

# Antibodies

 Course	 <a href="#">Essential Protein Structure and Function</a>
 Confidence	Confident
 Next Review	@April 29, 2024
 Last Edited	@May 1, 2024 5:52 PM

## Basics - What are antibodies?

Immunology as a subject started in 1796, when vaccination with cowpox by Jenner protected against smallpox.

- Two types of immune responses – adaptive and innate – that together provide a remarkably effective defence system.
  - Adaptive– production of antibodies against an infectious pathogen (viruses, bacteria, fungi, parasites). Antibodies are generated against vast range of substances (each called an antigen – antibody generation). First appeared in fishes.
  - Innate– inbuilt ability to combat a wide range of pathogens using proteins (eg: complement) and cells (eg: macrophages). Evolutionarily ancient (from insects onwards).
  - Adaptive immune system supplement the activity of innate system

### Roles of antibodies

- Three roles
  1. Neutralisation of bacterial toxins
  2. Helps phagocytosis of bacteria
  3. Triggers complement activation in plasma.
    - a. Ab acts as signals in the latter two roles
- Diseases involving IgG
  - IgG deficiency – patients are more likely to get infections
  - Autoimmune diseases (when IgG recognise self) include primary Sjogren syndrome, systemic sclerosis, systemic lupus erythematosus,

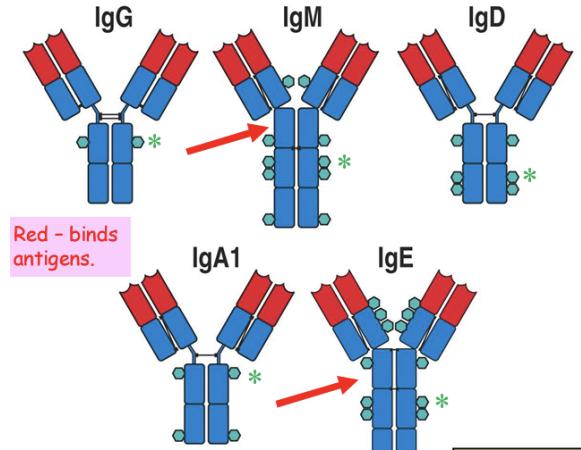
and primary biliary cirrhosis)

- IgG-related disease is a fibro-inflammatory condition that mimics malignant, infectious, and inflammatory disorders

### Generation of Ab

- B-cells from bone make antibody, produced by the bone marrow
  - B-cells circulate to lymph nodes where antigens activate them.
- Surface membrane-bound antibody on a single B-cell detects a foreign antigen.
- Clonal expansion of that single B-cell gives many B-cells.
- Antibody class switching occurs. Antibody is secreted in large amounts to combat the antigen.
- IgG circulates in high concentrations in plasma, being 75% of the antibody content in adults, and 20% of the total blood protein.
  - Haemoglobin accounts for a large proportion of plasma protein.
- The immune system (lymphocytes) is comparable in mass to the liver/brain.

### Five classes of human antibodies



- Antibodies are Y-shaped with 12 domains
  - Note that IgM and IgE have an extra pair of domains in the middle
- IgG is the most abundant followed by IgA and IgM, IgD and IgE are the least.
  - Also note different extents of glycosylation in the five classes. One site in CH2 is almost conserved.

- IgG, IgD, and IgE are monomers.

- IgM forms pentamers
- IgA forms dimers.

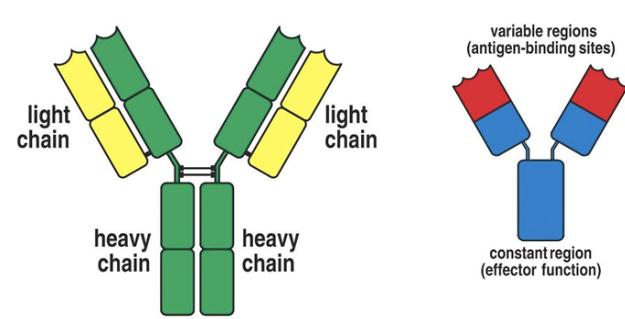
### Functions:

- IgM and IgD appear on B-cell surfaces to initiate clonal expansion.
- IgM pentamers (high avidity) are the first to appear after exposure to antigen, then replaced by IgG.
- IgA is found in mucus (secretions: external protection)
  - E.g. gut, nose, lung wall
- IgE is important for allergies (hay fever, degranulation, histamines)

## Protein structures of antibodies

### Primary structure of IgG

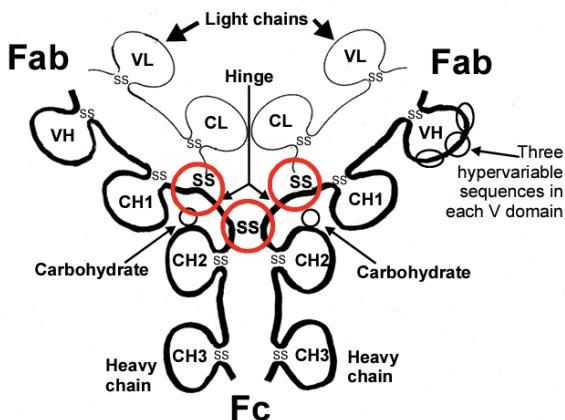
- Antibodies are conventionally drawn as Y-shaped molecules



- All have **four-chain structure** with 2 light chains and 2 heavy chain
  - Molecular weight ~150,000
- These are arranged as 2 Fab and 1 Fc fragments with recognition and effector roles respectively

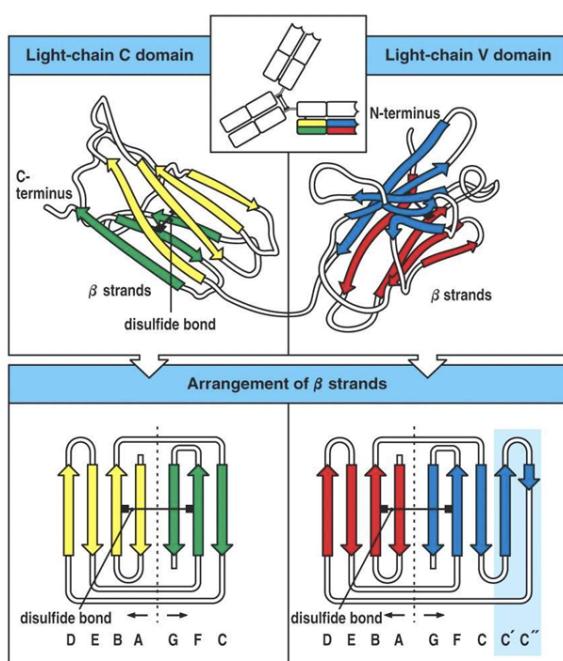
### Domain structure

- IgG consists of 12 domains
  - 4 variable domains (denoted as V), each of which contain three HV sequences.
    - Leads to bivalence - Bind simultaneously two antigens
  - 8 constant domains (denoted as C), each of which define the rest of the IgG structure.
    - Leads to effector

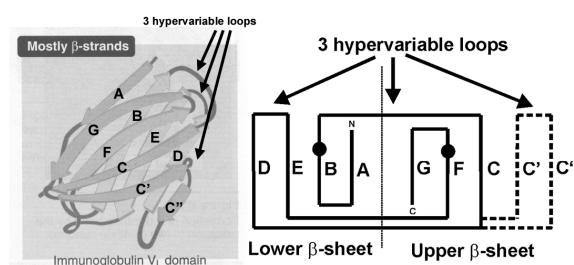


- Note the **12 intra-disulphide bridges (one per domain)** that stabilises each domain, as well as the **3 inter-disulphide bridges** that connect 2 light chains (denoted as L) and 2 heavy chains z (denoted H).
  - Total 15 disulphide bridges

## Secondary structure of IgG



- **All 12 domains** possess similar **2 beta sheet sandwich** structures.
  - This is the immunoglobulin fold **stabilised by a S-S bridge** between sheets (B and F).
- V domains contain 9 beta-strands in a V-type Ig fold (A to G)
- C domains contain 7 beta-strands in a C-type Ig fold (A to G) in which C' and C'' are absent.



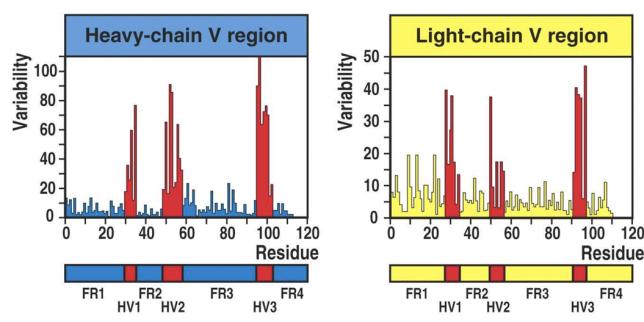
- Note that the **3 hypervariable sequences** are located at loops at one end of the two b-sheet structure. This defines the antibody binding site for epitopes on antigens

## Tertiary structure of IgG

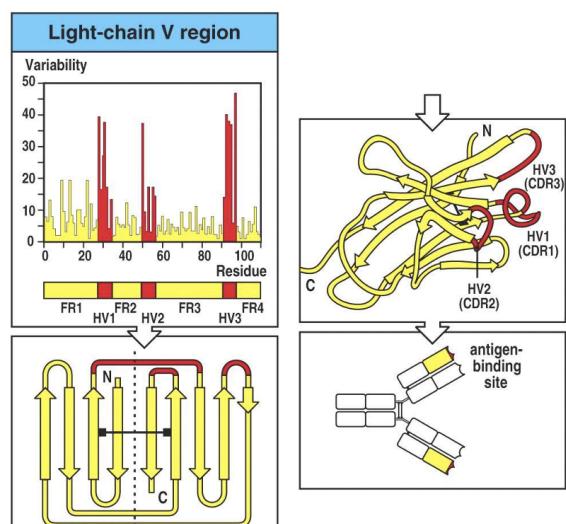
- Early experiments using papain and pepsin cleavages showed that there were two Fab regions and one Fc region
- Flexibility at the hinge region causes very few intact IgG molecules have been crystallised and solved, but the separable Fab and Fc regions are crystalisable
  - As the protein has to be in one conformation to be crystalise

## Antibody recognition of antigens

### Sequence variations



- Variable sequences in the VL and VH domains is not evenly distributed
- There are three hypervariable regions (HV1-3) and four framework regions (FR1-4)



- When the sequences were mapped onto the crystal structure of Fab regions, it was clear that the three hypervariable regions (HV1-3) were located on the loops at one end of the beta-sheet sandwich structure
  - They are better known as complementarity determining regions (CDRs)

### CDR structural shape variations

- There are 6 CDRs per antibody binding site
  - Each VH and VL domain contains three CDRs, making a total of six CDRs per Fab region
- Their shapes are complementary to the epitope of the antigen they bind

- E.g. a narrow groove, pocket, or a broad surface
- The binding is non-covalent
  - Electrostatic attraction between opposite charges
  - Hydrogen bonding
  - Hydrophobic interactions that exclude water

## Antibody genetics

### Generation of sequence diversity

- **Somatic recombination:** To generate as many as ~1 billion variable domains from DNA splicing, antibody DNA in B cells exists as gene segments that are rearranged.

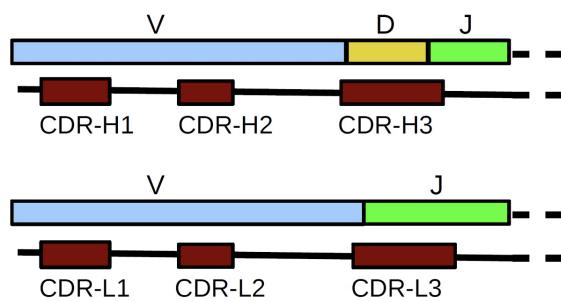
Number of functional gene segments in human immunoglobulin loci			
Segment	Light chains		Heavy chain
	$\kappa$	$\lambda$	H
Variable (V)	40	30	40
Diversity (D)	0	0	25
Joining (J)	5	4	6

- Light chains involve V and J segments that are merged with C segments at the DNA level
- Heavy chains involve V, D and J segments that are merged with C segments at the DNA level.
- There are many V, D, and J segments that create a very large number of antibody sequences
  - E.g. Kappa light chains have 40V and 5J segments in the genome.
- Antibody diversity is generated by other processes
  1. Combinatorial diversity
  2. Junctional diversity (adding/subtracting nucleotides)
  3. Somatic hypermutation (point mutations)

Kappa LC - 40V  $\times$  5J = 200 V domains  
 Lambda LC - 30V  $\times$  4J = 120 V domains  
 HC - 40V  $\times$  25D  $\times$  6J  $\times$  300 (frameshift and imprecise recombination of D) = 1,800,000 V domains

## Total of light and heavy chains combinations:

$1,800,000 \times 200 + 1,800,000 \times 120 = 576,000,000$  - or almost 1 billion ( $10^9$ ) combinations - and this is an underestimate. The antibody repertoire is probably over  $10^{15}$ .



When the V, D, and J genes are mapped onto the antibody sequences, it becomes clear that the site of D/J (heavy chain) or J (light chain) corresponds to the **third hypervariable region**. CDR is especially variable.

## Class switching in B cells

- To generate either the membrane bound IgM monomer (on B cells) or the secreted IgM pentamer, either a **secretion-coding (SC)** sequence or a **membrane-coding (MC)** sequence is spliced onto the end of the heavy chain sequence by alternative RNA processing.
  - IgM is always expressed first by B cells.

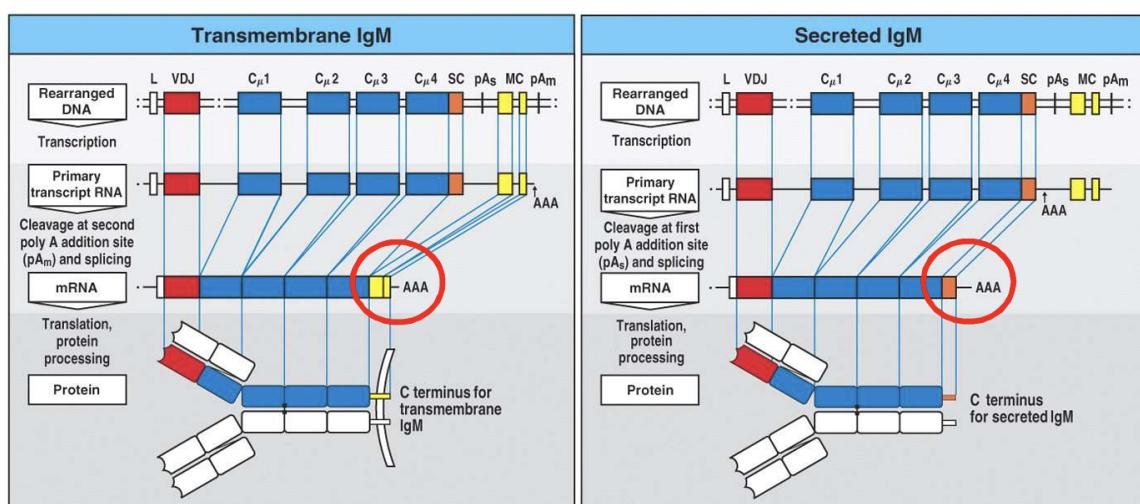


Figure 4-16 Immunobiology, 6/e. (© Garland Science 2005)

- The  $C_{\mu}$  and  $C_{\delta}$  exons are adjacent to each other. RNA processing will result in an mRNA that will give either IgM or IgD.

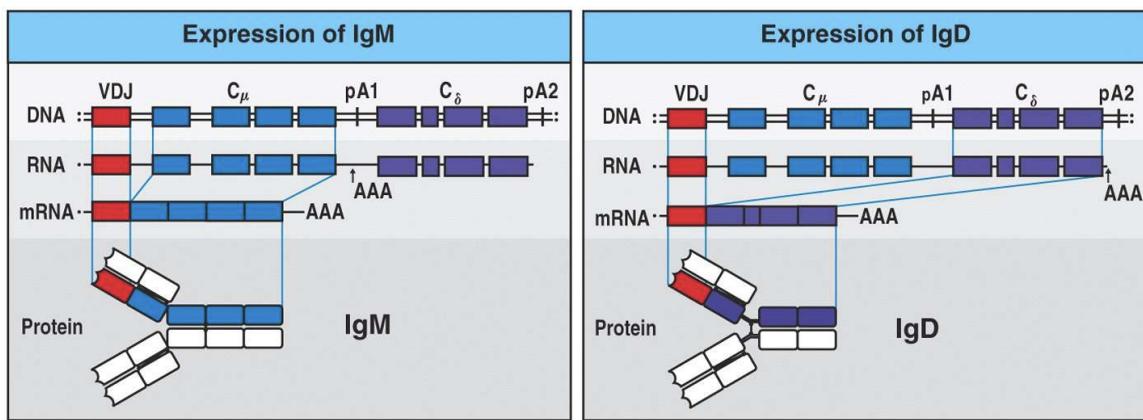


Figure 4-20 Immunobiology, 6/e. (© Garland Science 2005)

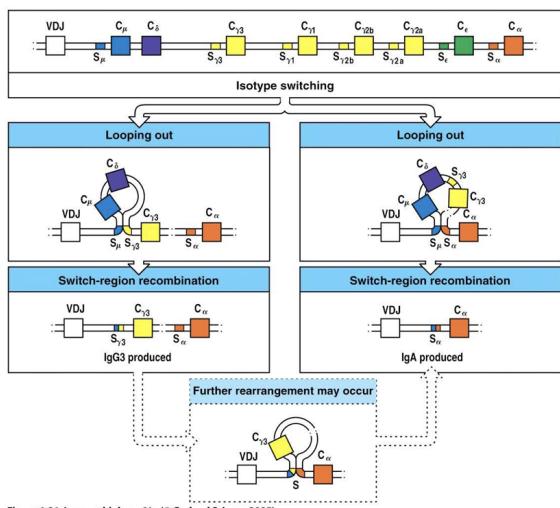


Figure 4-21 Immunobiology, 6/e. (© Garland Science 2005)

- C-gamma (IgG), C-epsilon (IgE), C-alpha (IgA) locates in this order from G theta
- By looping out the intervening DNA, immunoglobins of different classes with the same antigen specificity can be produced
- As shown for mouse antibody, either IgG3 is generated, which switches to IgA by looping out the intervening DNA, or IgA is generated by looping out more DNA.
  - Note that mouse antibody exists as different classes from human

## Summary

- In adaptive immunity, antibodies are generated in response to antigens by B cells
- Antibodies have flexible, bivalent Y-shaped structure that are composed of 2 Fab regions and one Fc region
- Exposed loops in the protein structure explains the sequence variability seen in the V domains

- Antibody genetics explain how hypervariable sequences are generated - and how antibody class switching takes place

# Antibodies as therapeutic proteins

## Monoclonals as therapeutics

### Why are antibodies so useful?

- Antibodies (Ab) are protein recognition molecules.
- High specificity and high affinities (dissociation constants  $K_d \sim$ nanomolar)
- Utility:
  - One third of drugs in development are antibodies, 85 antibody products approved (2019)
  - Laboratory reagents and clinical tests
    - E.g. ELISA

### Antibodies as drugs

1. As conventional drugs
  - Binding: block interactions / agonists / antagonists - to prevent binding
2. Immune activation
  - Complement dependent cytotoxicity (CDC)
  - Antibody-dependent cell-mediated cytotoxicity (ADCC) via Fc receptors
3. Targeting or delivery
  - Radioactive payloads (e.g. to target tumours for surgery or killing)
  - Chemical payloads (ADC - to kill tumour cells by internalisation of the Ab coupled to an anticancer drug)
  - Indirect delivery (ADEPT - antibody directed enzyme prodrug therapy - to selectively deliver chemotherapy to cancer sites)
4. Crosslinking
  - Bispecific antibodies (two Fab's have different antigen target)

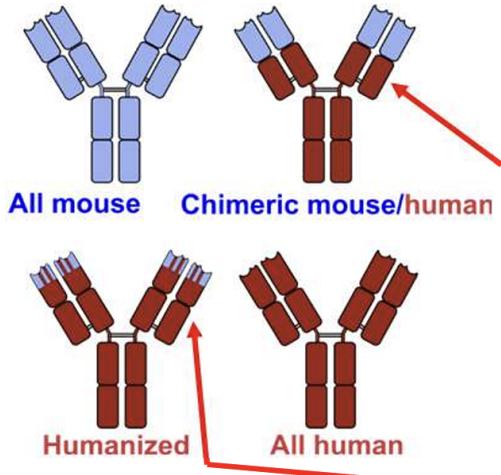
- Six best selling medicines target specific proteins involved in disease - monoclonal antibodies

## Monoclonal antibodies

- Normal antibodies are polyclonal.
  - Each B-cell that undergoes clonal expansion makes one pure antibody.
  - But there will be many antigenic targets (called "epitopes") on the foreign particle, so multiple antibodies will be generated.
  - Then all the antibodies in plasma are a mix of all these.
- A **myeloma** antibody is when a B-cell cancer gets out of hand and fills the patient's plasma with one antibody.
  - Much purer to work with, but is of limited utility or application.
- The major breakthrough came with monoclonal antibodies.
  - These can be purified in large amounts starting from a single B-cell making the desired mouse antibody.
  - This is immortalised by fusing it with a cancer cell line.
  - Grow up using cell culture.
  - Jerne, Köhler, and Milstein were awarded 1984 Nobel Prize on hybridoma technology
- Newer phage display methods lead to all-human monoclonals.
  - Prevent immune response
- Five stages
  1. Mice immunised with antigen A, to generate short-lived spleen B cells. Myeloma cells of the right type are secreted (no HGPRT enzyme, no Ab secretion).
  2. Fuse spleen cells and myeloma cells using polyethylene glycol (PEG)
  3. Transfer the hybrid cells to HAT medium
    - a. Only cells making HGPRT (immortal hybridomas) can proliferate in HAT medium
    - b. Mortal spleen cells and unfused HGPRT -ve myeloma cells die
  4. Screen the cells for antibody production specific for antigen A

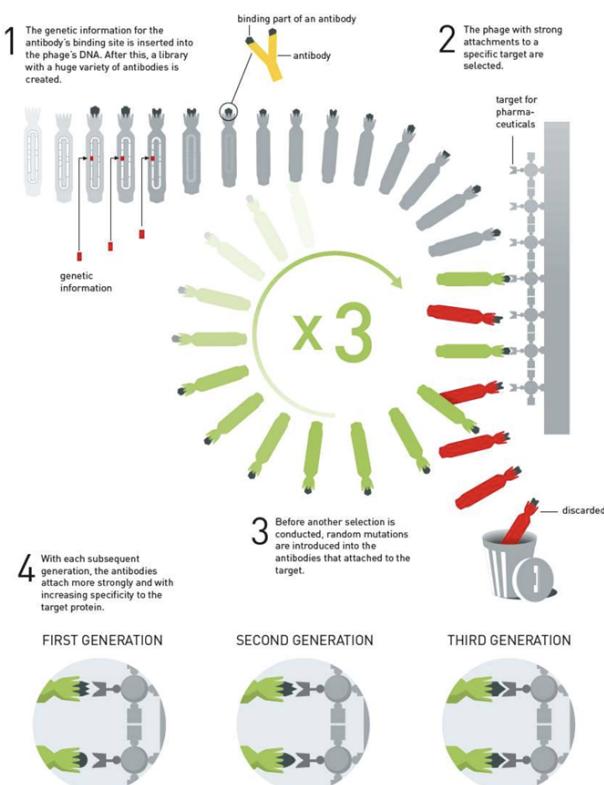
## 5. Clone or grow mouse cells up in bulk culture

- Note this procedure only makes mouse Ab, which creates immune issues



- Humanisation of monoclonals
  - Mouse antibodies result in an immune human anti-mouse antibody response (HAMA)
  - To get around this, chimeras have been made with mouse V domains and human C domains, reducing HAMA
  - Or mouse CDRs are grafted onto an otherwise all-human Ab. This is the famous Adair patent.

- 2018 Nobel prize to Arnold, Smith, and Winter on phage display technology to make human monoclonals
- A human phage display library is constructed by first isolating antibody RNA from a given source (E.g. sequencing from human peripheral blood mononuclear cells), followed by ligation into a phage display vector.
- These vectors can then be used for expression of human IgG on bacteriophage hosts to represent the entire immune repertoire from which RNA was isolated
- One can then screen a phage library in multiple



cycles for those which bind more strongly to the target antigen, and isolate the original IgG sequence

## Applications of antibodies

### ELISA assays

- An enzyme-linked immunosorbent assay (ELISA) is an antigen capture assay used to detect secreted products.
  - E.g. pregnancy test and HIV tests
- The antigen is attached to a solid support, and a antibody attached to an enzyme is added to the well plate
- After mixture, any unbound antigen (or antibody) is washed away
- Binding is detected by an enzyme-dependent colour-change reaction
- In HIV tests, a person's serum is allowed to react with HIV virus coat protein p24. If the person has been infected with HIV, the antibodies in the serum will bind to the HIV p24, and the extent of this binding can be measured

### Four types of ELISA

- Direct ELISA
  - Attachment of an antigen to a polystyrene plate, followed by an enzyme-labeled antibody that can react with the antigen and a substrate that can be measured.
  - Fast and simple, but less specific.
- Indirect ELISA
  - Attachment of an antigen to a polystyrene plate, followed by an unlabeled or primary antibody, followed by an enzyme-labeled antibody that can react with both the primary antibody and substrate.
- Sandwich ELISA
  - A capture antibody is attached to the polystyrene plate, then antigen is added that specifically attaches to the capture antibody. A second antibody (also specific for the antigen but not the same as the capture

antibody) is added and "sandwiches" the antigen. This second antibody is then followed by a third enzyme-labeled antibody specific for the second antibody that can react with a substrate that can be measured through a colour assay.

- High specificity and sensitivity.
- Competitive ELISA
  - This test is like the sandwich ELISA but involves the addition of competing antibodies or proteins when the second antibody is added.
  - This results in a decrease in the substrate signal generated.
  - This test gives good and highly specific results.

### **Pregnancy ELISA test**

- In the 1970s, the discovery of monoclonal antibodies led to the development of the relatively simple and cheap immunoassays, such as agglutination-inhibition-based assays that are used in modern home pregnancy tests.
- Home pregnancy test utilizes the principle of sandwich enzyme immunoassay, with a unique mono-mono antibody combination specific against hCG present in urine/serum.
- The patient's urine/serum specimen is allowed to react with the monoclonal antibody directed against hCG, coated on the microtiter wells and the monoclonal antibody - enzyme conjugate complex. If hCG is present in the test specimen, antibody-hCG/antibody-enzyme complex will be formed on the surface of the microtiter well.
- Washing the well under running tap water will clear off the unbound complex and the unreacted conjugate. Incubating the well with substrate reagent results in the development of blue colour.
- The intensity of the blue colour is proportional to the concentration of hCG present in the urine/serum specimen.

### **ELISA tests for Human Immunodeficiency Virus (HIV)**

- Most laboratories use an immunoassay for the HIV p24 antigen (the virus capsid protein) and HIV antibodies, followed by a confirmatory immunoassay to distinguish between HIV-1 and HIV-2. Early diagnosis makes possible early treatment. It is a routine procedure during pregnancy.

- In an ELISA test, a person's serum is diluted 400-fold and applied to a plate to which HIV antigens have been attached. If antibodies to HIV are present in the serum, they may bind to these HIV antigens.
- The plate is then washed to remove all other components of the serum. A specially prepared "secondary antibody" – an antibody that binds to human antibodies – is then applied to the plate, followed by another wash.
- This secondary antibody is chemically linked in advance to an enzyme. Thus the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence.

## Lateral flow tests

- Lateral flow tests can be designed to analyse various body fluids.
- Simple devices intended to detect the presence of a target substance in a liquid sample without the need for specialized and costly equipment. These tests are simple, economic and generally show results in around 5-30 minutes.
- Operate on the same principles as the ELISA. In essence, these tests run the liquid sample along the surface of a porous pad with reactive molecules that show a visual positive or negative result.
- The sample pad acts as a sponge and holds an excess of sample fluid. Once soaked, the fluid flows to the second conjugate pad. The conjugate pad contains the reagents required for a chemical reaction between the target molecule (e.g. an antigen) and its chemical partner (e.g. antibody) that has been immobilized on the particle's surface.
- Target particles are marked as they pass through the pad and continue across to the test and control lines. The test line shows a signal, often a color as in pregnancy tests. The control line contains affinity ligands which show whether the sample has flowed through and the bio-molecules in the conjugate pad are active.

## Quick COVID-19 tests

- In the case of COVID-19, most tests analyse material collected from the back of someone's nose and throat. The swab is then inserted into a tube of

liquid, after which a sample of this liquid is deposited on a small absorbent pad contained within the disposable testing kit.

- The liquid is drawn along the pad by capillary action, until it encounters a strip coated in antibodies which are specific to proteins, also known as **antigens**, from the **SARS-CoV-2** virus.
- If viral proteins are present, this will show up as a coloured line – much like a positive pregnancy test.
- Even though less accurate than PCR tests, their low cost, speed and ease of use make lateral flow tests particularly attractive. With their high specificity, existing COVID-19 lateral flow tests are potentially useful for confirming that someone suspected of having COVID-19 really has it. The UK is using lateral flow tests as a means of screening whole populations in order to reduce virus transmission.

### **Clearblue pregnancy test**

- The soluble anti- $\alpha$ hCG MAb is attached to a blue dye on latex particles. Also rabbit IgG-blue latex is there. Add urine.
- If pregnant, hCG is secreted by the implanting blastocyst and placenta. If hCG is present, this binds to the anti- $\alpha$ hCG MAb.
- If the hCG-MAb latex complex is present, this binds to the immobilized anti $\beta$ hCG Mab (on nitrocellulose). Blue line appears.
- The control line has goat anti-rabbit IgG antibody. The rabbit IgG-blue latex is there. If a second blue line appears, the test has progressed as expected.
- Example of an immunometric (sandwich) assay. Works >15 days after conception.

### **Lateral flow immunoassay (LFIA)**

**Principle:** based on the capillary action that draws a sample across a test strip containing antibodies or antigens.

- When the target analyte is present, it binds to labeled

### **ELISA**

**Principle:** ELISA relies on the binding of an antigen to antibodies attached to a solid surface (usually a microtiter plate). A second antibody, linked to an enzyme, binds to the antigen. The addition of a substrate leads to a colorimetric change that can be

antibodies, forming a complex that is captured at a specific zone on the strip, indicating a positive result.

#### **Features:**

- **Speed and Simplicity**
- **Portability**
- **Cost-effective**
- **Sensitivity and Specificity:**  
Typically less sensitive and specific than ELISA tests.

measured with a spectrophotometer, indicating the presence and quantity of the antigen.

#### **Features:**

- Sensitivity and Specificity
- Quantification: measuring the concentration of the analyte in the sample
- Complexity and Time
- Equipment Requirement e.g. spectrophotometer or microplate reader

## **Antibody effector functions**

- E.g. mediated through their Fc regions that bind to receptors and complement.