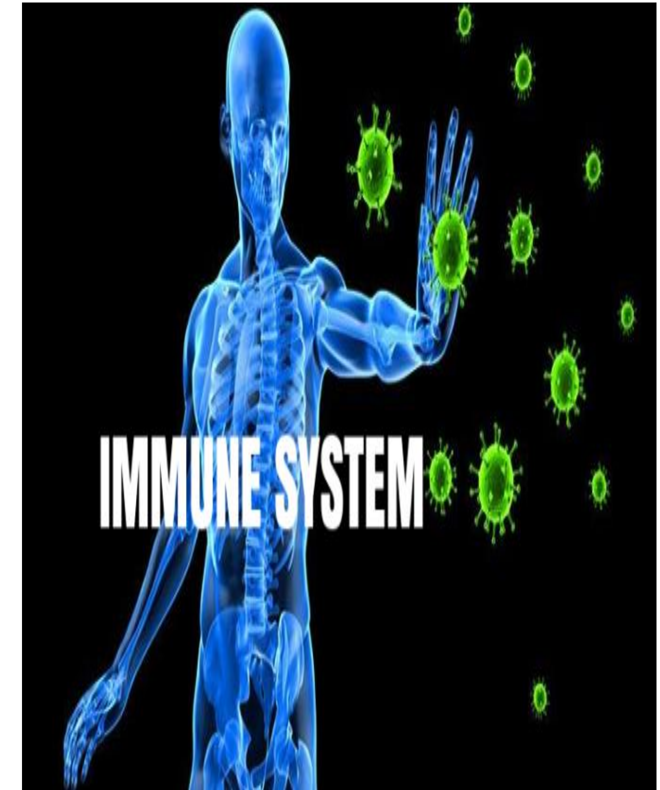
Serology *Lecture for Medical Laboratory Science students*

Chapter 1: An Overview of the Immune System



Yibeltal Akelew (MSc in Immunology)

Email: aayibeltal21@gmail.com

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Terminology

- **Immunity:** *A reaction to foreign substances* including microbes as well as macromolecules regardless of the physiologic or pathologic consequence of such a reaction
- **Immunology:** The *study of the cellular and molecular events that occur after organism encounters microbes and other foreign macromolecules*.

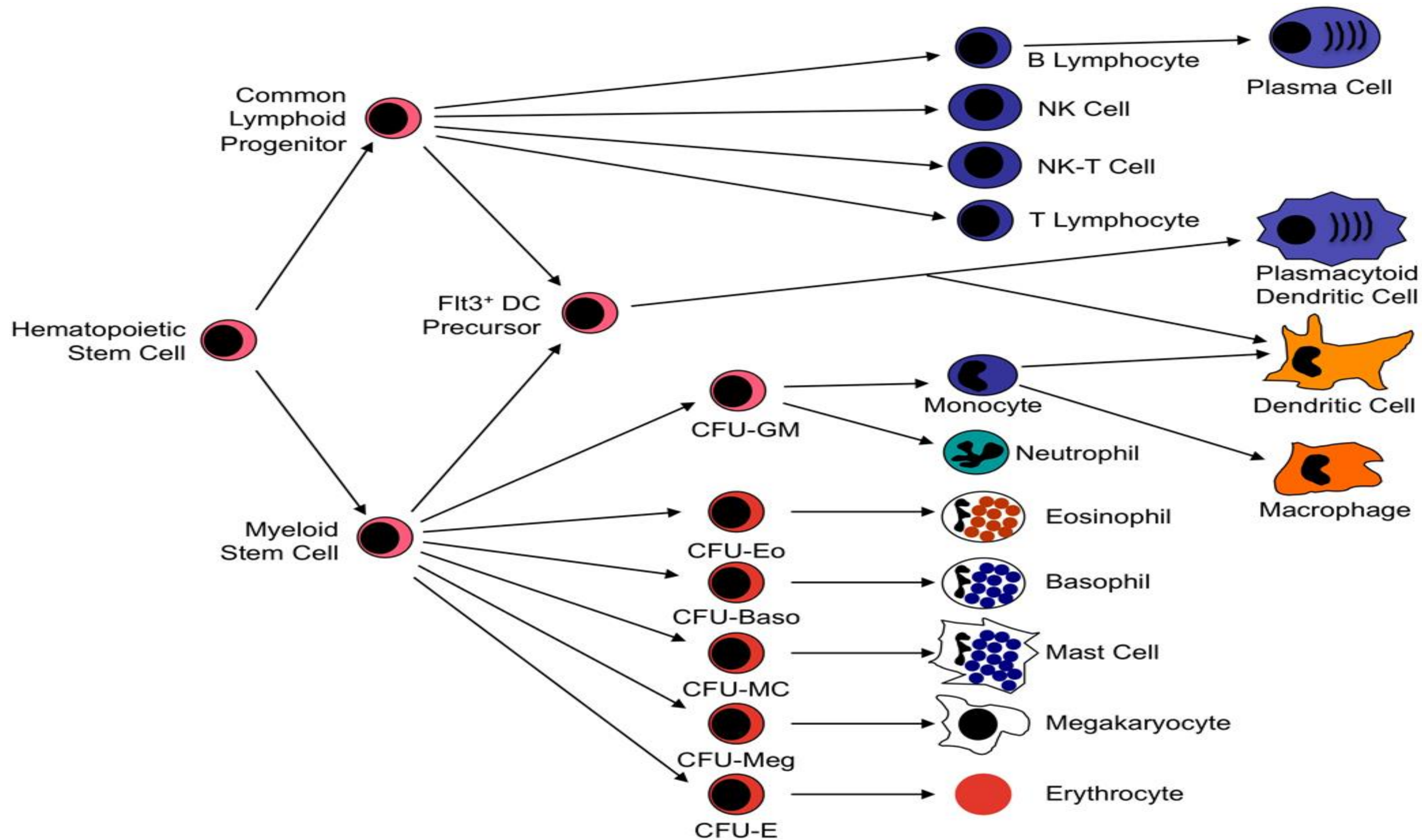
Cont…

- **Immune system:** system of biological structures and processes with in an organism that protects against disease
- **Immune response:** the collective and coordinated response to the introduction of foreign substances

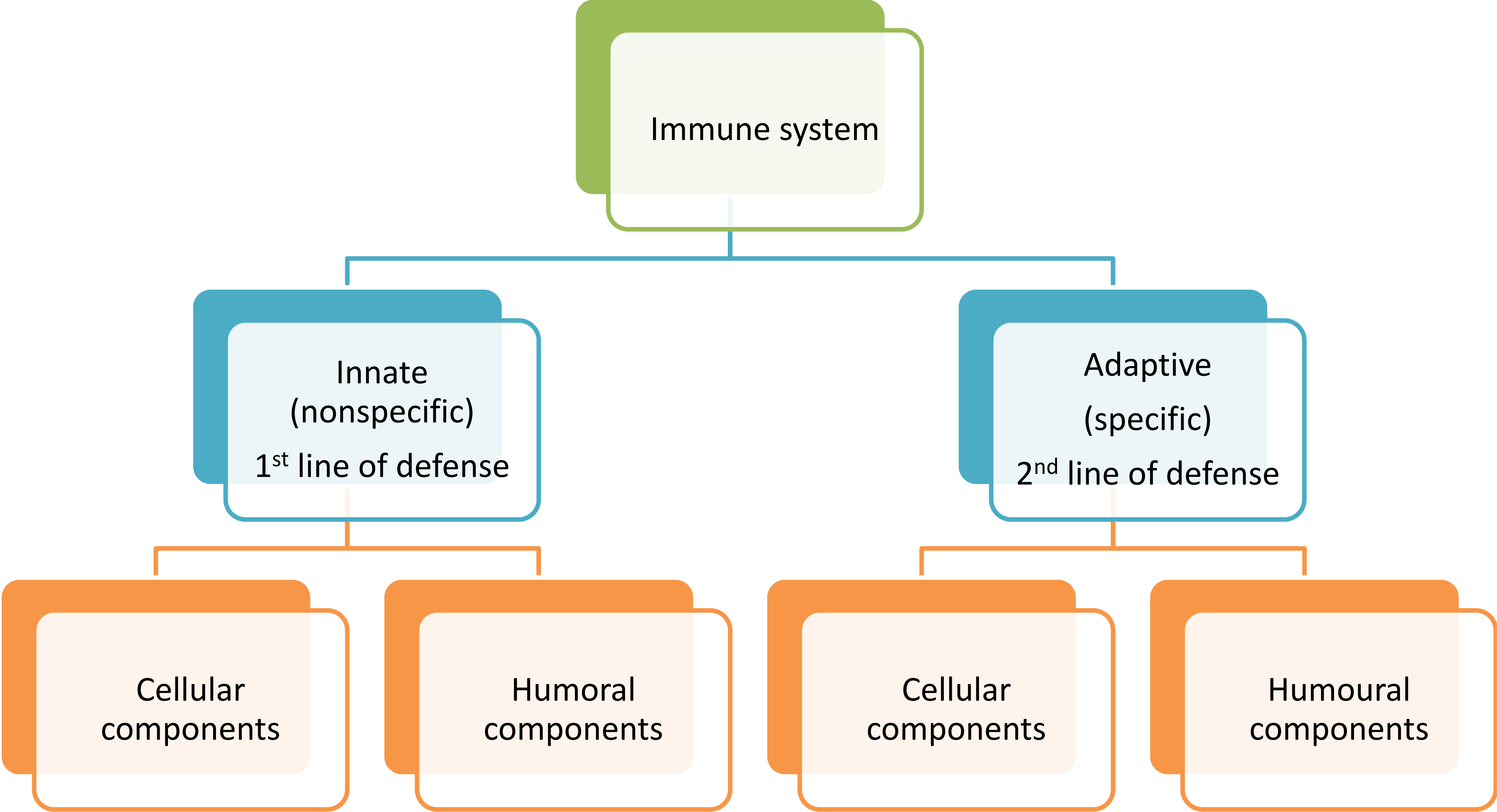
What does our immune system do?

- ✓ Protects us from infections
  - – Bacteria, viruses, parasites, fungi
- ✓ Promotes normal body functions
  - – Wound healing, tissue clean up
- ✓ Removes abnormal cells (including malignant ones)
- ✓ Can also cause disease (allergy, transplant rejection, autoimmune disease)

Overview of Hematopoietic Stem Cell-Derived Cell Lineages



Overview of the immune system



INNATE/NON-SPECIFIC

- Defense mechanisms one is born with
- Consistent within species
- Mechanisms stimulated *by common structures of microbes*
- Lacks ability to distinguish fine differences between foreign substances
- Generic response to all substances

Innate Host Defenses

- Anatomical barriers
  - Mechanical factors (Skin, Mucus Membrane)
  - Chemical factors (Sweat, HCl, Tears and Saliva)
  - Biological factors (Normal Flora in skin and mucus membrane)

- Humoral components
  - Complement
  - Coagulation system
  - Lysozyme
  - Cytokines

- Cellular components
  - Neutrophils
  - Monocytes and macrophages
  - NK cells
  - Eosinophils

- The components of innate immunity *recognize structures that are characteristic of microbial pathogens and are not present on mammalian cells*

- The microbial substances that stimulate innate immunity are called *pathogen associated molecular patterns (PAMPs),* and the receptors that bind these conserved structures are called *pattern recognition receptors*
- The innate immune system recognizes microbial products that are often essential for survival of the microbes.

Cell-associated pattern recognition receptors	Location	Specific examples and their PAMP ligands
Toll-like receptors	Plasma membrane and endosomal membranes of dendritic cells, phagocytes, endothelial cells, and many other cell types	TLRs 1-9: Various bacterial and viral molecules (see Fig. 2–2)
C-type lectins	Plasma membranes of phagocytes	Mannose receptor: Microbial surface carbohydrates with terminal mannose and fructose
		Dectin: Glucans present in fungal cell walls
Scavenger receptors	Plasma membranes of phagocytes	CD36: microbial diacylglycerides
NLRs	Cytoplasm of phagocytes and other cells	Nod1, Nod2 and NALP3: bacterial peptidoglycans
N-formyl Met-Leu-Phe receptors	Plasma membranes of phagocytes	FPR and FPRL1: peptides containing *N*-formylmethionyl residues

Soluble recognition molecules	Location	Specific examples and their PAMP ligands
Pentraxins	Plasma	C reactive protein (CRP): Microbial phosphorylcholine and phosphatidylethanolamine
Collectins	Plasma	Mannose-binding lectin (MBL): Carbohydrates with terminal mannose and fructose
	Alveoli	Surfactant proteins SP-A and SP-D: Various microbial structures
Ficolins	Plasma	Ficolin: *N*-acetylglucosamine and lipoteichoic acid components of the cell walls of gram-positive bacteria

Abbreviations: TLR, Toll-like receptor; PAMP, pathogen-associated molecular pattern; NLR, Nod-like receptor

Anatomical barriers- mechanical

System/Organ	Cell type	Mechanism
Skin	Squamous epithelium	Physical barrier Desquamation
Mucous membranes	Non-ciliated epithelium (e.g. GI tract) Ciliated epithelium (e.g. respiratory tract) Epithelium (e.g. nasopharynx)	Peristalsis Mucociliary elevator Flushing action of tears, saliva, mucus, urine

Anatomical barriers- chemical

System/Organ	Component	Mechanism
Skin	Sweat	Antimicrobial fatty acids
Mucous membranes	HCl (parietal cells), tears & saliva Defensins (respiratory & GI tract) Surfactants (lung)	Low pH Lysozyme & phospholipase A Antimicrobial Opsonin

Anatomical barriers- biological

System/Organ	Component	Mechanism
Skin and mucous membranes	Normal flora	Antimicrobial substances Competition for nutrients and colonization

Humoral components

Component	Mechanism
Complement	Lysis of bateria and some viruses Opsonin Increase in vascular permeability Recruitment and activation of phagocytic cells
Coagulation system	Increase vascular permeability Recruitment of phagocytic cells B-lysin from platelets – a cationic detergent
Lactoferrin and transferrin	Compete with bacteria for iron
Lysozyme	Breaks down bacterial cells walls
Cytokines	Various effects

Adaptive immunity

Humoral Immunity

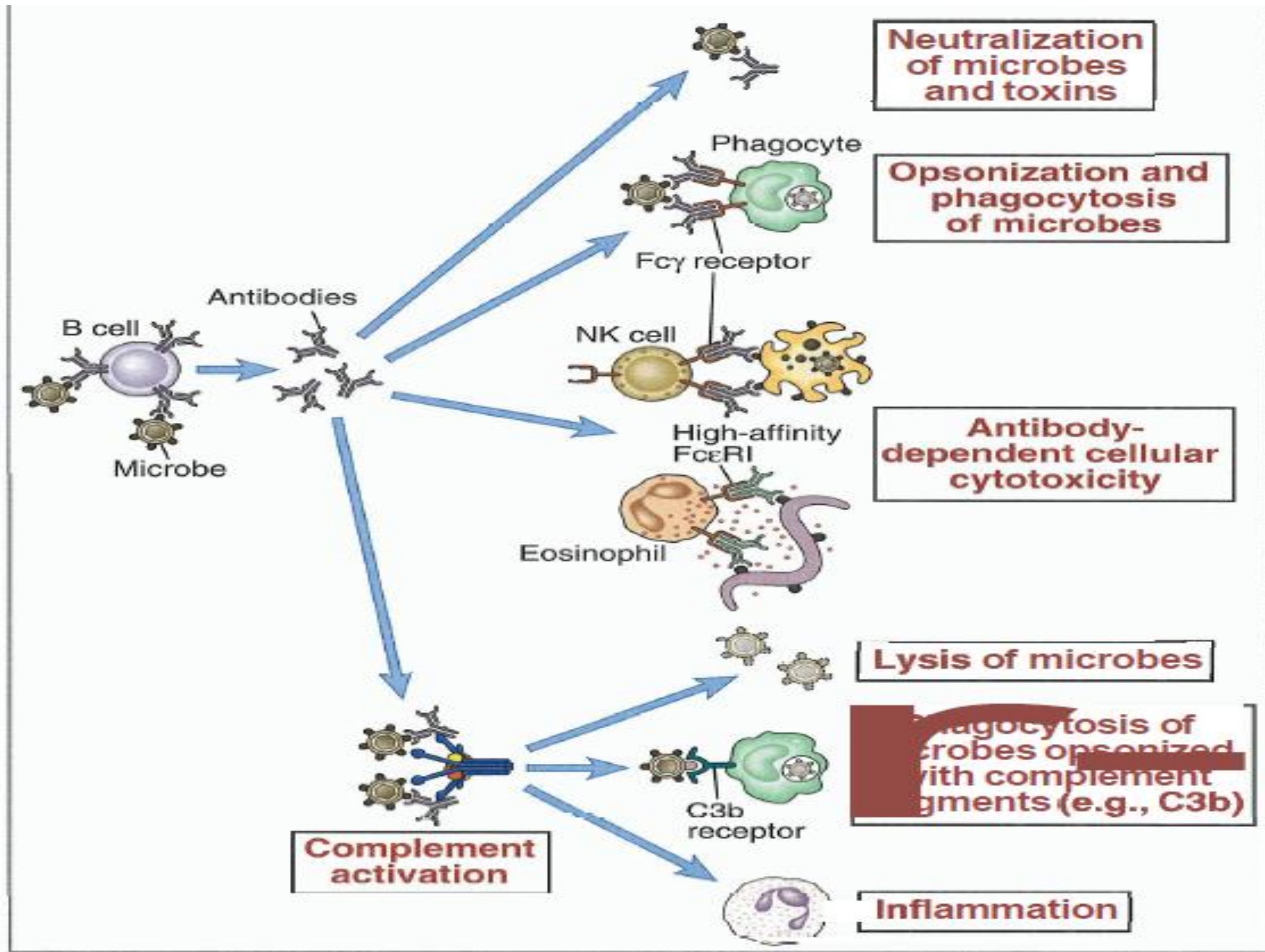
- Mediated by **antibodies** produced by Plasma cells to toxins or microbes

- Is important for **soluble antigens** and the destruction of **extracellular microorganisms**

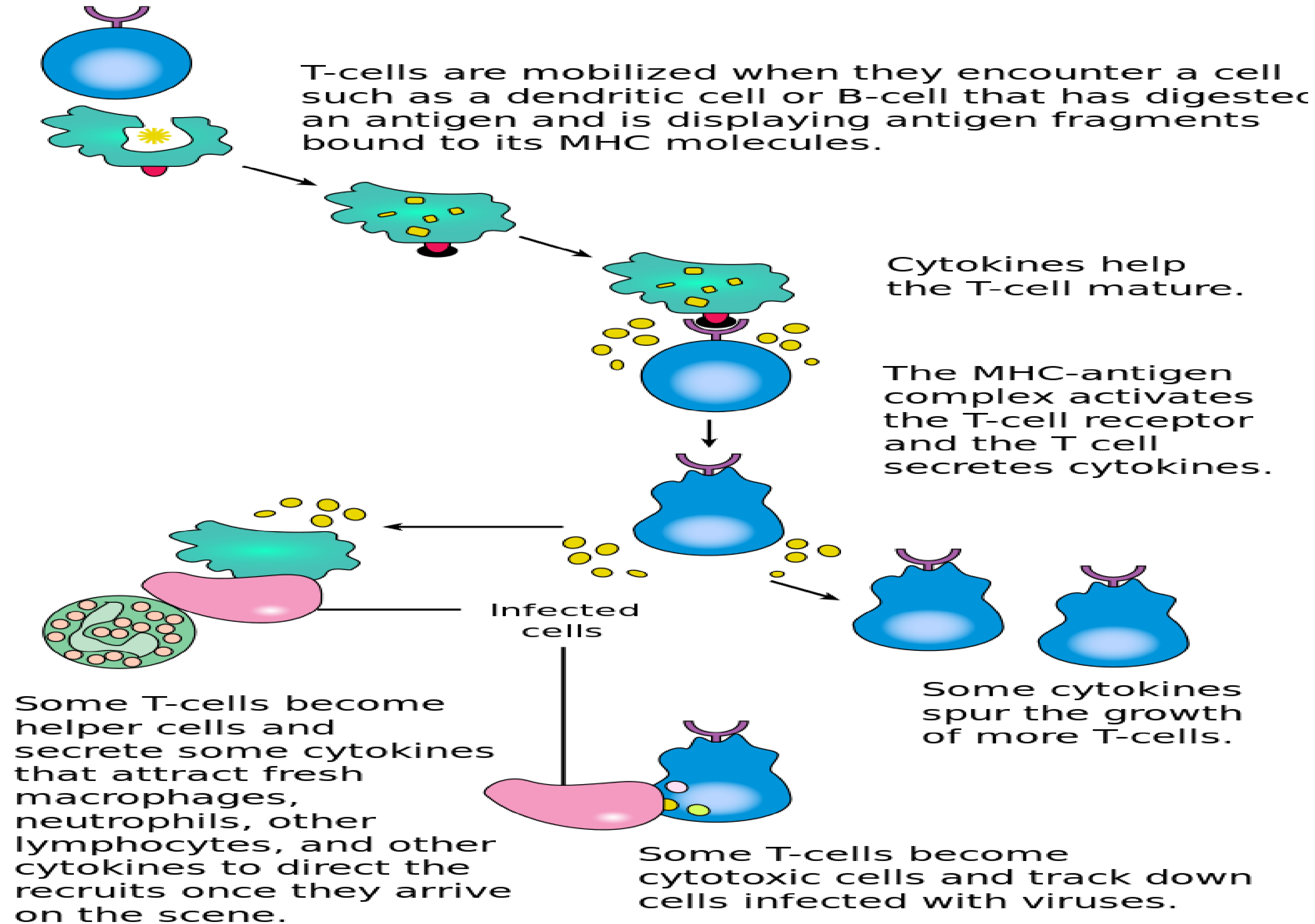
Cell mediated immunity

- Mediated by T-lymphocytes toward **intracellular microbes & viruses as well as damaged cells** .
- Some types of cell-mediated effector mechanisms depend on antibodies for target selection

Humoral immune reaction



The T lymphocyte activation pathway

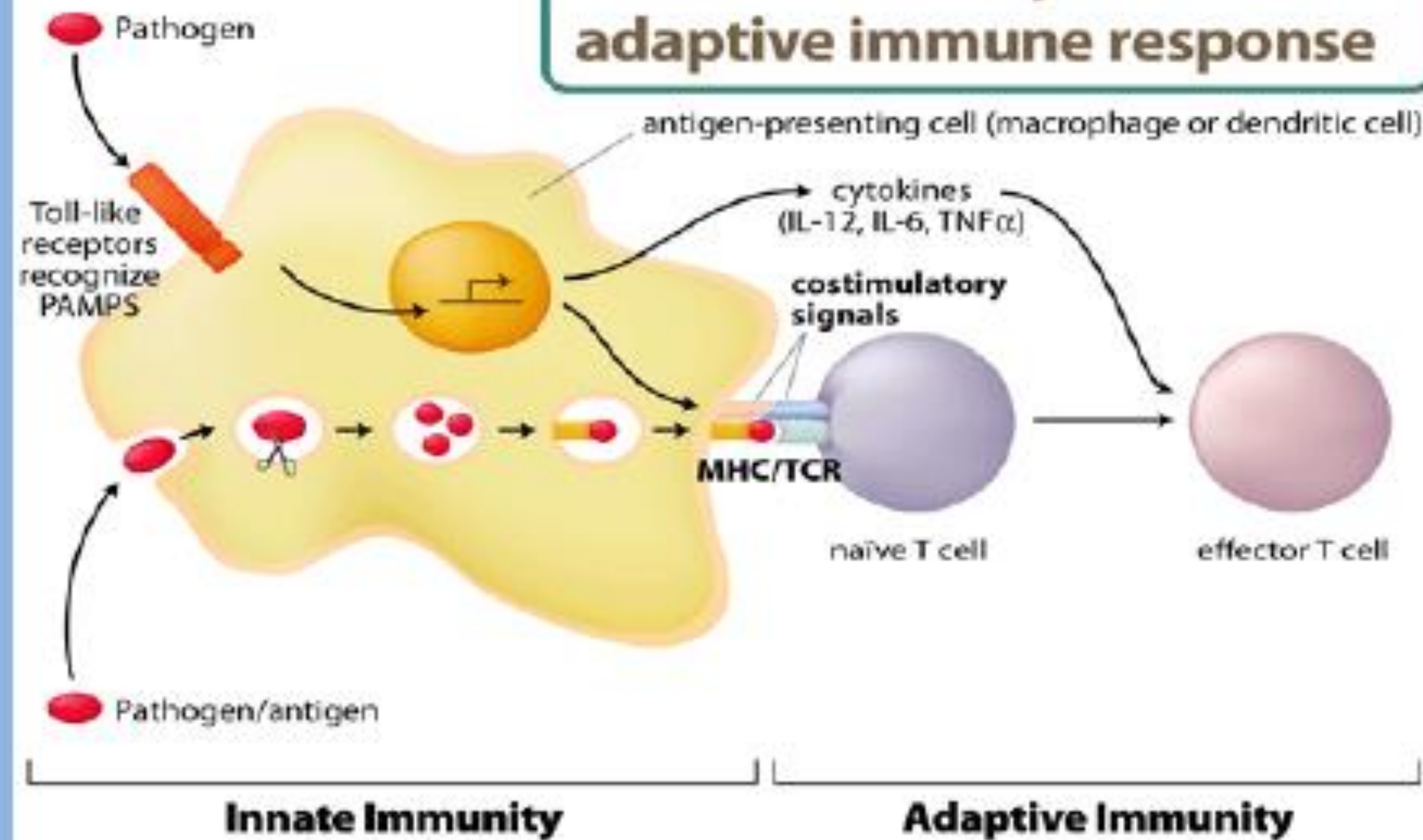


Link between Innate and adaptive immunity

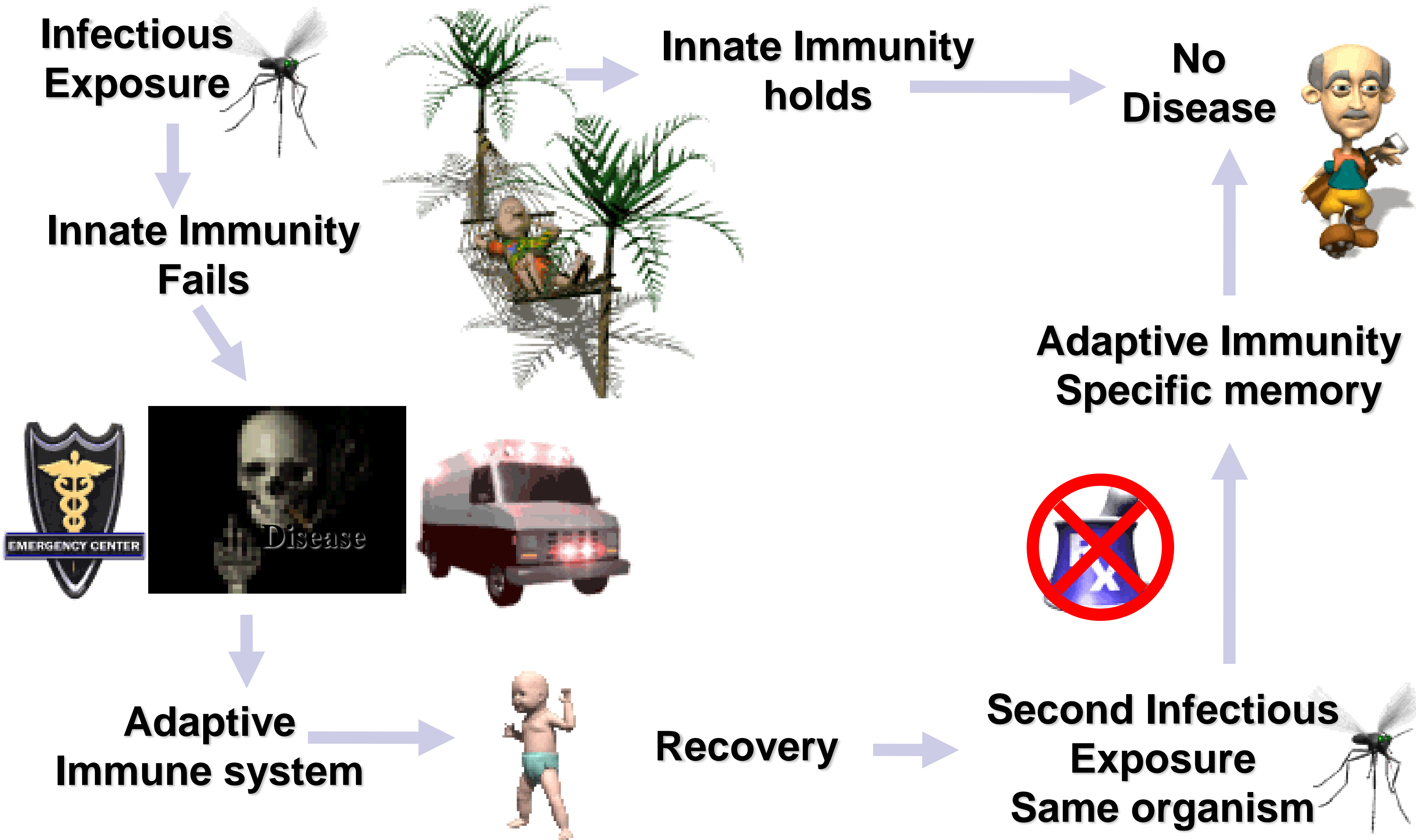
- Innate and adaptive immune responses function cooperatively.

- The innate immune response to microbes stimulates adaptive immune responses.
- Adaptive immune responses use many of the effector mechanisms of innate immunity to eliminate microbes

Crosstalk between Innate and adaptive Immune response



Adaptive and Innate - Interactions



Disorders of the immune system

- Immunodeficiency
- Autoimmunity
- Hypersensitivity
- Transplant and its rejection
- Types of rejection
- Rejection mechanisms
- Manipulation of the immune system
- Tumor immunology

Immunity and infection

– Immunity to viruses
– Immunity to bacteria
– Immunity to fungi
– Immunity to protozoa and worms
– Transplantation immunology

Chapter 2: Immunological Techniques

Outline

- **Introduction**
- **Immunological technique**
  - **primary binding sites**
    - ELISA
    - Immunoflurecence tests
    - RIA
  - **Secondary binding sites**
    - Agglutination tests
    - Precipitation tests
    - Complement fixation tests
  - **Tertiary binding tests**
- **Factors affecting antigen antibody reactions**

Serology

Definitions

- **Serology** is the scientific study of blood serum.

- In practice, the term usually refers to the diagnostic identification of antibodies in the serum.
  - Such antibodies are typically formed in response to an infection (against a given microorganism)

- against other foreign proteins (in response, for example, to a mismatched blood transfusion), or to one's own proteins (in instances of autoimmune disease).

Serology……………cont

- **Immune system:** the structures, cells, and soluble constituents that allow the host to recognize and respond to foreign stimulus.
- **Secondary immune response:** the cellular and humoral events that occur when an antigen is encountered for a second or subsequent time.
- **Serum:** the fluid portion of the blood after the blood clots.
- **Specificity:** the special affinity between an antigen and its corresponding antibody.

Application of serologic tests

- Serological tests may be performed for diagnostic purposes when an infection is suspected, in rheumatic illnesses, and in many other situations, such as checking an individual's blood type.
- Serology blood tests help to diagnose patients with certain immune deficiencies associated with the lack of antibodies.

In such cases, tests for antibodies will be consistently negative.

Introduction

Antibody molecules combine reversibly with antigens to form immune complexes.

The detection and measurements of these reactions form the basis of serology a sub discipline of immunology.

Introduction…….*cont*

- **Therefore;**
  - **Serology** - is the science of measuring antibody or antigen in *body fluids*.

Immunological techniques

- **Three** groups of immunological techniques are used to detect and measure antigen-antibody combination.
  - ***Primary binding tests***
  - ***Secondary binding tests and***
  - ***Tertiary binding tests.***

Immunological tech….*cont*

Primary binding tests ….cont

E.g.

- Enzyme linked Immunosorbent assay (ELISA) tests and
- Radioimmunoassay (RIA)

- Western blotting
- Northern blotting

- Southern blotting
- Fluorescence tests

Immunological tech....*cont*

- Widely used in the serological diagnosis of
  - *bacterial,*
  - *viral,*
  - *fungal, and*
  - *parasitic diseases.*

- They are usually sensitive and give reproducible results.

Enzyme Linked Immuno Sorbent Assay (ELISA) –a theoretical and practical guide

ELISA – WHAT DOES IT MEAN? WHAT IS IT USED FOR?

- **E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay
  - Immuno – Antibody/ Antigen
  - Sorbent – Absorbs / Attaches to surface
  - Enzyme linked – Enzyme attached to antibody

- Used for Detection and Quantification of analytes
  - Any protein: e.g. Cytokines, Antibodies, Hormones, etc.
  - Liquid form (e.g. Serum, Urine, Sputum, Cell culture supernatants, etc.)
  - Also, but less common and more difficult: carbohydrates, sugars

ELISA

- They are specific, sensitive, and require only a small amount of specimen

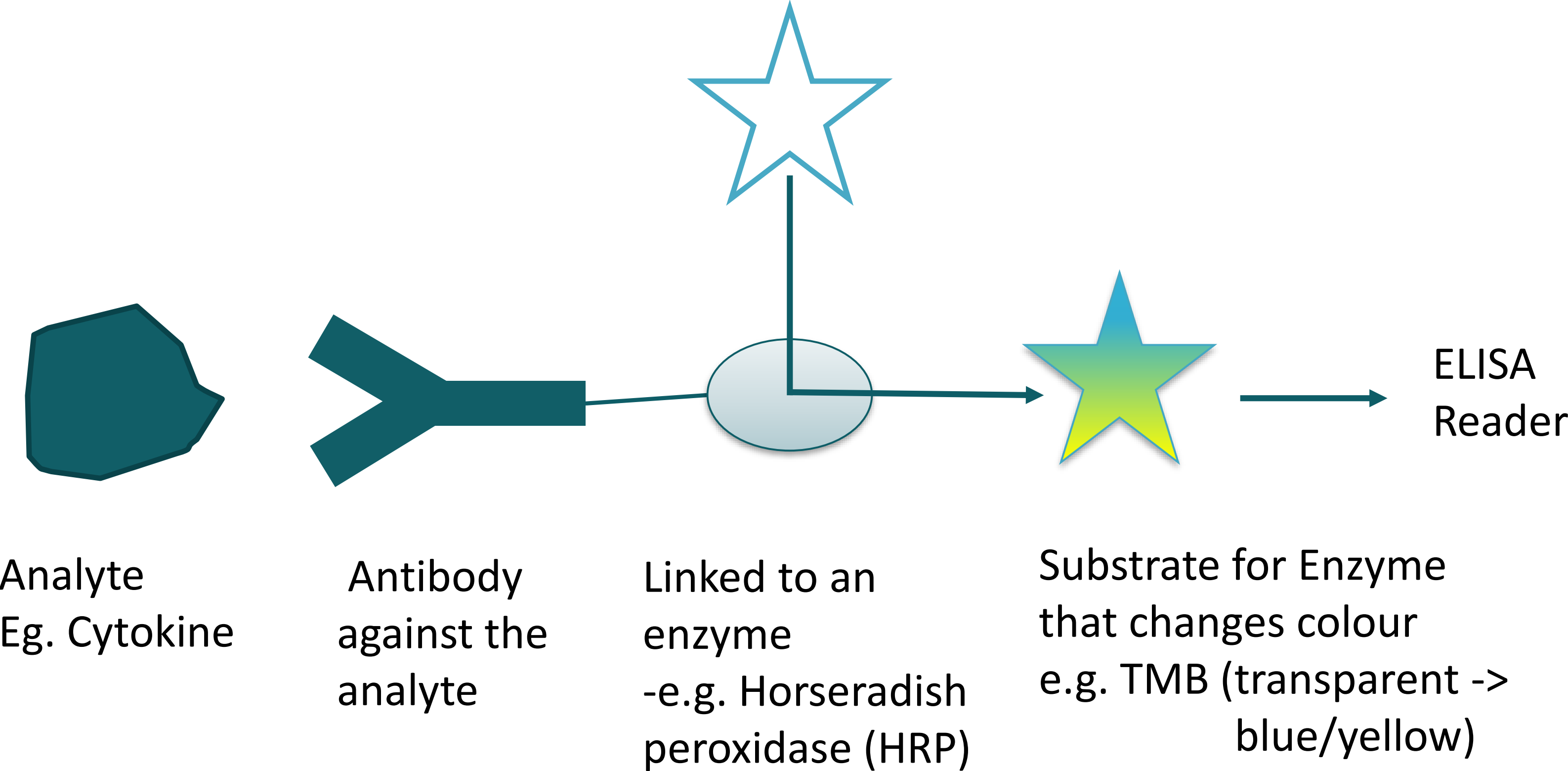
  –Reagents used in the ELISA are stable and have a long shelf life which makes for easy distribution to district laboratories

  –The results of qualitative ELISA techniques can be read visually

  –Large numbers of specimens can be tested at one time and the

- ELISA can be easily automated for use in epidemiological surveys.

Principle of ELISA



Assay Components

❑ Solid phase

- ➢ Plastic
  - Polystyrene/Polyvinyl chloride (Maxisorb)
- ➢ Nitrocellulose
- ➢ Agarose
- ➢ Polyacrylamid

❑ Coating of Solid Phase

- ❑ Coating Buffers:
  - PBS / PH:7.3
  - Carbonate /Bicarbonate/ PH:9.6
- Blocking (BSA 2%)
- Adding of sample

Specimen For ELISA

- Serum
- CSF
- Sputum
- Urine
- Semen
- Supernatant of culture
- Stool .....

Assay Components

- **Ag/Ab conjugate**
- **Enzyme**
  - Alkaline phosphatase
  - Horse radish proxidase
  - Galactose oxidase
  - Urease
- **Antibody**
  - **Whole polyclonal Ab**
  - **IgG fractions**
  - **$F(ab')_2$ fragments of polyclonal Ab**
  - **Affinity purified polyclonal Ab**
  - **Monoclonal Ab**

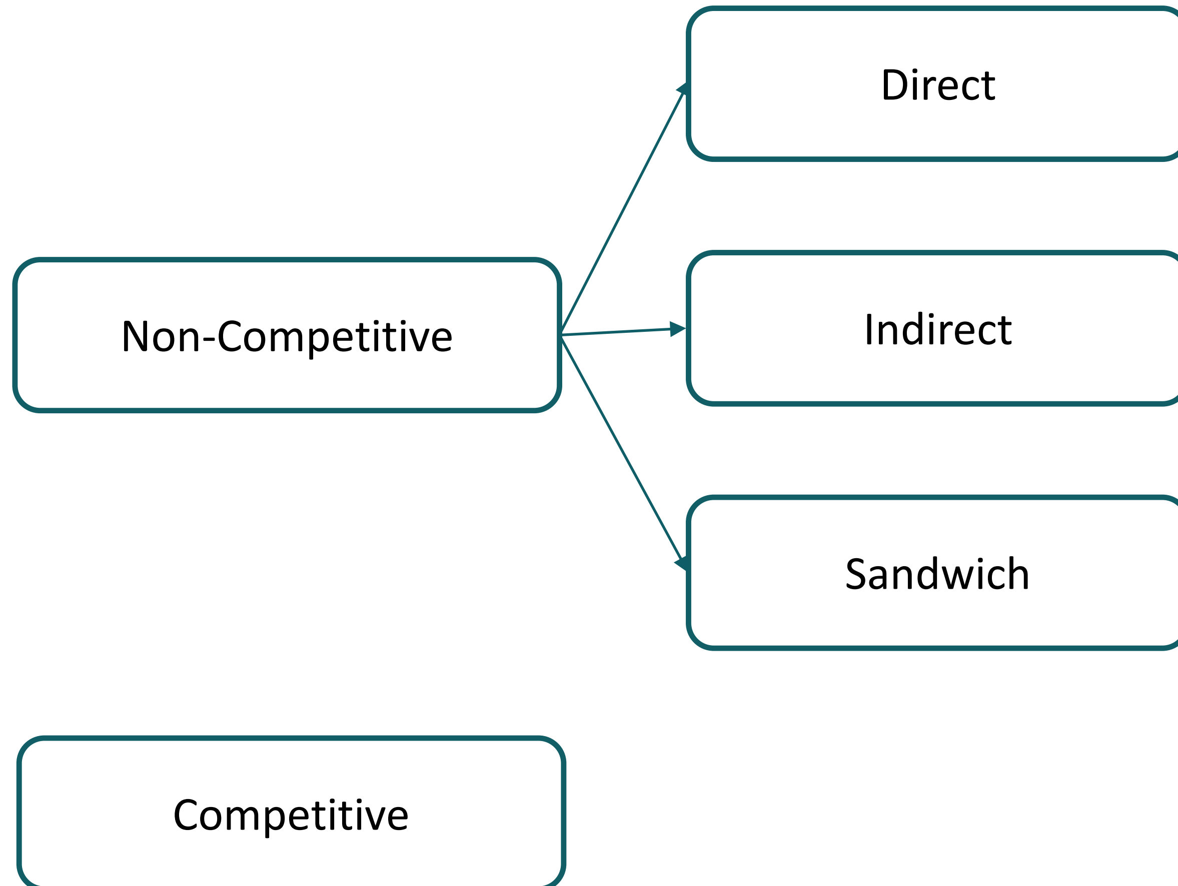
Substrate & Stopping Buffer

- Initially the substrate should be colorless.
  - After degradation by the enzyme it should be strongly colored or fluorescent

Colorimetric Substrate

ENZYME	SUBSTRATE	CHROMOGEN	STOPPING
AlkalinePhosphatase	p-NPP	P-NPP+diethanolamine +MgCl2	NaOH 1 M
Horse radishPeroxidase	H2O2	Tetramethylbenzidine+ Phosphate-Citrate buffer	H2SO4 1 M
Horse radishPeroxidase	H2O2	O-Phenylenediamine+HCl	HCl 1M

Different types of ELISA

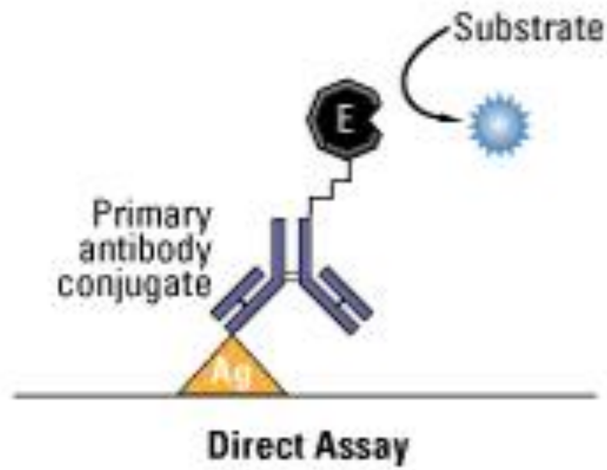


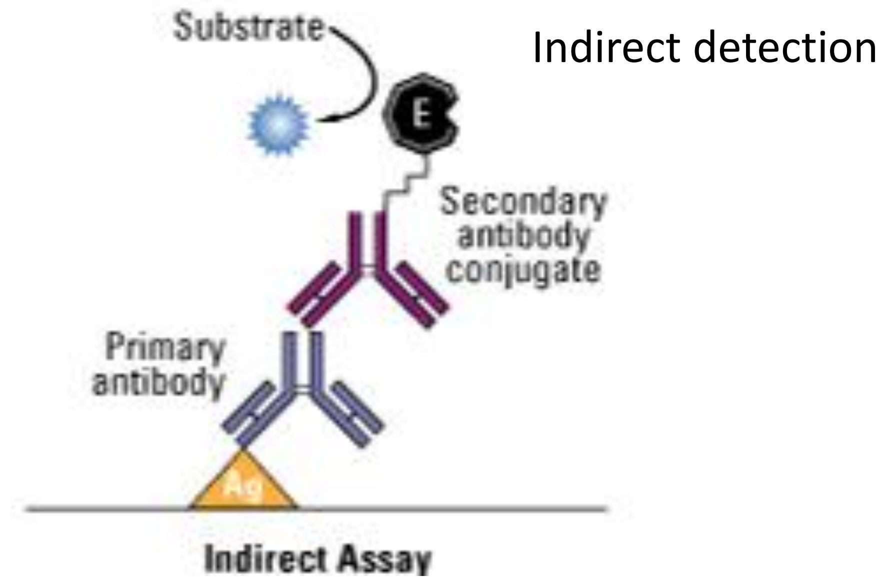
Indirect ELISA

- In this technique, known antigen is attached to the inside surface of the well and patient's serum is added.
- After incubation and washing, enzyme labeled antihuman globulin is reacted with the antibody that has attached to the antigen.
- The presence and concentration of antibody that has reacted with the antigen is shown by a change in color when the substrate is added
- The intensity of the color is directly proportional to the concentration of antibody in the serum.

Direct ELISA

Indirect ELISA





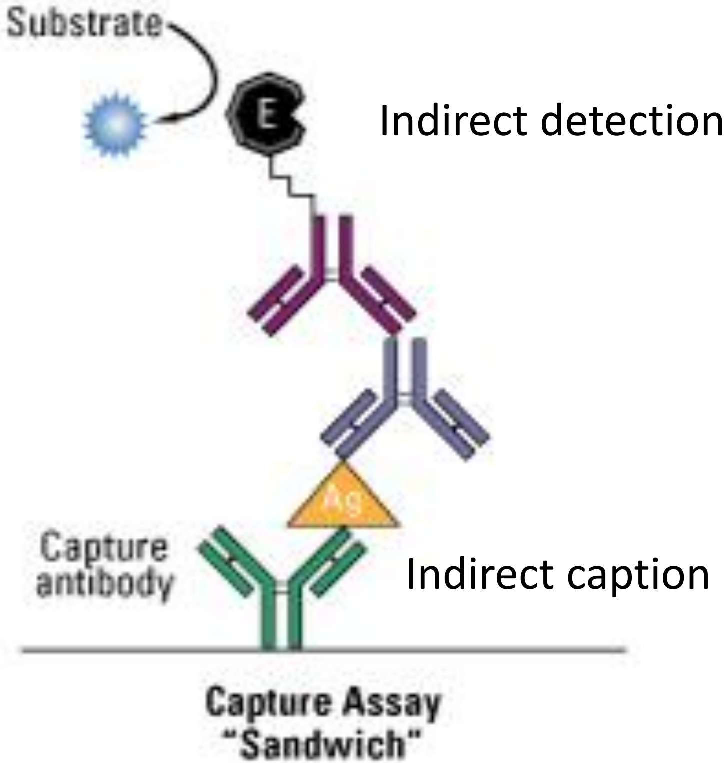
Indirect detection

Difference b/n direct and Indirect ELISA?

Advantages?

Disadvantages?

Sandwich ELISA



Direct vs. Indirect

Capture methods

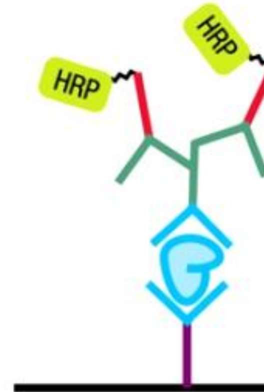
Direct capture

Indirect capture

Higher specificity!
Less antibody cross-reactivity

Detection methods

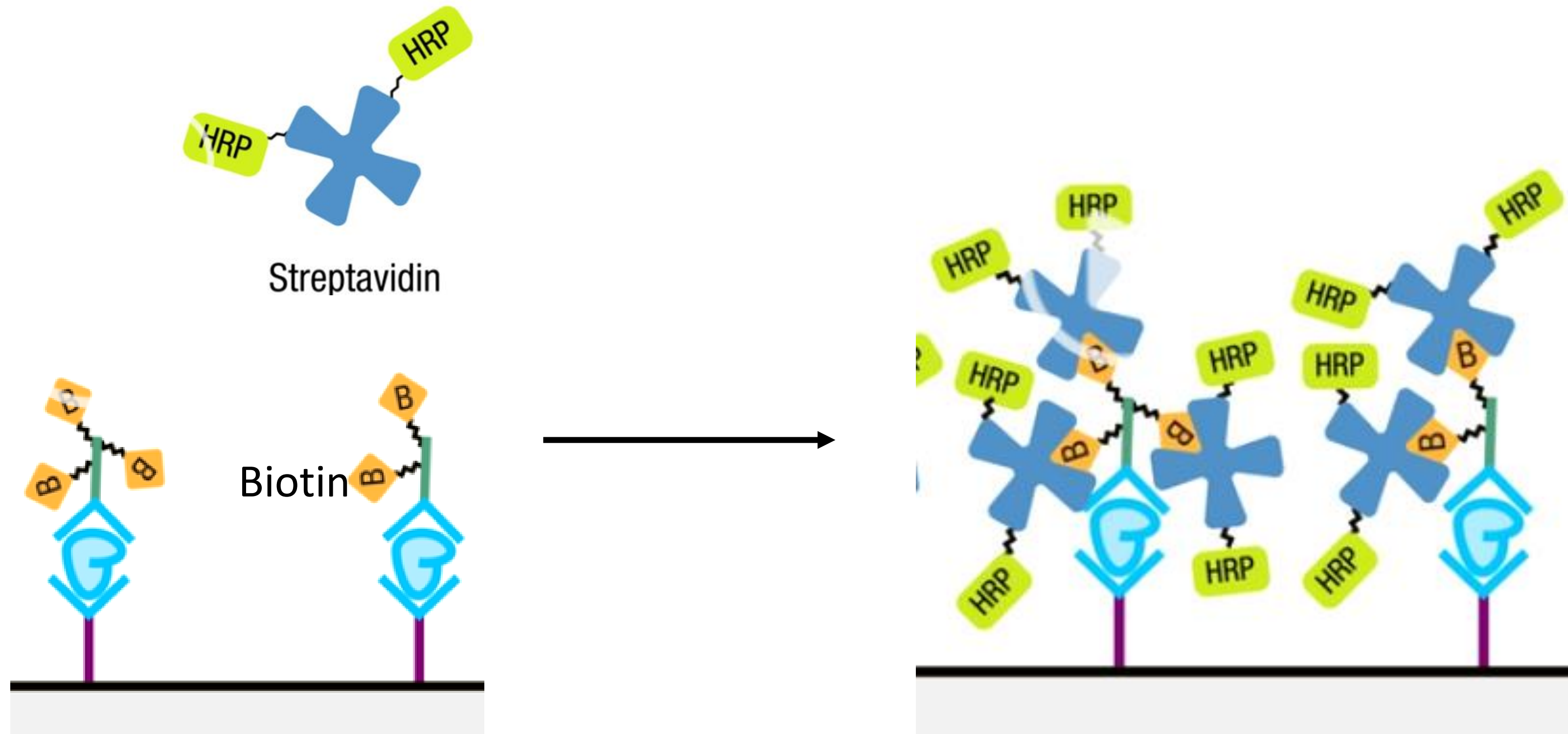
Direct detection



Indirect detection

Higher sensitivity!
Signal amplification!

Other indirect detection methods - Biotin-Streptavidin-HRP



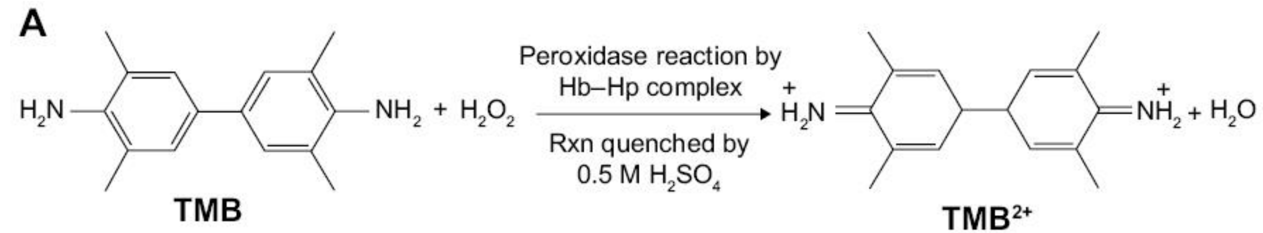
Even stronger signal amplification!

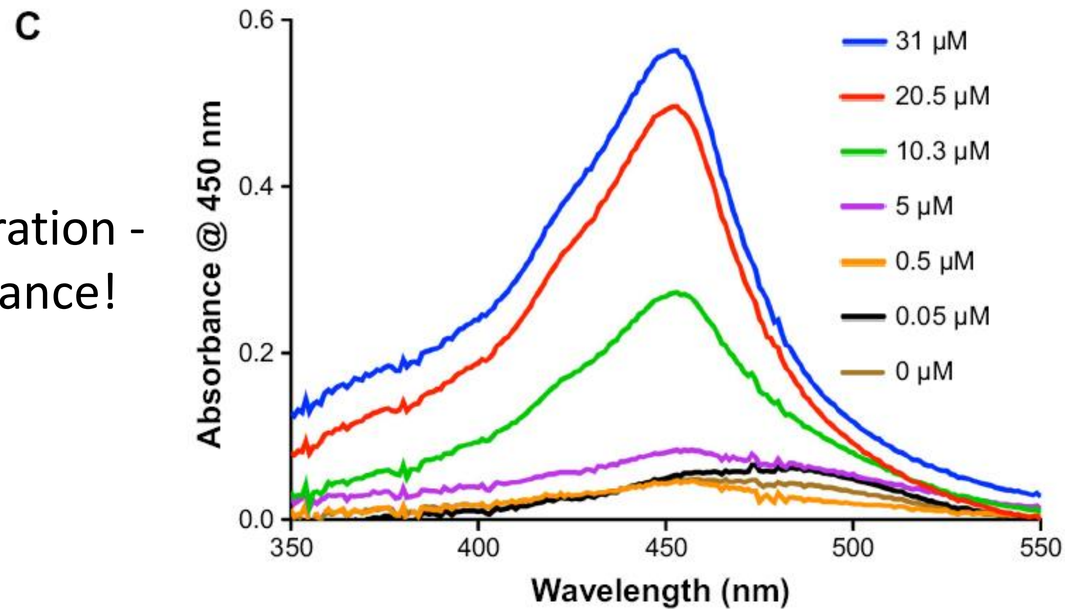
Detection

ELISA reader - Spectrophotometer

Determines:
Optical density (OD)
Or
Absorbance

Detection





Higher concentration -> higher absorbance!

The RAW Optical Density (OD) values

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.716	1.757	1.84	1.8	0.96	1.013	0.313	0.346	0.655	0.667	1.44	1.409
B	1.438	1.462	1.79	1.801	0.984	0.97	0.334	0.322	0.214	0.267	1.255	1.239
C	0.967	1.014	1.794	1.746	0.958	0.948	0.312	0.312	0.157	0.146	0.233	0.255
D	0.586	0.606	1.791	1.822	0.955	0.906	0.325	0.305	0.091	0.082	1.791	1.822
E	0.319	0.347	1.462	1.493	0.562	0.59	0.171	0.173	0.334	0.322	0.334	0.322
F	0.167	0.186	1.484	1.472	0.589	0.588	0.175	0.169	0.958	0.948	0.086	0.09
G	0.094	0.103	1.445	1.424	0.587	0.573	0.175	0.168	0.589	0.588	0.313	0.342
H	0.086	0.09	1.531	1.478	0.584	0.536	0.091	0.086	0.175	0.168	0.962	1.012

How to calculate concentrations (eg. ng/ml) from this?
Relative -> Absolute values

Standard curve!
Add the analyte you want to detect in different KNOWN concentrations
(usually included in ELISA Kits)

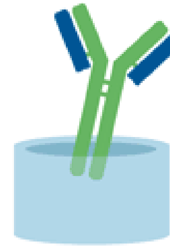
How to start?

The practical part!

The procedure – Sandwich ELISA

Day 1:

- Coat plate with Capture Antibody



Day 2:

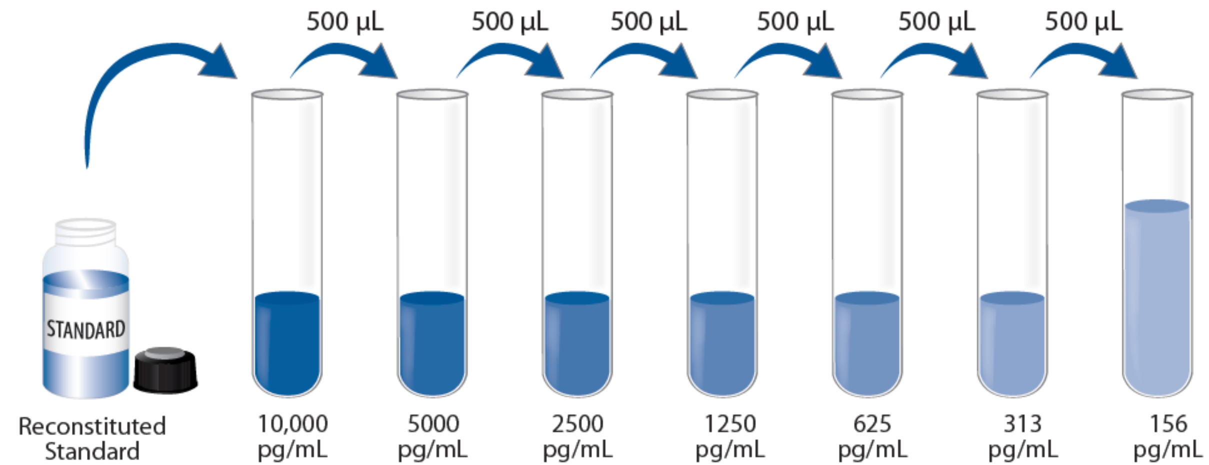
- Wash & block your plate
  - Wash: to remove non-bound capture antibody
  - Block: add protein (e.g. BSA) to occupy all plastic surface – no other antibodies/analytes can bind to the plate, only to capture antibody

- Prepare your samples
  - Do you have to dilute your samples or not?

    What is the detection range of your ELISA? – Check the manual!
    What is your expected analyte concentration? – Check the literature!

The procedure – Sandwich ELISA

- Dilute the standard



- Reconstitute - Check CoA
- Make 1ml of highest concentration

E.g. Two fold serial dilution

The procedure – Sandwich ELISA

Always do technical du- or triplicates!

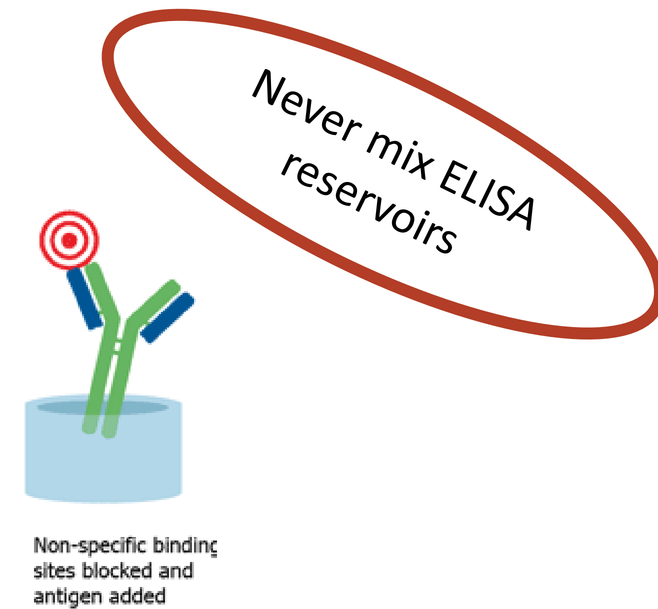
- Make a pipetting scheme

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	S1	S1	S9	S9						
B	156pg/ml	156pg/ml	S2	S2	…							
C	313	313	S3	S3								
D	625	625	S4	S4								
E	1250	1250	S5	S5								
F	2500	2500	S6	S6								
G	5000	5000	…									
H	10 000	10 000									S40	S40

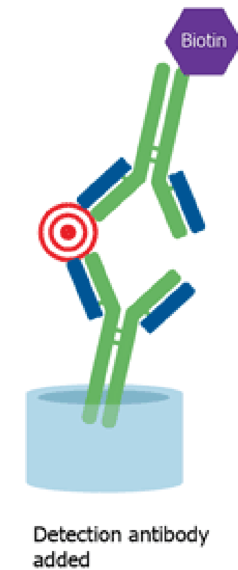
Yellow: standard; S1= sample 1; Everything in duplicates

The procedure – Sandwich ELISA

- After blocking – Wash 3x
- Add samples and standard to the wells
  - Incubate for 2h

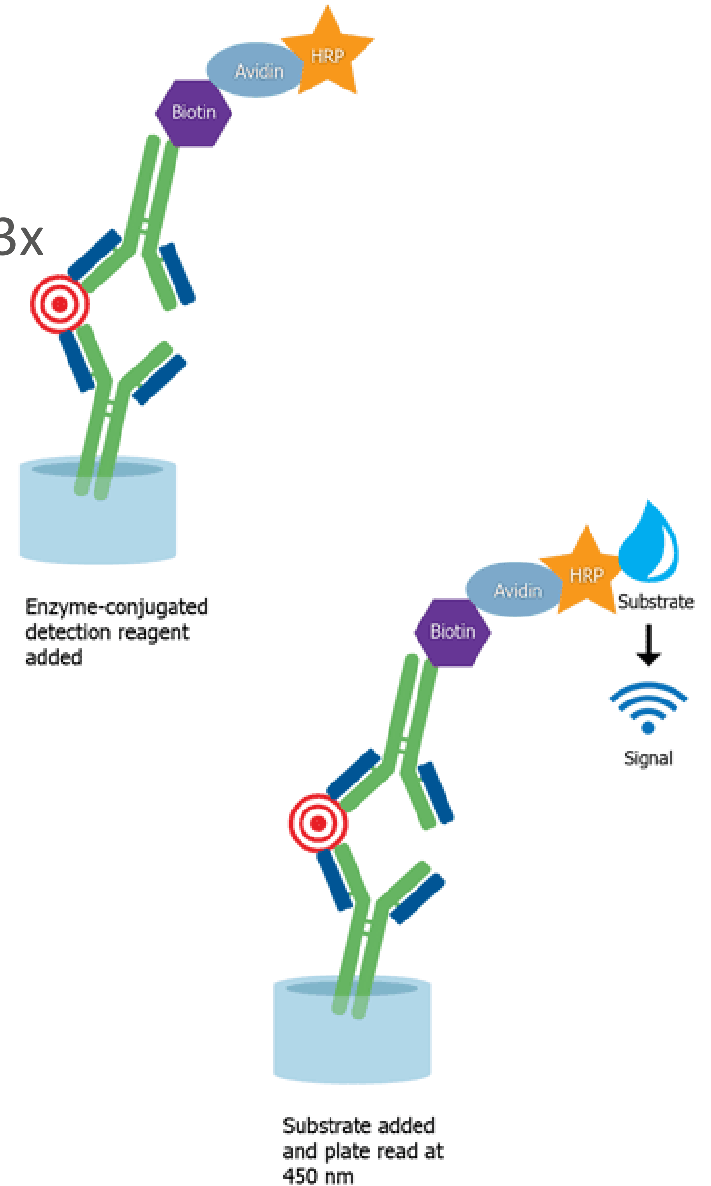


- Wash unbound proteins etc. away 3x
- Add detection (secondary) antibody
  - Incubate for 2h



The procedure – Sandwich ELISA

- Wash unbound secondary antibody away, 3x
- Add Streptavidin-HRP
  - Incubate for 20min
- Wash unbound Streptavidin-HRP away, 3x
- Add Substrate solution
  - Incubate for 20min in the dark!



The procedure – Sandwich ELISA

- Add stop solution (acid! H_2SO_4)
  - Stops the enzymatic reaction

- Read in Spectrophotometer at 450nm (Emission of TMB substrate)
  - Also read at 540 or 570nm – this is your background value, correct for optical imperfections in the plate.

Calculating your results

- Substract the 540/570nm values from the 450nm values
  - Sometimes automatically done by the machine

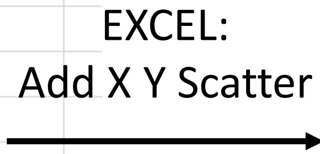
- The standard curve
  - calculate the average of the duplicates

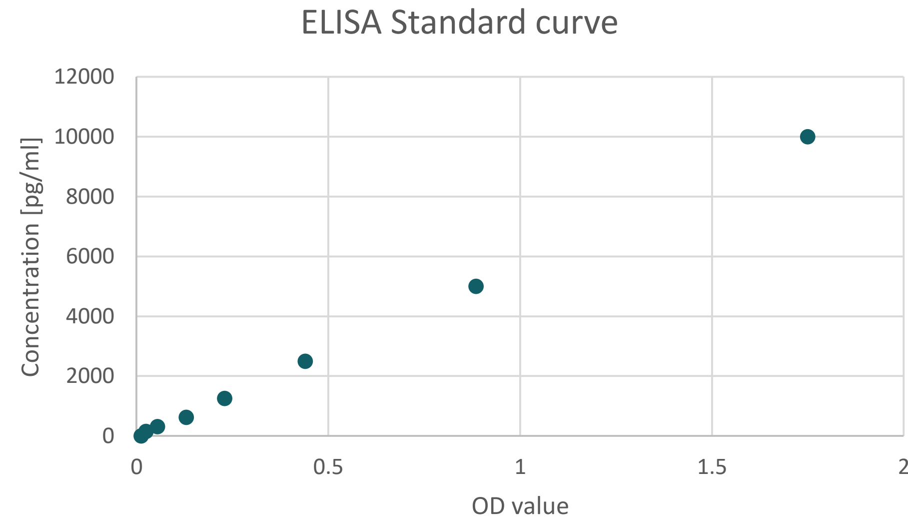
OD 1	OD 2	AVERAGE
0,015	0,01	0,0125
0,03	0,02	0,025
0,05	0,06	0,055
0,14	0,12	0,13
0,22	0,24	0,23
0,42	0,46	0,44
0,87	0,9	0,885
1,8	1,7	1,75

Calculating your results

- The standard curve
  - Plot OD (X) versus the concentration (Y)

Average OD	Conc (pg/ml)
0,0125	0
0,025	156
0,055	313
0,13	625
0,23	1250
0,44	2500
0,885	5000
1,75	10000

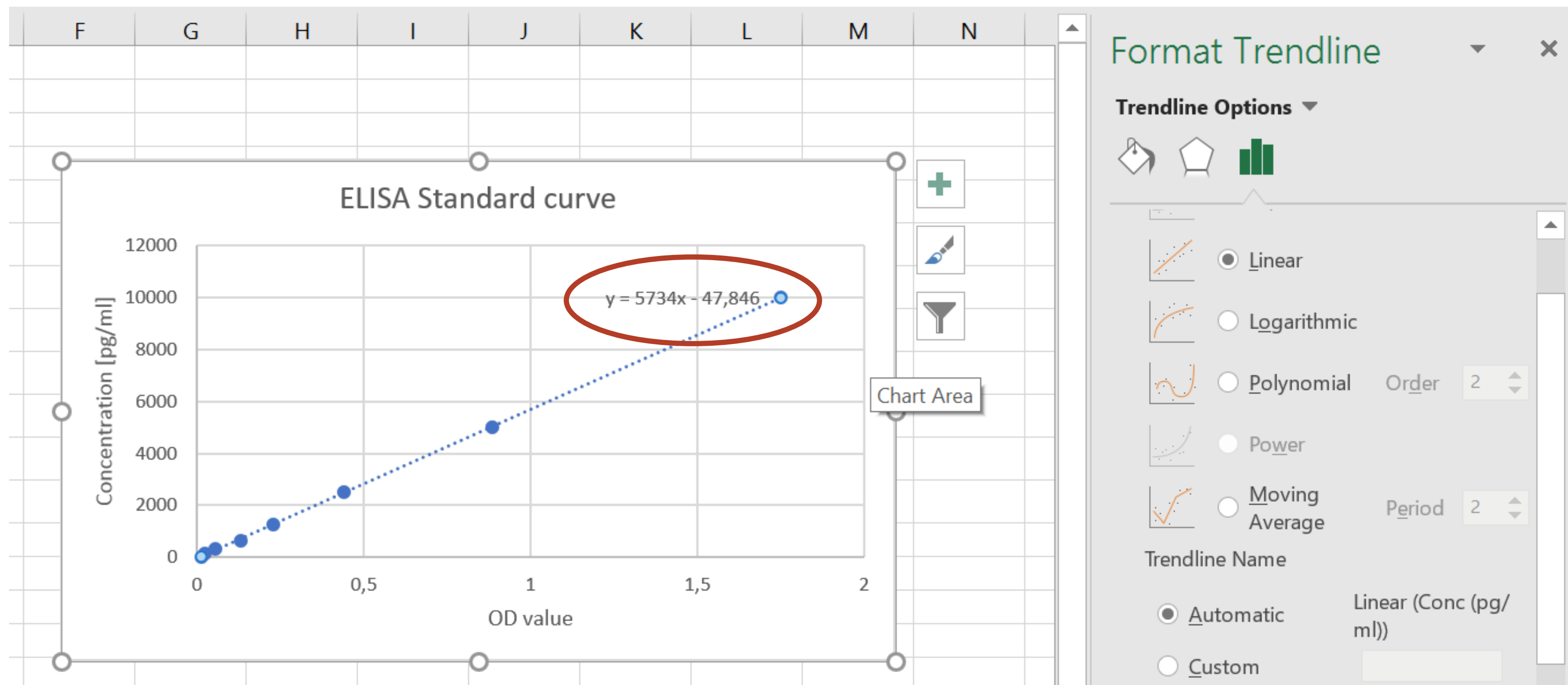




Calculating your results

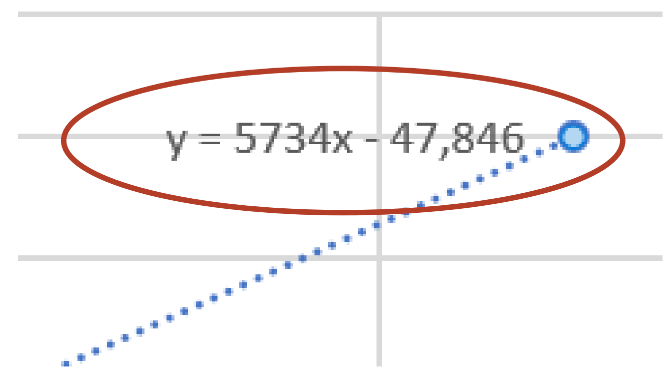
- The standard curve
  - Connect the OD to the concentration

EXCEL:
Right-klick on data points:
Add trendline
- linear
- display equation on chart



Calculating your results

- Calculate the concentration of your samples:
  - Calculate the average value
  - Insert your OD values in the equation (X value)



	OD samples	
Sample 1	1,5	=5734*B23 - 47,846
Sampe 2	0,6	
Sample 3	0,3	
Sample 4	2,4	
Sample 5	0,78	

→

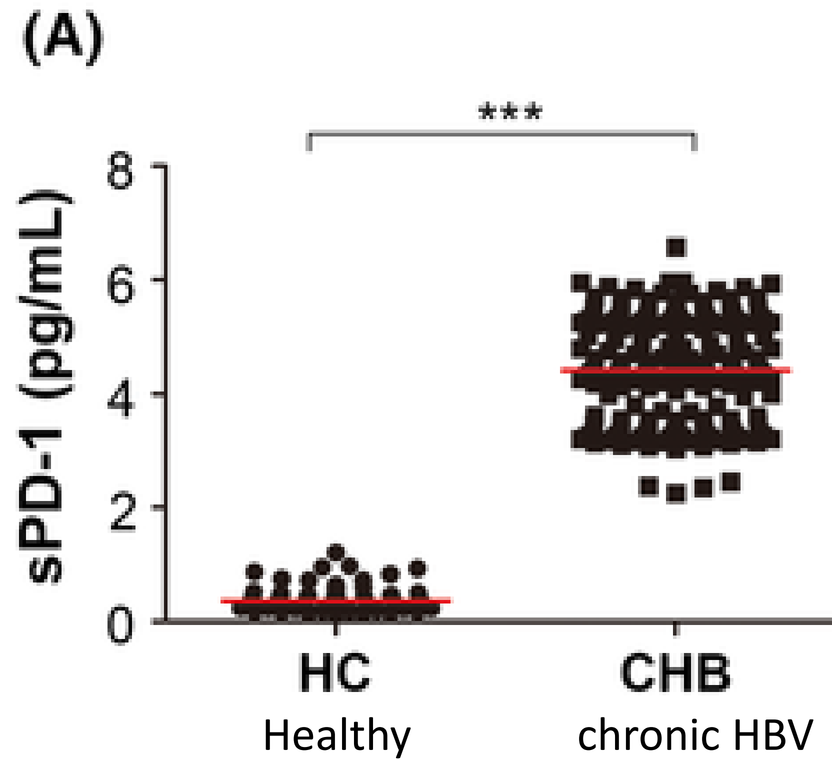
OD samples	
1,5	8553,154
0,6	
0,3	
2,4	
0,78	

→

8553,154
3392,554
1672,354
13713,754
4424,674

Analyte concentration in pg/ml

Displaying your results



Zhou et al. 2018, JVH

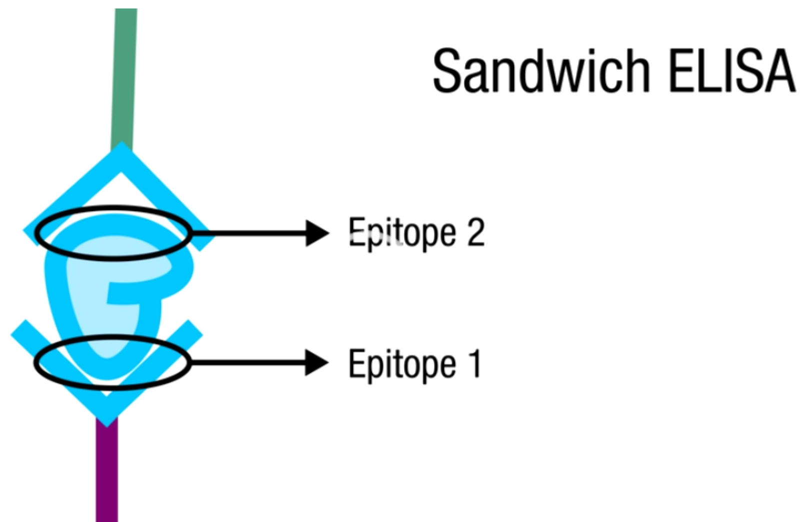
Competitive ELISA

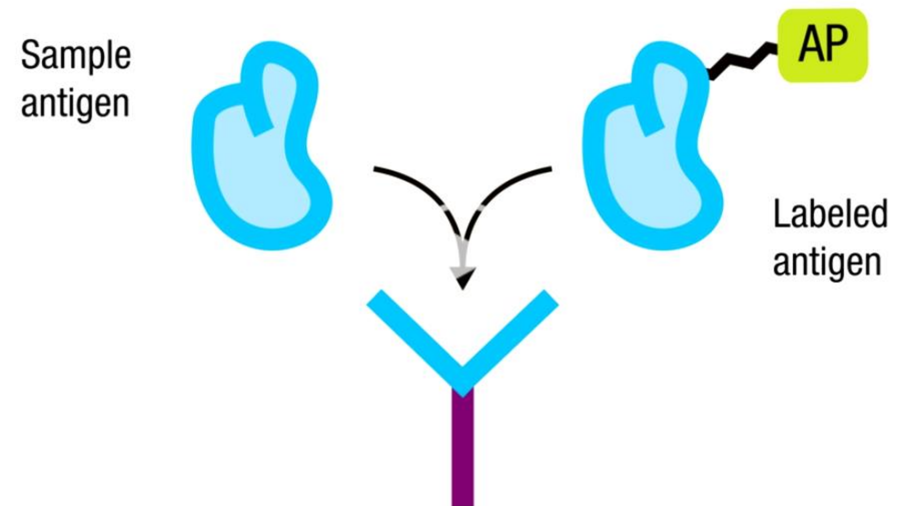
- In this technique, The labeled antigen competes for primary antibody binding sites with sample antigen (unlabeled).
- The more antigen in the sample the less labeled antigen is retained in the well and the weaker the signal).
  - The steps for this ELISA are somewhat different than the first two examples:
    - Unlabeled antibody is incubated in the presence of its antigen (Sample).
    - These bound antibody/antigen complexes are then added to an antigen-coated well

Competitive ELISA ….

- The plate is washed, so that unbound antibody is removed. (The more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence "competition.")
- The secondary antibody, specific to the primary antibody is added. This second antibody is coupled to the enzyme.
- A substrate is added, and remaining enzymes elicit a chromogenic signal.
- The reaction is stopped in order to prevent eventual saturation of the signal

Competitive ELISA





Competitive ELISA

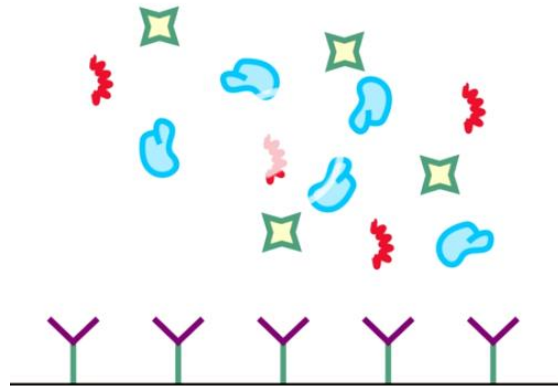
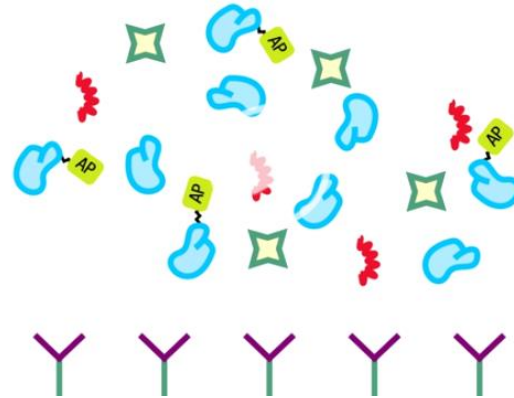
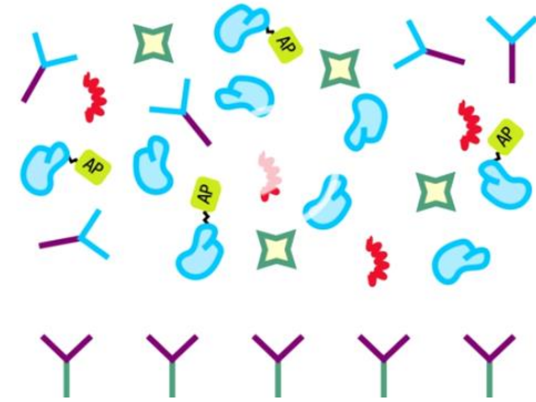


Plate with 2nd antibody –
Add sample

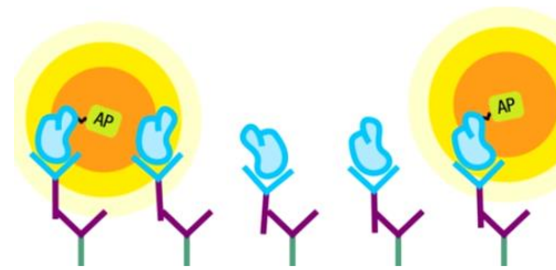


Add labelled analyte



Add 1st antibodies
„your“ analyte and the labelled analyte will compete for 1st AB

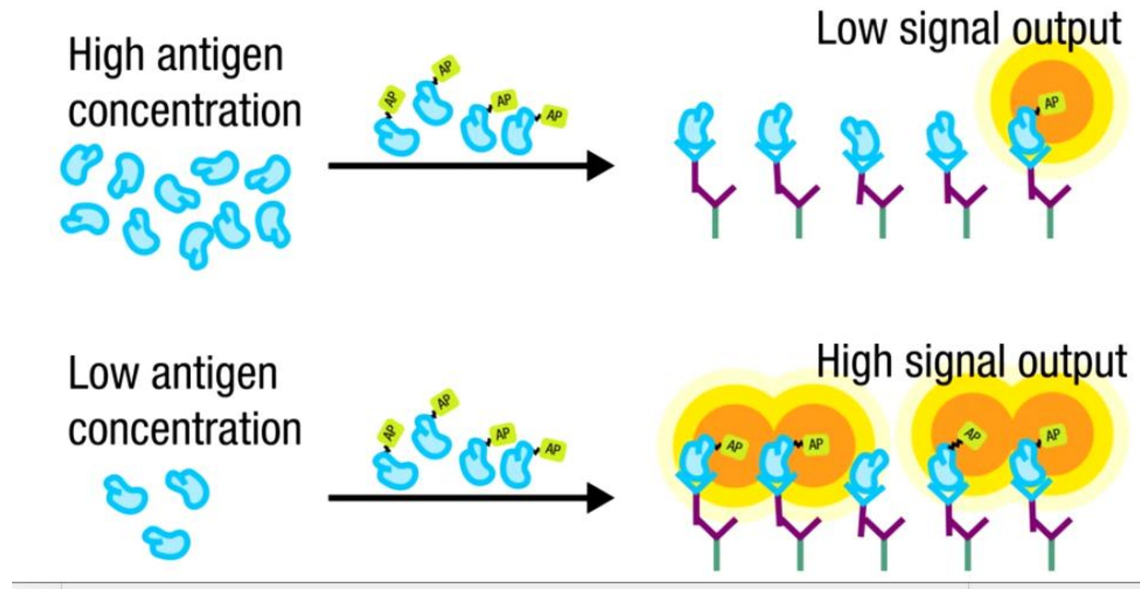
Binding of antibodies and analytes



Adding substrate + measuring

Competitive ELISA

Inverse correlation of „your“
analyte and signal!



Troubleshooting tips - ELISA

Positive results in negative control

- **Contamination of reagents/samples.**
- **Sandwich ELISA – detection antibody is detecting coating antibody.**
- **Insufficient washing of plates.**
- **Too much antibody used leading to nonspecific binding**

High background across entire plate

- Conjugate too strong or left on too long.
- Substrate solution or stop solution is not fresh.
- Reaction not stopped.
- Plate left too long before reading on the plate reader.
- Contaminants from laboratory glassware.
- Substrate incubation carried out in the light.
- Incubation temperature too high.
- Non-specific binding of antibody

Low absorbance values

- Target protein not expressed in sample used or low level of target protein expression in sample used
- Insufficient antibody
- Substrate solutions not fresh or combined incorrectly
- Reagents not fresh or not at the correct pH
- Incubation time not long enough
- Incubation temperature too low
- Stop solution not added.

High absorbance values

- High absorbance values for samples and/or positive control. Absorbance is not reduced as
  the sample is diluted down the plate
- The concentration of samples or positive control is too high and out of range for the sensitivity of the assay.
- Re-assess the assay you are using or reduce the concentration of samples and control by
  dilution before adding to the plate.
- Consider the dilution when calculating the resulting concentrations.

Inconsistent absorbance across the plate

- Plates stacked during incubations.
- Pipetting inconsistent.
- Antibody dilutions/reagents not well mixed.
- Wells allowed to dry out.
- Inadequate washing.
- Bottom of the plate is dirty affecting absorbance readings

Color developing slowly

- Plates are not at the correct temperature.
- Conjugate too weak.
- Contamination of solutions.

APPLICATIONS OF ELISA (Summary)

- **Diagnosis of infectious disease**

  – Detection of antibodies & antigens in blood sample and other specimens

  – Serum Antibody Concentrations

  – Serum Antigen Concentrations

- **Diagnosis of allergy**

  – Detecting potential food allergens (milk, peanuts, walnuts, almonds and eggs)

  – Detection of IgE

- **Measurement of hormones & cytokines**
- **Detections of drugs & vitamins, tumor markers and serum proteins**

And happy pipetting

… and washing… waiting…washing...waiting....Reading!

Next course: Immunofluorescence ? Radioimmunoassay?

Thank you and Keep safe !!