MOLECULAR DIAGNOSTICS: immunetechniques and molecular biology methods in routine laboratory

- Routine diagnostics, automatization
- Haemagglutination, Coombs-test
- Nephelometry, turbidimetry
- ELISA, RIA

Timea Berki MD, PhD

Branches of clinical immundiagnostics

- **Immunochemistry:**
  - Qualitative and quantitative detection of normal and abnormal proteins in body fluids using immunological detection method
  - Immunogen specific response
  - Allergen-specific reactions
  - Tumour antigen detection

- **Immunserology:** The branch of serology that involves studies of the immune response in body fluids

  - Infection serology: Immune response against microbes
  - Autoimmune serology: Autoantibody detection

- **Flow cytometry, tissue típying**
  - Phenotype analysis in blood and tissues
  - Ploidity detection
  - Immune response in transplanted patients
  - Cell sorting
  - MHC antigen detection
  - MHC sepcific antibody detection

Immunochemistry

- **Protein chemistry:**

  - serum protein ELFO, urine protein ELFO

  - measuring levels of specific proteins: IgG, IgA, IgM, kappa-lambda, light chains complement proteins (C3, C4), rheumatoid factor (RF), albumin, pre-albumin, haptoglobin, transzferrin, α1-antitrypsine, apolipoprotein A and B, ceruloplazmin, CRP, C1 inhibitor

  - Cryoglobulin, IgD, IgG and IgA subclasses

- **Specific antibody titers:**

  - serology: IgM, IgG and IgA antibodies as signs of infections (hepatitis, HIV, Toxoplasma, Rubeola, CMV, HSV, Borrelia, Mycoplasma, Yersinia, Helicobacter etc.)

  - autoimmunity: systemic: ANA, dsDNA, ANCA

    organ specific autoantibodies: GBM, TG-TPO, gliadin-TTG, ASCA, GPC, intrinsic factor IgG, IgA

- **Allergy:**

  - total IgE, allergen-specific IgE

Immunoassay

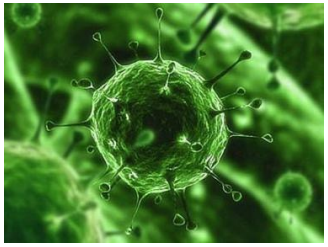
Main fields of application:

- medical diagnostics (pl. hormons, tumor markers, cardiological markers, etc.)
- pharmacology (pl. drug levels, drug research)
- toxicology (illegal drugs, doping agents)
- Environment protection (pesticides, hormons)
- analysis of food products (pl. micotoxins, prions, biogen amins, alkaloids)
- basic researches in physiological sciences

Basic terms

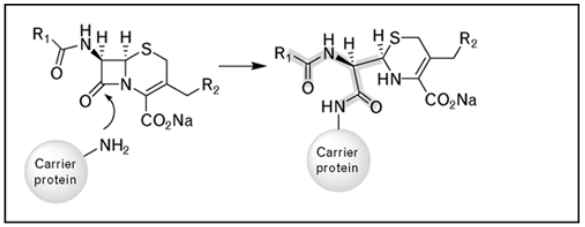
- Immunoassay:
  antigen + antibody → immunocomplex → signal
- „Analyte” → what is measured (antigen or antibody)
- Epitope = antigen determinant
- Monoclonal –polyclonal antibody

Immunoassays are analytical methods based on the reaction between antigen and antibody



antigen

Susbstance that provokes antibody production (usually large particles, eg.: virus, bakterium, pollen, antibody, cell, tissue, protein of foreign origin)

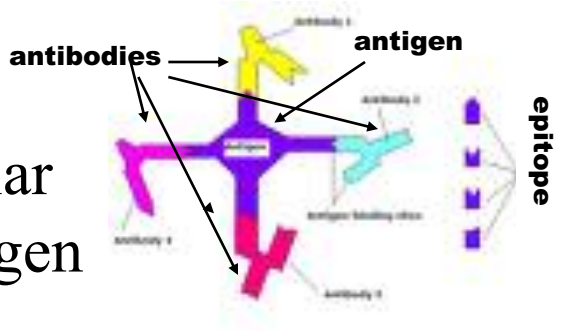


hapten

Small molecule, only capable of inducing antibody production if bound to a large molecule (carrier) (eg. drug molecules)

epitope

The part (molecular group) of the antigen that binds to the antibody



antibody

Produced by the immune system, helps the eliminiation of the antigen from the body by binding to it

Polyclonal - monoclonal

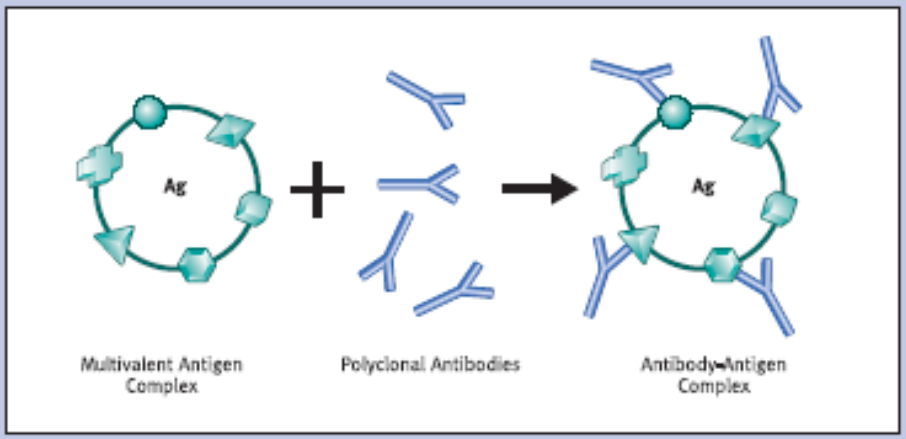


FIGURE 1-2 Multiple antigen specificities of polyclonal antibodies

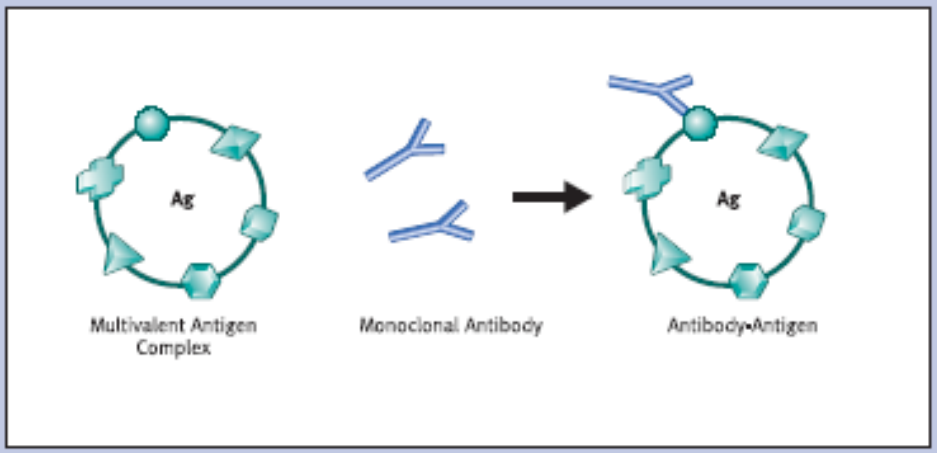


FIGURE 1-3 Uniform specificities of monoclonal antibodies

Specific features of antibodies – characteristics of the analytical methods

Very specific binding
(an antibody can only bind to one specific antigen)

⇒ **Selective**

Very high number of different antigens can be bound (approx. 10^{11} different antigens can be recognized)

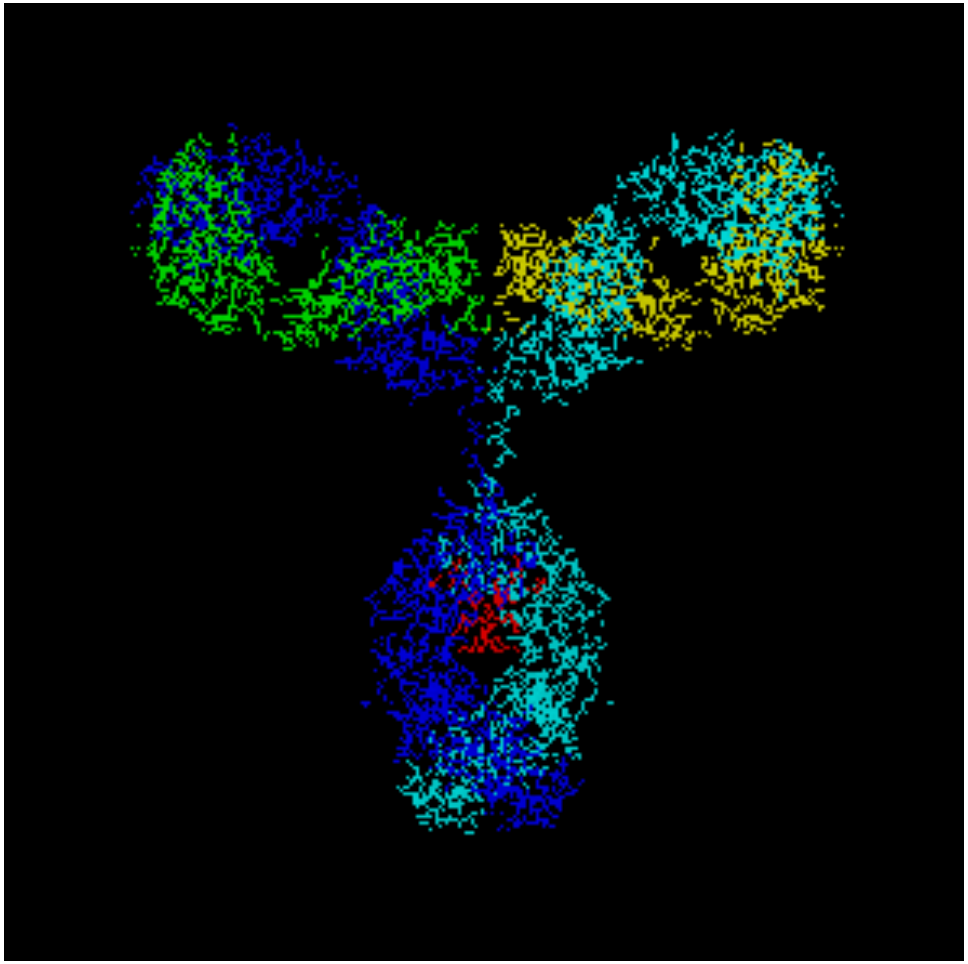
⇒ **Wide variety of substances can be measured**

Very strong binding with the specific substance

⇒ **Sensitive**

Antibody

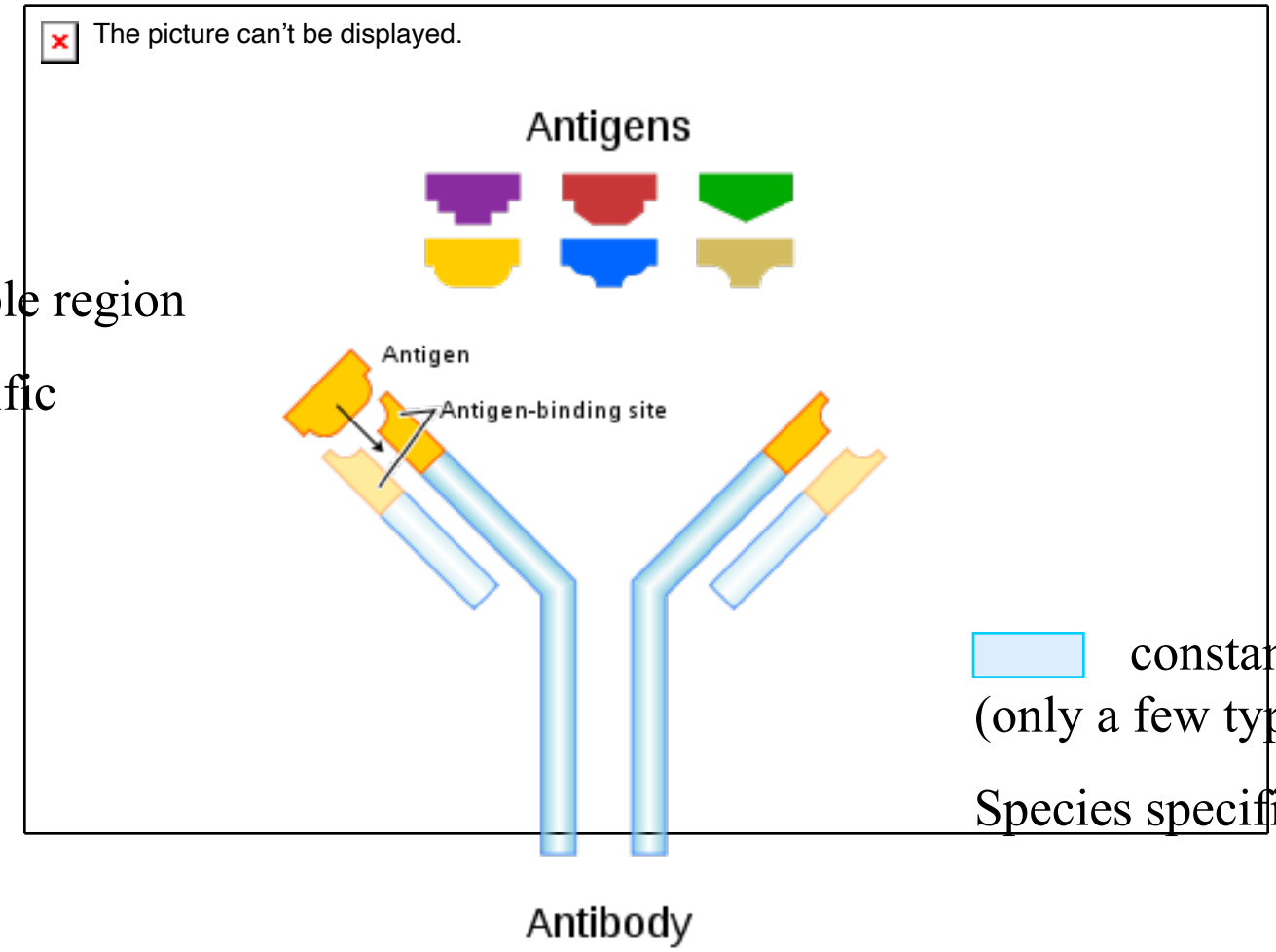
- Specific protein molecules - immunoglobulins
- Y shaped
- Two heavy and two light chains



Antigen-antibody complex

variable region

antigen specific



constant region
(only a few type exists)

Species specific

Immunological laboratory methods

- Separation-technical methods: ELFO, ImmunELFO, immunfixation, immundiffusion

- Optical methods: nephelometry, turbidimetry

  direct immunoassay: detection of soluble colloidal immuncomplexes (mg/l)

- Immunanalytical methods – indirect immunoassay: μg/l – ag/l (10^{-18} - attogramm)

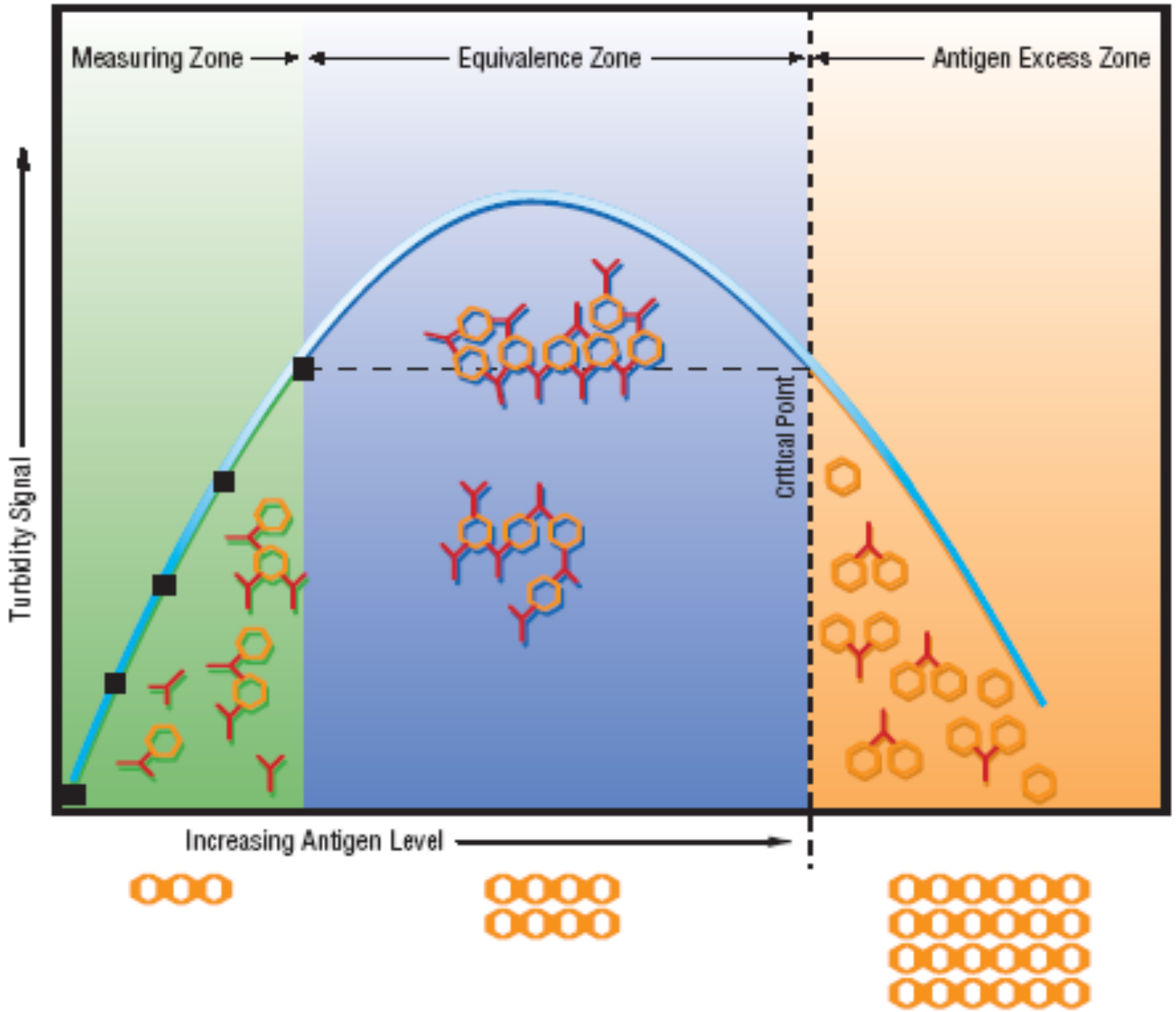
  enzyme - ELISA,

  radioactive – RIA

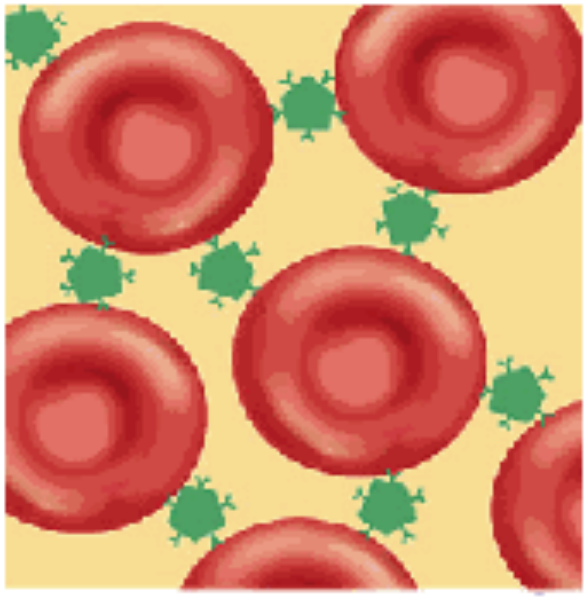
  fluorescent – FIA

  luminescent - LIA

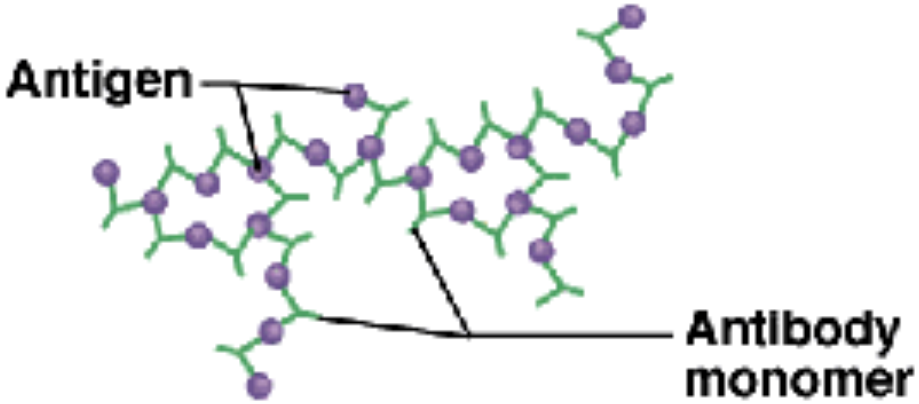
Heidelberger Curve



Agglutination and Precipitation



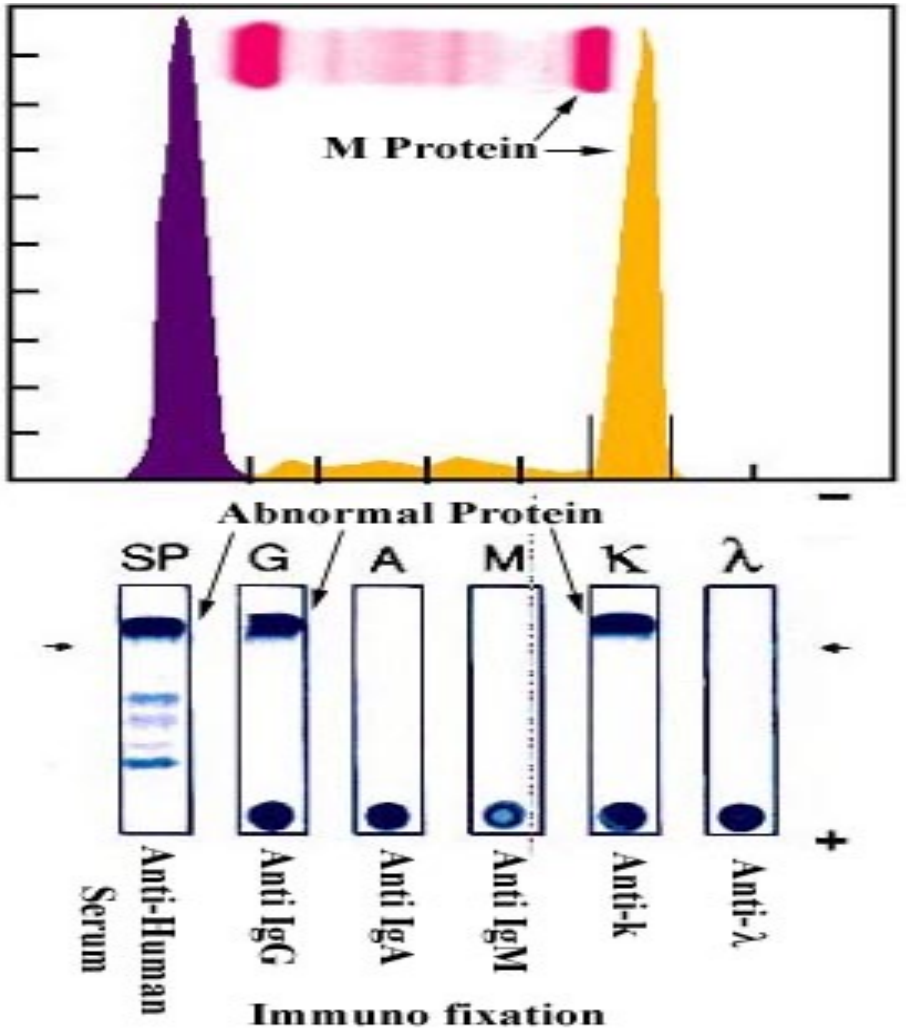
Agglutination by IgM



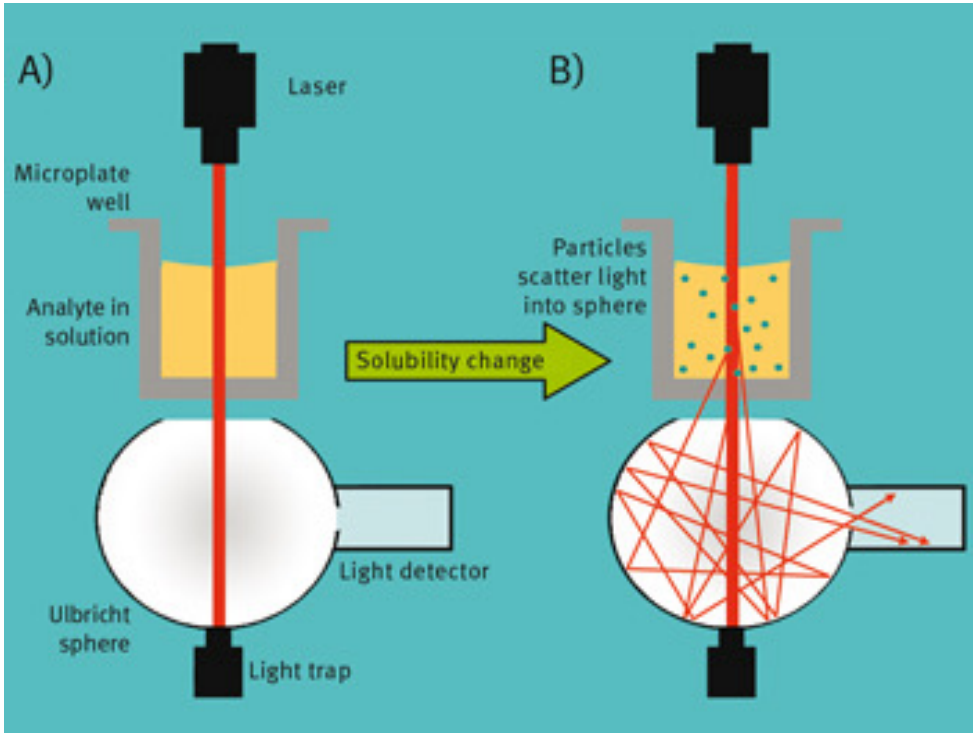
Precipitation of Antigen-Antibody Complex

Immuno fixation

- **Immunelectroforesis: characterisation of paraprotein.**
- **When an abnormal band appears on the serum ELFO we perform immunELFO with different antibodies against Ig subclasses and light chains.**



Nephelometry

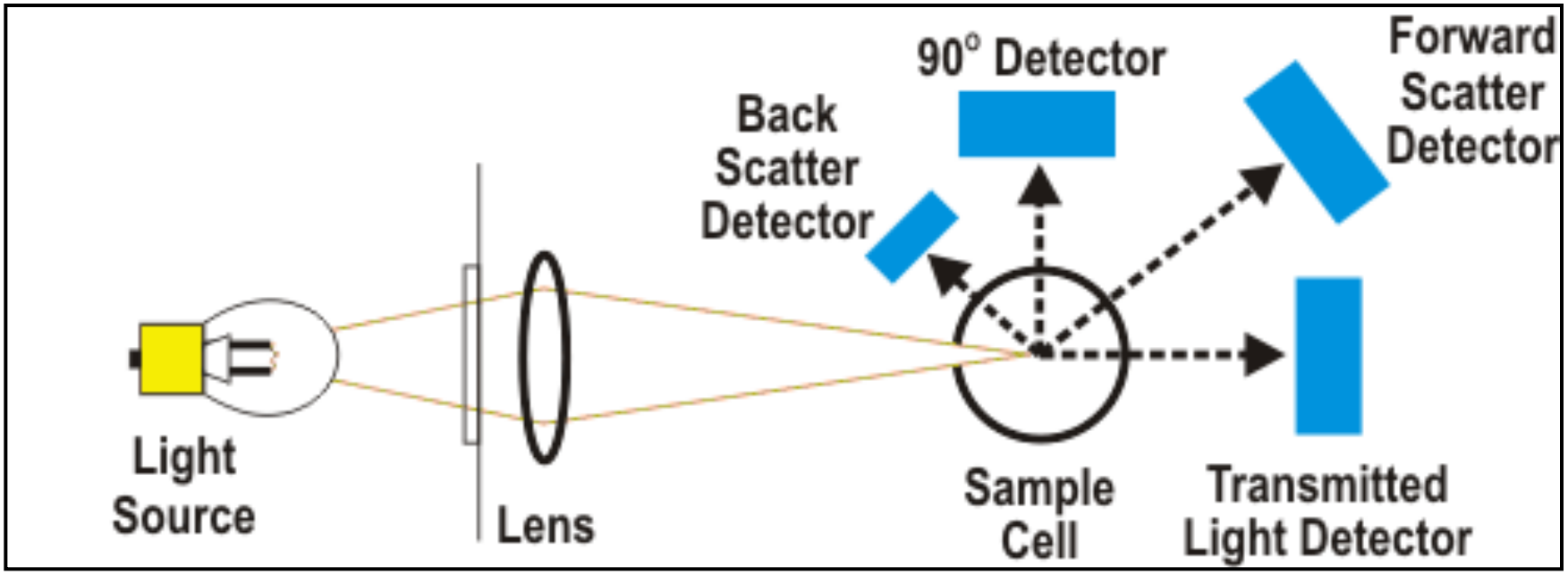


It is sued to determine the levels of several blood plasma proteins: IgG subclasses

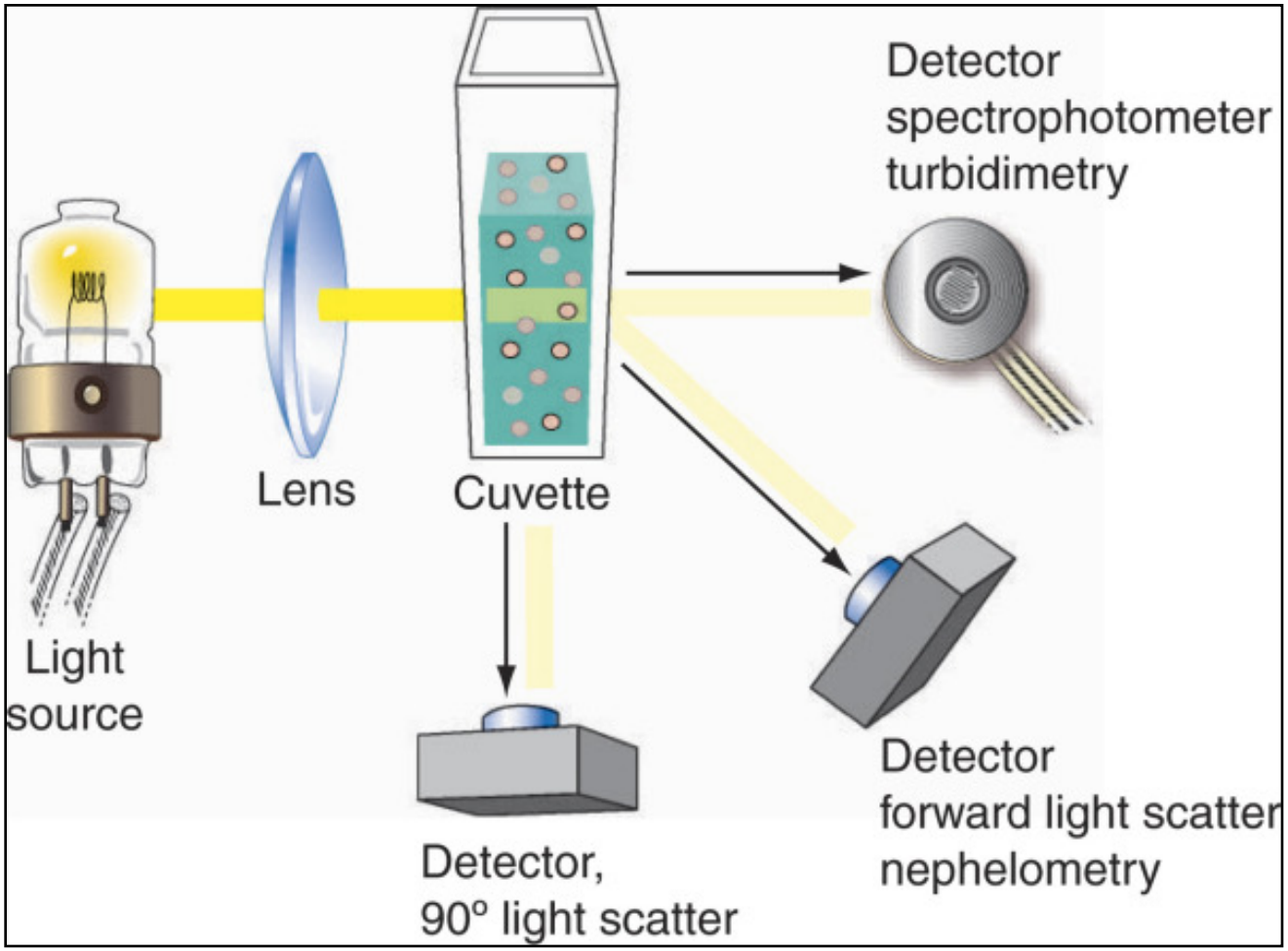
It is based on the principle that a dilute suspension of small particles will scatter light (usually a laser) passed through it rather than simply absorbing it. The amount of scatter is determined by collecting the light at an angle (usually at 30 and 90 degrees).

Schematic diagram of the measurement principle of the NEPHELOstar: A clear solution with minimal scattering results in low signal (A). A solution with particles scatters light and results in higher signal (B).

Turbidimetry – more detectors



Nephelometer – light scattering
Turbidimetry – light emission measurement



Signal detection:

- Signal generation with a molecule which is proportional to the immune reaction

- Labeling methods: - radioactive signal,
  - enzyme - substrate
  - light emission (e.g. fluorochrome)

- Coupling to antigen or antibody

The labeling method:

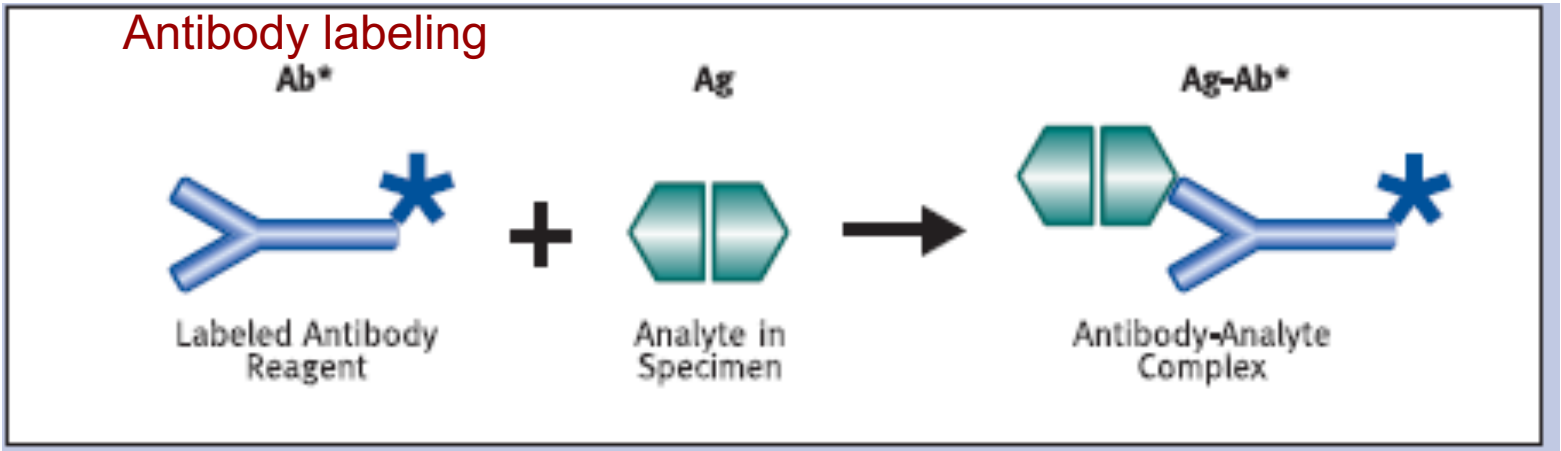


FIGURE 1-5 Labeled antibodies allow detection of antigen/antibody complexes in immunoassays

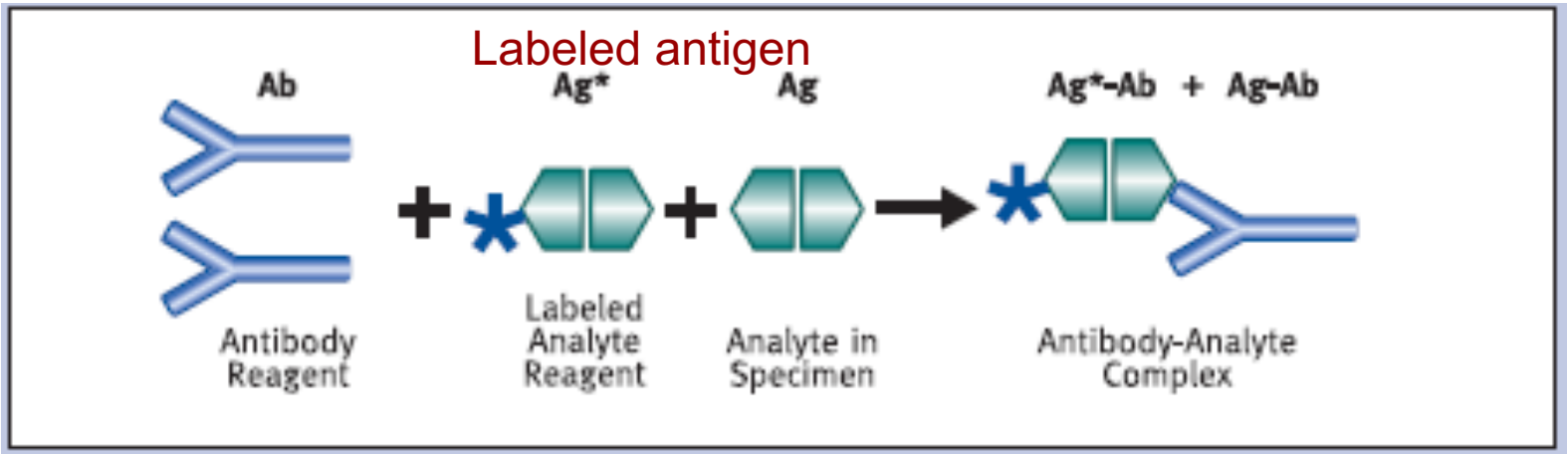


FIGURE 1-6 Labeled antigen also allows detection of antigen/antibody complexes in immunoassays

Types of immunoassays:

- Non-competitive
- Competitive

- Homogenous
- Heterogenous

One-step competitive assay

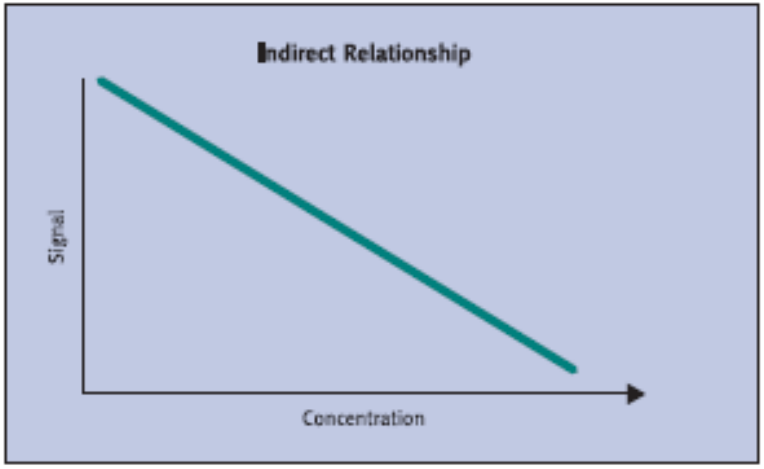


FIGURE 1-7
Amount of antigen is indirectly related to the amount of label (signal) in competitive formats

- The amount of analyte is inversely proportional to the signal

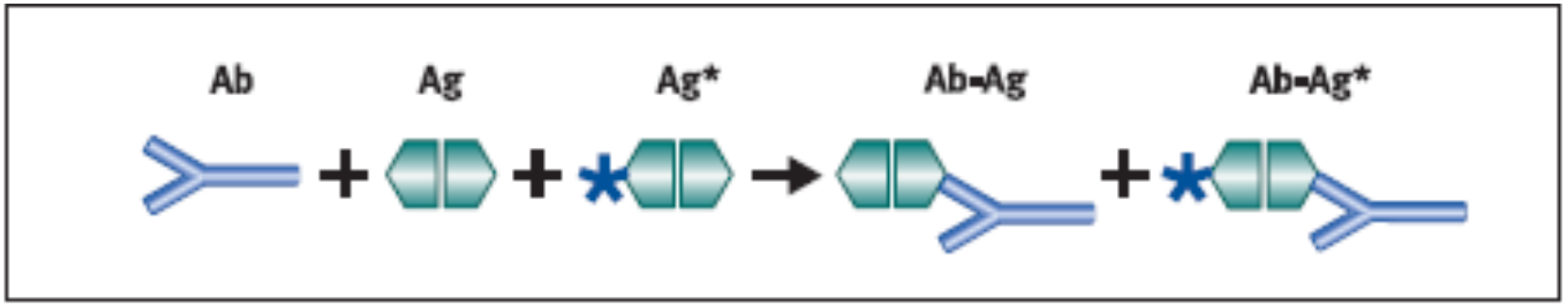


FIGURE 1-8 One step competitive immunoassay

Two-step, competitive assay

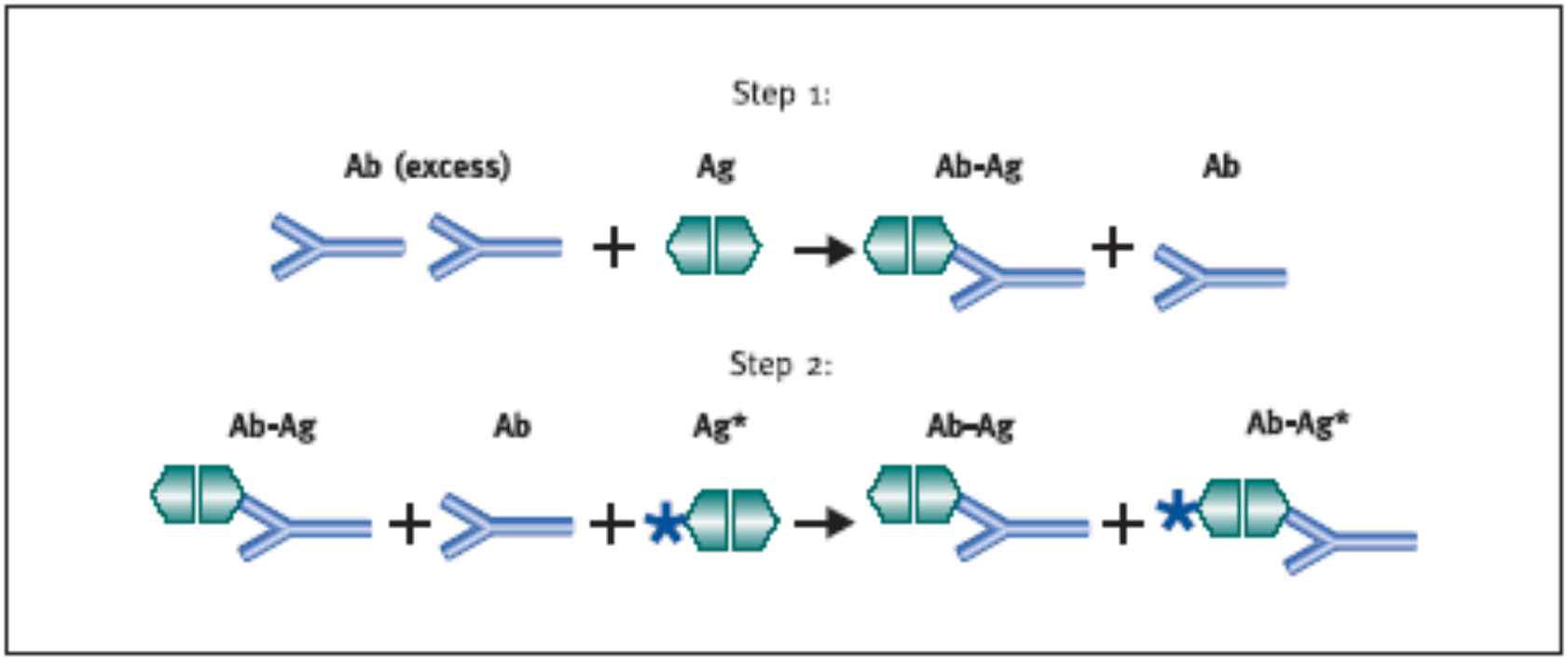


FIGURE 1-9 Two step competitive immunoassay

Non-competitive sandwich method

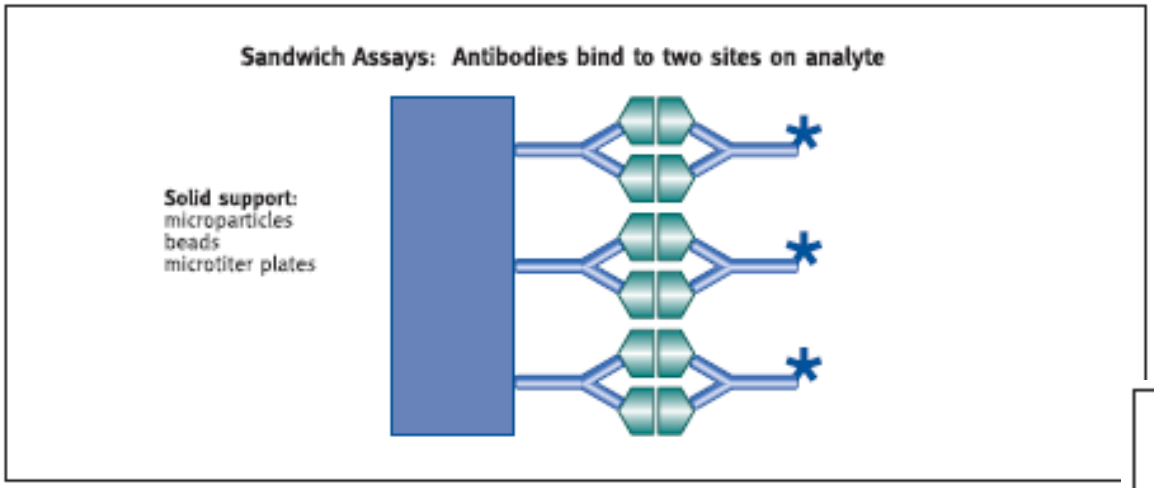


FIGURE 1-10 Noncompetitive sandwich method of immunoassay

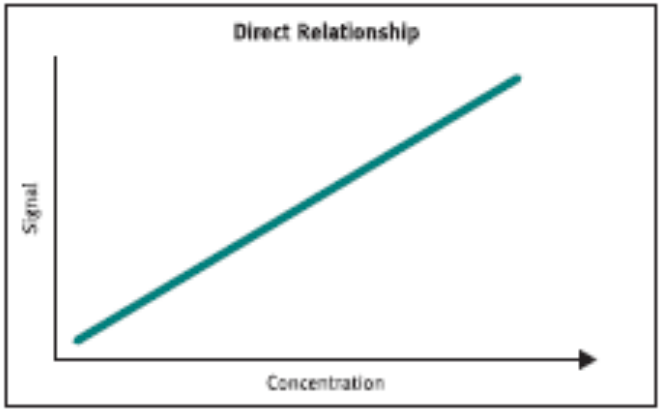


FIGURE 1-11 Amount of antigen is directly related to the amount of label (signal) in competitive formats

- Highest sensitivity and specificity
- The amount of antigen is directly related to the signal

Homogeneous and heterogeneous immunoassay

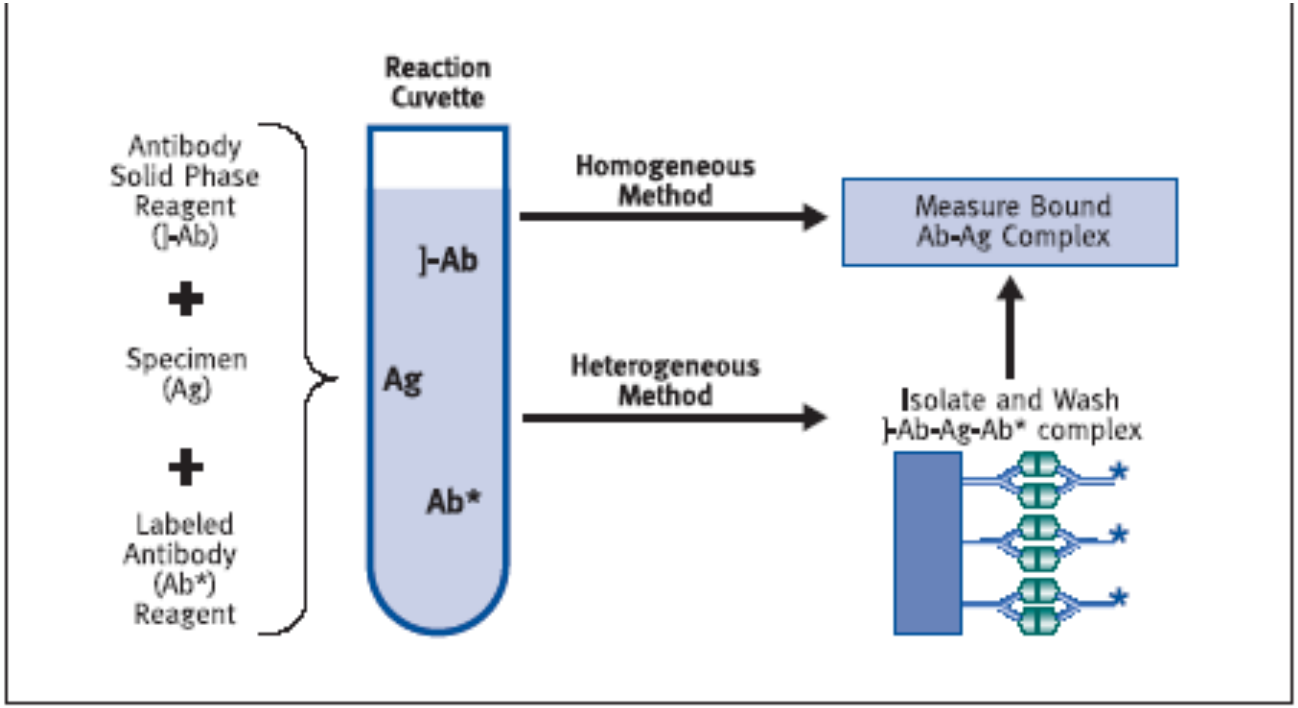


FIGURE 1-12 Homogeneous and heterogeneous immunoassays

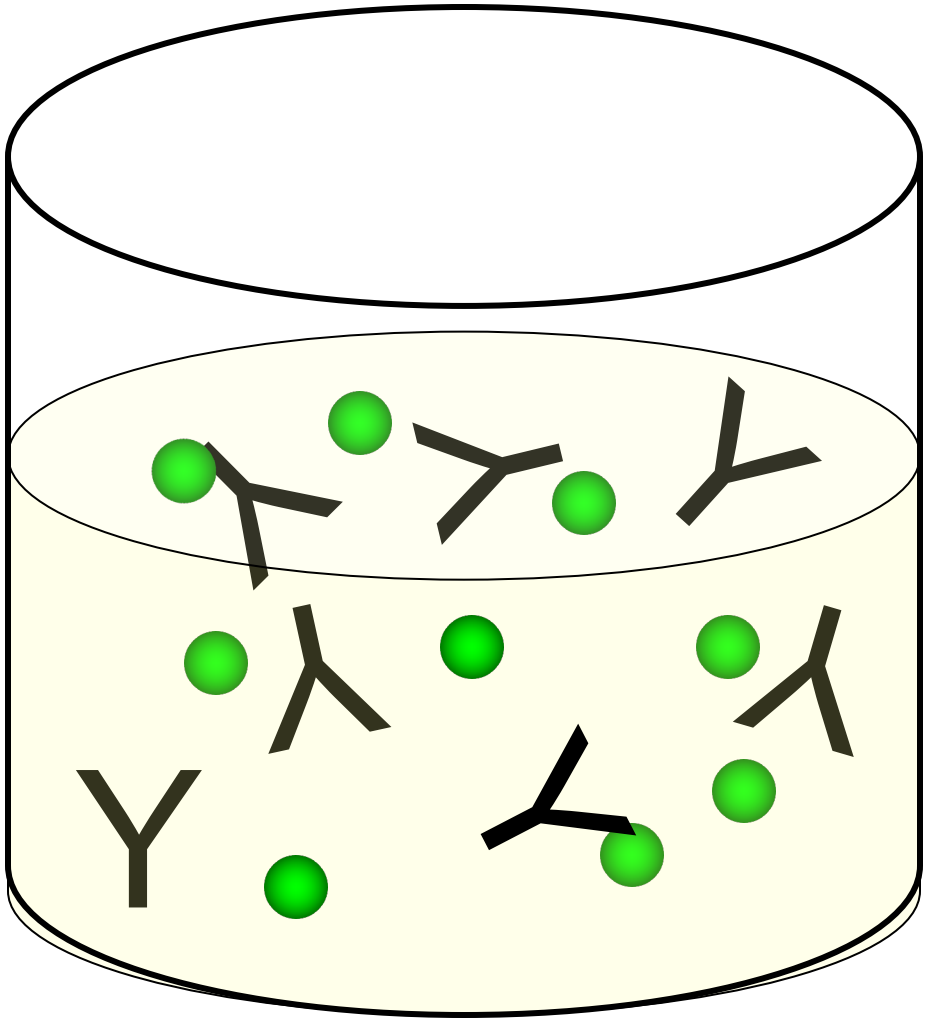
Ha az antigén-antitest komplex mérés előtti szeparálása szükséges → heterogén assay-ről beszélünk

Ha nincs szeparálás a méréshez → homogén assay

Homogenous immunoassay I.

Animáció/Slide Show üzemmódban

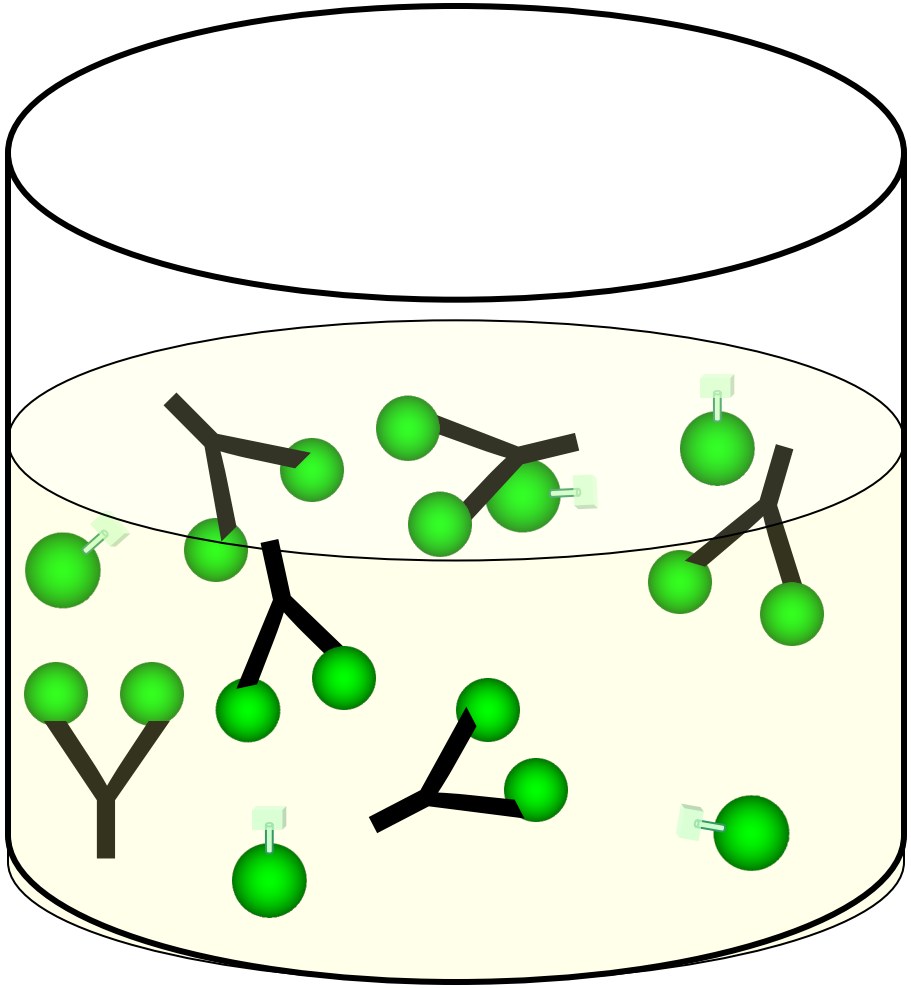
- ANTIGEN containing sample
- Addition of antibody
- Incubation



Homogenous immunoassay II.

Animáció/Slide Show üzemmódban

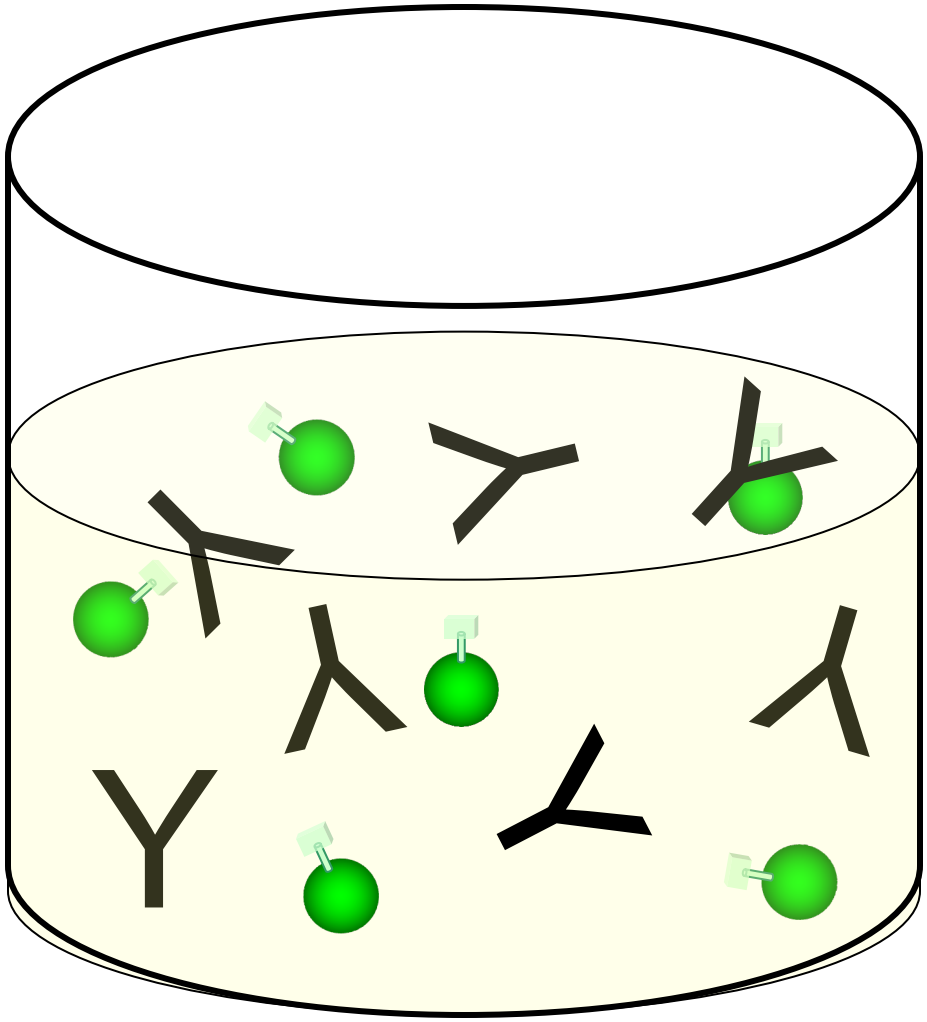
- Antibody bind to the antigens in the sample
- Addition of labelled antigen
- The labelled antigen binds to the free binding sites of the antibody
- Little signal



Homogenous immunoassay III.

Animáció/Slide Show üzemmódban

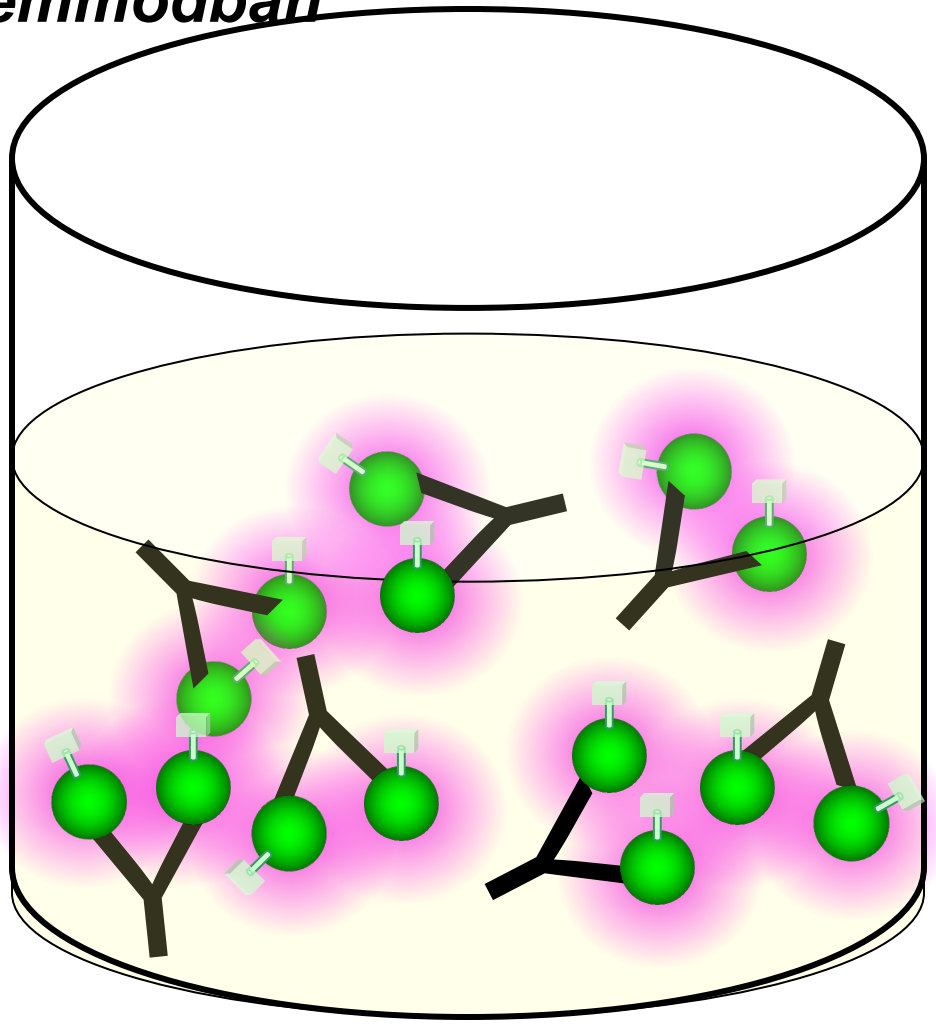
- No ANTIGEN in the sample
- Addition of antibody
- Incubation
- Addition of labelled antigen



Homogenous immunoassay IV.

Animáció/Slide Show üzemmódban

- Antibody binds to the labelled antigen
- The binding cause signal



Detection technologies:

- **RIA: Radioimmunoassay** – 1960.

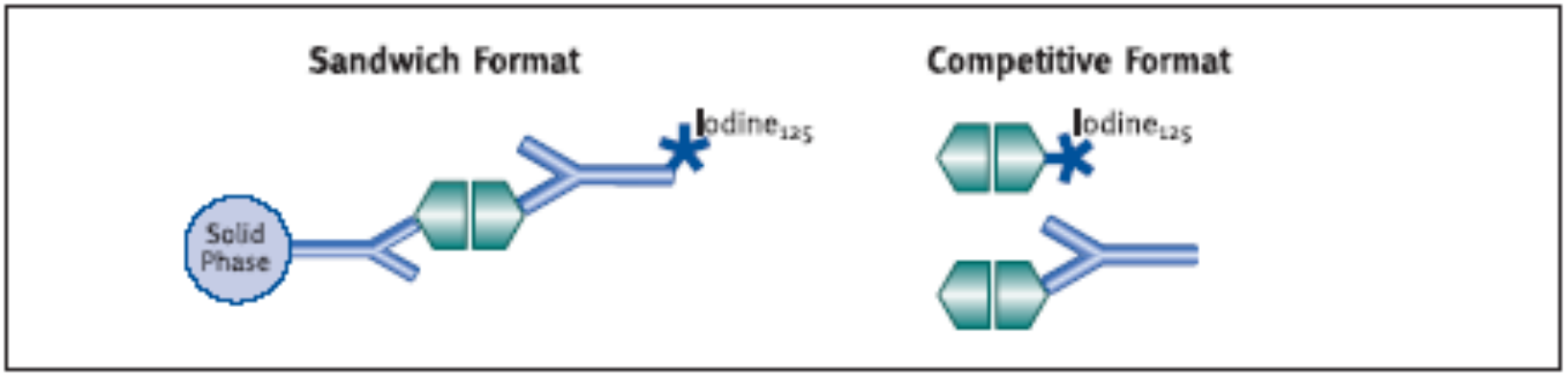


FIGURE 2-1 Radioimmunoassays (RIAs) utilize a radioactive isotope label

- **EIA: Enzyme-immunoassay** – HRPO, Phospahtase, β-galactosidase

→ Photometric detection of light or colour reaction

- **Fluorescent polarized immunoassay** - FPIA

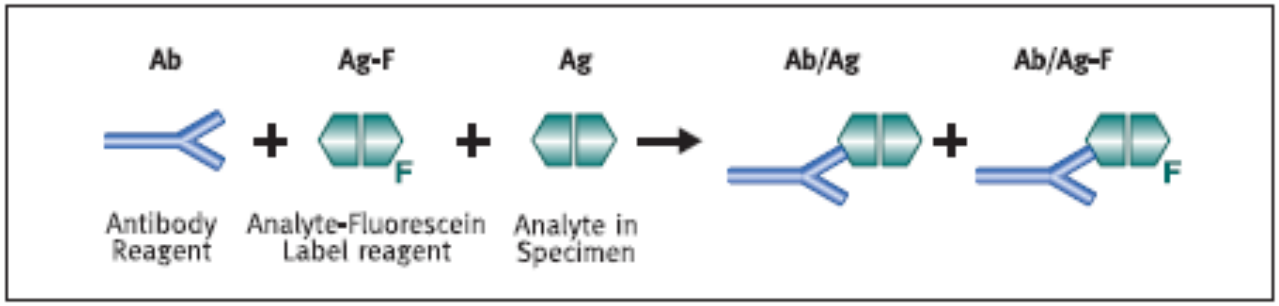


FIGURE 2-2 Competitive fluorescence polarization immunoassay (FPIA)

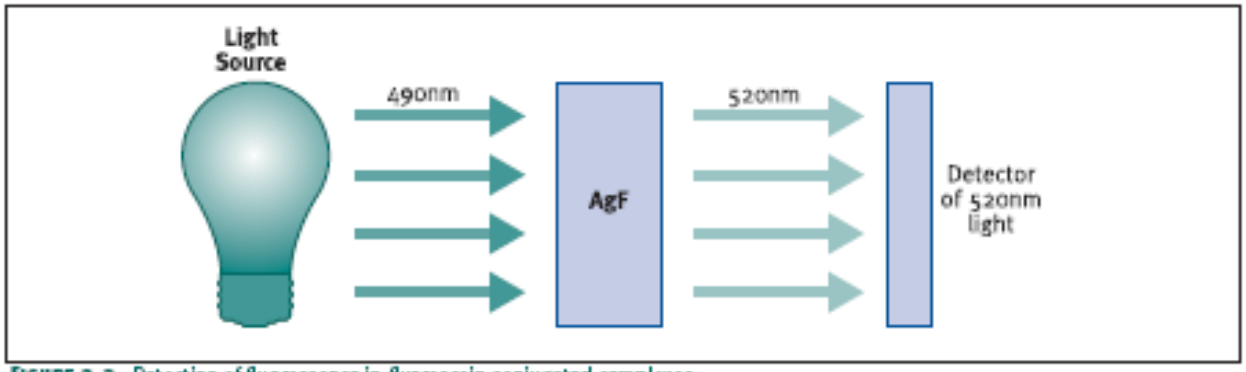


FIGURE 2-3 Detection of fluorescence in fluorescein-conjugated complexes

Homogeneous, competitíve immunoassay,

One-step reaction

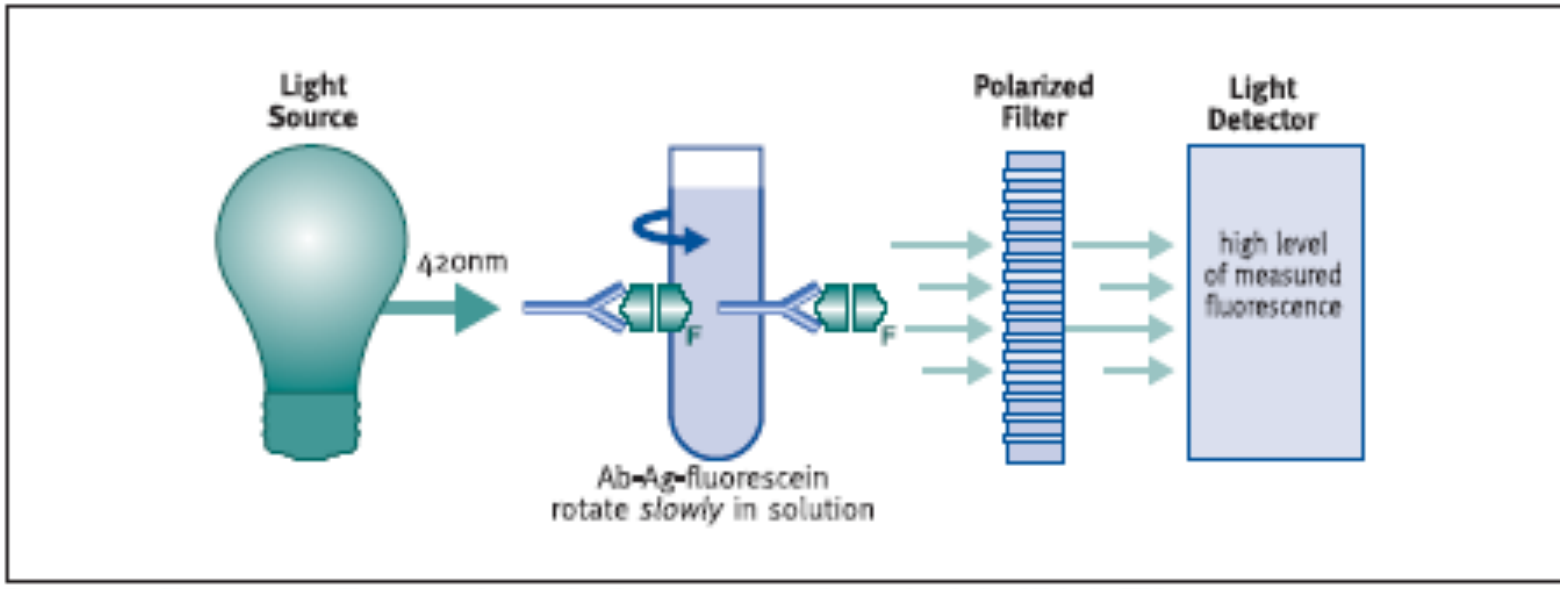


FIGURE 2-4 Measurement of large complexes using fluorescence, rotation, and polarized light in FPIA

Detection of small molecules: toxicology, drugs, hormones

Abbott TDx system → clinical automation

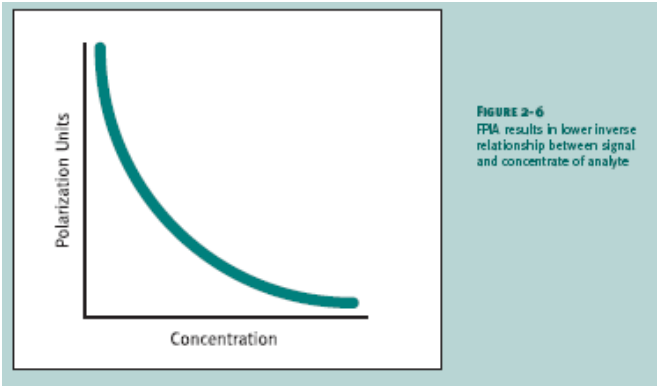


FIGURE 2-6
FPIA results in lower inverse relationship between signal and concentrate of analyte

- **Microparticle enzyme-immunoassay** - MEIA

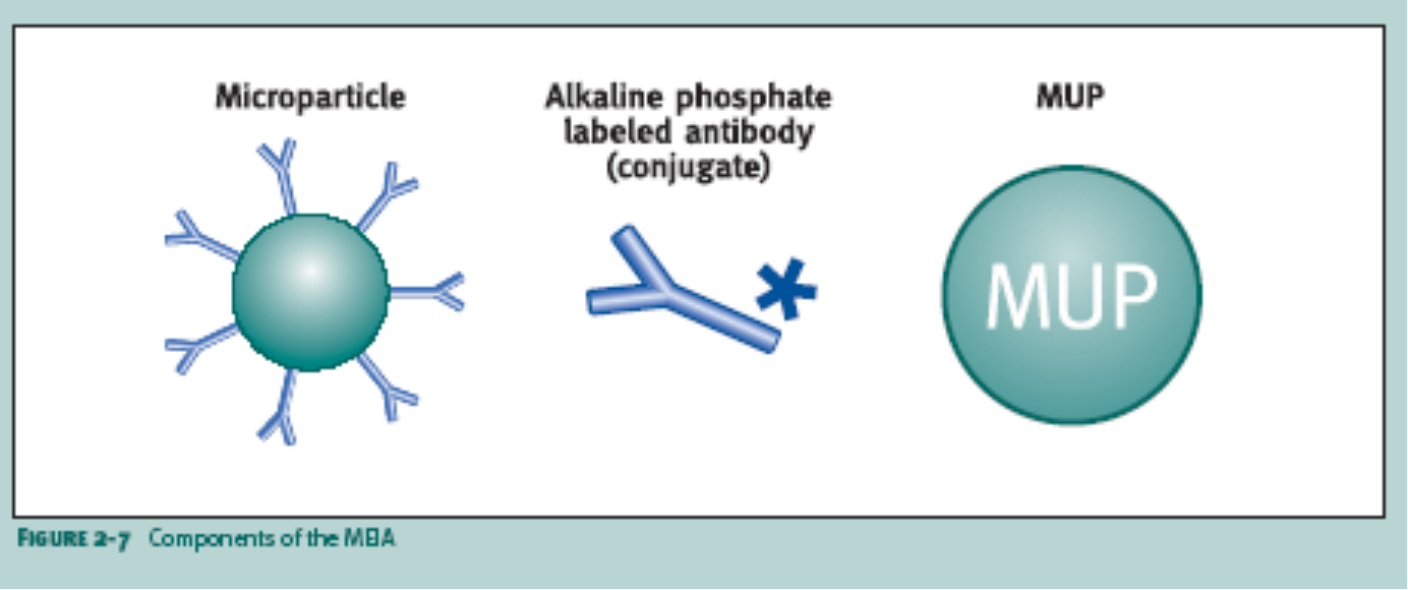


FIGURE 2-7 Components of the MEIA

MUP: Fluoreszcens 4-Methyl Umbelliferone Phospahate

Heterogen, non-competitíve immunoassay
Cllinical autoation for large molecule detection:

Heart, tumor markers, metabolites hepatitis, thyroid tests

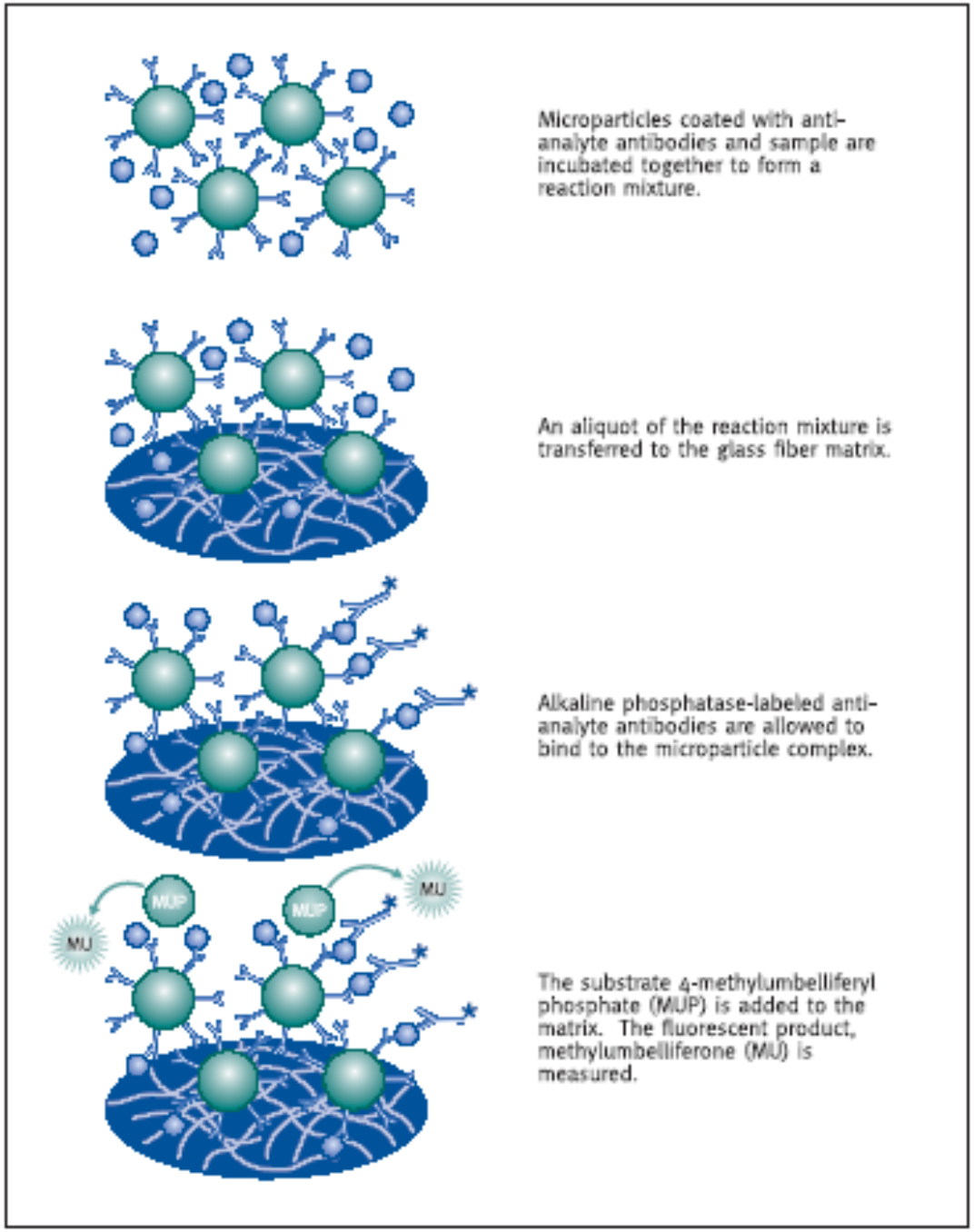


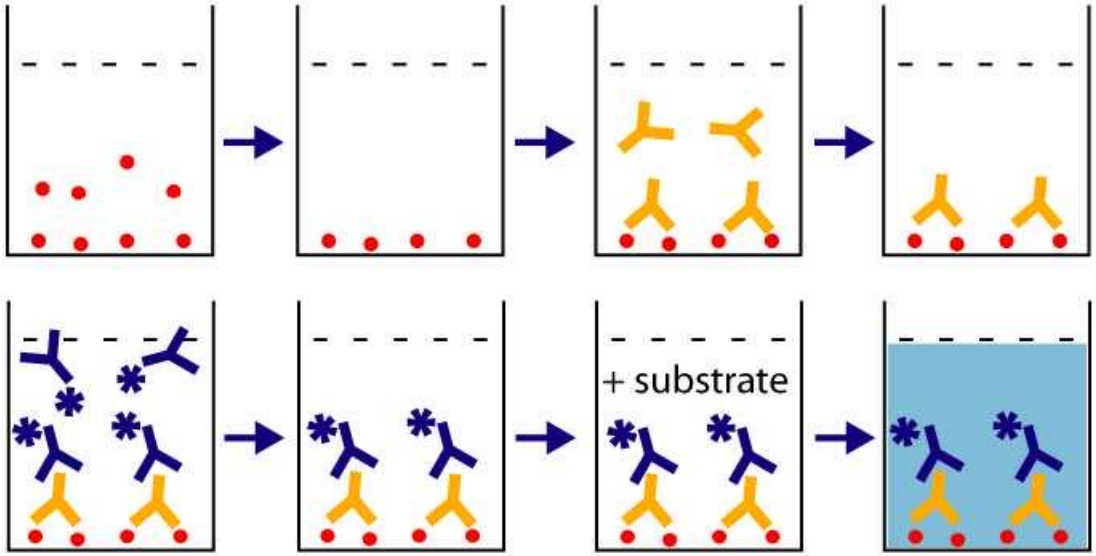
FIGURE 2-8 Process of the MEIA method

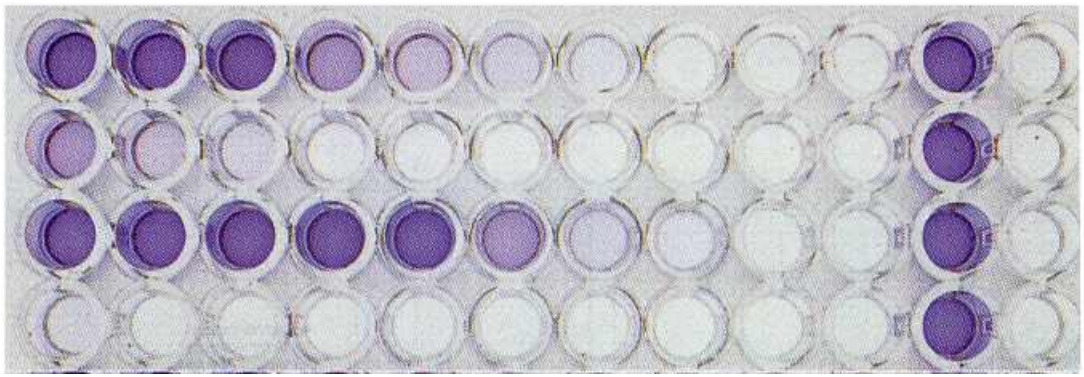
Példa heterogén fázisú kompetitív immunoassay-re

http://www.sumanasinc.com/webcontent/animations/content/ELISA.html

ELISA – Enzyme Linked Immunosorbent Assay

ELISA 5. – „simple indirect”



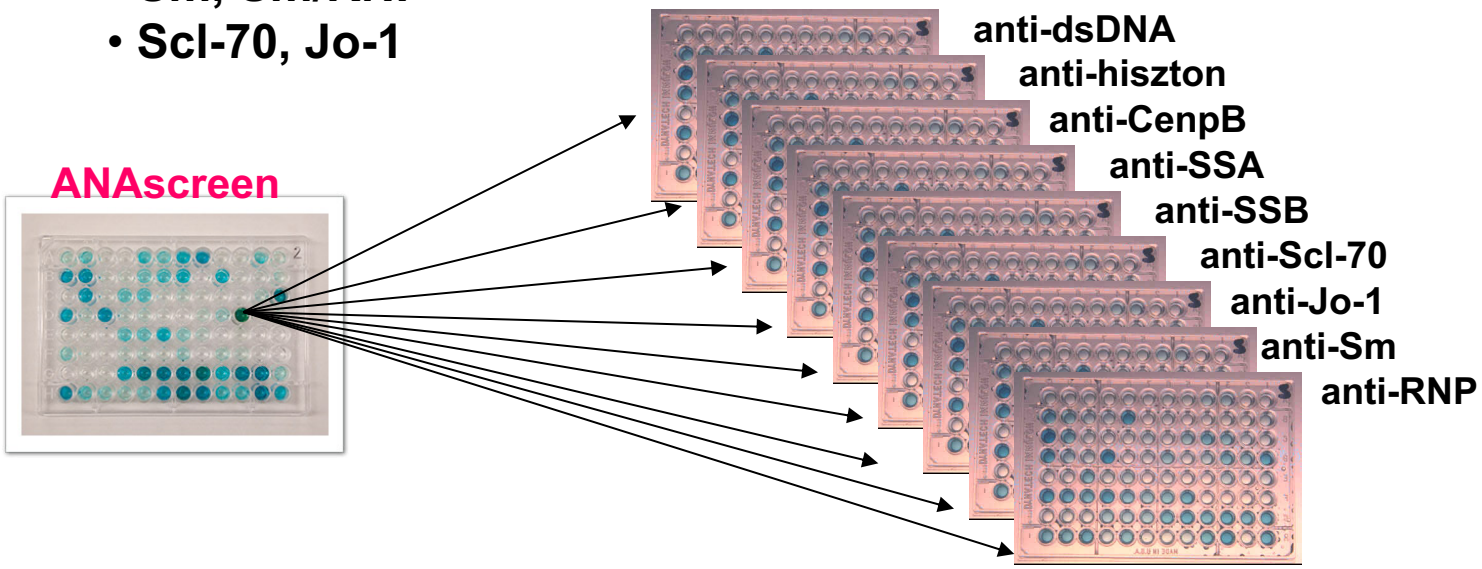


ANAcreen on microplate ELISA

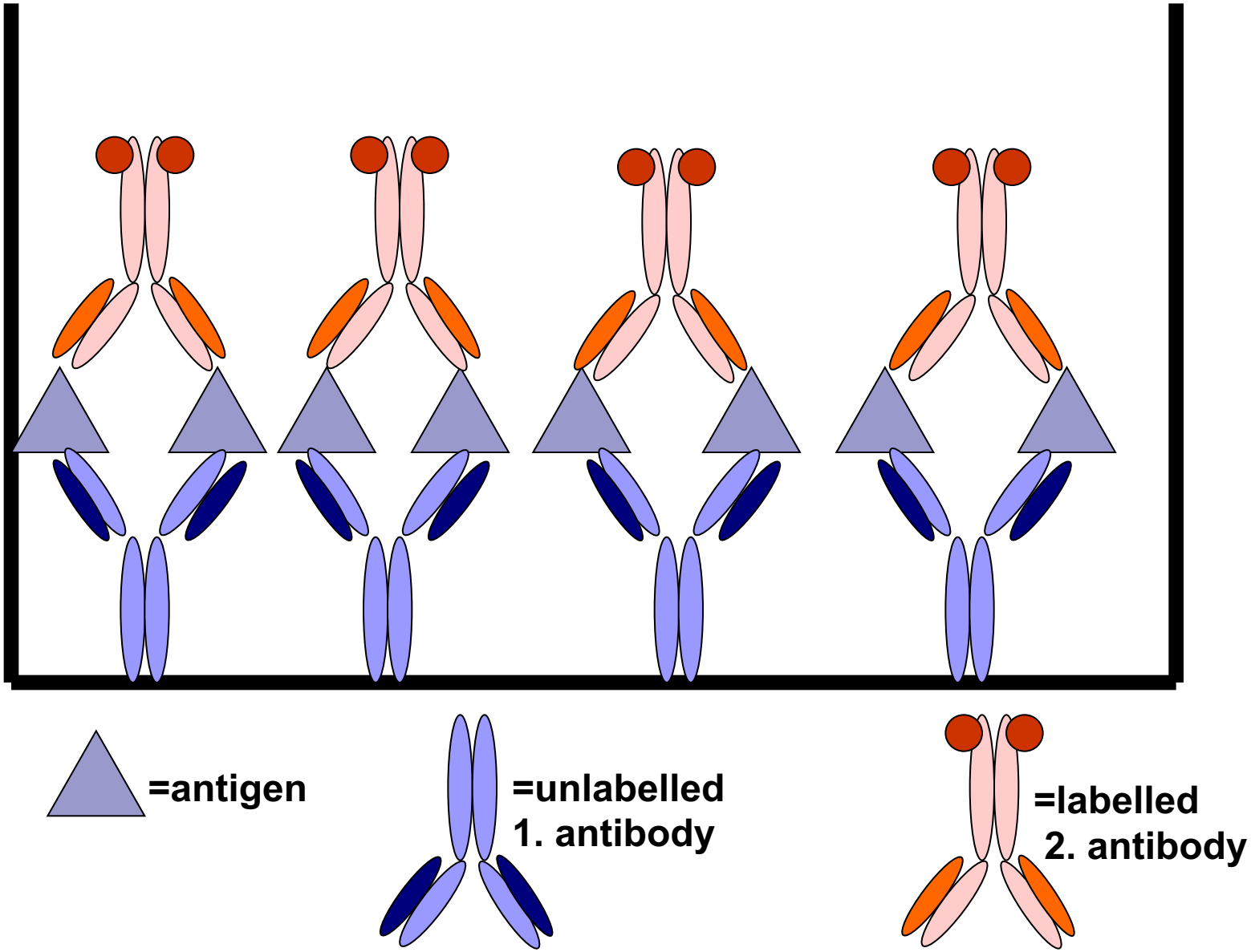
Az ELISA lemezt Hep2 sejtek magjából kivont antigének keverékével érzékenyítik:

- **dsDNA**
- **hiszton**
- **centromer**
- **SSA/Ro, SSB/La**
- **Sm, Sm/RNP**
- **Scl-70, Jo-1**

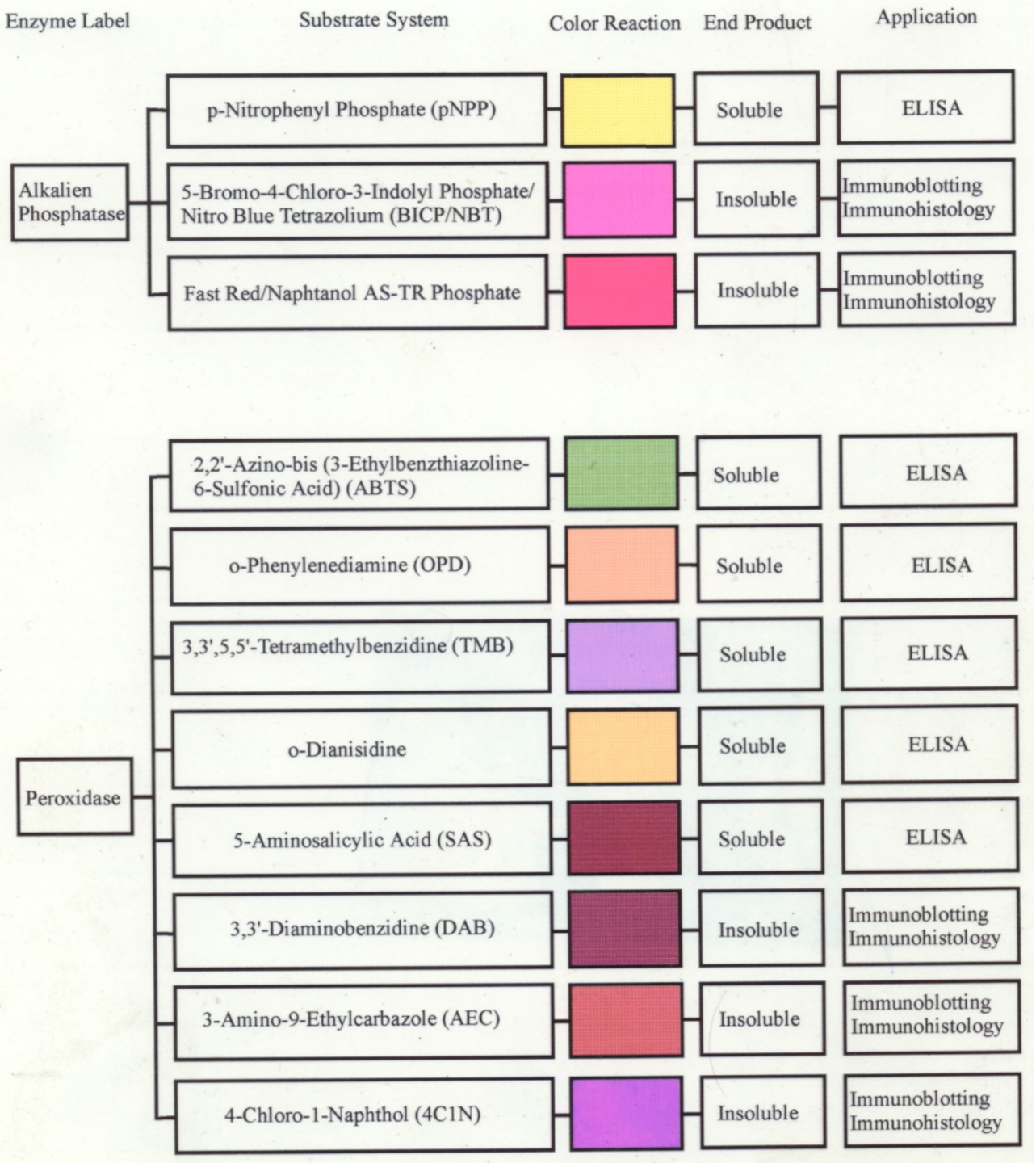
Pozitivitás esetén egy-egy antigénnel fedett külön ELISA lemezeken un. „kifejtő” vizsgálatok következnek



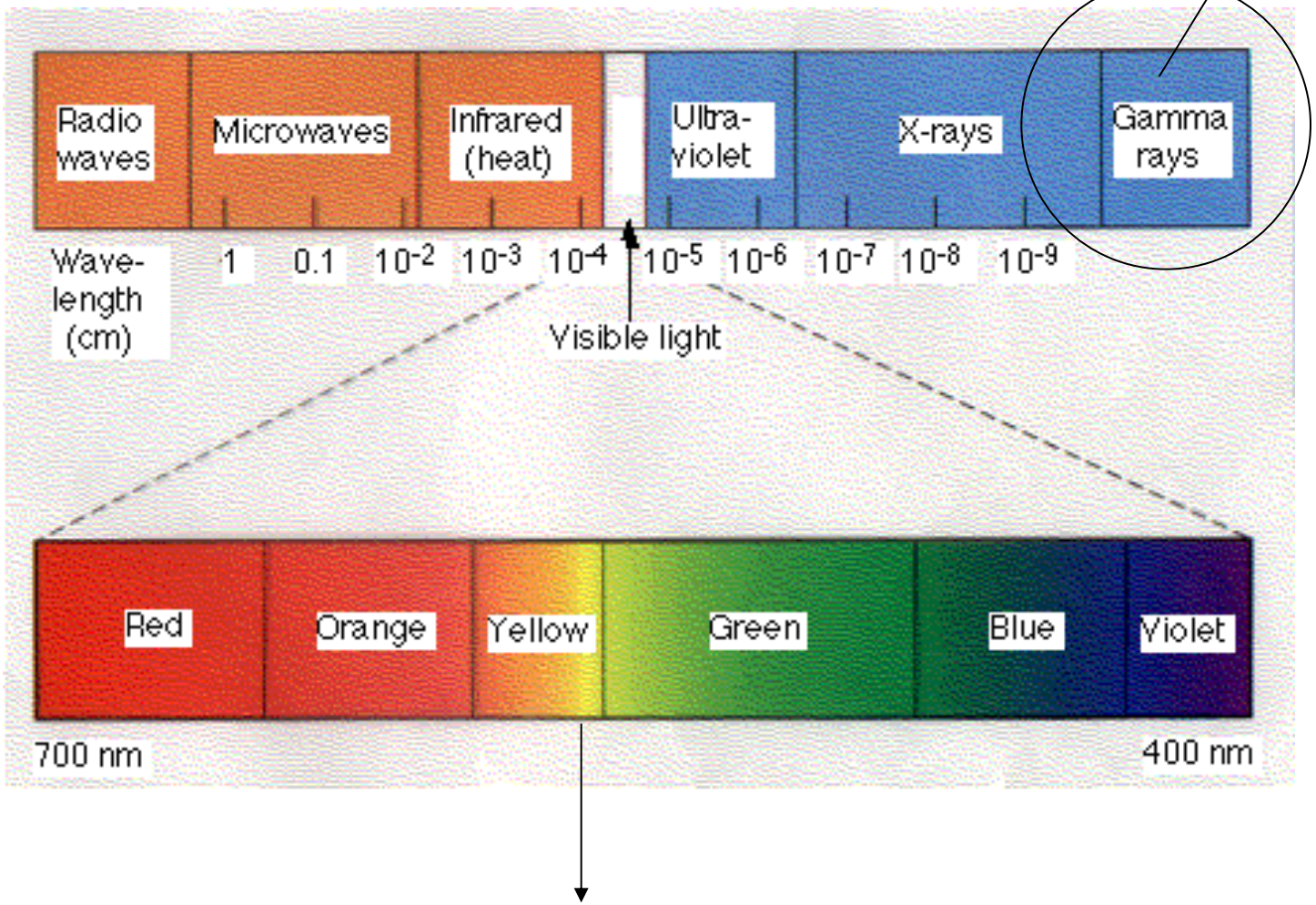
ELISA – sandwich



Substrate systems



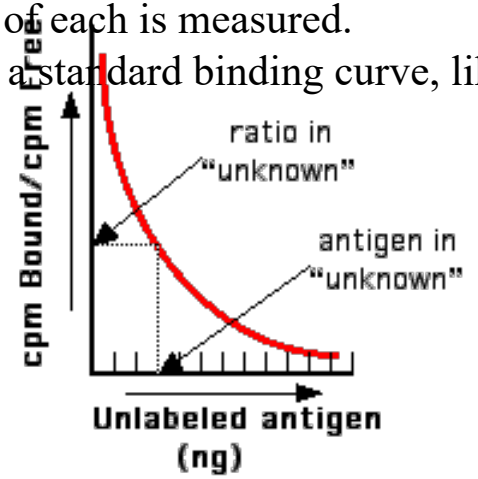
RIA

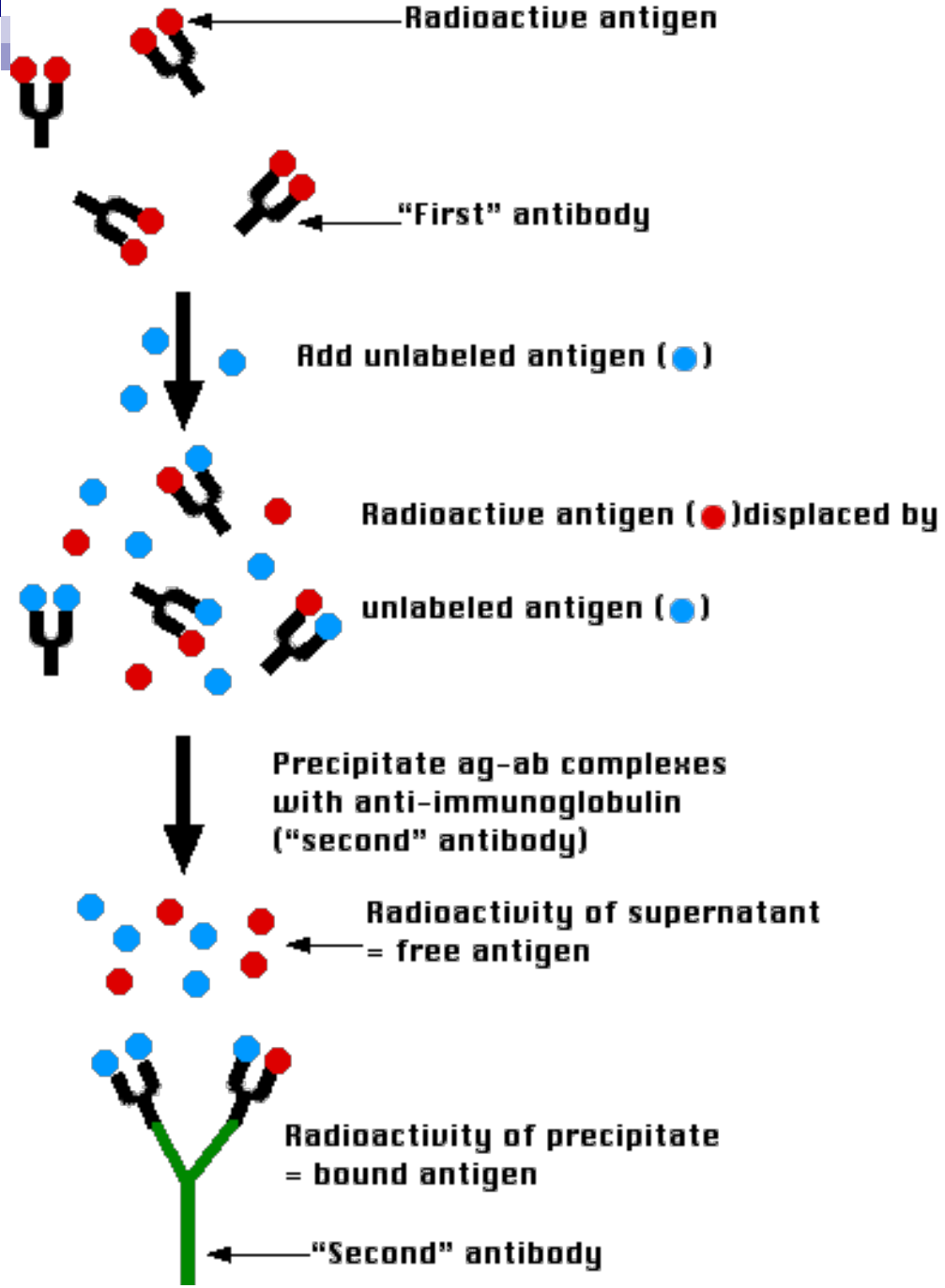

Radio waves
Microwaves
Infrared (heat)
Ultra-violet
X-rays
Gamma rays
Wave-length (cm)
1
0.1
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
Visible light
Red
Orange
Yellow
Green
Blue
Violet
700 nm
400 nm

ELISA

Radioimmunoassay: RIA

- The technique was introduced in 1960 by Berson and Yalow as an assay for the concentration of insulin in plasma. It represented the first time that hormone levels *in the blood* could be detected by an in vitro assay.
- mixture is prepared of
- radioactive antigen
  - Because of the ease with which iodine atoms can be introduced into tyrosine residues in a protein, the radioactive isotopes ^{125}I or ^{131}I are often used.
- antibodies against that antigen.
- Known amounts of unlabeled ("cold") antigen are added to samples of the mixture. These compete for the binding sites of the antibodies.
- At increasing concentrations of unlabeled antigen, an increasing amount of radioactive antigen is displaced from the antibody molecules.
- The antibody-bound antigen is separated from the free antigen in the supernatant fluid, and
- the radioactivity of each is measured.
- From these data, a standard binding curve, like this one shown in red, can be drawn.





- Both ^{125}I or ^{131}I emit gamma radiation that requires special counting equipment;

- The body concentrates iodine atoms — radioactive or not — in the thyroid gland where they are incorporated in thyroxine (T4)

Detection of small molecules:

- [3H]oestradiol, progesteron, testosterone,
- aldosteron, cortisol, T3, T4, FSH, LH,

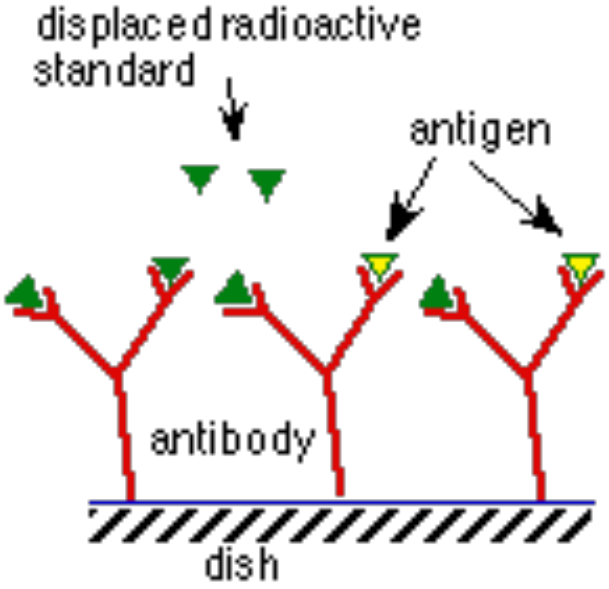
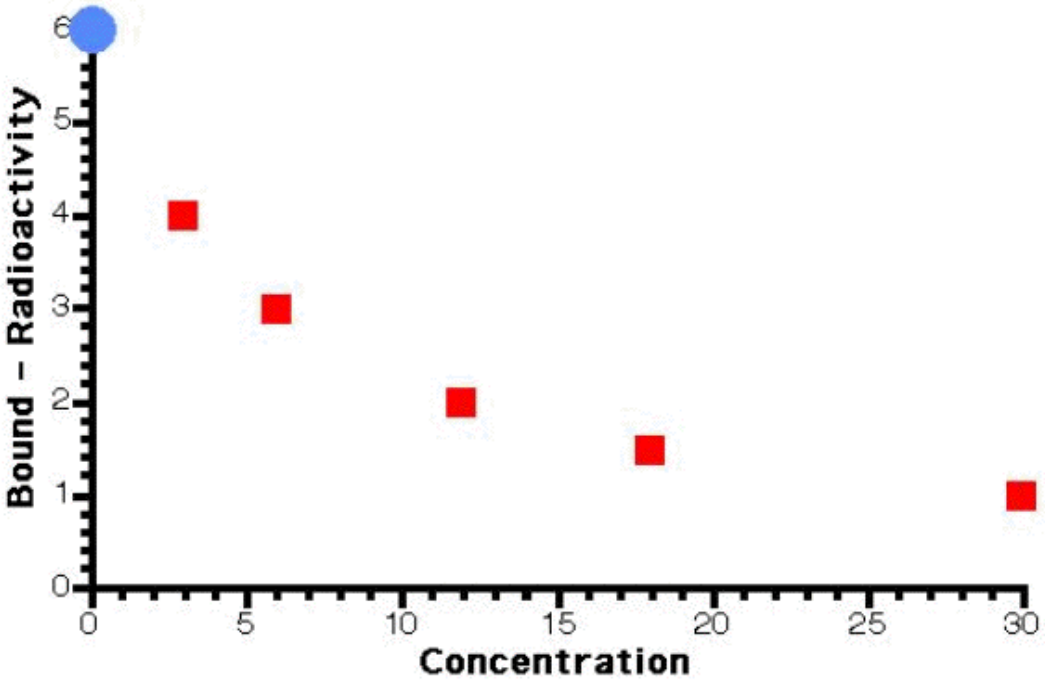
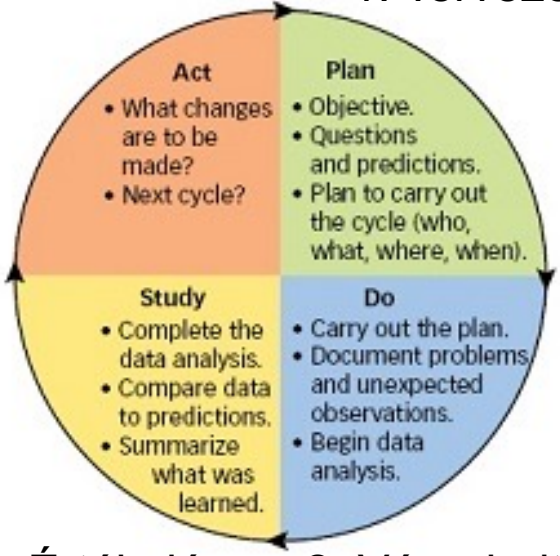


Figure 1: RIA



PDCA: plan–do–check–act/adjust = Deming ciklus

4. Változtatások 1. Tervezés



3. Értékelés 2. Végrehajtás,