

IMMUNOASSAY

A brief guide through its history, principles,
practice and future trends

Helena Fingerová

Palacký University Medical School, Olomouc, Czech Republic



IMMUNOASSAYS

- Highly specific *in vitro* tests that use antigen-antibody reaction to detect extremely low concentrations of a broad range of biologically important substances in blood and other body fluids
- Antigen-antibody reaction - known since the end of the 19th ct, precipitation in gel, agglutination or turbidimetry assays gradually developed until
- their potential has fully been appreciated since 1960 when higher sensitivity was achieved by
labeling one of the components

WHY?

Because the principle made possible to develop

- **simple**
- **precise**
- **sensitive** (nano- and picomolar range)
- **high throughput measurement**
of more substances than any other analytical technique

All immunoassay require the same **key reagents**

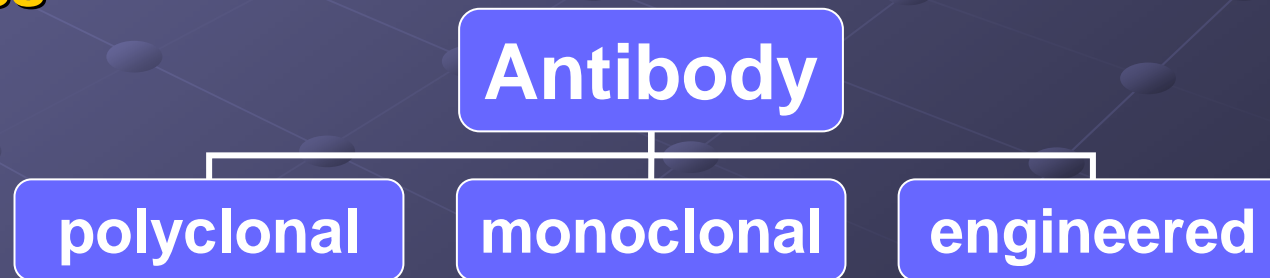
- One or more **antibodies** raised against epitopes believed to be specific to the analyte in question
- **A label (tracer)** producing a measurable signal
- **Calibrators** in a fluid (matrix) similar to the patient's sample

Antibody (antiserum)

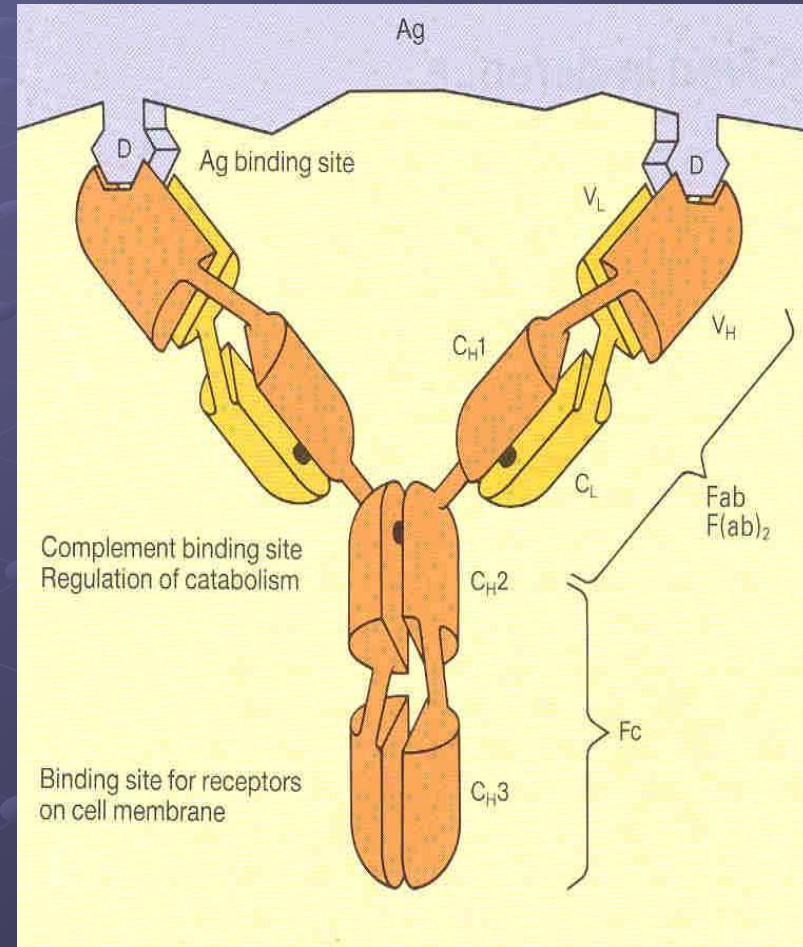
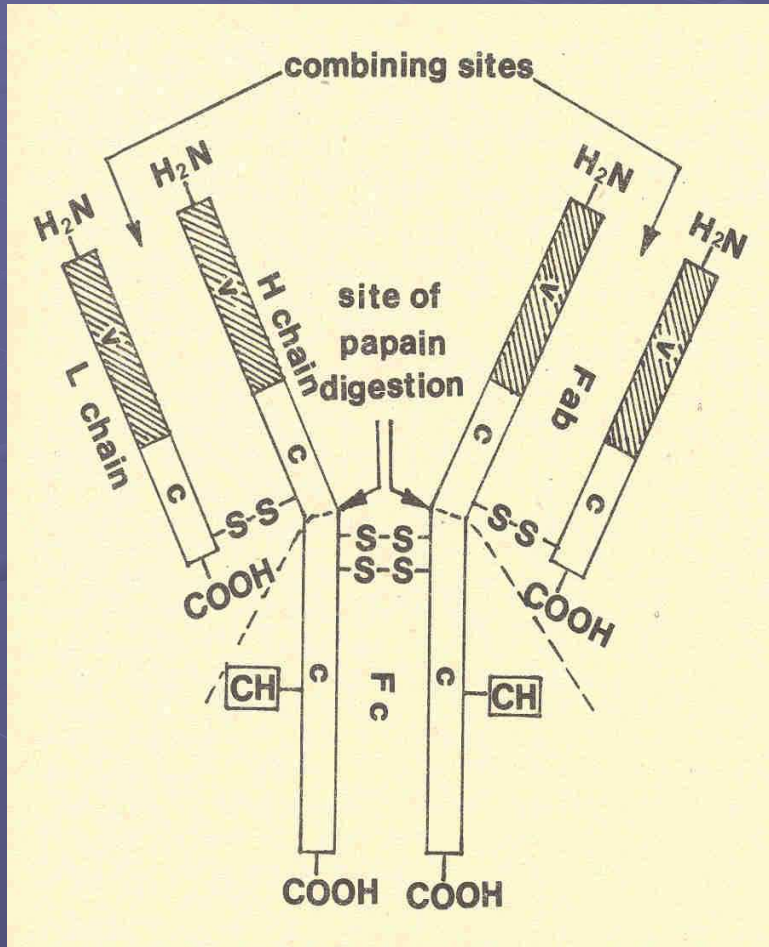
The antibody = immunoglobulin produced by the body in response to an „invading“ (foreign) substance as a part of **immune response**

Good antibodies possess high **specificity** and **affinity** for a specific antigen

The antibody used in immunoassay is usually of the **IgG class**

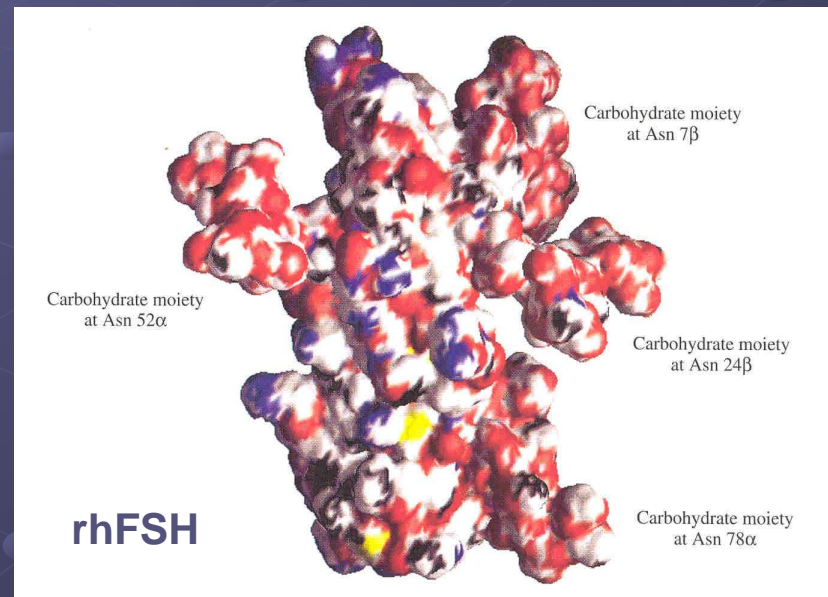
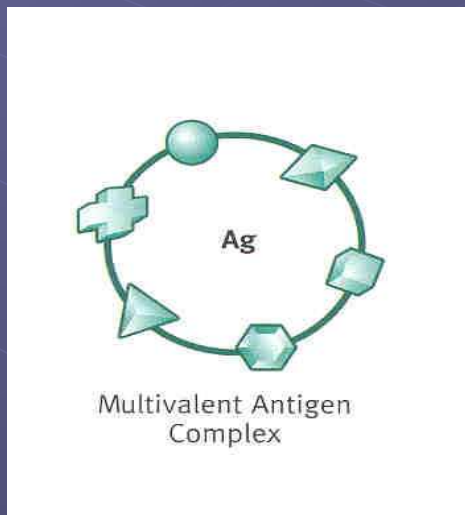


IgG structure



Natural Antigen

- Substance that **naturally elicit** immune response
- Usually a larger molecule (over 10 kD) with several **epitopes** (antigenic determinants)

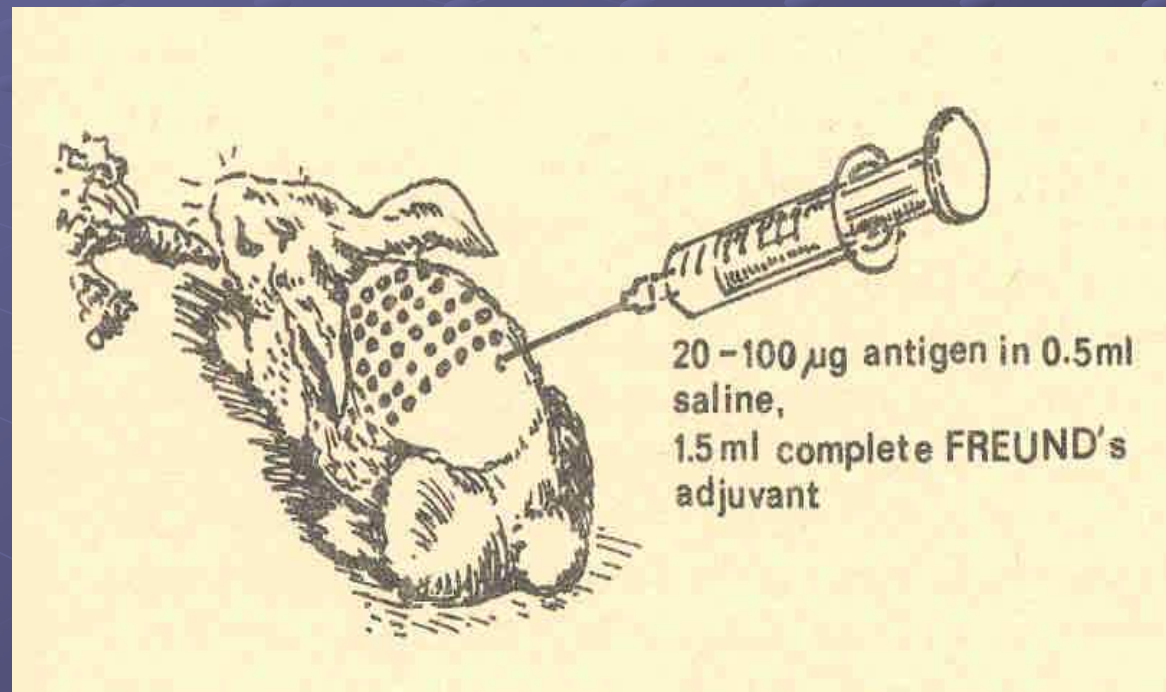


Conjugated haptens

- Smaller molecules (called **haptens**) are either weakly or not at all immunogenic
- To produce the antibody it is necessary to couple them to an **immunogenic carrier** (e.g. BSA, thyroglobulin..)
 - The optimal molar ratio (excess may range from 10 :1 to 80 :1) is important for production of good antisera

Polyclonal antibodies

- raised in animals (rabbits, sheep, goat...) by repeated immunization



- a mixture of antibodies which may bind to different epitopes of the immunogen with different avidities

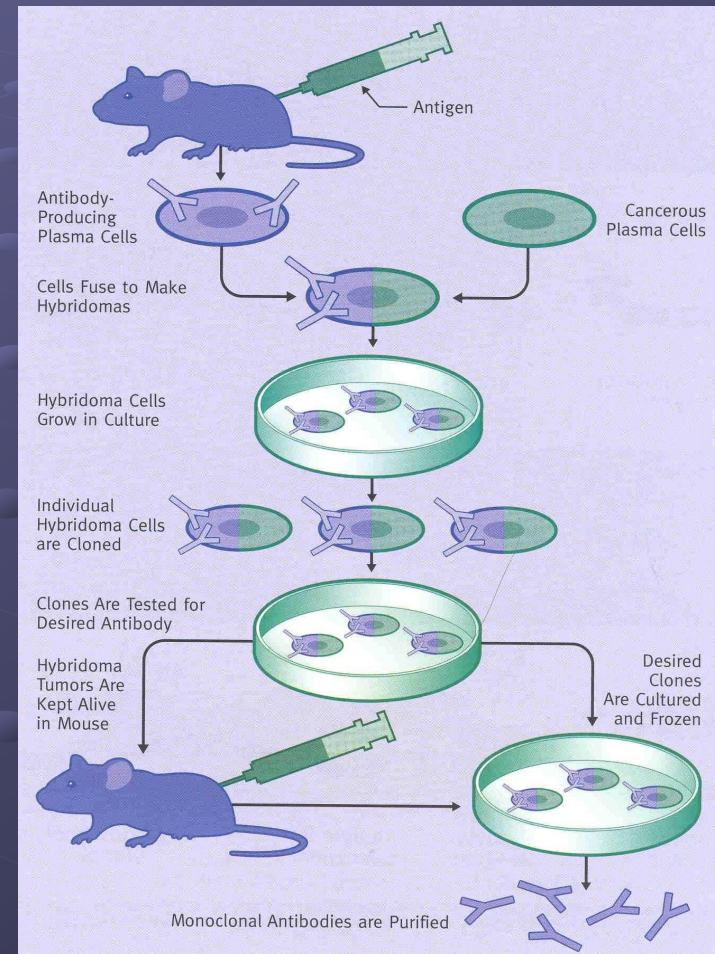
Double antibody

- Raised in **another species** to the primary antibody, e.g. sheep (goat, donkey...) anti rabbit (mouse, ...) IgG
 - added in much higher concentrations than primary antibody + normal rabbit (mouse,..) gamma globulin



Production of monoclonal antibodies

- Injecting an antigen into a host animal (typically a mouse)
- Isolating antibody-producing cells (B lymphocytes)
- Fusing immune cells to mouse myeloma cells
- Hybridomas are grown in culture and produce antibodies
- Selecting hybridomas that produce desired antibodies



Monoclonal antibodies

- derived from a **single cell line**, monoclonals are specific for a **single epitope** on a multivalent antigen
- hybridoma cell lines can produce the same antibody consistently and indefinitely, monoclonal antibodies **facilitated:**
 - manufacturing of immunodiagnostics
 - further development and automation of immunoassays



How does it work?

How does it work?



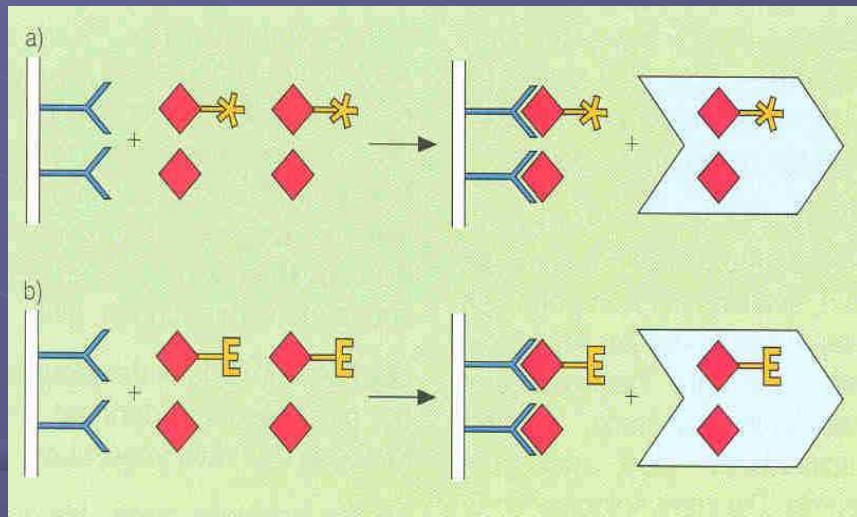
Photo: Z. Putz

DISCOVERY OF RIA

- In 1960 the first RIA for **insulin** (Berson & Yalow) used ^{131}I -insulin as a tracer, and gel filtration to separate the bound and free fraction
 - Paper was originally rejected by Science and J Clin Invest, but later accepted.
 - In **1977** R. Yalow, R. Guillemin and A.V. Schally shared **Nobel prize** „for the development of RIAs for peptide hormones“
- Principle: **competition** of unlabeled analyte in sample with fixed amount of radio-labeled analyte for **limited binding sites on a specific Ab**
- Probably **the most important advance** in biological measurement in the second half of the 20th century

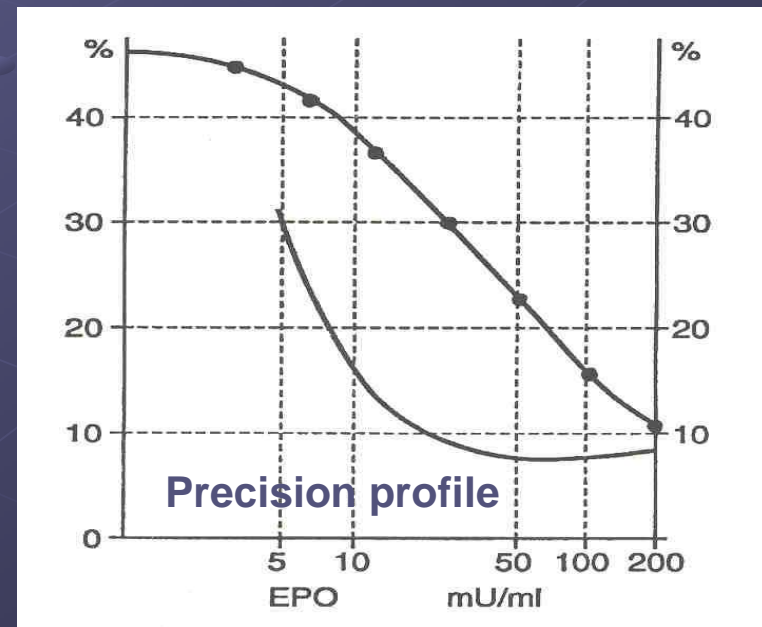
Competitive immunoassay

The less Ag in the sample,
the more **labeled Ag** can be bound by Ab



- a) Isotope label (RIA)
- b) Enzyme label (EIA)

Calibration curve



Competitive assay for antibody testing

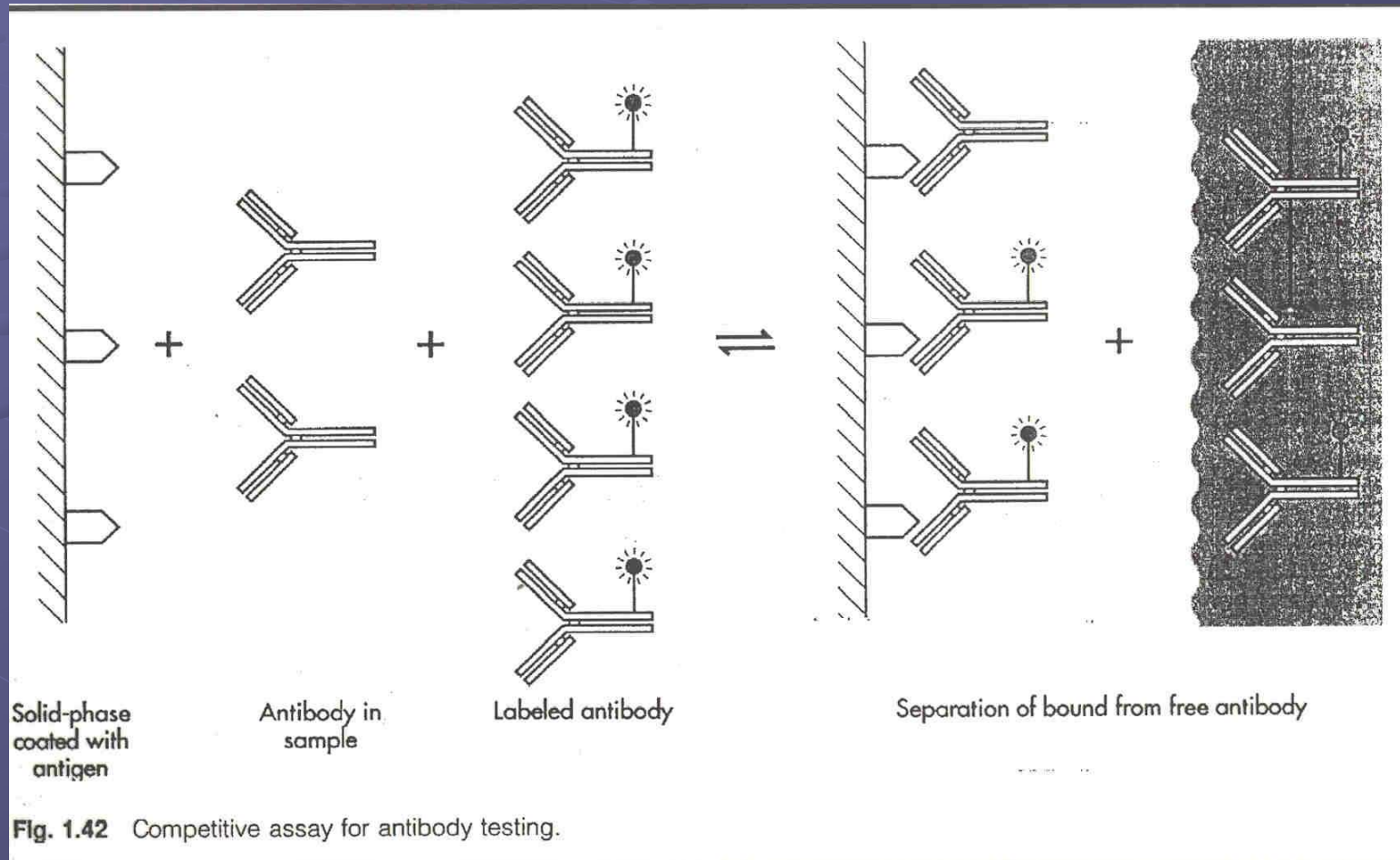


Fig. 1.42 Competitive assay for antibody testing.

Noncompetitive immunoassay

a new assay format in 1968

Analyte in the sample is bound to **excess of „capture antibody“** immobilized on solid phase (testtubes, microplates, etc.)

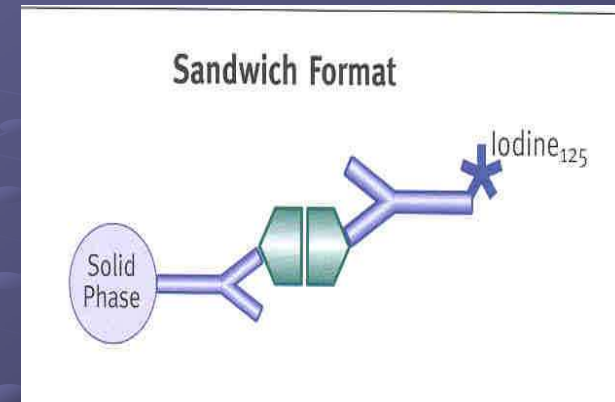
So that there always remain **unoccupied binding sites**

Only the **occupied binding sites** can be detected by **“labeled antibody“**

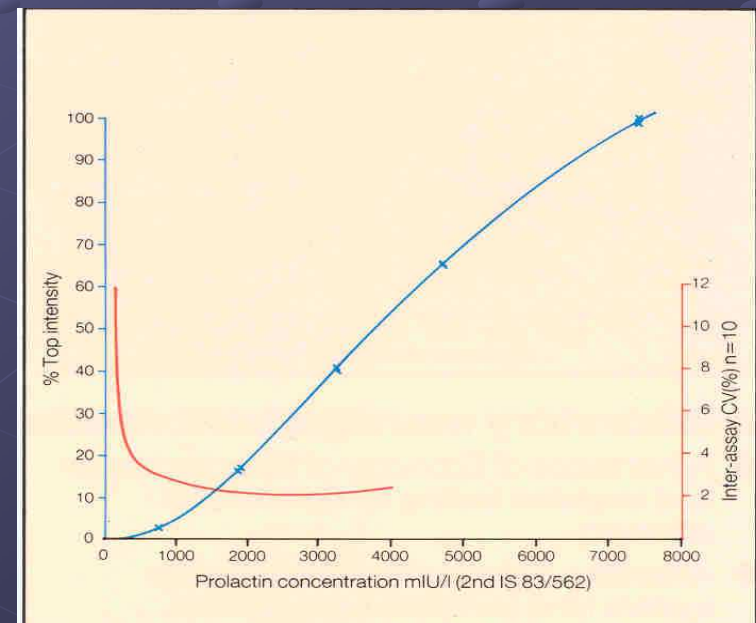
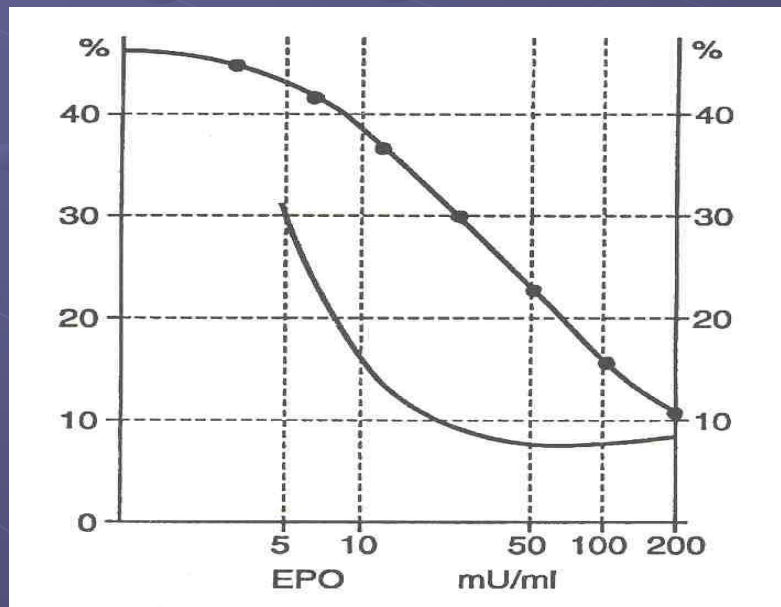
The amount of Ag in the sample is **directly related** to the „signal“, e.g. the amount of bound labeled Ab

Noncompetitive immunoassay

(also known as sandwich, immunometric, excess reagent assays)

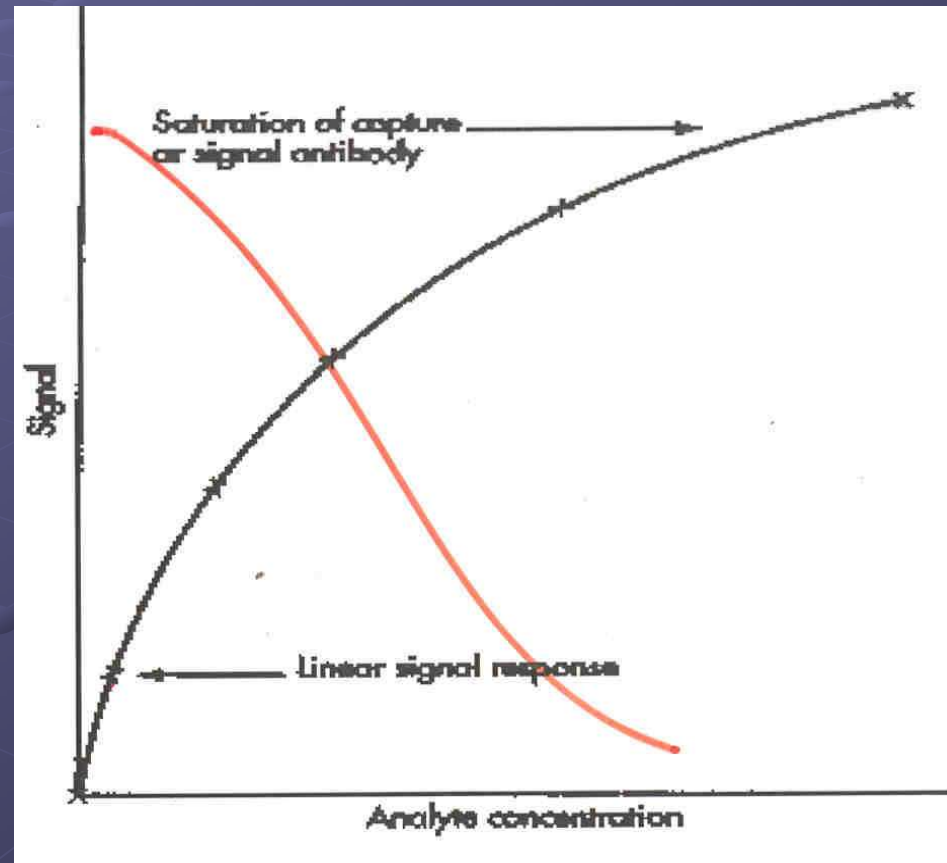


Competitive format



Why noncompetitive immunoassays are better than competitive assays?

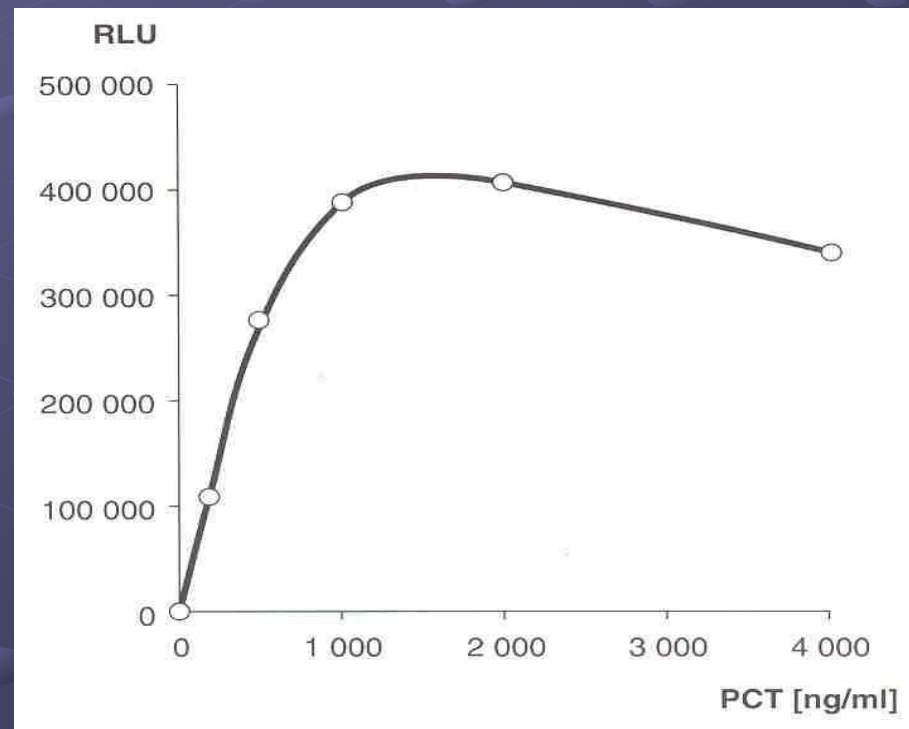
- higher sensitivity and specificity
- universal labeling procedure (IgGs)
- generally longer shelf-life of labeled antibodies
- extended working range



Limitations of **noncompetitive** immunoassays

- **not applicable to small molecules**
- more expensive (higher consumption of antibodies, isolation of pure immunoglobulin necessary)
- „**hook effect**“ (high dose hook effect) in some assays

● HAMA interference



Categories (formats) of immunoassay

- **Competitive immunoassays**

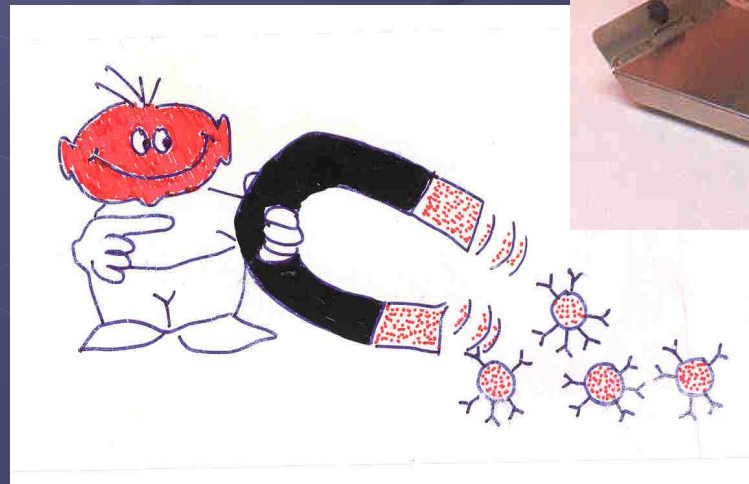
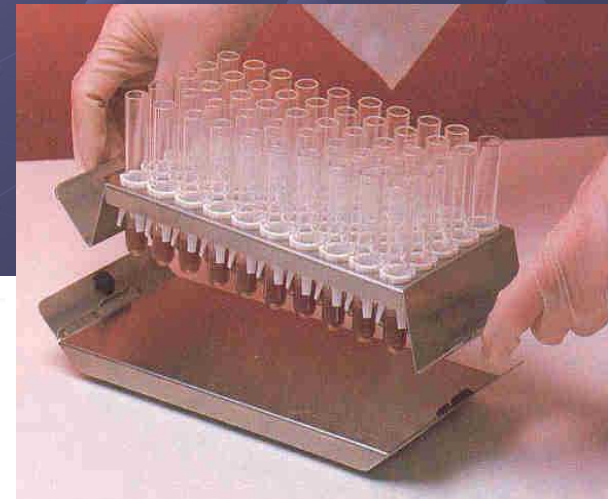
(limited reagent assays)

- **Noncompetitive or immunometric assays**

(excess reagent assay, sandwich assay)

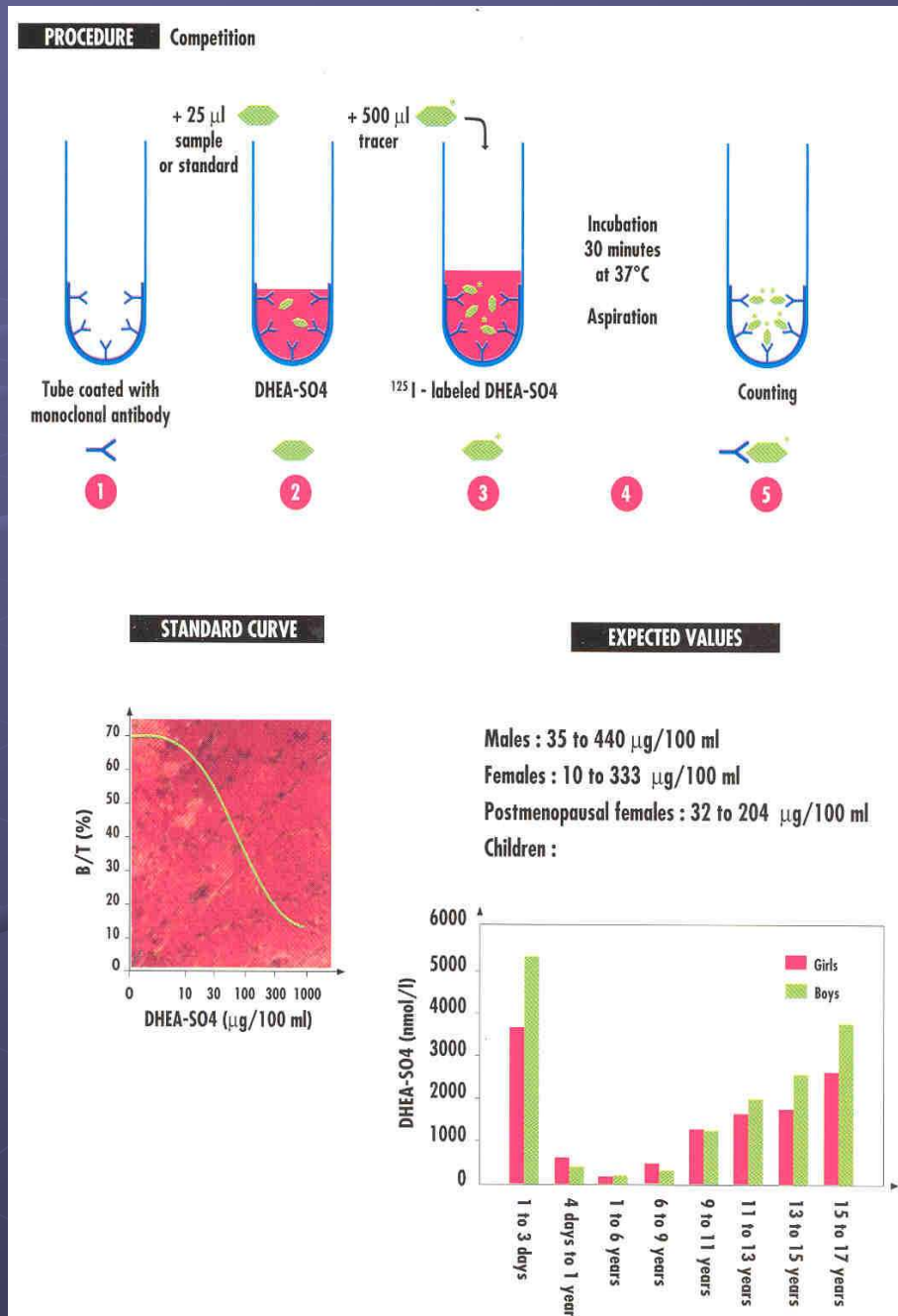
Heterogeneous Assays

- Always require **separation** of the label bound in the immune complex and the free label
 - **Double antibody + PEG**
 - **Solid phase systems**
 - Coated tubes, microplates, beads, etc
 - Magnetic particles



Solid phase RIA kit (DHEA-S)

procedure
 standard curve
 expected values
 in age and sex groups



Labels, tracers

● Radioactive isotopes

- ^3H used in competitive binding assays before the era of immunoassays
- ^{131}I used in the first RIAs ($t_{1/2}$ 8 days)
- ^{125}I with a longer half-life (60 days) soon replaced ^{131}I isotope for use in RIAs

RADIOACTIVE LABELS (^{125}I , ^3H)



- **High sensitivity of detection**
- **No interferences**
- Small molecular size
- Simple labeling
- Low cost



- Environmental risk (waste disposal)
- Dedicated instrumentation
- Separation step necessary
- **Short shelf-life**
- **Difficult to automate**



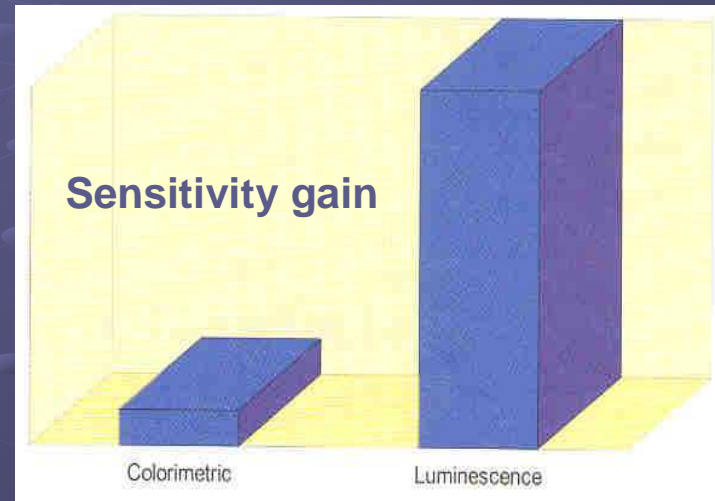
ALTERNATIVE LABELS

- **Enzymes** (alkaline phosphatase, horse raddish peroxidase and others)
- **Fluorescent substances** (fluorescein, lanthanide chelates)
- **Luminiscent substances** (substituted isoluminol, acridinium esters)
- **Particles** (latex particles, colloidal gold, Eu chelate nanoparticles)

Detection of enzyme labels

Low sensitivity: **Absorbance** measurement

- **chromogenic substrates**



High sensitivity: **Light emission** measurement

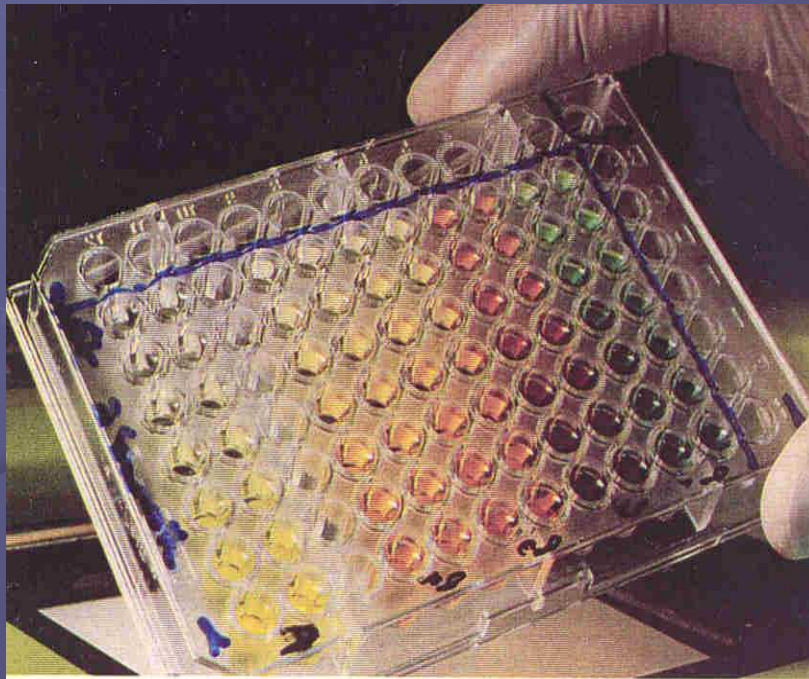
- **chemiluminiscent substrates** (peroxidase + luminol + enhancer) or alkaline phosphatase + adamantyl-1,2-dioxetane phenyl phosphate)
- nonfluorescent substrates that are converted to **fluorescent products** (4-methylumbelliferyl phosphate)

ELISA

Enzyme Labeled ImmunoSorbent Assay

probably the most popular format

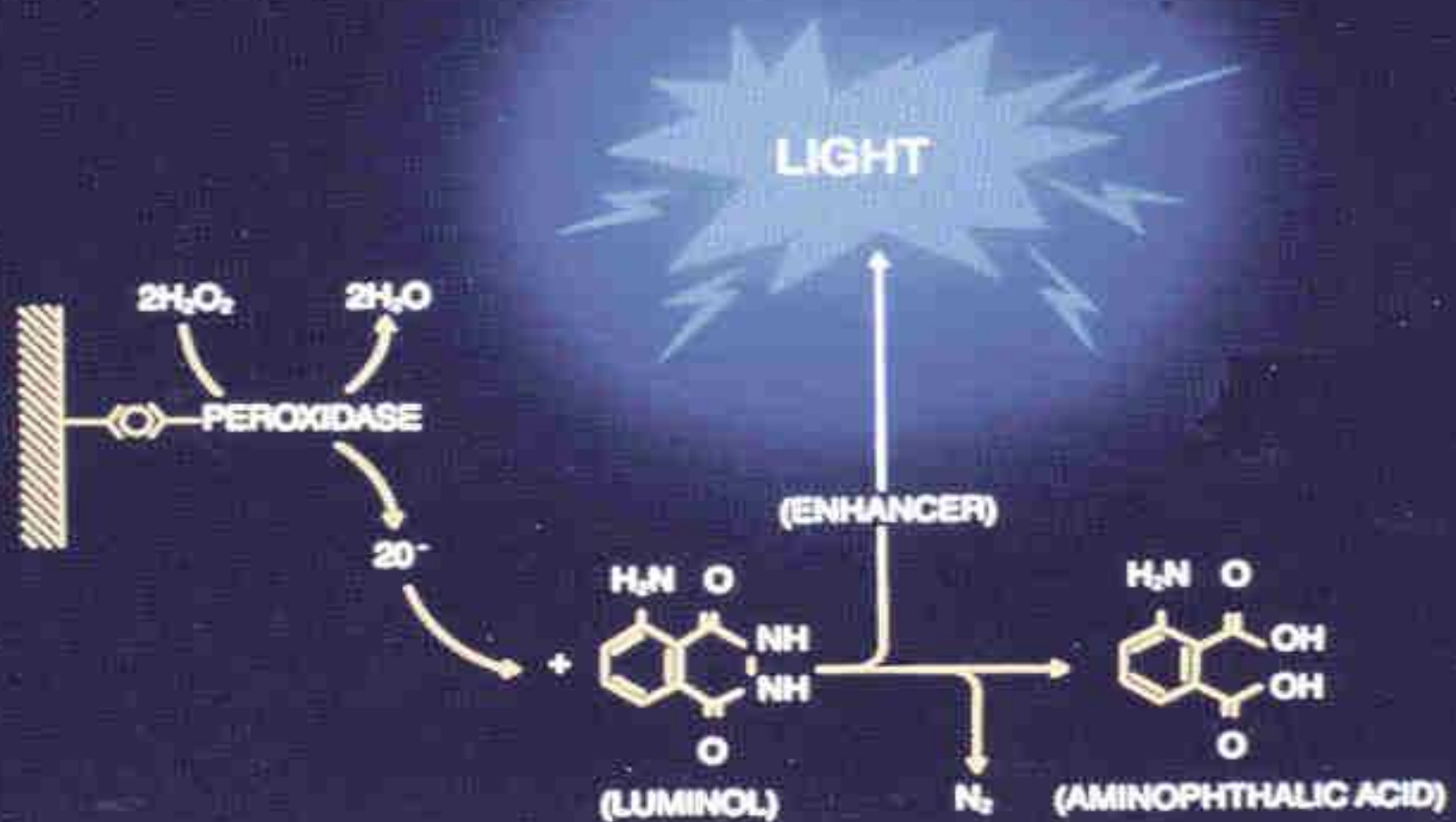
microplate



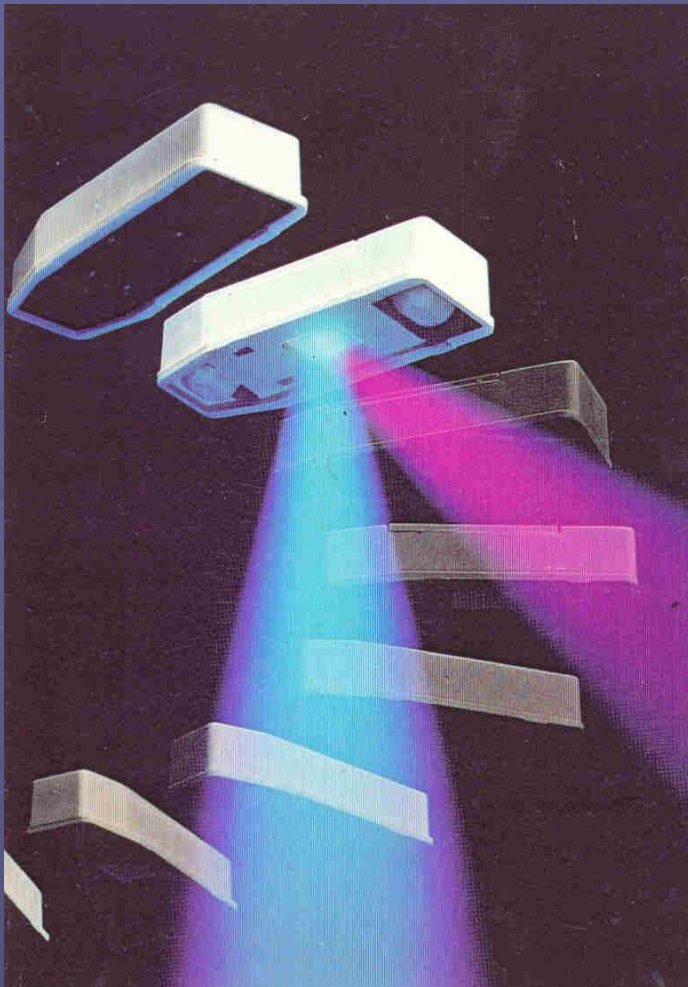
individual strips or wells
in a frame



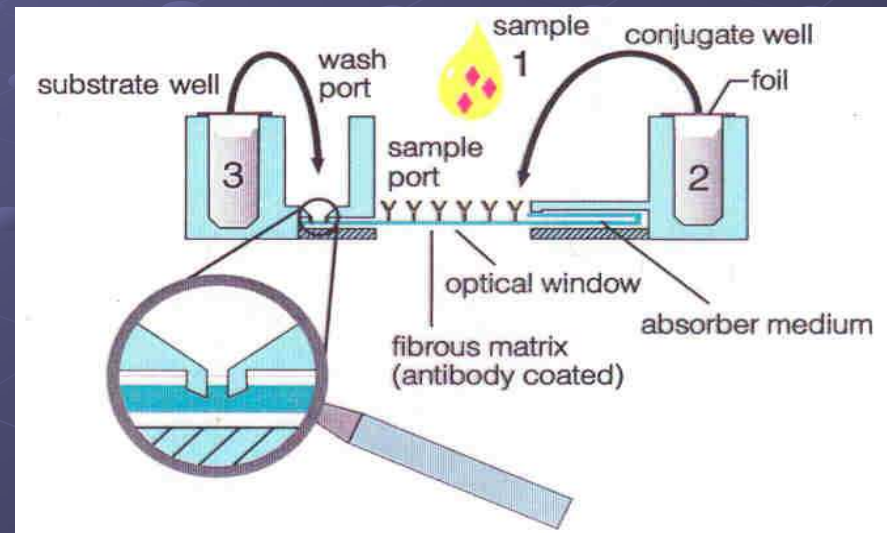
PRINCIPLE OF ENHANCED LUMINESCENCE ASSAYS



Fluorogenic ELISA



Test specific module

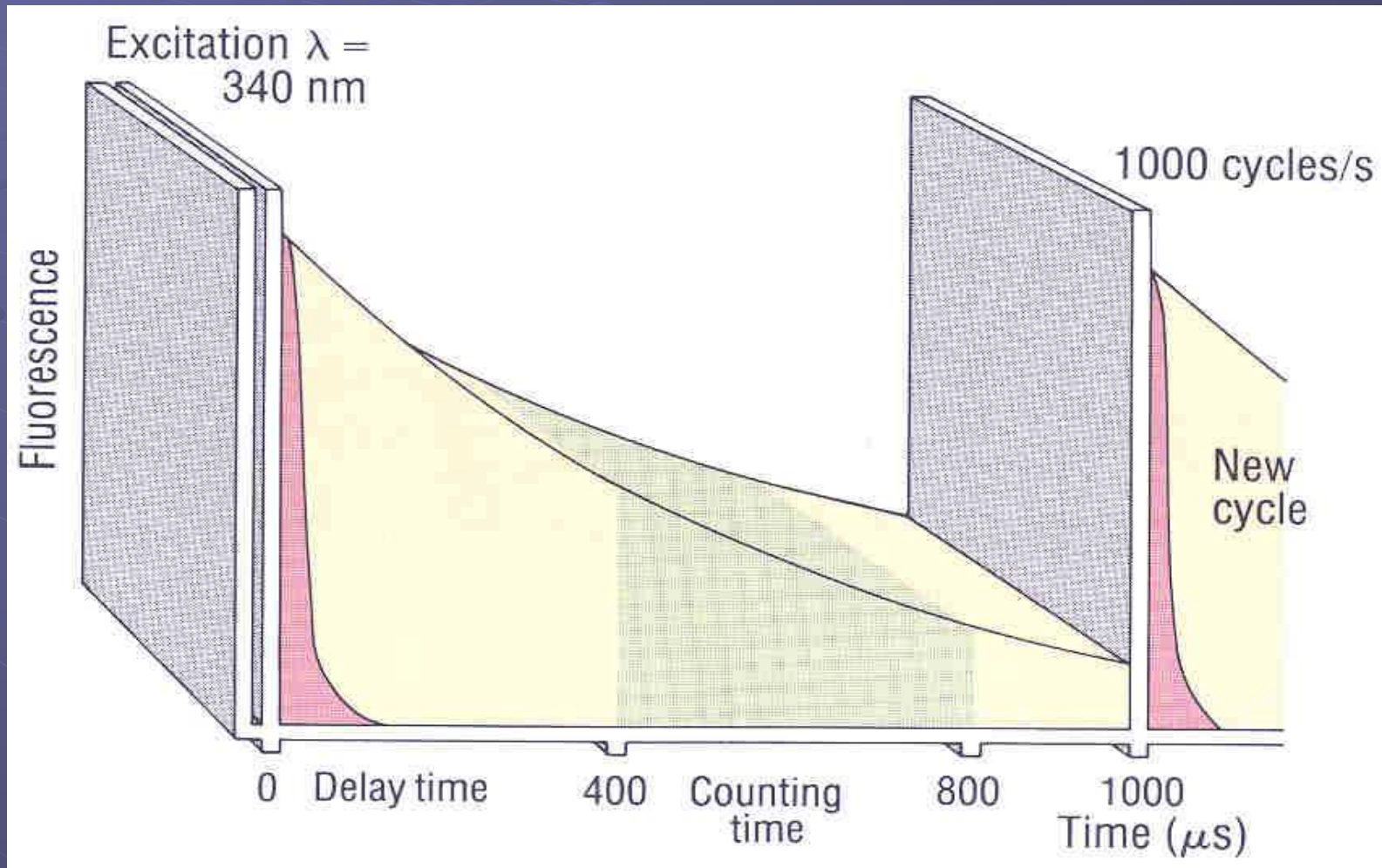


FLUORESCENT LABELS

- **Fluorescein** and **fluorescein isothiocyanate (FITC)** – used for labeling antibodies in **histochemistry**
- **Background fluorescence** is a problem in biological specimens
- **Solution: time-resolved fluoroimmunoassays** using long-lived fluorescence of lanthanide chelates (lifetime in **microseconds**) – label signal is measured after background fluorescence has decayed (lifetime in **nanoseconds**)

DELFLIA[®]

Dissociation Enhanced Lanthanide FluoroImmunoAssay



LUMINESCENCE HISTORY

- 1667 bioluminescence was recognized
- 1887 first luminiscent substances known
- 1947 first application of firefly luciferase
- 1967 first immunoassay utilizing luminol
- 1982 acridinium ester used for the first time as a label in a manual assay

MAGICLITE by Ciba Corning



In 1990, Ciba Corning launched the world's first fully automated immunoassay system with Random Access, **ACS:180**



ADVIA CENTAUR

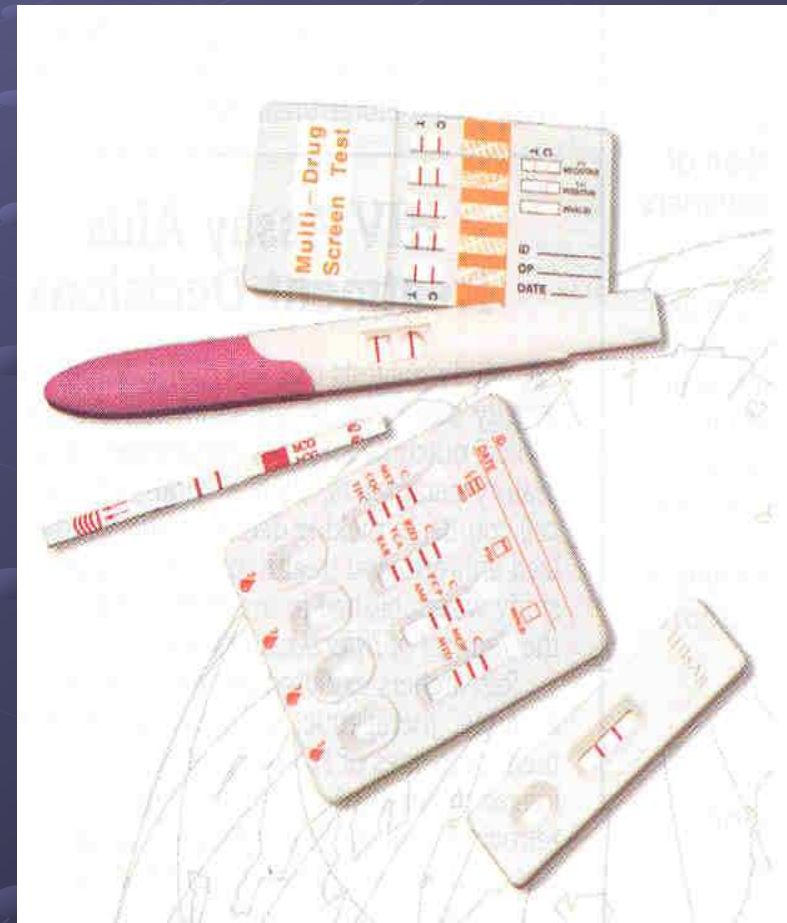
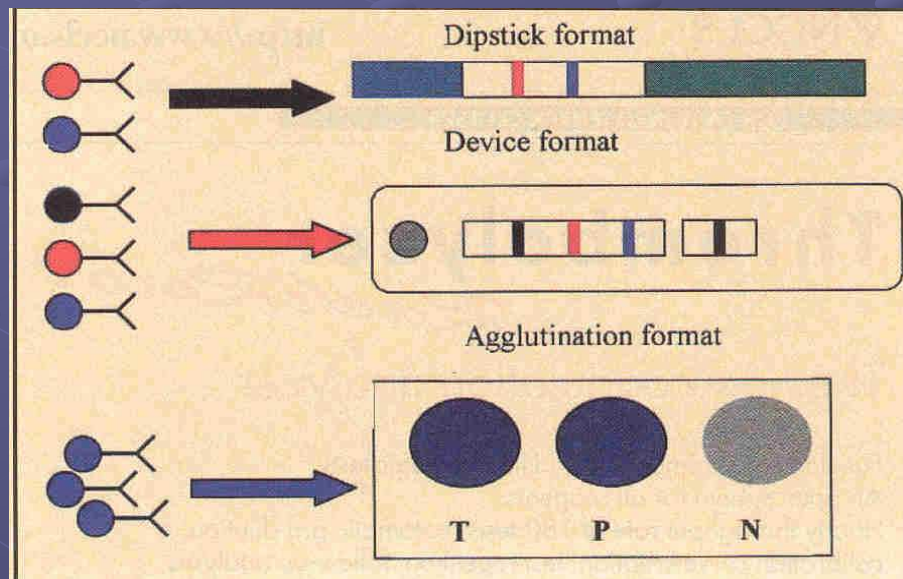
output 240 tests/hour



MODERN PARTICLE LABELS

Immunochromatographic tests, membrane tests, lateral flow or one step tests

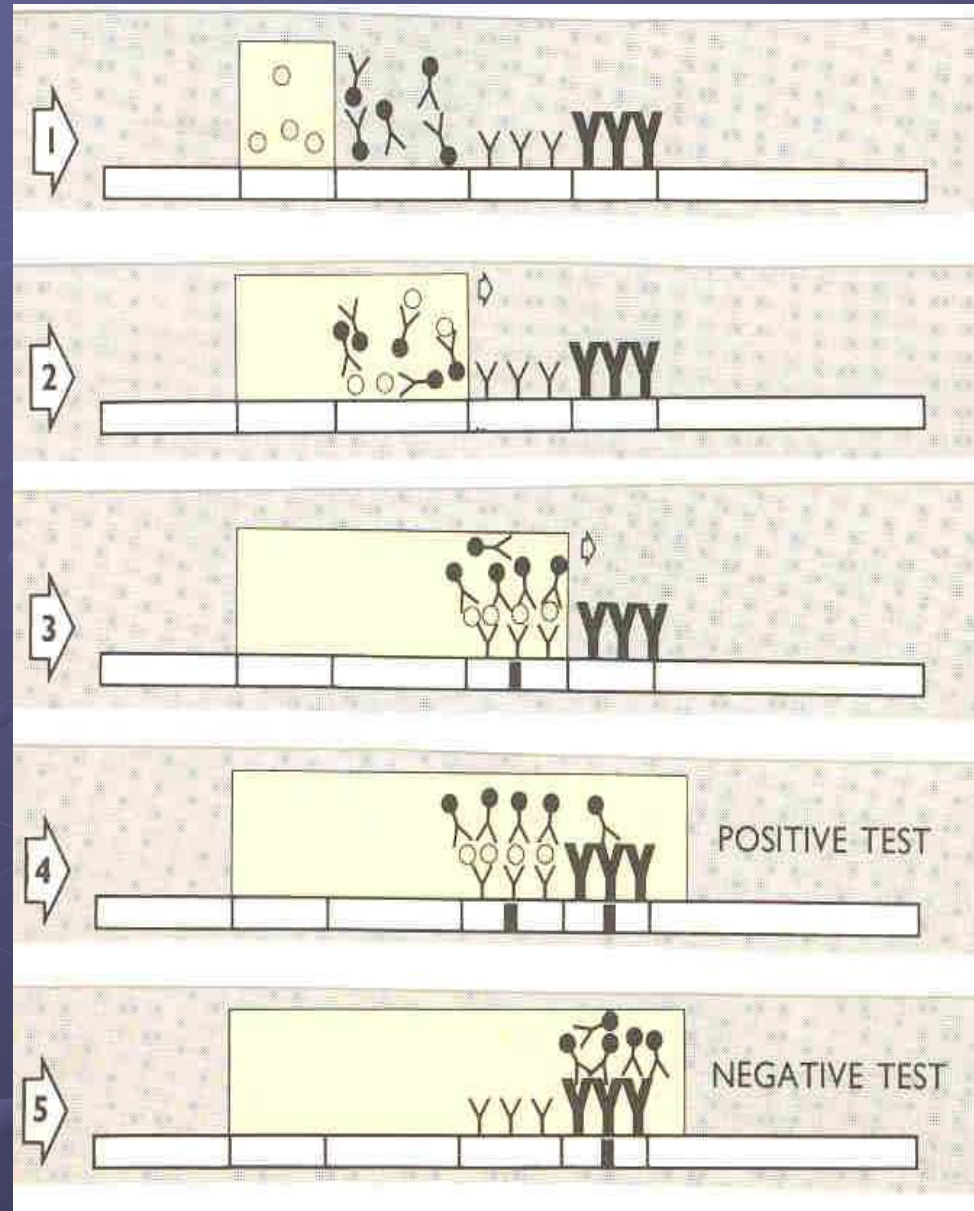
(in dipstick or device format)



How does it work?

Ab coated particle (coloured latex, colloidal gold) binds Ag and is captured by immobilised Ab

Excess of labeled Ab binds to the second Ab (anti-mouse Ab)



Some alternative labels
made it possible to develop

Homogeneous immunoassays

- **Heterogeneous assays** (require separation of bound Ab-Ag complex)
- **Homogeneous assays** (do not require this separation, as signal is changed when the label is bound in the Ab-Ag complex)

Moreless outdated HOMOGENEOUS ENZYME IMMUNOASSAYS

EMIT (Enzyme Modified Immunoassay Technology), the active site of the enzyme label is blocked when bound

FPIA (Fluorescence Polarization ImmunoAssay) rotation of fluorescent label is slower when bound

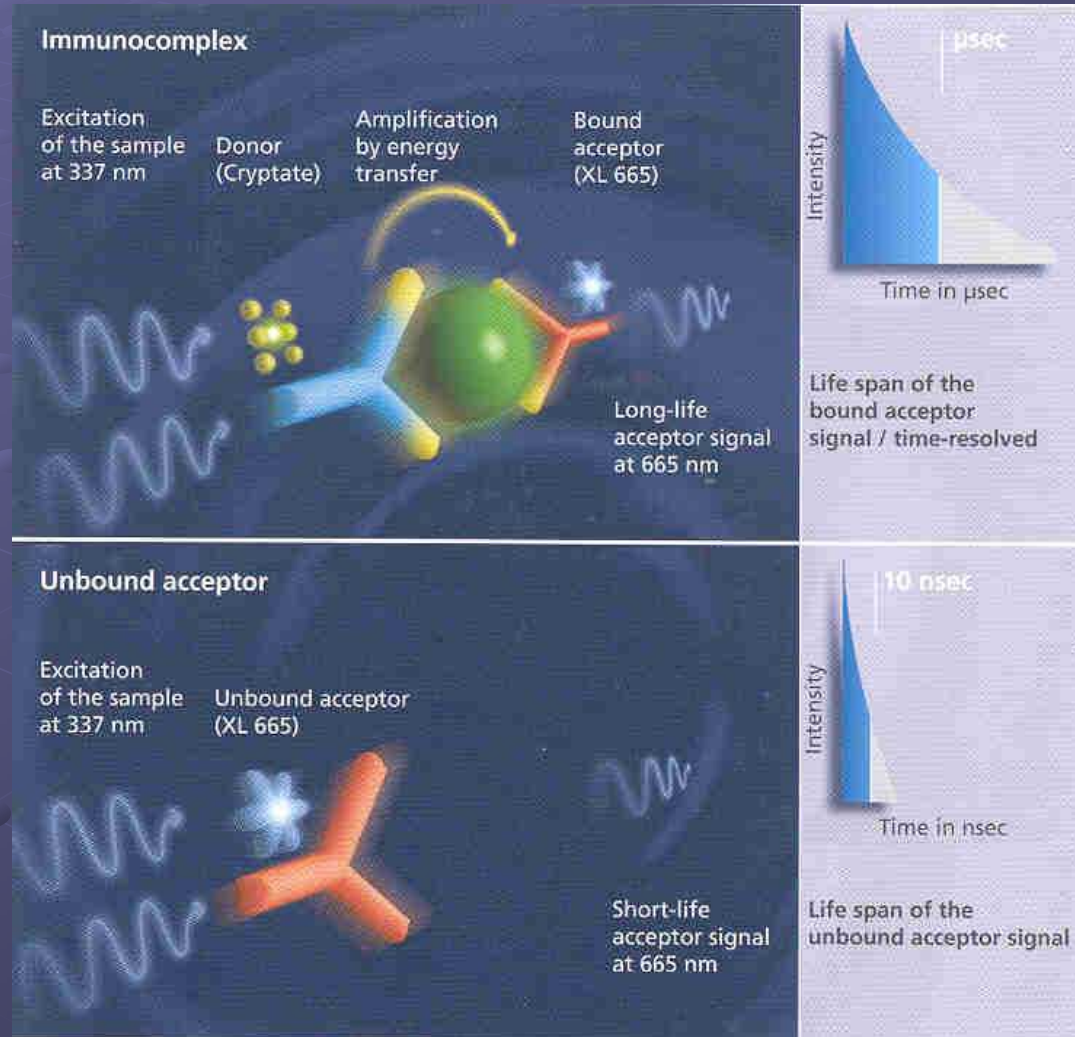
Competitive assays for small molecules in relatively high concentration – TDM

The only isotopic homogenous immunoassay:

SPA (Scintillation Proximity Assay), ^3H labelled small hapten gets into the proximity of a scintillator encapsuled in the solid phase with immobilized antibody

Modern HOMOGENEOUS ENZYME IMMUNOASSAY

automate
Cryptor



MESSAGE: IMMUNOASSAYS

- are **unique** in using antibodies as analytical reagents
- are **indirect** analytical tests
 - the intensity of a signal in a sample is compared with the signal generated by a simultaneously measured calibrator
- **calibrators** should be in a proper matrix to mimic the sample
 - **traceability** of calibrator to reference preparation should be documented
 - International Reference Preparations
- **reference methods** do not exist in many cases

● 1st generation immunoassays

- competitive immunoassay

- (RIA, EIA, FIA, LIA, often in-house, manual double antibody and solid phase assays, commercial kits)

● 2nd generation immunoassays

- noncompetitive immunometric assays

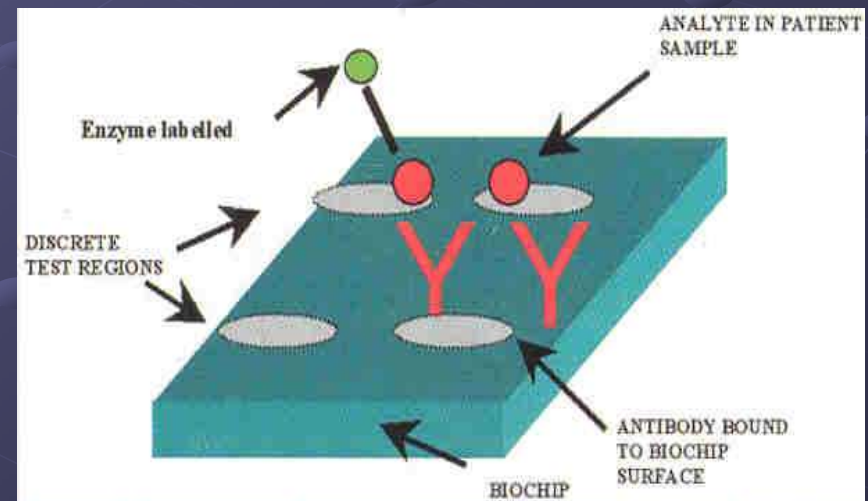
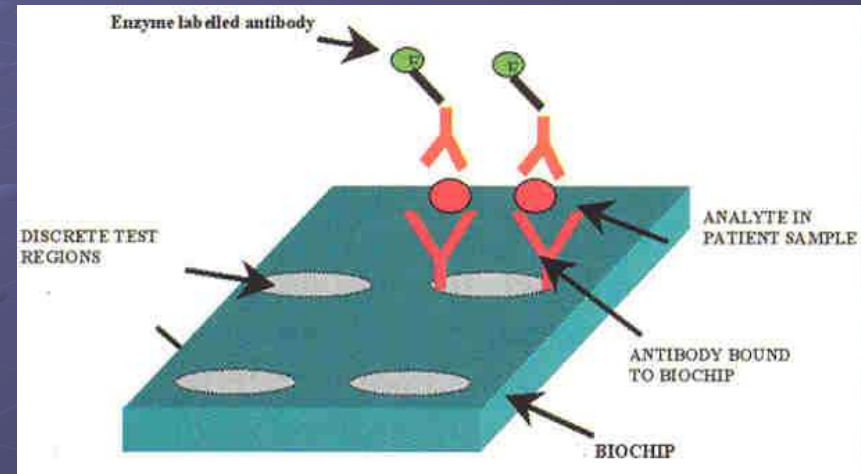
- (IRMA, ELISA, lateral flow assays, automated assays, random access automates, consolidation with clinical chemistry)

● 3rd generation immunoassays

- microspot analysis, biochip arrays, multiple parameter testing

3rd generation immunoassays

- multiple parameter testing
- nanotechnologies



Factors impacting analytical performance

● **Antibodies**

- specificity, avidity, type

● **Labels**

- sensitivity of detection, size, stability, interferences, background noise

● **Format** (competitive or noncompetitive)

● **Separation system** (in heterogeneous formats)

● **Automation**

- eliminating human error

MAIN INTERFERENCES IN IMMUNOASSAYS

● **Competitive assays**

- false positive results due to cross-reacting molecules (typical example: E₂ or testosterone direct assays)
- **remedy:** improving sensitivity of primary antibody

● **Noncompetitive assays**

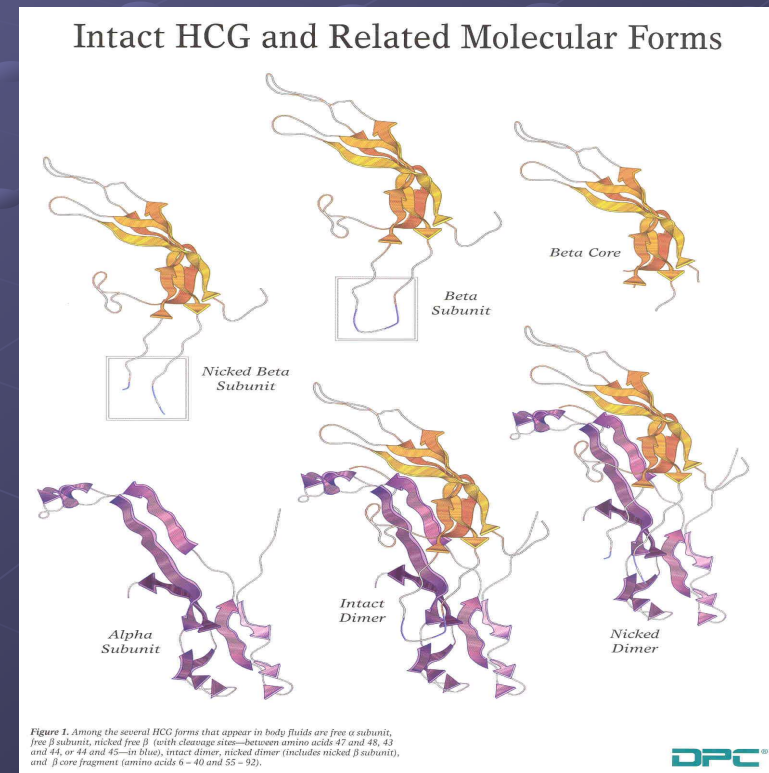
- HAMA, auto- and heterophilic antibodies, rheumatoid factors - both false positive and false negative results (typical examples: hCG, myoglobin)
- **remedy:** heterophil blocking reagent

Many analytes are heterogenous

Complexed (PSA), dimers (prolactin), fragments (hCG),
different degree of glycosylation, etc...

It may result in discrepant findings
between assays from different
manufacturers

Sometimes ability to measure
different molecular forms can
increase diagnostic value of the test
(PSA/freePSA, hCG subunits or
hyperglycosylated hCG)



Parameters of Assay Quality

● Analytical:

- **Accuracy:** ability to measure the correct concentration (comparison with reference method?)
- **Precision (reproducibility):** acceptable coefficient of variance
- **Detection limit (sensitivity):** lowest concentration different from zero

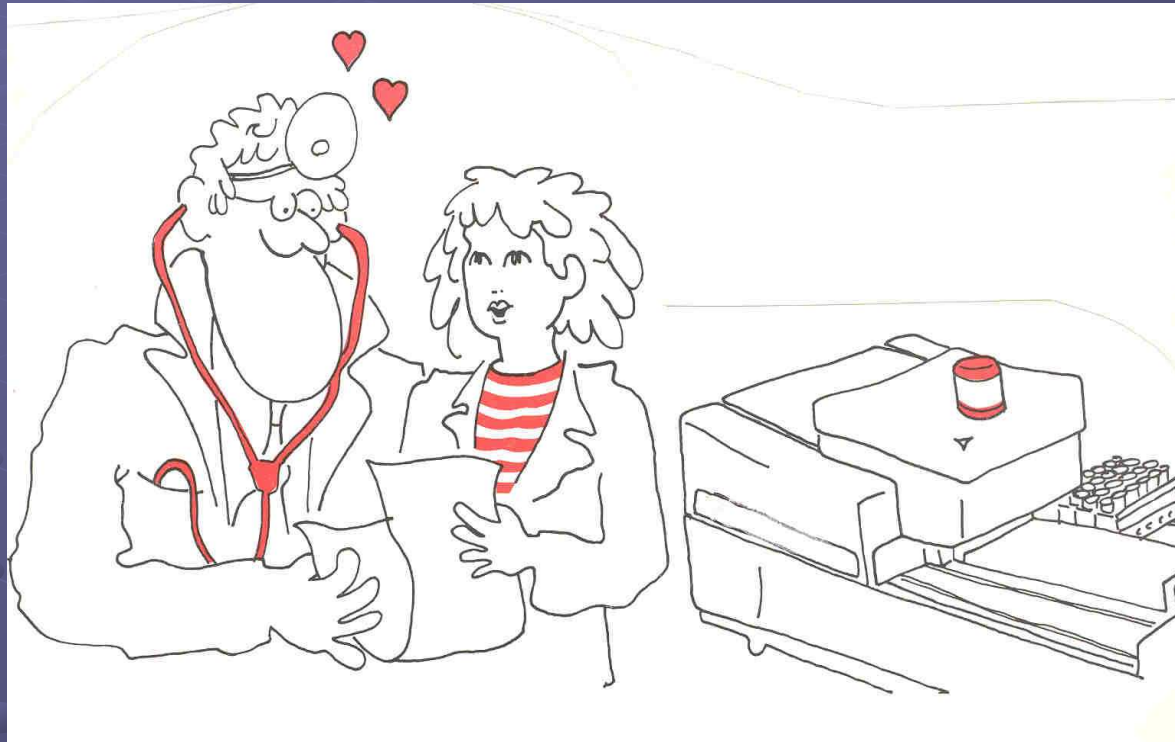
● Diagnostic:

- **Sensitivity:** % of true positive results
 - If high, then a negative result practically exclude the diagnosis (SnNout)
- **specificity:** % of true negative results
 - If high, then a positive result practically include the diagnosis (SpPin)

Immunoassays in the past



And today



**Communication between clinicians and
laboratorians is crucial for the benefit of patients!**