„LABORATORY IMMUNETECHNIQUES IN MOLECULAR BIOLOGY RESEARCH”
methodical course

9-13st December 2019

Introduction. Immunisation. Polyclonal and monoclonal antibody production, purification and labelling for practical use.

Course code: OPEL_B-139_NEP1

The competition of course equals to 6 ECTS credits.


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Immunological techniques

MICROANALITIKAL METHODS

- immunoserology
- immunohistochemistry

FUNCTIONAL TESTS

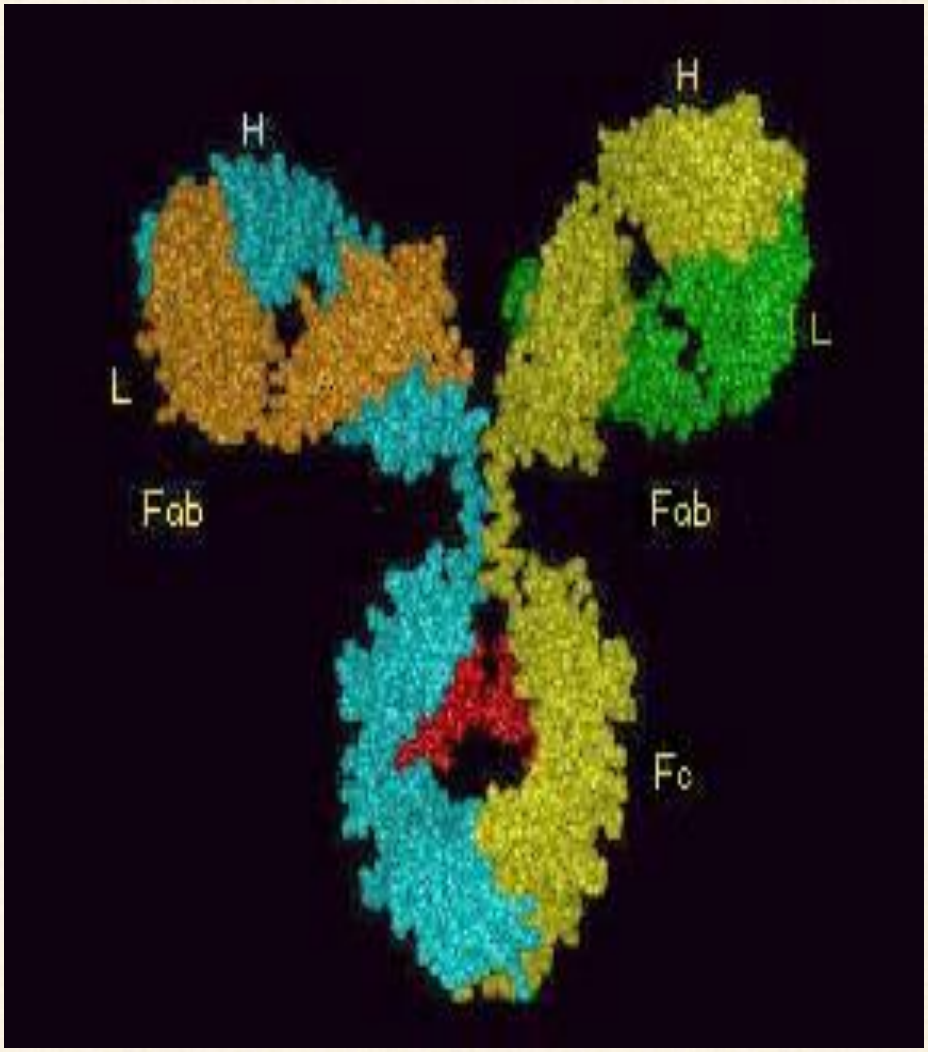
- *in vitro* methods (tissue cultures)
- *in vivo* methods

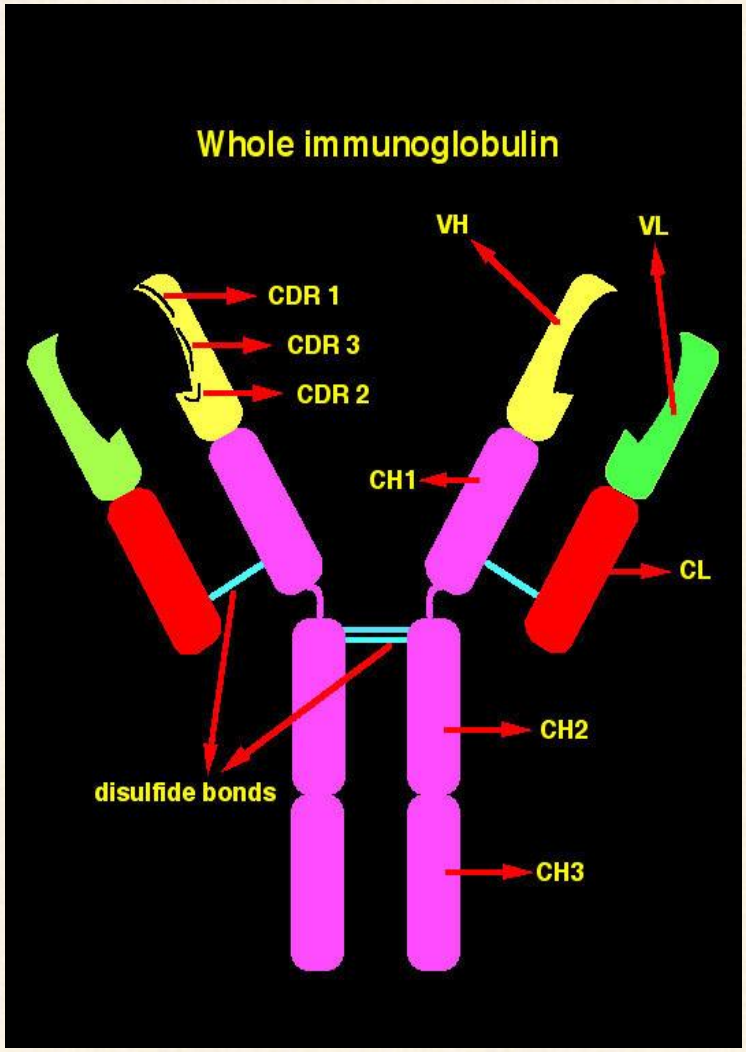
NUCLEIC ACID TECHNIQUES

Immunoserology

- **Methods based on immunoprecipitation**
  - Radial immunodiffusion (Mancini)
  - Radial double immunodiffusion (Ouchterlony)
  - IELFO
  - Nephelometria
- **Methods based on agglutination**
  - Coomb's test (direct, indirect)
  - Latex agglutination
  - Quick tests
- **Micro immunoassais**
  - RIA, ELISA, FIA, RIF

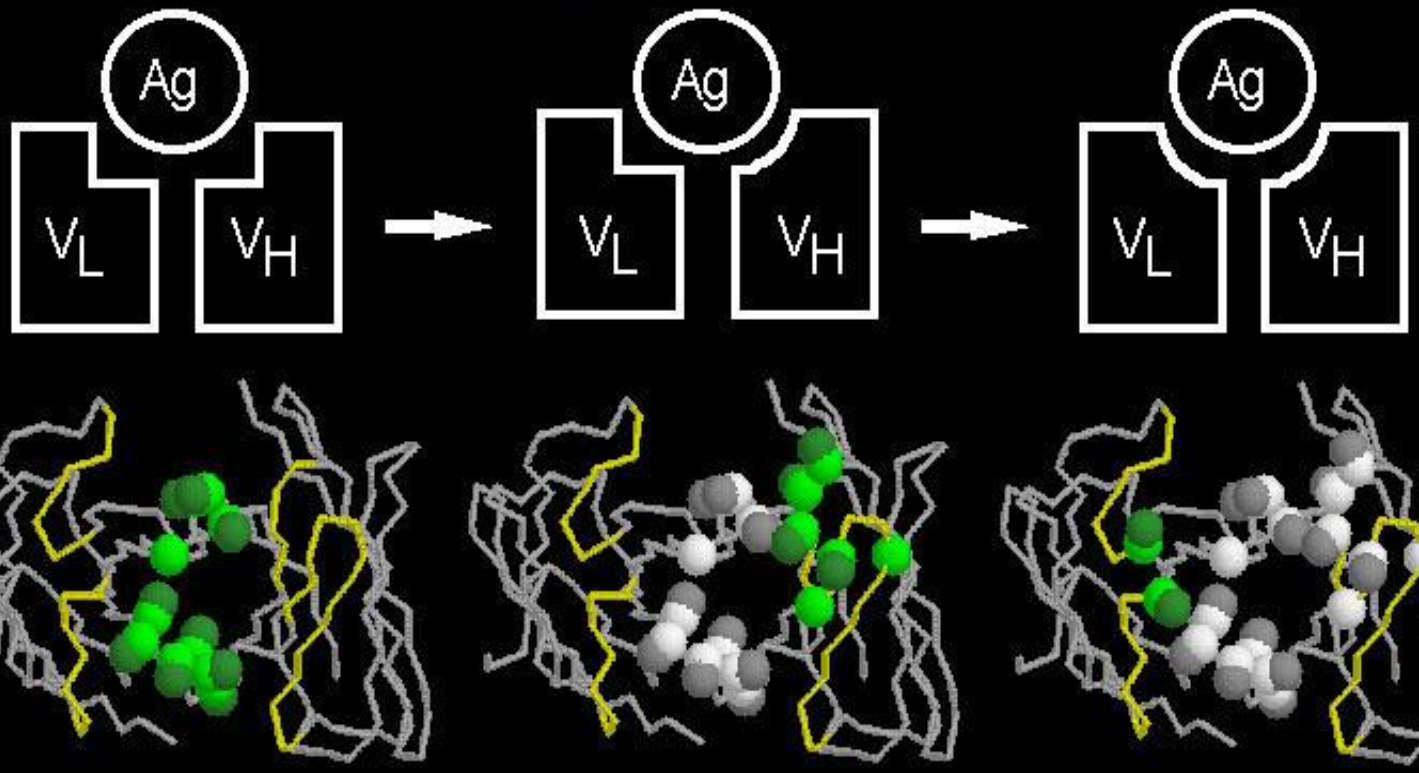
Structure of immunoglobulin G





Antibody affinity maturation

Pini et al. (1998) J. Biol. Chem. *273, 21769-21776*



1st library 2nd library 3rd library

Factors influencing the antibody production

- MHC haplotype of the recipient
- Nature and origin of the antigen
- Dose of the antigen
- Mode of the adminiostration
- Adjuvants
- Kinetics of the immunisation

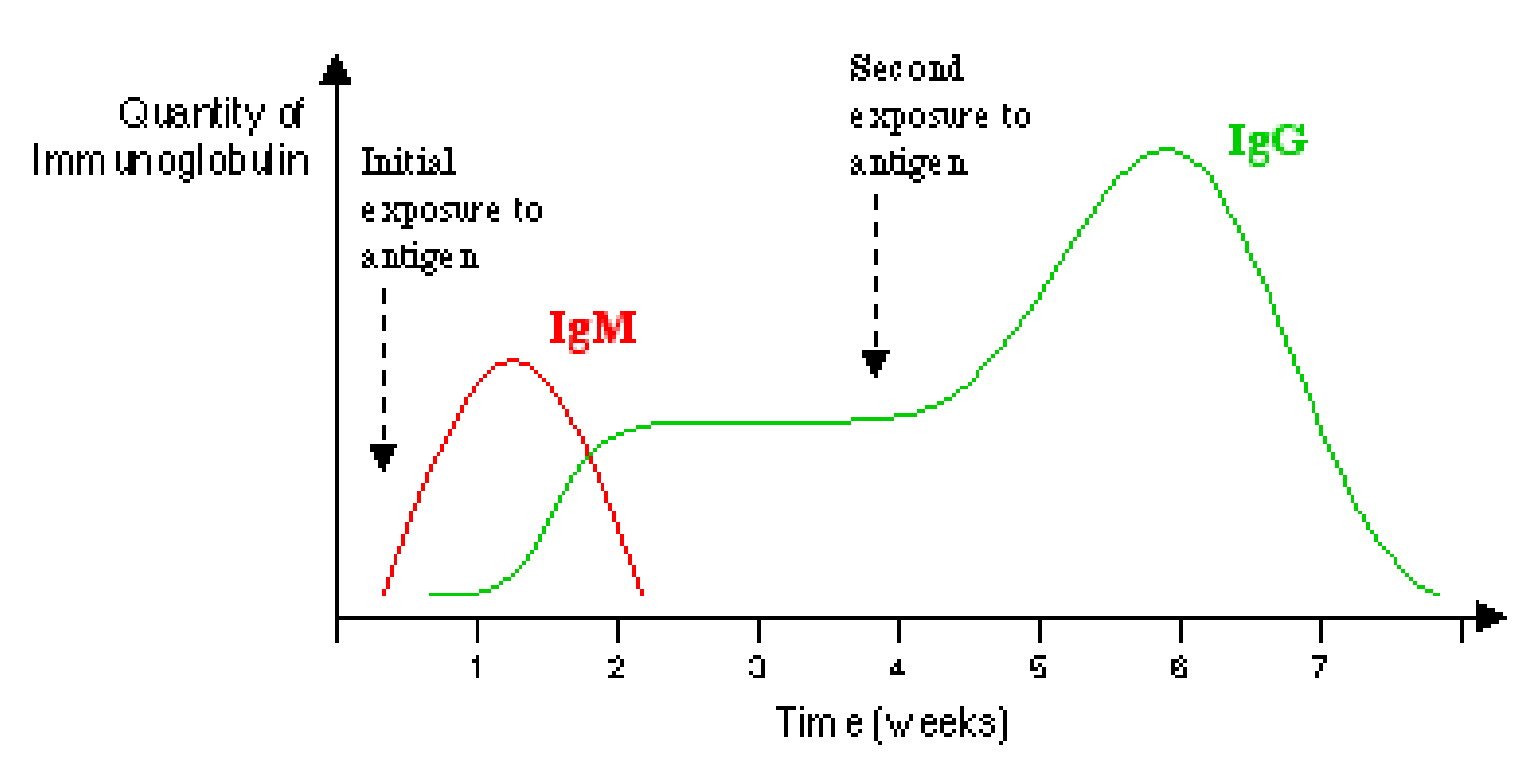
Production of the antibodies

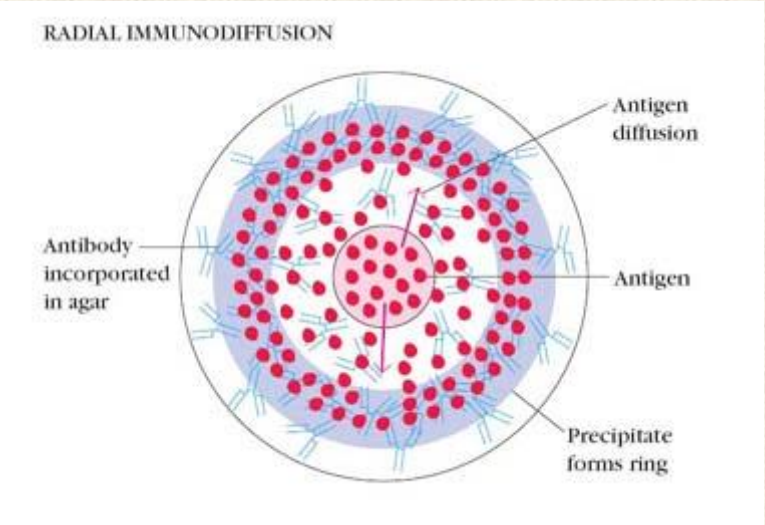
- Policlonal antibodies - antisera
  - immunisation
  - antibody purification
  - labeling for practical applications
- Hybridomas and monoclonal antibodies
  - antibody design and production
  - large scale fermentation of the best clones
  - labeling for practical applications

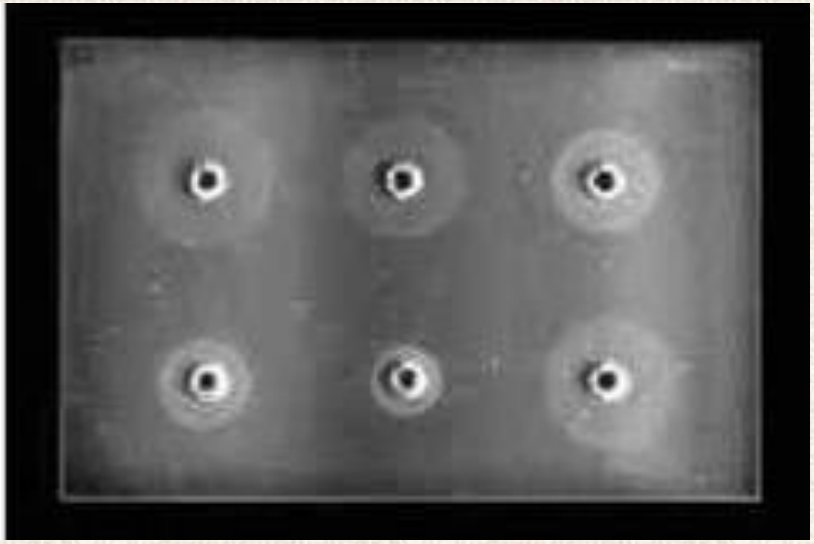
Animals for immunisation

FIG. 4
The production of an antiserum: bleeding an immunized horse from the jugular vein.

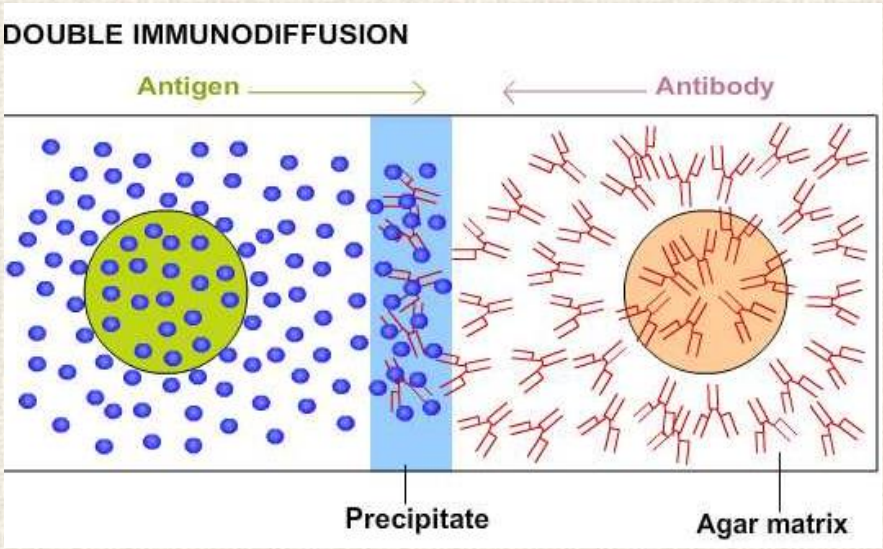
Kinetics of immunoglobulin production

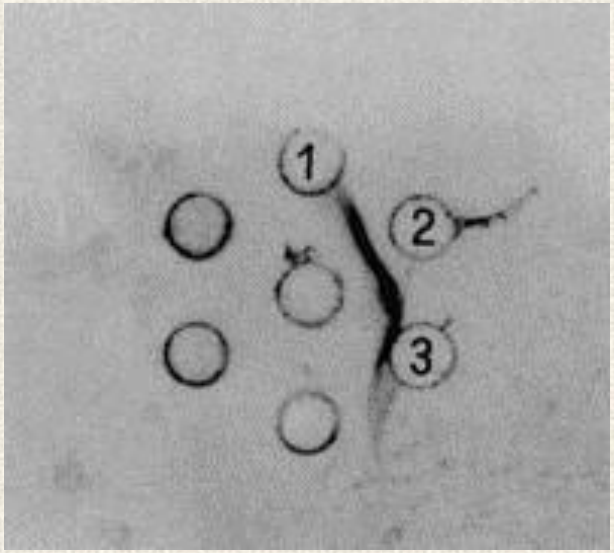




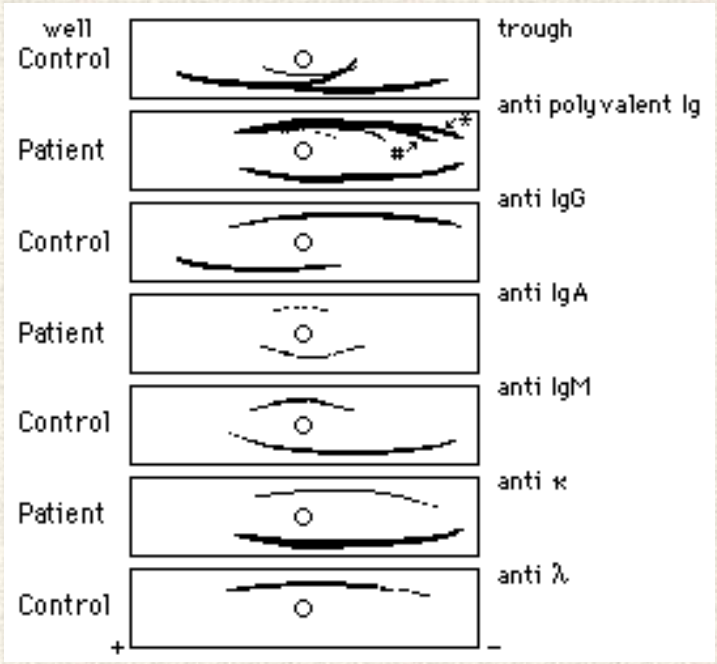


Radial immunodiffusion (Mancini technique)

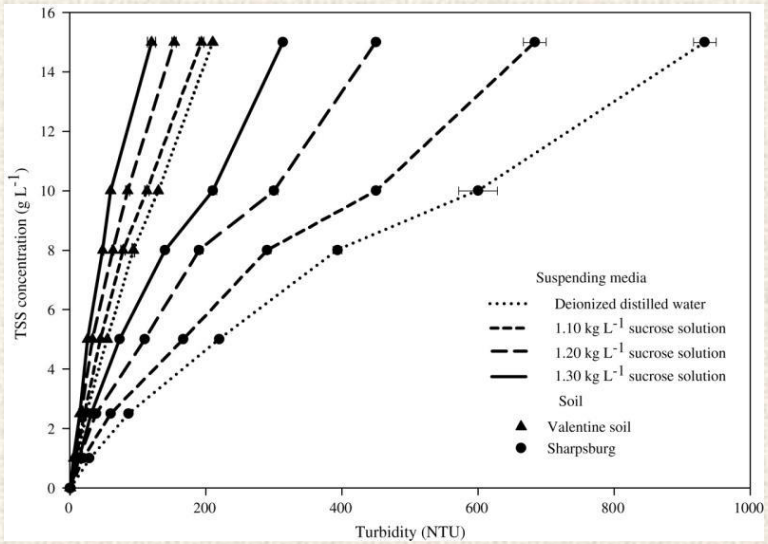




Double immunodiffusion (Ouchterlony technique)



Immunelectrophoresis



Nephelometric turbidity

DIRECT COOMB'S TEST

RBC + anti- Hu IgG Coomb's Reagent =

Patient Sample

Example: The baby's sample is positive for the presence of the mother's Ab on the surface of RBCs in erythroblastosis fetalis

Agglutination

INDIRECT COOMB'S TEST

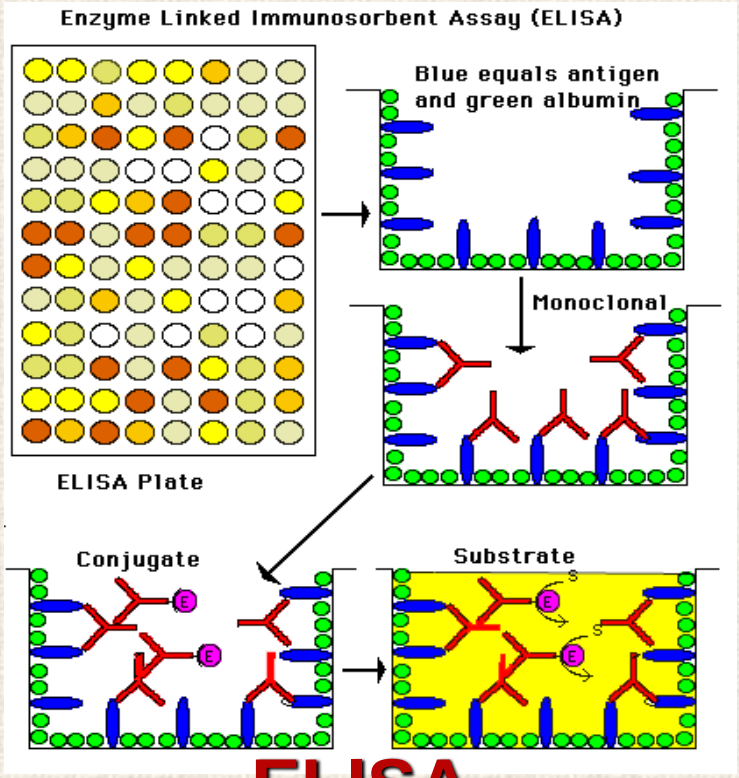
STEP 1: RBC + Patient Sample =

Rh^+ RBCs

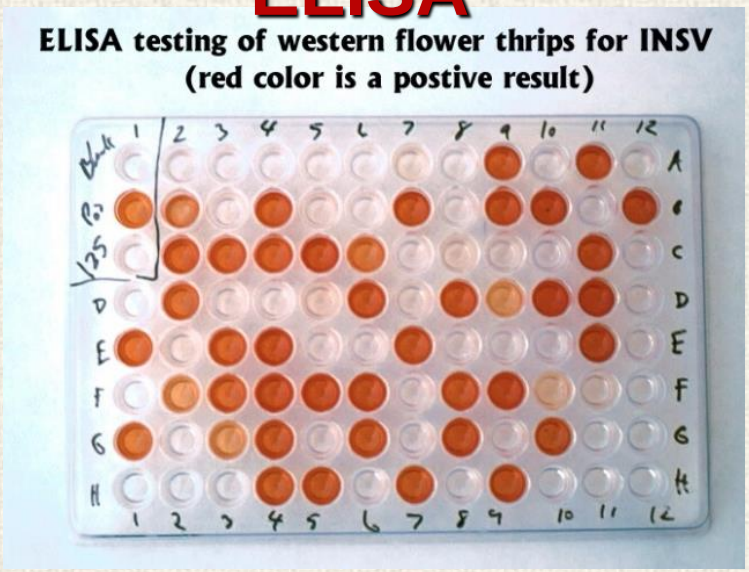
STEP 2: RBC + anti- Hu IgG =

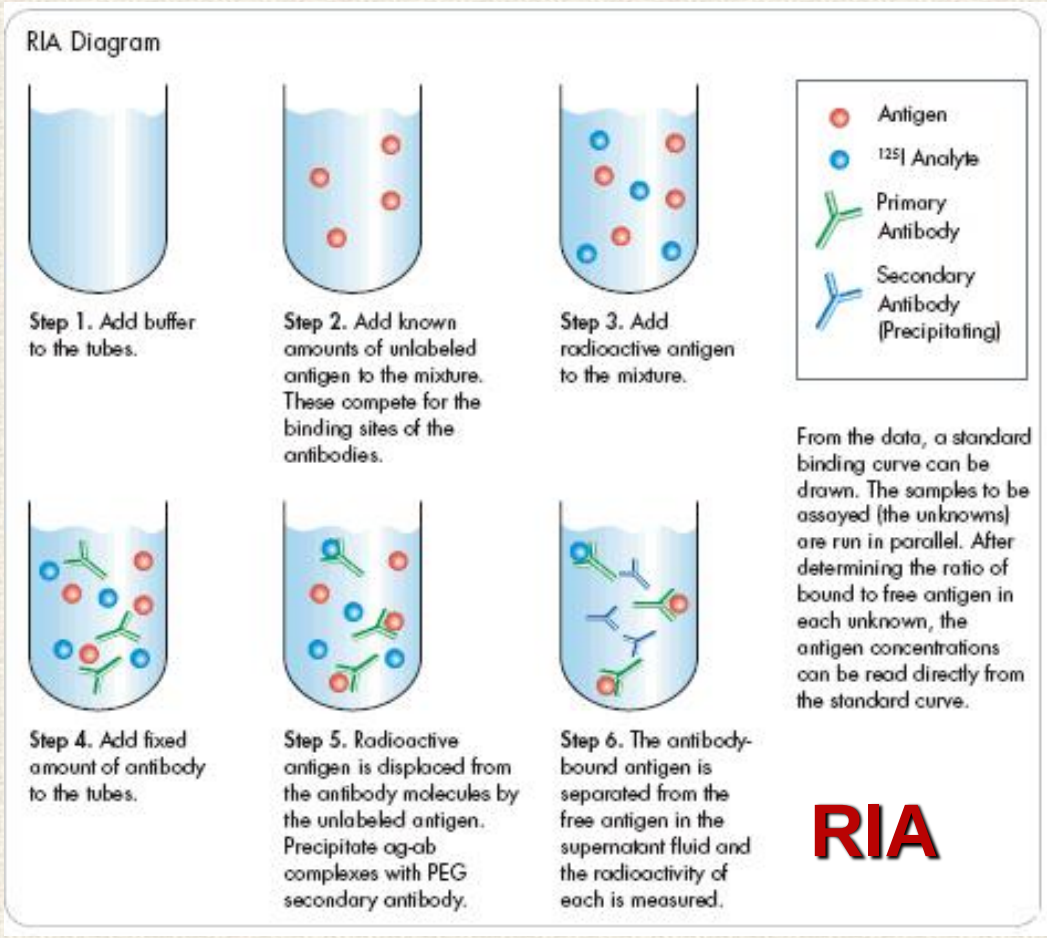
Example: A mother who is Rh^- can have prenatal testing to determine the presence of nonagglutinating Ab in her bloodstream that will attack the fetus's RBCs if the father is Rh^+

Agglutination

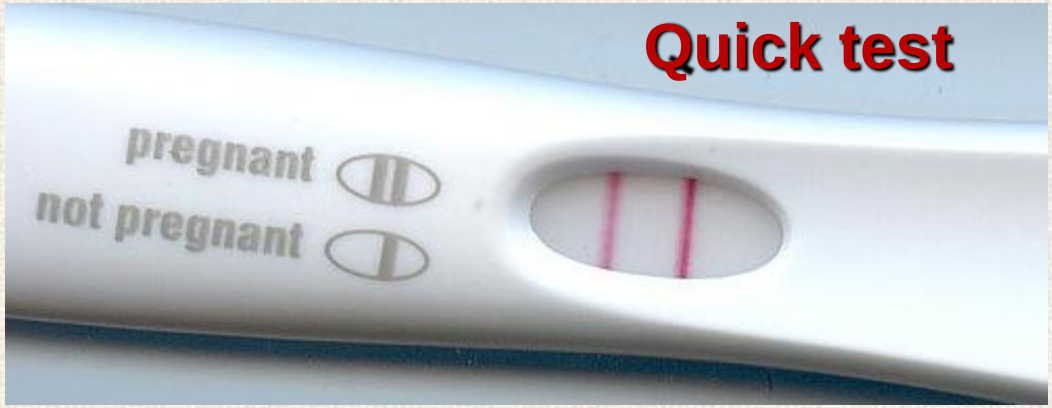

Enzyme Linked Immunosorbent Assay (ELISA)
Blue equals antigen and green albumin
Monoclonal
ELISA Plate
Conjugate
Substrate

ELISA


ELISA testing of western flower thrips for INSV
(red color is a postive result)


RIA Diagram
Step 1. Add buffer to the tubes.
Step 2. Add known amounts of unlabeled antigen to the mixture. These compete for the binding sites of the antibodies.
Step 3. Add radioactive antigen to the mixture.
Antigen
125I Analyte
Primary Antibody
Secondary Antibody (Precipitating)
From the data, a standard binding curve can be drawn. The samples to be assayed (the unknowns) are run in parallel. After determining the ratio of bound to free antigen in each unknown, the antigen concentrations can be read directly from the standard curve.
Step 4. Add fixed amount of antibody to the tubes.
Step 5. Radioactive antigen is displaced from the antibody molecules by the unlabeled antigen. Precipitate ag-ab complexes with PEG secondary antibody.
Step 6. The antibody-bound antigen is separated from the free antigen in the supernatant fluid and the radioactivity of each is measured.

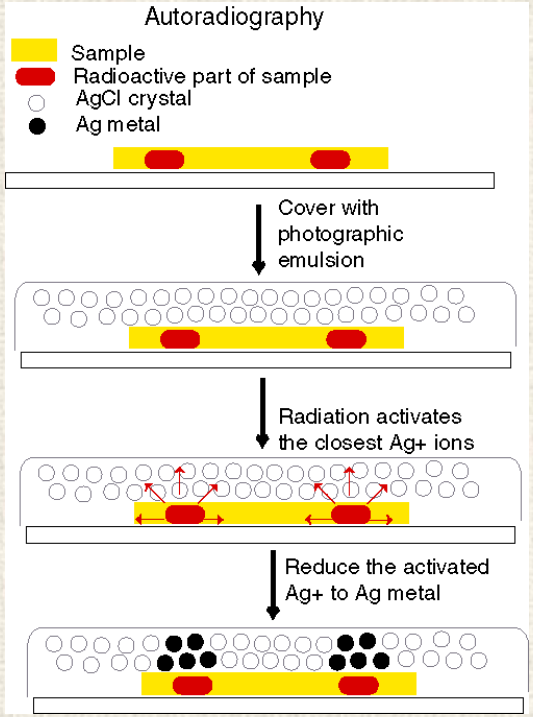
RIA

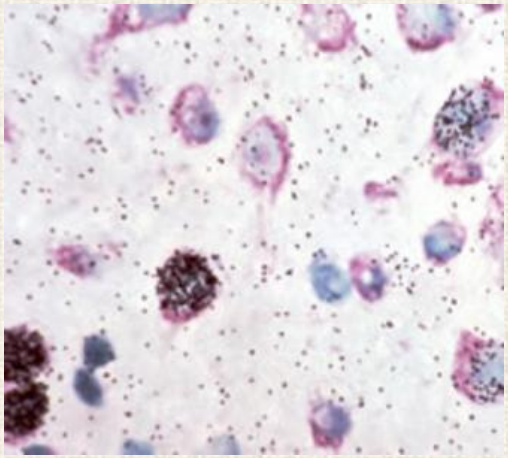

Quick test
pregnant
not pregnant

Immunohisto/cytochemistry

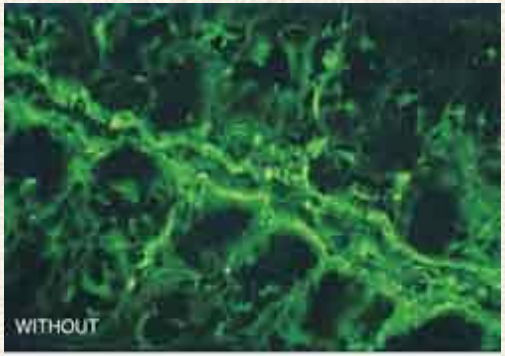
- **Light microscopic methods** (autoradiography, enzyme-immunocytochemistry)
  - Immunohistochemical imagine analysis
- **Fluorescent microscopic methods**
  - Conventional immunoflouresce
  - Laser scanning (confocal) microscopy
- **Flow cytometry**
  - Cell surface and intracytoplasmic labelling
  - Microbead technique (MMA)

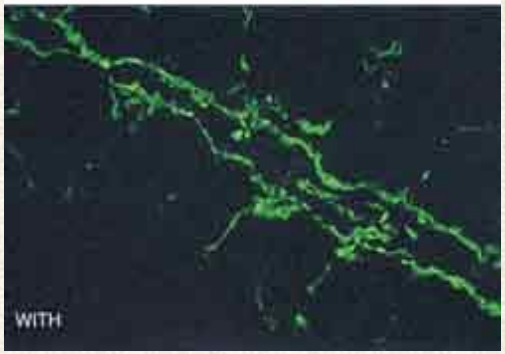
Immunohistochemical applications

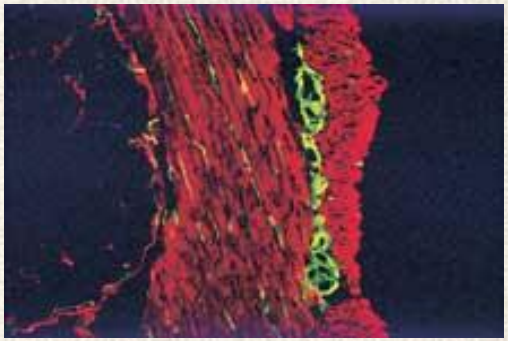




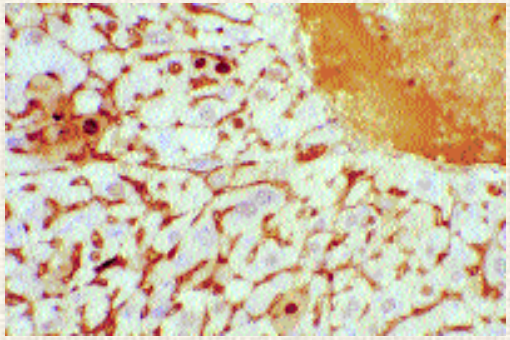
Autoradiography

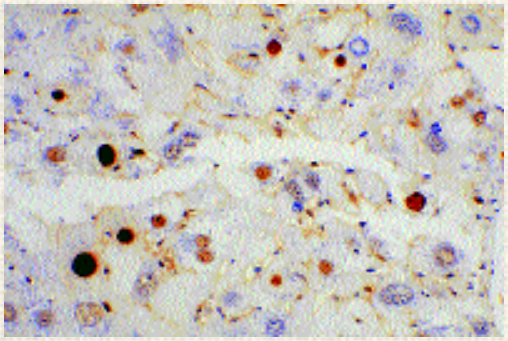


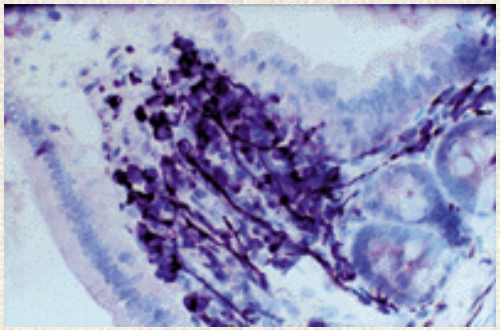




Immuno-fluorescence

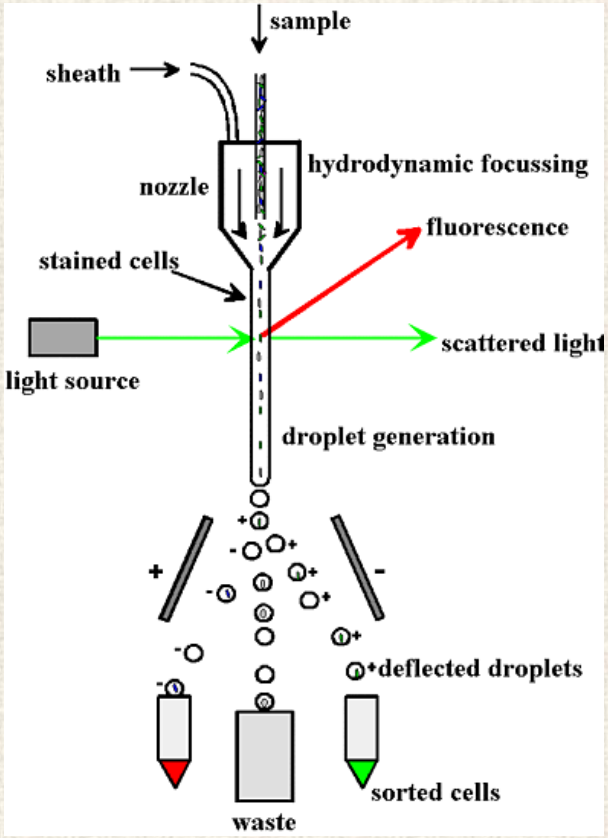


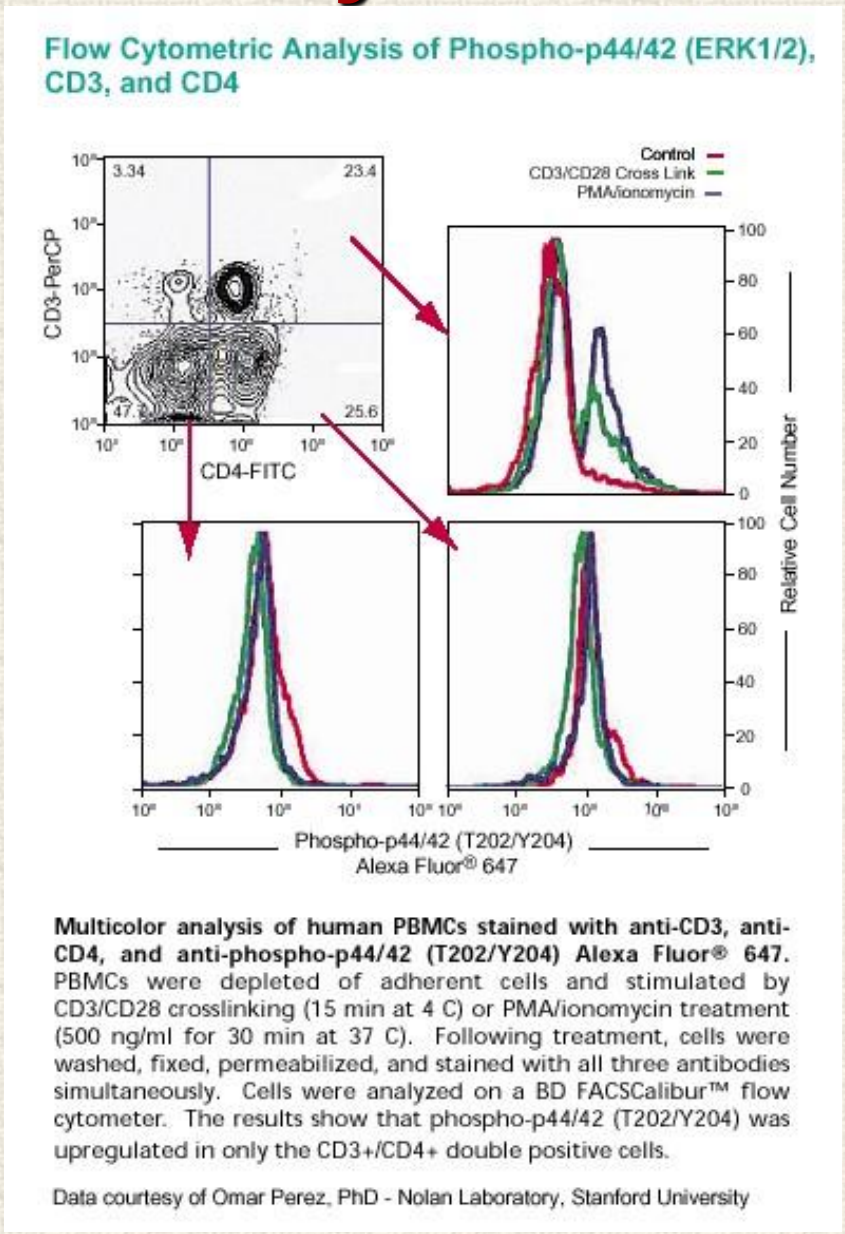




Enzyme immunohisto-chemistry

Flow cytometry



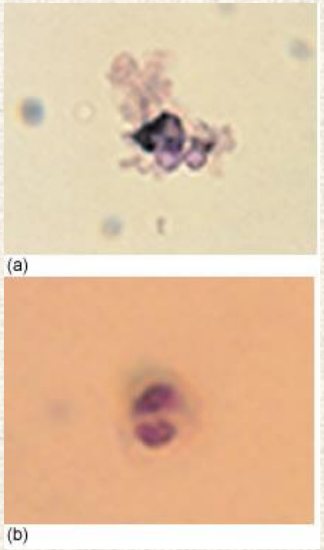


Multicolor analysis of human PBMCs stained with anti-CD3, anti-CD4, and anti-phospho-p44/42 (T202/Y204) Alexa Fluor® 647. PBMCs were depleted of adherent cells and stimulated by CD3/CD28 crosslinking (15 min at 4 C) or PMA/ionomycin treatment (500 ng/ml for 30 min at 37 C). Following treatment, cells were washed, fixed, permeabilized, and stained with all three antibodies simultaneously. Cells were analyzed on a BD FACSCalibur™ flow cytometer. The results show that phospho-p44/42 (T202/Y204) was upregulated in only the CD3+/CD4+ double positive cells.

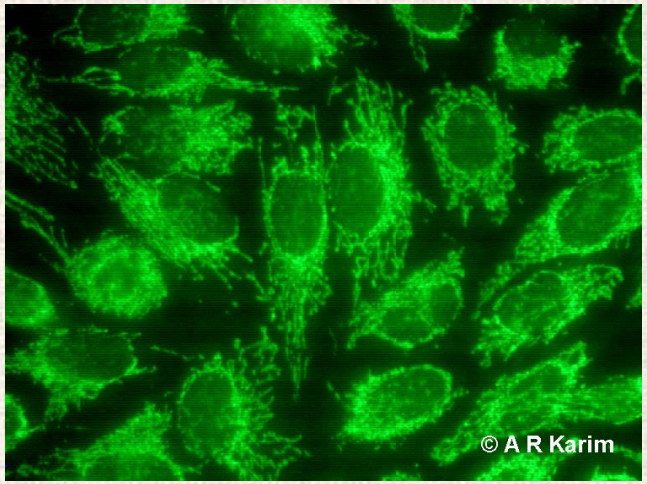
Data courtesy of Omar Perez, PhD - Nolan Laboratory, Stanford University

In vitro functional tests

- **Short term cultures**
  - analysis of cell migration
  - phagocytosis tests (sheep RBC, lisosyme, NBT)
  - activity analysis (mixed lymphocyte culture, cytotoxicity)

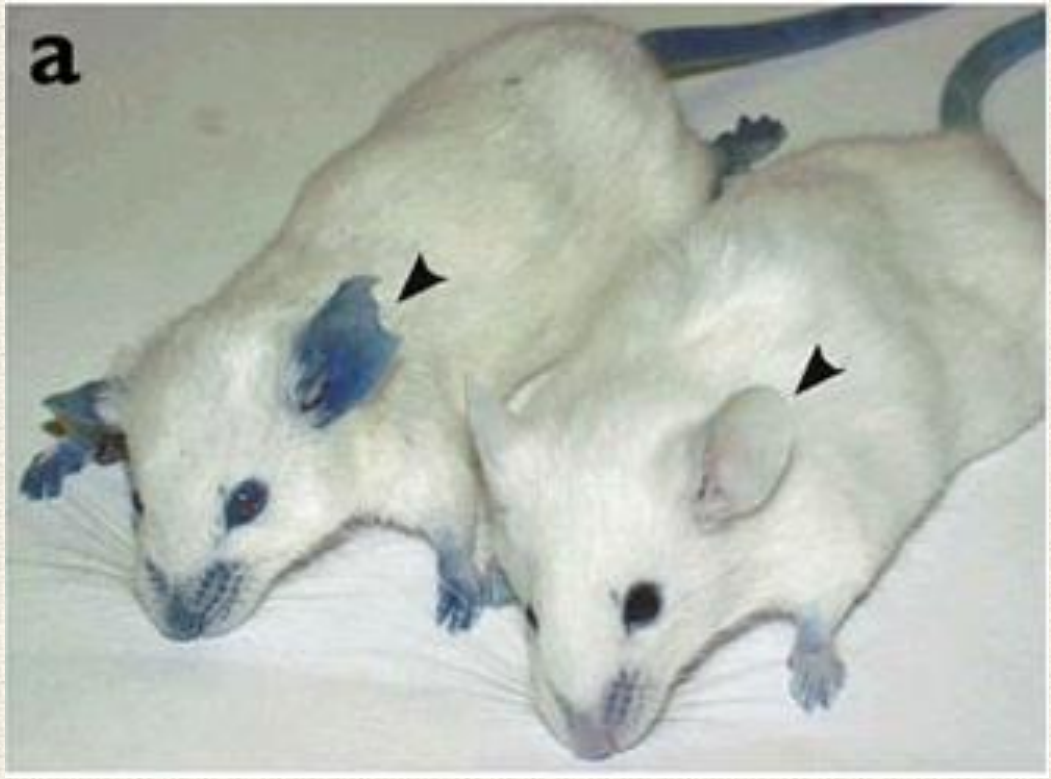


- **Continous cell cultures**
  - autoantibody analysis
  - cytotoxicity tests



In vivo functional tests

- Immunotoxicity tests
- Skin window test
- Passive cutan analphilaxis (PCA)



Characteristics of polyclonal antibodies

- Blood serum (variable idiotypes, different isotypes, mixture of antibodies with different affinity)
- Characterised by avidity
- Standard in bench

Immunoglobulin purification

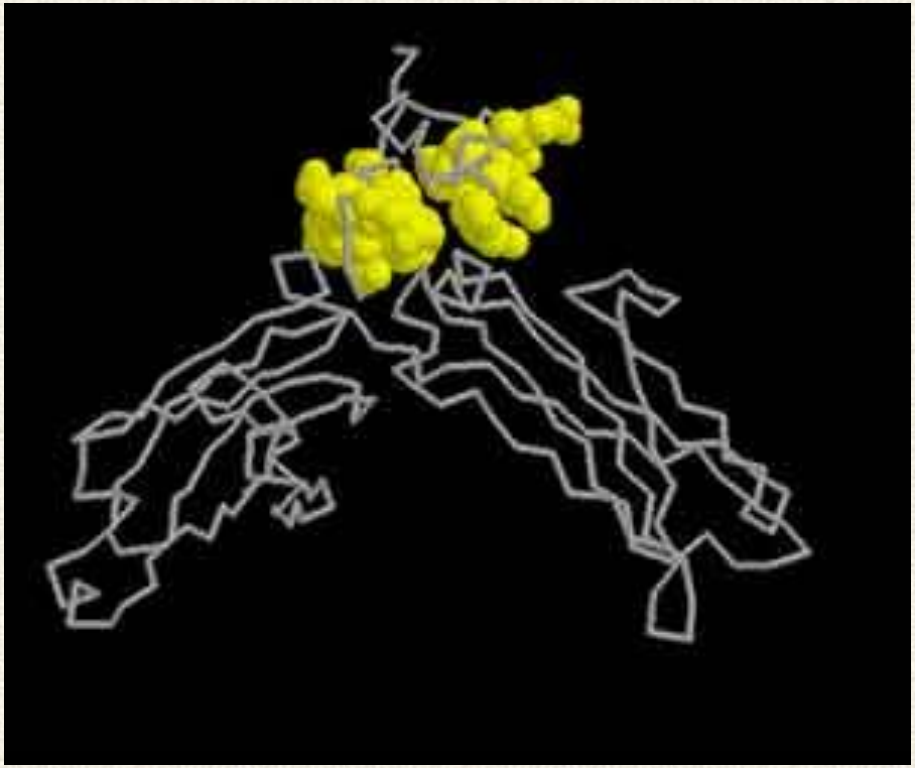
Salt precipitation $(NH_4)_2SO_4$

Liquid chromatography

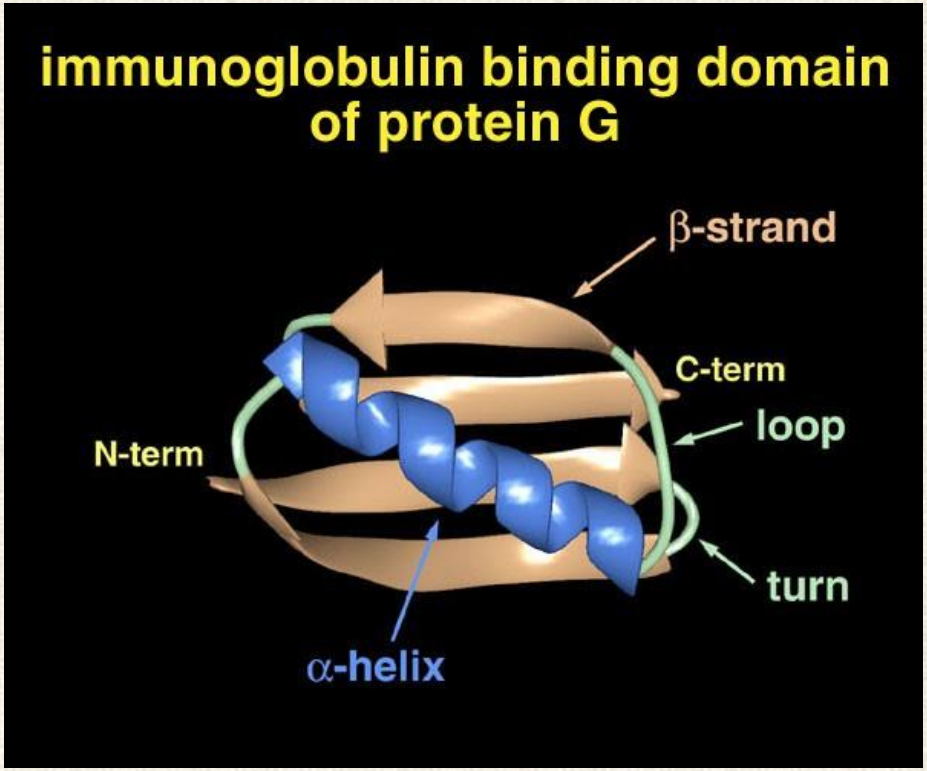
Affinity chromatography (Fc end, antigen)

Affinity purification

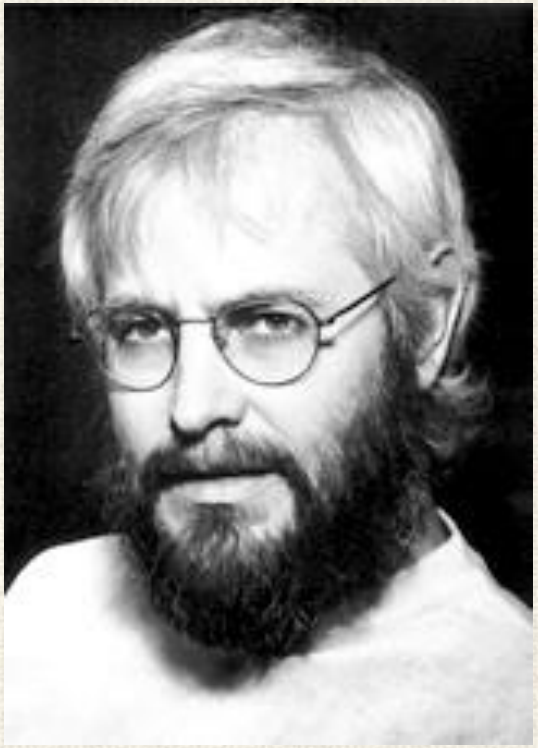
Protein A



Protein G



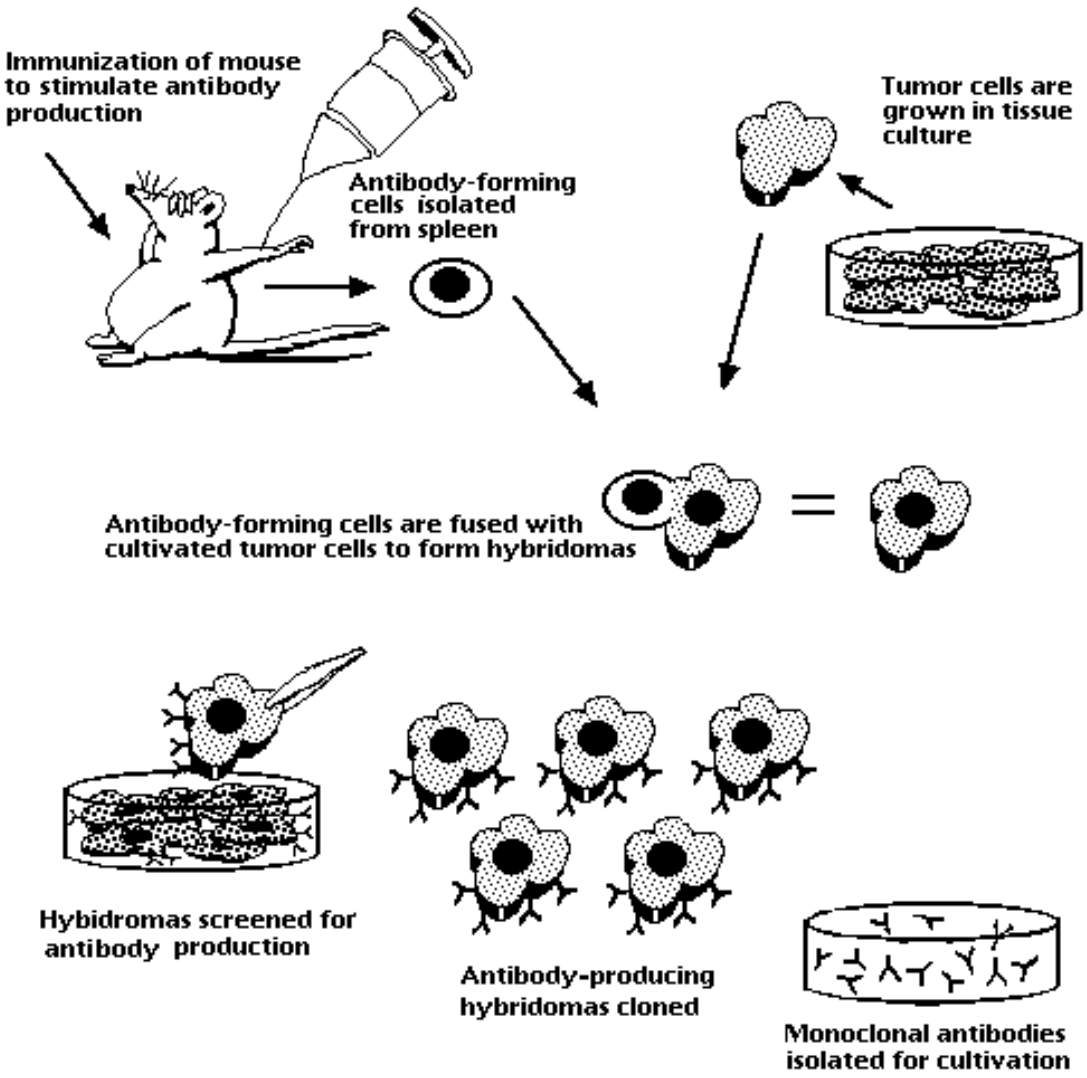
Hybridomas and monoclonal antibodies



César Milstein és Georg Köhler

Nobel prize,1984: ***"for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies"***

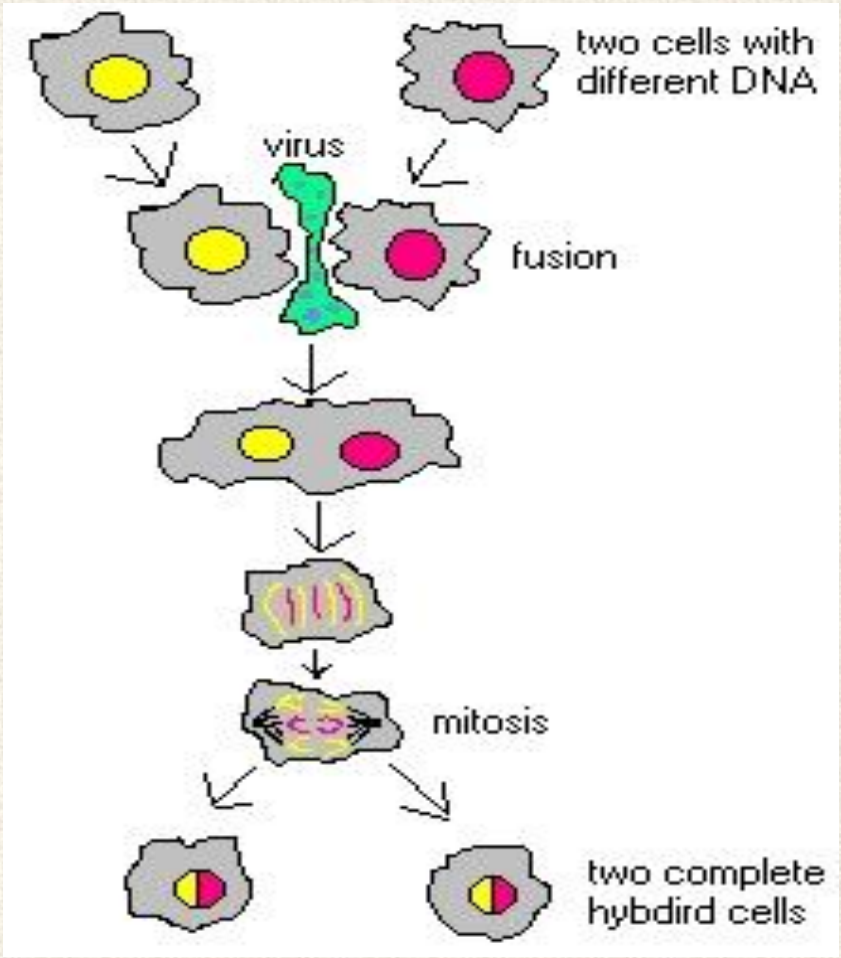
Monoclonal Antibody Production

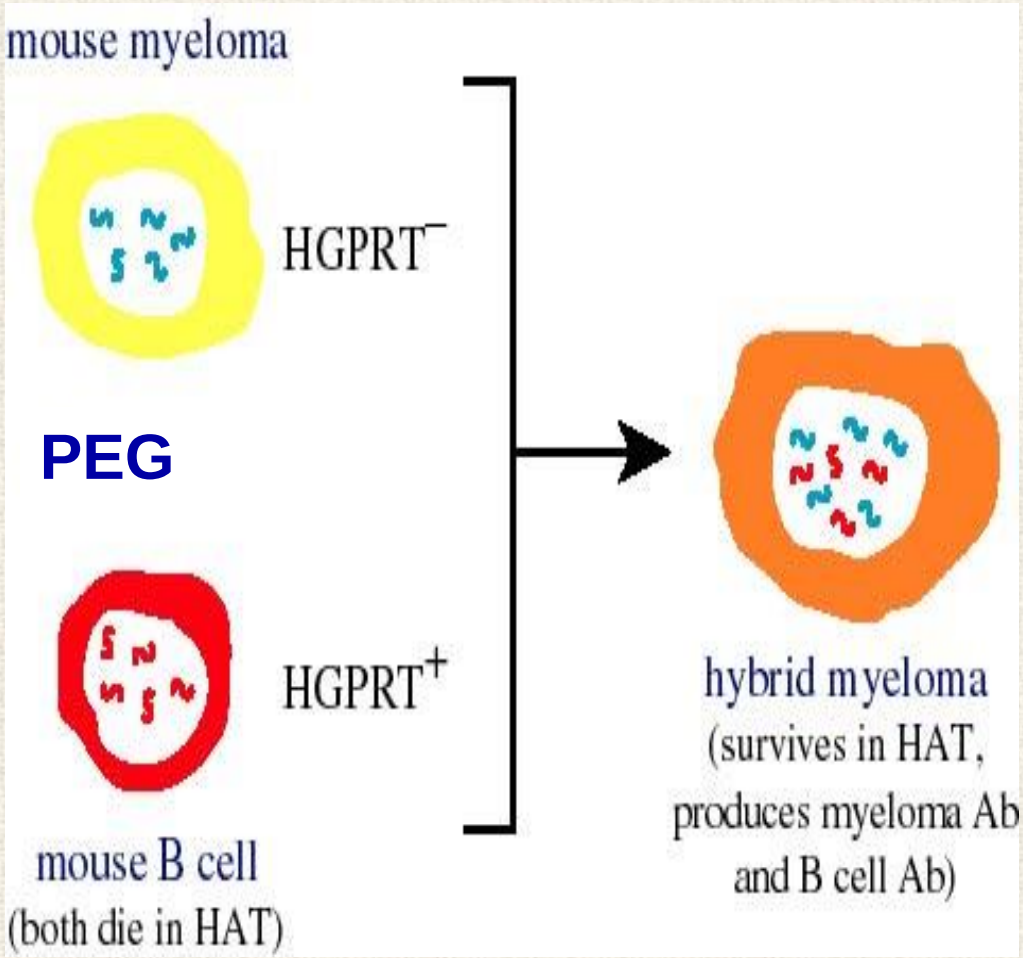


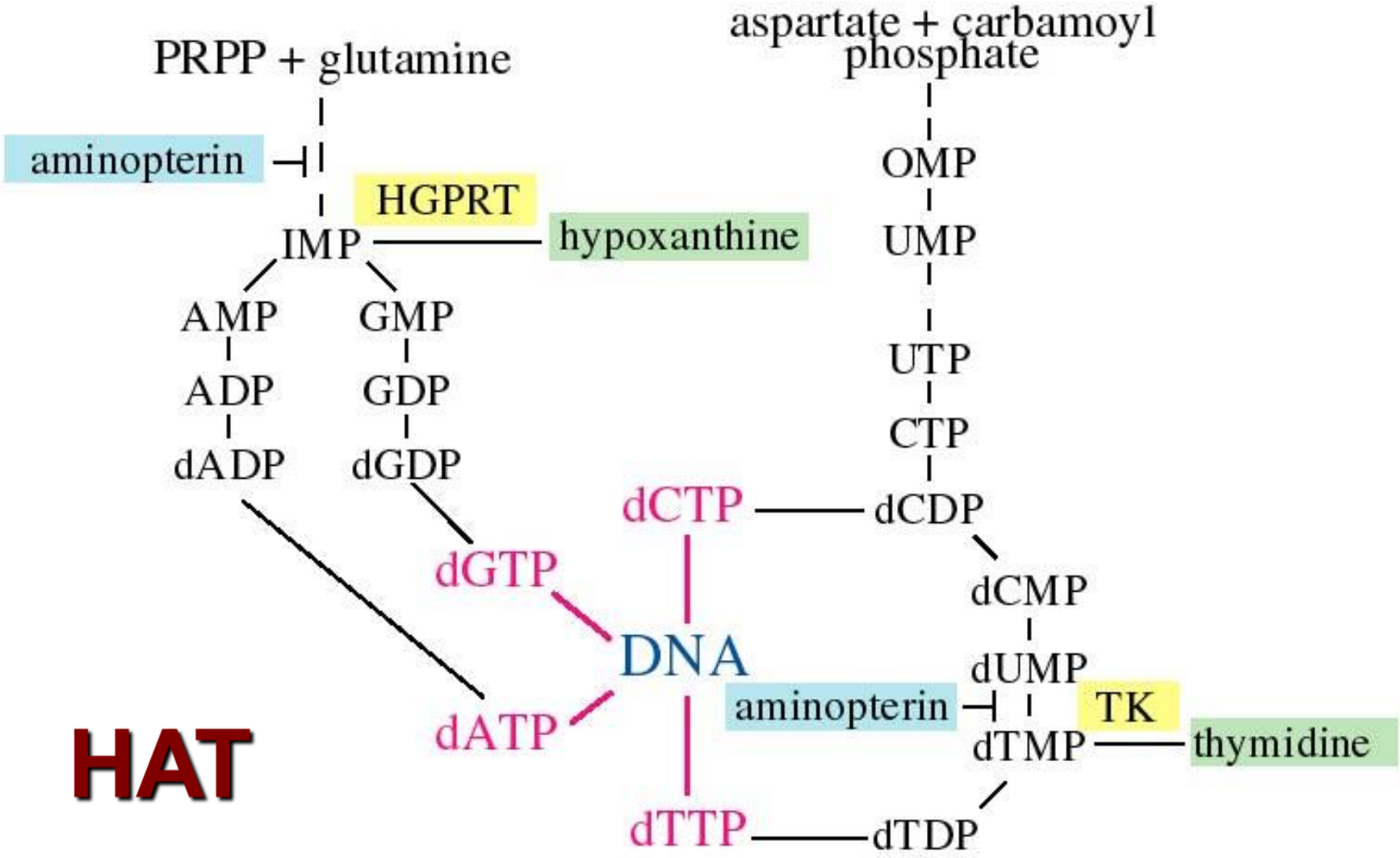
Characteristics of monoclonal antibodies

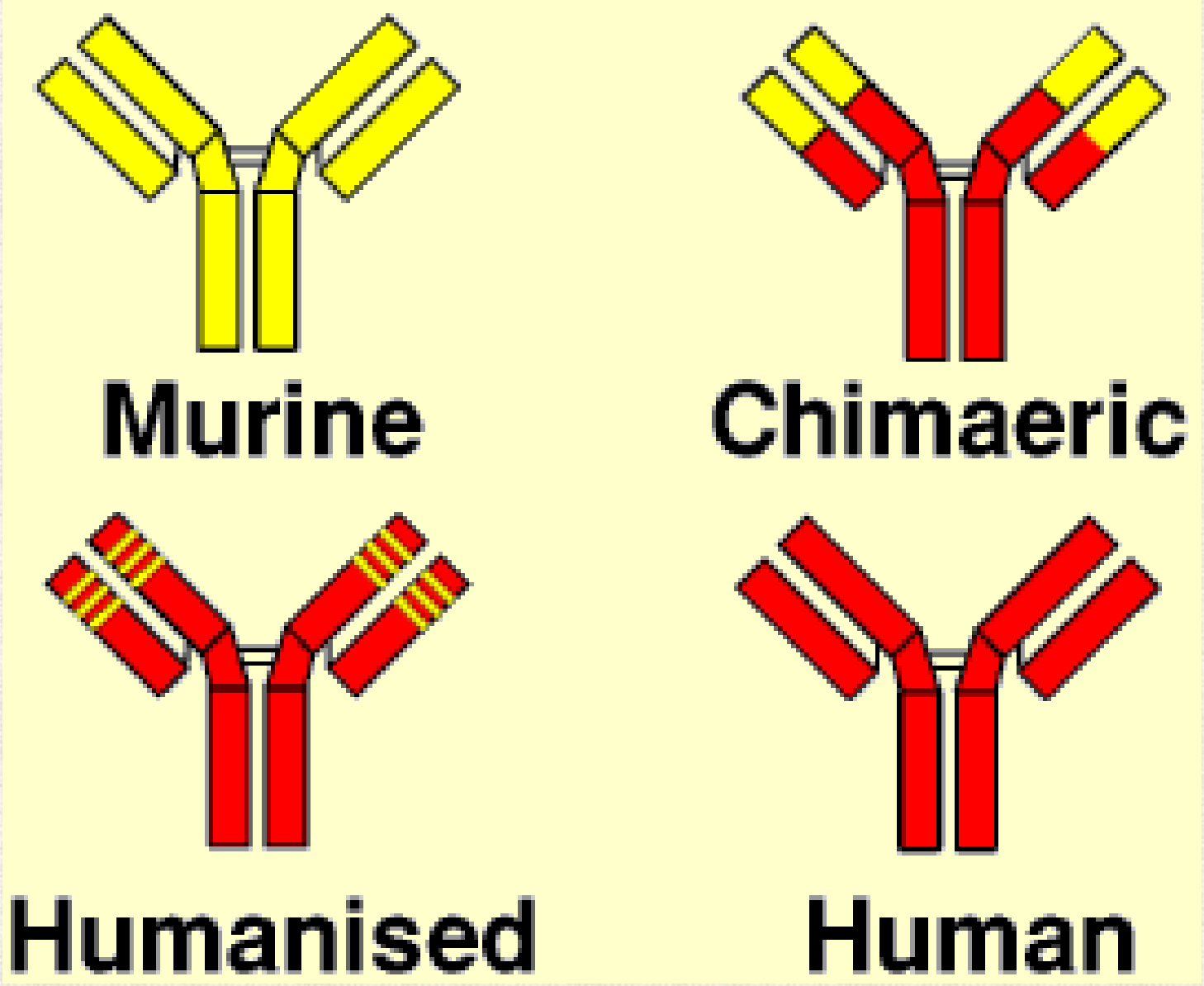
- Genetically engineered antibodies
- Specific in single epitope
- Uniform antibody molecules
- Characterised by chemical affinity
- Standard by the cell line

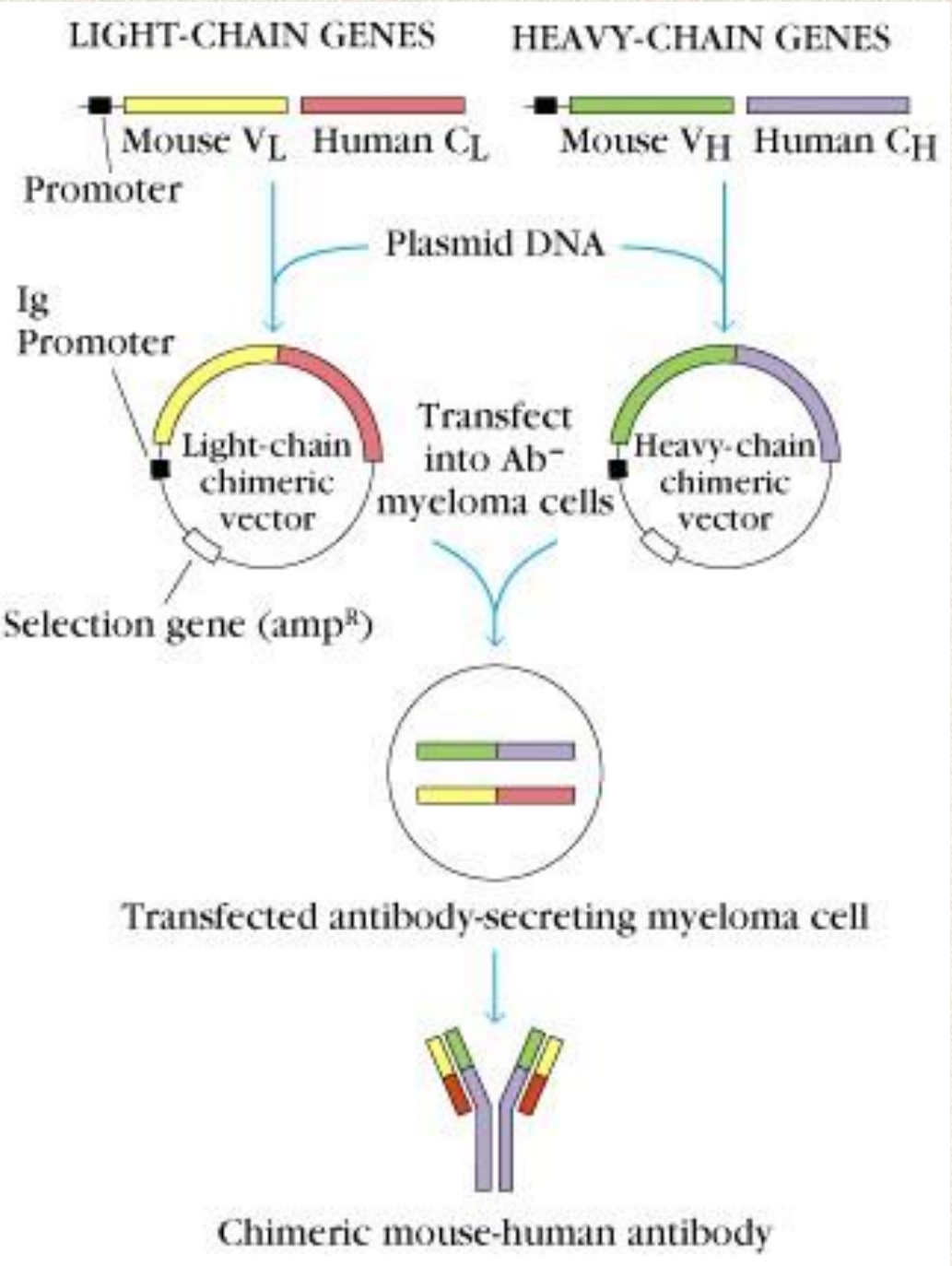
Somatic cell hybridisation and selection

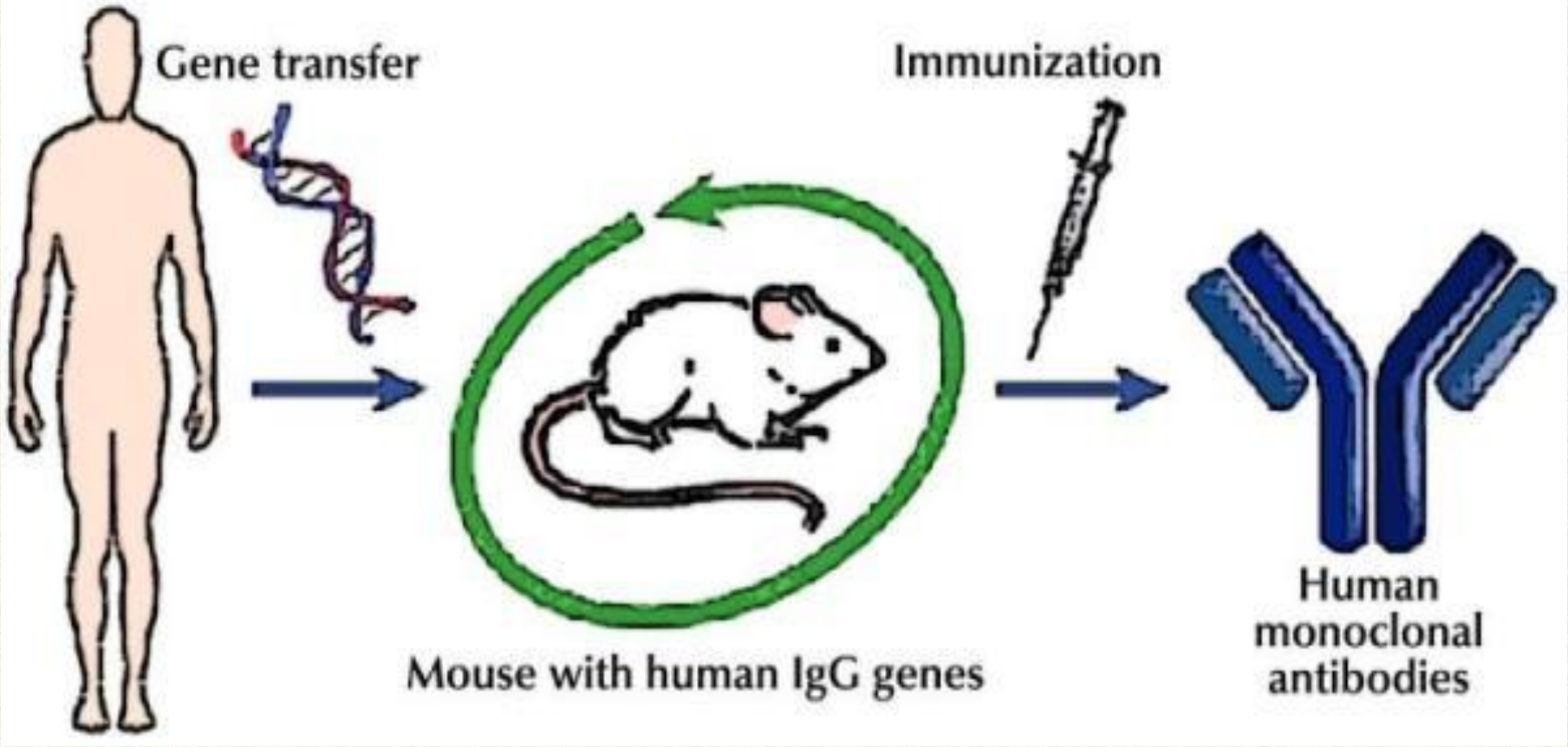


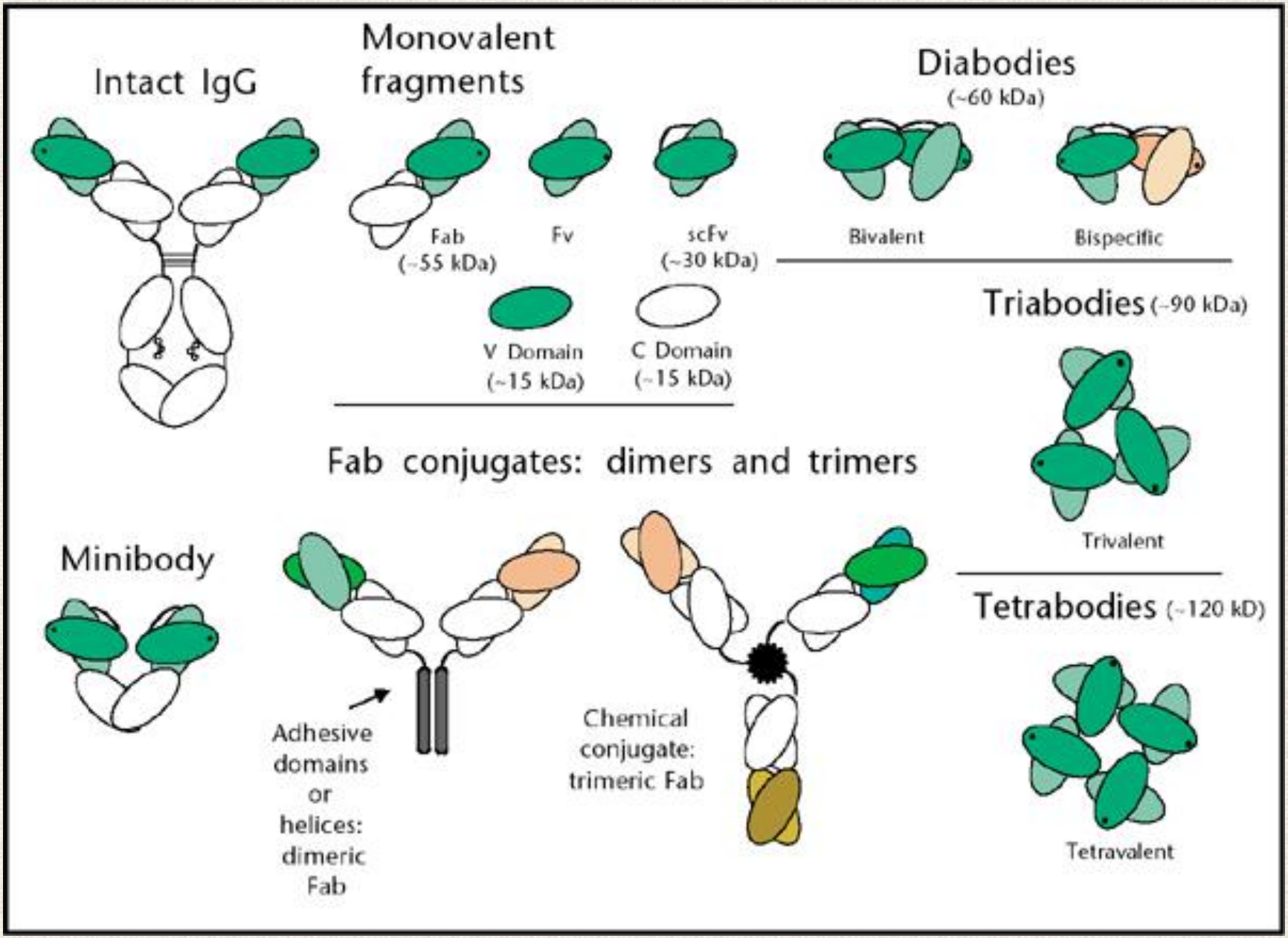



PRPP + glutamine
aspartate + carbamoyl phosphate
aminopterin
HGPRT
IMP
hypoxanthine
OMP
UMP
AMP
GMP
UTP
ADP
GDP
CTP
dADP
dGDP
dCTP
dCDP
dGTP
dCMP
DNA
dUMP
aminopterin
TK
dATP
dTMP
thymidine
dTTP
dTDP
HAT

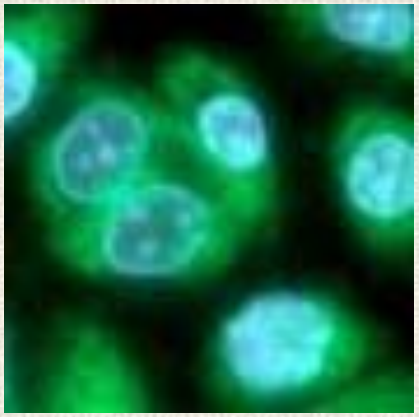

Murine
Chimaeric
Humanised
Human

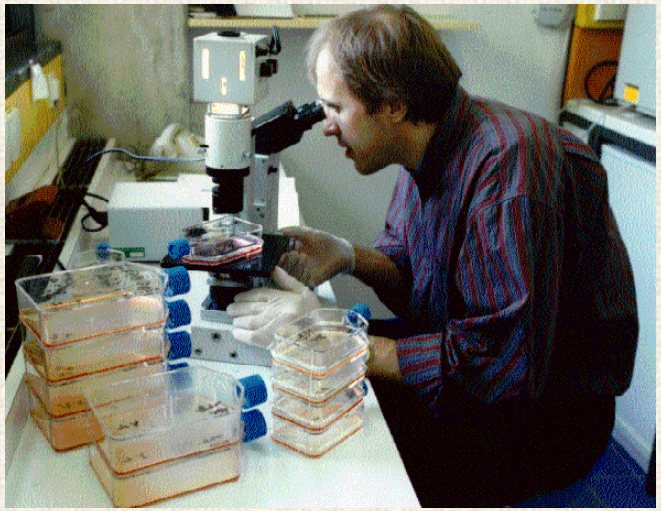

LIGHT-CHAIN GENES
HEAVY-CHAIN GENES
Mouse V_L
Human C_L
Mouse V_H
Human C_H
Promoter
Plasmid DNA
Ig
Promoter
Light-chain
chimeric
vector
Transfect
into Ab⁻
myeloma cells
Heavy-chain
chimeric
vector
Selection gene (amp^R)
Transfected antibody-secreting myeloma cell
Chimeric mouse-human antibody


Gene transfer
Immunization
Mouse with human IgG genes
Human monoclonal antibodies


Intact IgG
Monovalent fragments
Fab (~55 kDa)
Fv
scFv (~30 kDa)
V Domain (~15 kDa)
C Domain (~15 kDa)
Diabodies (~60 kDa)
Bivalent
Bispecific
Triabodies (~90 kDa)
Trivalent
Fab conjugates: dimers and trimers
Minibody
Adhesive domains or helices: dimeric Fab
Chemical conjugate: trimeric Fab
Tetrabodies (~120 kD)
Tetravalent

Hybridoma culturing





First monoclonal antibodies for therapeutic use

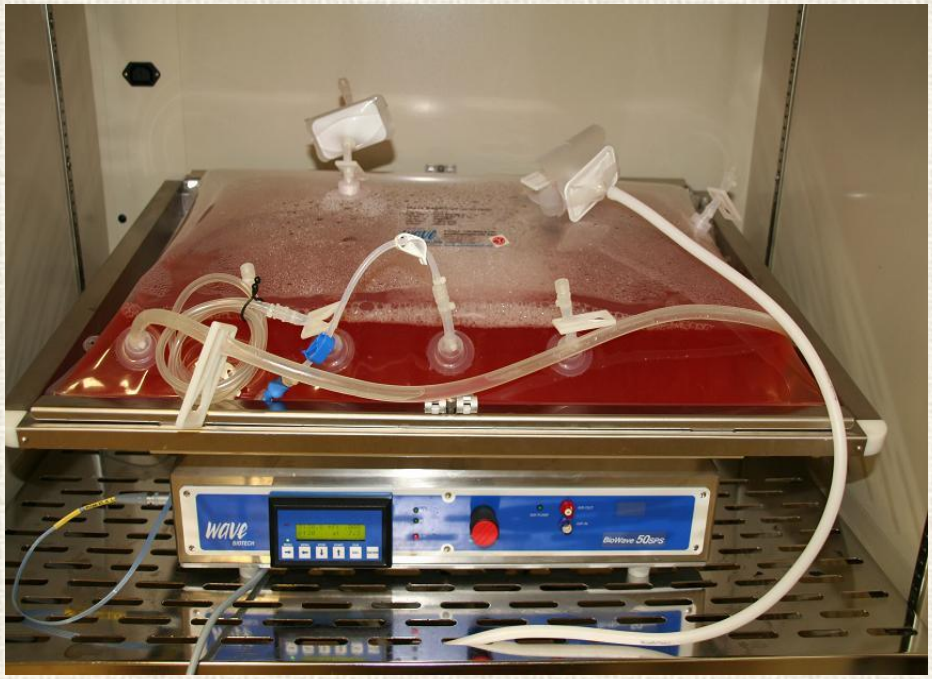
Generic name	Trade name	Sponsor company	Type	Approval date
Muromonab-CD3	Orthoclone	Ortho Biotech	Murine	1986
Abciximab	ReoPro	Centocor	Chimeric	1994
Rituximab	Rituxan	Genentech	Chimeric	1997
Daclizumab	Zenapax	Hoffman-La Roche	Humanized	1997
Basiliximab	Simulect	Novartis	Chimeric	1998
Palivizumab	Synagis	MedImmune	Humanized	1998
Infliximab	Remicade	Centocor	Chimeric	1998
Trastuzumab	Herceptin	Genentech	Humanized	1998
Gemtuzumab ozogamicin	Mylotarg	Wyeth-Ayerst	Humanized	2000
Alemtuzumab	Campath	Millennium/ILEX	Humanized	2001

Licences are expired in 2010 and 2011. Number of therapeutic monoclonal antibodies in the market is closed to 2000 including biosimilars.

November 10, 2017, **68** new monoclonal antibody products have been approved in the US or in the EU for the treatment of a variety of diseases. At the current approval rate of ~70 new original monoclonal antibody products will be on the market by 2020, and combined world-wide sales will be nearly $125 billion.


CELLMAX
SPECTRUM

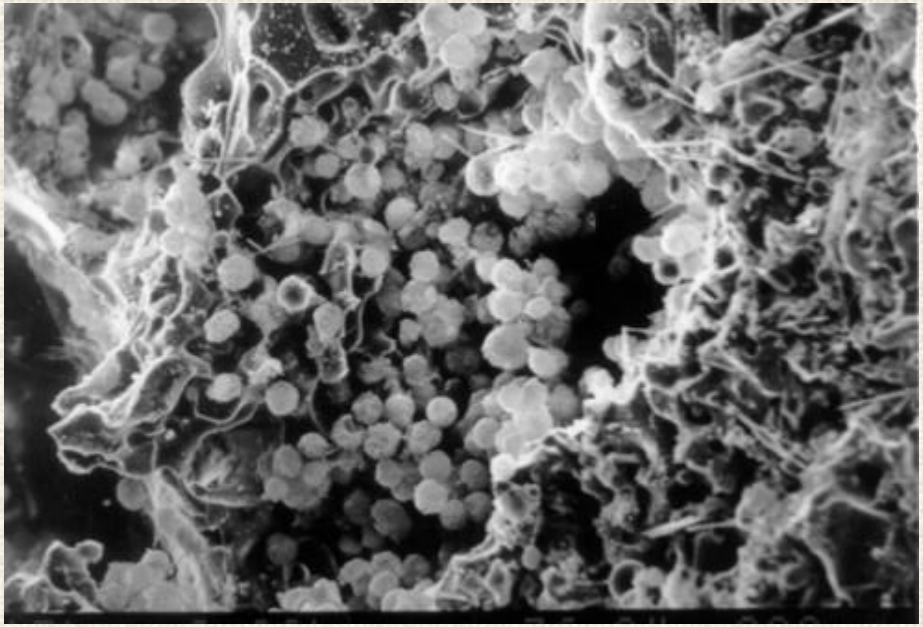

ns!
011
06 05


wave

Fermentation in industrial scale

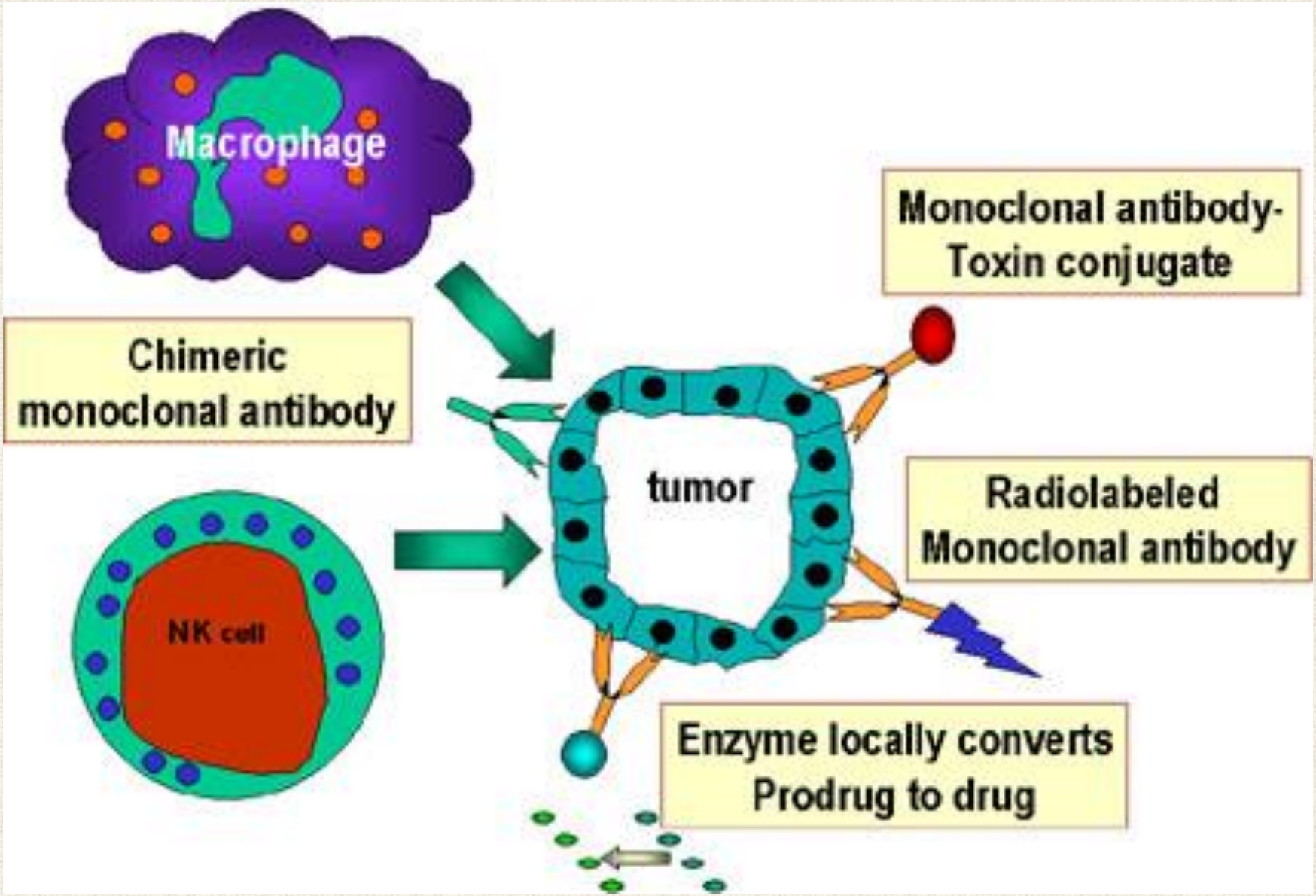

CMC

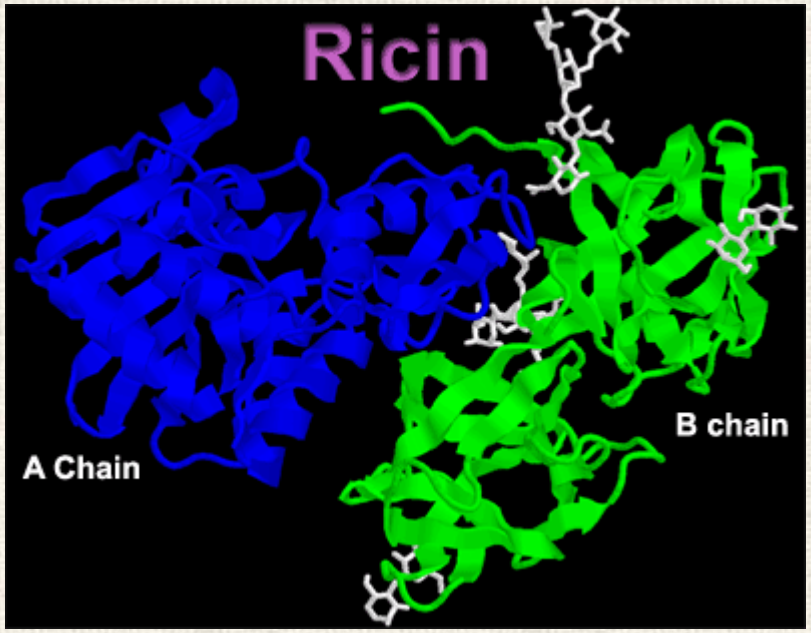

AMIT VERMA



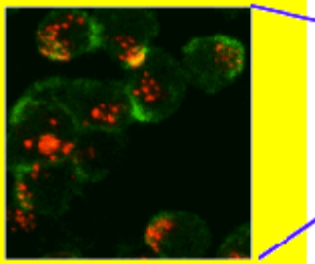
New production greenhouse facilities are also available to …… through a collaboration with the University of Arkansas at Fayetteville. These plant growth facilities will support cGMP compliant growth of **transgenic plants for the expression of monoclonal antibodies in plants.**

Immunotoxin therapy

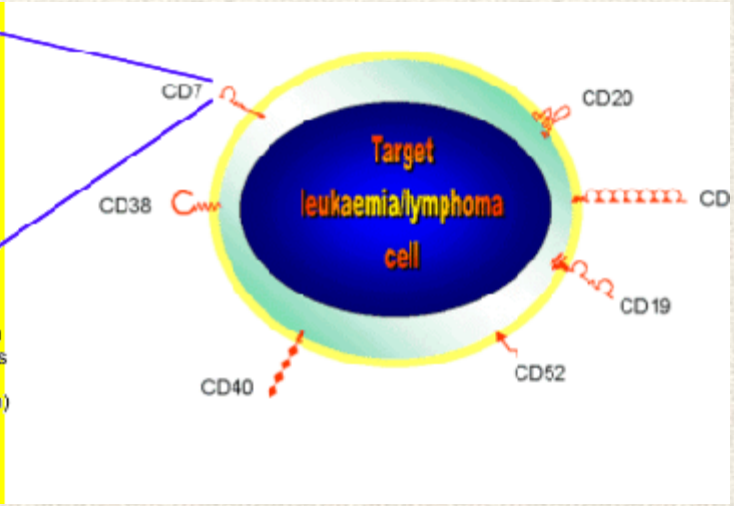


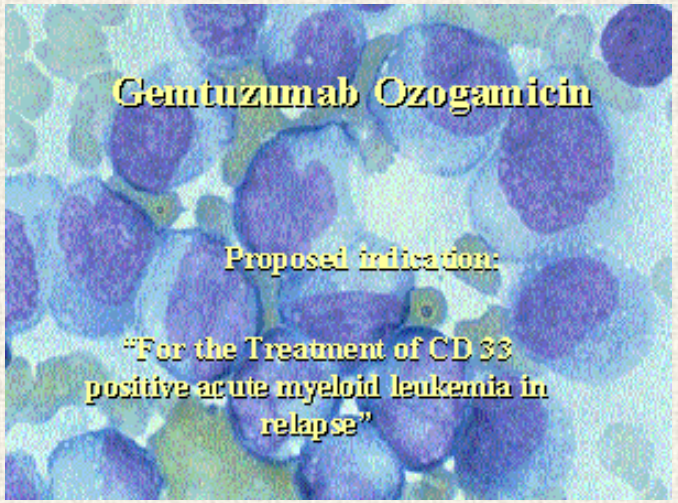


HER-2/neu

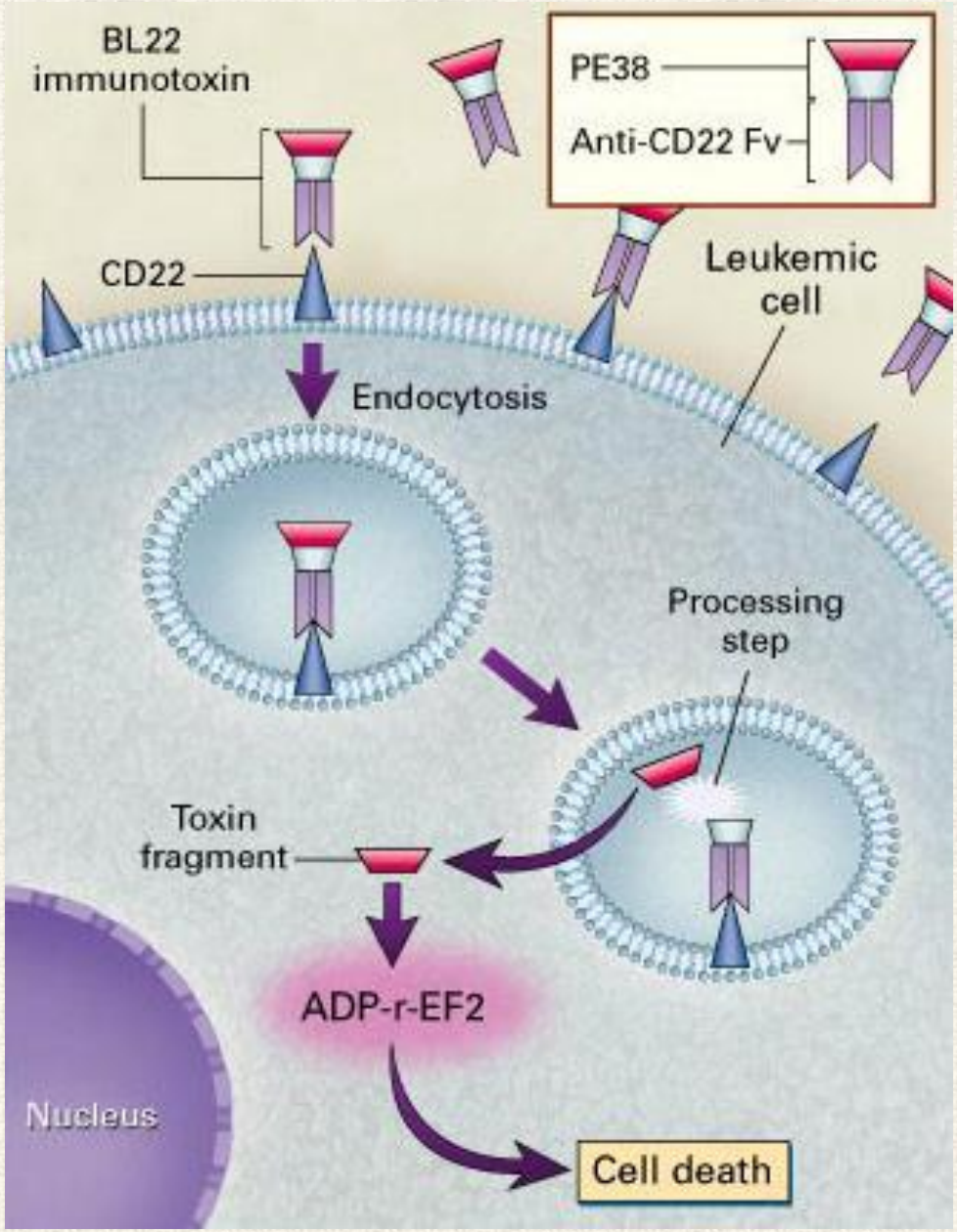


Confocal microscope image showing the internalisation of ar anti-CD7 saporin immunctoxin by human T- leukaemia cells into intracellular vesicles (green stain) n relation to the trans-golgi region (red stain)





Immunotoxin therapy of „Hairy Cell" leukaemia



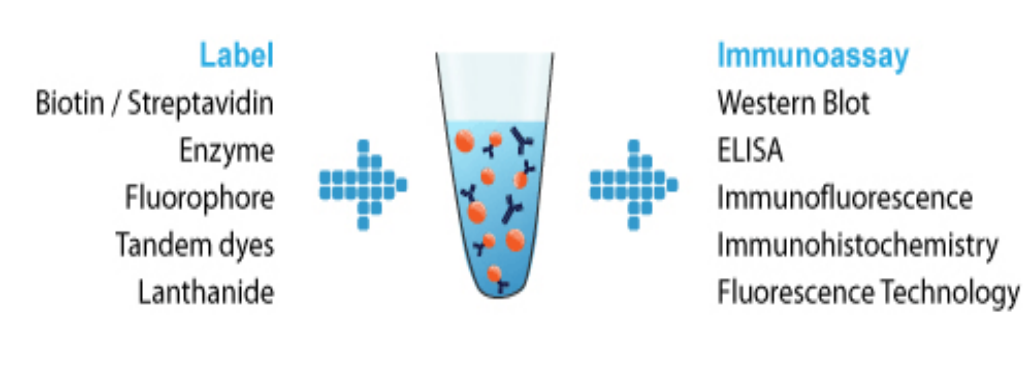
Nomenclature of therapeutic mononclonal antibodies

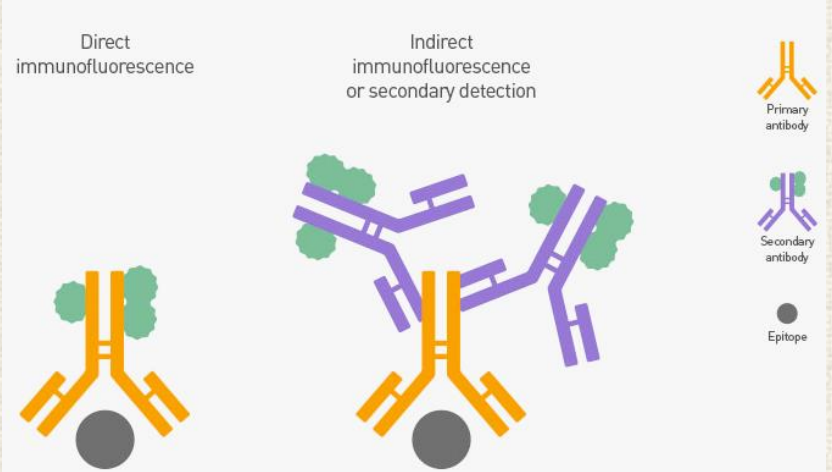
Prefix (variable) – **Target** – **Origin** – **mab**

(E.g. *anti*-CD20 *Ri tu xi mab*)

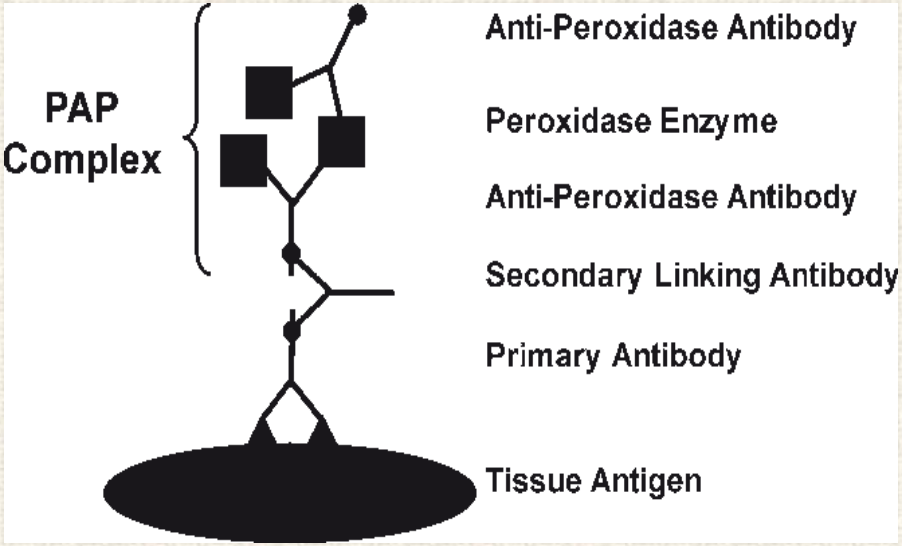
TARGET		ORIGIN	
b(a)	bacterium	-a-	rat
c(i)	circulatory system	-e-	hamster
f(u)	fungus	-i-	primat
k(i)	interleukin	-o-	mouse
l(i)	immune system	-u-	human
n(e)	nervous system	-xi-	chimeric
s(o)	bone	-zu-	humanized
tox(a)	toxin	-xizu-	chimeric/humanized hybrid
t(u)	tumor	-axo-	rat/mouse hybrid
v(i)	virus		

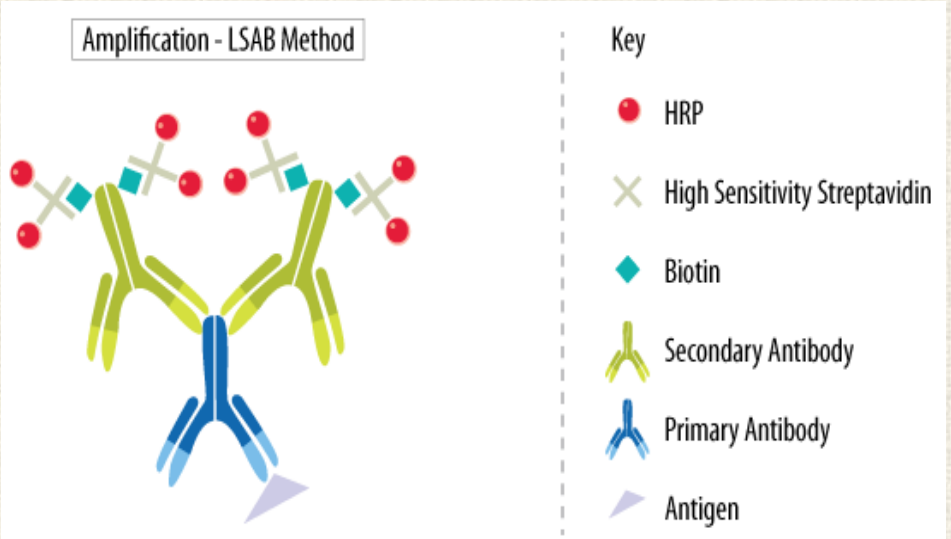
Antibody labelling for laboratory use





Amplification techniques

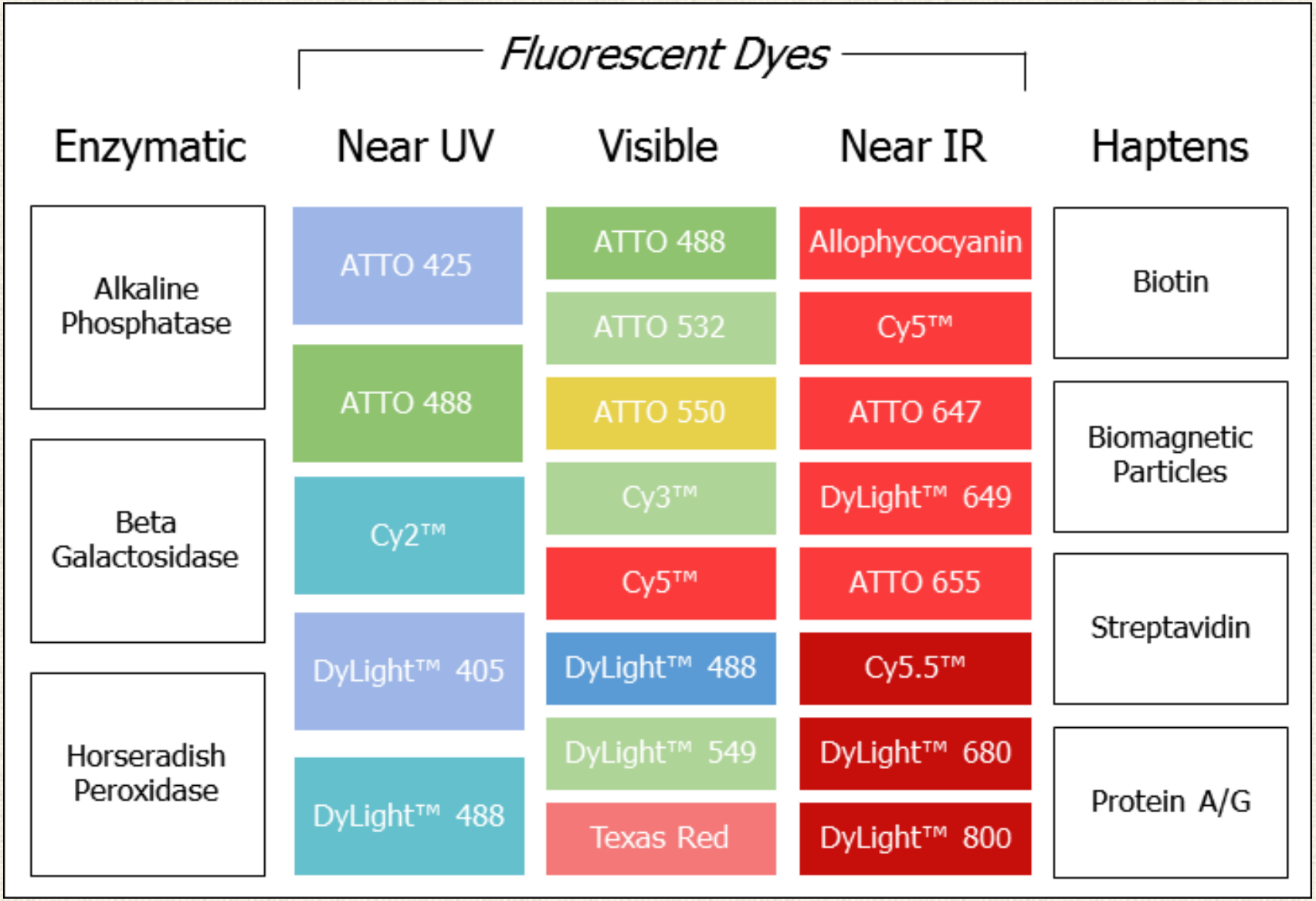




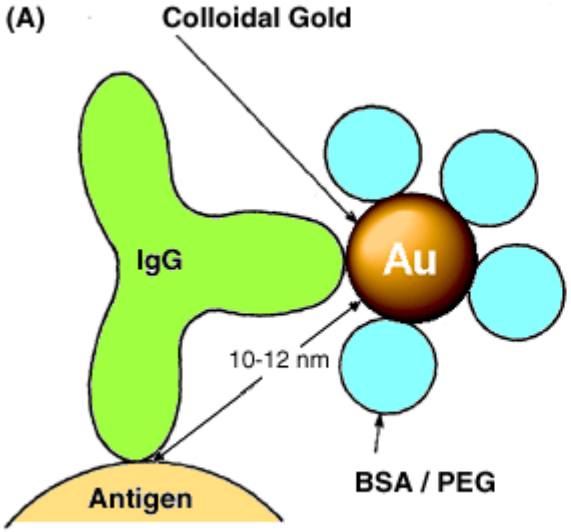
Fluorescent labelling

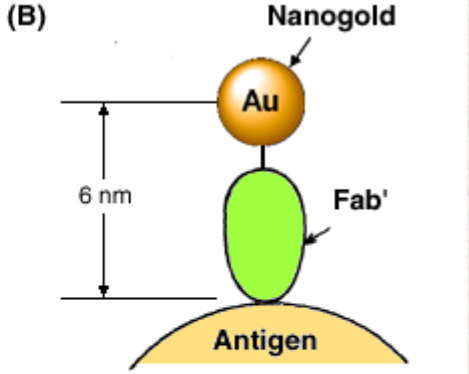
Excitation Wavelength	Fluorochromes/Dyes	Standard Filter	Optional Filter
488 nm	FITC, BB515, Alexa Fluor® 488, CFSE	533/30	–
	GFP	533/30	510/15
	JC-1, Fluo-4 AM, SYBR® Green	533/30	–
	YFP	533/30	540/20
	PE, JC-1, BD™ MitoStatus TMRE	585/40	–
	OFP	585/40	565/20
	Propidium Iodide	585/40, 670 LP	610/20
	BD Horizon™ PE-CF594	670 LP	610/20
	RFP, mCherry, dsRed	670 LP	610/20
	PerCP, PE-Cy™5	670 LP	–
	PerCP-Cy™5.5	670 LP	–
	7-AAD	670 LP	–
	PE-Cy™7	670 LP	780/60
640 nm	APC, BD™ MitoStatus Red, Alexa Fluor® 647	675/25	–
	APC-H7, APC-Cy7	670 LP	780/60

Combination of labelling methods



Gold labeling





Size comparison:
(A) conventional BSA-stabilized colloidal gold-IgG probe, vs.
(B) Nanogold-Fab' probe