DNA diagnostics and DNA microchips. Enzyme-Linked Immunosorbent Assay (ELISA)

Candidate of bio. Sci. **Meldebekova Aliya A.**

1. **DNA-Based Diagnostic Approaches**
2. **Immunological Approaches to Detect Protein Biomarkers**
    - Antibodies (structure, polyclonal, monoclonal)
    - Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

- The **success** of modern **medicine and agriculture** often **depends on** the ability of workers in these fields **to detect the presence of** specific viruses, bacteria, fungi, parasites, proteins, and small molecules in humans, animals, plants, water, and soil.

- For example, **the prevention, control, or treatment of infectious disease** is generally facilitated by the **early and accurate identification** of the causative **pathogenic organism**.

- **Many of** these **detection procedures** require the **growth in culture of the potential pathogen** and then the analysis of a spectrum of physiological properties that facilitate its identification.

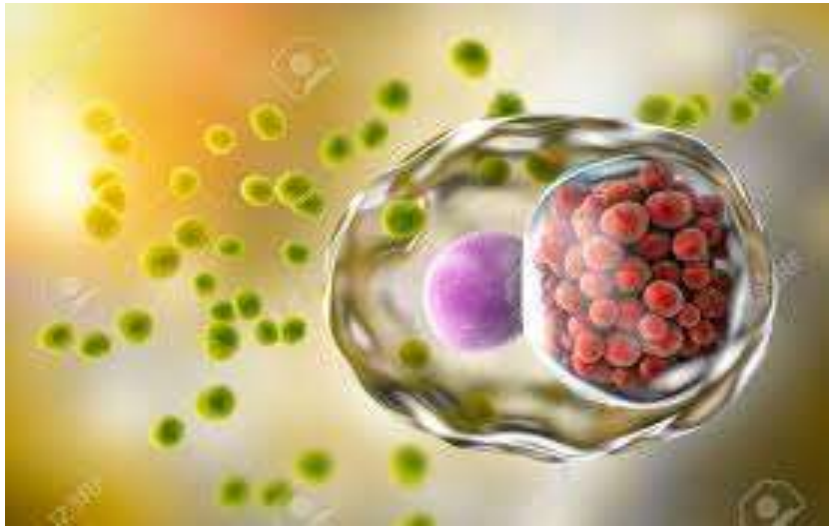
- Although tests of this type are effective and reasonably specific, they are often slow and costly.
- These constraints apply to the identification of both bacterial and parasitic (Table 1) organisms.
- In addition, **if the pathogenic organism does not grow well** or cannot be cultivated, **the opportunity to detect** the disease-causing organism **is severely limited**.

TABLE 9.1 A comparison of some of the methods used to diagnose parasite infection

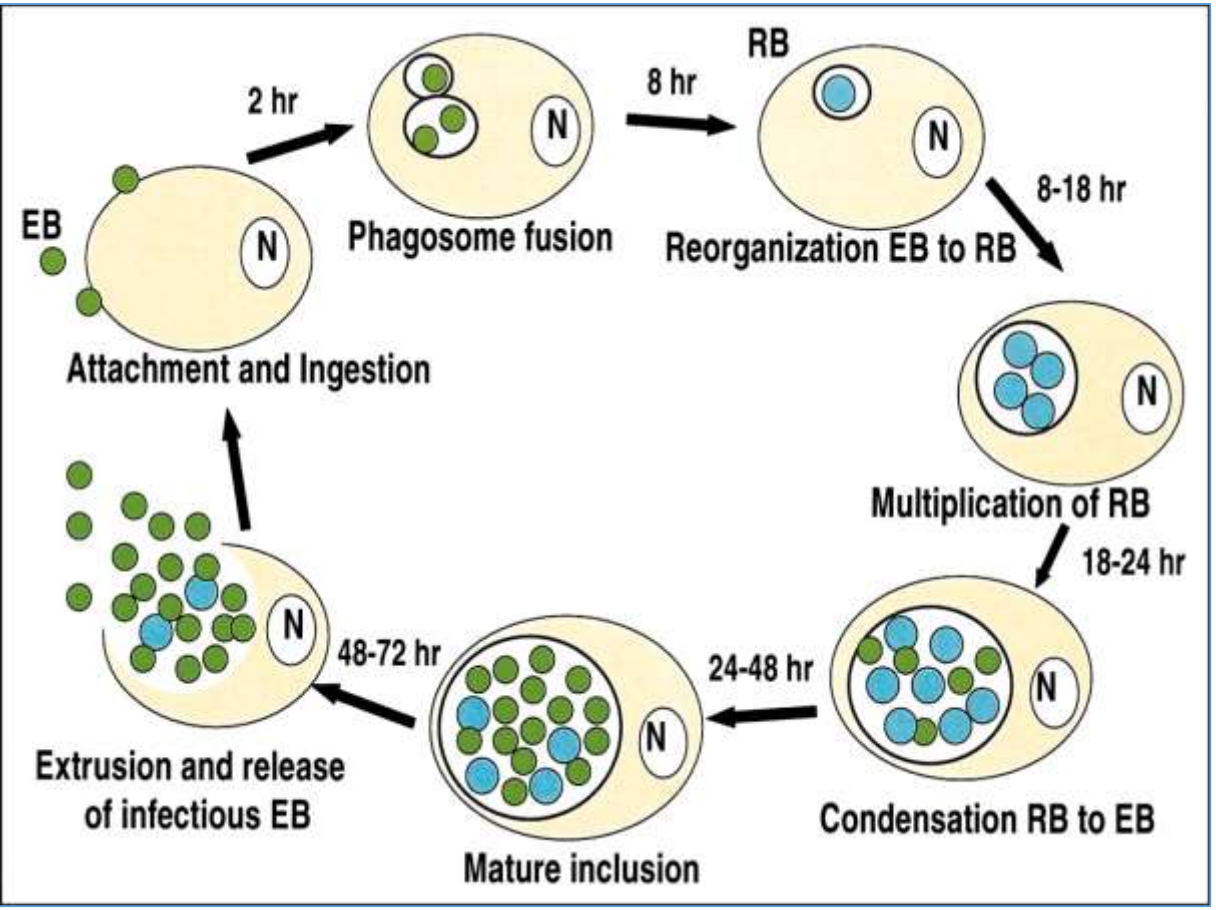
Method	Advantages	Disadvantages
Microscopic examination	Simple Direct detection of parasite Differentiates morphologically distinct organisms	Slow, laborious, and tedious Low sensitivity Cannot discriminate between similar organisms Requires a high skill level
In vitro culture and mouse inoculation	Detects only viable parasites Measures virulence and infectivity	Slow and expensive Different strains show a range of responses Parasite may lose its viability in the specimen Uses animals
Detection of antibodies in serum	Simple and fast Automatable Can be used to screen a large number of samples	Not always specific Does not distinguish between active and latent infections
DNA hybridization and PCR	Fast, sensitive, and specific Detects parasite directly Can distinguish different species Independent of previous infections Parasites need not be viable Automatable	Expensive and multistep Does not distinguish between live and dead organisms Possible false positives and false negatives

Adapted from Weiss, *Clin. Microbiol Rev.* 8:113–130, 1995.

For example, ***Chlamydia trachomatis***, an obligately **intracellular bacterium**, **causes a sexually transmitted disease** prevalent in North America and Europe.

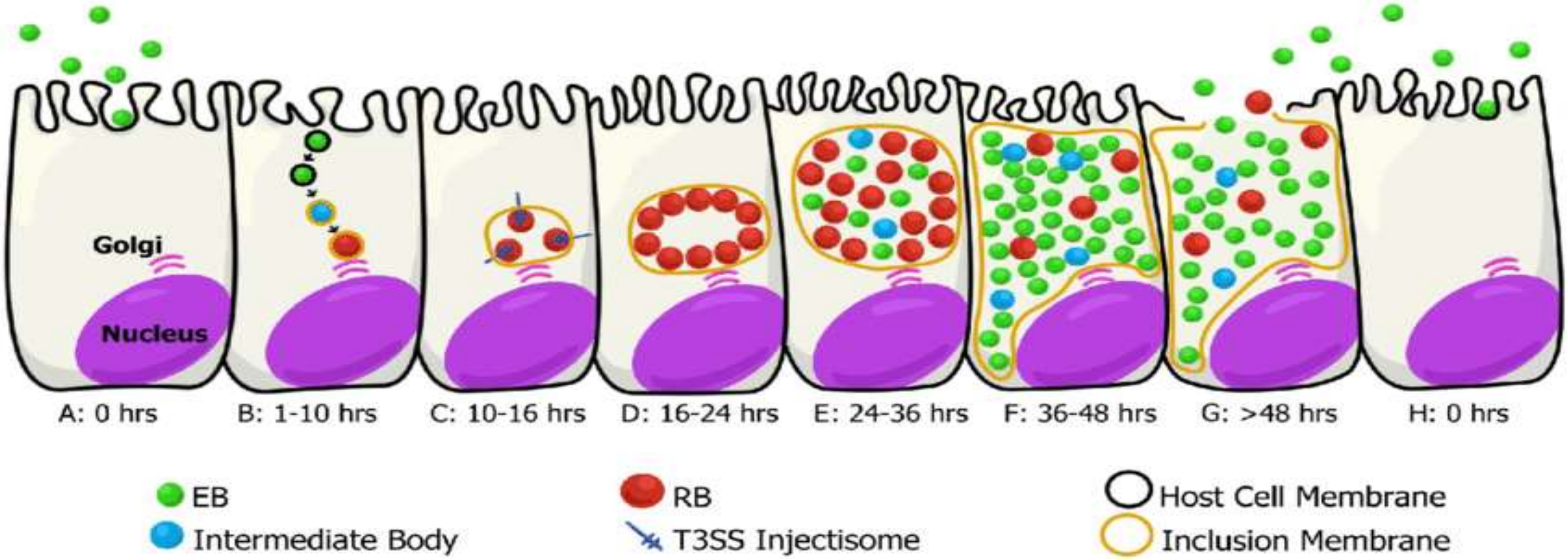


Clinical diagnosis of chlamydial infection is difficult, because **long-term cell cultivation** is required. **Frequently, false-negative results** (i.e., the diagnosis of the absence of the organism is erroneous) are obtained, and. **consequently, adequate treatment procedures will not be implemented.** Certainly, if **growth were required** for detection, then **at best only a few of** all **known pathogenic organisms** could ever be routinely **identified**.
To overcome this major constraint, **molecular diagnostic procedures** using either immunological or DNA detection methodologies have been devised.



Host	Infection*	Complication*
Men	Urethritis Postgonoccal urethritis Proctitis Conjunctivitis Pharyngitis	Epididymitis Prostatitis Reiter's syndrome Sterility?
Women	Cervicitis Urethritis Proctitis Conjunctivitis Pharyngitis	Salpingitis Endometritis Perihepatitis Ectopic pregnancy Infertility Dysplasia? Postpartum endometritis? Prematurity? Stillbirth? Neonatal death?
Infants	Conjunctivitis Pneumonia Asymptomatic pharyngeal carriage Asymptomatic gastrointestinal tract carriage Otitis media?	

* Question marks indicate that relationship has not been firmly established.


Golgi
Nucleus
A: 0 hrs
B: 1-10 hrs
C: 10-16 hrs
D: 16-24 hrs
E: 24-36 hrs
F: 36-48 hrs
G: >48 hrs
H: 0 hrs
EB
Intermediate Body
RB
T3SS Injectisome
Host Cell Membrane
Inclusion Membrane

In general, **any useful detection strategy** must be:

- **specific,**
- **sensitive,**
- **simple**.

Specificity means that the assay must yield a positive response for **only the target organism** or **molecule**.

Sensitivity means that the diagnostic test must identify **very small amounts of the target organism** or **molecule**, even in the presence of other potentially interfering organisms or substances.

Simplicity is required for the test to be run **efficiently, effectively**, and **inexpensively on a routine basis**.

DNA-Based Diagnostic Approaches

- DNA-based diagnostic tests **detect** the **existence of specific nucleotide sequence**s, for example, **mutations** associated with a genetic disease sequences indicative of the presence of a pathogen.
- They are highly sensitive and specific and can detect variations among individuals in a single nucleotide at a specific position in the genome (single nucleotide polymorphism, SNP).
- The ability to diagnose diseases in humans at the genetic level makes it possible to determine the cause of an illness and to predict whether individuals or their offspring are predisposed to the disease.
- Because a DNA based test does not require expression of a gene, in contrast to diagnostic detection of proteins, DNA analysis can be used for the identification of asymptomatic carriers of hereditary disorders, for prenatal diagnosis of serious genetic conditions, and for early diagnosis before the onset of symptoms.

Hybridization Probes

- **Hybridization** is the formation of **hydrogen bonds** between **two complementary strands** of nucleic acids. Steps:

- the cells in a sample such as infected or biopsied tissue are lysed,
- the genomic DNA in the lysed cells is denatured by treatment with a strong alkali to generate single-stranded target DNA,
- an excess of **labeled oligonucleotide probe** is added under appropriate conditions of temperature and ionic strength to promote base-pairing between the probe and the target DNA.
- Unbound probe DNA is removed by washing,
- and hybridization is detected by measuring the activity of the reporter molecule attached to the probe.

Hybridization probes are often used to detect the presence of microbial pathogens.

- Malaria, caused by the parasite ***Plasmodium falciparum***.

Application of Hybridization probes

- Hybridization probes are often used to detect the presence of microbial pathogens. More than 100 different DNA diagnostic probes have been developed for the detection of various pathogenic strains of bacteria, viruses, and parasites.

- Malaria, caused by the parasite ***Plasmodium falciparum***.

Probes have been developed for the diagnosis of human bacterial infections caused by:

- ***Legionella pneumophila*** (causative agent of pneumonia),
- ***Salmonella enterica*** serovar Typhi (food poisoning),
- **enterotoxigenic E.coli** (gastroenteritis), and
- ***Neisseria gonorrhoeae*** (gonorrhea, a sexually transmitted infection).
- In principle, nearly all pathogenic organisms can be detected by this procedure.

DNA-Based Diagnosis of Cystic fibrosis

About 1,900 different mutations are reported to occur in the CFTR genes of patients with cystic fibrosis. However, some of the mutations that cause cystic fibrosis are much more common than others (Table 4.2).

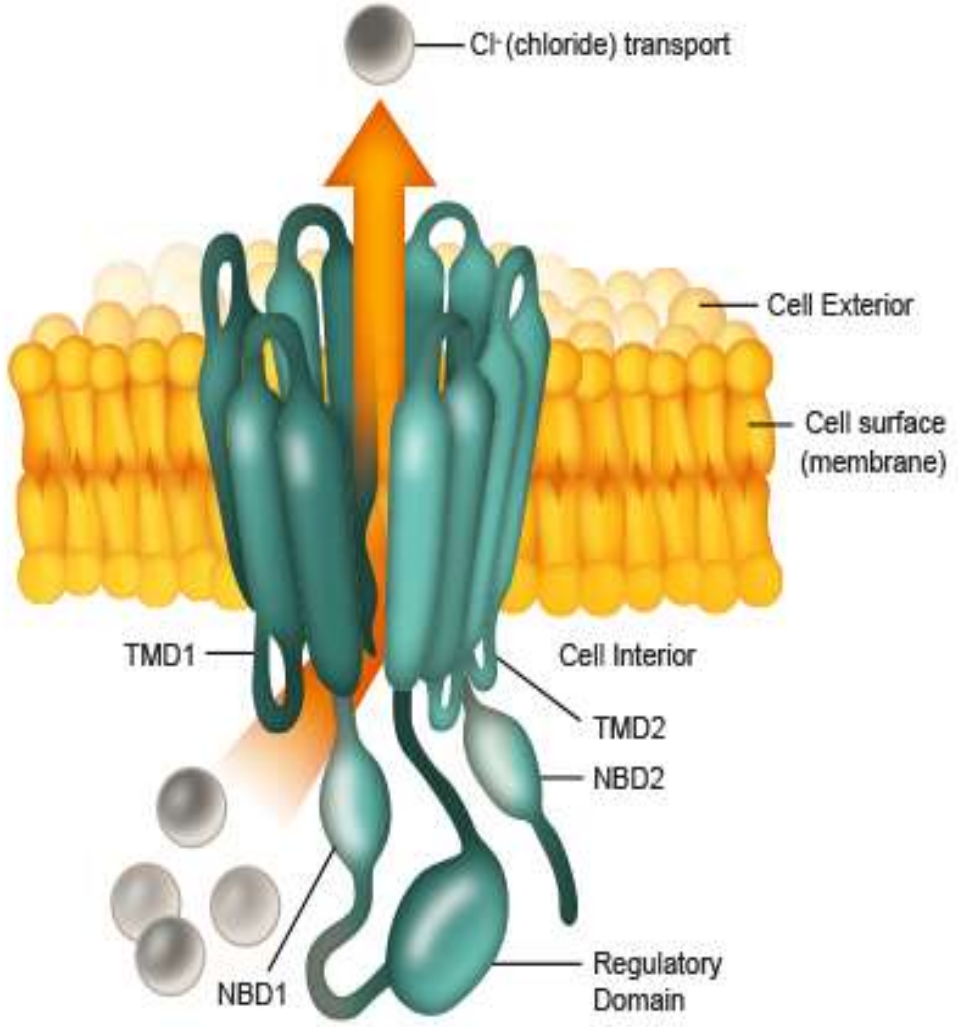
Table 4.2 The most common mutations of the CFTR protein that lead to cystic fibrosis

Mutation designation	Amino acid change to the CFTR protein
ΔF508	Deletion of phenylalanine at position 508
G542X	Replacement of glycine at position 542 by a stop codon
W1282X	Replacement of tryptophan at position 1282 by a stop codon
N1303K	Replacement of asparagine at position 1303 by lysine
1717-1G>A	Replacement of glycine by alanine at the last nucleotide in the intron proceeding nucleotide 1717 in the cDNA
R553X	Replacement of arginine at position 553 by a stop codon
I148T	Replacement of isoleucine at position 148 by threonine
3120+1G>A	Replacement of glycine by alanine at the first nucleotide in the intron following nucleotide 3120 in the cDNA

Adapted from Eshaque and Dixon, *Biotechnol. Adv.* **24**:86–93, 2006.
Amino acids are numbered starting at the N-terminal end of the protein.

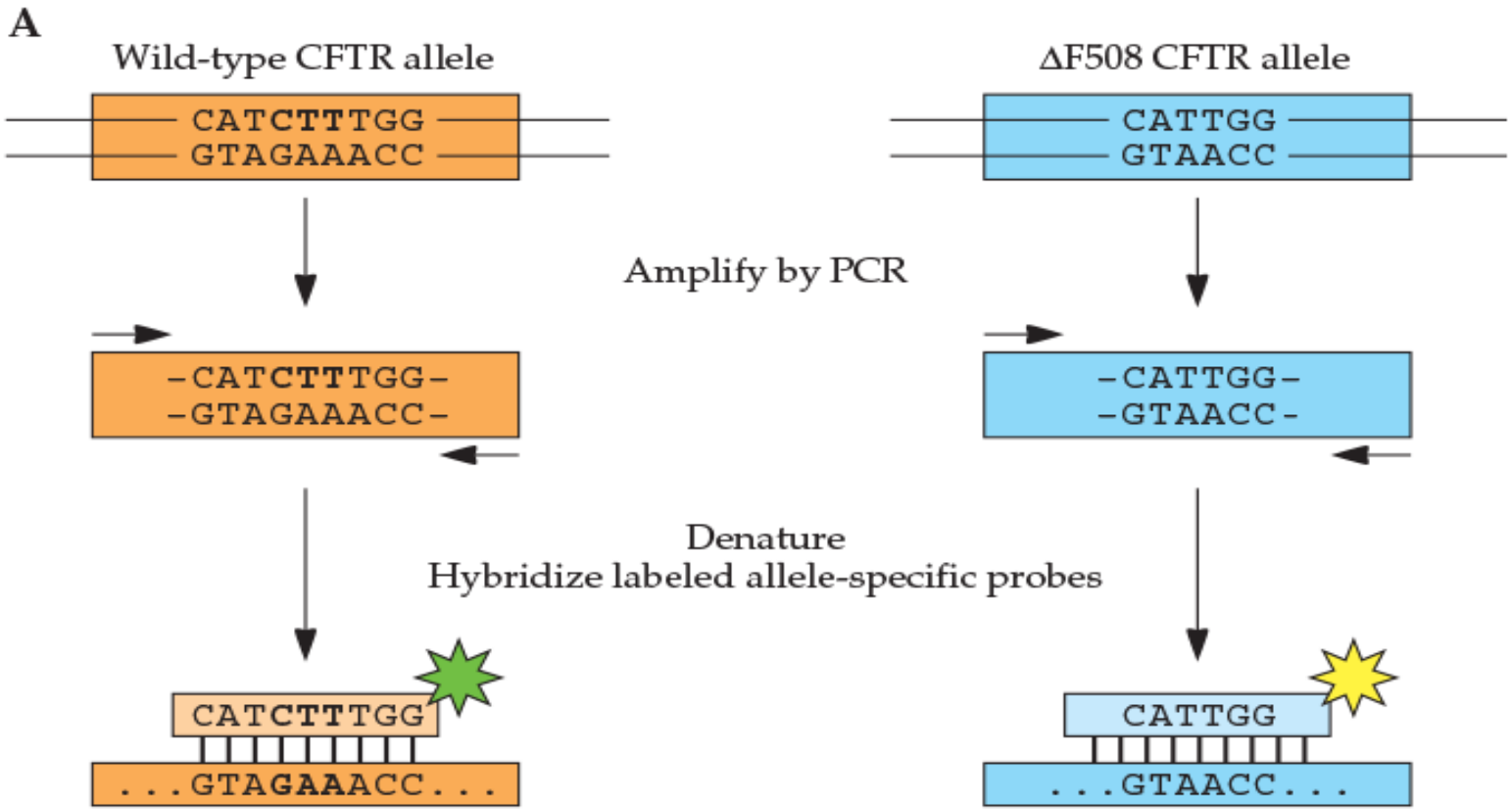
- Cystic fibrosis is a progressive, genetic disease that causes persistent lung infections and limits the ability to breathe over time. It is caused by a defective cystic fibrosis trans membrane conductance regulator protein (CFTR).
- Current diagnostic tests for cystic fibrosis include several different techniques.
- One of the most widely used methods **is allele-specific oligonucleotide dot blots** (**also called allele-specific hybridization**). With this technique, genomic DNA or cDNA from an individual is amplified by PCR and, following transfer to a membrane, is hybridized (separately) to labeled oligonucleotide probes for the mutant (usually ΔF508) and wildtype genes (Fig. 9.26).
- In this way, it is possible to distinguish between normal individuals, cystic fibrosis carriers, and cystic fibrosis-affected individuals (Fig. 9.27).
- With this technique, the probe or the probe–target complex may be labeled in a variety of ways, including the use of radioactivity, enzymes that produce color change when acting on certain substrates (see the discussion of the ELISA procedure above), and fluorescent dyes. This technique may be automated and is currently commercially available in a kit form that can detect 12 frequent and 17 rare cystic fibrosis mutations.

Cystic fibrosis is a progressive, genetic disease that causes **persistent lung infections** and limits the ability to breathe over time. It is caused by a **defective cystic fibrosis transmembrane conductance regulator protein (CFTR)**.



CFTR - a protein, involved in **chloride ion transport out of cells**;

- if its defective, Cl^- builds up inside cells and **draws water inside,** this results in a sticky, sugar-rich extracellular **mucus**.



B

Source of blood sample	Hybridization test results
Normal individual	
Cystic fibrosis carrier	
Cystic fibrosis-affected individual	

Allele-specific hybridization to screen for cystic fibrosis

An individual's CFTR gene is amplified by PCR and then hybridized to labeled oligonucleotide probes for the mutant (e.g., ΔF508) and wild-type genes, separately (Fig. A).

In this way, it is possible **to distinguish between:**

- healthy individuals with **two wild-type alleles**,
- cystic fibrosis **carriers** with **one mutant allele** and **one wild-type allele**, and
- **cystic fibrosis-affected individuals** with **two mutant alleles** (Fig. B).

DNA Fingerprinting and **Forensics**

- Humans are 99.9% identical
- But of course, we are unique – in a genome of three billion letters, even a 0.1% difference translates into three million differences.
- These differences (or **polymorphisms**) reside in several places in the genome, often in **microsatellites**.
- Examples of such polymorphisms include VNTRs, STRs, RFLPs and SNPs
- VNTR - **variable number tandem repeat**
- STR – short tandem repeats
- RFLP - Restriction fragment length polymorphism
- SNP – Single Nucleotide Polymorphism

DNA Fingerprinting

- Focuses on the 0.1-1.0% of human DNA that is unique
- First described in 1985 by Dr. Alec Jeffreys in England
- DNA evidence is admissible in courts

Uses of **DNA Fingerprinting**

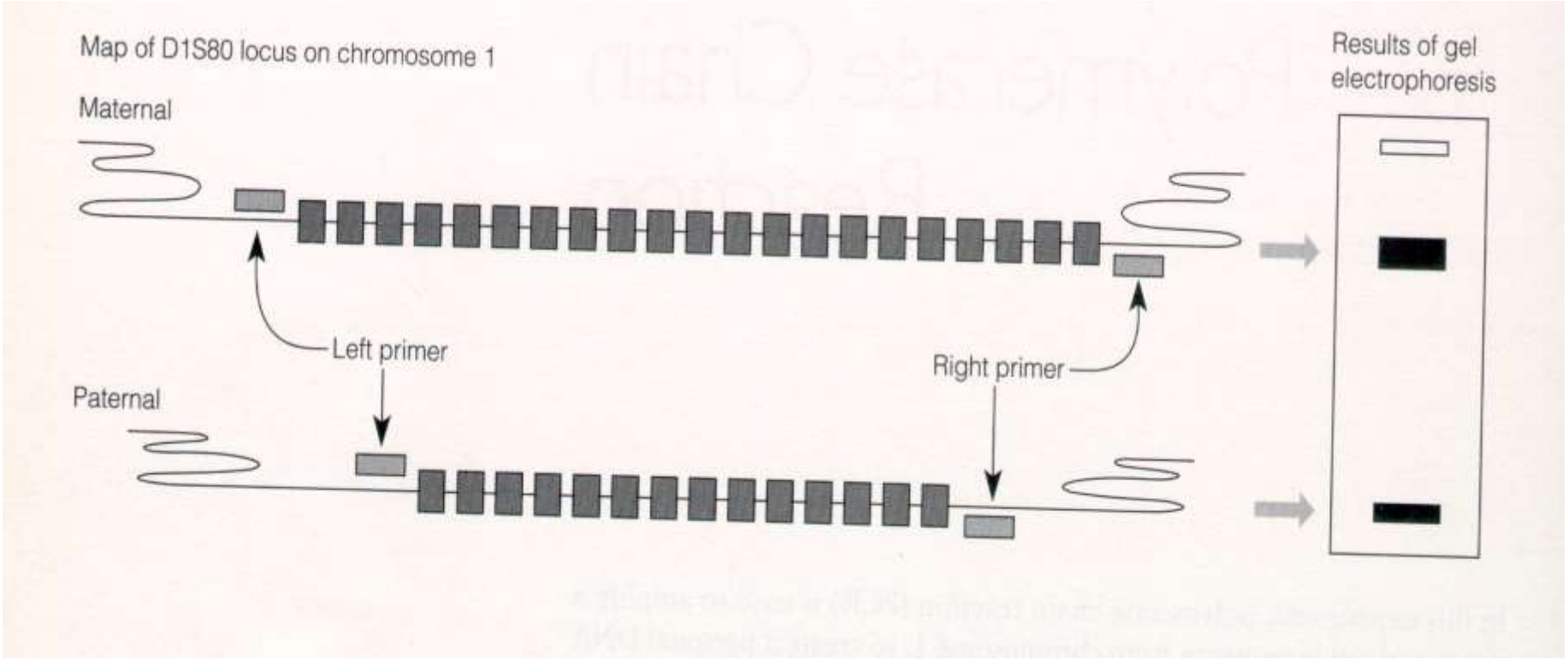
- Paternity testing
- Identification of criminals (e.g. murderers, rapists, letter bombers)
- Immigration disputes (family relationships)
- Identification of deceased individuals with mutilated or decomposed bodies (e.g., the military, victims, etc.)

How is DNA fingerprinting done?

- DNA obtained from hair, semen, blood, sweat, saliva, bone or any other tissue (often found at a crime scene)
- DNA fingerprints/polymorphisms are revealed by PCR using appropriate STR primers
- STRs – short tandem repeats; composed of 2-7 bp repeat units (e.g., [AC]n) which are tandemly repeated so that the overall length is less than 1 kb
- Can use single locus primers or multilocus primers
- Amplified DNA can be resolved on a gel or by a capillary electrophoresis system

DNA fingerprinting: an example

- D1S80, a VNTR located on human chromosome 1, contains a 16 bp repeat unit.
- The number of repeats varies from one individual to the next, and is known to range from 14-41.



DNA Microarrays for Health and Ancestry Determination

- A DNA Microarray Test is used to reveal person's unique set of SNPs
- Such SNPs are associated with diseases & populations
- Several companies offer such services
  - **23 and me** (https://www.23andme.com/) and (https://www.youtube.com/watch?v=0gC8RQ7PemM)
  - **Ancestry.com** (https://www.ancestry.com/)

- Whole Genome Sequencing to Access Genetic Risk and Ancestry may replace the DNA Microarray Test

Genetic Testing for Traits, Diseases, and Ancestry (SNPs, PCR, DNA Sequencing)

TreeGene (Kazakhstan)
https://treegene.kz/uslugi/ychromosome-heritage/

23 and me
https://www.23andme.com/
FamilyTreeDNA
https://www.familytreedna.com/
Ancestry.com
http://www.ancestry.com/
MyHeritage
https://www.myheritage.com/
Sure Genomics
http://www.suregenomics.com/

Immunological Diagnostic Procedures

- Many immunological detection methods are sensitive, specific, and simple.
- They can be used for a wide range of applications, including:

✓drug testing,
✓assessment and monitoring of various cancers,
✓ detection of specific metabolites,
✓pathogen identification, and
✓monitoring infectious agents.

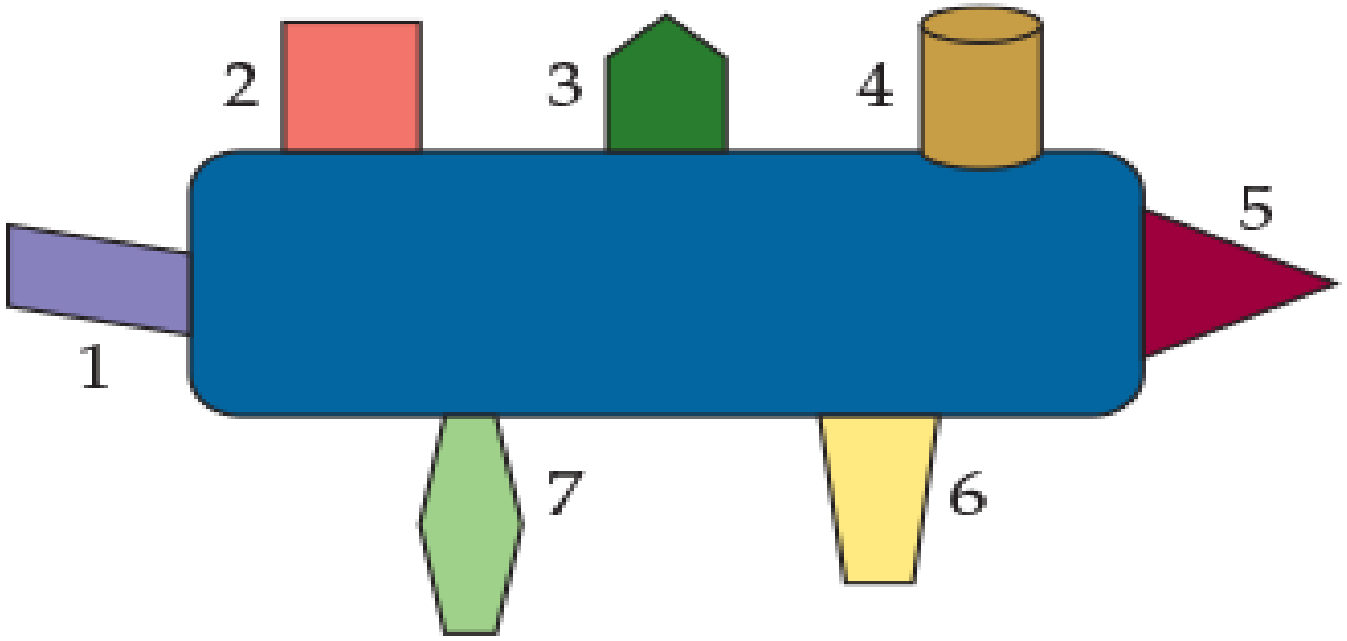
However, **there are limitations**.
For example, **if the target is a protein**, then the use of antibodies **requires** that the **genes** contributing to the presence of the target site **be expressed** and that the target site not be masked or blocked in any way that would prevent the binding of the antibody.

Immunological Diagnostic Procedures

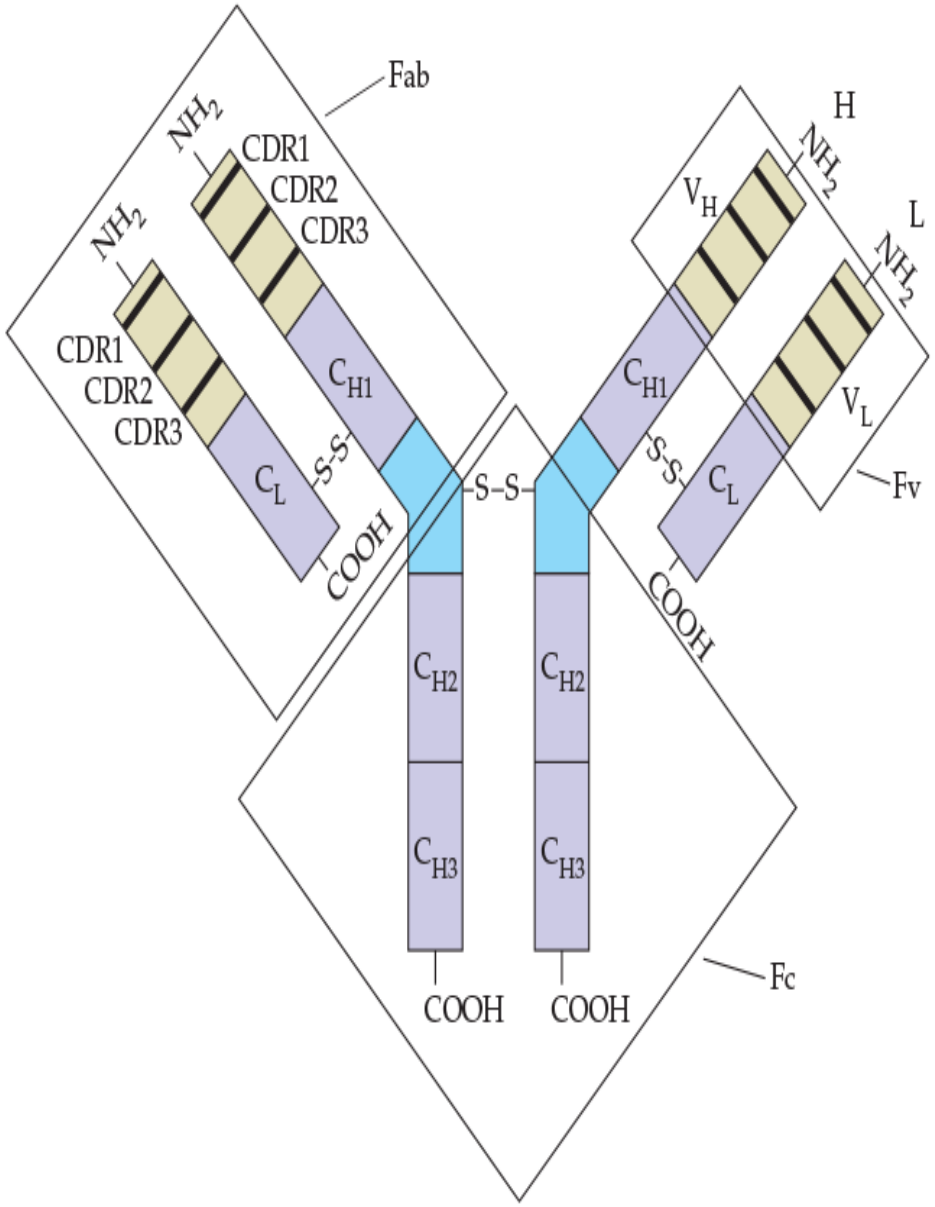
- For example, some infectious agents produce distinctive biochemical molecules.
- **The problem is how to determin**e when the **identifying component is presen**t in a biological sample.

- Often, such a marker molecule can be identified directly in a specialized biochemical assay that is very specific for the **marker molecule**.

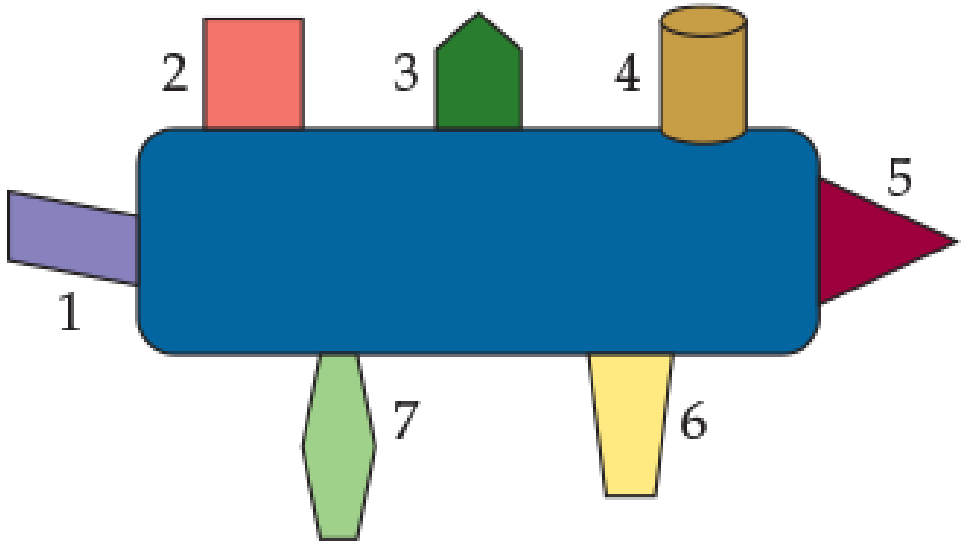
Schematic representation of a target antigen.
The surface of this antigen has **7** different **antigenic determinants** (**epitopes**).



When this antigen is used to immunize an animal, **each** antigenic **determinant (epitope)** elicits the **synthesis of** a different **antibody**.
Together, the different antibodies that interact with an antigen constitute **a polyclonal antibody** directed against that antigen.



Structure of an **antibody** molecule



Polyclonal antibodies are made against and react with multiple antigenic sites (epitopes) on a target antigen.

Monoclonal antibodies are directed against a particular antigenic site (epitope).

ELISA (Enzyme-Linked Immunosorbent Assays) method

There are a number of different ways to determine whether an antibody has bound to its target antigen.
The enzyme-linked immunosorbent assay (ELISA) is one method, and it is frequently **used for diagnostic** detection.
The ELISA procedure may be either indirect (Fig. 9.1A) or direct (Fig. 9.1B).

A generalized **indirect ELISA protocol** (Fig. 9.1**A**) **has** the **following steps**:

1. **Bind the sample** being tested for the presence of a specific molecule or pathogenic organism **to a solid support**, such as a plastic **microtiter plate**, which usually contains 96 sample wells. **Washing** the support **to remove unbound molecules**.

2. Add a marker-specific antibody (primary antibody directed against the target antigen) to the bound material, and then wash the support to remove unbound primary antibody.

3. **Add a second antibody** (secondary antibody) that binds specifically to the primary antibody and not to the target molecule. An **enzyme** (such as **alkaline phosphatase**, peroxidase, or urease) **is bound (conjugated) to the secondary antibody** that catalyze a reaction **that converts a colorless substrate into a colored product**. Wash the mixture to remove any unbound secondary antibody-enzyme conjugate.

4. Add the colorless substrate.

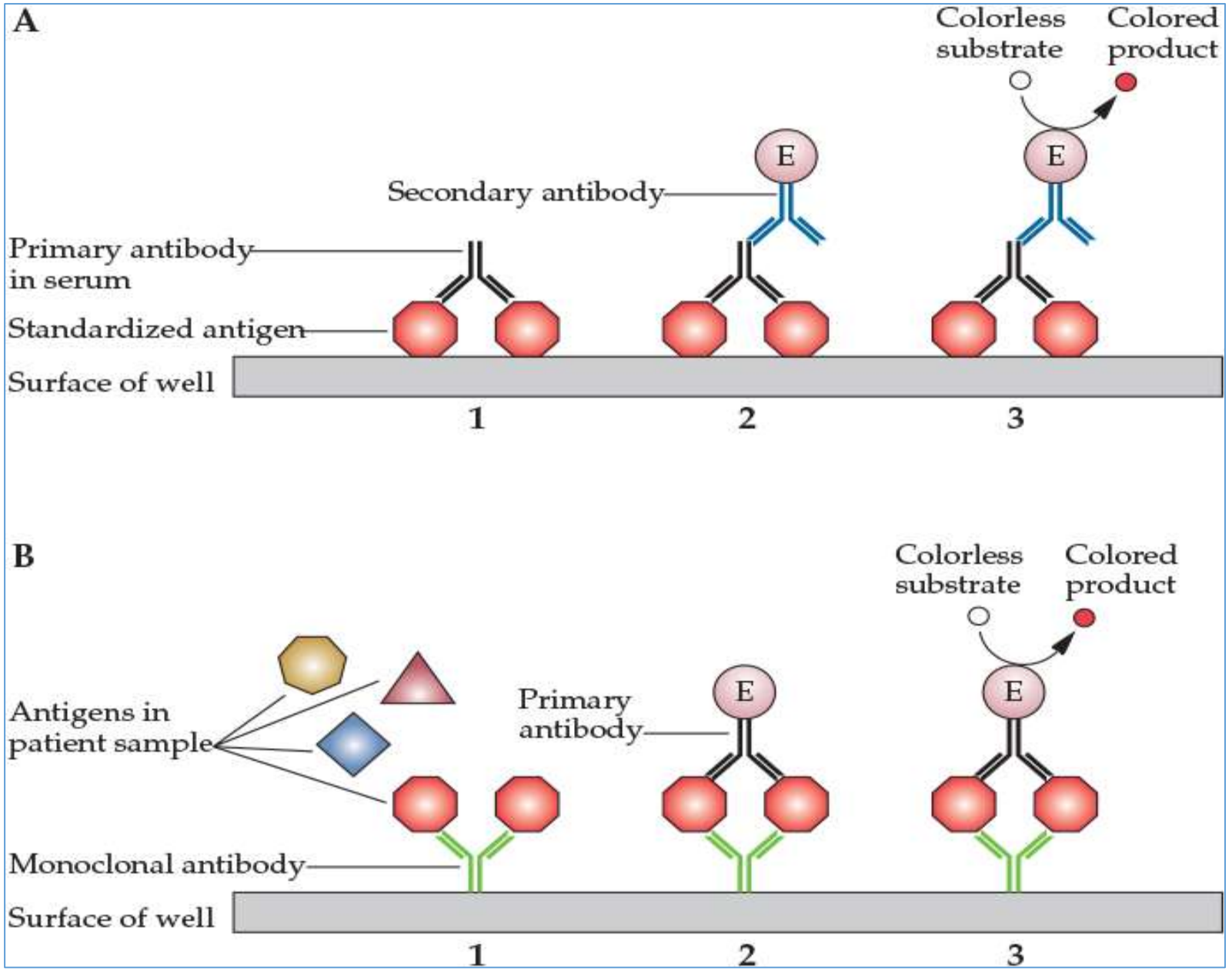
5. Observe or measure the amount of colored product.

Enzyme-Linked Immunosorbent Assays (ELISAs).

A). **Indirect ELISA**.

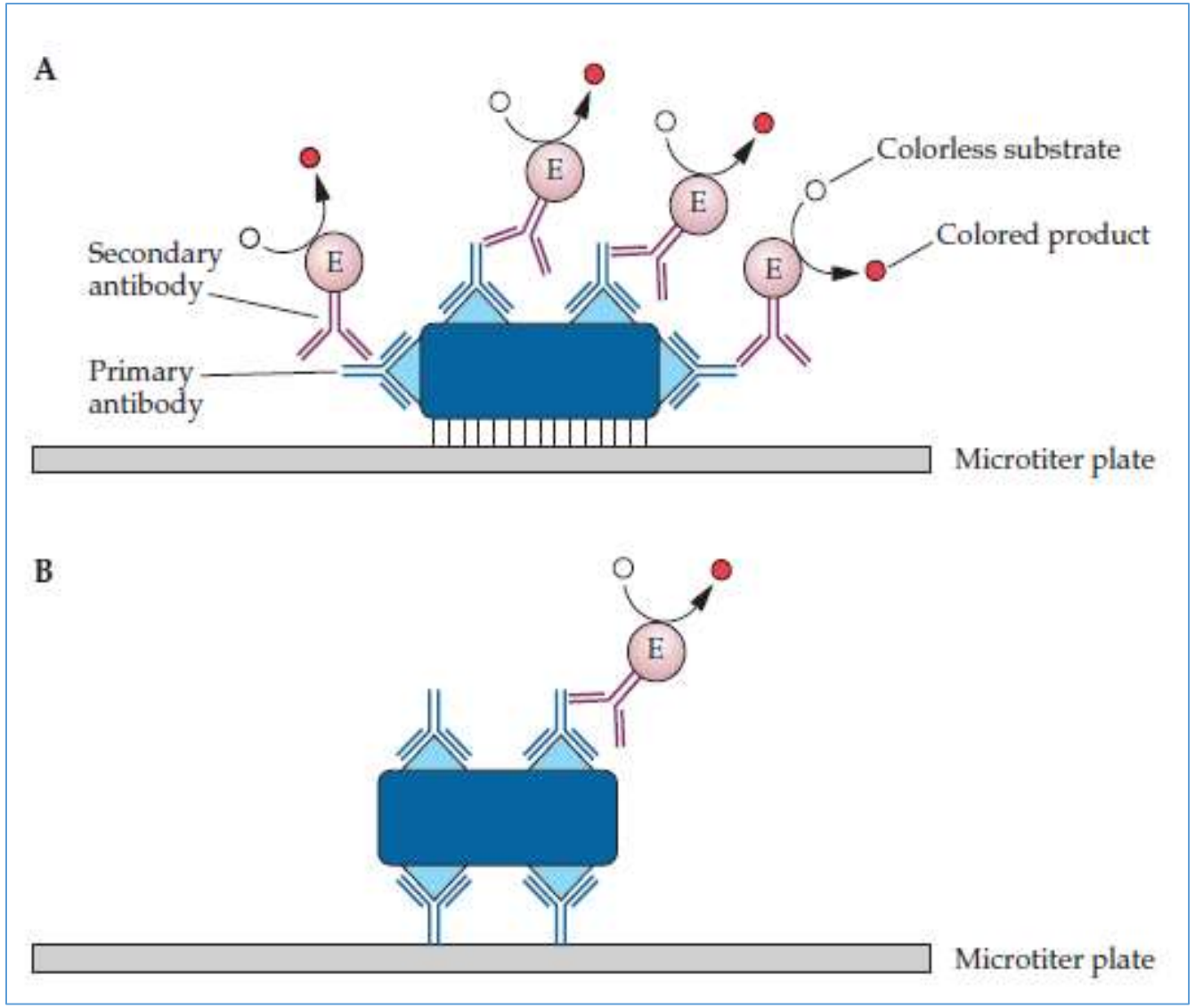
B). **Sandwich ELISA**

- if the target site is present in the sample, then the primary antibody binds to it,
- the secondary antibody binds to the primary antibody, and
- the attached enzyme catalyzes the reaction to form an easily detected colored product.



- Conversely, if the primary antibody does not bind to a target site in the sample, the second washing step removes it.

- Consequently, the secondary **antibody-enzyme conjugate** has nothing to bind to and is removed during the third washing step, and the final mixture remains colorless.



Generalized ELISA protocol for detecting a target antigen. The primary antibody is often obtained from rabbits that have been immunized with the target antigen, while the secondary antibody is from goats immunized with rabbit antibodies. The enzyme (E) is conjugated to the secondary antibody. **(A)** Indirect ELISA; **(B)** direct ELISA.

Sandwich ELISA directly detects a particular antigen in a complex sample.

To capture the antigen,

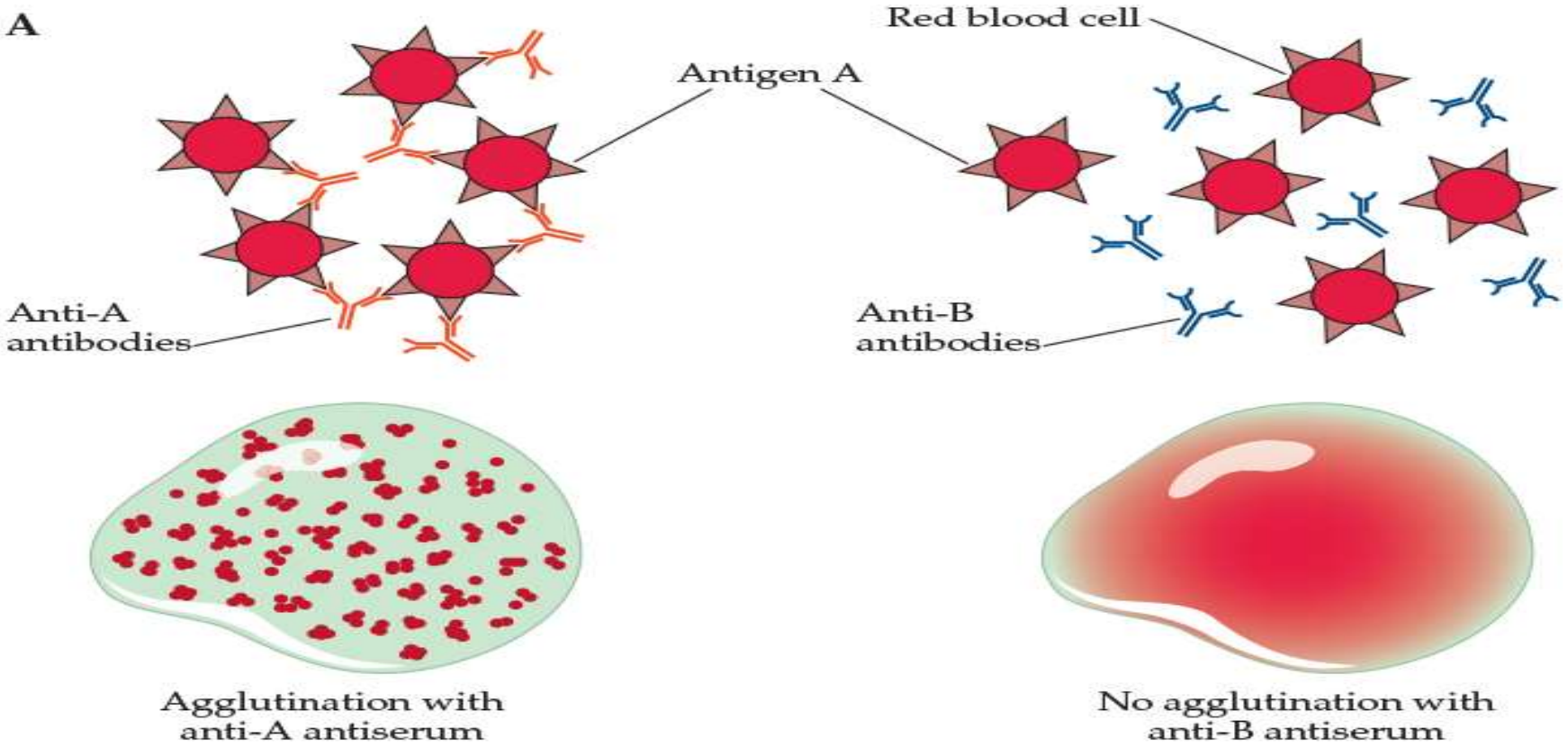
- a monoclonal antibody that is specific for the target antigen is first bound to the surface of a microtiter plate.
- The sample is then added to a well;
- if the specific antigen is present in the sample, it binds to the immobilized antibody,
- A labeled primary antibody is added to detect the presence of bound antigen. The labeled antibody may detect a different epitope on the same antigen.

For some diagnostic assays, **the use of polyclonal antibodies** has **two disadvantages**:

(1) the amounts of the different antibodies within a polyclonal preparation may vary from one batch to the next, and

(2) polyclonal antibodies cannot be used to distinguish between two similar targets, e.g., when the difference between the pathogenic form (target) and the nonpathogenic one (nontarget) is a single determinant.

However, these problems can be solved, because it is now possible to generate an antibody preparation that is directed against a single antigenic determinant, namely, a monoclonal antibody.



B

Blood type	Agglutination with serum	
	Anti-A	Anti-B
A	+	−
B	−	+
AB	+	+
O	−	−

Agglutination (clumping) test.

For example, **a Hemagglutination test** to determine blood type.

Sandwich ELISA is used in Pregnancy test

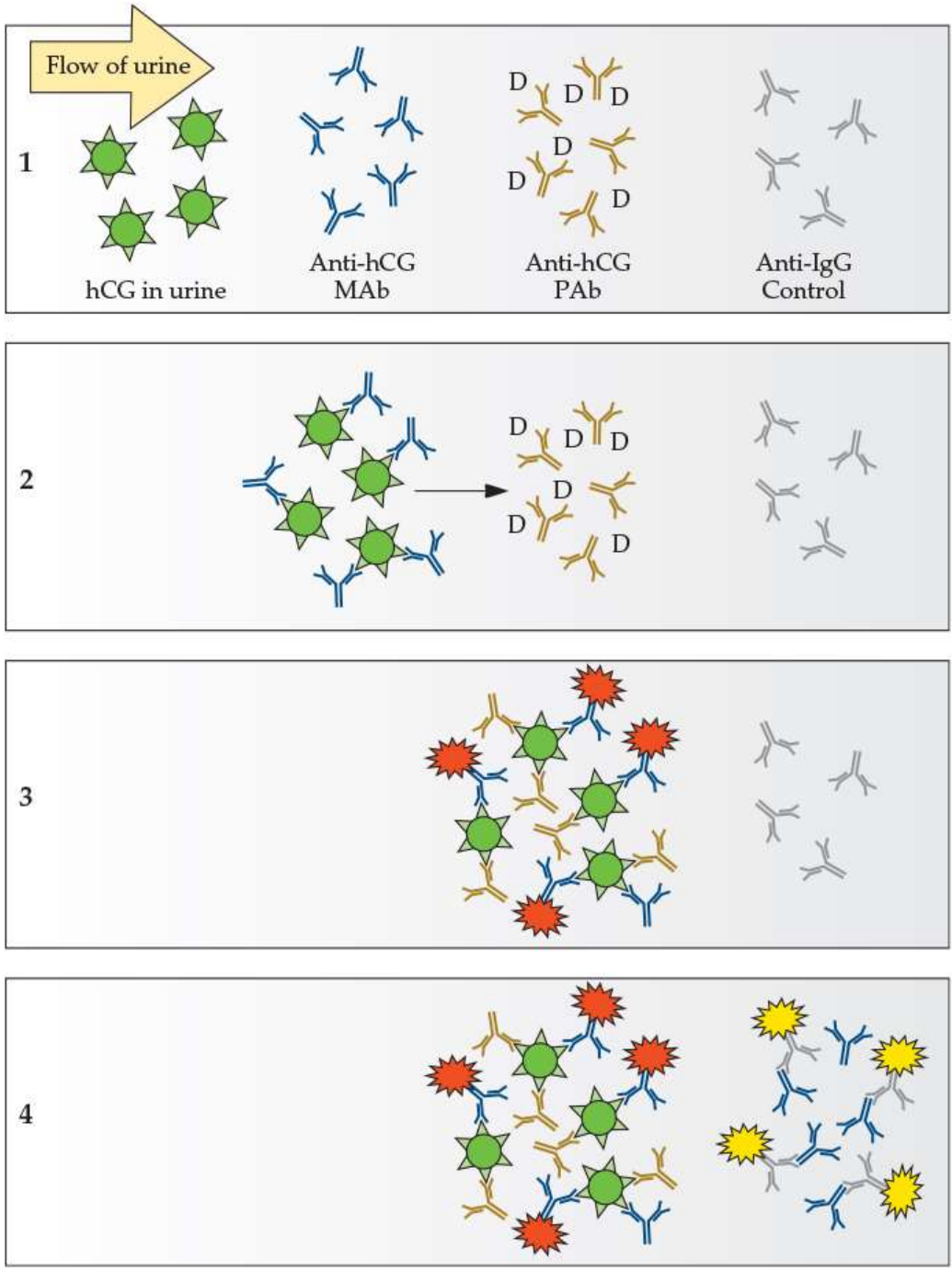
(human Chorionic Gonadotropin (hCG) pregnancy test: https://www.youtube.com/watch?v=aOfWTscU8YM http://www.sumanasinc.com/webcontent/animations/content/pregtest.html)

Urine is applied to one end of the membrane and is drawn through the membrane by capillary action to the region containing the anti-hCG monoclonal antibodies.

If hCG molecules are present, they bind to the monoclonal antibodies. The antibodies with bound hCG are carried with the urine through the membrane to the site of the immobilized anti-hCG polyclonal antibodies.

The polyclonal antibodies capture the hCG-monoclonal antibody complexes.

Monoclonal antibodies that are not bound to hCG are carried away with the urine by capillary action. The monoclonal antibodies are conjugated to an enzyme that reacts with dye molecules contained in the region with the polyclonal antibodies. Formation of a colored product indicates a positive pregnancy test result.



Measuring Disease-Associated Proteins by Sandwich ELISA

- **Ovarian cancer** is a devastating disease that kills over 15,000 women a year in the United States. More than 22,000 new cases are diagnosed each year, most at an advanced stage when the survival rate is less than 20%. An immunoassay widely used to monitor progression and recurrence of ovarian carcinoma measures levels of the protein CA125 in serum. CA125 is a high-molecular-weight glycoprotein that is present at higher levels in 50% of women with ovarian cancer compared to healthy women.
- A useful biomarker for ovarian cancer is a protein that is secreted specifically by ovarian tumors. **Human epididymis protein 4 (HE4)** was identified as a promising new biomarker that was expressed in ovarian carcinomas.
- Mouse monoclonal antibodies were generated against two different HE4 epitopes and used to develop an ELISA. HE4 was found to be elevated in sera from patients with early and late-stage ovarian cancer compared to sera from healthy women and from women with benign ovarian tumors.
- The U.S. FDA has approved the use of a HE4 sandwich EUSA to monitor the recurrence or progression of ovarian cancer in women who are being treated for the disease.

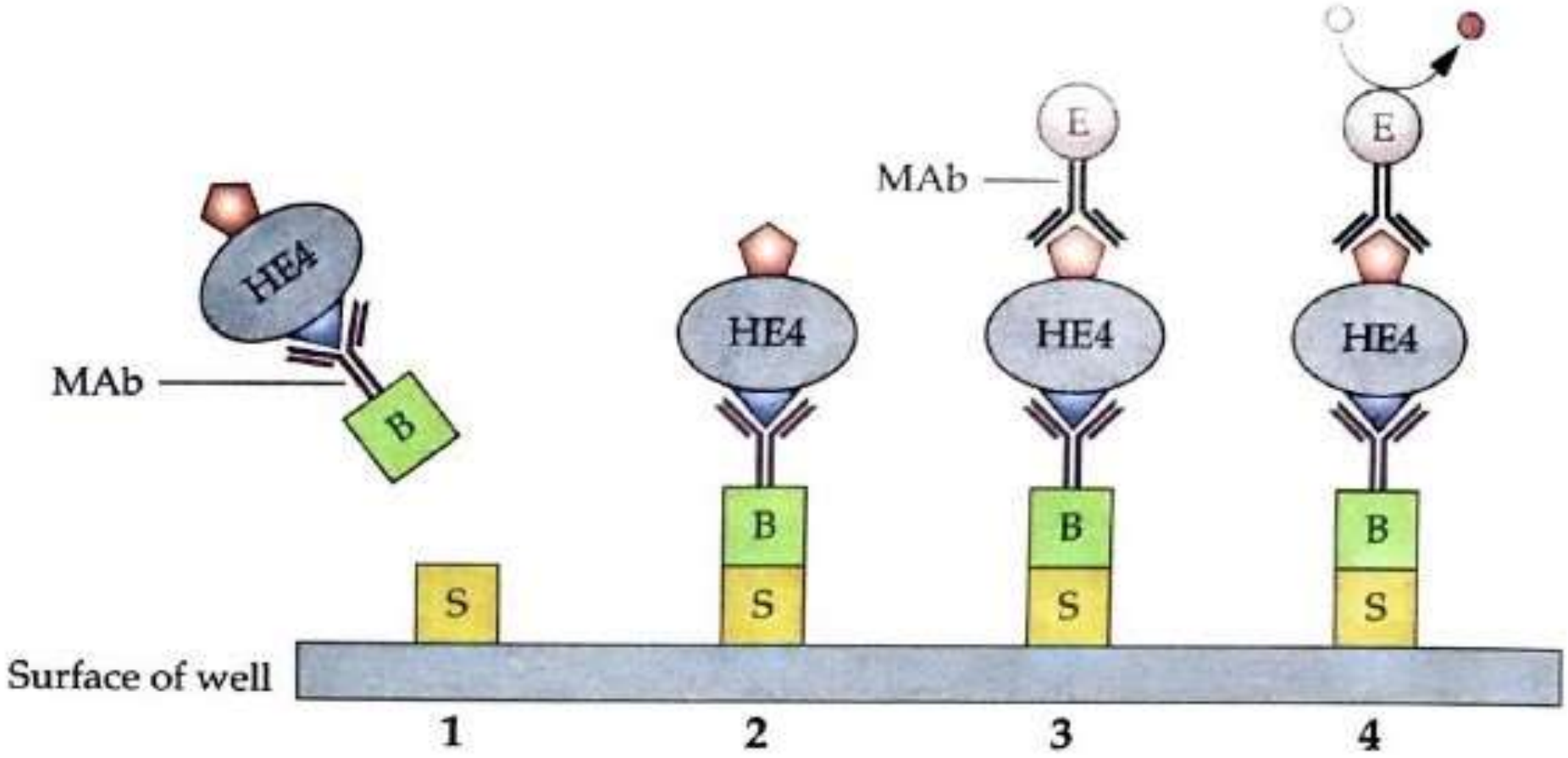
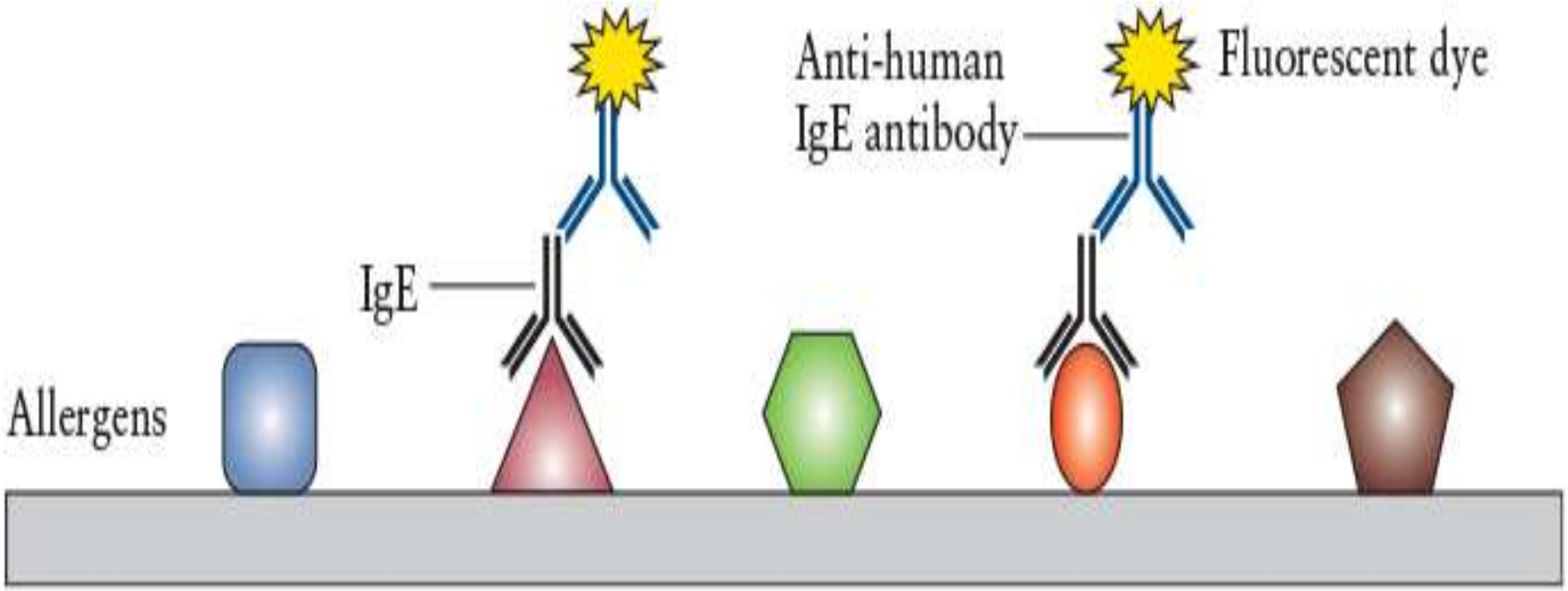
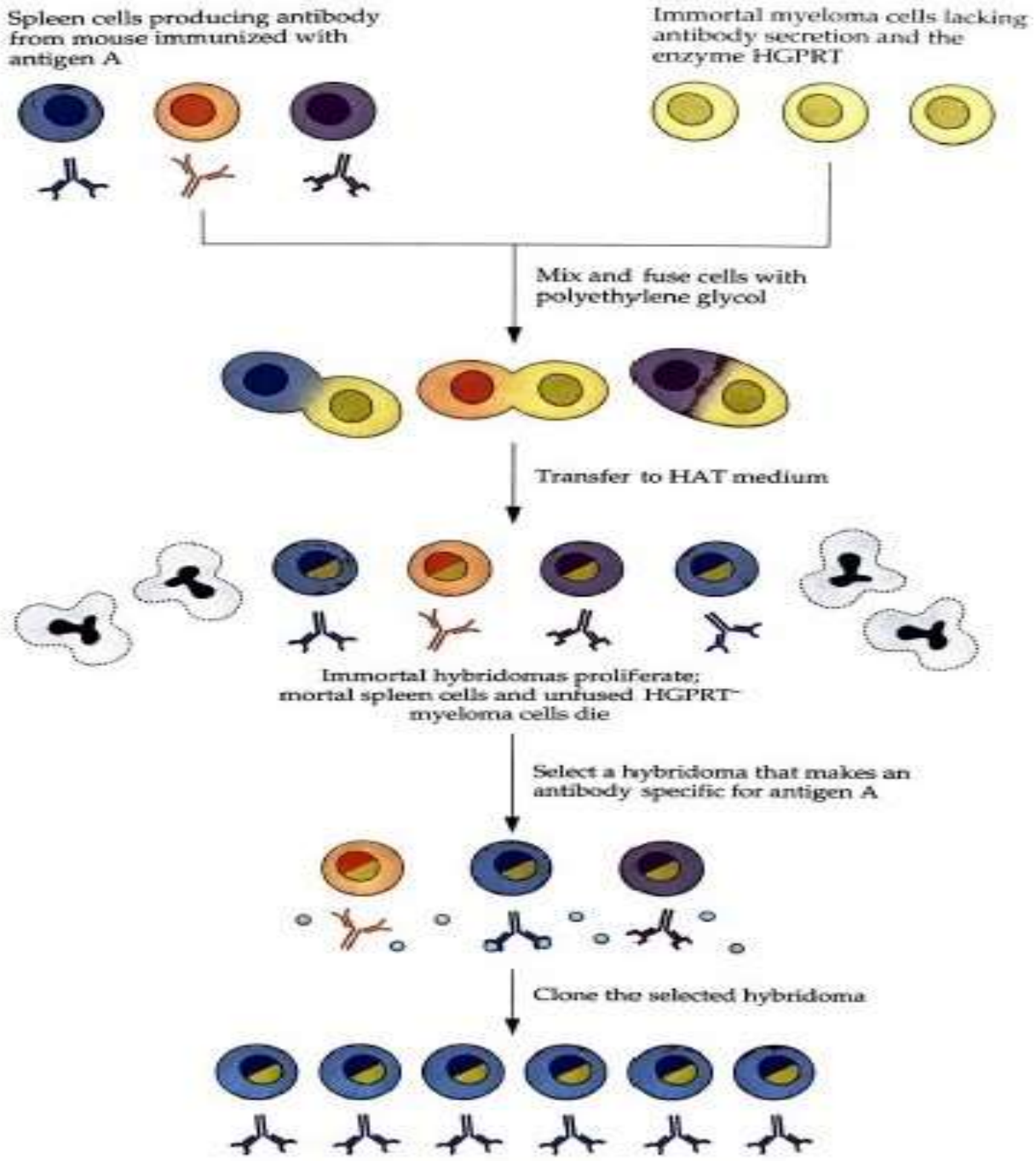


Figure 4.7 Sandwich ELISA to monitor progression or recurrence of ovarian cancer. (1) Patient samples are incubated with biotinylated (B) monoclonal antibodies (brown MAb) generated against one HE4 epitope (blue). (2) Biotin, together with the MAb–HE4 complex, binds with high affinity to streptavidin (S) that coats the wells. (3) A second antibody (black MAb) that binds to a different HE4 epitope (red) is added, and after generation of a colored product by horseradish peroxidase (E) conjugated to the antibody (4), HE4 is quantified spectrophotometrically.



Diagnostic allergen microarray.
Purified **allergens are arrayed on a solid support** and probed with **serum from an allergic patient**.
IgE antibodies (which mediate **type I allergies**) are detected with an **anti-human IgE antibody**.



Production of monoclonal antibodies

To avoid probems with reproducibllity of polyclonal antisera, it was desirable to have an unlimited supply of antibody molecules of homogeneous structure (**derived from a single clone of B cells**), high affinity, and known specificity for a specific target antigen.

Georges Köhler and César **Milstein** described the **hybridoma technique** or **production** of **monoclonal antibodies**.

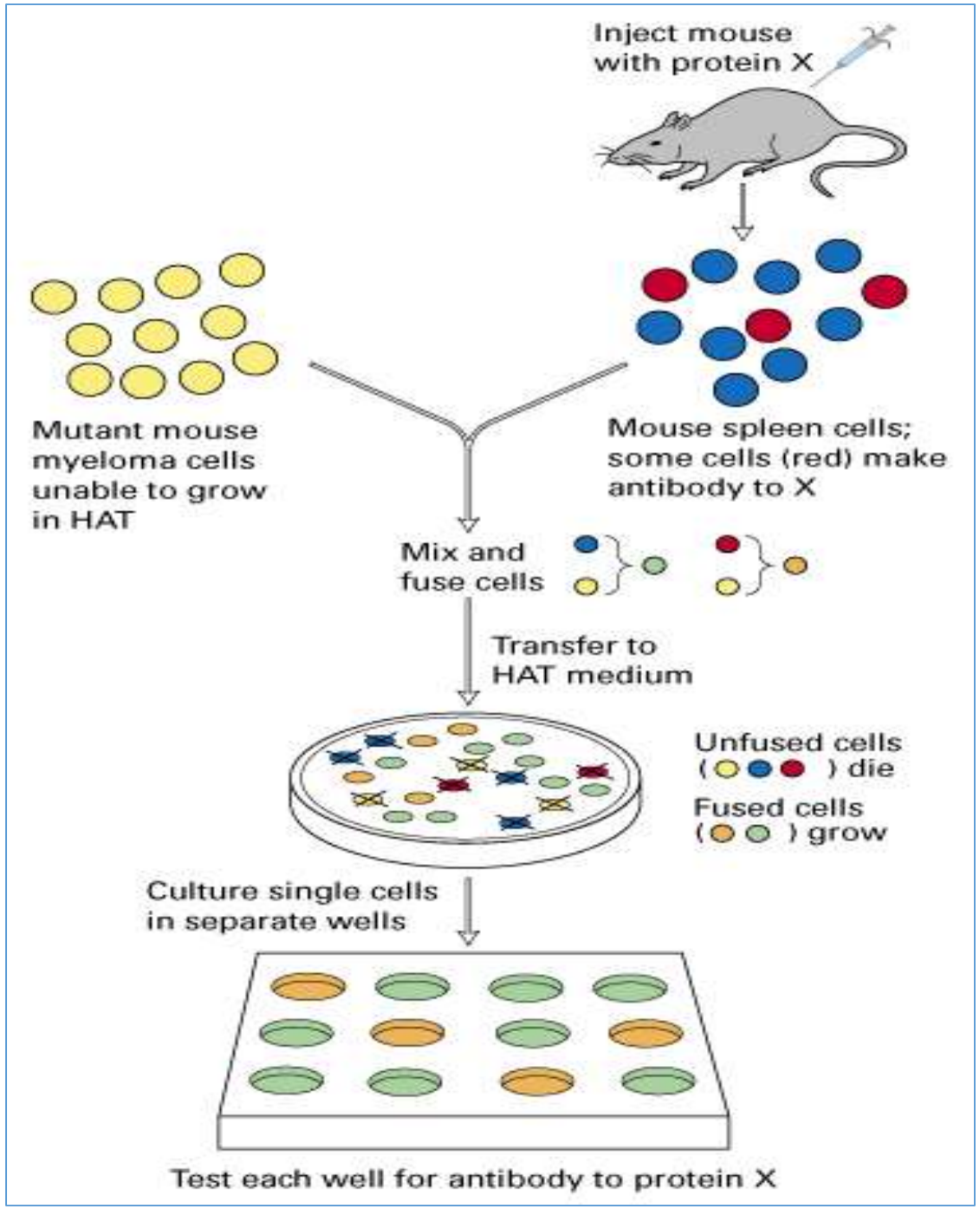
Procedure for producing a monoclonal antibody to protein X (or other molecule of our interest)

B lymphocytes or B cells **produce antibodies** but they do not reproduce in culture **(nutrient medium)**.

Some B cells can become cancerous and are known as **myelomas which can reproduce (multiply) in culture**.

https://www.youtube.com/watch?v=O-SrPqJuEVg

https://www.youtube.com/watch?v=0A99pk6kpS4



The polyethylene glycol treatment facilitates fusion between cells.
Nevertheless, the **fusion events are rare and random**.

There will be:

- nonfused **myeloma cells**,
- nonfused **spleen cells**,
- myeloma-spleen fused cells (hybridoma),
- **myeloma-myeloma** fusion cells, and
- **spleen-spleen** fused cells in the mixture.

The **HAT medium**, however, allows only the **myeloma–spleen fusion cells to grow**, because none of the other cell types can proliferate in this medium.

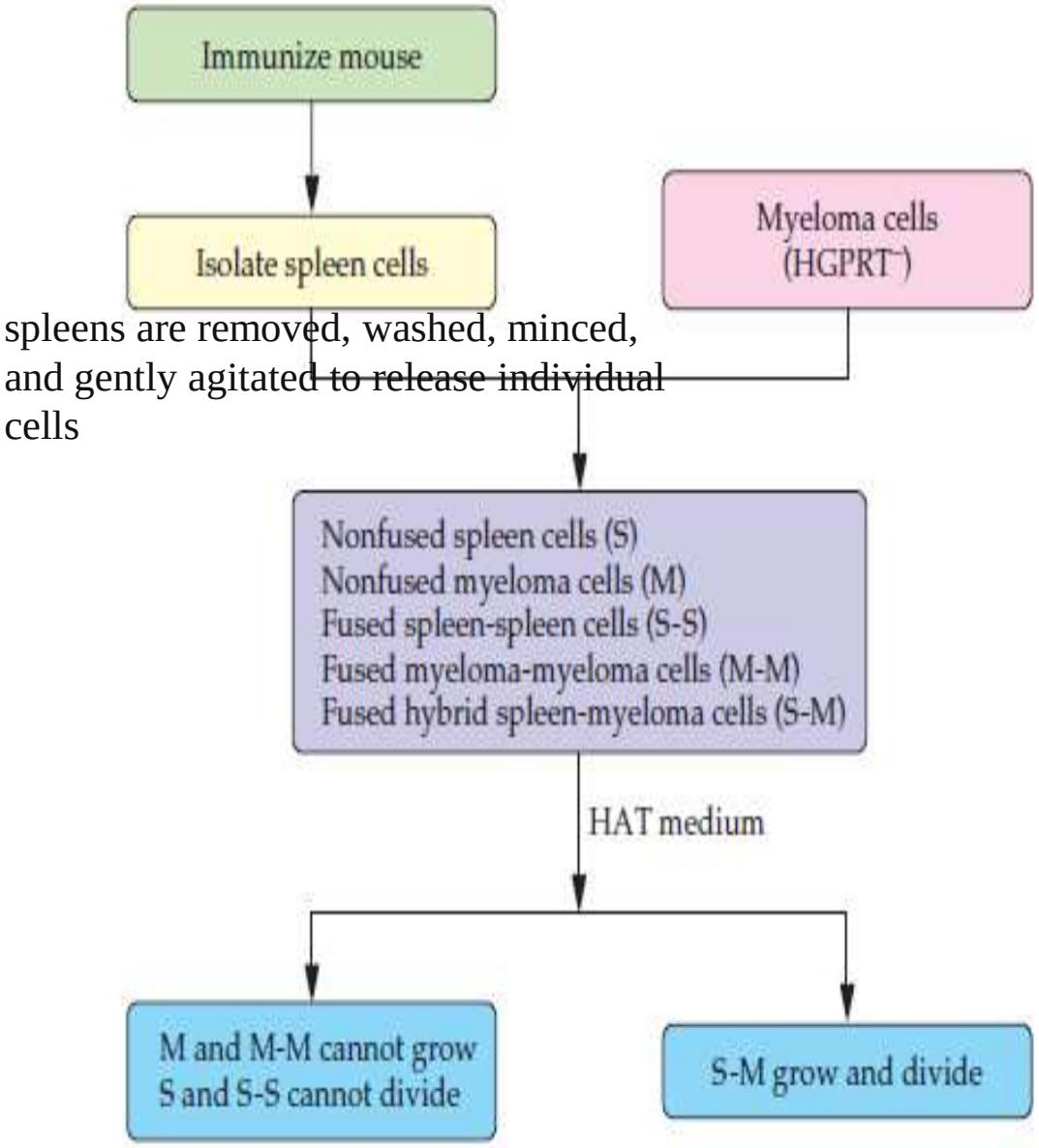
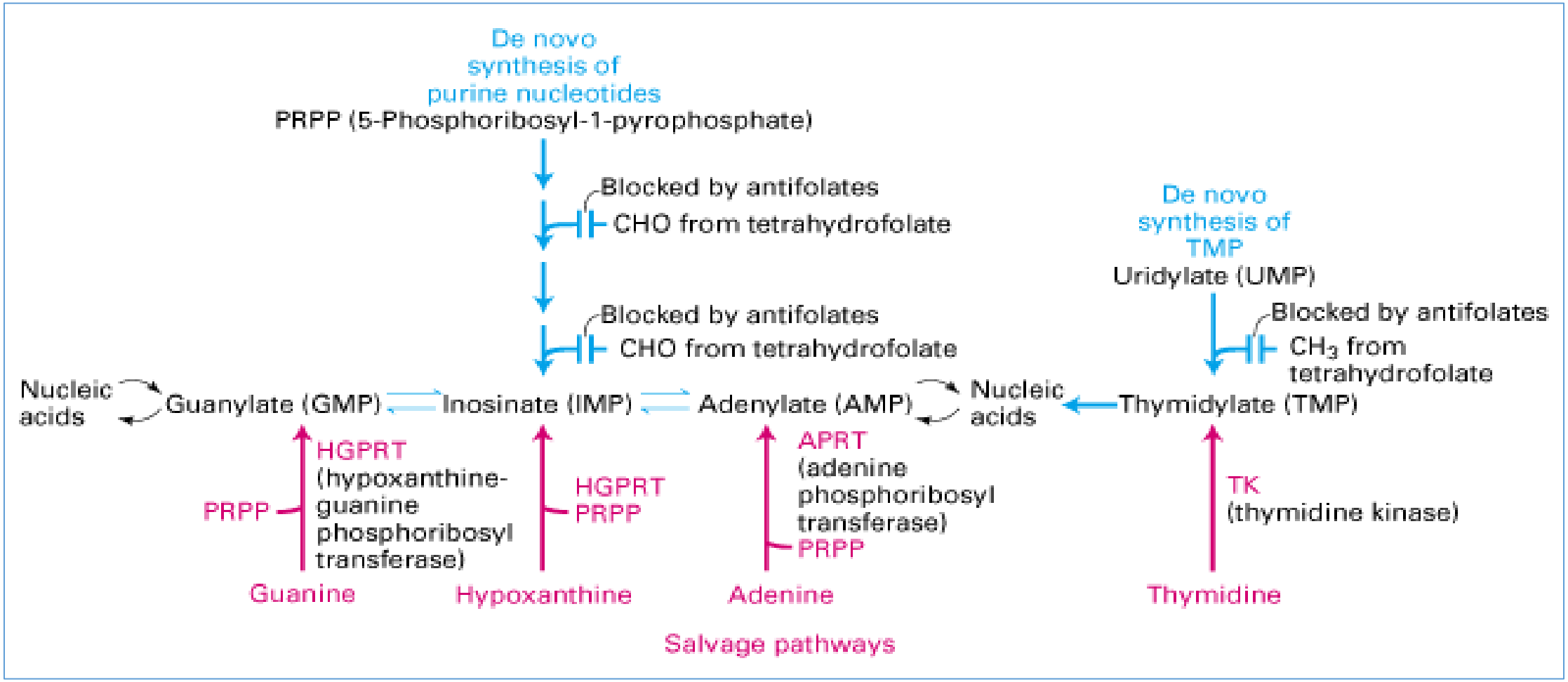


FIGURE 9.3 The HAT procedure for selecting hybrid spleen–myeloma (hybridoma) cells.

- The **HGPRT enzyme deficient** myeloma and the myeloma-myeloma fusion cells **cannot use hypoxanthine** as a precursor for the biosynthesis of the **purines guanine and adenine**.
- However, they have a second (alternative), natural pathway for purine biosynthesis that utilizes the enzyme **dihydrofolate reductase**. Therefore, **aminopterin** is added in the medium because it inhibits dihydrofolate reductase activity. Hence, HGPRT-deficient myeloma and myeloma-myeloma fusion cells are unabe to synthesize purines in HAT medium, so they die.

- The spleen-myeloma fusion cells survive in HAT medium because the spleen cell has a functional HGPRT enzyme, which can utilize the hypoxanthine in the medium even though purine production by means of dihydrofolate reductase is blocked by aminopterin, and because the cell division functions of the myeloma cell are active. Thymidine is provided to overcome the bock in pyrimidine production that is caused by the inhibition of dihydrofolate reductase by aminopterin.
- About 10 to 14 days after the fusion treatment, only spleen-myeloma fusion cells have survived and grown in the HAT medium.

How HAT medium (selective medium) works



Myeloma cells are HGPRT- (defective) and will die on **HAT media** containing **hypoxanthine**, **aminopterin** (**an antifolate**), and **thymidine**.

Spleen cells are **HGPRT+** , so spleen-myeloma (hybridoma) cells can grow on HAT. (Note: spleen cells by themselves cannot grow in culture).