ELISA
(ENZYME LINKED IMMUNOSORBENT ASSAY)

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- ELISA assays use antibodies or antigens covalently bound to enzymes.
- The conjugated enzymes are selected on the basis of their ability to catalyze the conversion of a substrate into a colored, fluorescent, or chemiluminescent product.
- A number of variations of the basic ELISA assay have been developed.
- Each type of ELISA can be used qualitatively to detect the presence of antibody or antigen.

- Indirect ELISA:

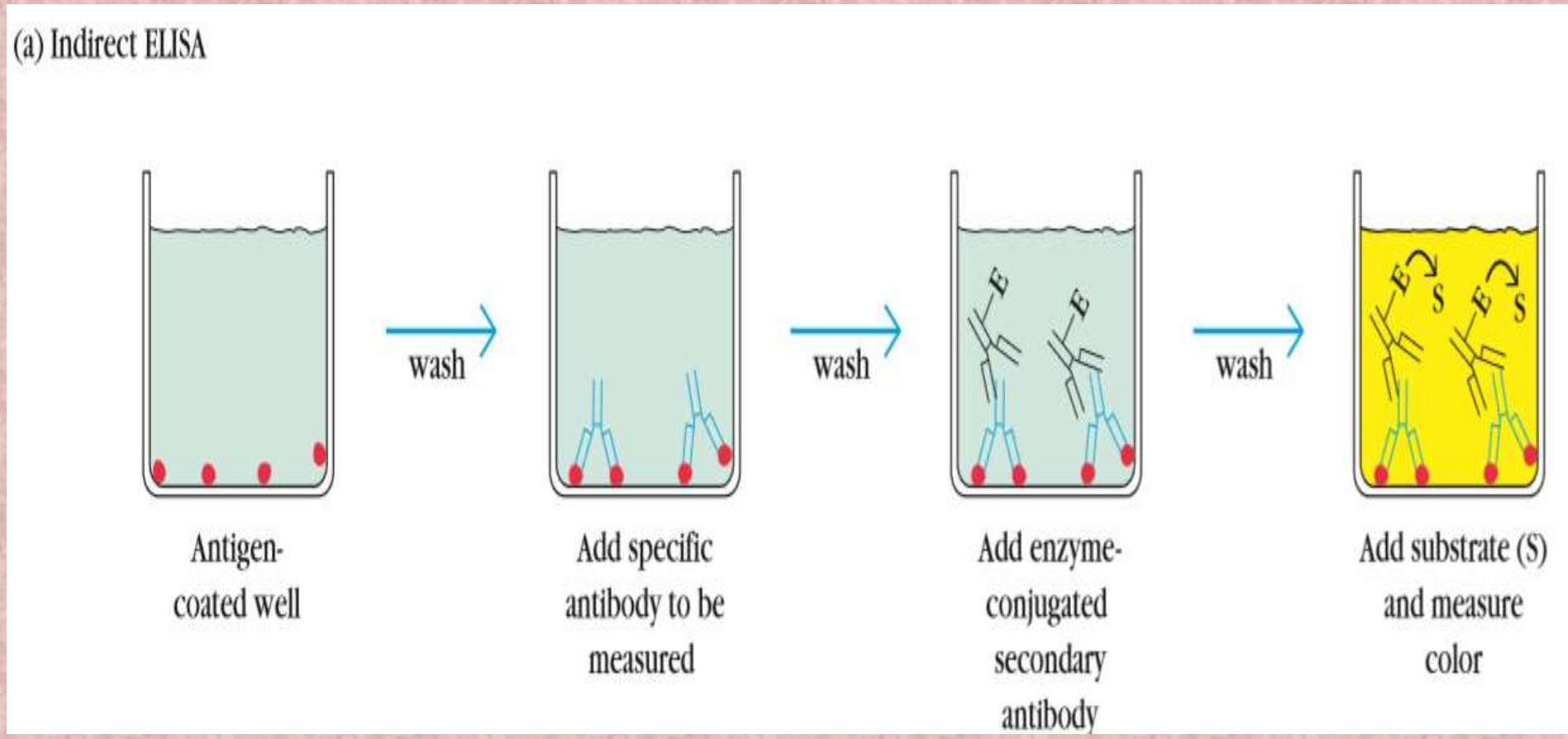
• Antibody can be detected, or its concentration determined with an indirect ELISA assay (see Figure 20-7a).

• Serum or some other sample containing primary antibody (Ab_1) is added to an antigen-coated microtiter well and allowed to react with the antigen attached to the well.

• After any free Ab_1 is washed away, the antibody bound to the antigen is detected by adding an enzyme-conjugated secondary antibody (Ab_2) that binds to Ab_1.

• Any free Ab2 is again washed away, and a substrate for the enzyme is added.
• The amount of colored, fluorescent, or luminescent reaction product that forms is measured using a specialized plate reader.
• This version of ELISA is the method of choice to detect the presence of serum antibodies against human immunodeficiency virus (HIV), the causative agent of AIDS.
• In this assay, recombinant envelope and core proteins of HIV are adsorbed as solid-phase antigens to microtiter wells.

• Individuals infected with HIV will produce serum antibodies to epitopes on these viral proteins.
• Generally, serum antibodies to HIV can be detected by ELISA within 6 weeks of infection.



- Sandwich ELISA:

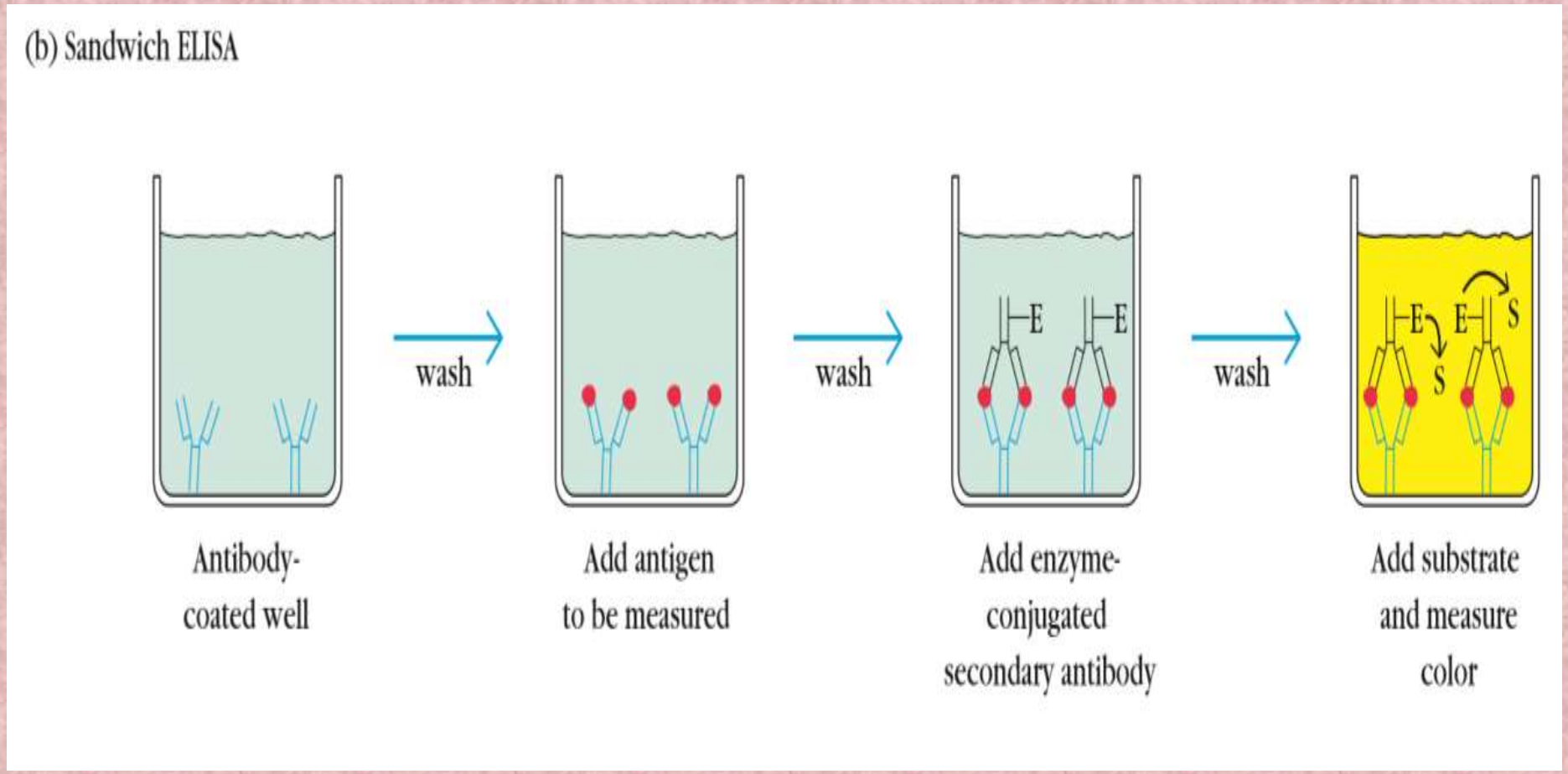
• Antigen can be detected or measured by a sandwich ELISA (see Figure 20-7b).
• In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well.
• A sample containing unknown amounts of antigen is allowed to react with the immobilized antibody.
• After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen.

• After any free second antibody is removed by washing, substrate is added, and the colored reaction product is measured.

• A common variant on this assay uses a biotin-linked second antibody and then adds enzyme-linked avidin in an additional step.

• Sandwich ELISAs have proven particularly useful for the measurement of soluble cytokine concentrations in tissue culture supernatants, as well as in serum and body fluids.

• Note that, for this assay to work, the two antibodies used for the antigen immobilization (capture) and detection phases respectively must bind to different determinants (epitopes) on the antigen.

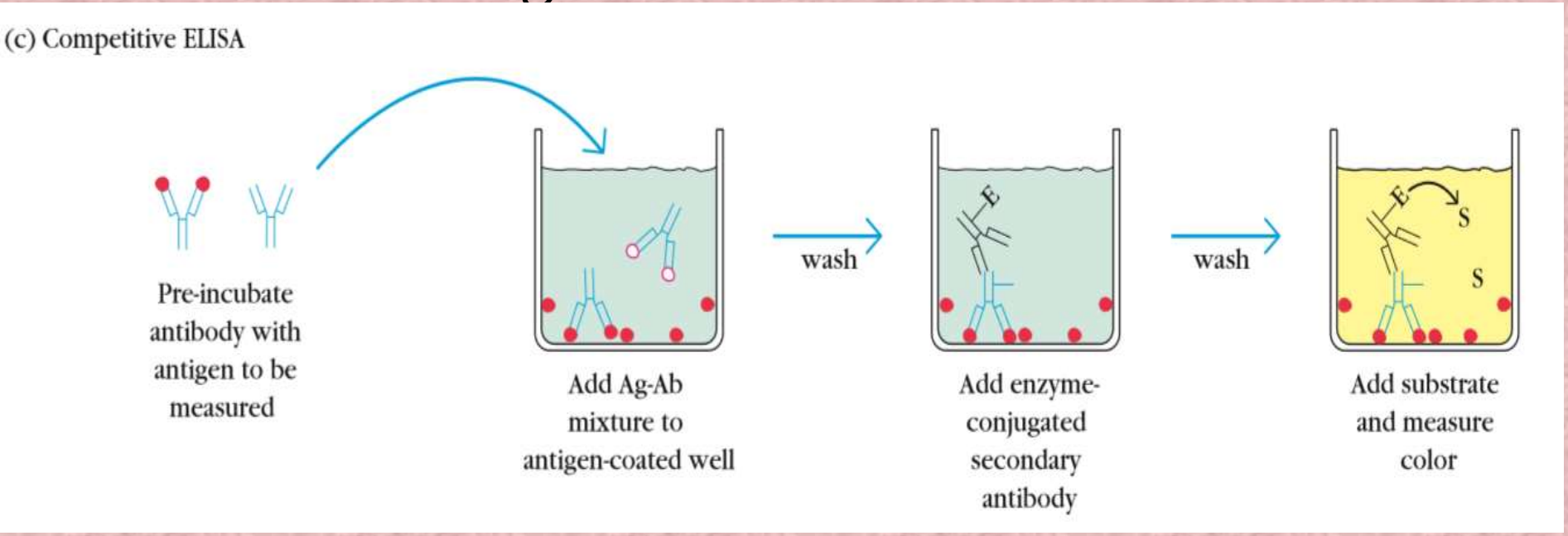


- Competitive ELISA

• The competitive ELISA provides another extremely sensitive variation for measuring amounts of antigen (see Figure 20-7c).
• In this technique, antibody is first incubated in solution with a sample containing antigen.
• The antigen-antibody mixture is then added to an antigen-coated microtiter well.
• The more antigen present in the initial solution-phase sample, the less free antibody will be available to bind to the antigen-coated well.

•After washing off the unbound antibody, an enzyme-conjugated Ab_2 specific for the isotype of the Ab_1 can be added to determine the amount of Ab_1 bound to the well.

• In the competitive assay, the higher the concentration of antigen in the original sample, the lower the final signal



❑ Available Enzyme Systems for ELISA assays:

• The three enzymes commonly used in ELISA assays are alkaline phosphatase, β-galactosidase, and horseradish peroxidase (HRP), each of which can be used with chromogenic substrates (substrates which are colorless but give rise to a product that absorbs light in the visible range).

• Of these three, β galactosidase is the least frequently employed.

▪ Chromogenic, Fluorogenic, or Chemiluminogenic Substrates :

• As indicated above, chromogenic substrates that give rise to a colored product are particularly useful for “yes or no” assays, as they can be read by the naked eye.
• They are quick to use and do not require costly instrumentation.
• In contrast, fluorogenic and chemiluminogenic substrates offer significant advantages in terms of sensitivity, but they require specialized equipment.

➢Hybridoma technology: Monoclonal antibody

• From the early days of immunology, investigators and clinicians have made use of the ability of animals to respond to immunization with the production of antibodies directed toward injected antigens, such as viruses, bacteria, fungi, or simple chemicals from the laboratory shelf.

• Antibodies harvested from the serum of immunized animals are the secreted products of many clones of B cells and are thus referred to as polyclonal antibodies.

- Polyclonal antibodies are generated by immunizing an experimental animal or a human subject with antigen one or more times, bleeding the subject, and purifying the antibodies from the subject's serum.
- Because polyclonal antibodies are a mixture of antibodies directed toward a variety of different epitopes of the immunizing antigen, they are particularly useful for techniques such as agglutination or immunoprecipitation, which rely on the ability of the antibody to form a large antigen-antibody complex.

• The disadvantage of using a polyclonal preparation is that some of the antibodies in the mixture may have ill defined cross-reactivities with related antigens.

• The disadvantages of unforeseen cross-reactivities or variations in the fine specificity of polyclonal antibodies are eliminated when using monoclonal antibodies (mAbs), which are the product of a single, stimulated B cell.

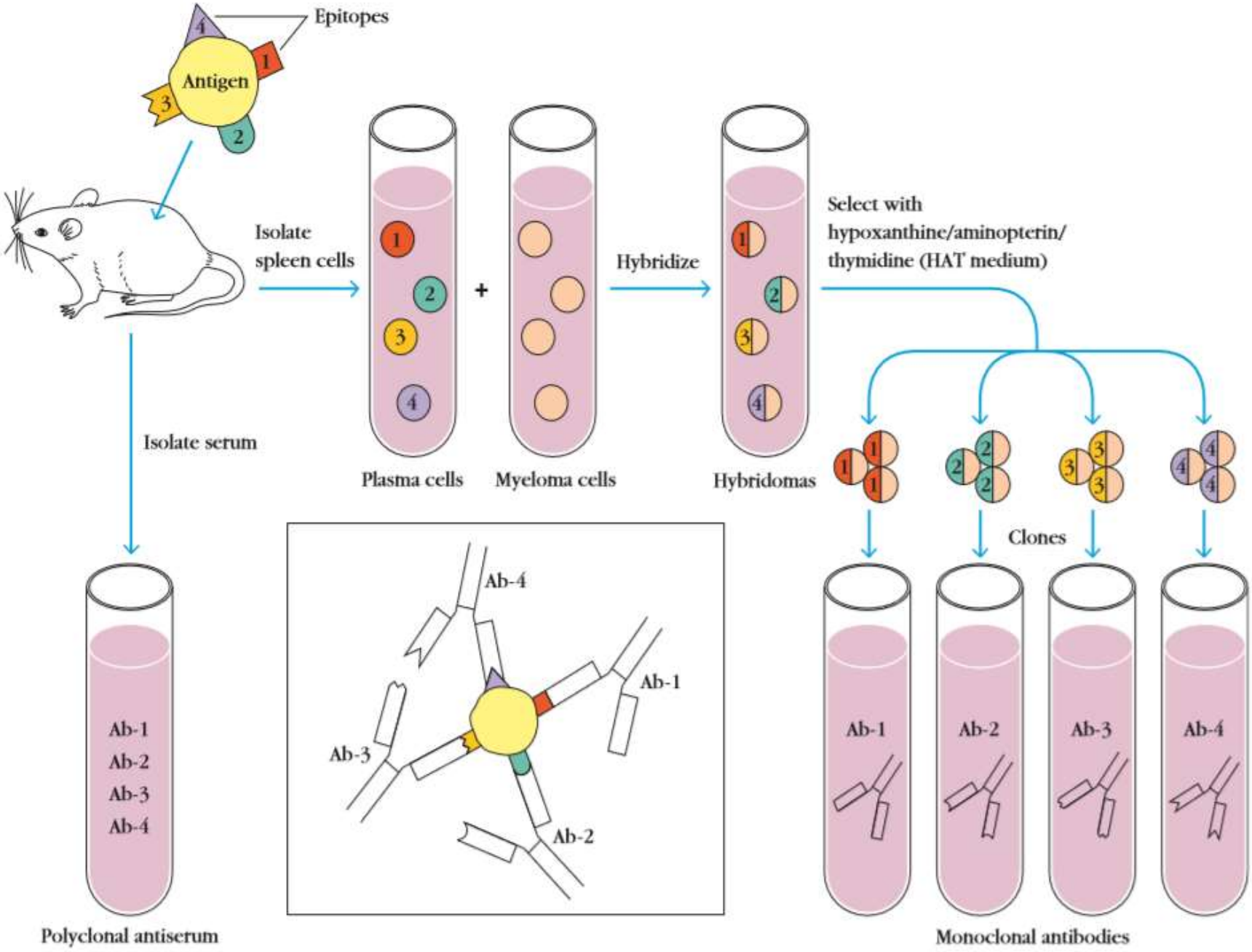
• In 1975, Georges Köhler and Cesar Milstein figured out how to generate large quantities of antibodies derived from a single B-cell clone.

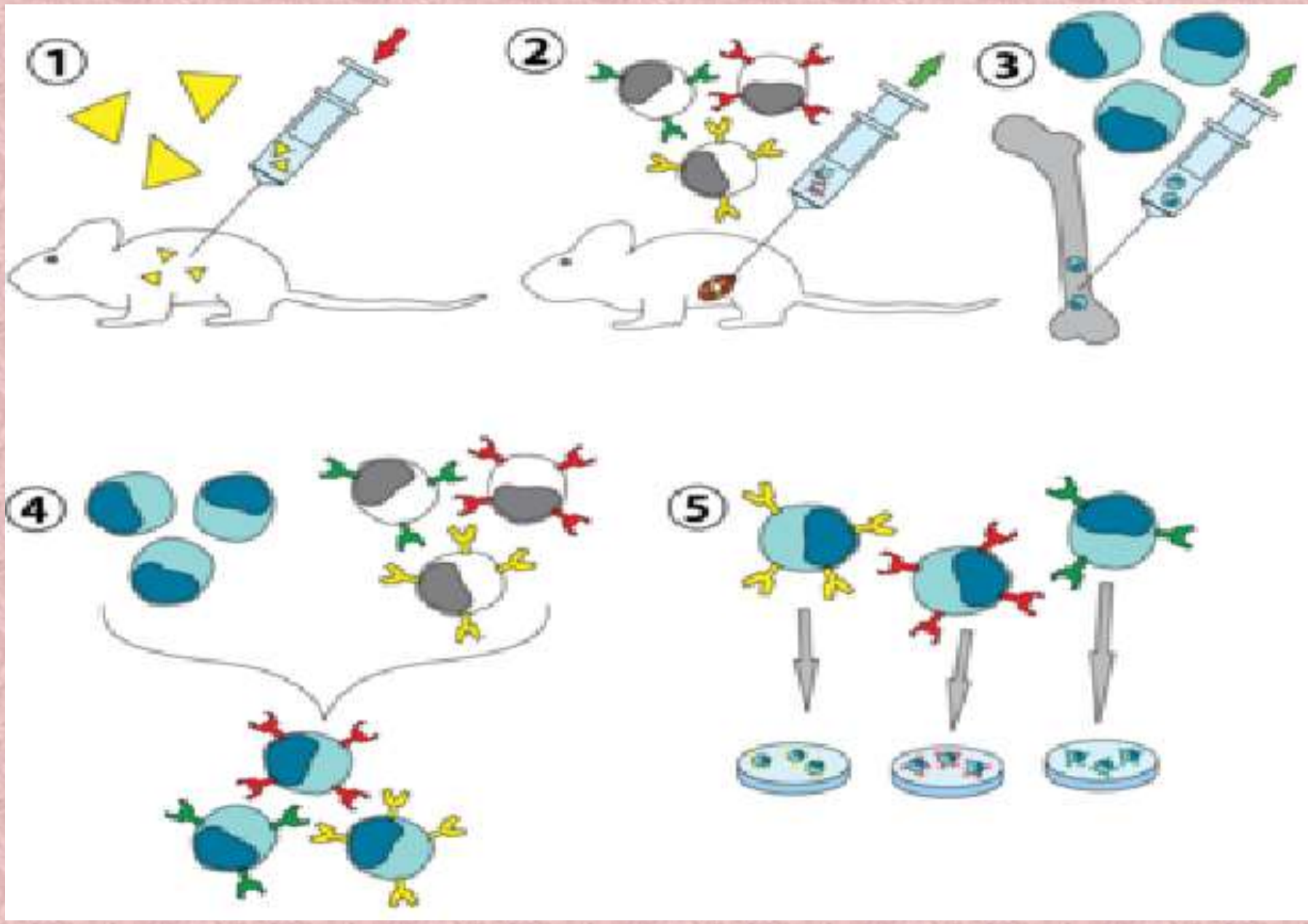
• By fusing a normal, activated, antibody-producing B cell with a myeloma cell (a cancerous plasma cell), they were able to generate a hybridoma that possessed the immortal growth properties of the myeloma cell parent and secreted the unique antibody produced by the B-cell parent.

• Over time, myeloma-cell partners were generated that had lost the ability to synthesize their own immunoglobulin, thus ensuring that the only antibodies secreted into the culture medium were those from the B-cell fusion partner.

• The original fusions used Sendai virus to disrupt the plasma membrane of the cells; nowadays, chemical fusogens such as polyethylene glycol are used instead.
• Hybrids formed by the fusion of two antibody-producing B cells will not grow out of these cultures because B cells have a relatively short half-life in vitro.
• However, hybrids formed by the fusion of two or more cancer cells would have the potential to grow out from the initial fusions, and compete successfully for nutrients with the B cell-myeloma hybrids.

• A method therefore had to be devised to eliminate these tumor-tumor hybrids from the cultures of fused cells.
• Köhler and Milstein solved this problem by using myeloma cells lacking the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT).
• HGPRT is necessary for one of the two potential pathways of DNA synthesis, the salvage pathway.
• The alternative pathway of DNA synthesis, the de novo pathway, can be inhibited by the antibiotic aminopterin.


Epitopes
Antigen
Isolate spleen cells
Plasma cells
Myeloma cells
Hybridize
Hybridomas
Select with hypoxanthine/aminopterin/ thymidine (HAT medium)
Isolate serum
Clones
Ab-1
Ab-2
Ab-3
Ab-4
Polyclonal antiserum
Monoclonal antibodies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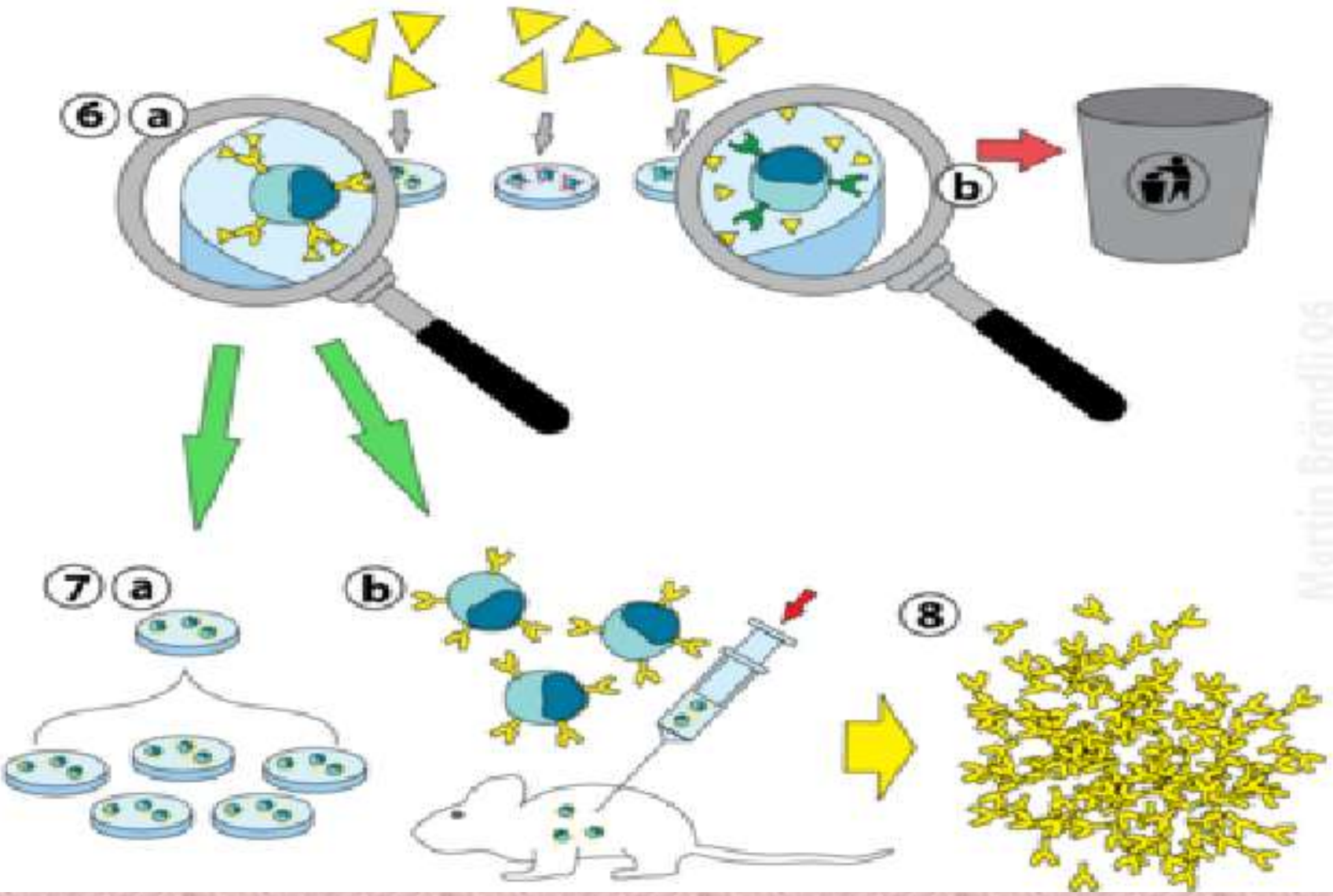
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a
b
7
a
b
8

(1) Immunisation of a mouse
(2) Isolation of B cells from the spleen
(3) Cultivation of myeloma cells
(4) Fusion of myeloma and B cells
(5) Separation of cell lines
(6) Screening of suitable cell lines
(7) *in vitro* **(a)** or *in vivo* **(b)** multiplication

• Köhler and Milstein reasoned that, if they grew the hybrid cultures in the presence of aminopterin, the mutant tumor cells and tumor-tumor hybrids would be unable to synthesize new DNA by either the salvage or the de novo pathways and would eventually die.

• However, in the hybridomas formed by fusion between B cells and tumor cells, the B-cell parent would provide the HGPRT, and so these hybrids would survive in the selection medium. Because the medium containing aminopterin is normally supplemented with hypoxanthine and thymidine to support nucleotide synthesis, the selective medium is known as “HAT medium.”

• The resulting clones of hybridoma cells randomly lose chromosomes over the first few days following fusion, but eventually they stabilize and can be cultured indefinitely, secreting large quantities of mAbs of predefined specificity and known cross-reactivity.

• The importance of hybridomas to the biological sciences was recognized when Köhler and Milstein were awarded the Nobel Prize in Physiology or Medicine in 1984. The precise specificity, affinity, and cross-reactivity of mAbs are entirely stable with time, and they are particularly useful for diagnostic purposes.

❖ Application of hybridoma technology

Monoclonal antibodies are proving to be very useful as diagnostic, imaging, and therapuetic reagents in clinical medicine.

Many monoclonal antibody diagnostic reagents now available are products for detecting pregnancy, diagnosing numerous pathogenic microorganisms, measuring blood levels of various drugs, and detecting antigens shed by certain tumors.

➢ Advantages of using Monoclonal Antibodies:

- Hybridoma serves as an immortal source of monoclonal antibody.
- Same quality of the antibody is maintained amongst the different production batches.
- Highly reproducible and scalable, unlimited production source.
- Speed and sensitivity and specificity of assays.