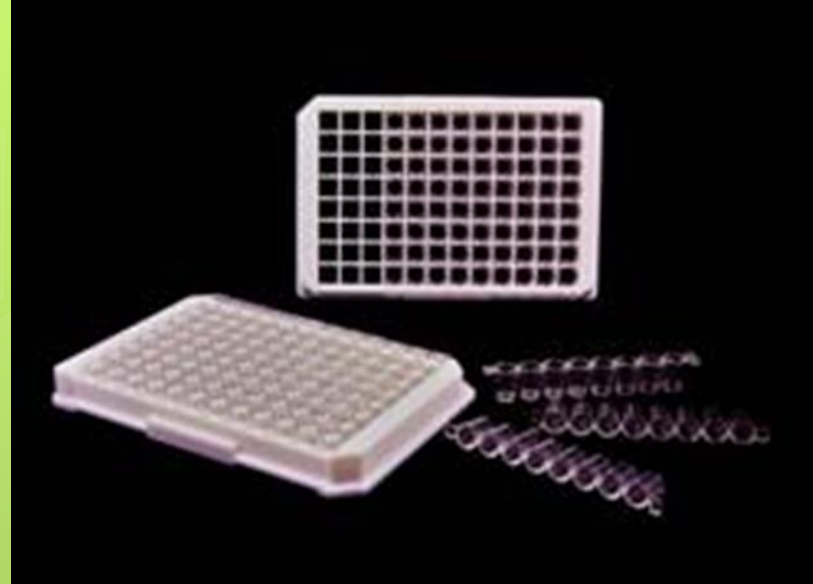

Lab 3 ELISA Practical immunology

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Labeled immunoassay

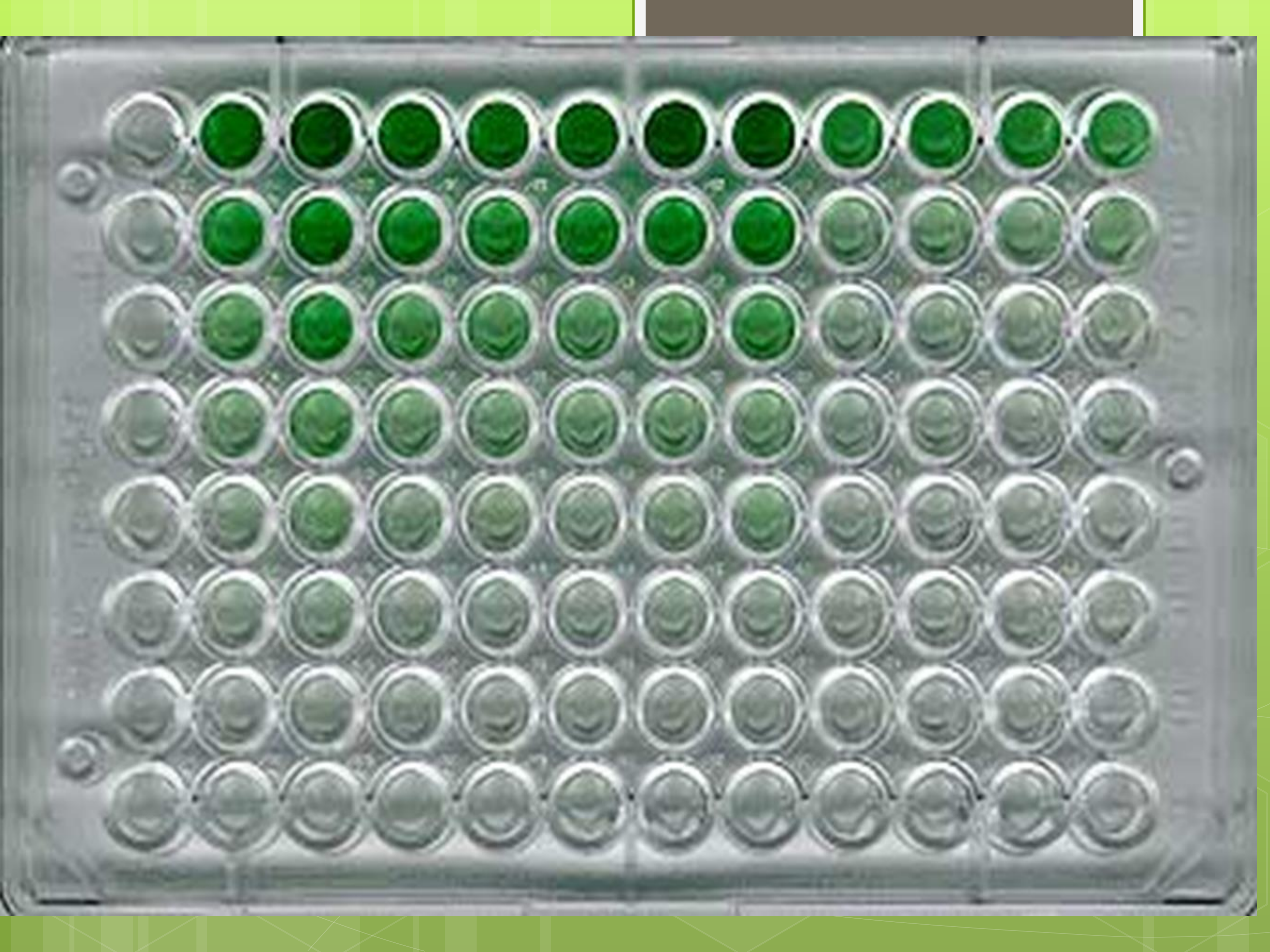
- **The technique of labeled immunoassay using labeled reagents for detecting antigens and antibodies**

- **Labeled immunoassay employing ligand labeled with:**

1. Enzymes (Enzyme-linked immunosorbent assay; ELISA)
2. Fluorscent dyes (Fluorscent immuno assay).
3. Radioisotopes (Radioimmuno-assay)

Enzymes immunoassay
Enzyme-linked immunosorbent assay; ELISA

- is a very sensitive immunochemical technique which is used to access the presence of specific protein (antigen or antibody) in the given sample and it's quantification.
- ELISA (Enzyme-linked immunosorbent assay) is one of immunoassay method used to detection of 1-Antibodies 2-Proteins 3-Peptides 4-Biomolecules
- It is also called solid-phase enzyme immunoassay as it employs an enzyme linked antigen or antibody as a marker for the detection of specific protein.



Why known as ELISA

- 1. Antigen/antibody of interest is absorbed on to plastic surface („sorbent‟).
- 2. Antigen is recognized by specific antibody („immuno‟).
- 3. This antibody is recognized by second antibody („immuno‟) which has enzyme attached („enzyme-linked‟).
- 4. Substrate reacts with enzyme to produce product, usually colored.

APPLICATIONS OF ELISA

- Serum Antibody Concentrations
- Detecting potential food allergens

 (milk, peanuts, walnuts, almonds and eggs)

- Disease outbreaks- tracking the spread

of disease e.g. HIV, bird flu, common, colds, cholera, STD etc

- Detections of antigens

 e.g. pregnancy hormones, drug allergen,

- Detection of antibodies in blood sample for

 past exposure to disease e.g. Lyme Disease, trichinosis, HIV, bird flu

- **Principle of ELISA**
- ELISA is a plate-based assay technique. Along with the enzyme-labelling of antigens or antibodies, the technique involves following three components (1-Antibody (antiserum) 2-Antigen 3-Labeling materials)

in combination which make it one of the most specific and sensitive than other immunoassays to detect the biological molecule:

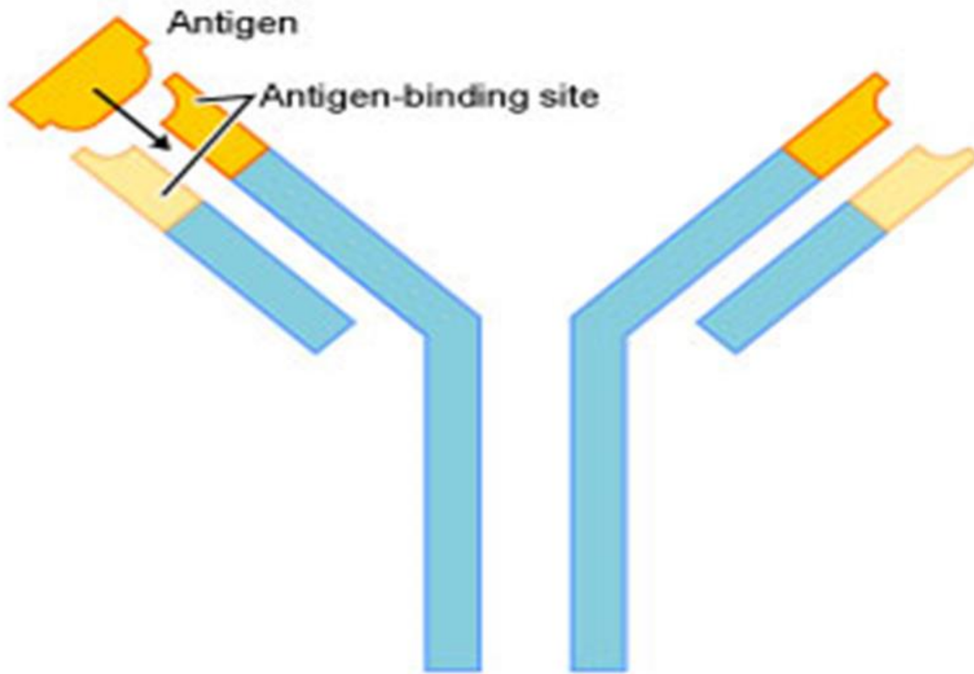
Important components of

- 1-Antibody (antiserum) 2-Antigen 3-Labeling materials
- Antibody: proteins produced by the immune system which help defend against antigens

The variable regions are though to be the place for recognition and binding with the antigen.

- Antigen: Any molecule that induces production of antibodies when introduced in the body is called antigen. OR □ Any “thing”, foreign to the immune system. e.g. bacteria, viruses, (or their parts), pollen, etc.

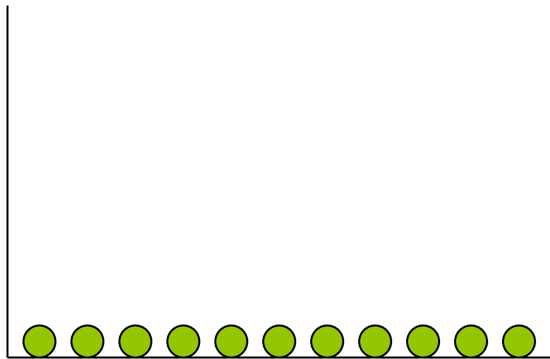
Antigens


Antigen
Antigen-binding site
Antibody

Coating

1.Coating: Polystyrene plate coated with Ags or Abs

Coat solid phase with antigen when analyzing antibody and antibody when analyzing antigen



Analyte = antibody

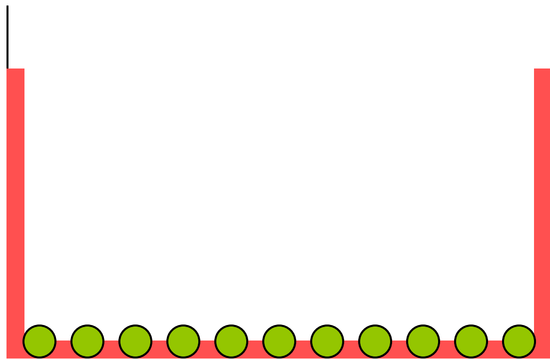
Analyte = antigen

Incubate, wash

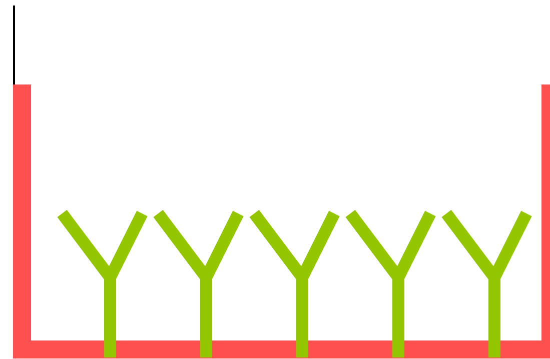
Blocking protein

- A concentrated solution of non-interacting protein, such as bovine serum albumin (BSA) or casein, is added to all plate wells. This step is known as blocking, because the serum proteins block nonspecific adsorption of other proteins to the plate

2. Blocking: Block free binding sites. Incubate. Wash.



Analyte = antibody



Analyte = antigen

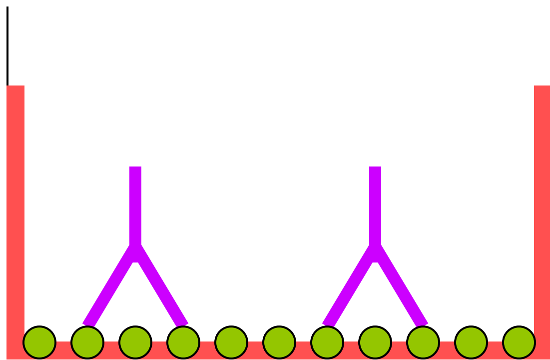
2-SAMPLE and CONTROLS

- samples may be added directly or in some cases may be diluted in a given ratios before adding them in the wells
- Dilute in buffer-Tween 20

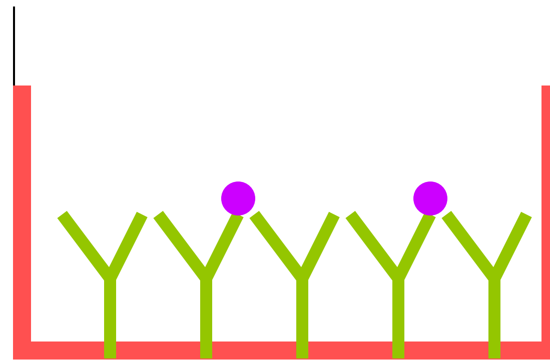
- Standards and Controls: are references against which the value of the analyte in the samples is estimated, they include known positive and negative controls

- standards usually are recombinant protein

3. Add sample. Incubate. Wash



Analyte = antibody

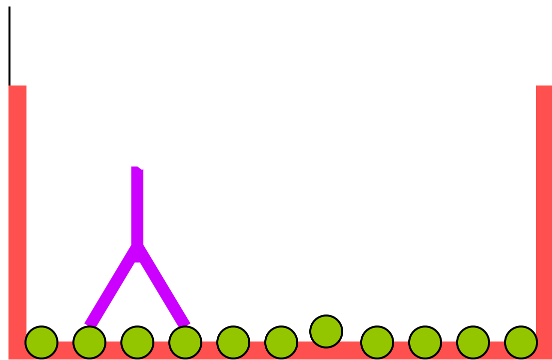


Analyte = antigen

Enzymatic Detection

- Two enzymes are commonly used in ELISA applications.
- Alkaline Phosphatase (AP) is a large enzyme used in a minority of assays. Its size (140 KDa) makes it difficult to conjugate more than one or two molecules of the enzyme to each molecule of an antibody, and this limits the amount of signal that can be generated. AP is also prone to stability issues unless stored and handled correctly.
- Horseradish Peroxidase (HRP) is a more commonly used enzyme. Its small size (40KDa) allows more molecules to be coupled to antibodies, and this can boost signal generation. HRP is the enzyme of choice for most researchers performing ELISAs and can be used with a variety of substrates.

Detection

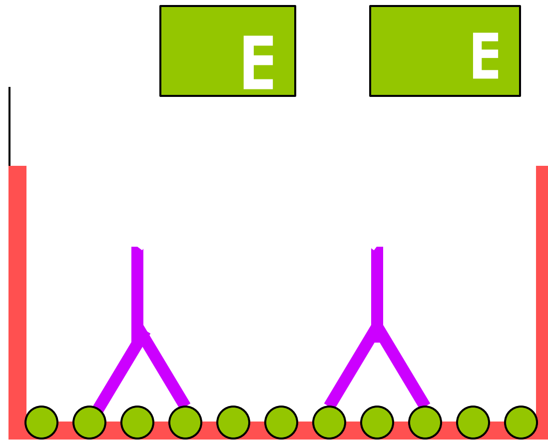


3- conjugate : it is an either Ag or Ab tagged(labeled) with an enzyme.

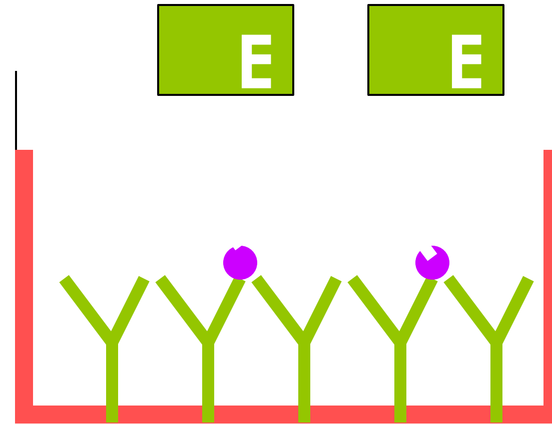
Different types of enzyme s used in labeling:

- Alkaline phosphatase
- Horseradish peroxidase
- G-6-PD
- Glucose oxidase

4. Add conjugate. Incubate. Wash.

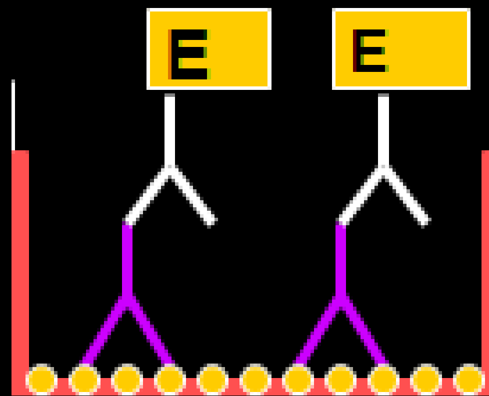


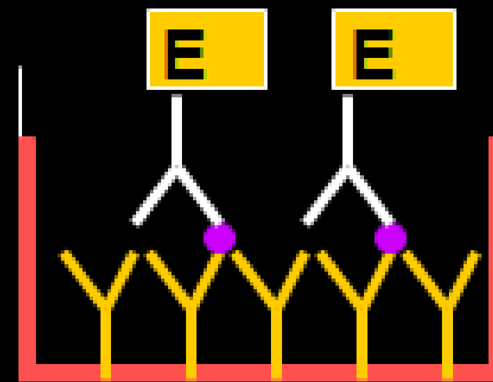
Analyte = antibody



Analyte = antigen

4. Add conjugate. Incubate. Wash.





Analyte = antibody

Analyte = antigen

- **Substrates**
- Enzymatic signal generation requires the catalysis of a substrate to produce a colored or fluorescent compound or chemiluminescence (visible light). Colorimetric substrates are available for both horseradish peroxidase (TMB, OPD, ABTS) and alkaline phosphatase (PNPP).

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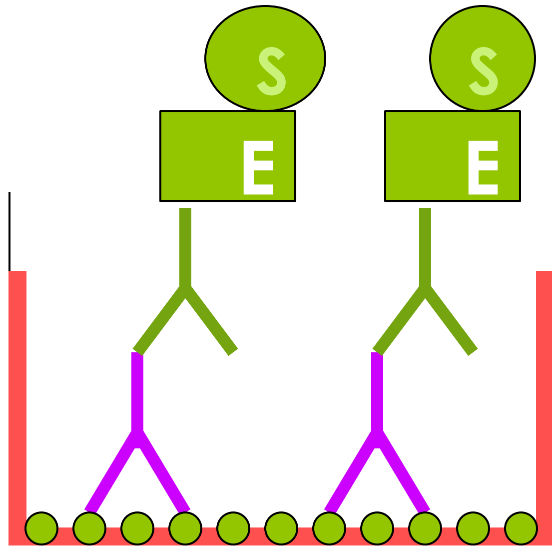
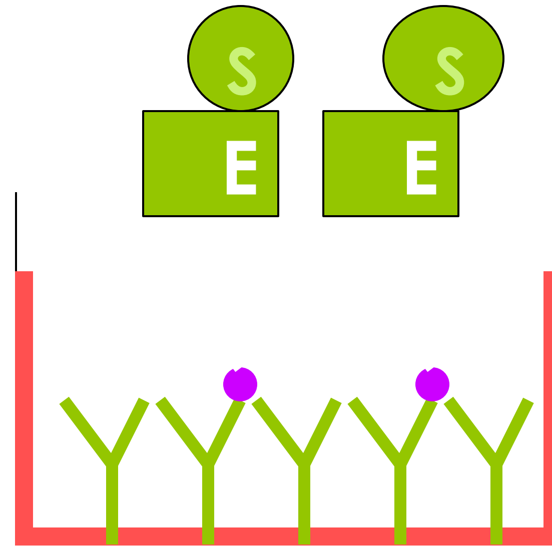
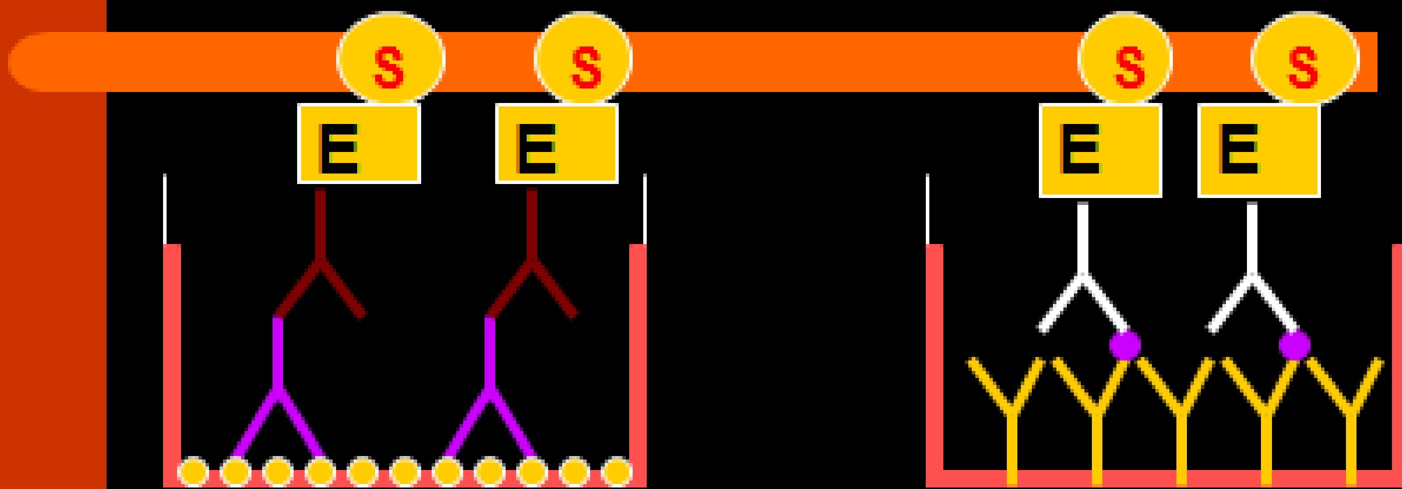


Analyte = antibody



Analyte = antigen



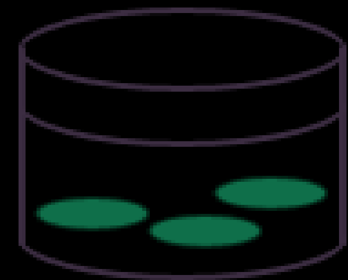
Analyte = antibody

Analyte = antigen

- Components of Kit
- Pre-Coated, Stabilized 96-well Microtiter Plate.
- Sample Diluent
- Standards and controls
- Conjugated Detection Antibody . An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and B-galactosidase
- 10X Wash Solution
- Substrate
- Stop Solution

COATING

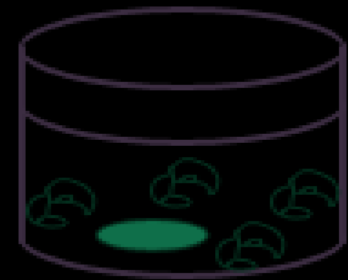
Polystyrene plate is treated with a solution of either antigen or antibody.



remove liquid and wash plate

BLOCKING

An unrelated protein-based solution is used to cover all unbound sites on the plates



remove liquid and wash plate

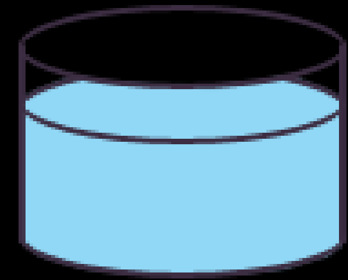
DETECTION

Enzyme-conjugated antibody or antigen binds specifically to the target antigen or antibody

remove liquid and wash plate

READ RESULTS

Substrate is added and the signal produced by the enzyme-substrate reaction is measured



Advantages of ELISA

- Reagents are relatively cheap & have a long shelf life
- ELISA is highly specific and sensitive
- No radiation hazards occur during labelling or disposal of waste.
- Easy to perform and quick procedures
- Equipment can be inexpensive and widely available.
- ELISA can be used to a variety of infections.
- can be automated

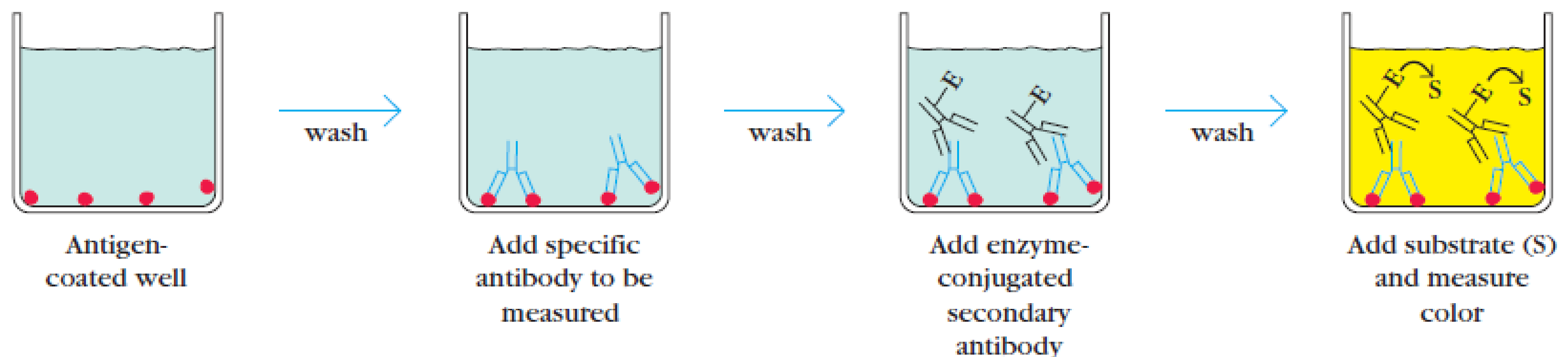
Disadvantage of ELISA

- Measurement of enzyme activity can be more complex than measurement of activity of some type of radioisotopes.
- Enzyme activity may be affected by plasma constituents.
- Kits are commercially available, but not cheap
- Very specific to a particular antigen. Won't recognize any other antigen
- False positives/negatives possible, especially with mutated/altered antigen

- A number of variations of ELISA have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody.

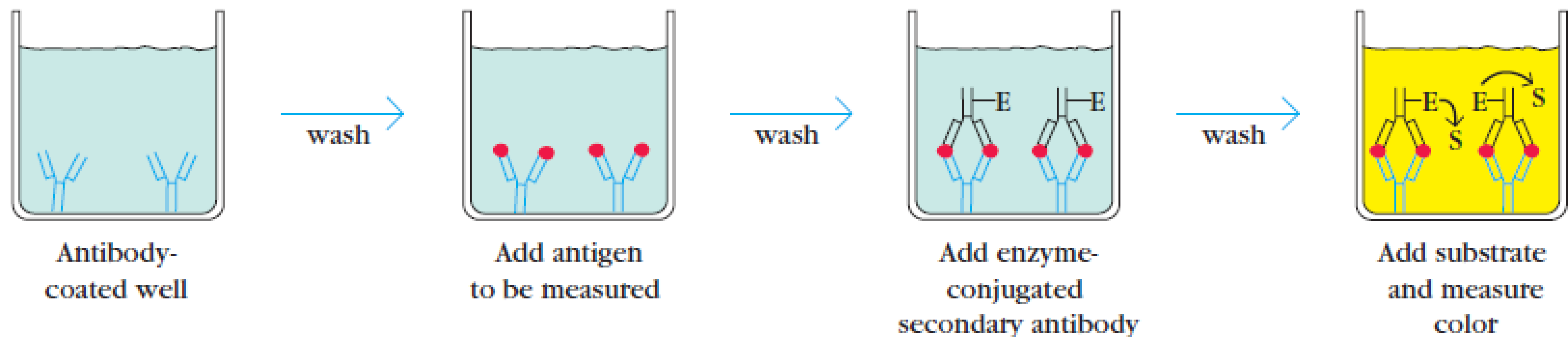
The indirect ELISA

- detects the presence of **antibody** in a sample. The antigen for which the sample must be analyzed is adhered to the wells of the microtiter plate. The primary antibody present in the sample bind specifically to the antigen after addition of sample. The solution is washed to remove unbound antibodies and then enzyme conjugated secondary antibodies are added. The substrate for enzyme is added to quantify the primary antibody through a color change. The concentration of primary antibody present in the serum directly correlates with the intensity of the color.



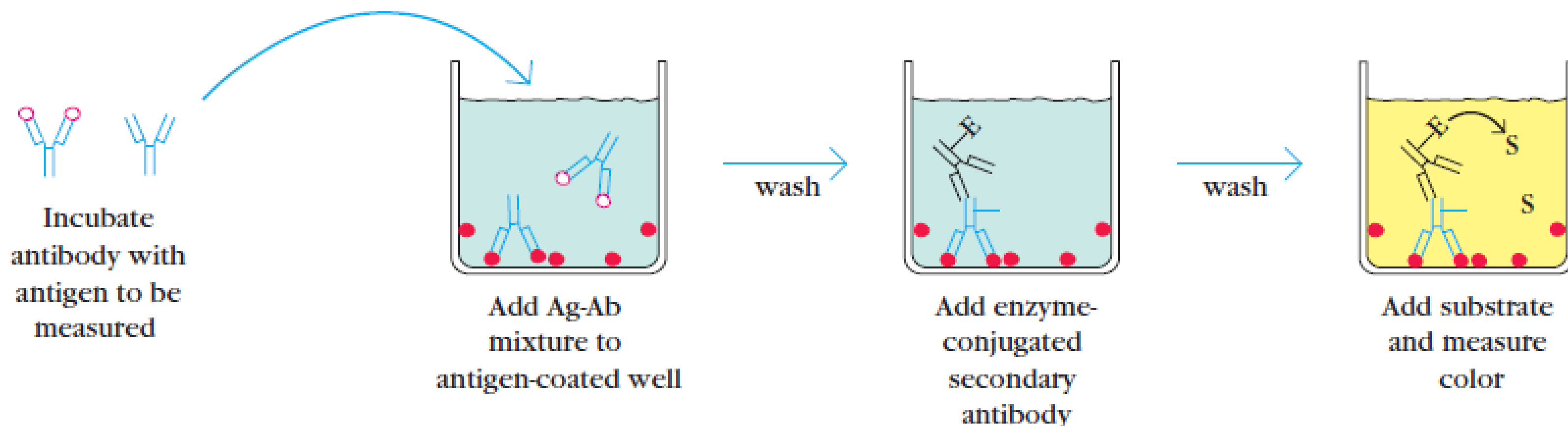
The sandwich ELISA

- is used to identify a specific sample **antigen**. The wells of microtiter plate are coated with the antibodies. Non-specific binding sites are blocked using bovine serum albumin. The antigen containing sample is applied to the wells. A specific primary antibody is then added after washing. This sandwiches the antigen. Enzyme linked secondary antibody is added that binds primary



Competitive ELISA

- This type of ELISA depends on the competitive reaction between the sample antigen and antigen bound to the wells of microtiter plate with the primary antibody.
  First, the primary antibody is incubated with the sample. This results in the formation of Ag-Ab complex which are then added to the wells that have been coated with the same antigens. After an incubation, unbound antibodies are washed off. The more antigen in the sample, more primary antibody will bind to the sample antigen. Therefore there will be smaller amount of primary antibody available to bind to the antigen coated on well. Secondary antibody conjugated to an enzyme is added, followed by a substrate to elicit a chromogenic signal. **Concentration of color is inversely proportional to the amount of antigen present in the sample.**



- **ELISA Data Interpretation**
- The ELISA assay yields three different types of data output:

1. **Quantitative:** ELISA data can be interpreted in comparison to a standard curve (a serial dilution of a known, purified antigen) in order to precisely calculate the concentrations of antigen in various samples.
2. **Qualitative:** ELISAs can also be used to achieve a yes or no answer indicating whether a particular antigen is present in a sample, as compared to a blank well containing no antigen or an unrelated control antigen.
3. **Semi-Quantitative:** ELISAs can be used to compare the relative levels of antigen in assay samples, since the intensity of signal will vary directly with antigen concentration.

- 1. Coating of Wells with Antibody 100 µL of antibody diluted in buffer is added to each well. Cover the plate and incubate at 4 °C overnight.
- 2. Washing wash manually 3 times as follows: Empty the plate by inversion over a sink. Tap the inverted plate against some layers of soft paper tissue to remove residual liquid. Wash the plate by filling the wells by immersion in buffer B. Leave on the table for 3 minutes. Empty the plate as described above and repeat washing two more times.

- 3. Incubation with Test Samples. 100 µL of test sample or standard diluted in buffer is added per well. Cover the plate and incubate at room temperature for 2 hours.
- 4. Wash as described in step 2.
- 5. Incubation with enzyme- Conjugated Antibody. 100 µL of enzyme-conjugated antibody diluted in buffer is added to each well. Cover the plate and incubate at room temperature for 1 hour. The enzyme-conjugated antibody should be directed against the antigen to be determined.

- 6. Wash as described in step 2.
- 7. Colour Development 100 µL of chromogenic substrate is added to each well. Cover the plate and incubate for 15 minutes, or until a suitable colour has developed. The plate should preferably be protected against light during this incubation.
- 8. Stopping the Colour Development Stop the reaction by adding 100 µL 0.5 M H_2SO_4 to each well.
- 9. Reading of Results Read results directly through the bottom of the microwell plate using an automated or semiautomated photometer (ELISA-reader). The subtraction of the absorbance at a reference wavelength (between 620 and 650 nm) is recommended.

PRECAUTIONS

- Negative control with strong signal The excessive background signal can be caused by inadequate rinsing of plates, reagents not sufficiently diluted, inadequate blocking of plates or non-specific binding of enzyme conjugate. The appearance of color in negative control wells may also indicate cross-reactivity of secondary antibody with components in the antigen sample.
- Positive control with no signal Microwell plates not coated properly. Reagents applied in wrong order or step omitted. Secondary antibody not matched to the species of primary antibody. Enzyme conjugate defective or inhibited by contaminant.
- ELISA with weak signal Wash buffer not adequately drained after every wash step. Inadequate incubation times. Enzyme conjugate defective or inhibited by contaminant. Substrate defective or contaminated. Microwell plates poorly coated. Loss of capture antibody during blocking/washing.

Applications

- Screening donated blood for evidence of viral contamination by HIV-1 and HIV-2 (presence of anti-HIV antibodies) Hepatitis C (presence of antibodies) Hepatitis B (testing for both antibodies and a viral antigen)
- Measuring hormone levels HCG (as a test for pregnancy) LH (determining the time of ovulation) TSH, T3 and T4 (for thyroid function)
- Detecting infections Sexually-transmitted agents like HIV, syphilis and chlamydia Hepatitis B and C Toxoplasma gondii
- Detecting illegal drugs.
- Detecting allergens in food and house dust

ELISA instrument

- Elisa instrument composed of three units:
- **Incubator & shaker**
- **Washer**
- **Reader**