

BIOMAN 2015

IMMUNOASSAYS

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Immunoassays

An **immunoassay** is a biochemical test that measures the presence or concentration of a molecules (typically macromolecules) in a solution through the use of an antibody or immunoglobulin.

The molecule detected by the **immunoassay** is often referred to as an "analyte" and is in many cases a protein or polysaccharide.

What is antigen

An antigen is defined as any substance that can bind to a specific antibody.

All antigens therefore have the potential to elicit synthesis of the specific antibodies, but some need to be attached to the immunogen (activator of the immune system) in order to do so.

Antigen can be a large or small molecule, or a complex containing several molecules.

Antigen can have one or more different epitopes (antigenic determinants), the places the antibodies can (bind) attach themselves to.

What Is Antibody

Antibody:

An immunoglobulin, a specialized immune protein, produced because of the introduction of an antigen (immunogen) into the body, and which possesses the remarkable ability to combine with the very antigen that triggered its production.

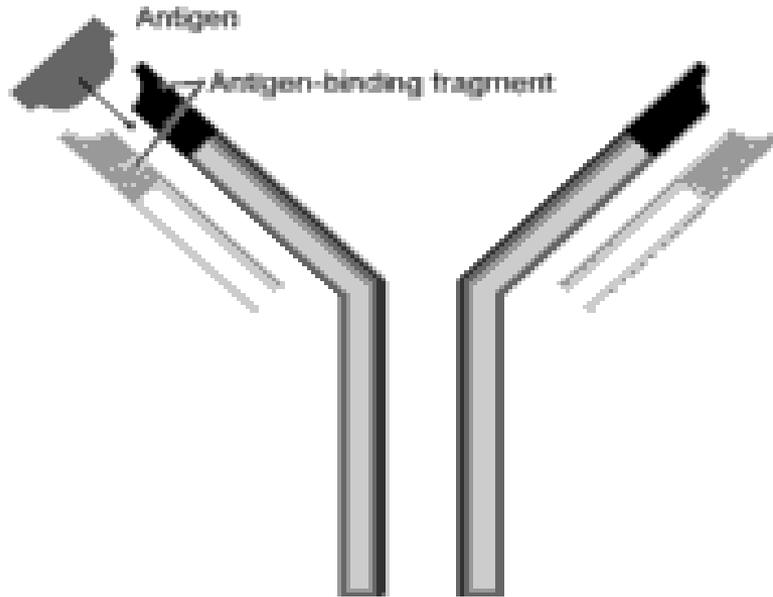
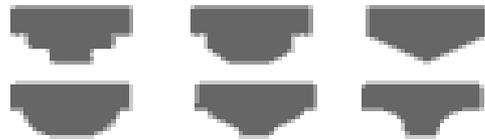
The production of antibodies is a major function of the immune system and is carried out by a type of white blood cell called a B cell (B lymphocytes), upon stimulation by T lymphocytes.

Antibodies can be triggered by and directed at foreign proteins, polysaccharides, microorganisms, toxins and many more molecules.

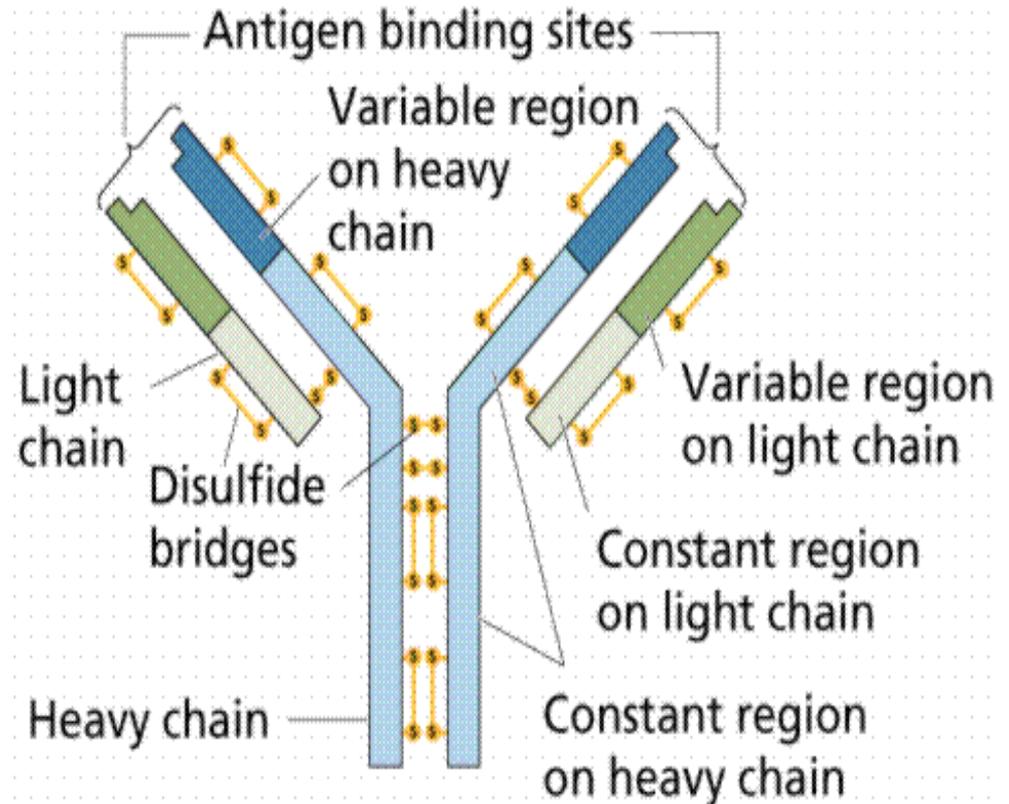
According to <http://www.medicinenet.com/script/main/hp.asp>

What Is Antibody

Antigens



Antibody



Immunoglobulins

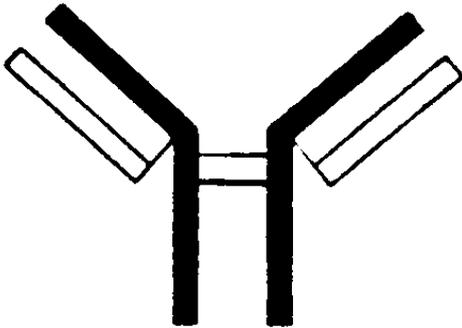
- Immunoglobulins – Antibodies
 - IgD** surface of many cells (mostly B)
 - IgM** surface of B cells
 - IgG** blood; the most abundant Ab
 - IgA** digestive and respiratory systems
 - IgE** skin, digestive and respiratory tract;
responsible for allergic reactions

IgG: immunoglobulin G

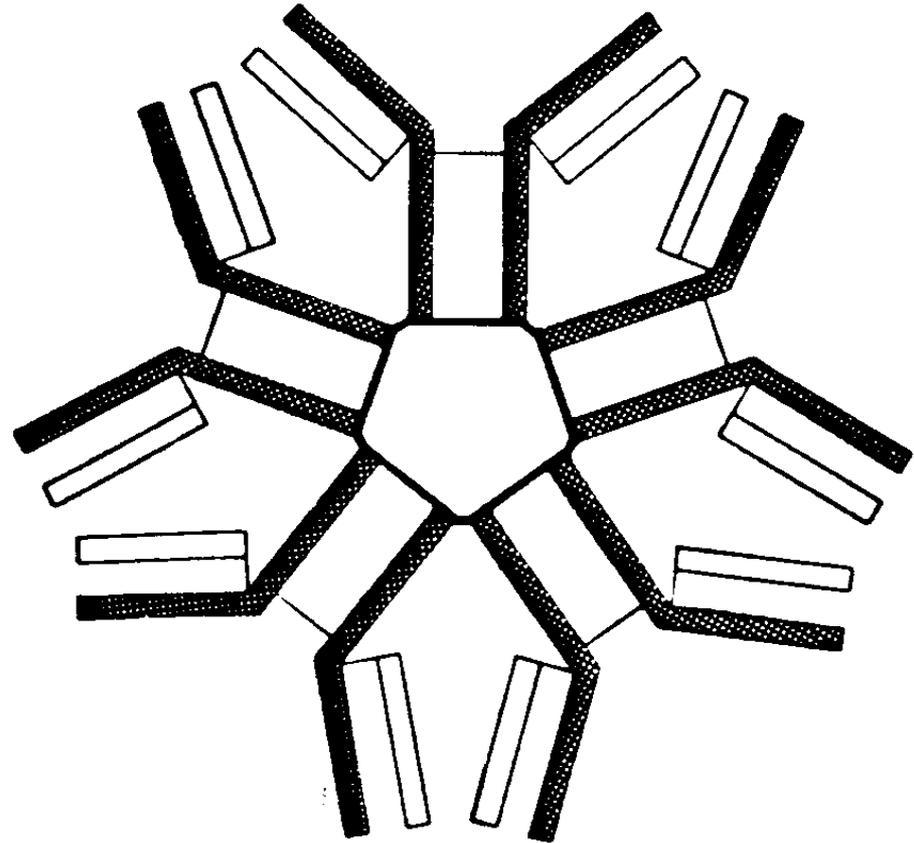


Antibodies

IgG



IgM

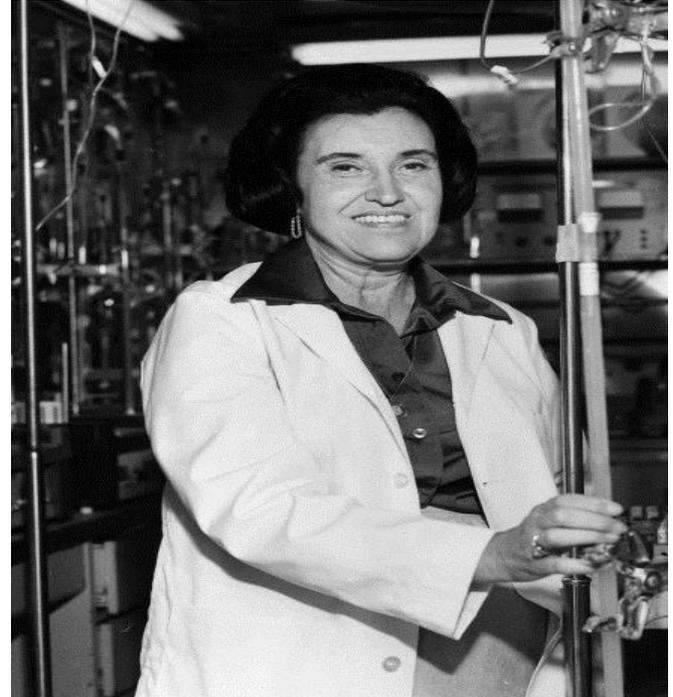


Antibodies: Applications

- **Diagnostic tests;** example the pregnancy test
- **Research tool;** visualization of the cell components
- **Drug delivery system;** example chemotherapy drugs can be delivered directly to tumors due to antibodies ability to find tumor cells and attach to them

History and Background

- In the year 1959, Drs. Rosalyn Yalow & Soloman Berson invented the radioimmunoassay, which applied the use of radioisotopes in the measurement of insulin.
- The RIA is the predecessor of modern immunoassays.



Dr. Rosalyn Yalow became the first female to win a Nobel Prize with her work on the radioimmunoassay.

Immunoassays

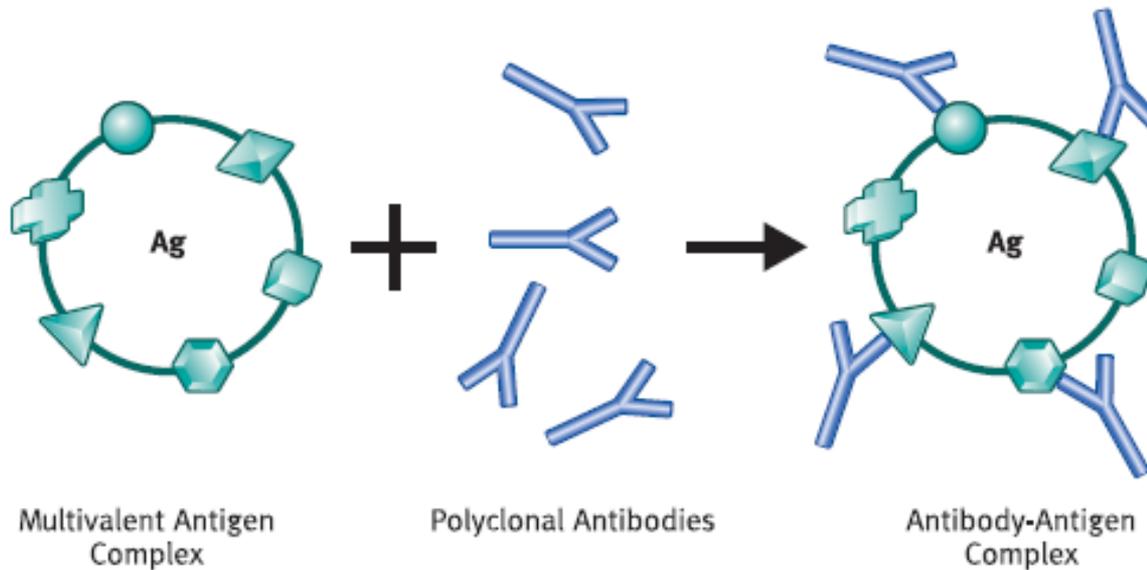
- Immunoassays are excellent diagnostic and research tools.
- Antibodies can specifically recognize the molecule of interest even amongst thousands of other molecules and will bind to the molecule of interest very tightly thus making it possible to tag and detect the analyte of interest.
- They allow to detect very minute amounts of the analytes (molecules of interest)
- Immunoassays are highly sensitive and very specific
- Multibillion dollar business
- Examples: HIV test, hepatitis test, tests for numerous infectious diseases and other disorders (thyroid dysfunction), test for *H. pylori*, numerous QC tests.

Antibodies types

Polyclonal Antibodies recognize the same antigen but different epitopes (antigenic determinants).

They are heterogeneous proteins (mix), made by B lymphocytes upon stimulation by T lymphocytes

Polyclonal Ab are naturally made by many vertebrates such as mammals and birds.

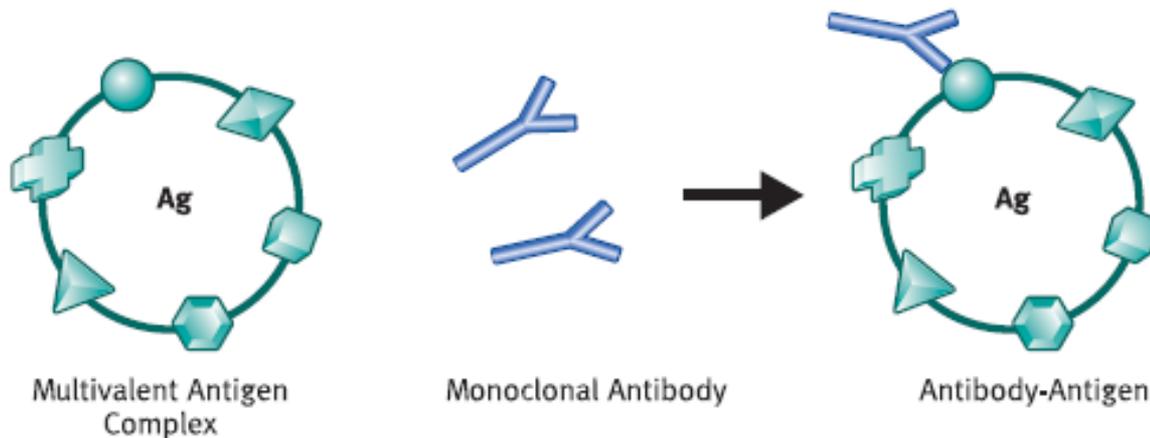


Antibodies types

Monoclonal Antibodies recognize the same antigen and the same epitope.

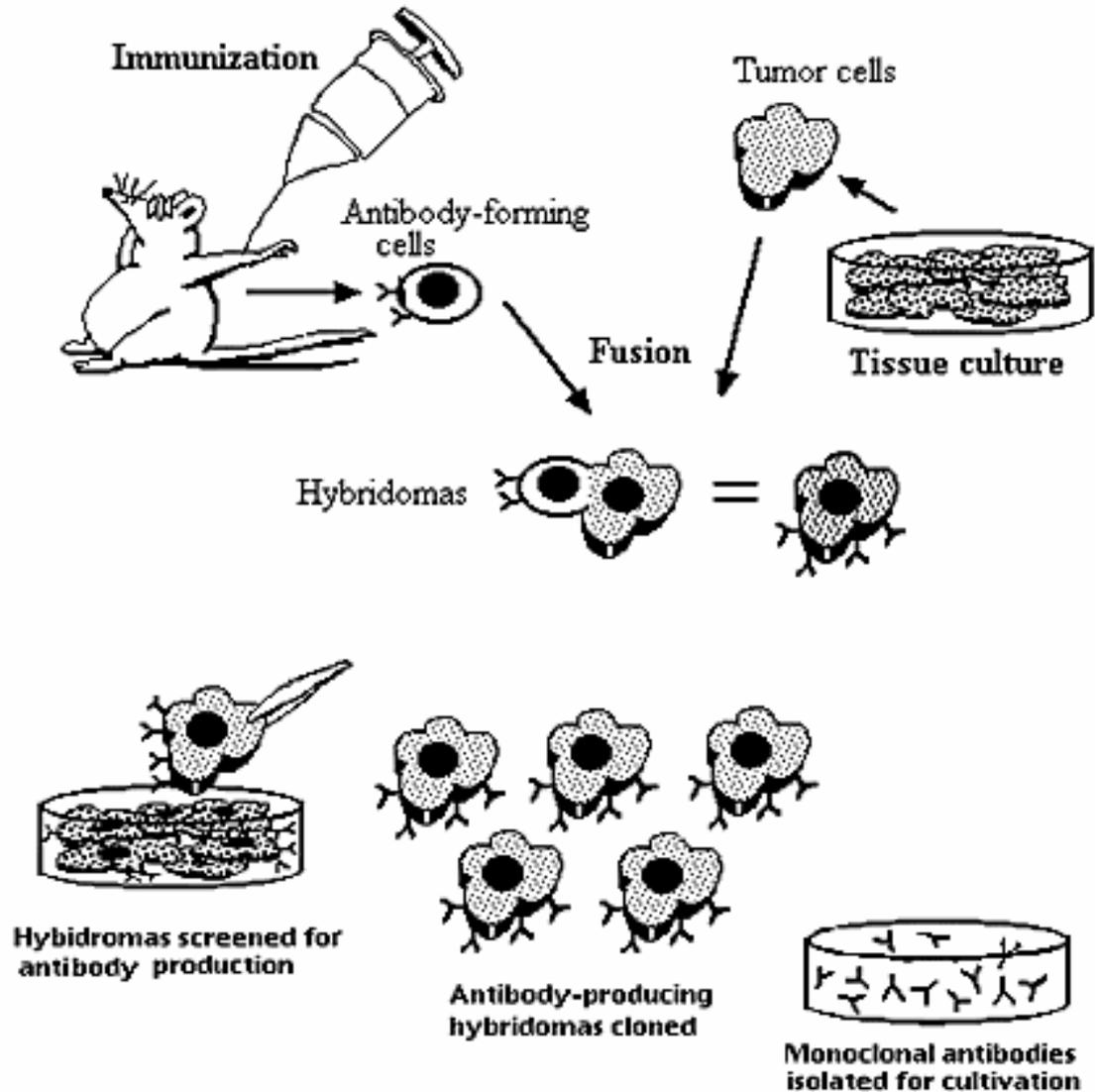
They are homogenous clones derived from one cell.

Monoclonal Ab are made artificially by the genetically modified cells lines grown in a culture; hybridomas



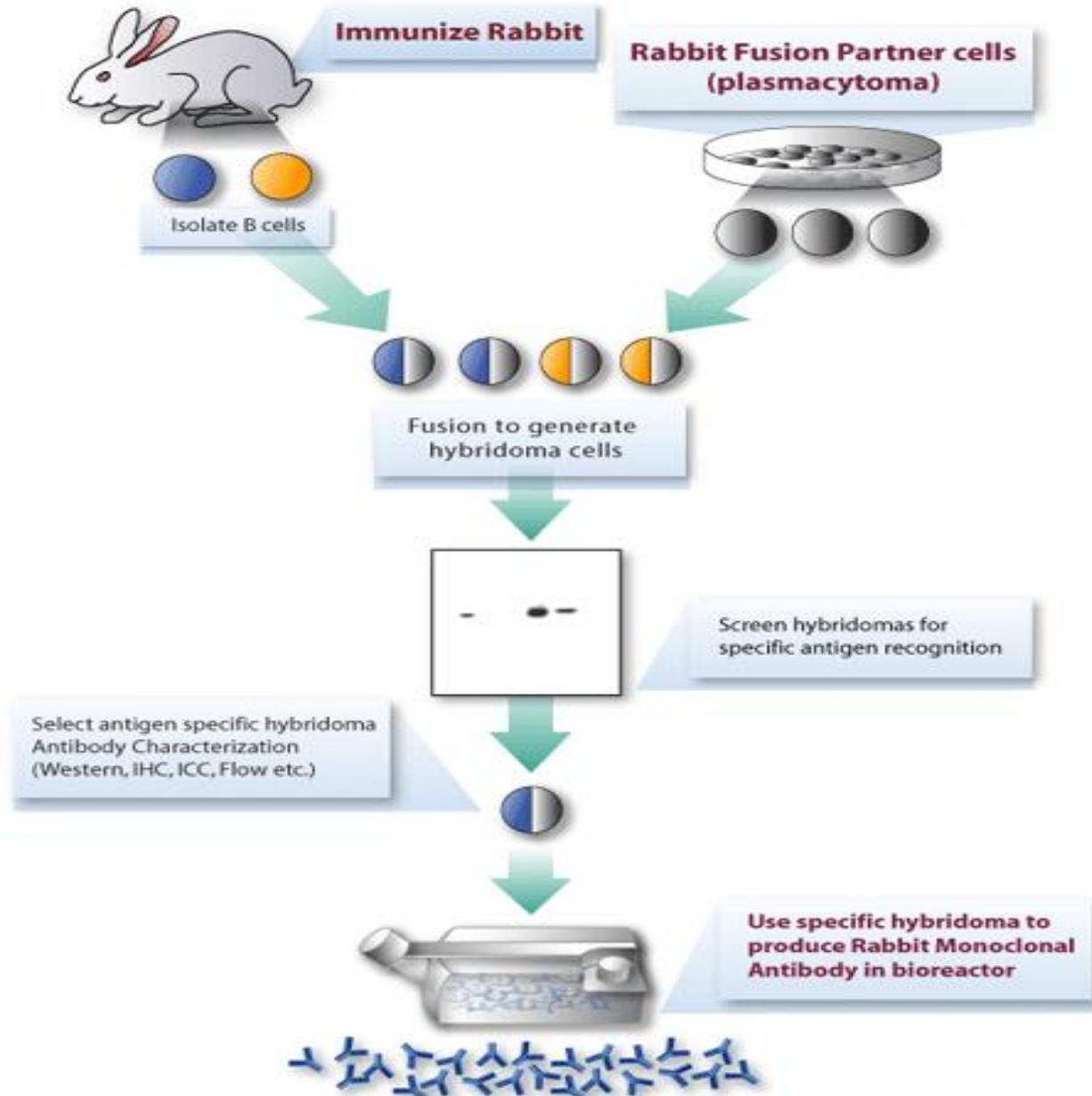
Steps in monoclonal Ab production.

1. Mouse is injected with the antigen and her spleen makes antibodies.
2. Spleen is removed from the mouse .
3. Mouse Ab producing cell are fused with the immortal cancer cells .
4. Hybridomas are screen for the Ab production and cultivated (individual cells are cloned) as long as Ab are needed.



Monoclonal Antibody Production

RABBIT MONOCLONAL ANTIBODY



Types of immunoassays

BASIC METHODOLOGIES

- Homogenous assays: easy to do, not very sensitive
- Heterogeneous assays: most assays on the market
- Non-competitive assay: suitable for large molecules
- Competitive assay: suitable for small molecules

Basic Methodologies

- **Homogeneous** immunoassays do not require separation of unbound antibody-antigen complexes from the bound complexes, and thus are faster and easier to perform than heterogeneous immunoassays.
- **Heterogeneous** immunoassays require the separation of unbound complexes, often utilizing a solid phase reagent such as a magnetic particle or plastic bead.

Basic methodologies: Competitive Format

- In competitive formats,
- The amount of unlabeled (native) analyte (usually antigen) in the test sample is measured by its ability to compete with labeled antigen in the immunoassay.
- The unlabeled antigen blocks the ability of the labeled antigen to bind because that binding site on the antibody is already occupied.

Noncompetitive format

- Noncompetitive assay formats can utilize either one step or two step methods.
- The two step assay format employs wash steps in which the sandwich binding complex is isolated and washed to remove excess unbound labeled reagent and any other interfering substances.
- The two step noncompetitive format usually offers the highest specificity and sensitivity of all the assay formats discussed here.

Types of immunoassays

TAGS

- **ELISA: enzyme linked immuno-sorbent assay**
- Radio-immunoassays
- Immunoassays with color or fluorescent tags (colloidal gold, polymers, crystals)

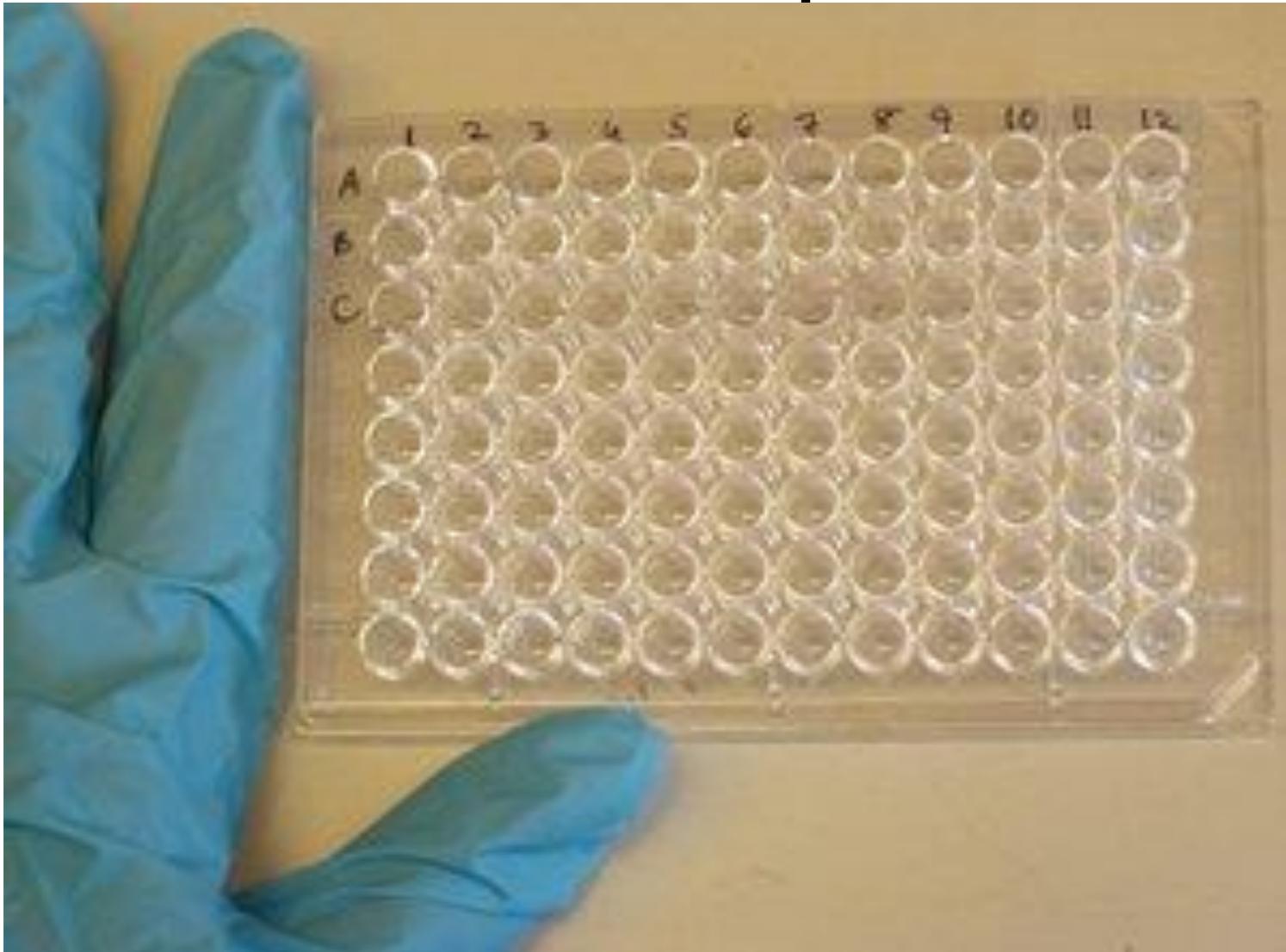
Type of detection antibody (conjugate)

- Direct assay
- Indirect assay

Basic formats

- Sandwich assay: several variations
- Multiplex assay
- Rapid assay: lateral flow chromatography

Microtiter plate



Basic definitions

- **Primary antibodies** bind directly to the antigen/s, assay can have one or two primary antibodies.
- **Capture antibody** is attached to the bottom of the well and binds or captures the antigen.
- **Detection antibody** is covalently linked to the tag, can bind directly to the antigen or the primary antibody
- **Secondary antibody** binds to the primary antibody (typically always detection Ab)
- **Conjugate** is a complex made of the antibody or the antigen and the tagging agent. The link is usually covalent.

Direct ELISA

The direct ELISA uses the method of directly labeling the primary antibody itself.

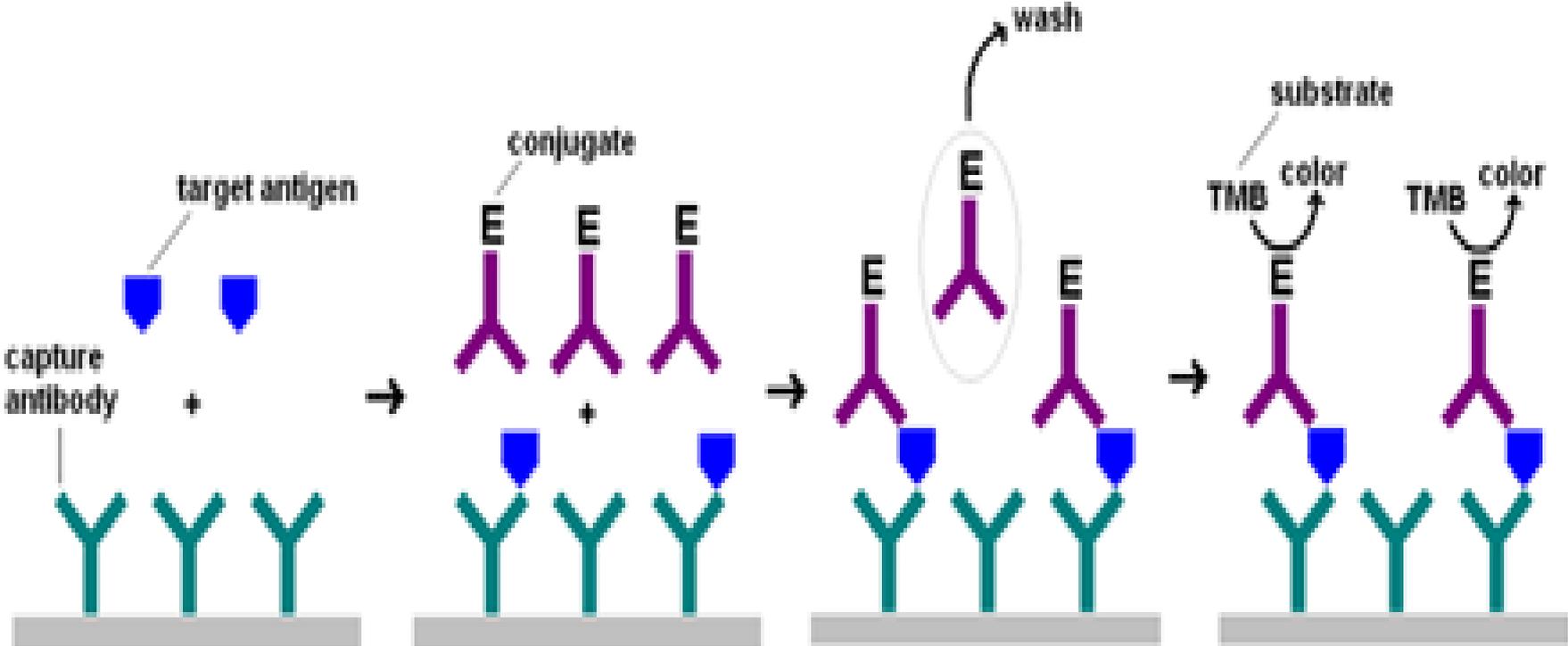
Since the secondary antibody step is omitted, the direct ELISA is relatively quick, and avoids potential problems of cross-reactivity of the secondary antibody with components in the antigen sample.

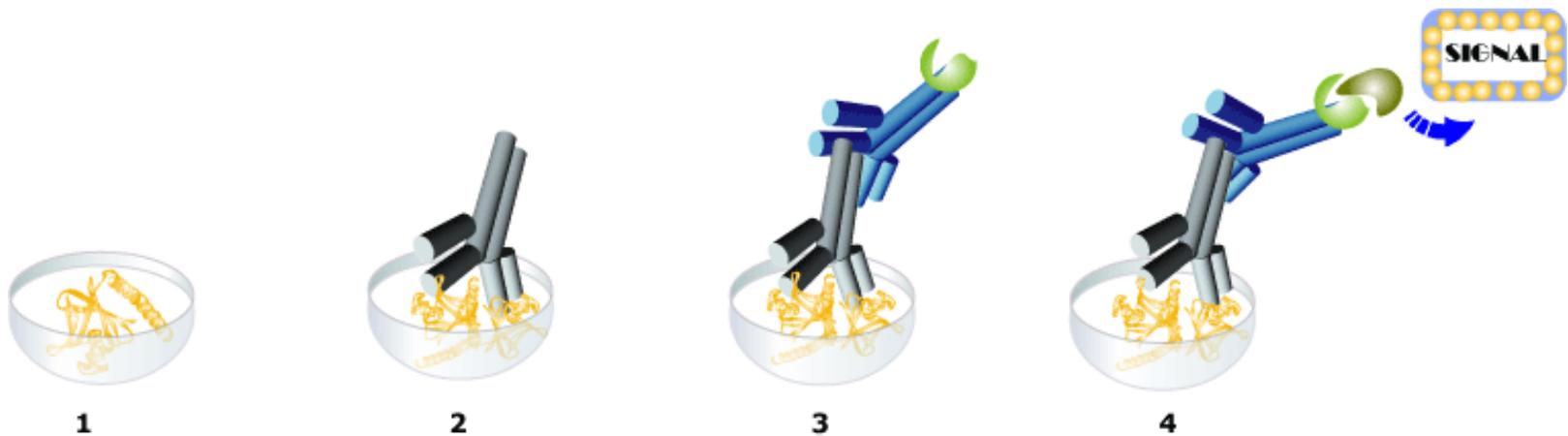
However, the direct ELISA requires the labeling of every antibody to be used, which can be a time-consuming and expensive proposition.

In addition, certain antibodies may be unsuitable for direct labeling.

Direct methods also lack the additional signal amplification that can be achieved with the use of a secondary antibody.

Direct sandwich ELISA steps





Indirect ELISA

The indirect, two-step method uses a labeled secondary antibody for detection.

First, a primary antibody is incubated with the antigen.

This is followed by incubation with a labeled secondary antibody (detection antibody conjugate) that recognizes the primary antibody.

Secondary antibody is responsible for generating a detection signal.

Sandwich ELISA

The sandwich ELISA measures the amount of antigen between two layers of antibodies.

The antigens to be detected must contain at least two antigenic sites (epitopes), capable of binding to the antibody, since at least two antibodies act in the sandwich.

For this reason, sandwich assays are restricted to the quantitation of multivalent antigens such as proteins or polysaccharides.

To utilize this assay, one primary antibody (the “capture” antibody) is bound to a solid phase typically attached to the bottom of a plate well.

Antigen is then added and allowed to complex with the bound primary antibody.

Unbound products are then removed with a wash, and a labeled second primary antibody (the “detection” antibody) is allowed to bind to the antigen, thus completing the “sandwich”.

Sandwich ELISA

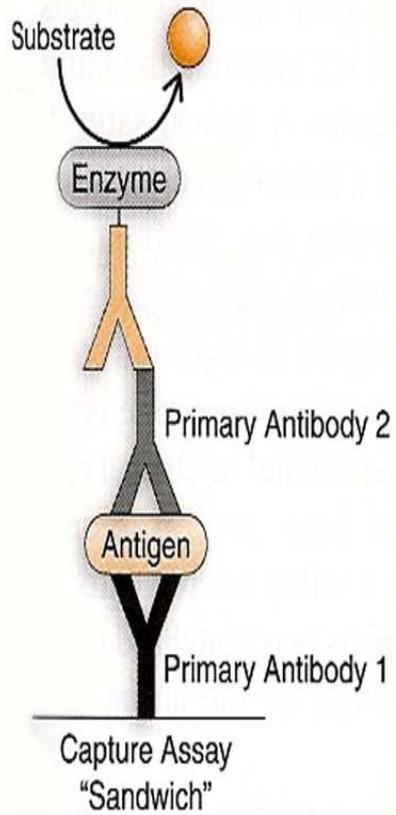
The assay is then quantitated by measuring the amount of labeled second primary antibody bound to the matrix, or the amount of secondary antibody bound to the second primary Ab, through the use of a colorimetric substrate.

Major advantages of this technique are that the antigen does not need to be purified prior to use, and that these assays are very specific.

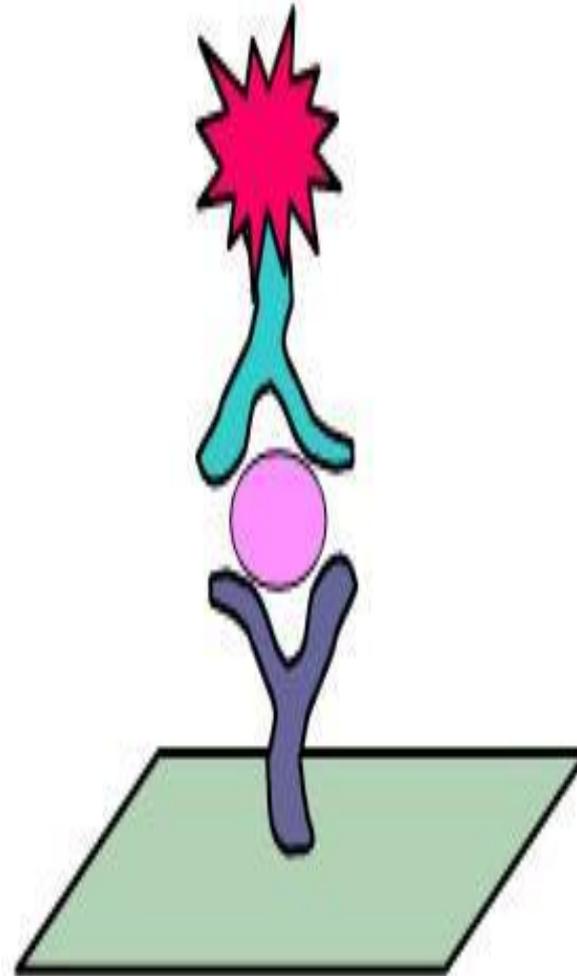
However, one disadvantage is that not all antibodies can be used.

Monoclonal antibody combinations must be qualified as “matched pairs”, meaning that they can recognize separate epitopes on the antigen so they do not hinder each other’s binding.

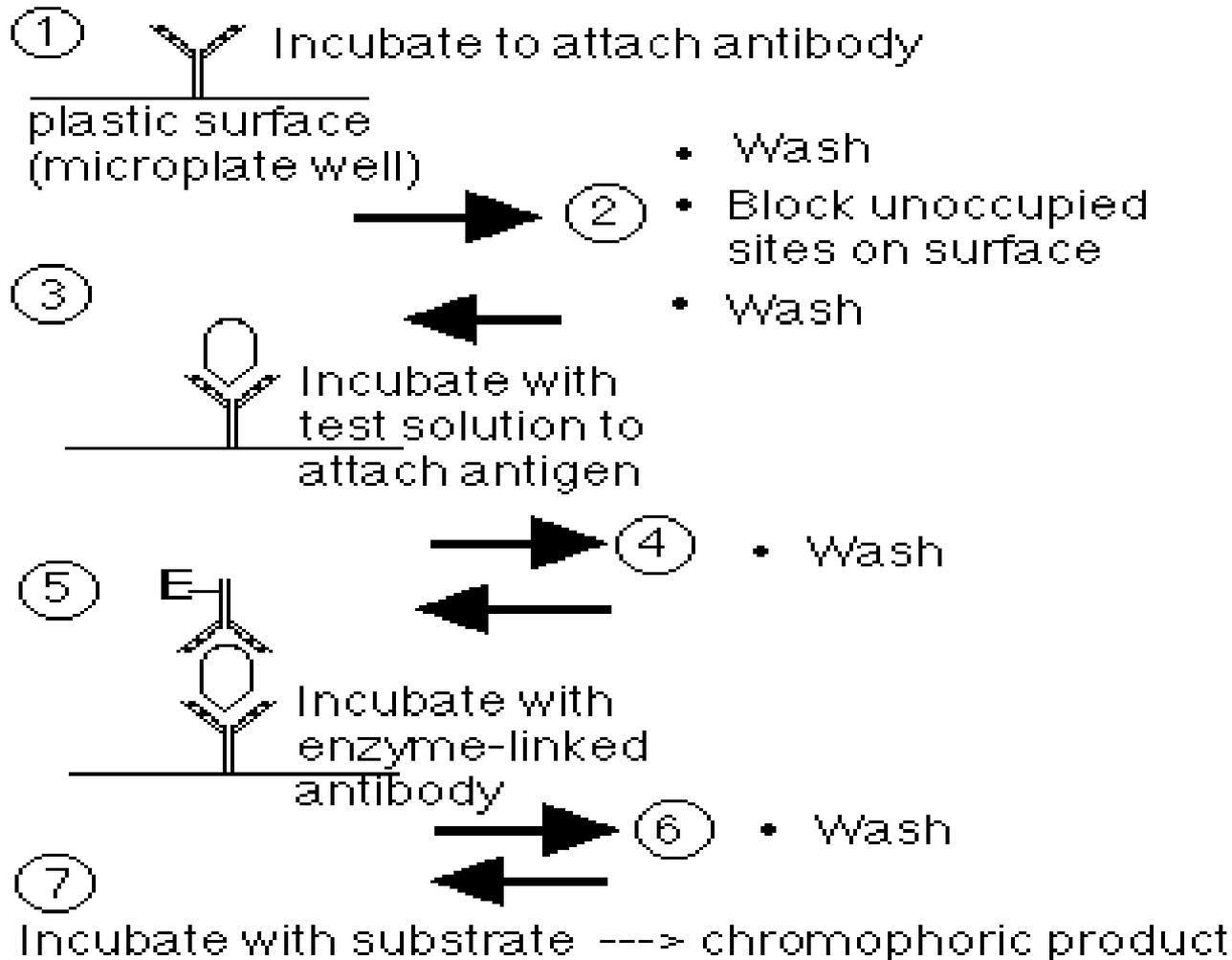
Indirect sandwich ELISA



Direct sandwich ELISA

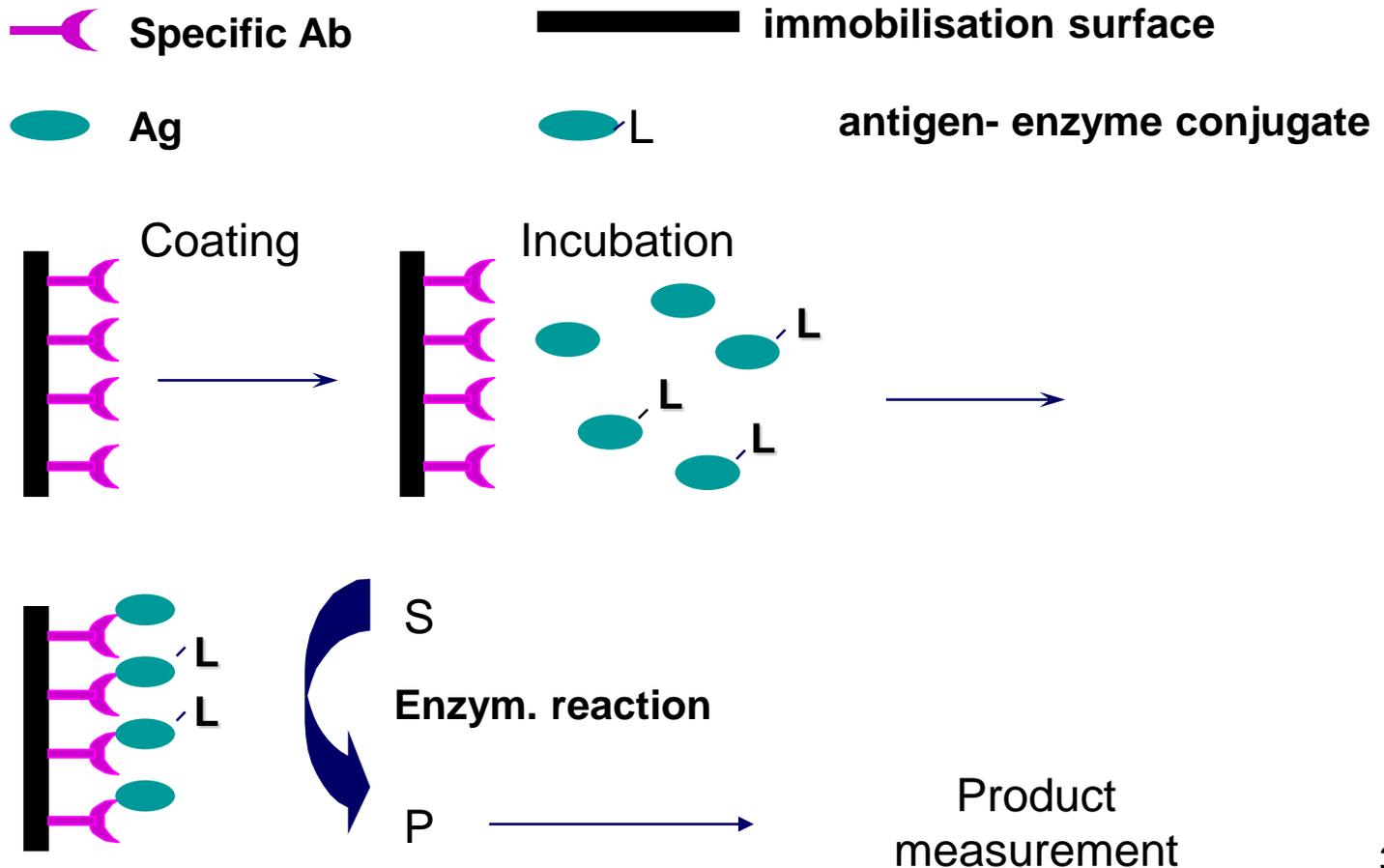


ELISA: Enzyme Linked Immunosorbent Assay protocol



Competitive Immunoassays

In competitive formats, unlabelled analyte (usually antigen) in the test sample is measured by its ability to compete with labeled antigen in the immunoassay.



Multiplex ELISA

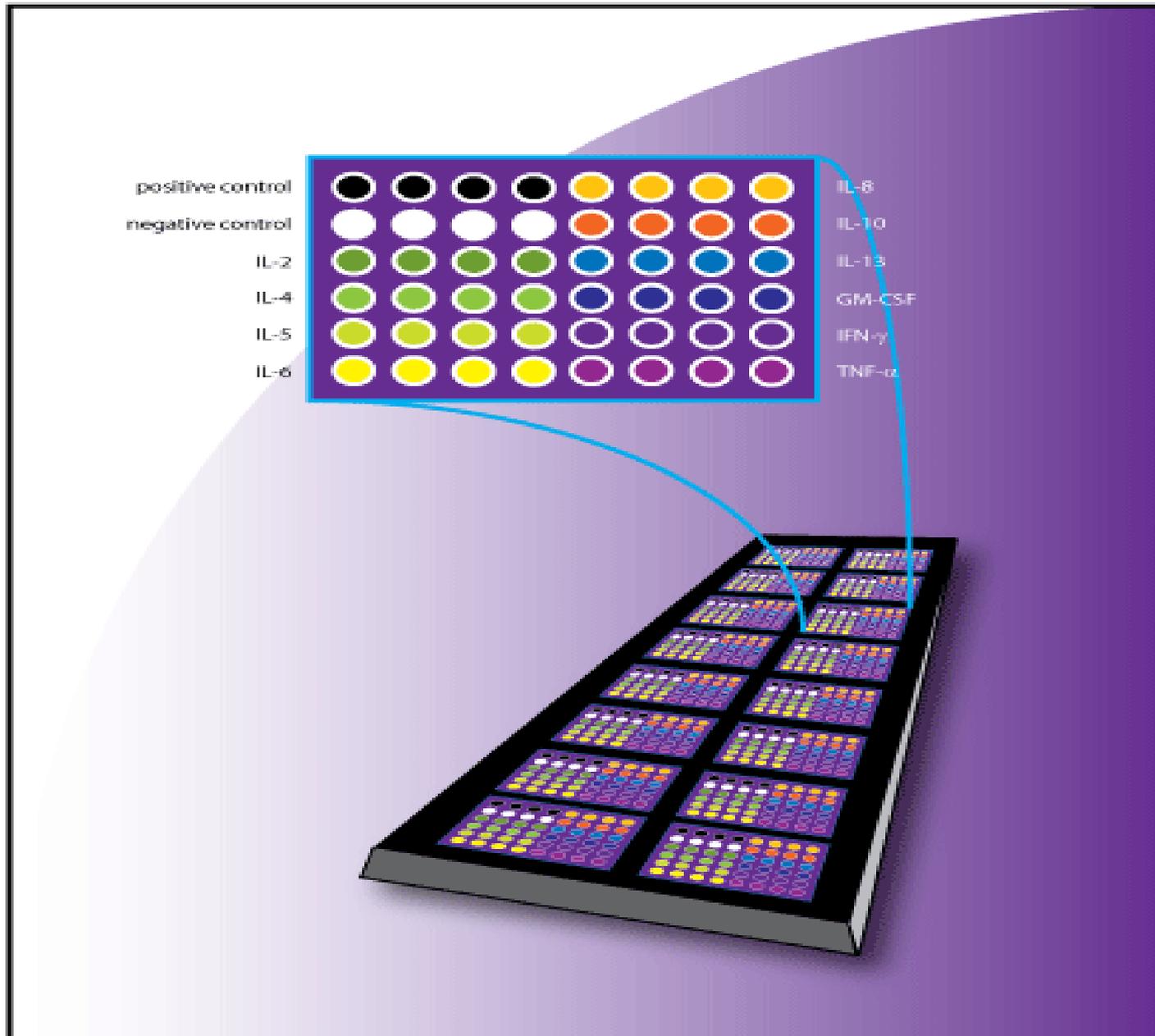
A logical progression of the widely used microtiter plate ELISA is toward a protein array format that allows simultaneous detection of multiple analytes at multiple array addresses within a single well.

Different types of multiplex ELISA have been developed.

Generally, multiplex ELISA can be achieved through antibody array, where different primary antibodies are printed on glass plate to capture corresponding antigens in a biological sample such as plasma, cell lysate, or tissue extract.

Detection method can be direct or indirect, sandwich or competitive, labeling or non-labeling, depending upon antibody array technologies.

Multiplex ELISA protein array



Rapid test



bird flu test H5N1



POSITIVE NEG ATIVE INVALID

Rapid Test device components

Porous materials

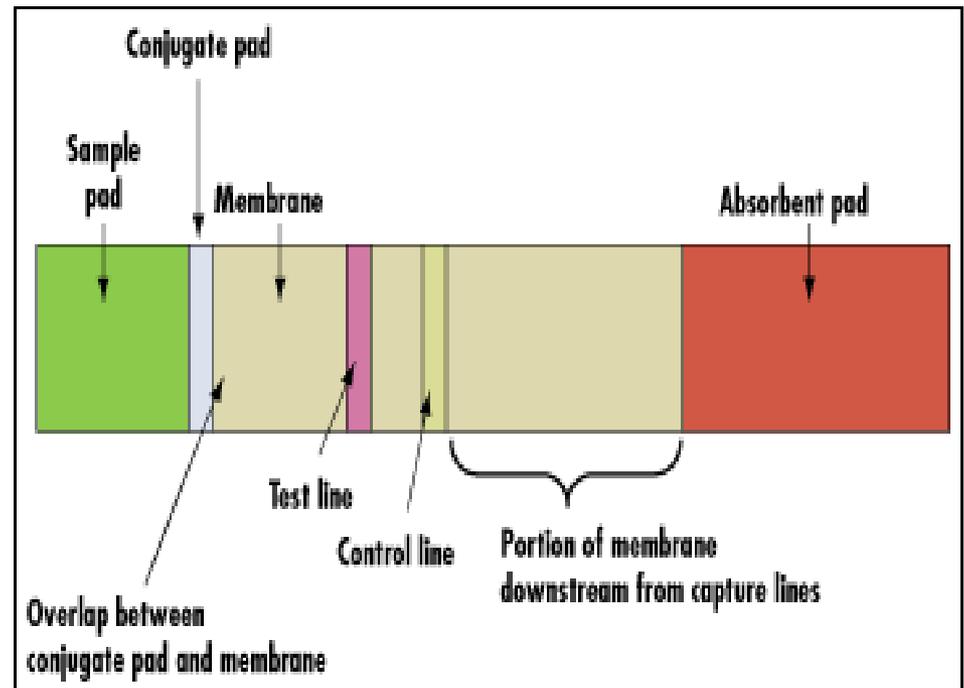
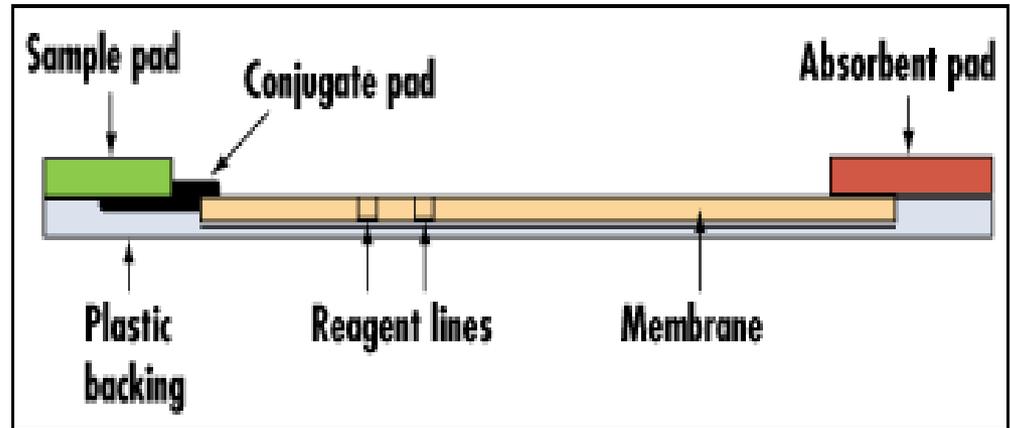
- Sample pads
- Conjugate pads
- Nitrocellulose membranes
- Absorbent pads

Reagents

- Capture antibodies and/or antigens (test line and control line)
- Conjugate (antibody or antigen)
- Detector particle (e.g., colloidal gold)
- Blocking agents, detergents, surfactants, stabilizers, buffers, etc.

Housing and lamination materials

- Back laminate (for holding porous components together)
- Top laminate (optional, to act as a "splash guard" or prevent evaporation and back-migration of detector reagent)



Pregnancy test

(A)

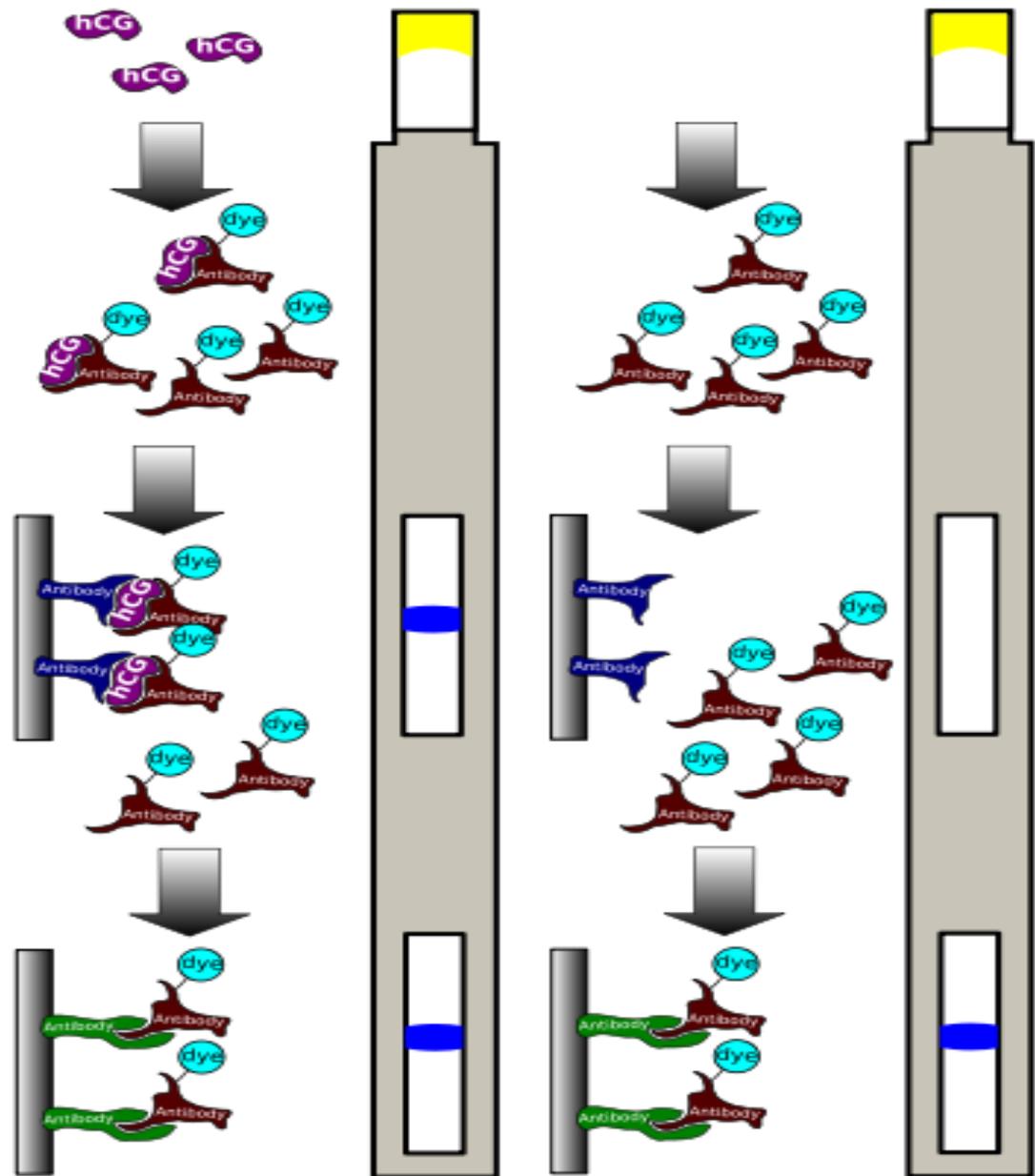
Pregnant

Not pregnant

(B)

(C)

(D)



Specifications for a pregnancy test

Following are the development guidelines given to the research and development manager responsible for designing, developing, and scaling up to manufacturing a hypothetical lateral-flow diagnostic test device for detecting human chorionic gonadotropin (hCG) in urine.

The hCG hormone is measured in milli-international units per milliliter (mIU/ml).

- More than 99% of specimens containing 25 mIU/ml will produce a visible signal at the test line.
- More than 99% of specimens containing 5 mIU/mL will not produce a visible signal at the test line.
- The test will have a control line that will always produce a visible signal if the test is performed correctly and if all of the test components are functional.
- If the sample is positive (contains 25 mIU/ml hCG), the test must produce a visible signal at the test line within 3 minutes of sample addition.
- The test result must be stable (not change) for at least 30 minutes after the sample is added.
- The test device will be contained in a plastic housing and designed to be compatible with "in-stream" sampling protocols.
- The packaged (unused) product must be stable for at least 18 months at ambient conditions (15°–30°C).
- Total incremental manufacturing cost per test (excluding licensing and royalties) can not exceed \$0.25 US.

Rapid test for drugs of abuse, competitive

Advanced biotechnology: **Negative** **Positive**

