibody present: Y/N) and quantitative analysis (how strong is the signal)



For reliable results:

- May use blocking agents to prevent nonspecific
- binding of antibodies to the plastic plate (e.g. detergent)
- Usually repeat all tested samples and controls in duplicate or triplicate wells

For accurate quantitative results:

- May need several positive controls with a range of known analyse concentrations (draw a calibration curve)
- Compare test sample colour density to controls
- May try diluted vs non-diluted samples
- Use a plate reader to quantify colour changes

Controls

Controls are always run side by side with actual samples to make sure that the procedure is working correctly and controls must be included in any valid ELISA.

- For the **negative control**, the antigen or primary antibody can be omitted
- The **positive control** always contains the target analyte

- A negative sample that gives a positive assay result is called a **false positive**.
- A positive sample that gives a negative assay result is called a **false negative**.

Many diagnostic assays give a percentage of false positive or false negative results, so confirmation of diagnosis by a second type of assay is important.
For example, immunoassays for antibodies to human immunodeficiency virus (HIV) can give either false positive or false negative results. False positives can result from recent vaccinations, and false negatives can result from immunosuppression (e.g., from drugs given after transplants) or from administering the test too soon after infection with HIV. (Antibodies against HIV do not appear until some weeks after HIV infection; the appearance of specific antibodies is called seroconversion.)

ELISA – applications

- pregnancy test – uses sandwich ELISA for antigen detection – uses specific antibody to look for the presence of the beta subunit of **hCG** or human chorionic gonadotropin in the blood or urine
- HIV Rapid tests – look for HIV antigen OR look for HIV antibodies
- Other rapid tests: Look for other viral antibodies e.g. West Nile virus
- Food industry – look for potential allergens (antigens) such as nuts
- Toxicology – rapid screen for certain drugs
- Test for antibody titre. Test to see if someone is immune to a disease

Question : how can we use ELISA to detect / quantify ANTIBODIES in a human sample?

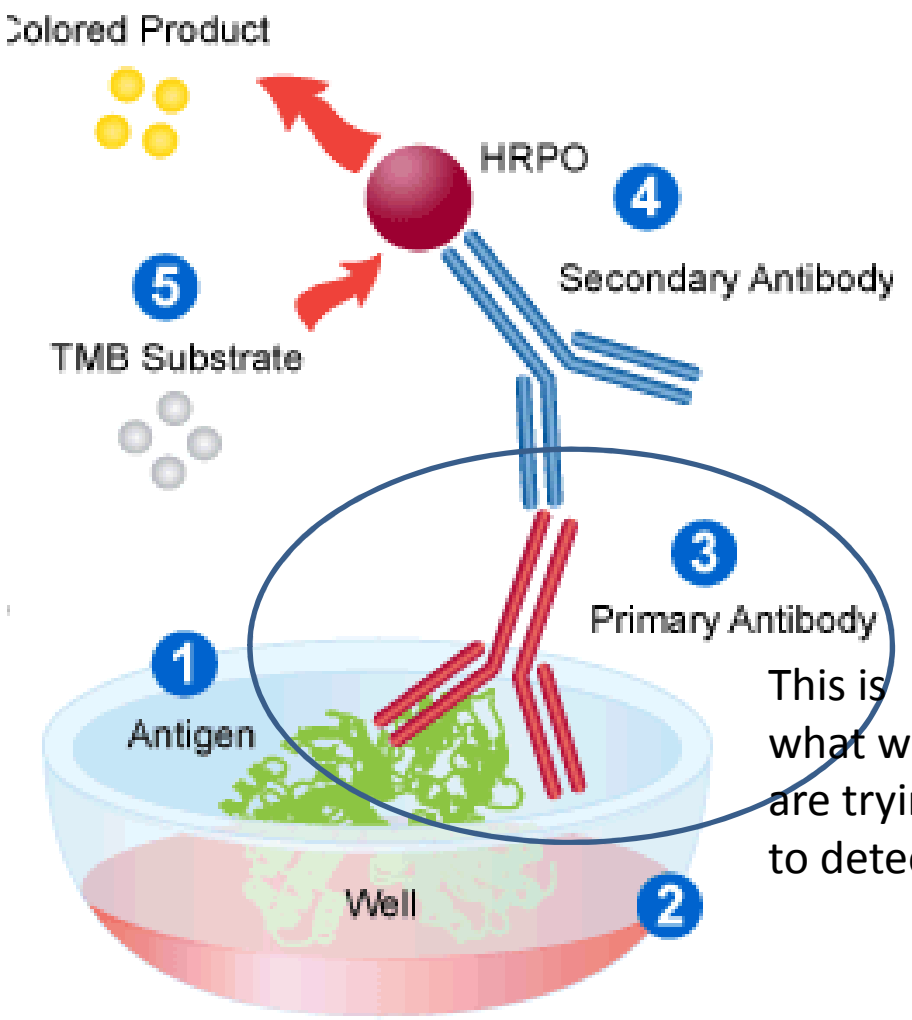
In this case we are not trying to detect a protein analyte /antigen
We want to measure an antibody titre e.g. to decide if a person is immune to a disease

ELISA for antibody detection – usually indirect method

1. Coat all wells with antigen; this is not what we are trying o detect, this will capture any antibodies in the test sample

2. Add sample (e.g. serum) –if antibody is present: binds to antigen
3. Add secondary antibody – enzyme conjugate
4. Add substrate - enzyme drives substrate colour change
5. Colour indicates presence of antibody

(if no antibodies ins ample – secondary antibody wont be able to stick = no colour change)



ELISA

Advantages

✓ Quick and convenient

✓Antigens of very low or unknown concentration can be detected

✓Generally safe

✓Many applications

✓Can measure specific analytes within a crude preparation.

Disadvantages

Uses a sample of many mixed cells :results are not cell type specific

Only monoclonal antibodies can be used (recognize one specific epitope) which cost more than polyclonal antibodies & are more difficult to find

✓Negative controls may indicate positive results if blocking solution is ineffective

✓Enzyme/substrate reaction is short term / colour change can be light-sensitive, so plates must be read as soon as possible