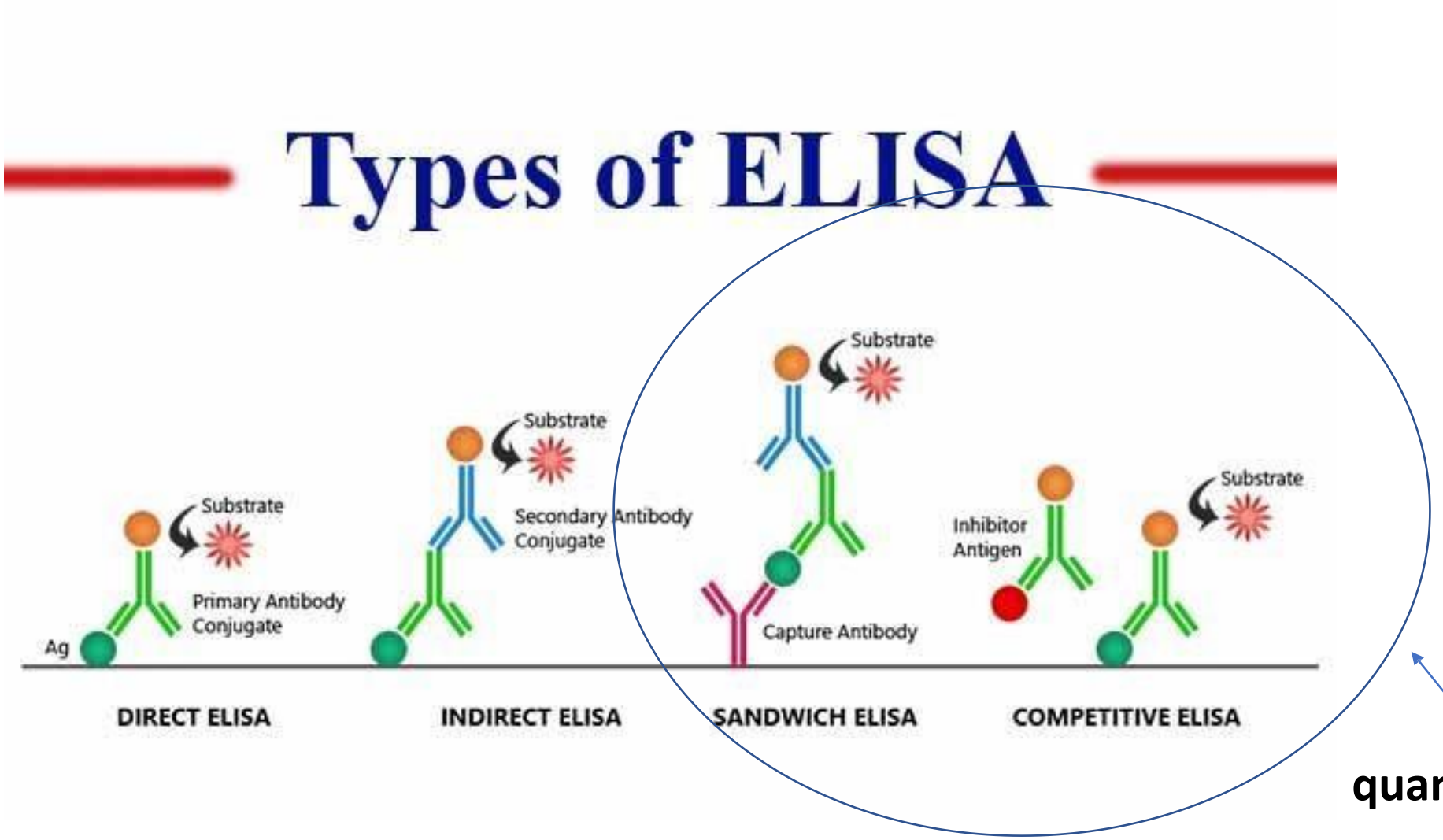
IMMUNOASSAYS

Use antibodies for analytical purposes

Most used type of assay that use a label to have an analytical signal

- **Radio immunoassay (a radioactive label, i.e I_{125} for thiroyd hormones)**
- **Fluorimetric assays (a fluorescent label, i.e fluorescein)**
- **Chemiluminometric assays (a chemiluminescent label is used)**
- **Enzyme immunoassays (or ELISA) (an enzyme is the label and the product of the reaction can be detected colorimetrically, fluorimetrically or by chemiluminescence)**

Enzyme Linked Immuno-Sorbent Assay (ELISA)



quantitative

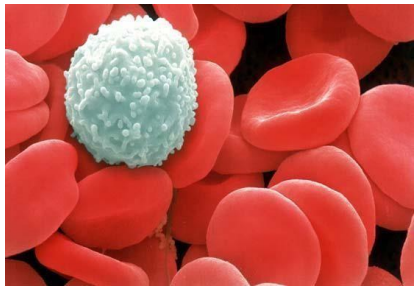
Antigeni (Ag)

- **L’antigene e una sostanza capace di interagire in maniera specifica con i prodotti finali della risposta immunitaria (anticorpi).**
- **L’immunogeno e quella sostanza che, dopo essere penetrata nei tessuti di un vertebrato superiore, e in grado di indurre una specifica risposta di difesa immunitaria con formazione di uno o prn anticorpi.**
- **La capacita di indurre una risposta immunitaria e/o cellulare, coinvolgendo cellule del sistema reticolo-linfocitario, viene dehnita immunogenicita.**
- **Attenzione: non tutti gli antigeni hanno capacita immunogenica (ad es. piccole molecole PM <3000 Da) .**
- **Gli antigeni interagiscono con gli anticorpi utilizzando piccole zone superficiali specifiche dette determinanti antigenici o epitopi.**

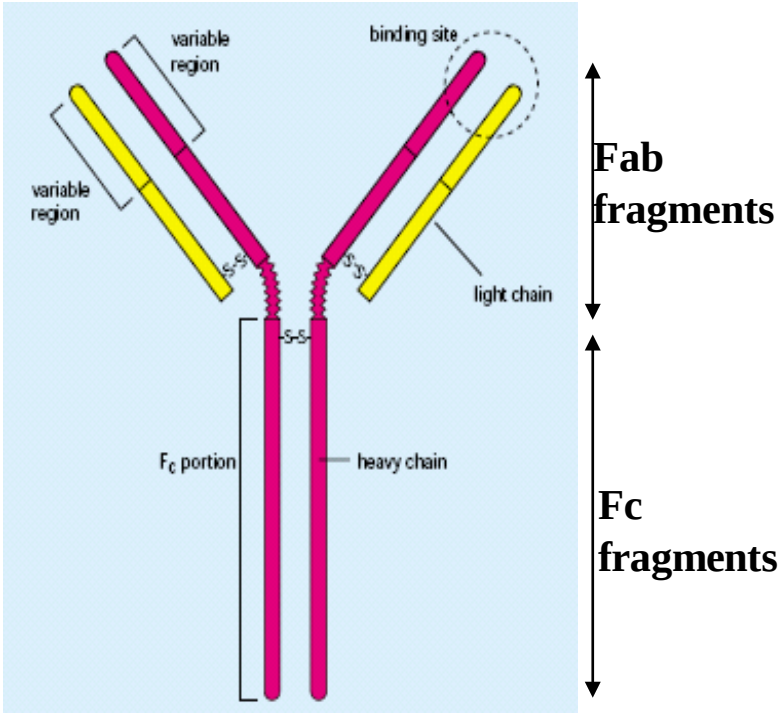
• Attenzione (!): in alcuni casi antigenicità ed immunogenicità vengono usati come sinonimi.

Antibodies

- Proteins secreted by B-lymphocytes (type of white blood cell), in vertebrates.



- Recognise and bind to molecules (**antigens**) on foreign particles, marking them for destruction by T-lymphocytes.

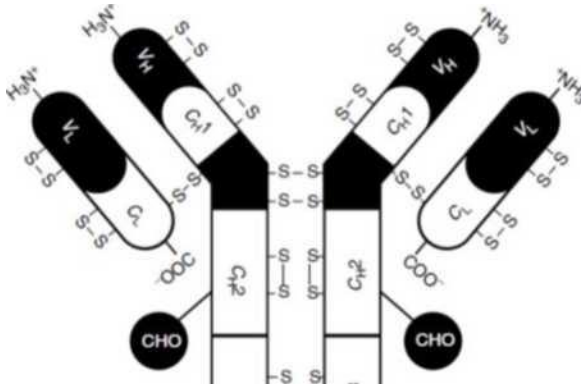


IgG molecule

- Each antigen may generate several antibodies for different sites (**epitopes**) on antigen.

Anticorpi

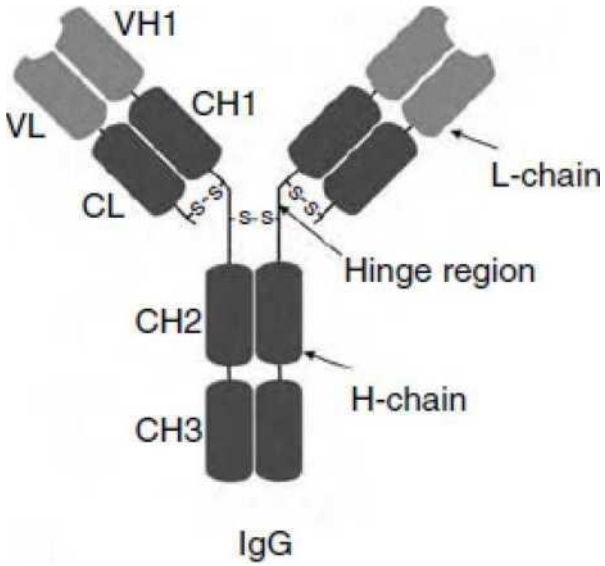
- **Gli anticorpi o immunoglobuline (Ig) sono una famiglia di glicoproteine plasmatiche, appartenenti alla classe delle Y-globuline,**
- **Vengono prodotti dai linfociti B maturi, appartenenti alla categoria dei globuli bianchi, che hanno il compito di difendere l'organismo da agenti esterni tramite la risposta umorale.**



V V

OOC COO

Anticorpo (Ab)



IgG

2 catene pesanti (H-chain) ca. 50 kDa

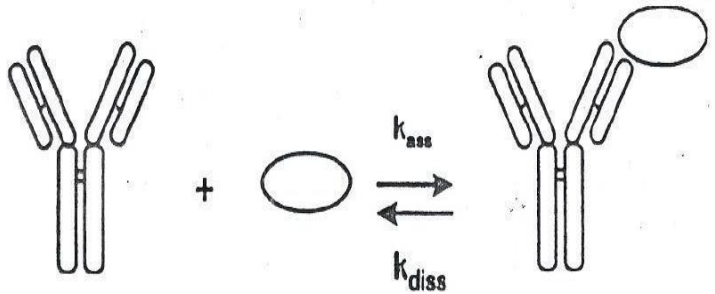
2 catene leggere (L-chain) ca. 26 kDa

Domini variabili: V_H e V_L

Domini costanti: C_H e C_L

Le H-chain definiscono l'isotipo (o classe) anticorpale: IgA, IgD, IgE, IgG, IgM

Antibody - Antigen interaction

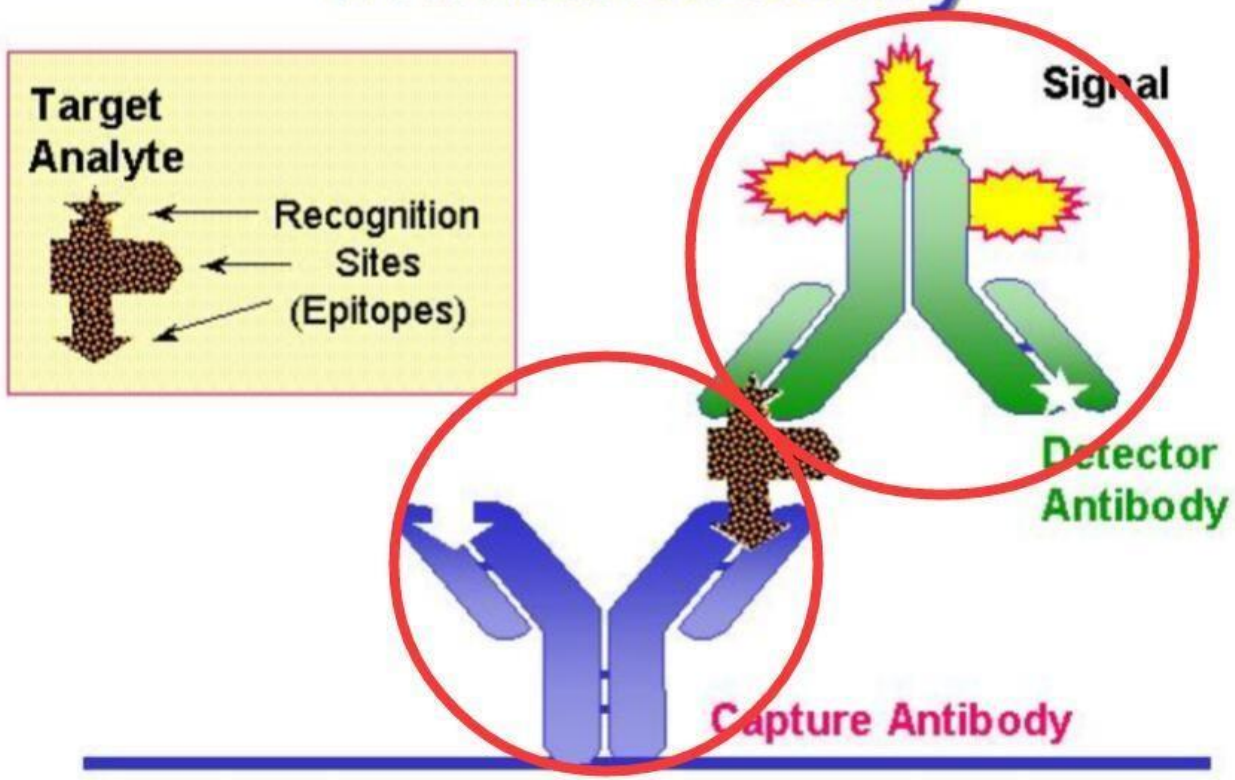


- Non - covalent
- Highly specific
- $k_{ass} \approx 10^6 - 10^8\ M^{-1}s^{-1}$
- $k_{diss} \approx 10 - 10^{-4}\ s^{-1}$
- $K_{aff} \approx 10^6 - 10^{12}\ M^{-1}$

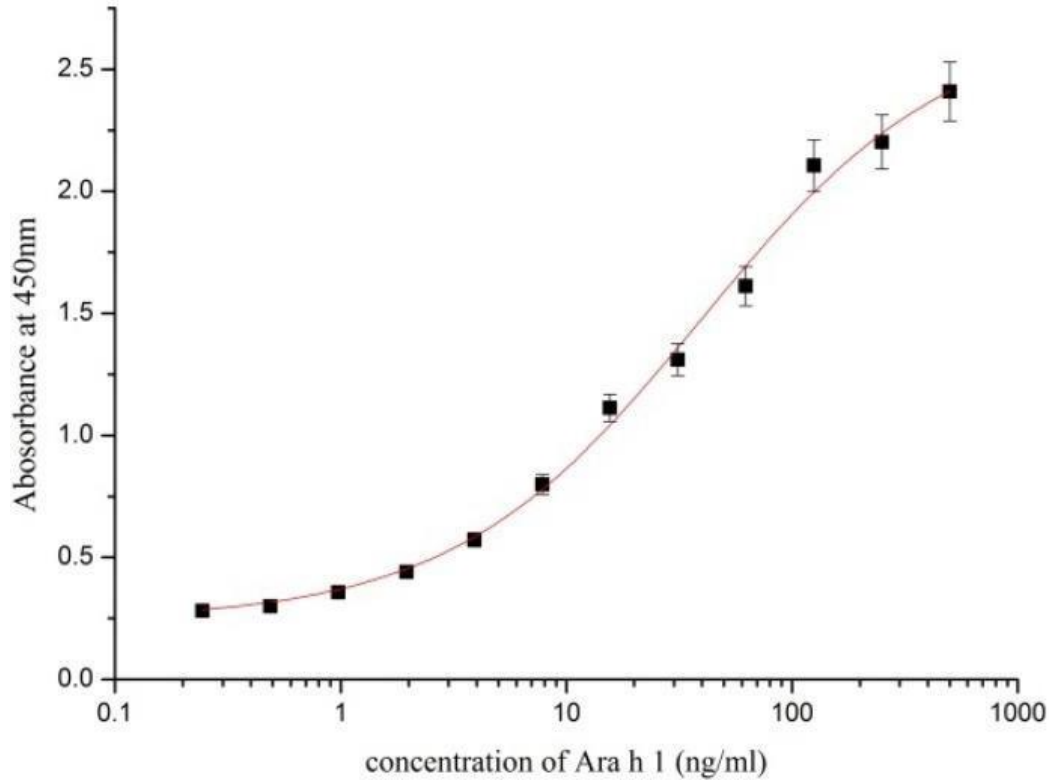
$$v = \frac{d[Ab:Ag]}{dt} = k_{ass}[Ab][Ag] - k_{diss}[Ab:Ag]$$

Equilibrium: $\frac{d[Ab:Ag]}{dt} = 0$ and $K_{aff} = \frac{k_{ass}}{k_{diss}} = \frac{[Ab:Ag]}{[Ab][Ag]}$

Double Antibody Sandwich Immunoassay



Dosaggio di un allergene (proteina) di arachide



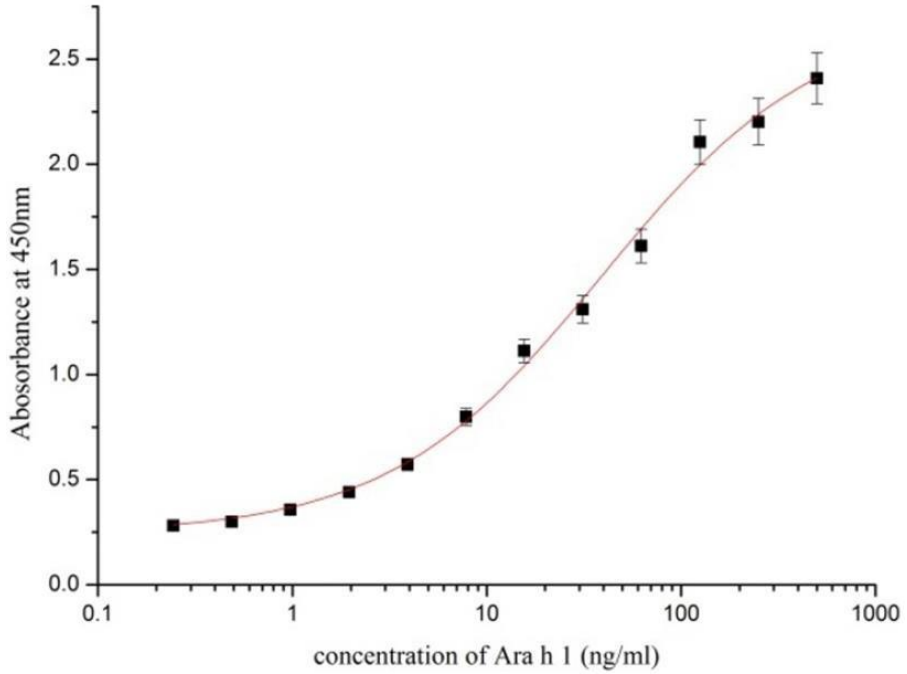
Tumor necrosis factor alpha (TNF-α), also known as cachectin and TNFSF1A, is the prototypic ligand of the TNF superfamily (1). It is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism (2-5). TNF-α is also involved in a number of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, neuropathic pain, obesity, type 2 diabetes, septic shock, autoimmunity, and cancer (5-11).

Human TNF-α is synthesized as a 26 kDa type II transmembrane protein that consists of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 177 aa extracellular domain (ECD) (12, 13). Within the ECD, human TNF-α shares 97% aa sequence

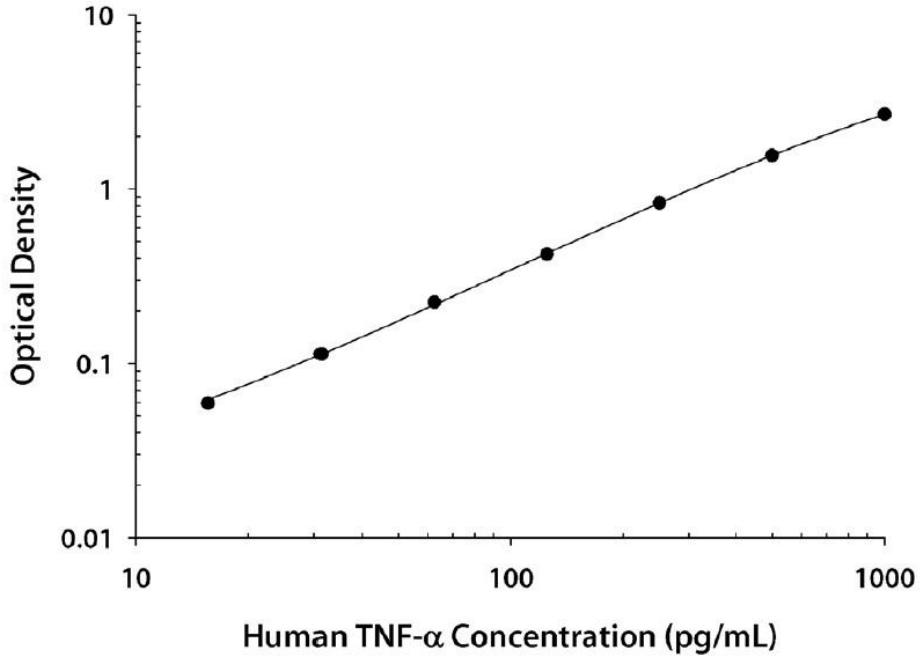
This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TNF-α bound in the initial step. The color development is stopped and the intensity of the color is measured.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the TNF-α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

Segnale vs. log concentrazione maggiore accuratezza!!



CALIBRATOR DILUENT RD6-35



Clinica Chimica Acta 510 (2020) 73–78

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Comparison of test performance of commercial anti-SARS-CoV-2 immunoassays in serum and plasma samples

Verena Haselmann*, Maximilian Kittel, Catharina Gerhards, Margot Thiaucourt, Romy Eichner, Victor Costina, Michael Neumaier

Department of Clinical Chemistry, University Medicine Mannheim, Medical Faculty Mannheim of the Uni

At the end of 2020, over 100 SARS-CoV-2 antibody assays have been CE-marked under EU Directive 98/79/EC . The available test systems can be discriminated into rapid diagnostic tests (RDT), either antigen- or antibody based, enzyme-linked immunosorbent assays (ELISA) and chemiluminescent immunoassays (CLIA).

Journal of Clinical Virology 139 (2021) 104821

Contents lists available at ScienceDirect

Journal of Clinical Virology

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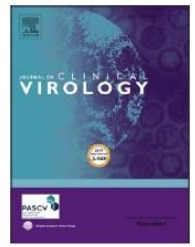
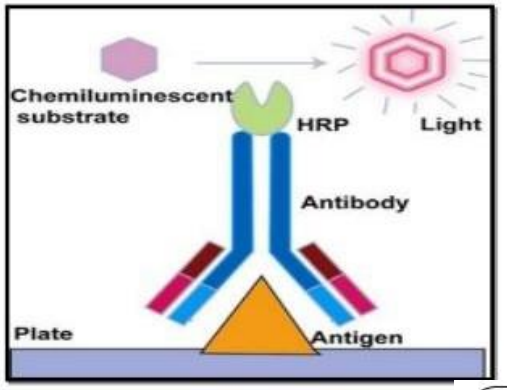


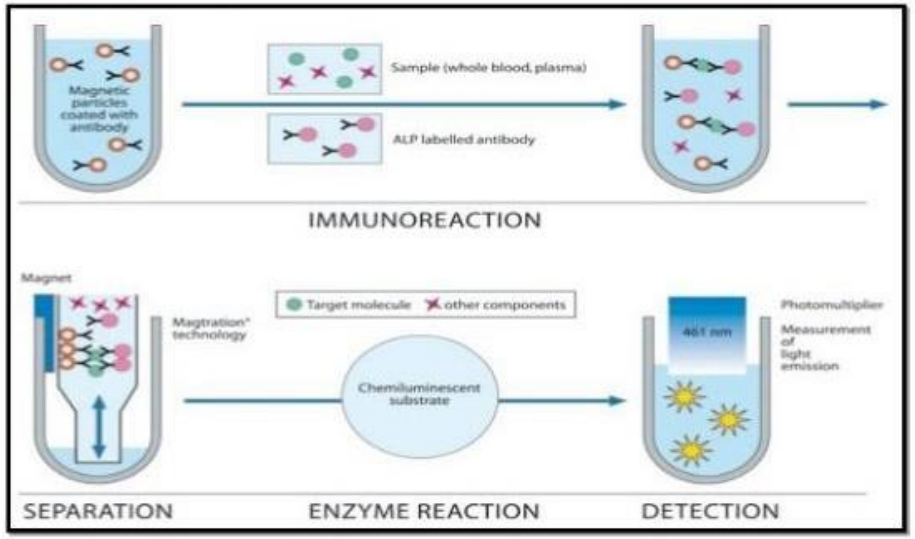
Head-to-head validation of six immunoassays for SARS-CoV-2 in hospitalized patients

Rens Zonneveld[a,*,1], Suzanne Jurriaans[a,1], Tom van Gool[a], Jorrit J. Hofstra[a], Thecla A. M. Hekker[a], Pien Defoer[a], Patricia E. Broekhuizen-van Haaften[a], Ellen M. Wentink-Bonnema[a], Lynn Boonkamp[b], Charlotte E. Teunissen[b], Annemieke C. Heijboer[c], Frans Martens[c], Godelieve de Bree[d], Michele van Vugt[d], Robin van Houdt[a], Amsterdam UMC COVID-19 Biobank

Chemiluminescence Immunoassay (CLIA) Technique



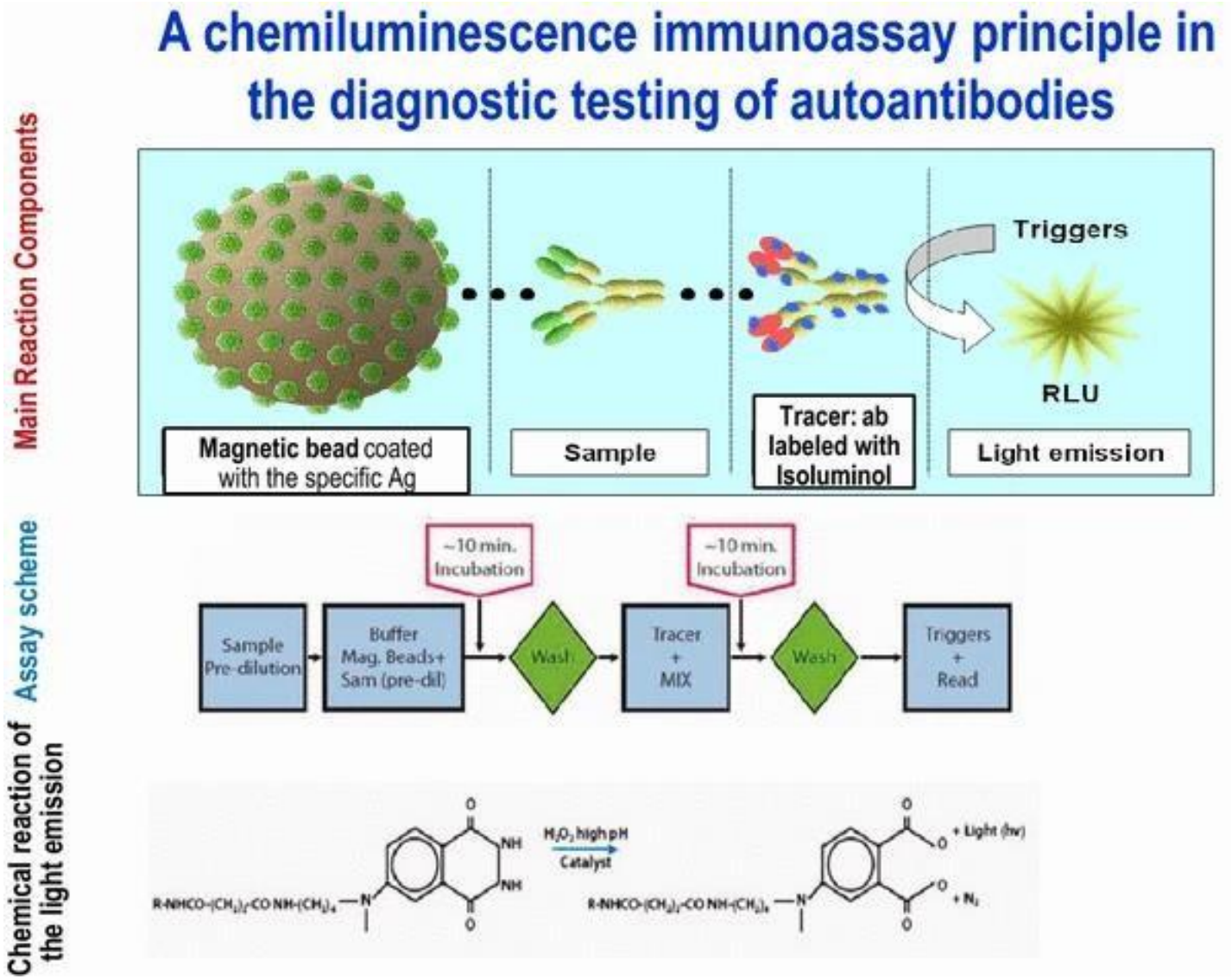
Principle:

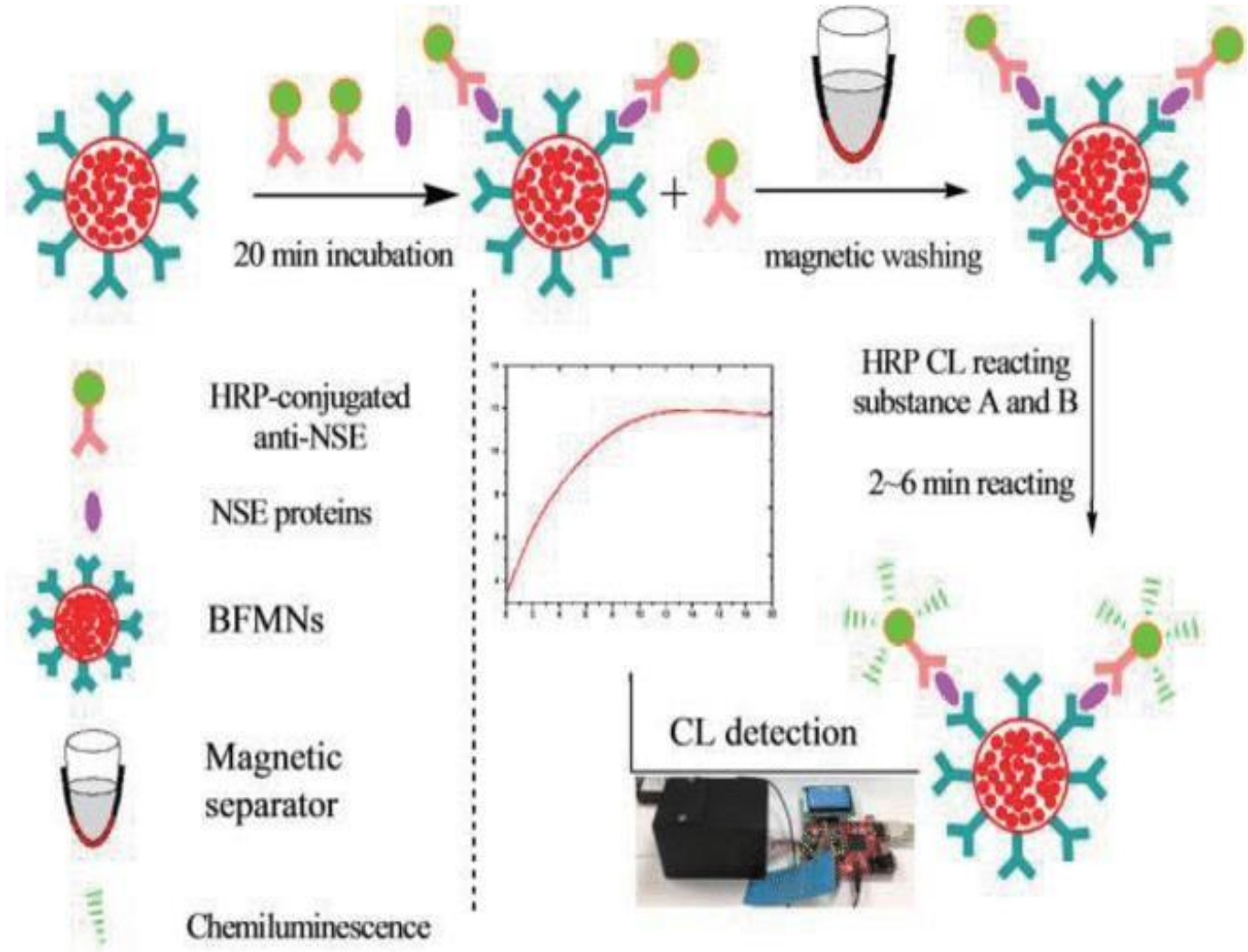


TYPES LUMINESCENCE

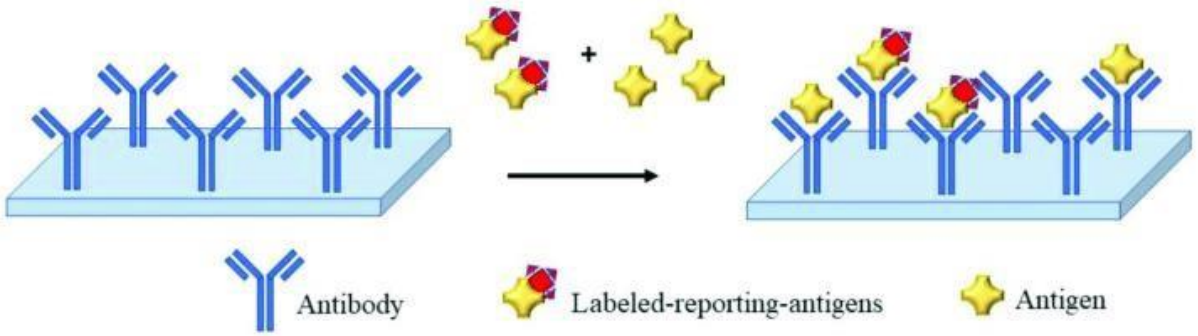
Excitation event		process
Chemicals	Luminol Isoluminol acridinium ester	**Chemiluminescence**
Biochemical	Luciferin aequorin	**Bioluminescence**
Electromagnetic	Ruthenium Tris (bipyridly) chelate	**Electroluminescence**
Photons	inorganic phosphors	**Photoluminescence**

Magnetic Microbeads are very often used for the separation step in immunoasssays

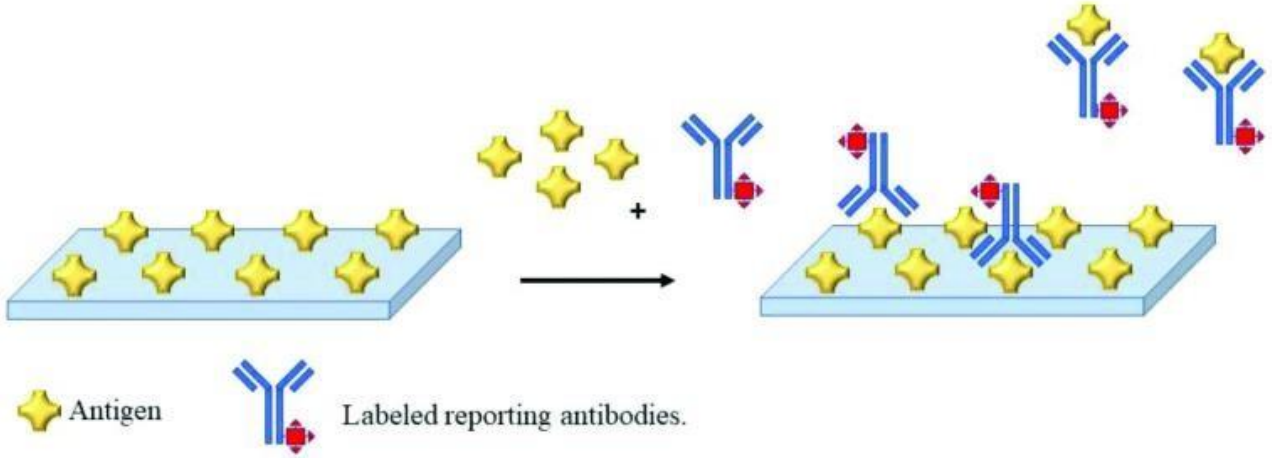



20 min incubation
magnetic washing
HRP CL reacting
substance A and B
2~6 min reacting
HRP-conjugated
anti-NSE
NSE proteins
BFMNs
Magnetic
separator
Chemiluminescence
CL detection

Competitive immunoassays

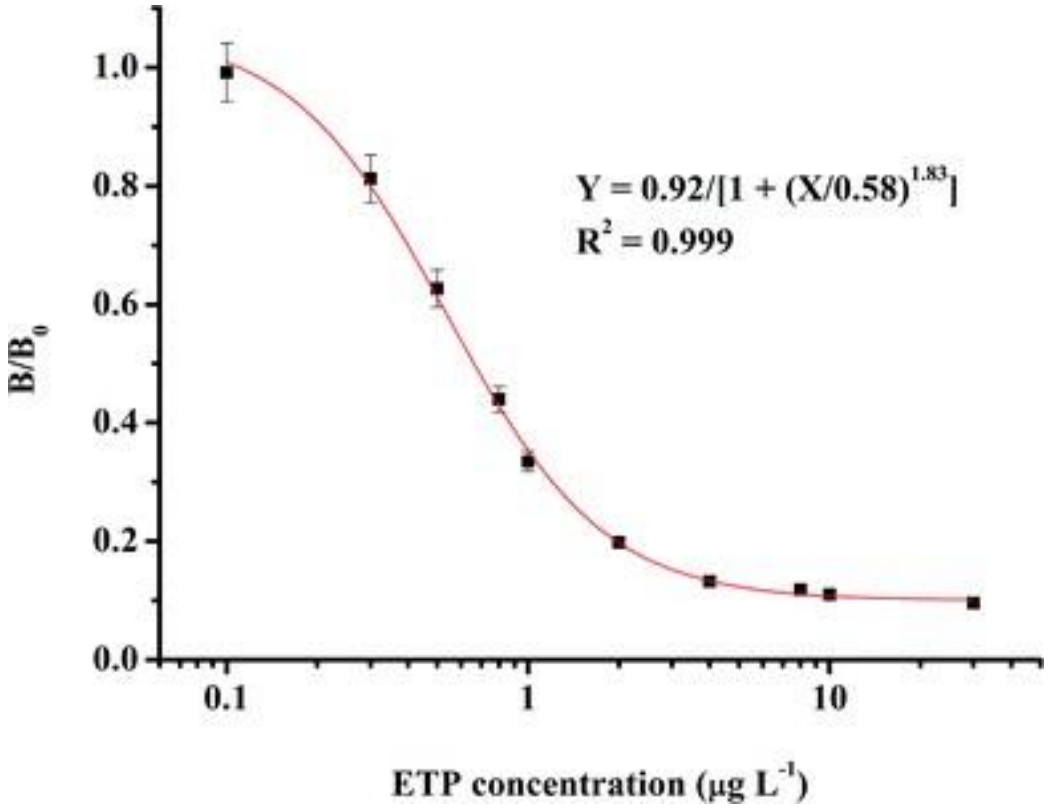


(a)



(b)

Calibration curve for ethopabate (veterinary drug)



$$y = \frac{a-d}{(1+(x/c)^b)} + d$$

a = (theoretical) response at low concentration/dilution
b = absolute value of the slope at the inflection point
c = value of x at inflection point
d = (theoretical) response at high concentration/dilution
x = concentration or dilution
y = response (OD)

Typical calibration curve for immunoassay

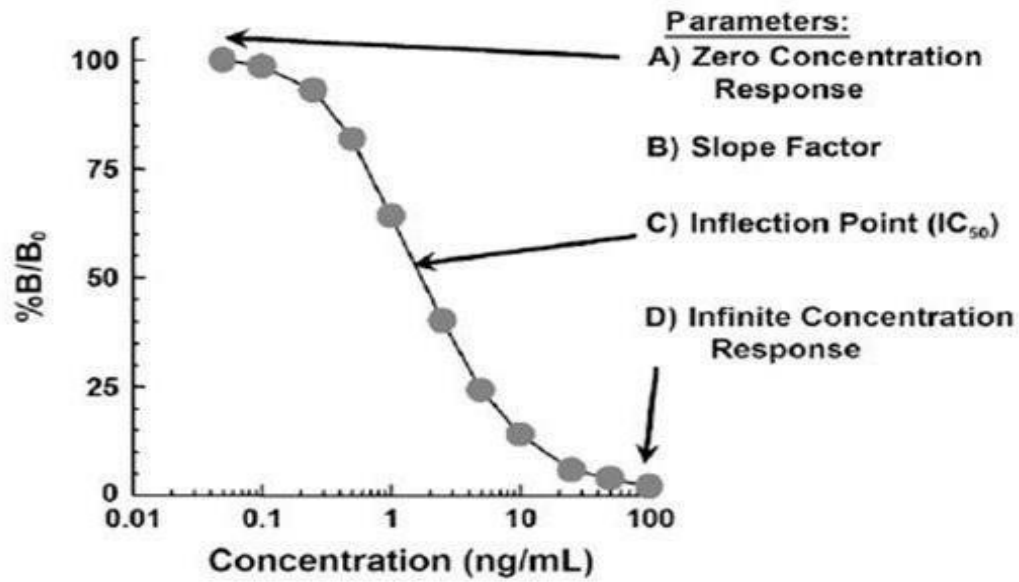
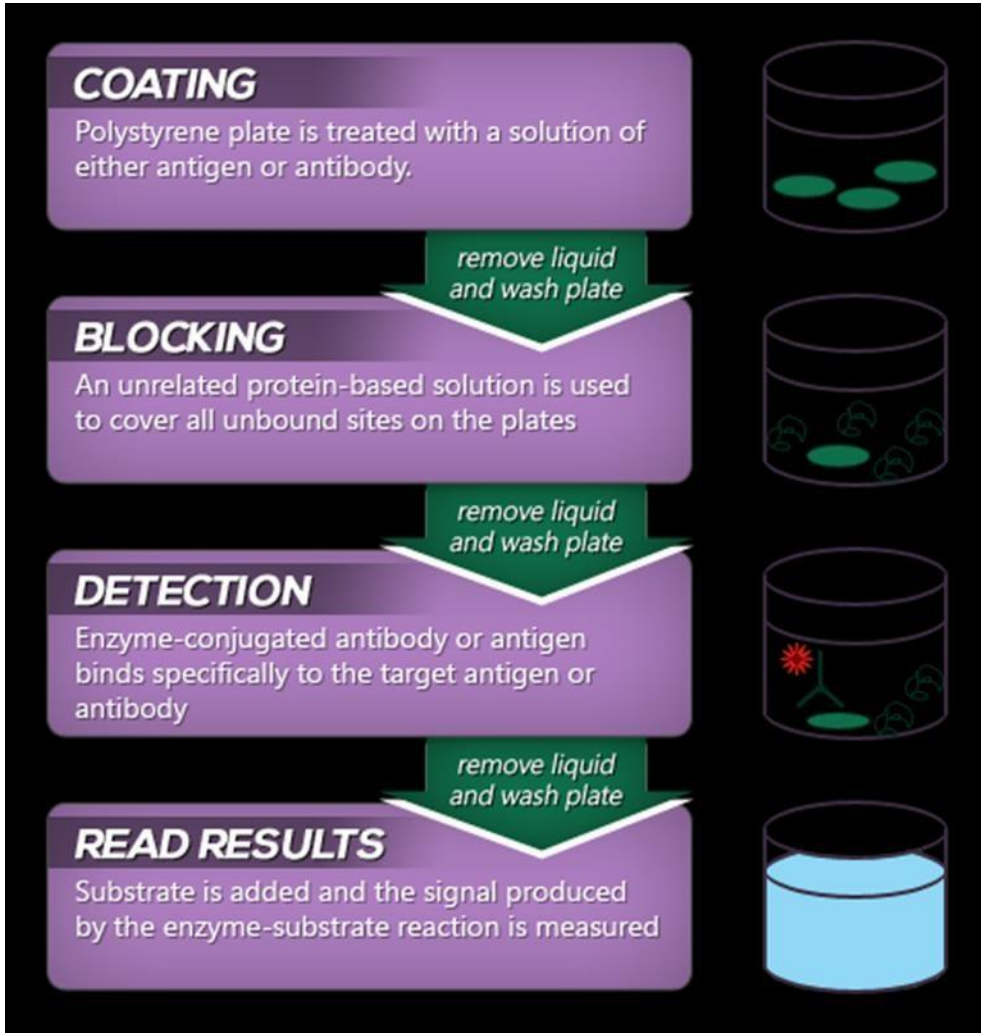


Figure 1. Typical 4-parameter logistic graph for a competitive-format immunoassay.

Enzymes of ELISA

Enzyme*	Source	Specific Enzyme activity (unitsΔ/mg)
Alkaline phosphatase	Calf intestine	400
Beta Galactosidase	E.coli	400
Glucose oxidase	Aspergillus Niger	200
Glucose -6-phosphate dehydrogenase	Leucon Stoc mesenteroides	250
Peroxidase	Horseradish	900

Δ A unit of Enzyme activity represents the conversion of 1 µmol of Enzyme substrate to product per minute .



ENZYME SUBSTRATE

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

ENZYME	SUBSTRATE	CHROMOGEN	STOPPING
Alkaline Phosphatase	p-NPP	p-NPP+ diethandamine+Mg Cl2	1 M NaOH
Horse radish Peroxidase	H2O2	Tetramethylbenzidine + Phosphate – Citrate buffer	1 M H2SO4
Horse radish Peroxidase	H2O2	O – Phenylenediamine + HCl	1 M HCl

14

Horseradish peroxidase and alkaline phosphatase retain high activity when conjugated with antibodies or antigens and have different types of substrates useful for different assays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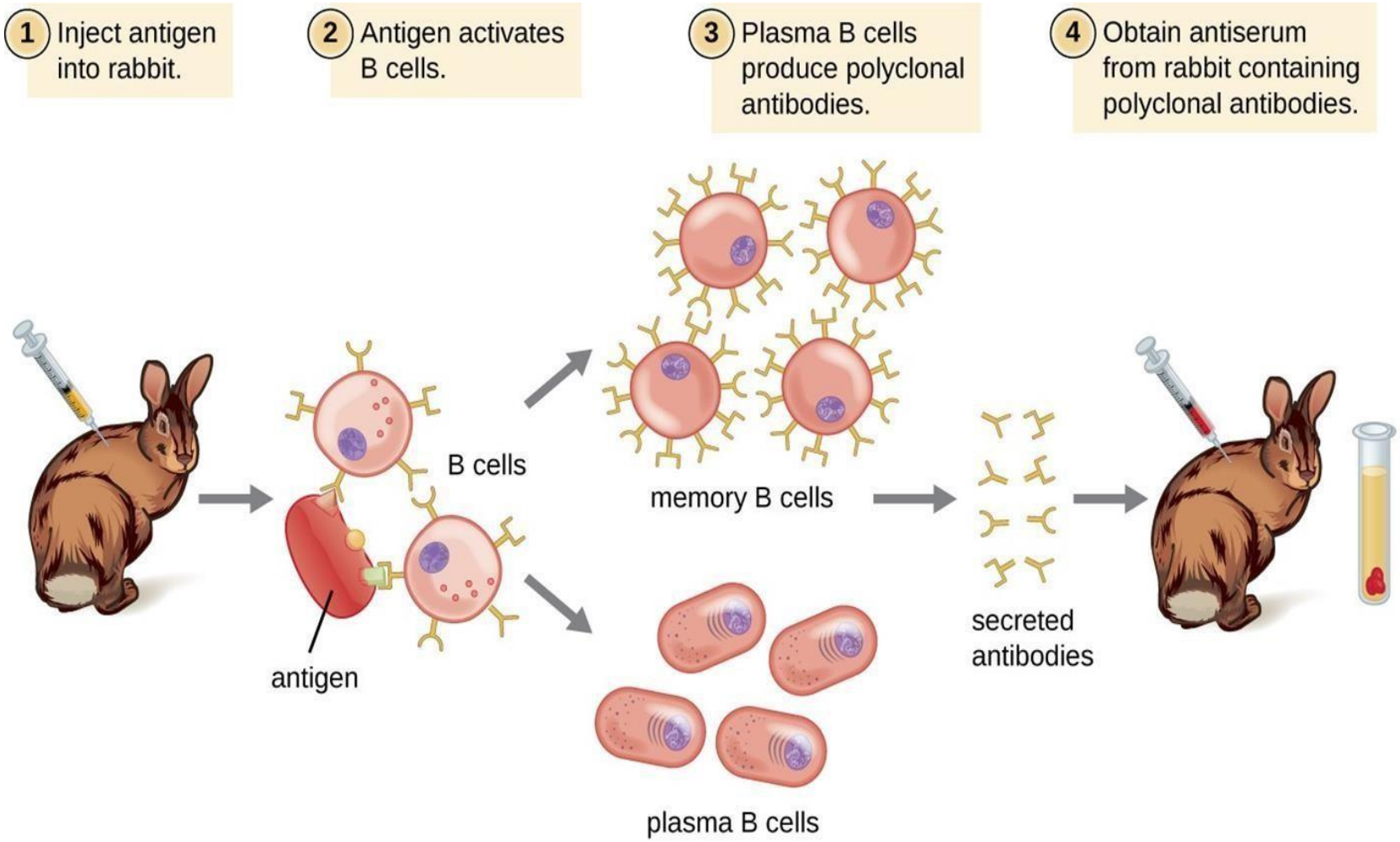
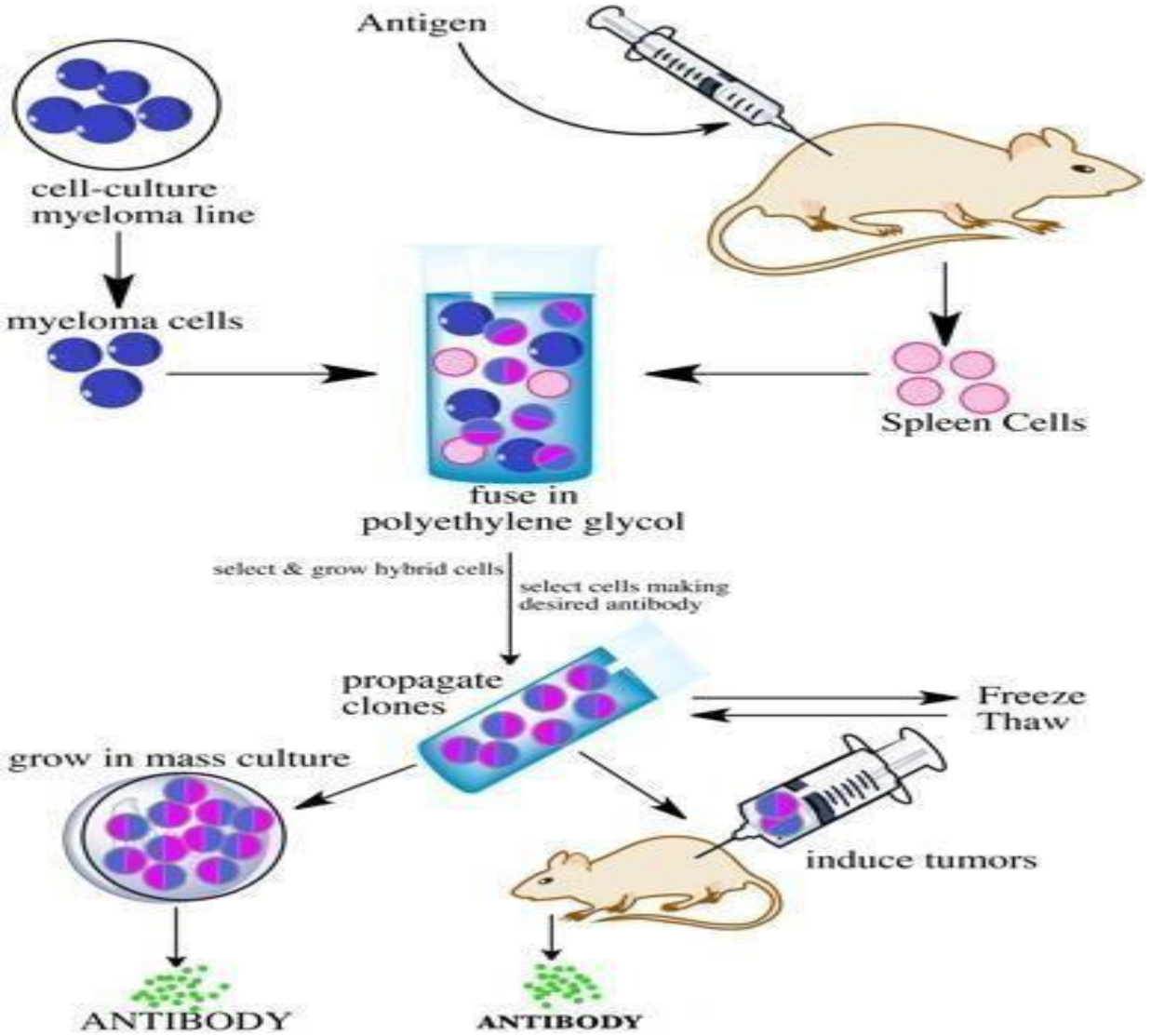
Buckwheat
Casein
Crustacean
Egg
Gluten
Hazelnut
Lupin
Mustard
Peanut
Sesame
Soy

Polyclonal antibodies production



Monoclonal antibodies



Recombinant antibodies

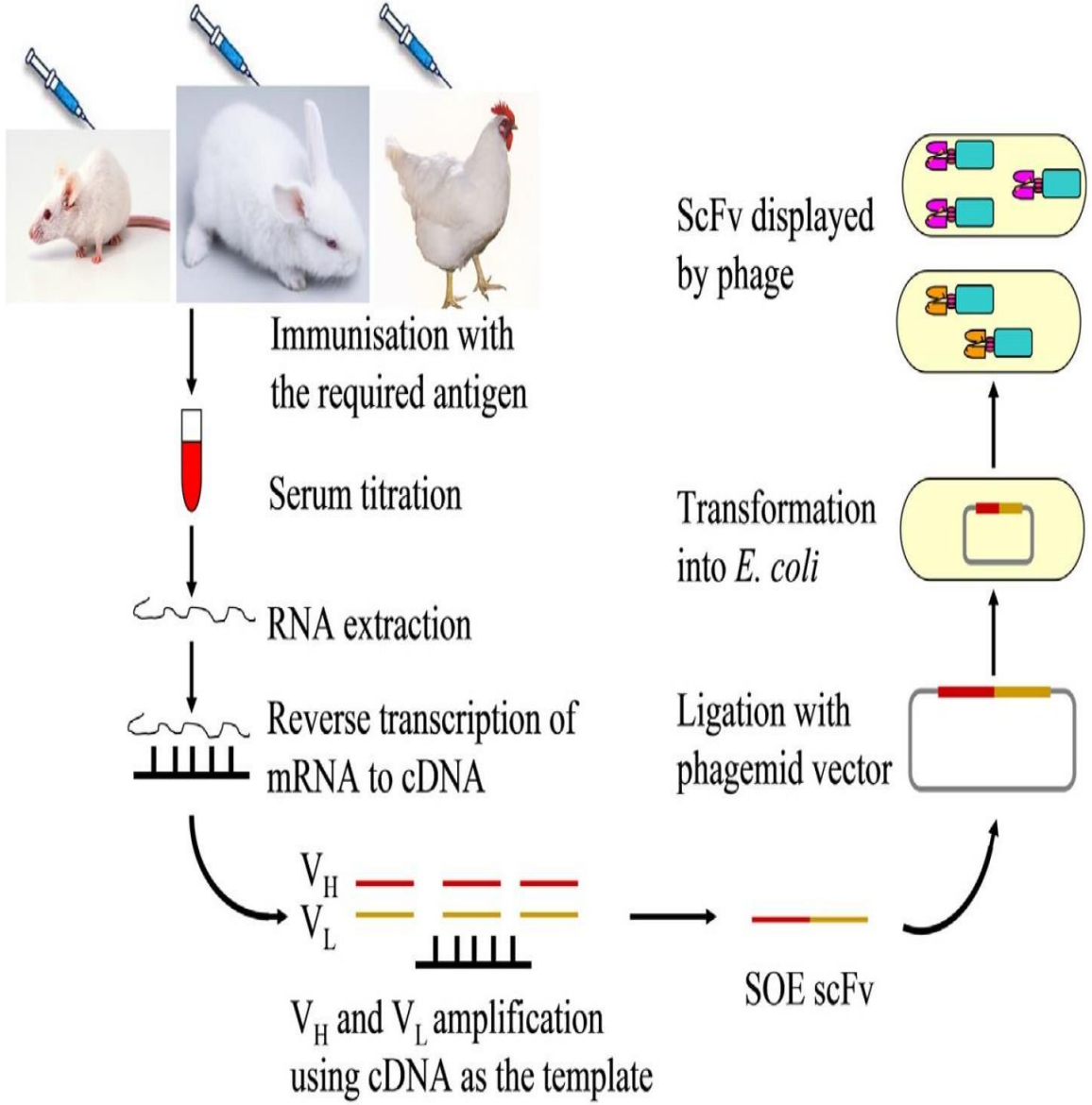


Fig. 1. Illustration of scFv library generation.

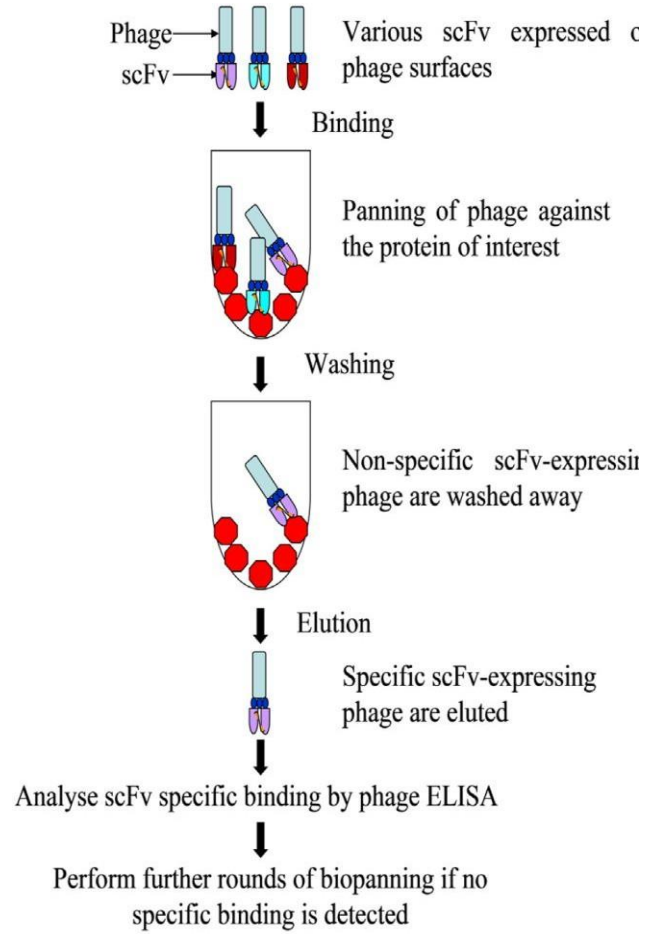
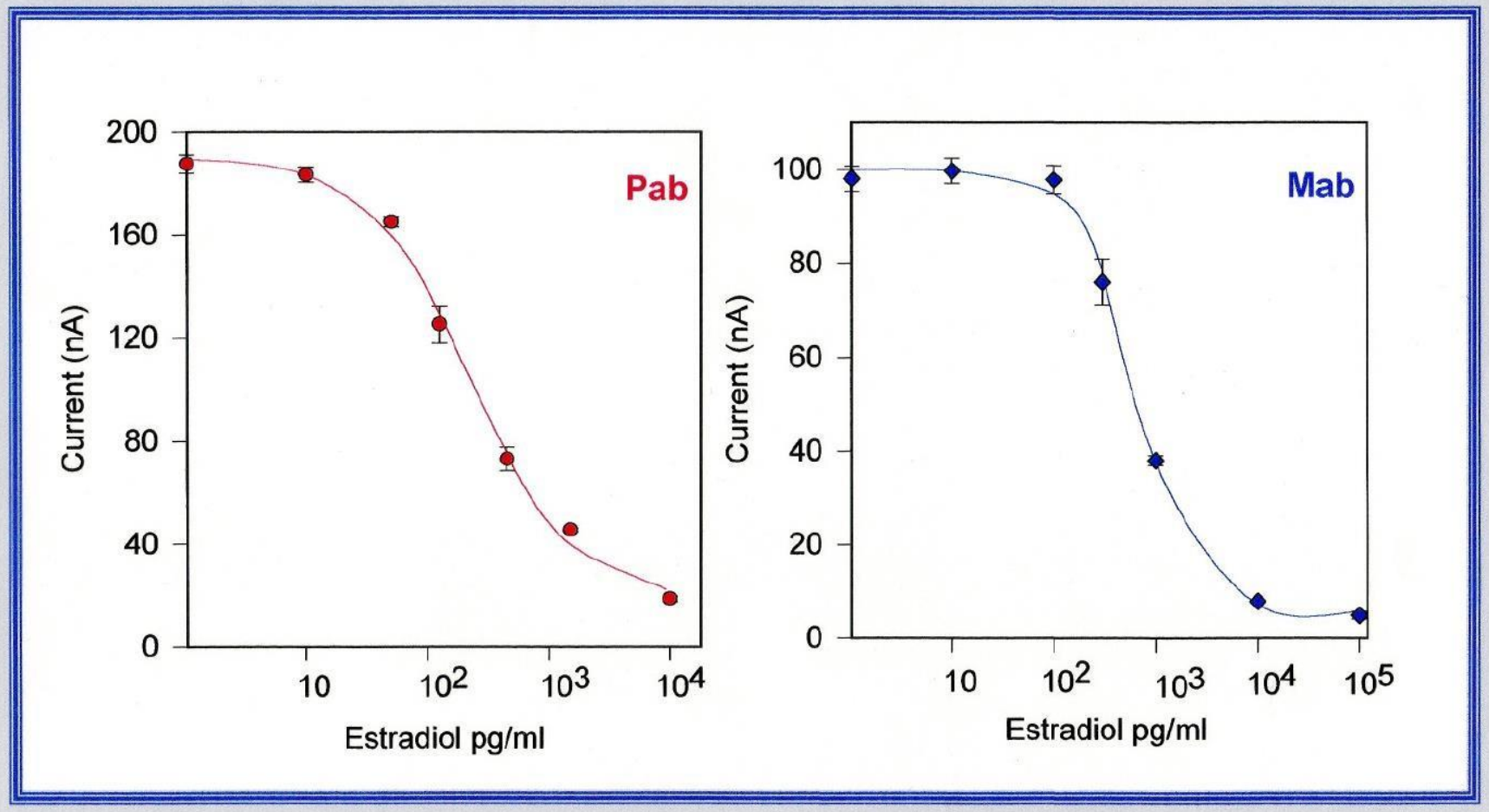


Fig. 2. Illustration of a typical panning cycle.

Enzyme Linked Immuno-Sorbent Assay

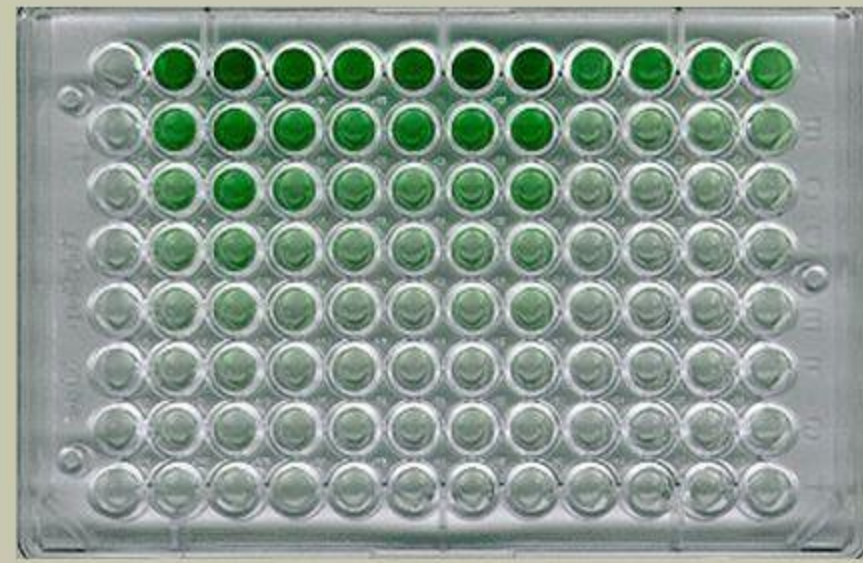
ELISA elettrochimico



Pab have wider analytical range sensitivity depends on K_{aff}

ELISA

- **Enzyme-linked immunosorbent assay** (**ELISA**) is a test that uses antibodies and color change to identify a substance.



Electrochemical immunosensors (labeled)

Chronoamperometry

Differential pulse voltammetry (DPV)

- **Enzymes and substrates:**

Alkaline phosphatase

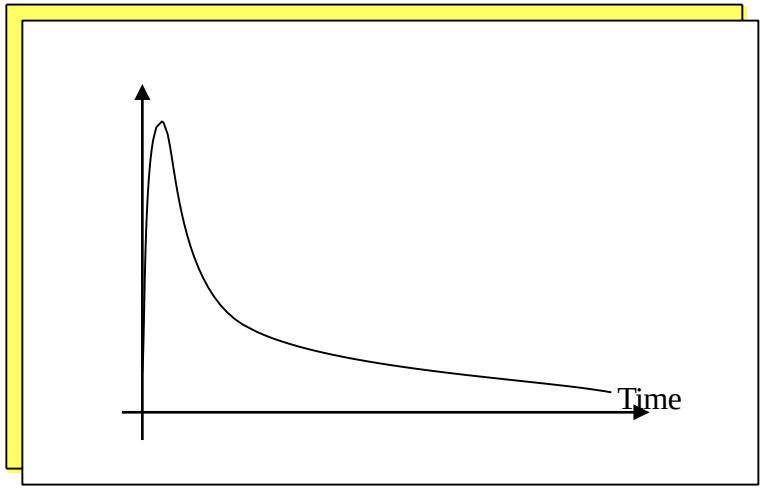
1-naphtyl-phosphate

Horseradish peroxidase

- TetramethylBenzidine + H_2O_2
- $[K_4Fe(CN)_6]$ + H_2O_2

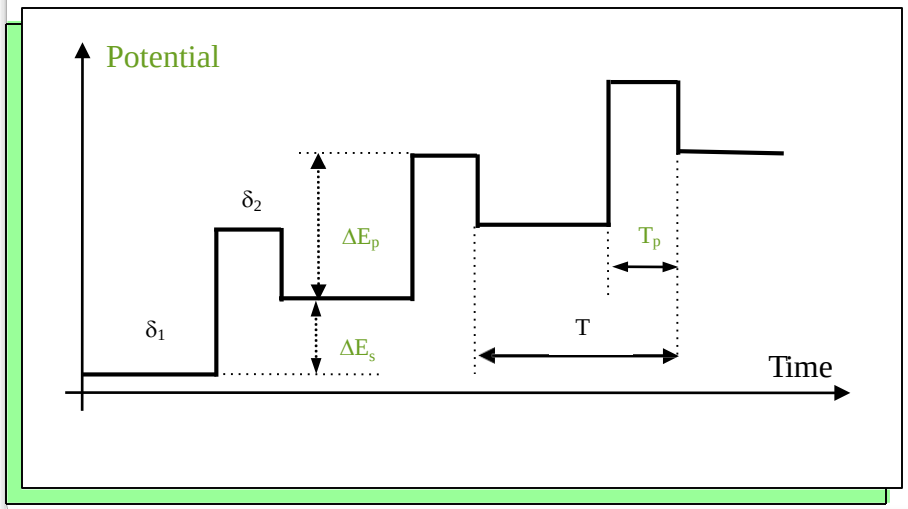
electrochemical detection:

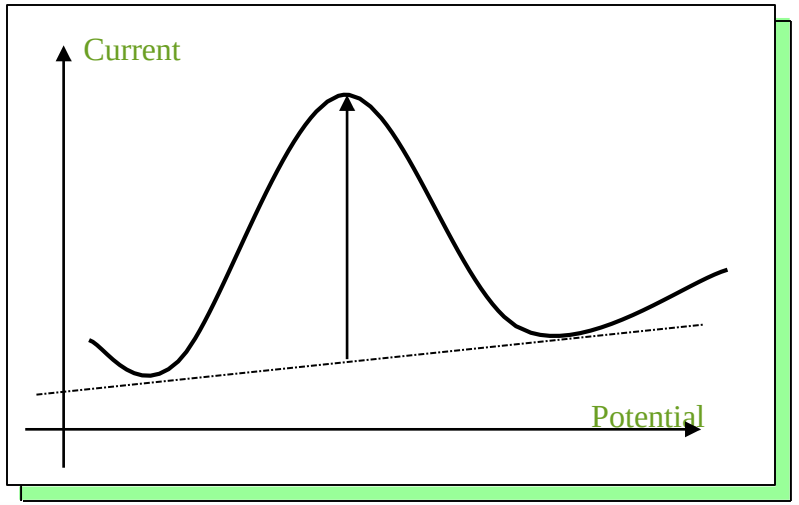
chronoamperometry and differential pulse voltammetry (DPV) :

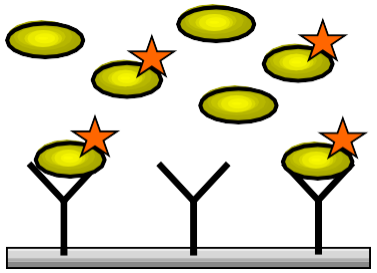


δ_2= 60 ms

ΔE_p= 5-100 mV







saggio diretto

Anti OTA-Ab

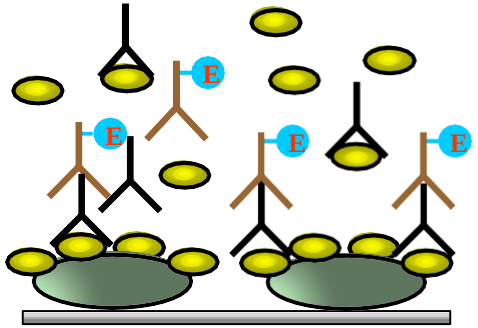
OTA

OTA-ALP

BSA-OTA

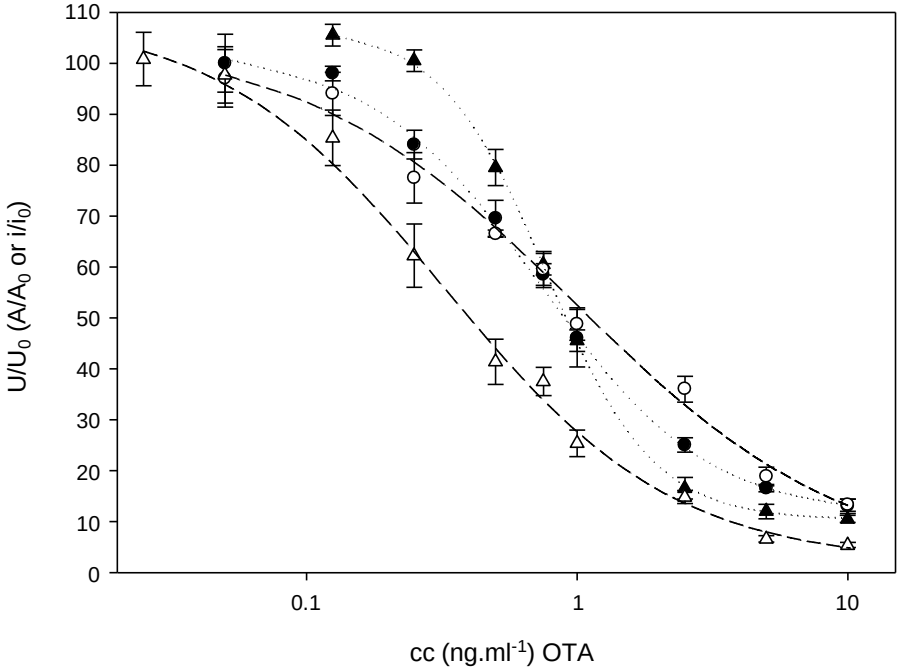
SAb-ALP

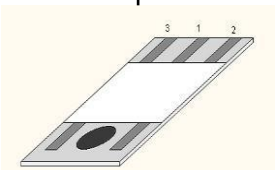
Anti OTA-Ab

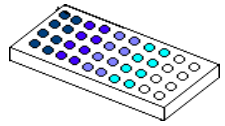


saggio indiretto

Ocratossina -OTA







$$f(x) = \{(a - d)/[1 + (x/c)b]\} + d$$

	Competition curve parameters				Linear regression
	a (A or nA)	*b* (nA.ng.ml^{-1})	*c* (ng.ml^{-1})	*d* (A or nA)	
ic **spettr**	1.220 ± 0.053	1.40 ± 0.50	0.80 ± 0.22	0.129 ± 0.077	$f(x) = 49.3\ (\pm 0.8) - 57.5\ (\pm 0.1)\ x$ [$r = 0.991$]
ic **amp.**	6019 ± 118	0.90 ± 0.22	0.93 ± 0.10	176 ± 30	$f(x) = 52.5\ (\pm 0.4) - 43.7\ (\pm 0.5)\ x$ [$r = 0.994$]
dc **spettr**	1.392 ± 0.061	2.17 ± 0.15	0.80 ± 0.14	0.132 ± 0.071	$f(x) = 47.4\ (\pm 0.7) - 86.0\ (\pm 0.3)\ x$ [$r = 0.993$]
dc **amp.**	707 ± 56	1.10 ± 0.10	0.35 ± 0.04	16 ± 13	$f(x) = 34.9\ (\pm 0.6) - 52.2\ (\pm 0.9)\ x$ [$r = 0.992$]

Immunoassay	**Working Range** (ng/ml)	**L.O.D.** (Blank – 3 σ) (ng/ml)
ic spettr	0.20 – 2.5	0.150
ic **amp.**	**0.10 – 7.5**	**0.120**
dc **spettr**	0.10 – 10	0.080
dc **amp.**	**0.05 – 2.5**	**0.060**

Immunosensor procedure:

✓Pre-coating: 6 μl of rabbit anti IgG (4° C overnight)

✓Blocking: 6 μl of 1 % PVA (polyvinyl alcohol) (30 min)

✓Coating: 6 μl of anti-OTA Ab (1 h)

✓Competition: 6 μl of OTA-AP + standard/sample (30 min)

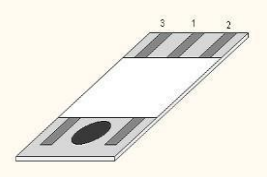
✓Detection: 100 of 5 mg/ml 1-Naphtylphosfate (2 min) + DPV

washings: 150 μl phosphate buffer pH 7.4

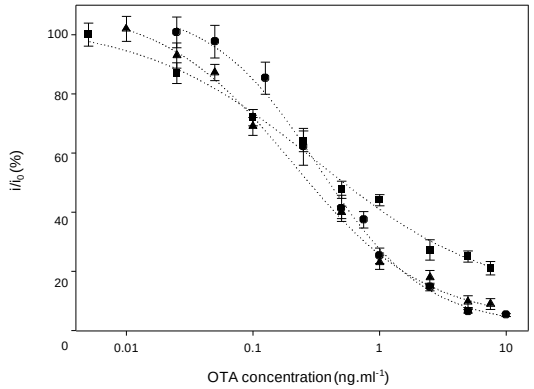
effect of extraction solvent

Activity of an electrode modified with IgG-ALP after 30 min incubation with 1:9 - 9:1 solutions (1:1 in DPBS) acetonitrile:water → 95-108%

Sensitivity of the calibration curve ~ 50%



25 g in 100 mL di ACN:H_2O



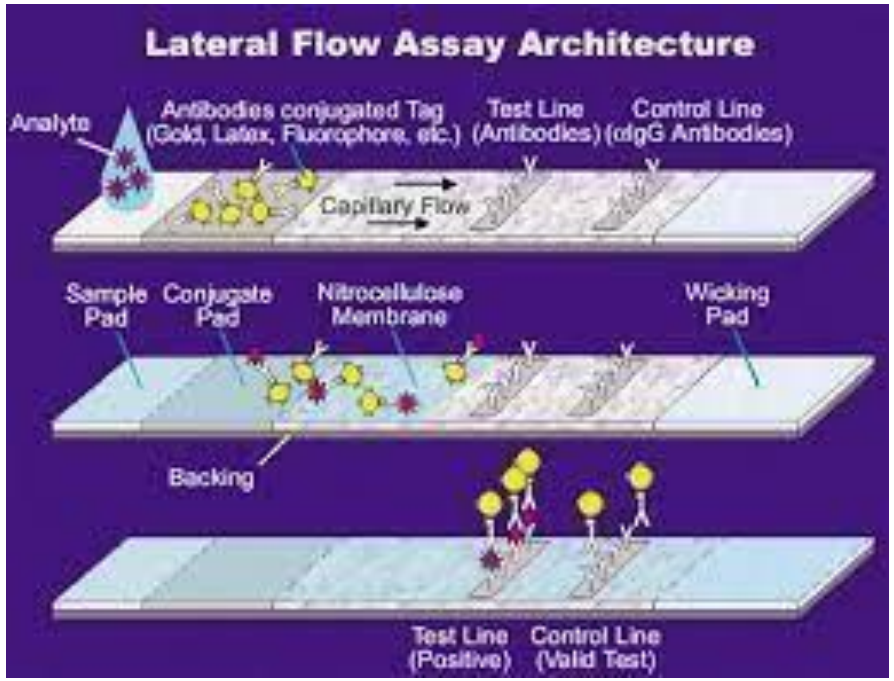
Final dilution 1:8

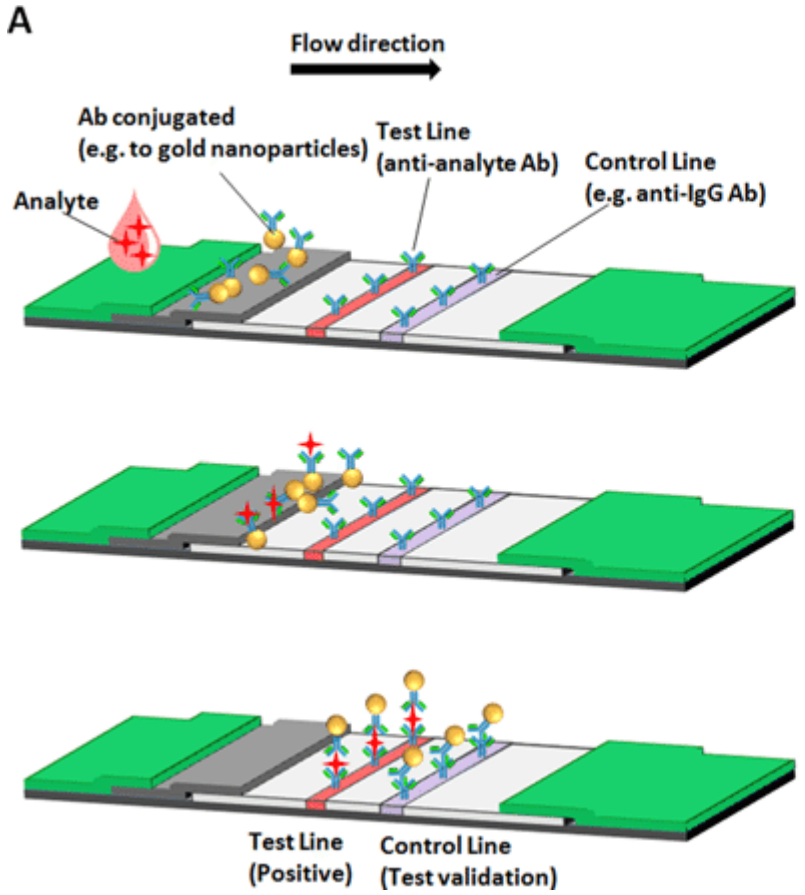
Maximum Residue Limit = 3 ng/g

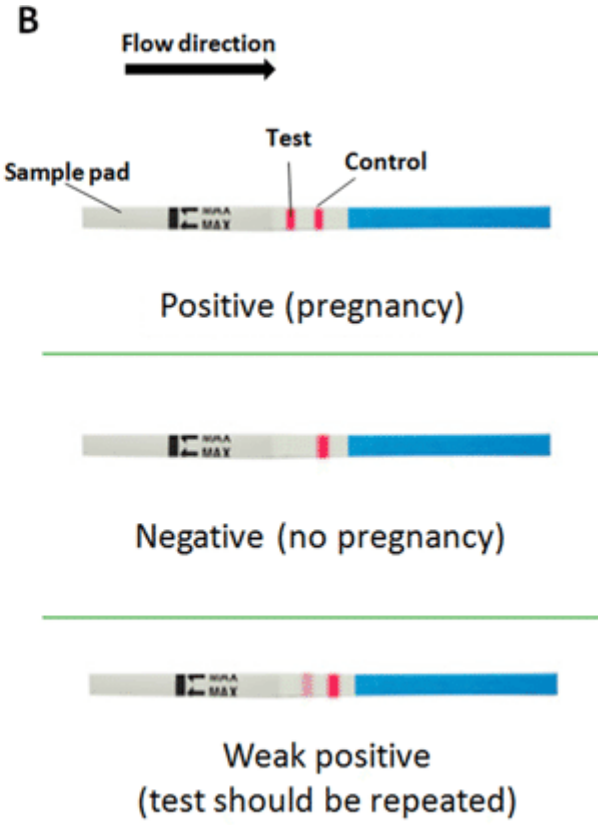
I_{50} = 1.6 ng/g

Parameters		0.1 PBS Buffer ●	ACN:H_2O (6:4) ▲	Wheat Extract (blank) ■
a	(nA)	707 (± 56)	260 (± 12)	408 (± 72)
b	(nA.ng.ml^{-1})	1.1 (± 0.1)	0.62 (± 0.03)	0.8 (± 0.1)
c (I_{50})	(ng.ml^{-1})	0.35 (± 0.04)	0.32 (± 0.02)	0.20 (± 0.03)
d	(nA)	16 (± 13)	24 (± 8)	13 (± 15)
w.r.	(ng.ml^{-1})	0.05 – 2.5	0.02 – 5.0	0.05 – 2.5
L.O.D.	(ng.ml^{-1})	0.06	0.015	0.05
Lin.	**Reg.**	30.9 (± 0.6) – 52.2 (± 0.9) *x*	42.3 (± 0.3) – 25.4 (± 0.6) *x*	23.5 (± 0.1) – 41.1 (± 0.5) *x*

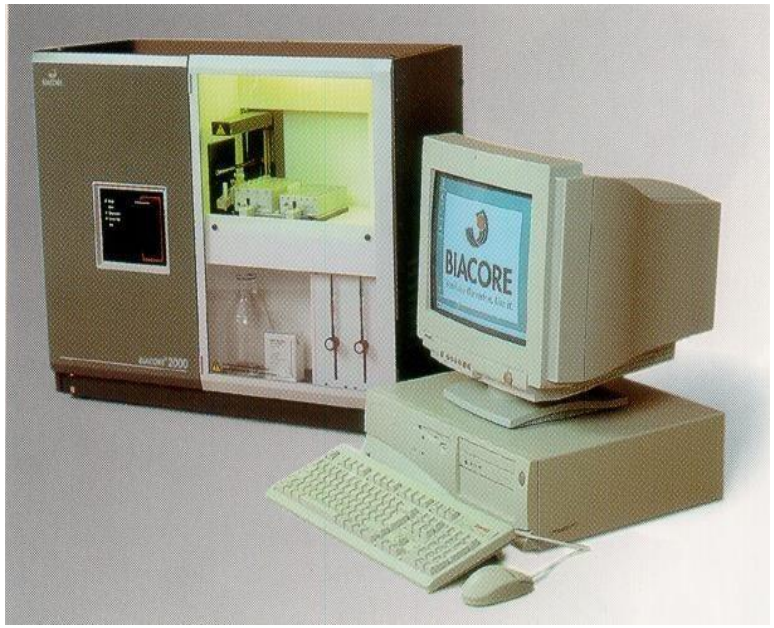
Lateral flow immunoassays

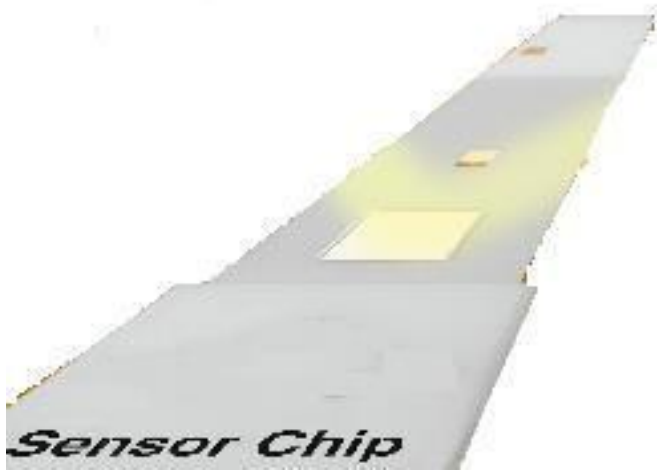


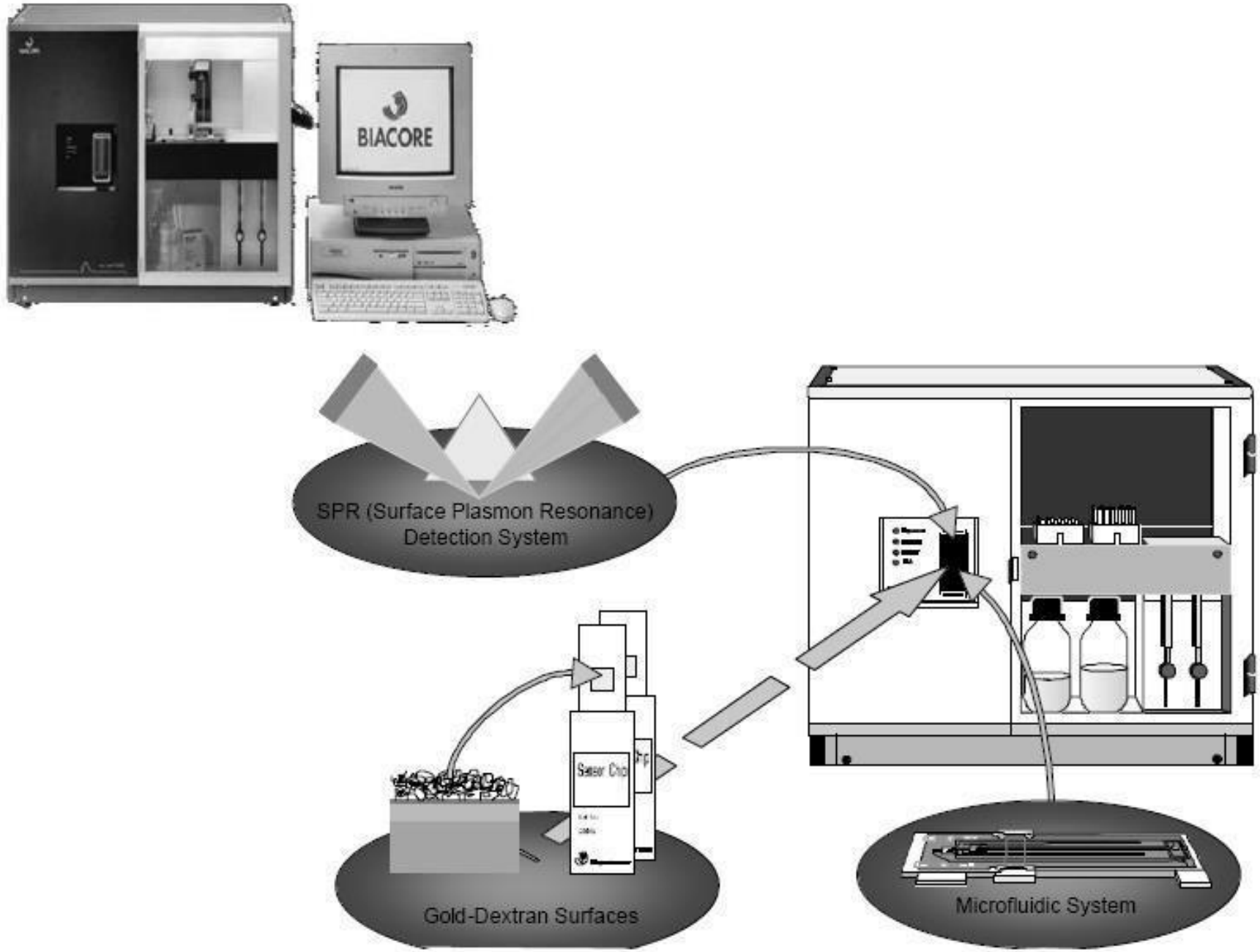




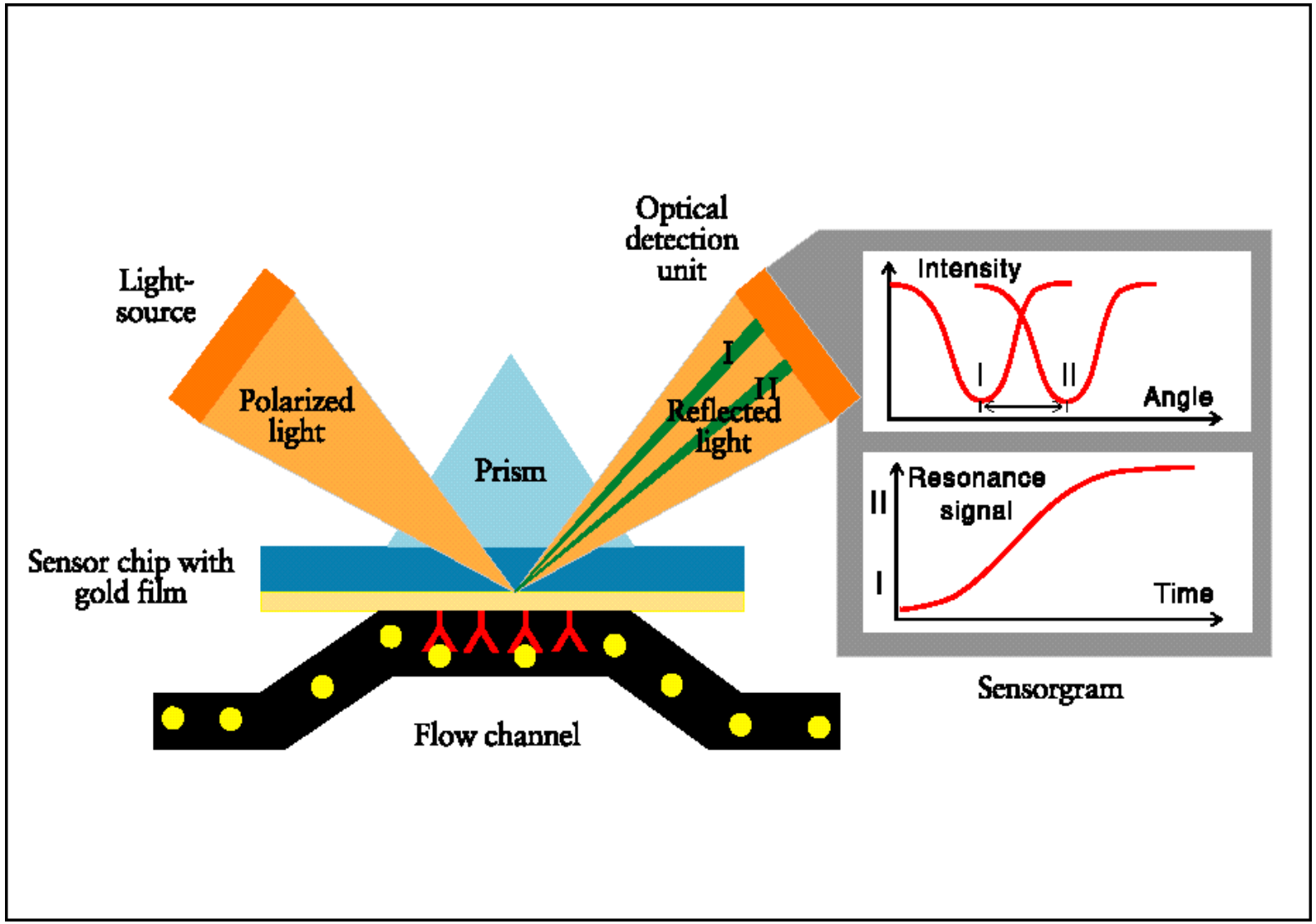
Biacore



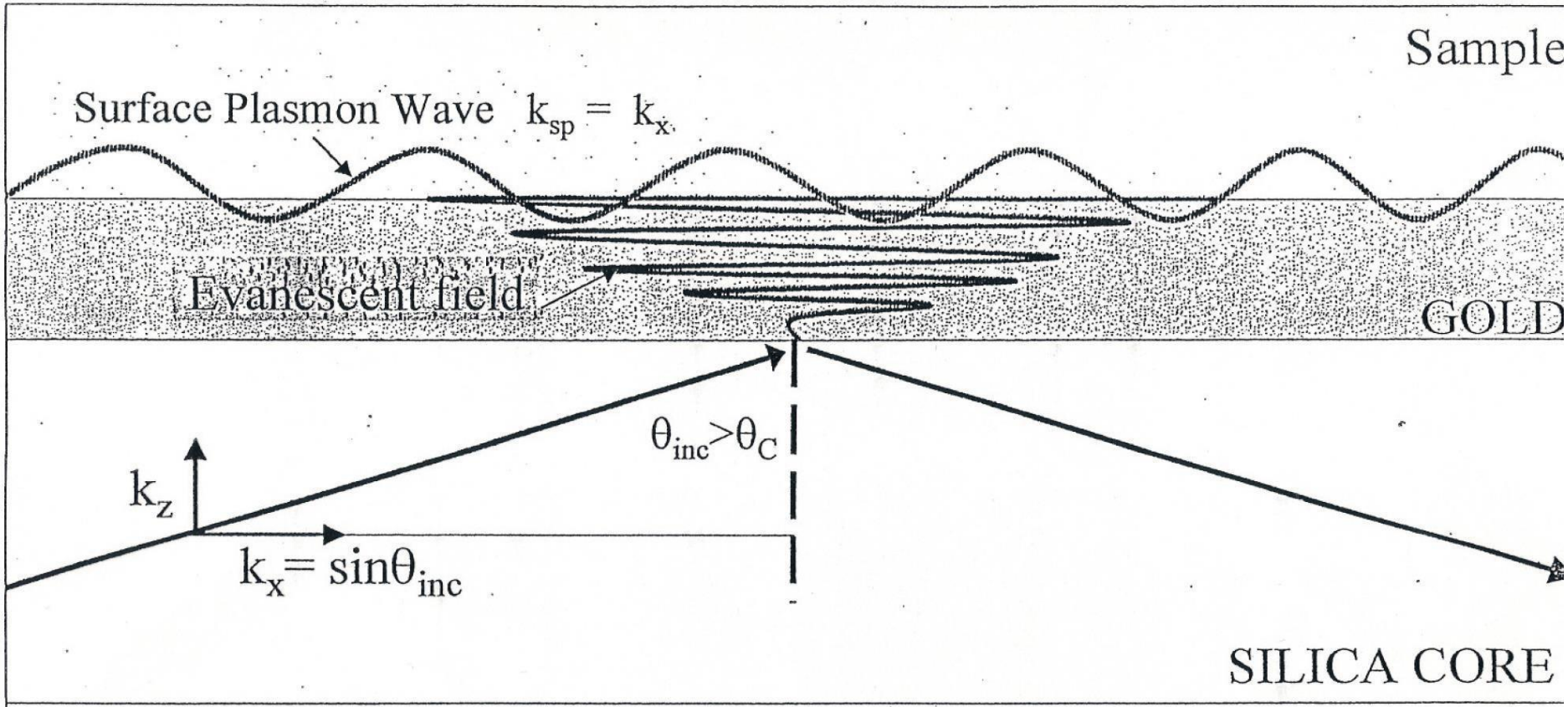



BIACORE
SPR (Surface Plasmon Resonance)
Detection System
Gold-Dextran Surfaces
Microfluidic System

SPR Biosensors_label-free!!



Surface Plasmon Resonance



θ_{inc} - angle of incident light

λ_{inc} - wavelength of incident light

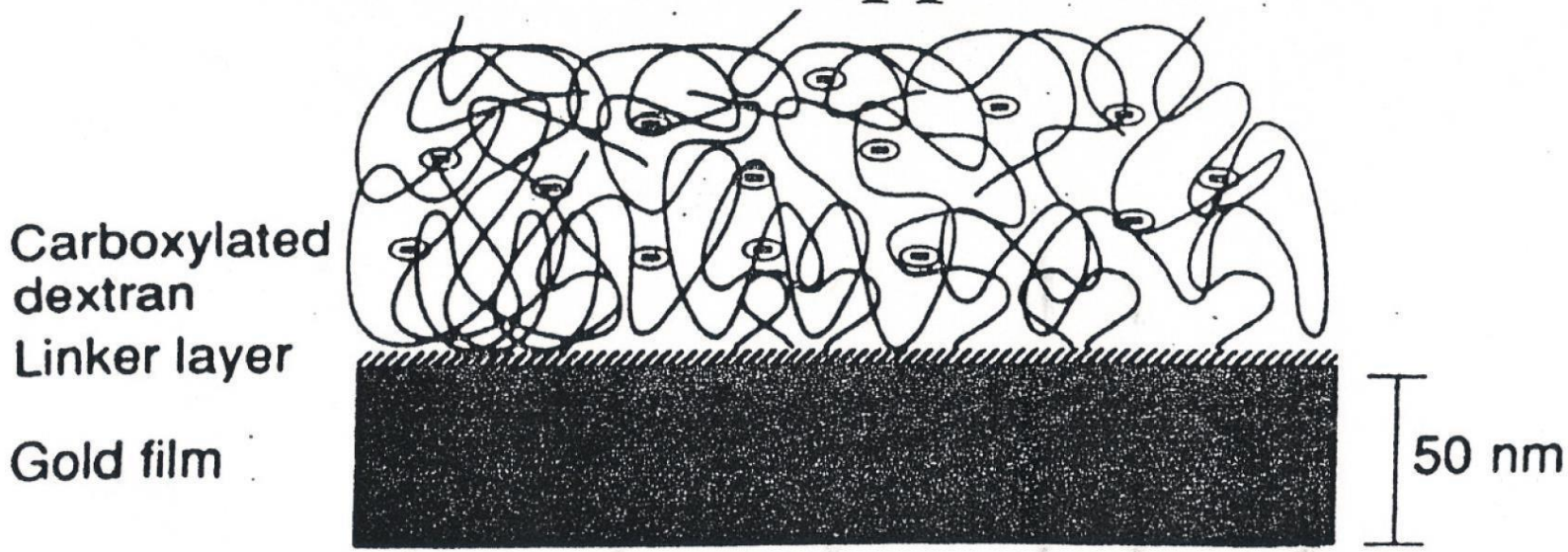
n_{glass} - Refractive Index of glass

n_{metal} - Refractive Index of metal

n_{sample} - Refractive index of sample

t_{metal} - Thickness of metal

BIACORE approach



dextran hydrogel

open structure (good accessibility)

no denaturation

- **enhancement of the capacity of the interaction layer**
- **stagnant layer / mass transport flow needed (µl/min)**
- **negative charge**
- **regenerable (up to 100 x)**

SPR principles

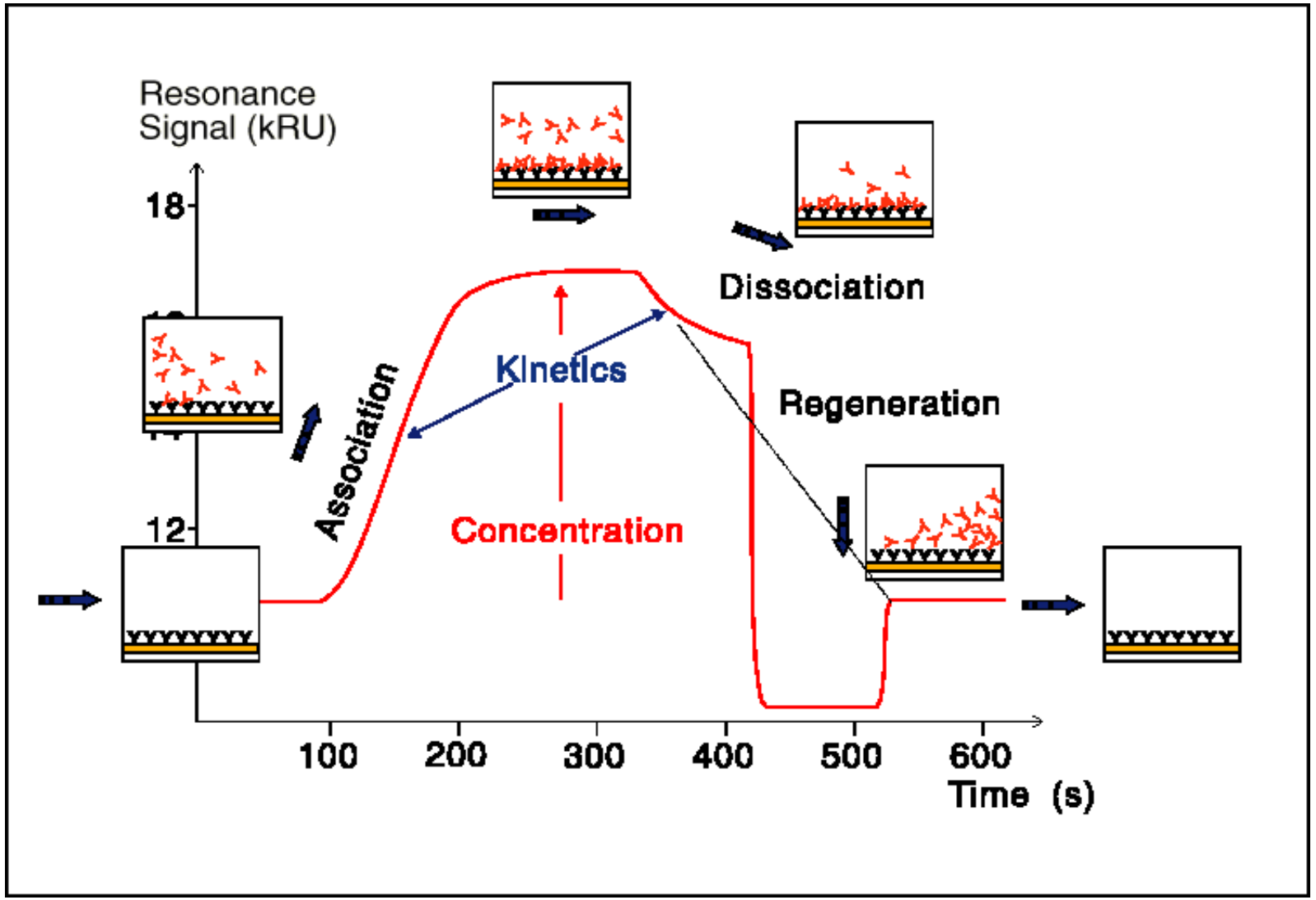
Surface plasmon resonance (SPR) arises when light is reflected under certain conditions from a conducting film at the interface between two media of different refractive index. the media are the sample and the glass of the sensor chip, and the conducting film is a thin layer of gold on the chip surface. SPR causes a reduction in the intensity of reflected light at a specific angle of reflection. This angle varies with the refractive index close to the surface on the side opposite from the reflected light.

When molecules in the sample bind to the sensor surface, the concentration and therefore the refractive index at the surface changes and an SPR response is detected. Plotting the response against time during the course of an interaction provides a quantitative measure of the progress of the interaction. This plot is called a sensorgram.

What Biacore actually measures is the angle of minimum reflected light intensity. The light is not absorbed by the sample: instead the light energy is dissipated through SPR in the gold film. Thus the light used to detect interaction processes never enters the sample.

SPR response values are expressed in resonance units (RU). One RU represents a change of 0.0001° in the angle of the intensity minimum. For most proteins, this is roughly equivalent to a change in concentration of about 1 pg/mm2 on the sensor surface. The exact conversion factor between RU and surface concentration depends on properties of the sensor surface and the nature of the molecule responsible for the concentration change.

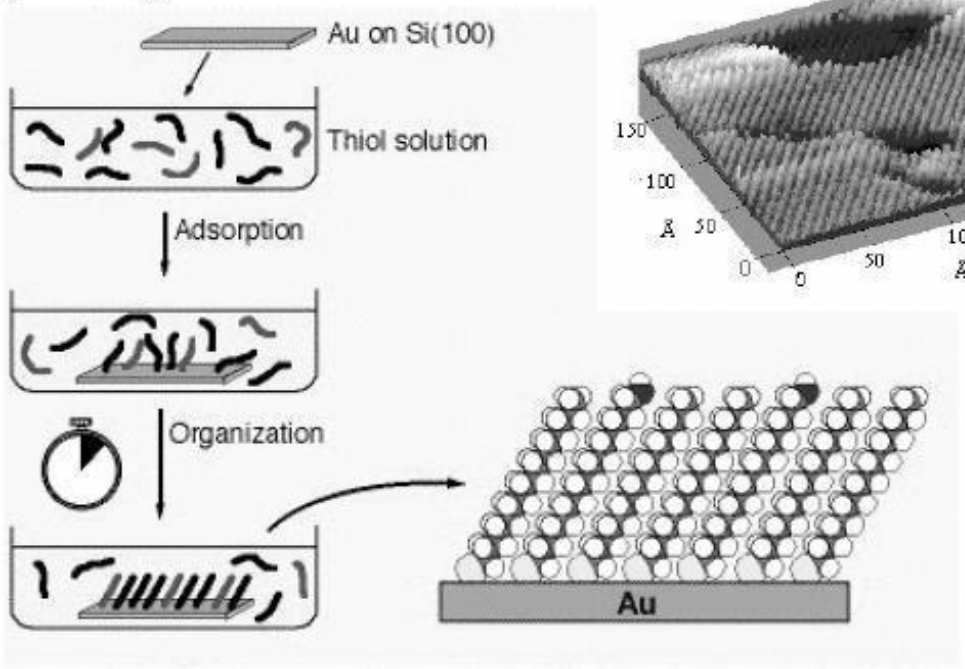
Sensorgram

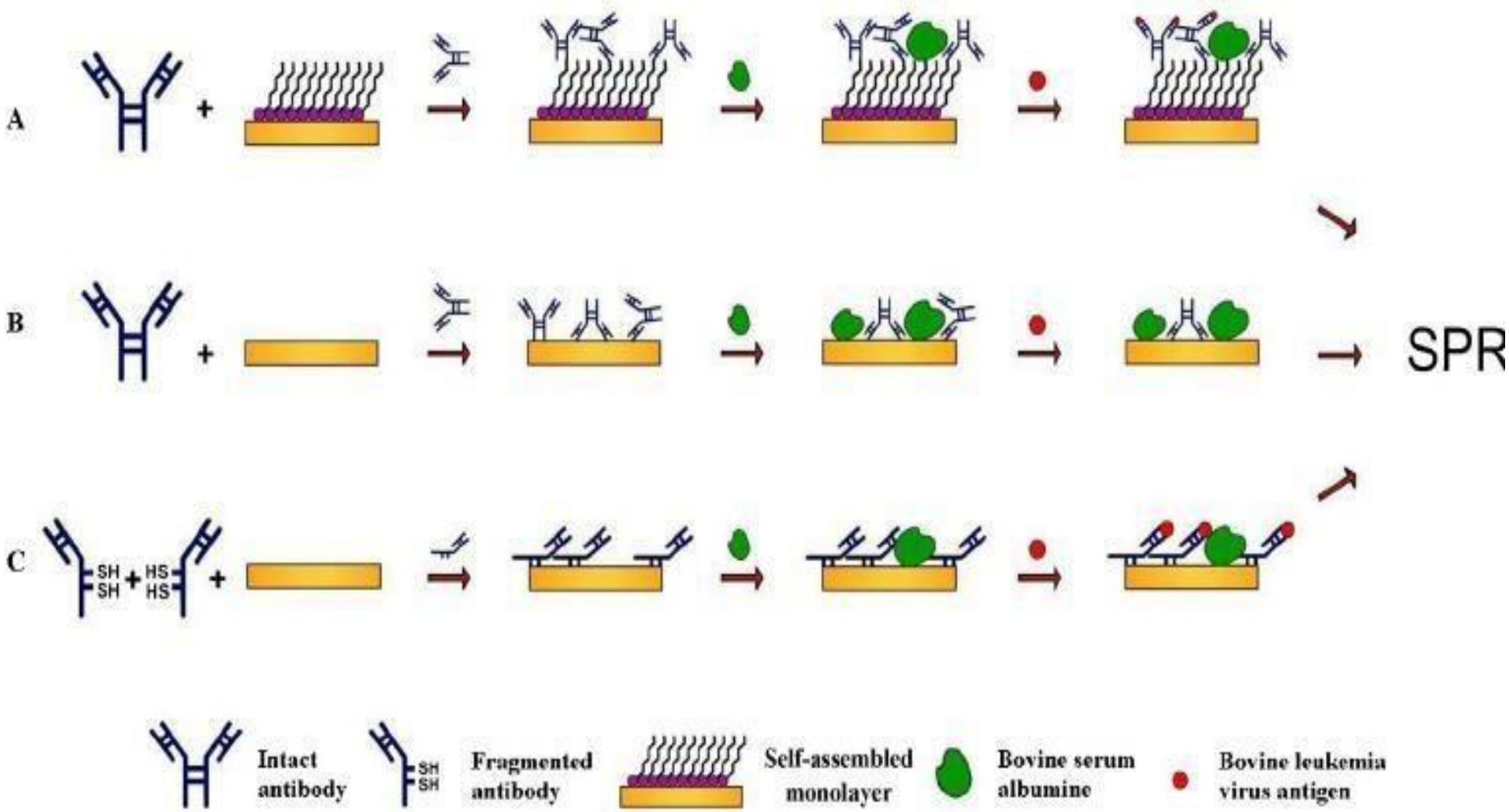

Resonance
Signal (kRU)
18
12
Association
Kinetics
Concentration
Dissociation
Regeneration
100
200
300
400
500
600
Time (s)

Immobilisation of organic molecules on gold

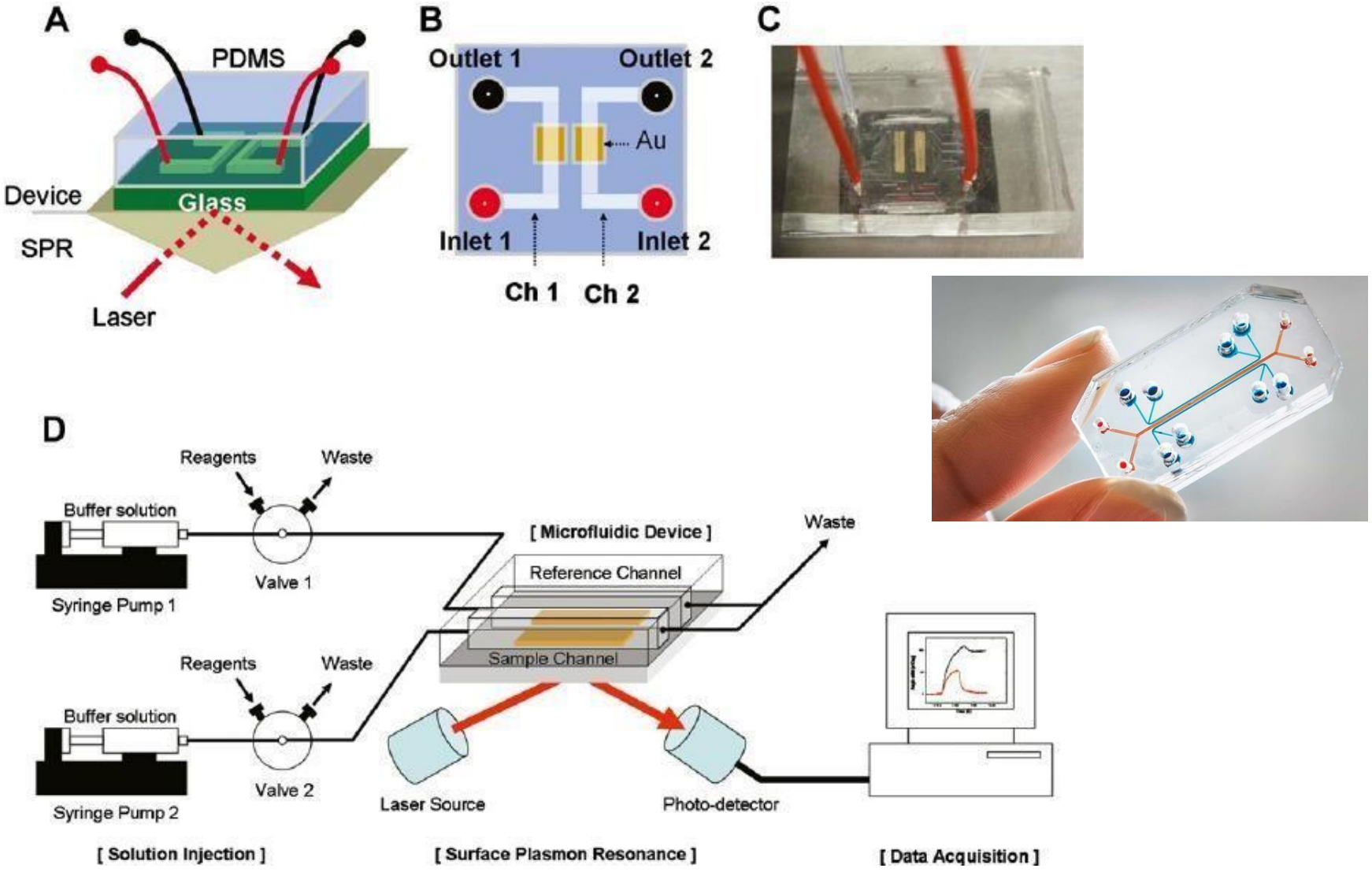
Self Assembled Monolayers (SAM)

Disulphides (R-S-S-R)
Sulphides (R-S-R)
Thiols (R-SH)



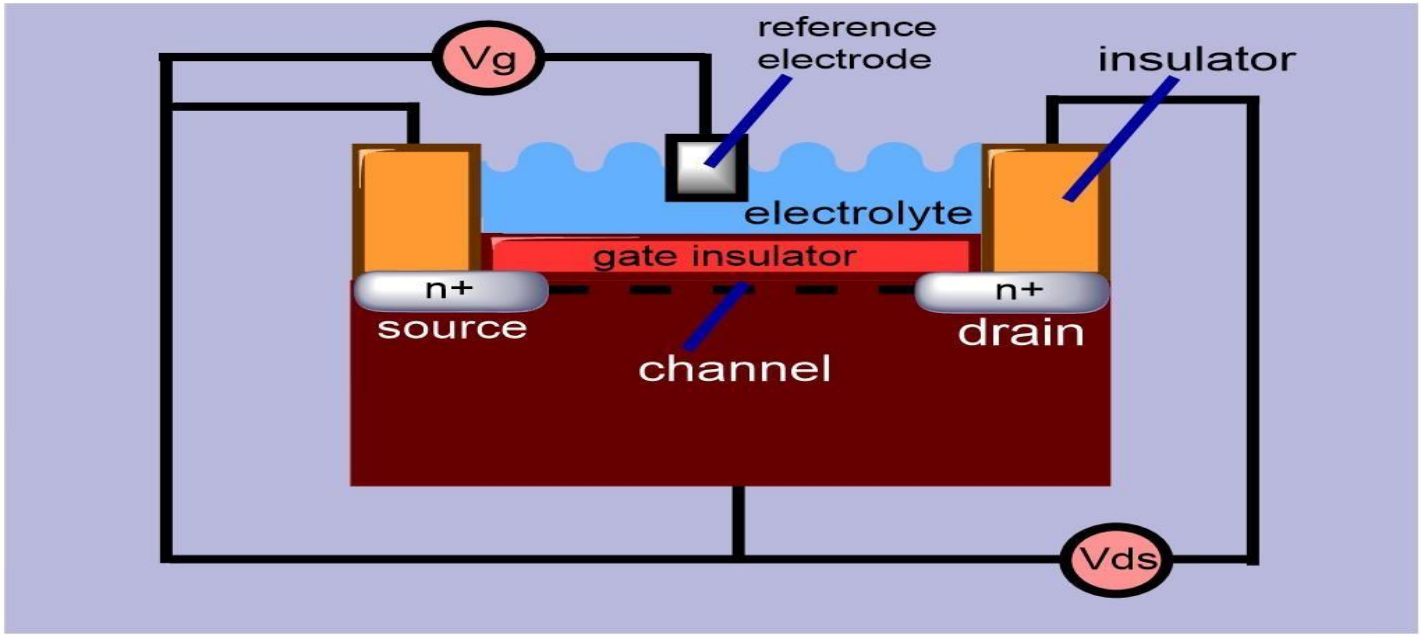

A
B
C
SH
SH
HS
HS
SPR
Intact antibody
Fragmented antibody
Self-assembled monolayer
Bovine serum albumine
Bovine leukemia virus antigen

Spr sensors and microfluidics

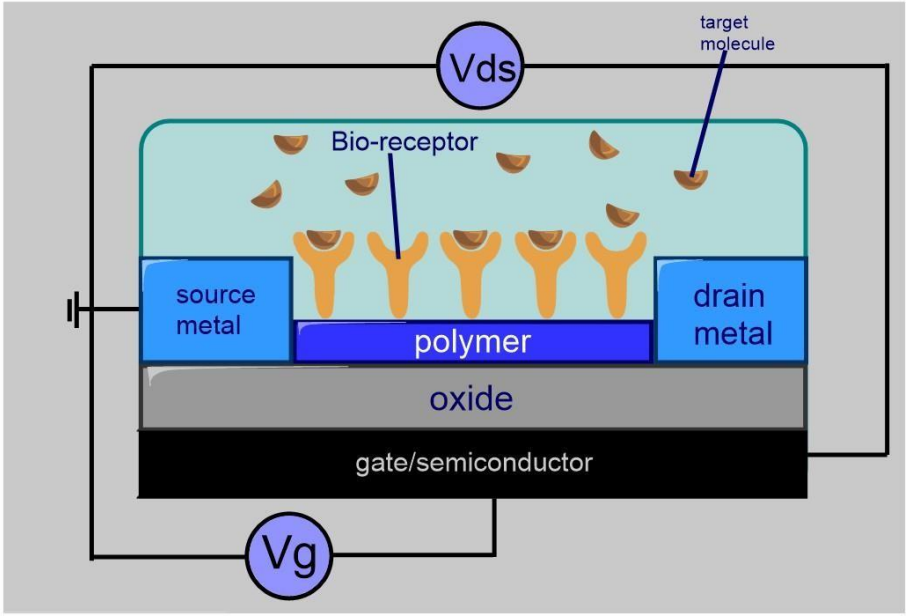


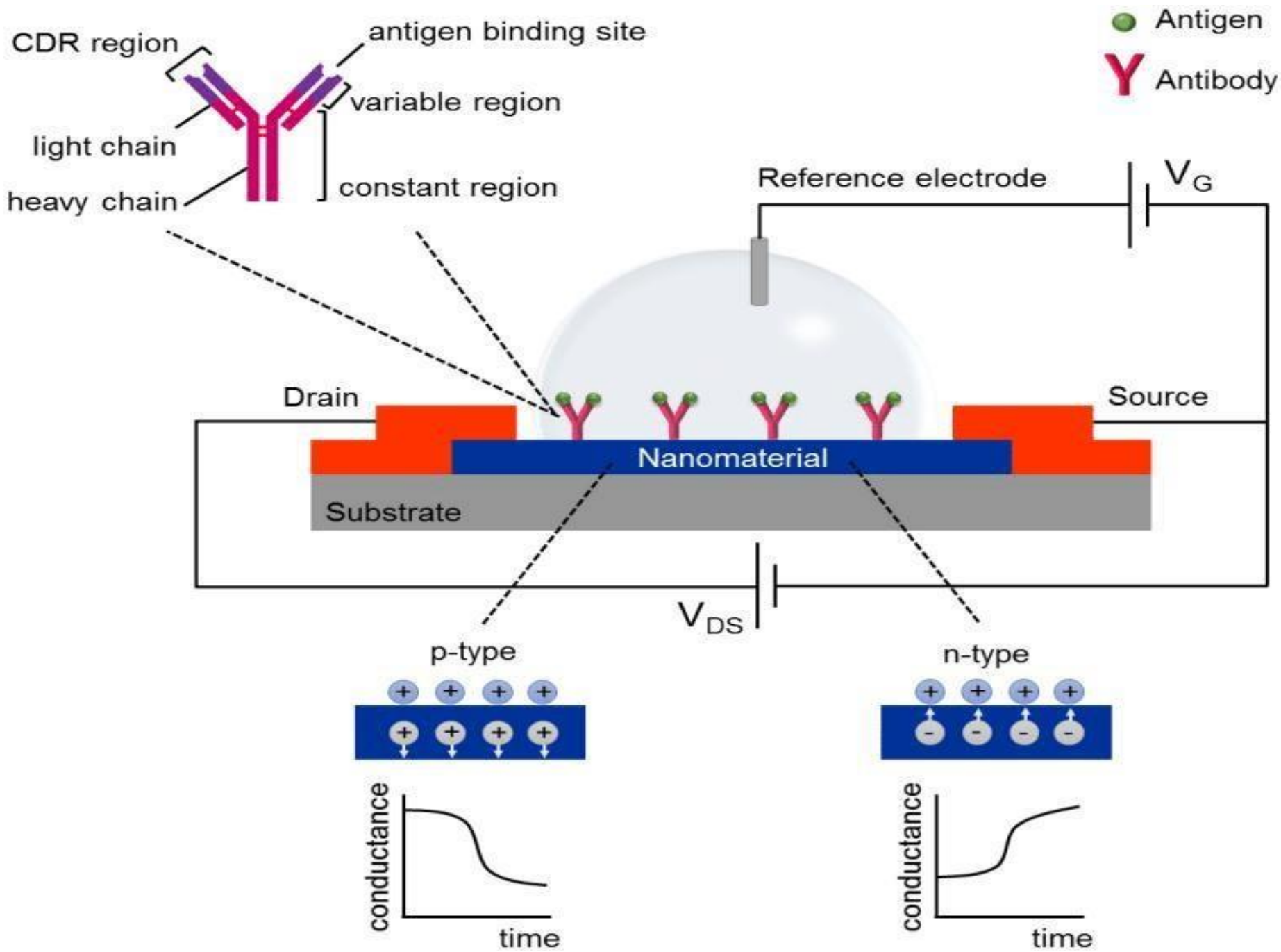
BIO(immuno)FETs label-free!

An **ion-sensitive field-effect transistor** (**ISFET**) is a field-effect transistor used for measuring ion concentrations in solution; when the ion concentration (such as H^+, see pH scale) changes, the current through the transistor will change accordingly. Here, the solution is used as the gate electrode. A voltage between substrate and oxide surfaces arises due to an ion sheath. It is a special type of MOSFET (metal-oxide-semiconductor field-effect transistor),[1] and shares the same basic structure, but with the metal gate replaced by an ion-sensitive membrane, electrolyte solution and reference electrode.[2] Invented in 1970, the ISFET was the first biosensor FET (BioFET) source wikipedia

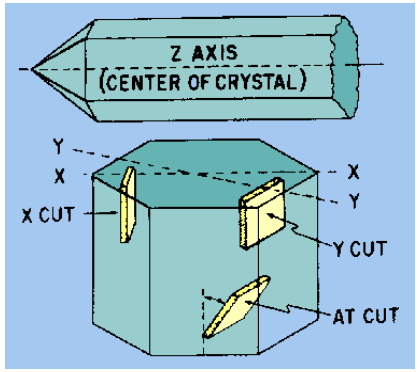


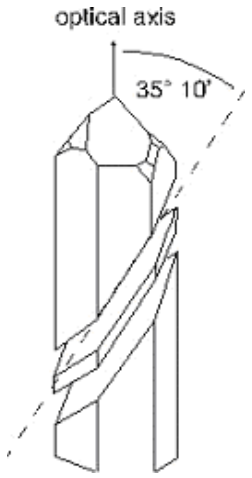
Bio-FETs couple a transistor device with a bio-sensitive layer that can specifically detect bio-molecules such as nucleic acids and proteins. A Bio-FET system consists of a semiconducting field-effect transistor that acts as a transducer separated by an insulator layer (e.g. SiO_2) from the biological recognition element (e.g. receptors or probe molecules) which are selective to the target molecule called analyte.[8] Once the analyte binds to the recognition element, the charge distribution at the surface changes with a corresponding change in the electrostatic surface potential of the semiconductor. This change in the surface potential of the semiconductor acts like a gate voltage would in a traditional MOSFET, i.e. changing the amount of current that can flow between the source and drain electrodes.[9] This change in current (or conductance) can be measured, thus the binding of the analyte can be detected. The precise relationship between the current and analyte concentration depends upon the region of transistor operation (source Wikipedia)

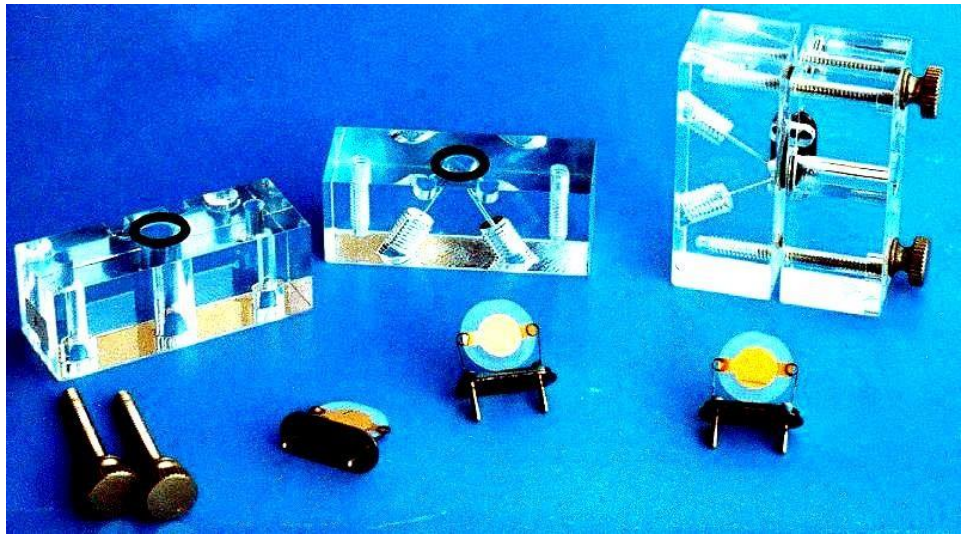


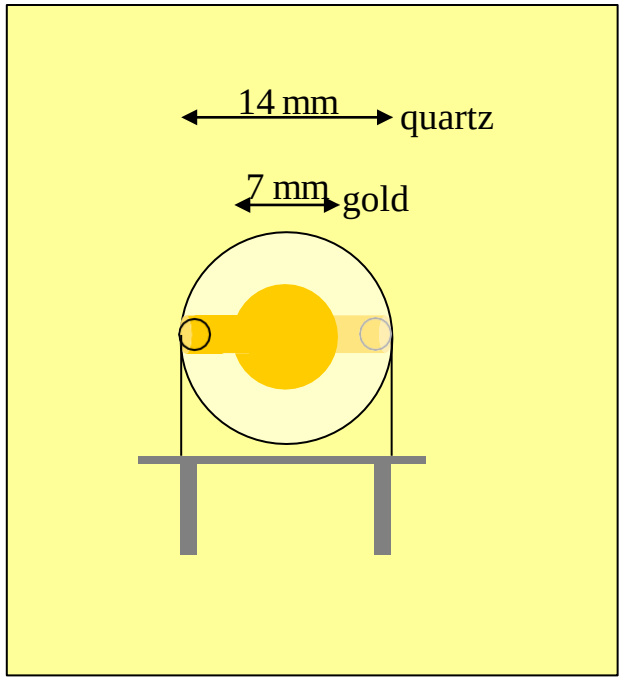

CDR region
antigen binding site
variable region
light chain
heavy chain
constant region
Antigen
Antibody
Reference electrode
V_G
Drain
Source
Nanomaterial
Substrate
V_{DS}
p-type
n-type
conductance
time
conductance
time

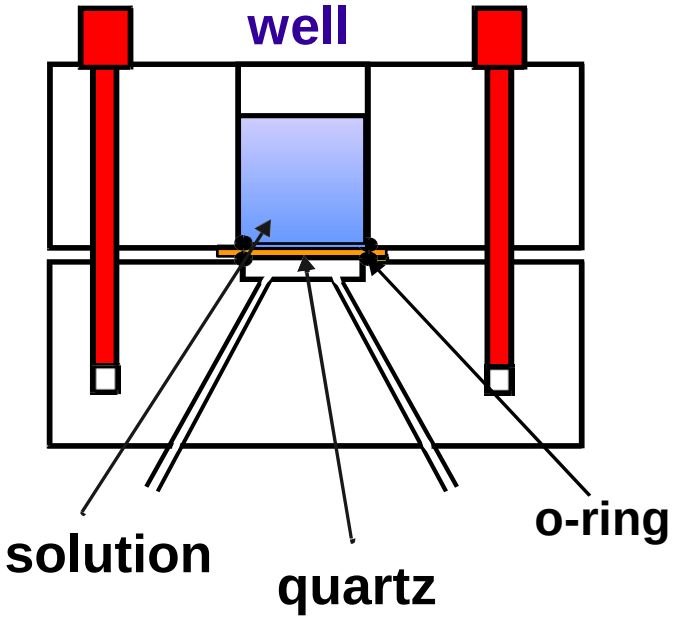
Piezoelectric Biosensors label-free!

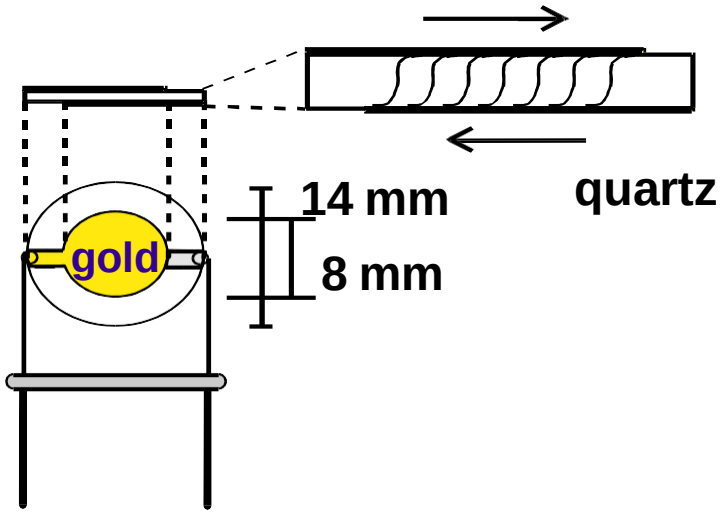






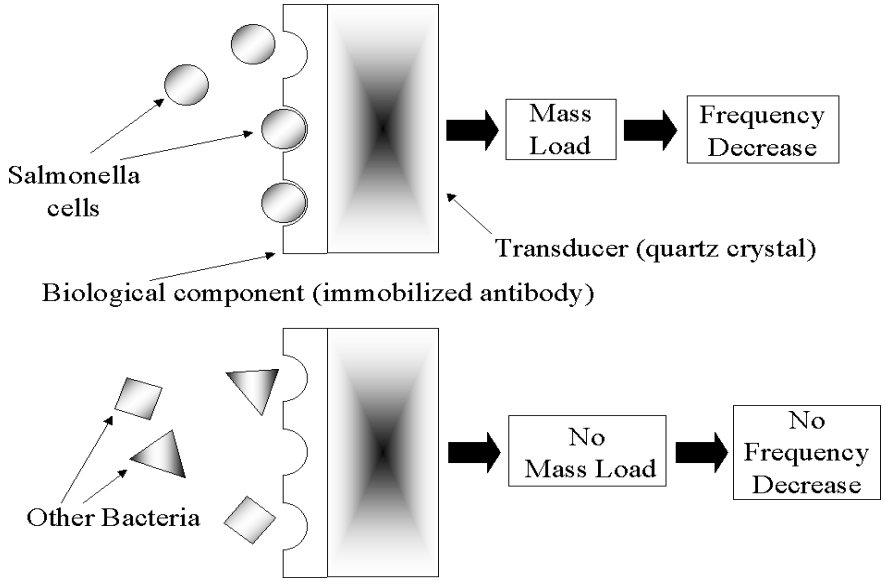






The standard QCM measures the mass of a material deposited on a quartz crystal surface as a linear function of a change in the oscillating crystal resonant frequency

The mass-loading frequency effects of the transducer are based upon Sauerbrey's equation



QCM-Mass

$$\Delta F = (-2.3 \times 10^{-6})\, F^2\, \Delta M/A$$

ΔF (Hz) = frequency shift of the coated crystal

F (Hz) = resonance frequency of the crystal

ΔM (g) = increase in mass loading

A (cm^2) = area of the coated crystal

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Short Communication

Piezoelectric Immunosensor for the Determination of C-Reactive Protein

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E-mail: miroslav.pohanka@gmail.com

C-Reactive protein is a marker of inflammation

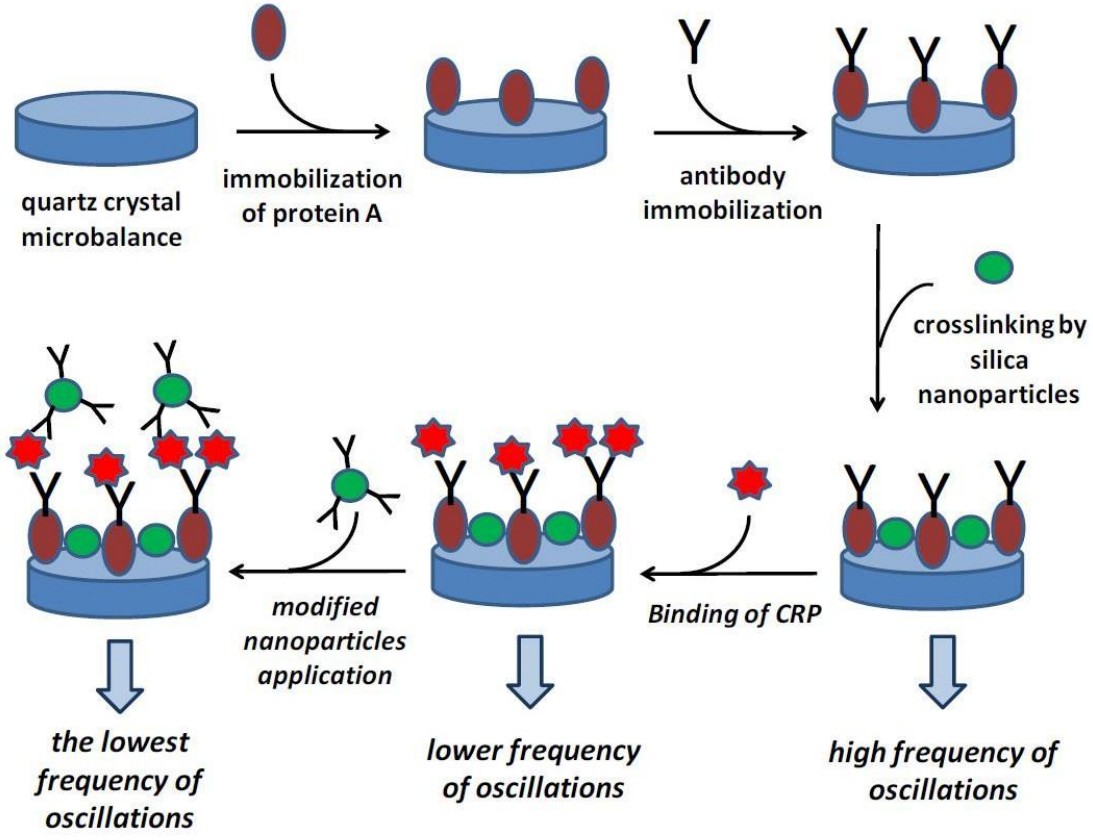
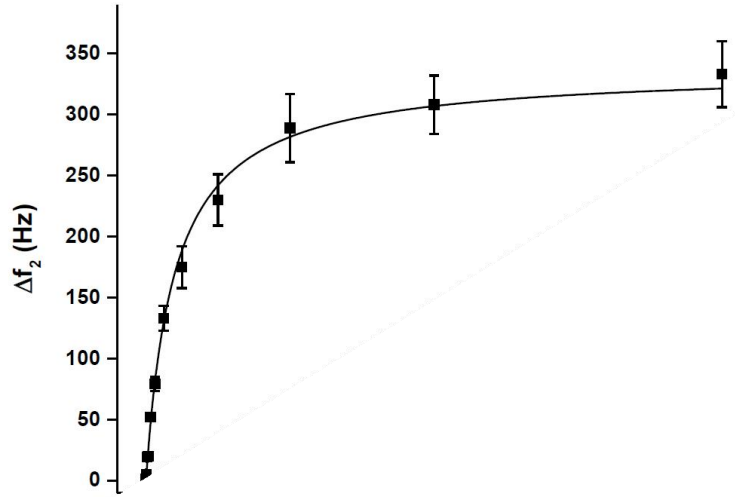


Figure 1. Principle of CRP assay by the proposed biosensor.



Figur

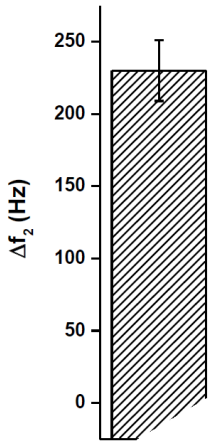


Figure 3. B