Principles of Immunoassays

SIPS522 Principles of Physiological Experimentation

Thursday, 29 January 2026, 1-3 pm

Assoc. Prof. Dr. Chantacha Sitticharoon

Department of Physiology,
Faculty of Medicine Siriraj Hospital, Mahidol University
Email: chantacha.sit@mahidol.ac.th

Assays

- Analytic procedure for qualitatively assessing the presence or quantitatively measuring the amount or the functional activity of the substance
  - bioassay
    - determines potency/concentrations (quantitative) or biological activity (qualitative) of a substance on living organism
  - biochemical assay
    - one that uses immunological reaction → immunoassay

Immunoassays

- in vitro test
- bioanalytical methods
- Quantitation of analyte (substance undergoing analysis by a laboratory test) depends on the reaction of
  - an antigen (Ag)(analyte)
    - any substance that causes the immune system to produce antibody to against it
  - an antibody (Ab)
    - proteins that are normally produced by the immune system in response to a foreign substance (Ag)

Use

- diagnosis of diseases
- therapeutic drug monitoring
- clinical pharmacokinetic
- bioequivalence studies in drug discovery
- pharmaceutical industries
- measurement of very low concentrations of low molecular weight substances
- macromolecular biomolecules of interest
- metabolites
- biomarkers which indicate disease diagnosis or prognosis

Criteria for effective assays

- sensitivity = lowest detectable dose, detection limit
  - smallest amount that can accurately be measured
  - % of TP/(TP+FN)
- specificity = least interference in the assay system,

  Do other substances interfere in the measurement?
  - minimal to no cross reactivity
  - % of TN/(FP+TN)

Real/Test	Positive	Negative	
Positive	TP Sensitivity	FN	TP+FN Sensitivity
Negative	FP	TN Specificity	FP+TN Specificity
			Accuracy

Criteria for effective assays

- accuracy = the ability to measure the correct concentration compared with reference method, true value, no bias (system error)
  - =TP+TN/(TP+TN+FP+FN)
  - Are the concentrations correct?
- precision = reproducibility (random error), acceptable coefficient of variance
  - How variables of the sample are?, within and between batches

Quality control: Intra-/Inter-assay variation

Table Title									
Well ID	Name	M 450	Mean	Std Dev	CV (%)	M 450 Corr.	Mean	Concentrations	Mean
SPL1		1.875	1.837	0.054	2.965	1.79	1.752	0.486	0.502
		1.798				1.713		0.519	

- to express the precision, or repeatability
  - 2 measures of the coefficient of variability (CV)
    - defined as the standard deviation of a set of measurements divided by the mean of the set
    - %CV = SD*100/Mean
    - internal QC (intra –assay variation)
      - general acceptable < 10%
    - external QC (inter –assay variation)
      - general acceptable < 15%

$$\mathrm{SD} = \sqrt{\frac{\sum (x_1 - x_2)^2}{2n}}$$

$$\mathrm{Mean} = \frac{\sum (x_1 + x_2)}{2n}$$

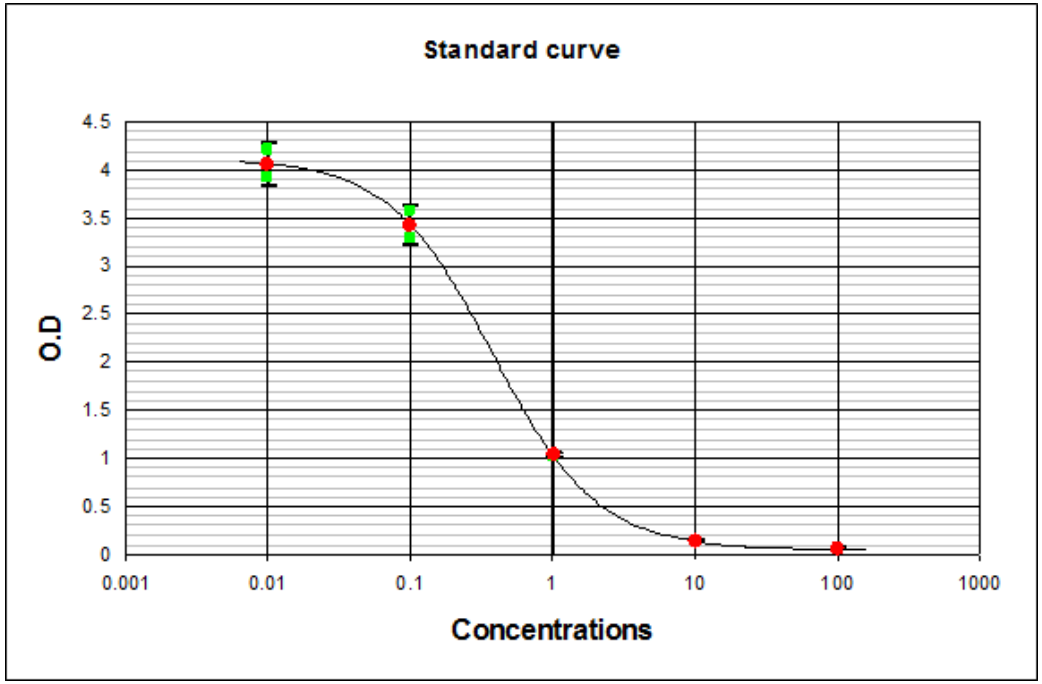
$$\mathrm{CV}(\%) = 100 \times \frac{\mathrm{SD}}{\mathrm{Mean}}$$

https://www.medcalc.org/manual/_help/formula/cvfomduplicates-formula.png

Criteria for effective assays

linearity = Whether the dose–response of the analyte is linear in a particular diluent within the range of the standard curve

- **Is the correct result obtained when we dilute the sample?**



http://www.phoenixpeptide.com/catalog/repository/EIA/EK_049_03.pdf

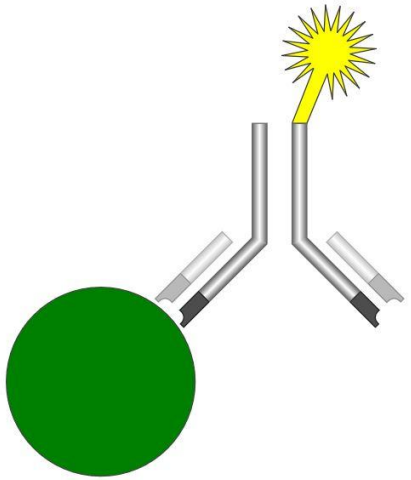
Characteristics of immunoassay

- simple
- high sensitivity (ability to detect very small concentrations, nano- and picomolar range)
- high specificity (capability of measure only an expected analyte)
- precise
- high-throughput measurement (of more substances than any other analytical technique)
- wide range of analytes in biological samples

Basic requirements for immunoassays

- ligand
  - Ag molecule
- binder
  - Ab
  - binding protein e.g. TBG, SHBG
  - cell receptors (receptor assay)

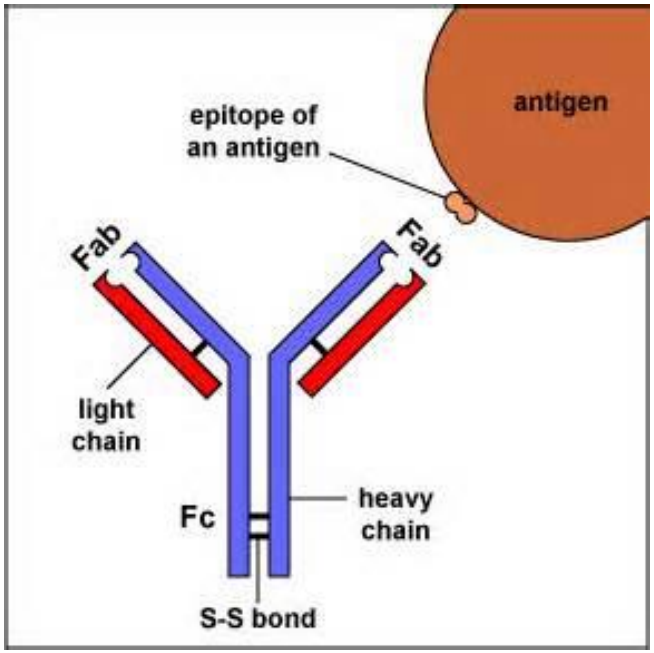
https://upload.wikimedia.org/wikipedia/commons/3/3c/Immunoassay.svg



- label (tracer)
  - an identifying marker incorporated to ligand/binder in order to produce a measurable signal
- separation system
  - to separate free ligand from bound ligand
- quality control
  - to monitor the reliability of the measurement

Ag

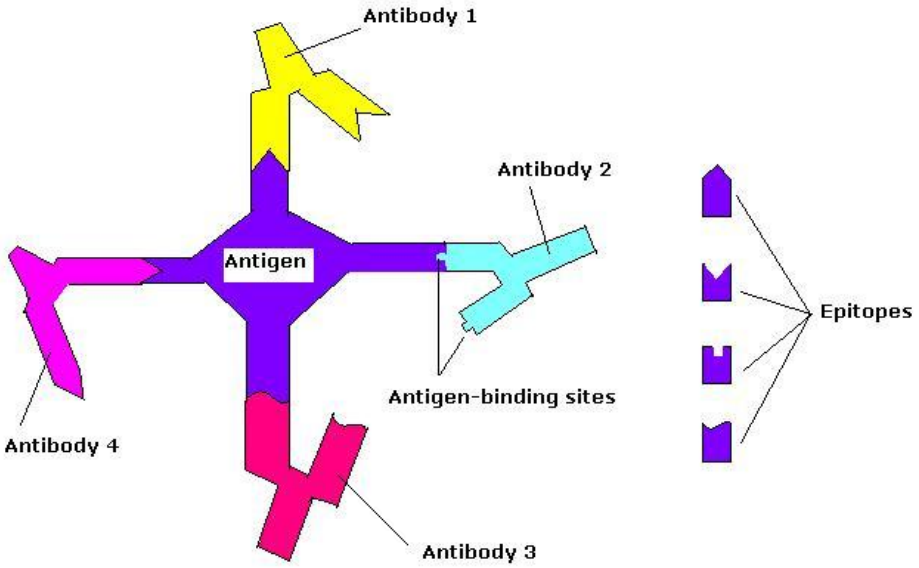
- molecule capable of inducing Ab (immunogen) or
- any substance that can be bound specifically to Ab
- natural or synthetic
- proteins, sugars, lipids, nucleic acids, etc.
- A site on an Ag molecule to which an Ab molecule binds is call epitope.



http://faculty.ccbcmd.edu/~gkaiser/SoftChalk%20BIOL%20230/Adaptive%20Immunity/antigens/u3fig9a.jpg

Natural Antigen

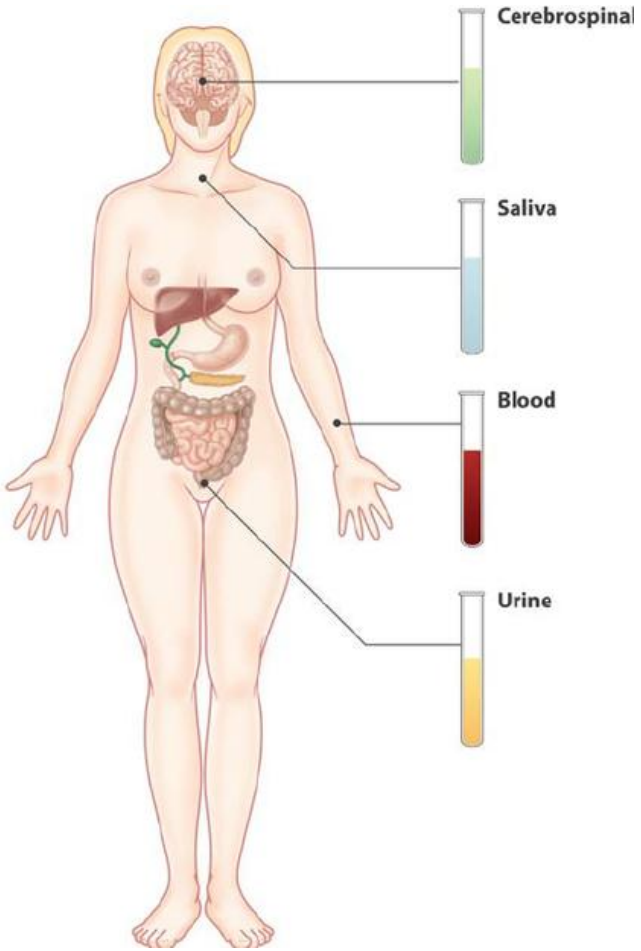
- naturally elicits immune response
- a larger molecule (over 10 kd)
- usually has several different epitopes (antigenic determinants)



https://upload.wikimedia.org/wikibooks/en/c/c8/Chem114A_epitopes.jpg

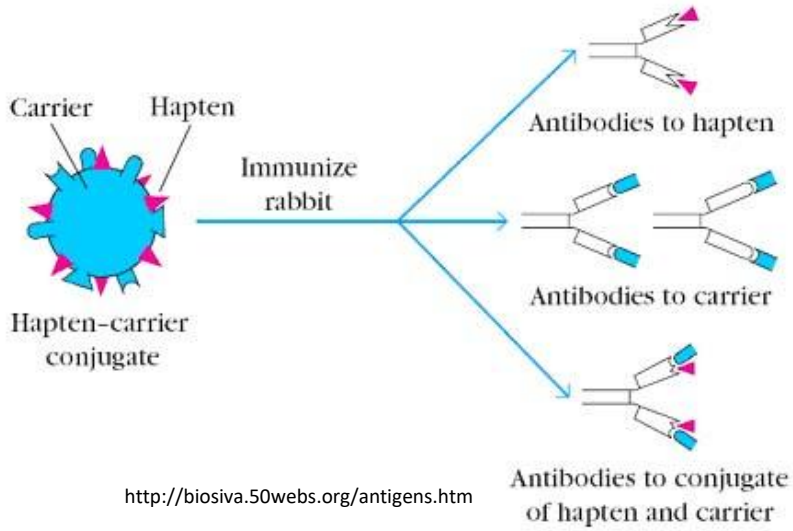
Biological samples

- blood, plasma, serum
- sweat
- saliva
- cerebrospinal fluid (CSF)
- urine
- synovial fluid
- others
- supernatant/media of cell culture
- extracted tissue/cell protein



Conjugated haptens

- smaller molecules (haptens)
  - either weakly or not at all immunogenic
- to produce the antibody
  - 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.



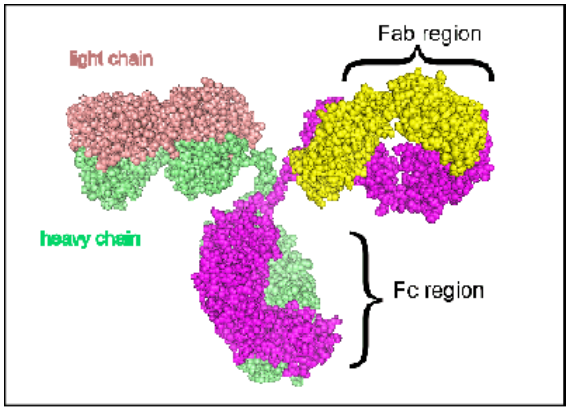
http://biosiva.50webs.org/antigens.htm

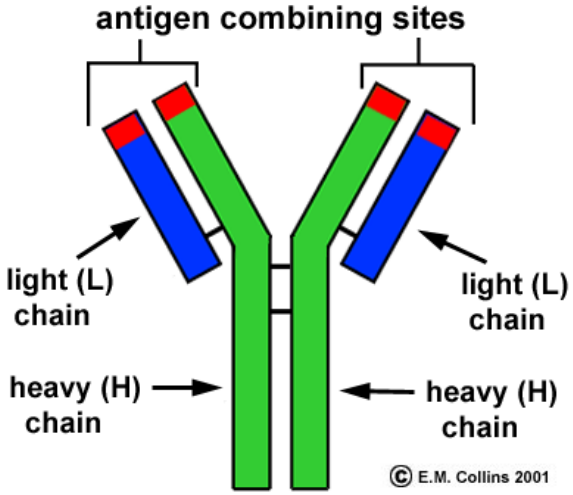
Ab

- Immunoglobulin (proteins) produced by the immune system in response to an invading (foreign) substance (Ag/analyte)
- high specificity and affinity for a specific Ag
- also an Ag
- usually the lgG class
- categories
  - polyclonal
  - monoclonal
  - engineered

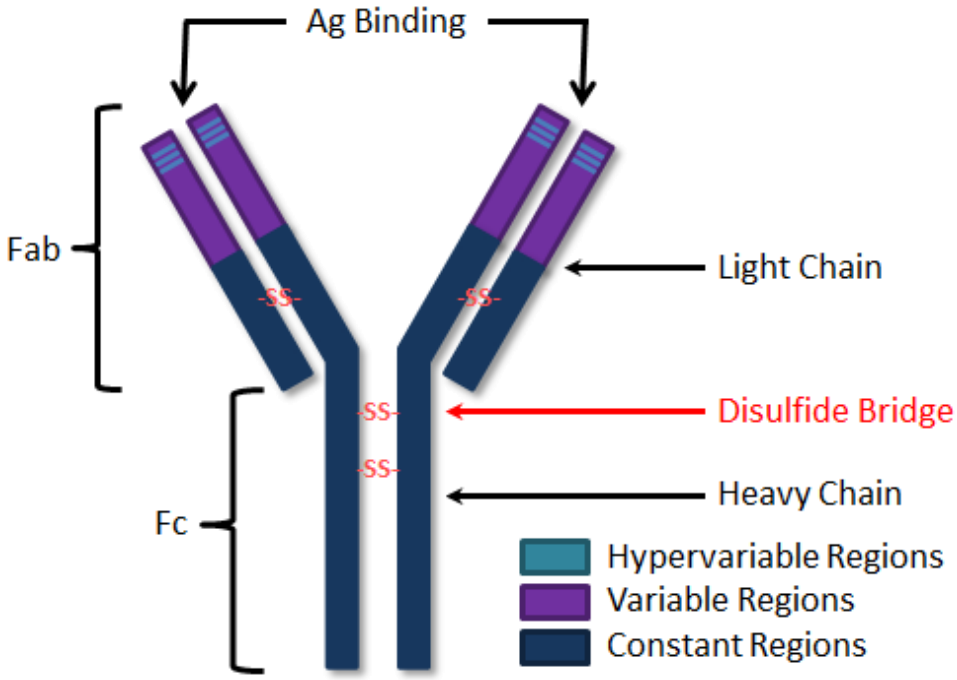
IgG structure

https://rostlab.org/services/epitome/images/Fig.%201.gif





Heavy chain determines the immunoglobulin isotype (IgA, IgD, IgG, IgE, IgM).



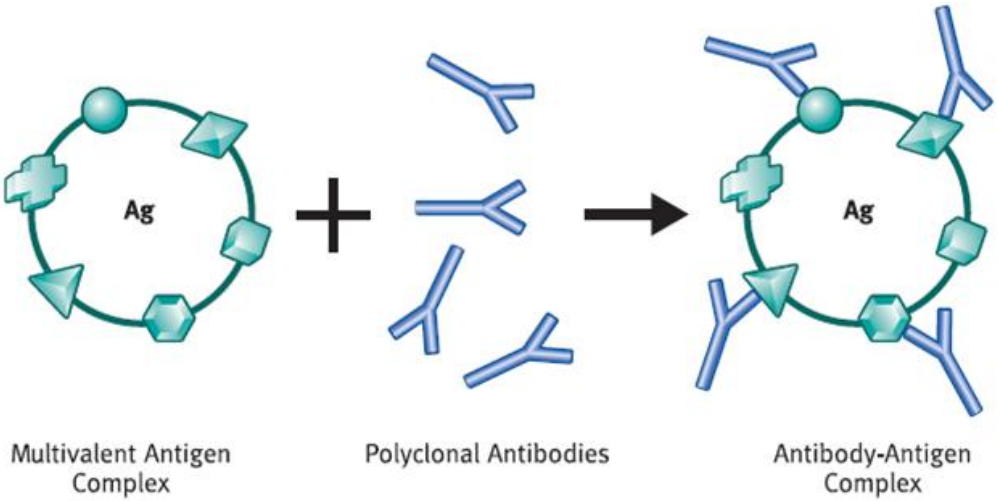
http://myplace.frontier.com/~dffix/medmicro/pix/Ig.png

Fab = fragment antigen binding
= antigen specificity
Fc = fragment crystalline
= the antibody's class effect, biological activity mediation

http://waynesword.palomar.edu/images/antibod1a.gif

Polyclonal Ab

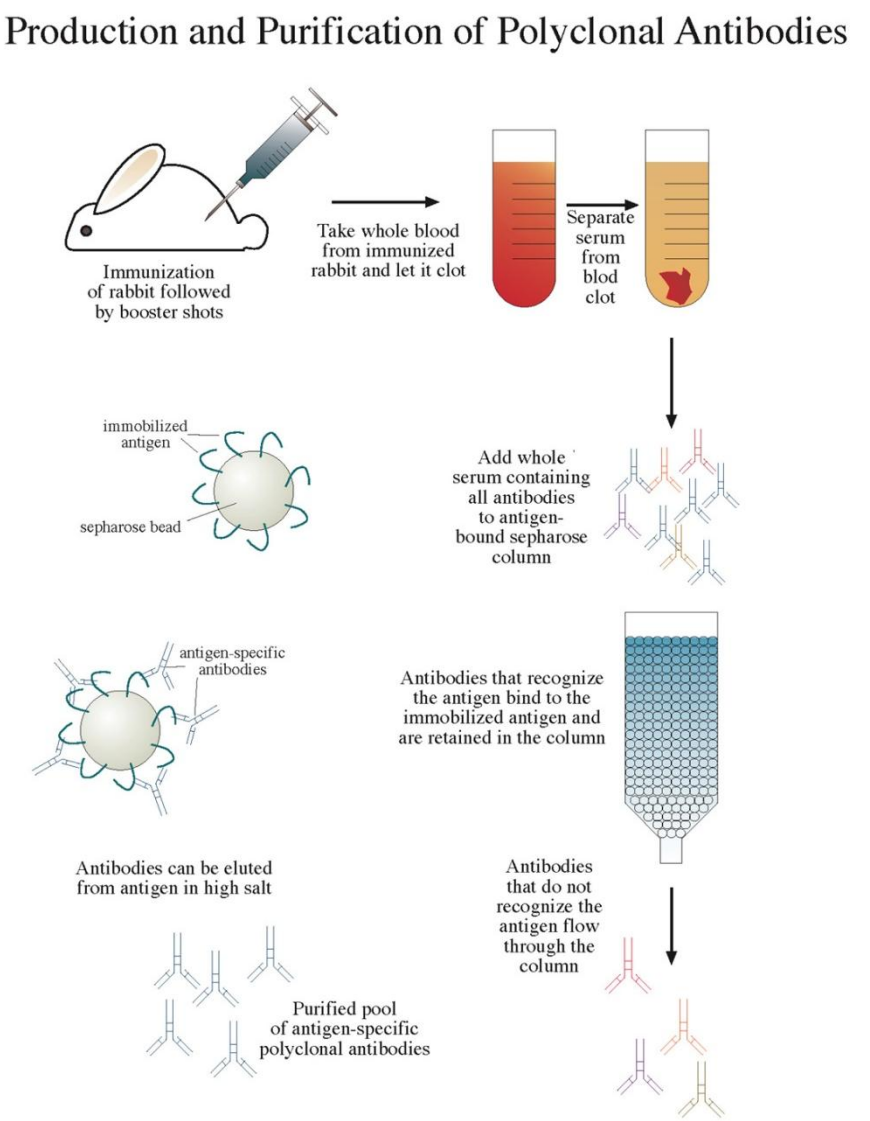
- a mixture of antibodies
  - may bind to different epitopes of the immunogen with different strength of binding



https://classconnection.s3.amazonaws.com/193/flashcards/975193/png/figure_1-21355400097482.png

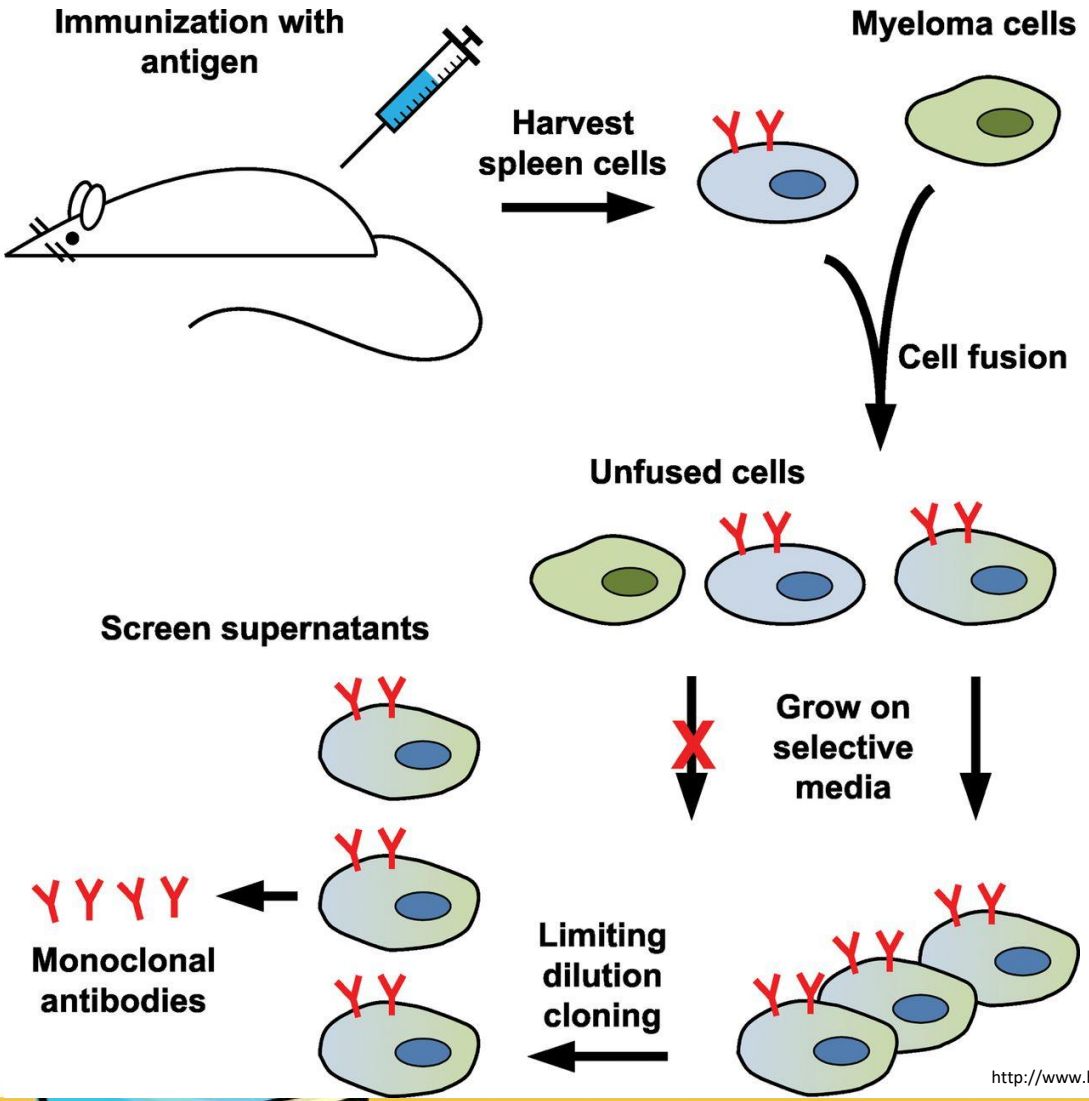
- raised in animals (mouse, rabbits, sheep, goat, etc.) by repeated immunization
- more likely to produce a false positive
  - less specific to antigen
  - probably binding to molecules similar to their antigens

Polyclonal Ab



http://www.cytographica.com/overheads/antibody.jpg

Production of monoclonal Ab



- 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

http://www.biochemj.org/content/ppbiochemj/436/1/1/F1.large.jpg?width=800&height=600&carousel=1

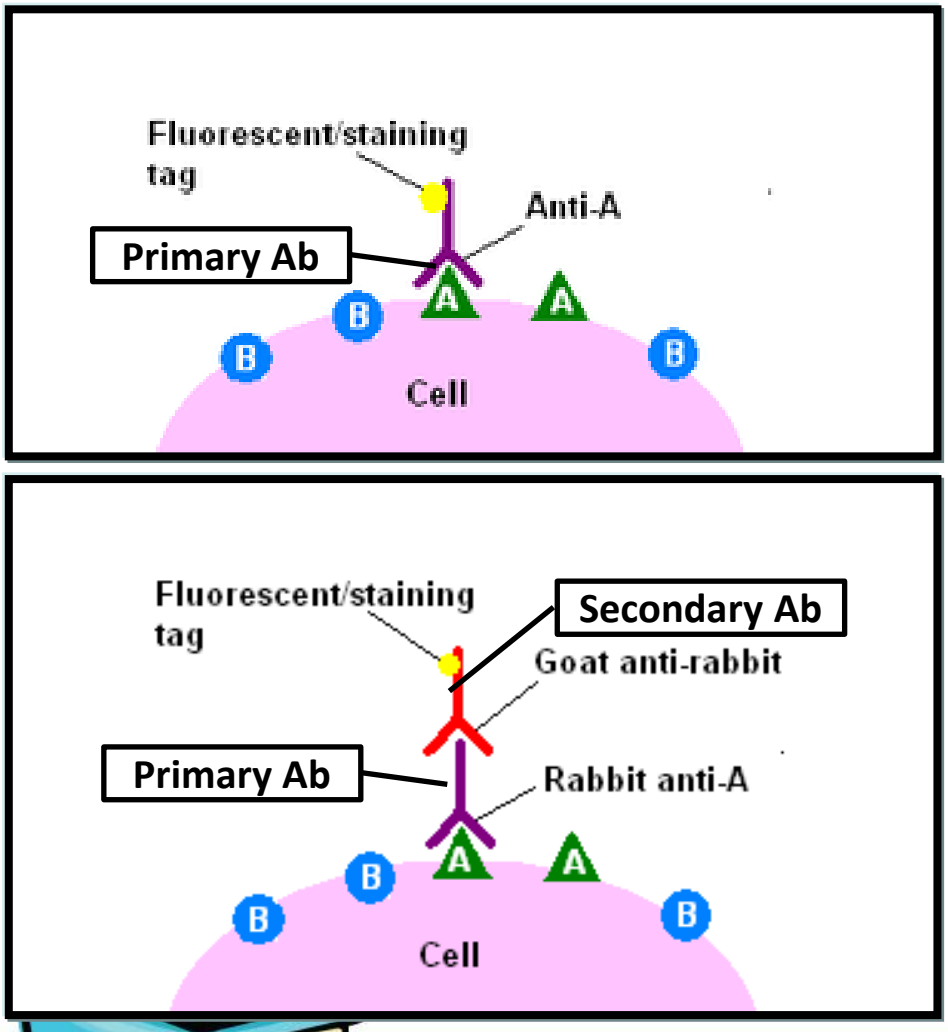
Monoclonal Ab

- specific for a single epitope on a multivalent antigen
- derived from a single myeoloma cell line
  - Hybridoma cells can produce the same antibody.
    - consistently (identical over long time)
    - indefinitely (large, unlimited quantity)

Disadvantages

- expertise required
  - contamination
  - Success rate of fusion step is important.
- lack of normal precipitating behavior as polyclonal Ab

Primary and Secondary Ab



- primary ab
  - can bind to a specific protein or a biomolecule of interest
- secondary ab
  - binds with a primary antibody
  - more sensitive and flexible in labeling and detection
  - The selection of the type of secondary antibody is based on the class/species of the primary antibody.

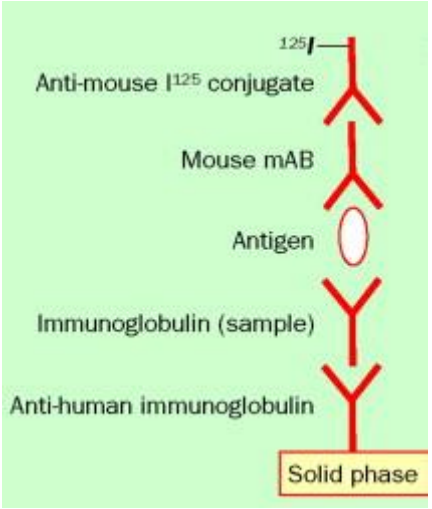
Labels in immunoassay

- a molecule that produce a signal that can be measured

- solid particles, RBC (agglutination assay)
- microparticles (latex particles, gold, EU chelate nanoparticles polystyrene, magnetic particles)(agglutination assay PIA = particle immunoassay, MIA = metalloimmunoassay)
- radioisotope (^{32}P,^{125}I,^{3}H) (RIA = radioimmunoassay)
  - an element having an unstable nucleus and emitting characteristic (alpha, beta or gamma) radiation during its decay to a stable form
  - alpha, beta or gamma counter
- enzyme (alkaline phosphatase, horseradish peroxidase, and others) (EIA = enzyme immunoassay)
  - light absorbance
  - spectrophotometer
- fluorophore (fluorescein, lanthanide chelates) (FIA = fluoroimmunoassay)
  - light emission
  - spectrofluorometer
- luminophore (substituted isoluminol, acridinium esters)(LIA = luminescence immunoassay)
  - light emission
  - luminometer

Radioactive isotopes in RIA

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



http://middleeast.thelancet.com/cms/attachment/2001035156/2003942903/gr3.jpg

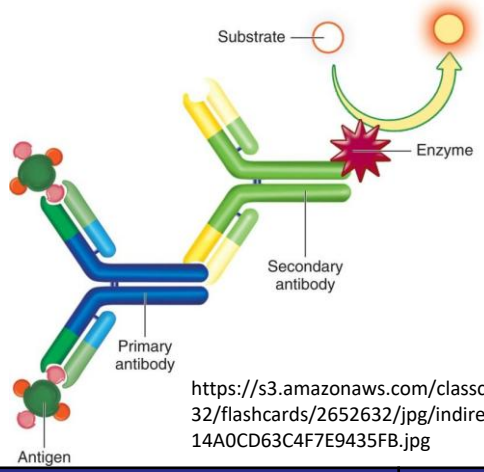
Advantages

- high sensitivity of detection
- no interferences
- small molecular size
- simple labeling
- low cost

Disadvantages

- environmental risk (waste disposal)
- dedicated instrumentation
- separation step necessary
- short shelf-life
- difficult to automate

Enzyme commonly used in EIA



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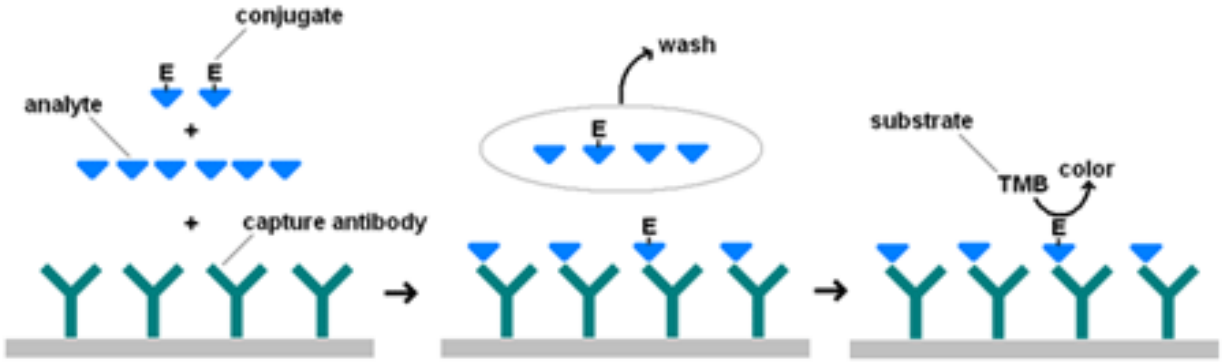
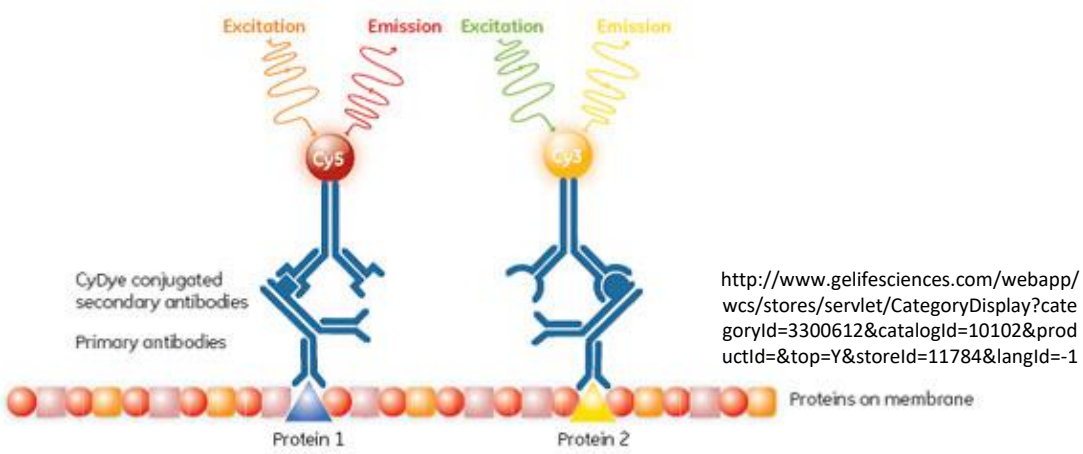


http://3.bp.blogspot.com/--gtO_WDg1vo/UCofxnKUPAI/AAAAAAAAAns/8AUDvg_6XAY/s1600/immunoassay6.gif

colorimetric or chemiluminescence

enzyme	substrate	color
alkaline phosphatase	p-nitrophenylphosphate	yellow
horseradish peroxidase	o-phenylenediamine 3,3'-diaminobenzidine (DAB) 3,3',5,5'-tetramethylbenzidine (TMB)	brown dark brown blue
beta-galactosidase	o-nitrophenylphosphate	yellow

Fluorescent-labeled immunoassay



http://www.gelifesciences.com/webapp/wcs/stores/servlet/CategoryDisplay?categoryId=3300612&catalogId=10102&productId=&top=Y&storeId=11784&langId=-1

- **Fluorescence occurs when molecules called fluorophores absorb light.**
  - **In the ground state, fluorophores do not emit light.**
  - **When subjected to light (excitation), their energy levels are raised to a brief but unstable excited state.**
  - **As fluorophores return to their ground state, they release light at a lower energy, higher wavelength (emission) than that of the excitation light.**
- **If selected fluorescent dyes are spectrally resolvable (i.e., emit light of different wavelengths), they can be used as labels to allow multiplexing – the simultaneous detection of more than one target in a single.**

Luminescent-labeled immunoassay

http://www.scienceinschool.org/content/living-light-chemistry-bioluminescence

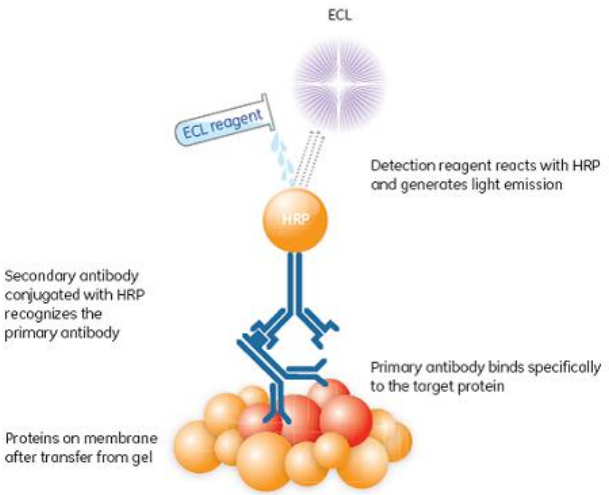
https://s-media-cache-ak0.pinimg.com/474x/b2/97/96/b2979633f50a35632ab56b2ab411b8f7.jpg

http://www.quantum-immortal.net/physics/images/firefly.bmp

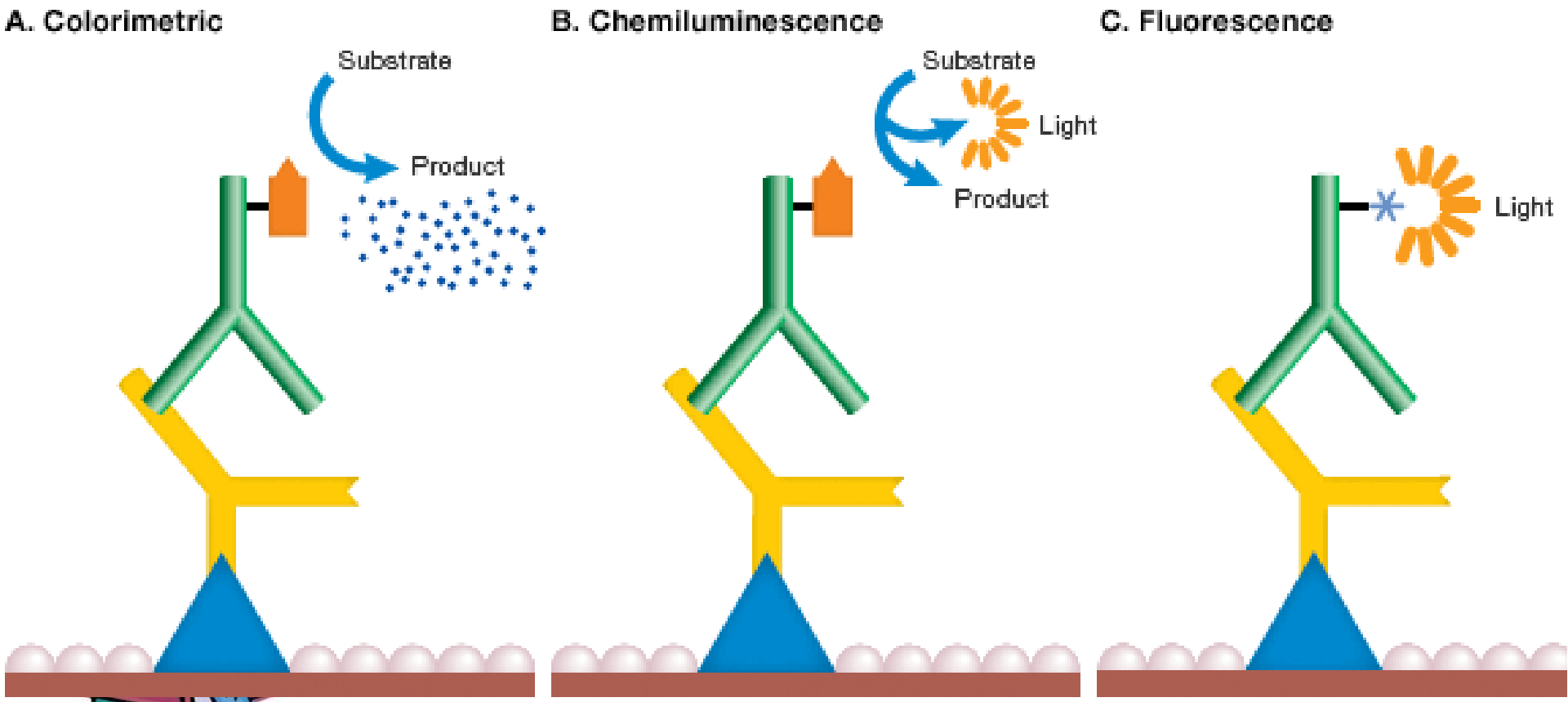
- **bioluminescence**
  - **the production and emission of light by a living organism**
  - **occurs widely in marine vertebrates and invertebrates, as well as in some fungi, microorganisms including some bioluminescent bacteria and terrestrial invertebrates such as fireflies.**

Luminescent-labeled Immunoassay

- Chemiluminescence
  - chemical reaction
  - occurs when a chemical reagent containing stored energy releases light
  - The reagent is normally stable and does not emit light.
  - The reagent can be converted into a light emitting product, for example after interaction with a specific enzyme.
  - In most contemporary, the enzyme horseradish peroxidase (HRP) conjugated to a secondary antibody is utilized to convert the substrate, producing light.
- Advantages
  - ultra sensitivity
  - rapid
  - diversity of analytical application
  - broad linear range
  - no intrinsic background
  - elimination of radioactive material



Labeled immunoassay



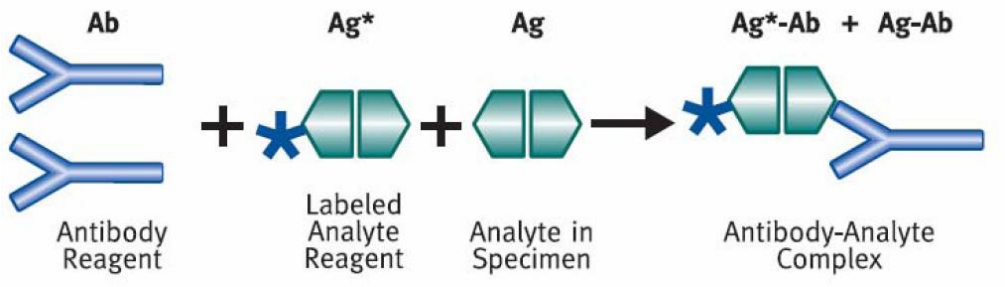
http://www.bio-rad.com/webroot/web/images/lsr/solutions/technologies/protein_electrophoresis_blotting_and_imaging/western_blotting/technology_detail/pet2522_5pt6.gif

Separation of bound and free fractions

- physical methods
  - filtration
  - chromatography
  - electrophoresis
  - charcoal-dextran adsorption
  - adsorption on ion-exchange resin
- chemical methods
  - organic solvents-ethanol
  - dioxane
  - polyethylene Glycol (PEG)/salts-sodium
  - zinc
  - ammonium sulphate
- solid phase system
  - plastic surface
    - discs, tubes
  - cellulose particles, beads
  - magnetic particles, beads
- secondary ab
  - soluble, solid phase
  - quite specific (precipitate bound)
  - often add normal serum to form bulky pellet

Type of immunoassays

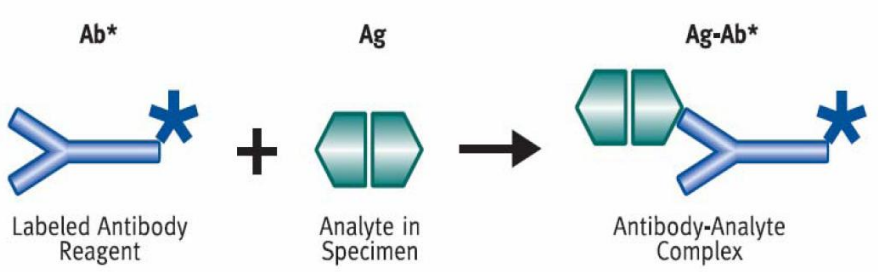
competitive



homogeneous

- do not require separation of bound Ab-Ag* complex before measuring the label

noncompetitive



heterogeneous

- require separation of bound Ab-Ag* complex before measuring the label

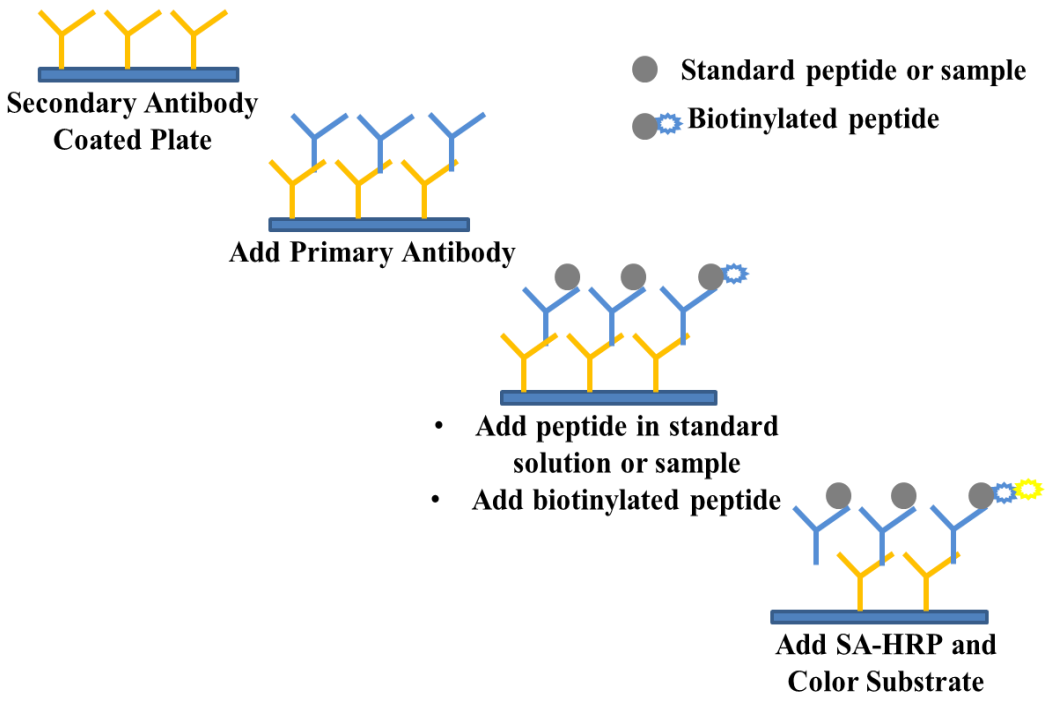
Development of Immunoassays

- 1st generation immunoassays
  - competitive immunoassay (limited reagent assays)
    - RIA, EIA, FIA, LIA, in-house, manual double antibody, solid phase assays, etc.
- 2nd generation immunoassays
  - noncompetitive immunoassay or immunometric assays (excess reagent assay, sandwich assay)
    - immunoradiometric assay (IRMA), enzyme-linked immunosorbent assay (ELISA), lateral flow assays, automated assays, random access automates, etc.
- 3rd generation immunoassays
  - microspot analysis, biochip arrays, multiple parameter testing, nanotechnologies

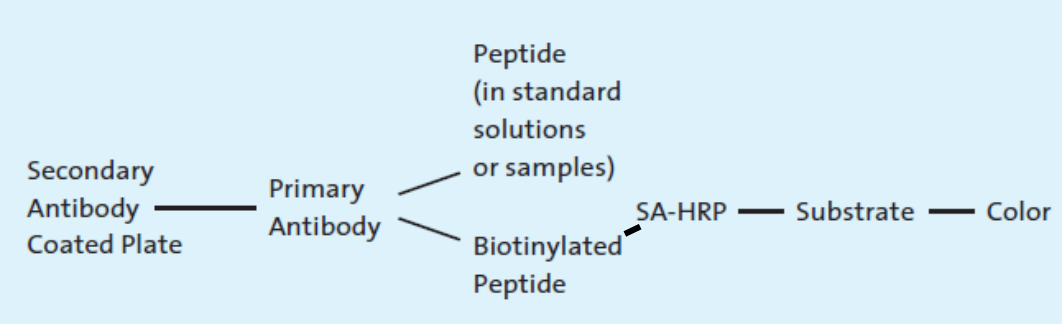
Competitive immunoassays

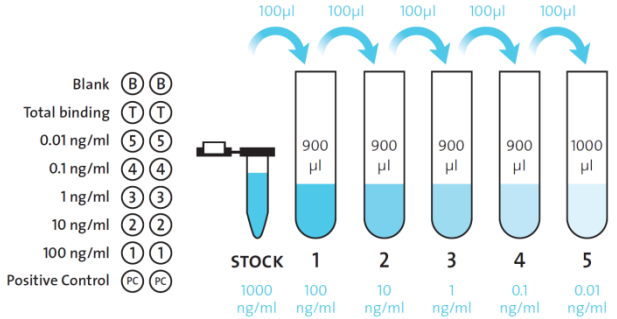
- the less Ag in the sample, the more labeled Ag can be bound by Ab
- RIA, EIA, FIA, LIA

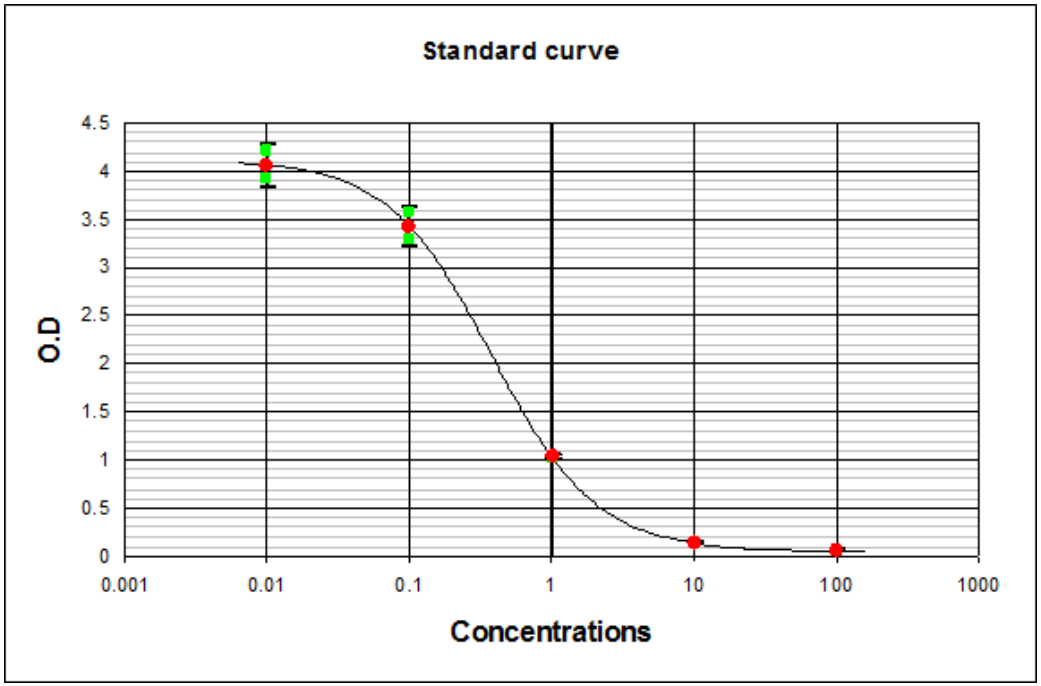
http://www.raybiotech.com/eia-competition-based-elisa-en.html



Competitive immunoassays

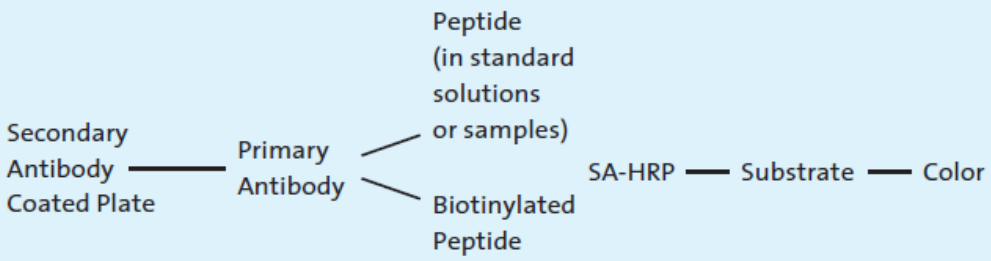


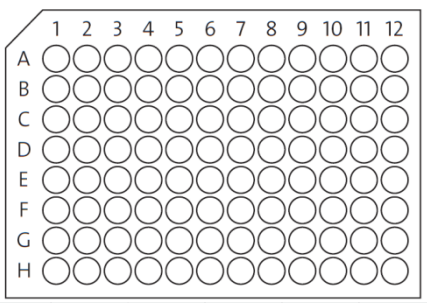


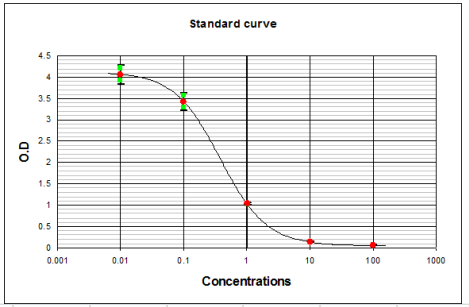


http://www.phoenixpeptide.com/catalog/repository/EIA/EK_049_03.pdf

Competitive immunoassays







SUMMARY OF ASSAY PROTOCOL

Add 50µl/well of standard, sample, or positive control, 25µl primary antibody and 25µl biotinylated peptide.

▼

Incubate at room temperature (20-23°C) for 2 hours

▼

Wash immunoplate 4 times with 350µl/well of 1x assay buffer

▼

Add 100µl/well of SA-HRP solution

▼

Incubate at room temperature (20-23°C) for 1 hour

▼

Wash immunoplate 4 times with 350µl/well of **1**x assay buffer

▼

Add 100µl/well of TMB substrate solution

▼

Incubate at room temperature (20-23°C) for 1 hour

▼

Terminate reaction with 100µl/well of 2N HCl

▼

Read absorbance O.D. at **450**nm and calculate results

Plate Title

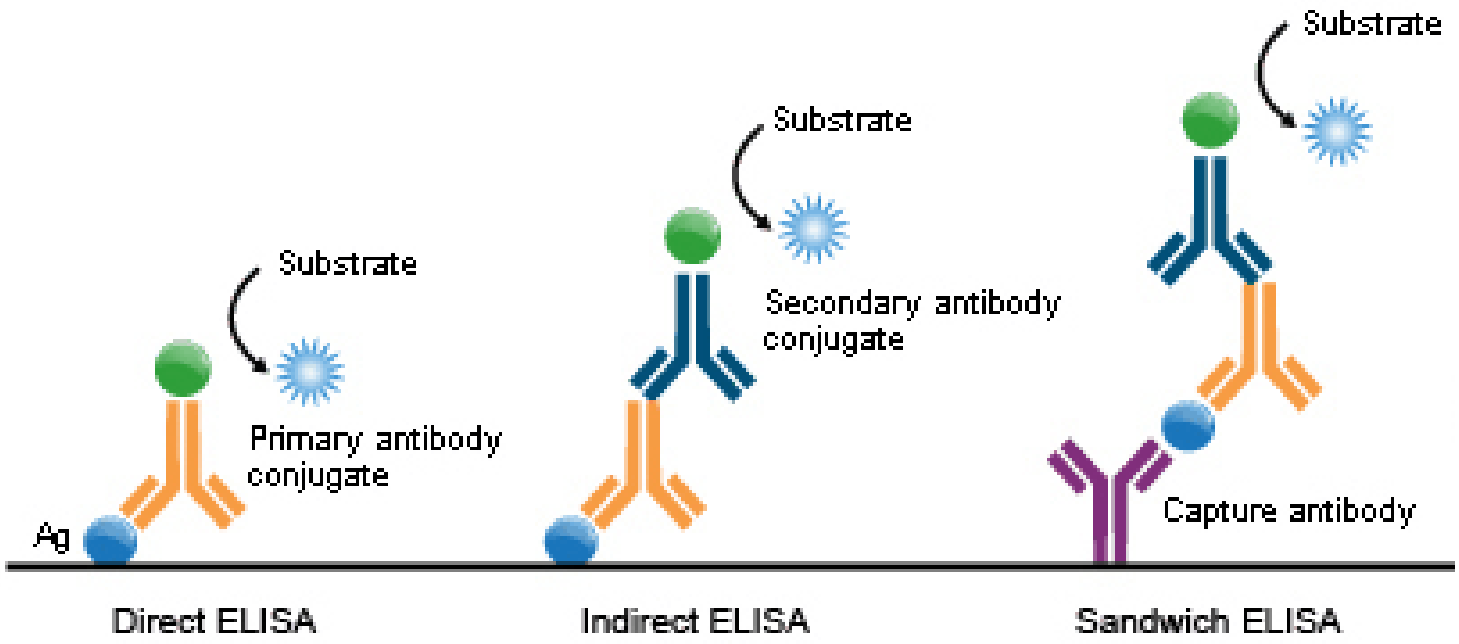
	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	SPL1	SPL1	SPL9	SPL9	SPL17	SPL17	SPL25	SPL25	SPL33	SPL33
B	SPLC1	SPLC1	SPL2	SPL2	SPL10	SPL10	SPL18	SPL18	SPL26	SPL26	SPL34	SPL34
C	STD1 0.01	STD1 0.01	SPL3	SPL3	SPL11	SPL11	SPL19	SPL19	SPL27	SPL27	SPL35	SPL35
D	STD2 0.1	STD2 0.1	SPL4	SPL4	SPL12	SPL12	SPL20	SPL20	SPL28	SPL28	SPL36	SPL36
E	STD3 1	STD3 1	SPL5	SPL5	SPL13	SPL13	SPL21	SPL21	SPL29	SPL29	SPL37	SPL37
F	STD4 10	STD4 10	SPL6	SPL6	SPL14	SPL14	SPL22	SPL22	SPL30	SPL30	SPL38	SPL38
G	STD5 100	STD5 100	SPL7	SPL7	SPL15	SPL15	SPL23	SPL23	SPL31	SPL31	SPL39	SPL39
H	CTL1	CTL1	SPL8	SPL8	SPL16	SPL16	SPL24	SPL24	SPL32	SPL32	SPL40	SPL40

Table Title

Well ID	Name	M 450	Mean	Std Dev	CV (%)	M 450 Corr.	Mean	Concentrations	Mean
SPL1		1.875	1.837	0.054	2.965	1.79	1.752	0.486	0.502
		1.798				1.713		0.519	
SPL2		1.714	1.904	0.268	14.079	1.629	1.819	0.558	0.481
		2.093				2.008		0.405	
SPL3		1.826	1.58	0.348	22.019	1.741	1.495	0.506	0.65
		1.334				1.249		0.793	
SPL4		1.661	1.766	0.148	8.371	1.576	1.681	0.584	0.536
		1.87				1.785		0.488	

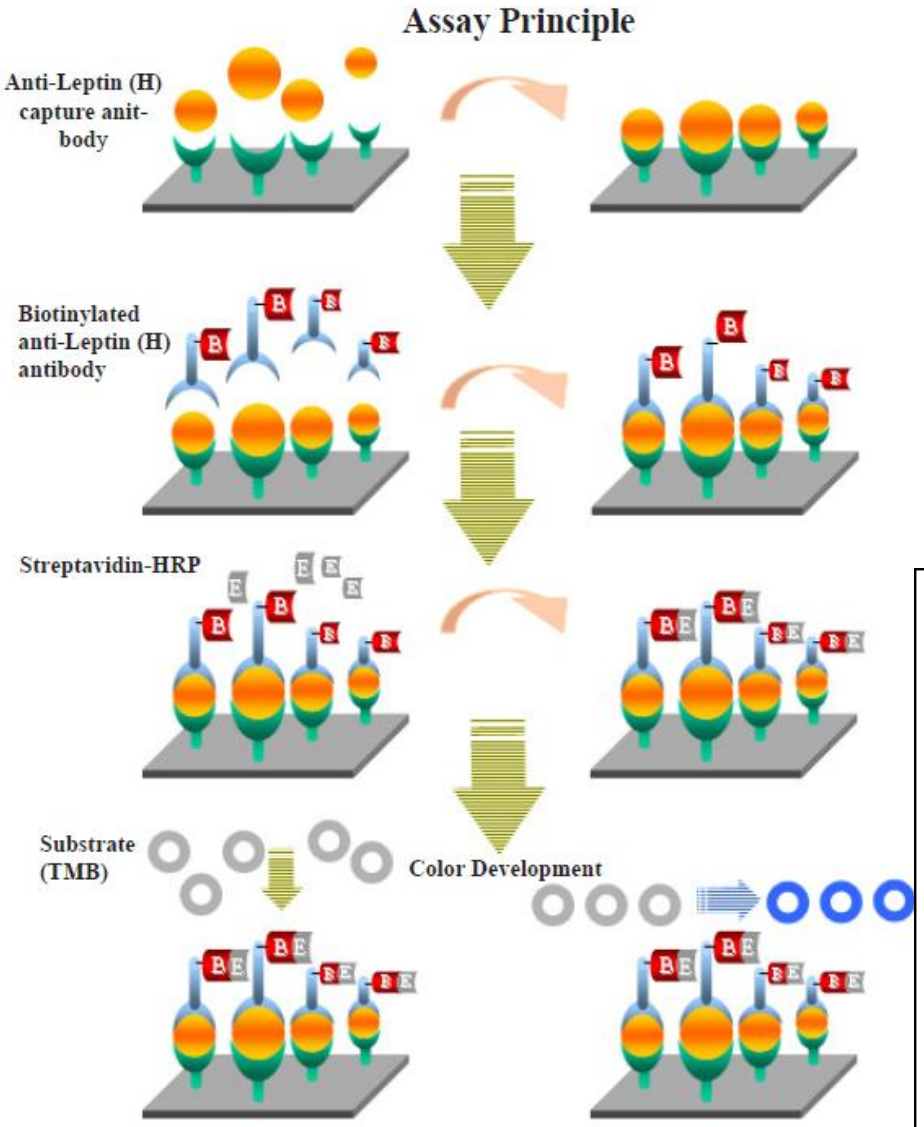
Noncompetitive immunoassays

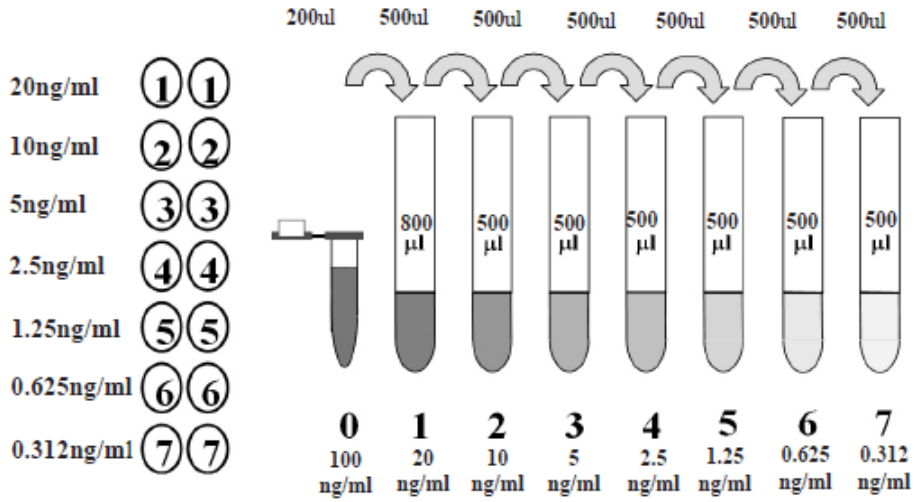
- also known as sandwich, immunometric, excess reagent assays
- immunoradiometric assay (IRMA), enzyme-linked immunosorbent assay (ELISA)

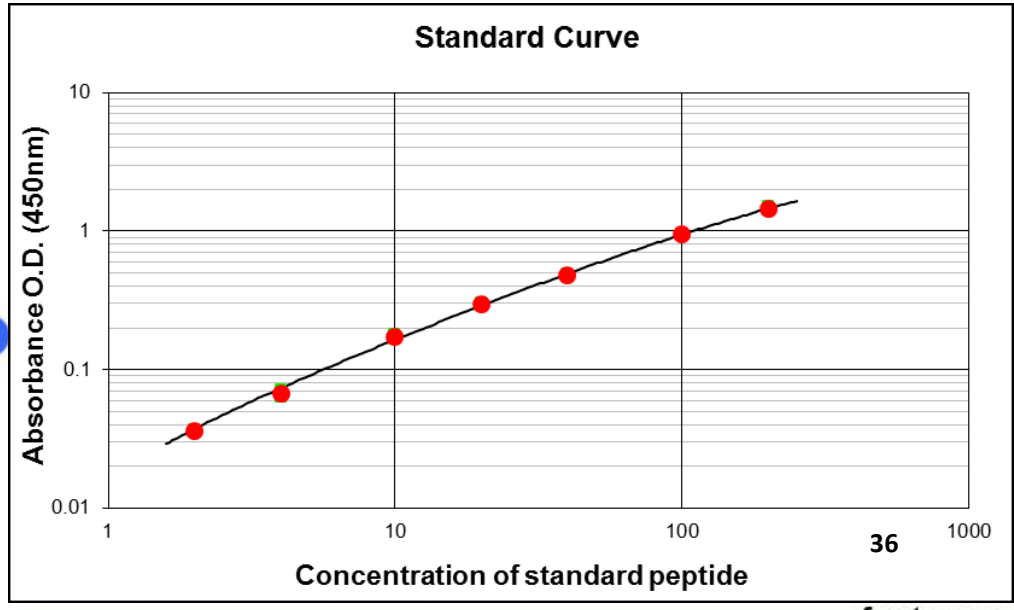


http://www.abnova.com/support/resources.asp?switchfunctionid=%7B70196CA1-59B1-40D0-8394-19F533EB108F%7D

Noncompetitive immunoassays







http://www.phoenixpeptide.com/catalog/repository/EIA/EK_003_12.pdf

Noncompetitive compared to competitive immunoassays

advantages

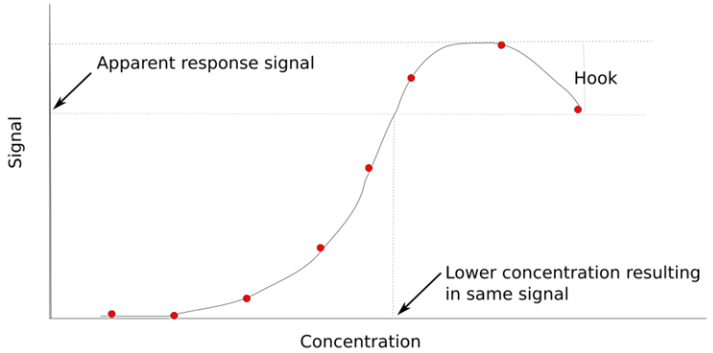
- higher sensitivity and specificity
- universal labeling procedure (IGgs)
- generally longer shelf-life of labeled antibodies
- extended working range

disadvantages

- not applicable to small molecules or small concentrations
- more expensive (higher consumption of antibodies, isolation of pure immunoglobulin
- “hook effect” (high dose hook effect) in some assays
- HAMA interference

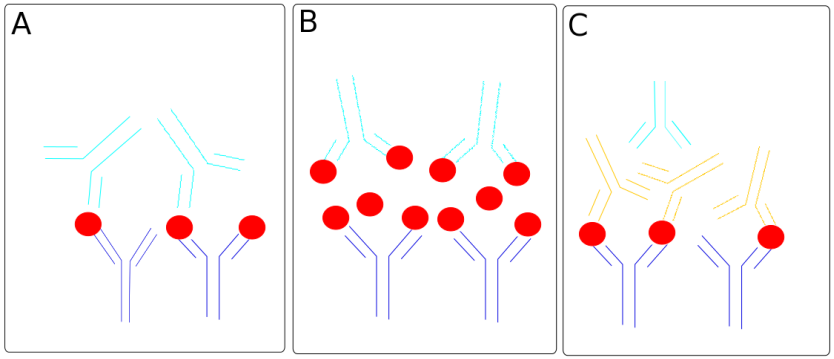
Interference in immunoassay

- **the hook or the prozone effect**
  - a type of interference which plagues certain immunoassays resulting in false negatives or inaccurately low results
  - in Ag excess
    - both the capture and detection Ab → saturated high Ag concentration
    - No sandwich can be formed.
- **antibody interference**



A dose response assay illustrating the hook effect. At high concentrations of analyte, a lower signal is observed. This shows that using this assay to determine the concentration of analyte in a sample containing very high levels would produce a lower signal. Since this signal normally correlates to a lower concentration, an inaccurately low result is recorded.

https://upload.wikimedia.org/wikipedia/commons/d/d5/Hook_effect.png



Panel A: Normal sandwich elisa. The capturing antibody is shown in purple, antigen in red and detection antibody in turquoise.
Panel B: Excessive antigen binds up sites on both capturing and detection antibodies, causing reduced detection levels.
Panel C: If blocking antibodies are present (in orange), they compete with detection antibodies for antigen binding. Reduced detection levels results.

https://upload.wikimedia.org/wikipedia/commons/c/ce/Illustration_of_the_effects_of_excess_antigen_and_blocking_antibodies_on_immunoassays.png

Human Anti-animal Ab (HAAA)

- high affinity, specific polyclonal Ab produced against a specific animal IgG or IgM
  - If it is produced in a high titre
    - compete with the test antigen by cross-reacting with reagent antibodies of the same species to produce a false signal
  - are most commonly human anti-mouse Ab (HAMA), but also include antibodies to rats, rabbits, goats, sheep, cattle, etc. and are reported to occur in 30-40% of patient samples

Human Anti-animal Ab

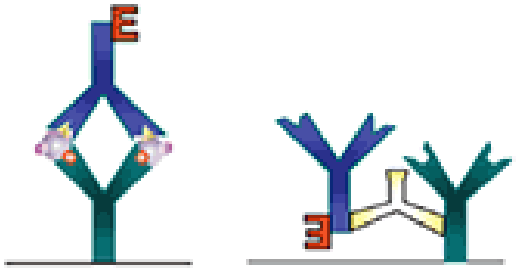


Fig A: False positive result due to interfering Ab

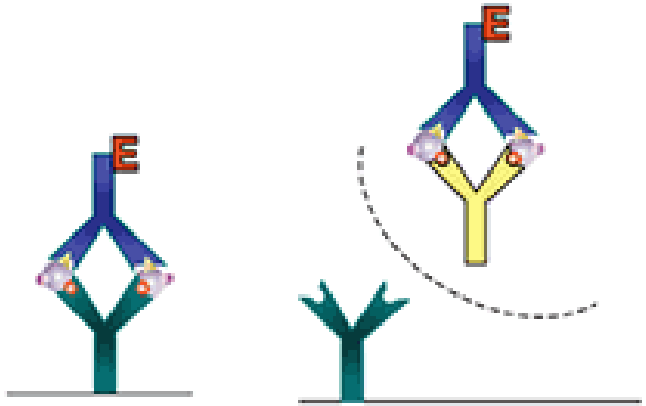
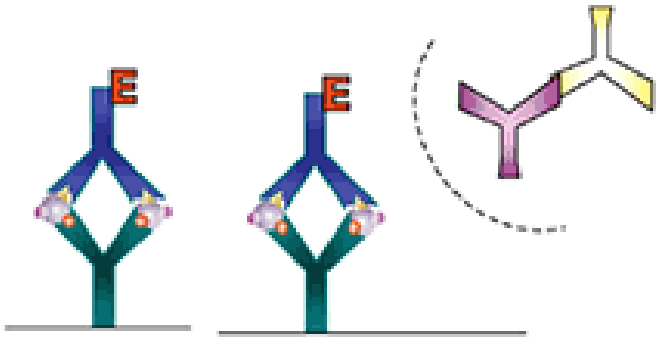


Fig B: False negative result due to interfering Ab



HAMA Blockers

Interferences can cause false positive and false negative.

http://www.diagnostics.us.tosohbioscience.com//Image%20Library/Unassigned/interferences_in_immunoassays3.gif?code=32d217fc-c2ec-4ab5-96ee-6ef94337b144

Cross reactivity

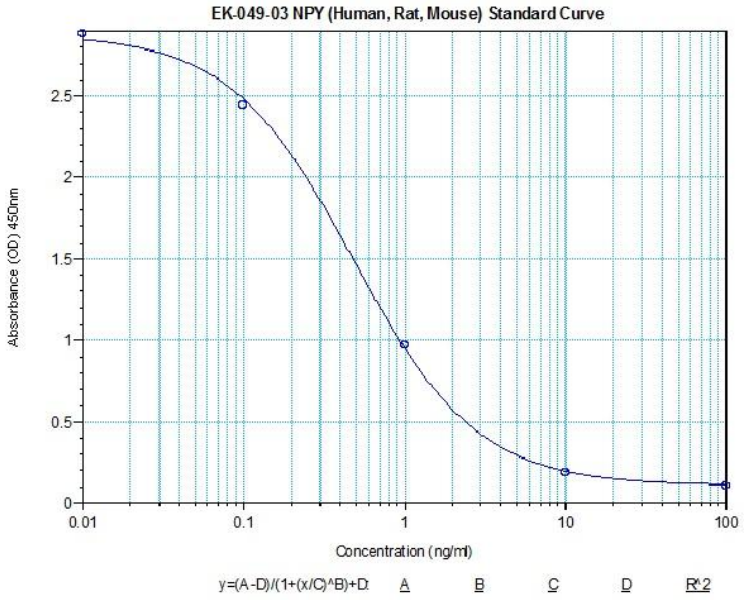
- the reaction between an Ab and an Ag that differs from the immunogen

http://www.adventuresofaglutenfreemom.com/wp-content/uploads/2011/08/Gluten-Cross-Reactivity-Model1.jpg

Sensitivity	Minimum Detectable Concentration = 0.09 ng/ml
Range	0-100ng/ml
Linear Range	0.09-1.43 ng/ml

Cross Reactivity

Peptide	*%Cross-reactivity*
Neuropeptide Y (Human, Rat, Mouse)	100
Neuropeptide Y (Porcine)	100
Neuropeptide Y (3-36) (Human, Rat, Mouse)	14.3
Peptide YY (Human)	0
Peptide YY (Rat, Mouse, Porcine, Canine)	0
Pancreatic Polypeptide (Human)	0
VIP (Human, Rat, Porcine)	0
Insulin (Human)	0
Amylin (Human)	0
Somatostatin (Human, Rat, Mouse, Porcine)	0



http://www.phoenixpeptide.com/catalog/product_info.php?products_id=4313

Other consideration

- **Positive control**
  - are groups where a phenomenon is expected
  - They ensure that there is an effect when there should be an effect.
  - by using an experimental treatment that is already known to produce that effect
  - then comparing this to the treatment that is being investigated in the experiment
  - are often used to assess test validity
- **Negative control**
  - are groups where no phenomenon is expected
  - They ensure that there is no effect when there should be no effect.

Other consideration

- equipment
- price
- technique/expertise
- disposal system

Reference

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http://careerconfidential.com/wp-content/uploads/2015/02/ThankYou2.jpg