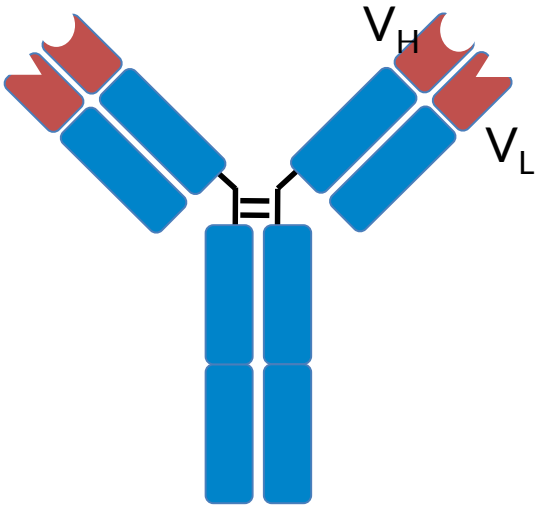


poetz@nmi.de
poetz@signatope.com

Oliver Pötz
CEO, SIGNATOPE GmbH

November 20th 2017

Laboratory Techniques and Medical Device Approvals: Immunoassays

- Glossar
- Antibodies and other capture molecules
- Competitive immunoassay
- Non-competitive immunoassay
- Direct immunoassay
- Detection systems
- Clinical sensitivity and specificity
- Biomarker discovery: an example

Glossar I

Affinity: Describes strength of antibody-antigen binding

Antigen: Molecule which is capable of triggering antibody production. Antigen is often used synonymously with analyte.

Antibodies (Immunglobuline): Plasma proteins which are generated by vertebrates to neutralize xenobiotics, bacteria and viruses. Antibodies bind specifically towards their antigen. Several classes of antibodies (IgE, IgG, IgM) and subclasses thereof do exist. IgGs are the major antibody class which are employed in immunoassays.

Assay: Test

Crossreactivity: Binding of an antibody to molecules which comprise similar or identical structures (epitopes) e.g.

RAUM***SCHIFF***WERFT and
DONAUDAMPF***SCHIFF***FAHRTSKAPITAENSMUETZE

Glossar II

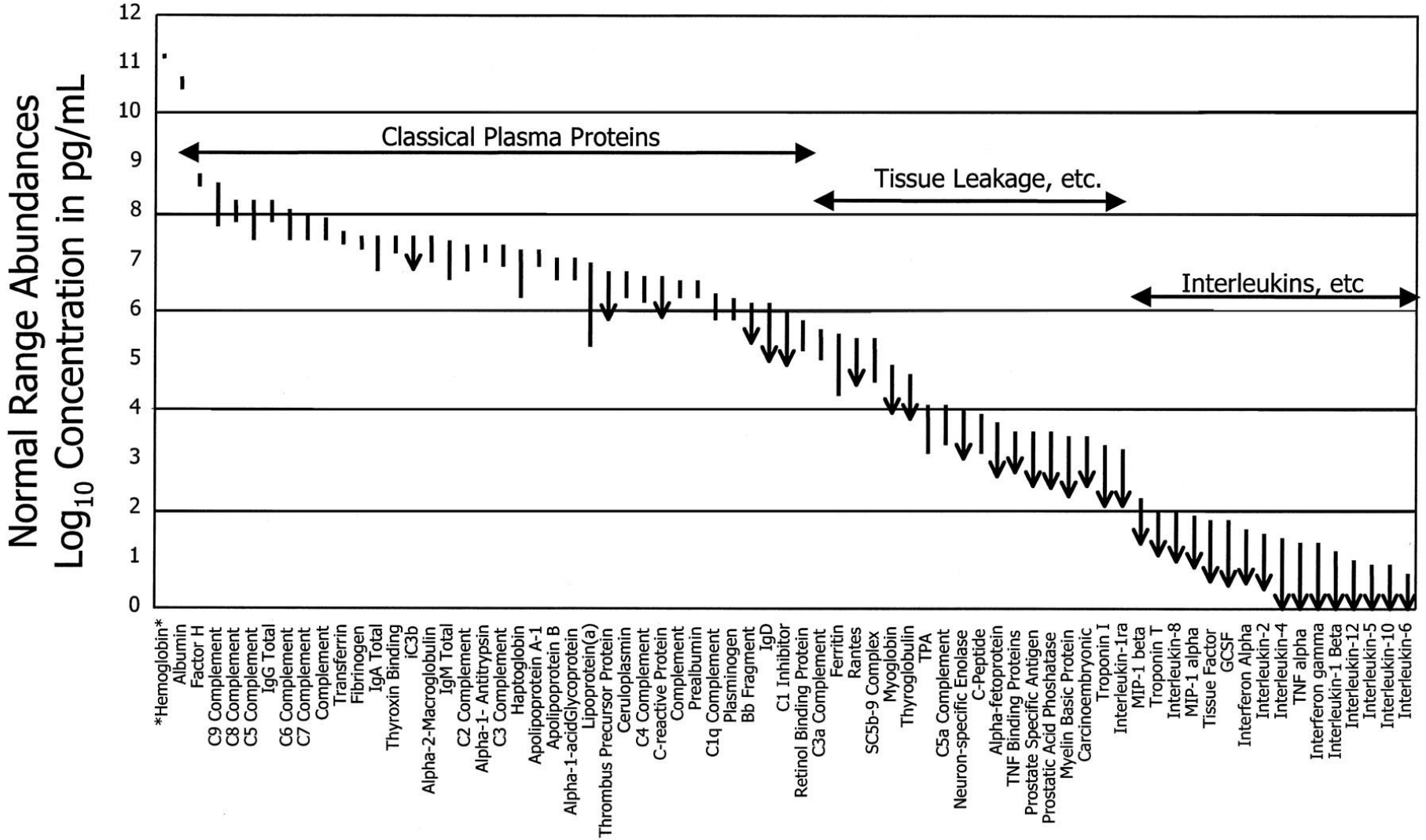
Epitope: Part of antigen which interacts with the antibody

Immunoassay: Every bioanalytical test in which an antibody-antigen reaction is employed for molecule detection.

Paratope: Part of antibody which interacts with the antigen

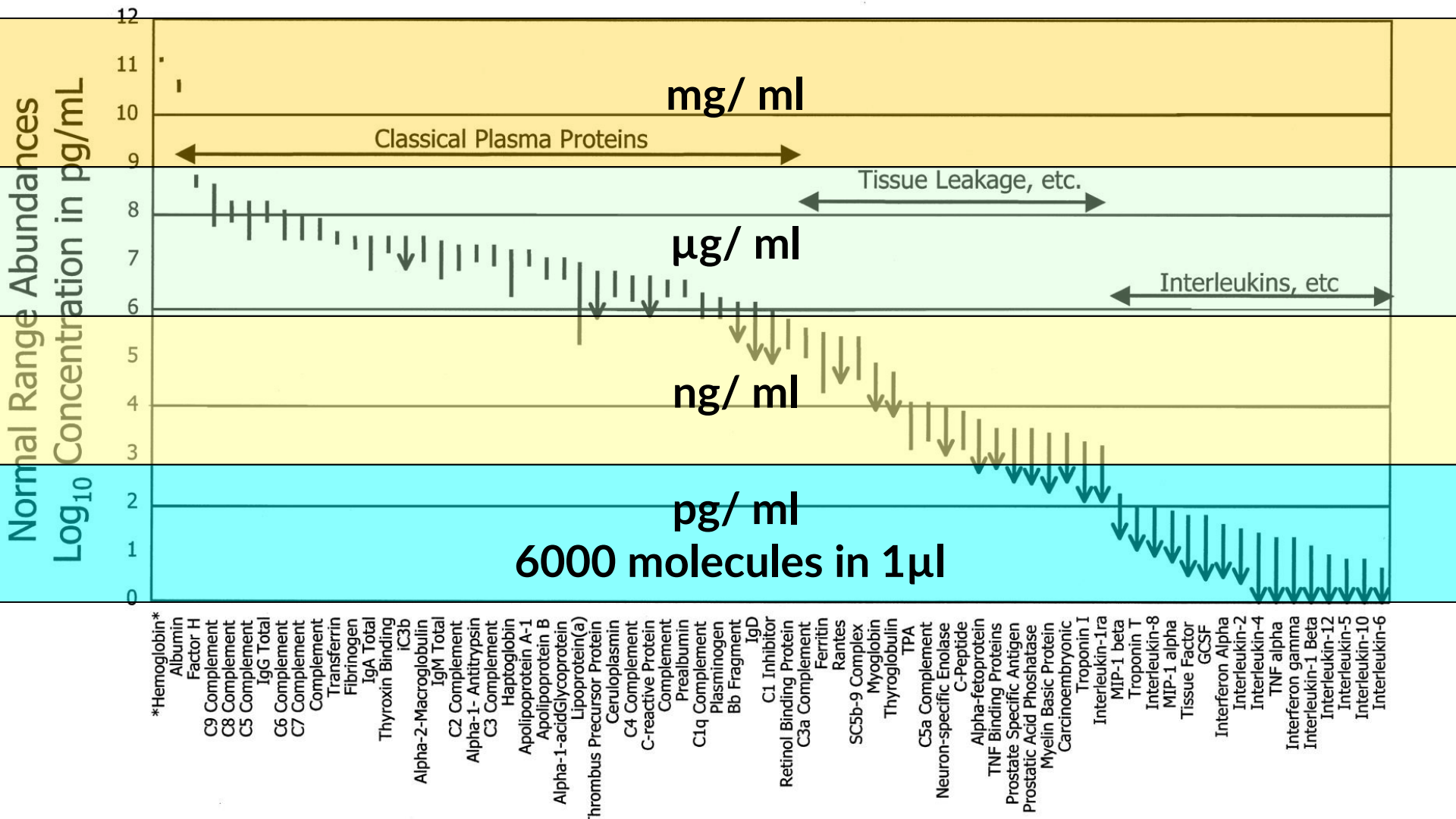
Specifity (in terms of antibody): Capability of an antibody to discriminate between its antigen and other molecules.

Plasma Biomarker – The Challenge



N. L. Anderson, N. G. Anderson, *Mol Cell Proteomics 2002, 1. 845-67*

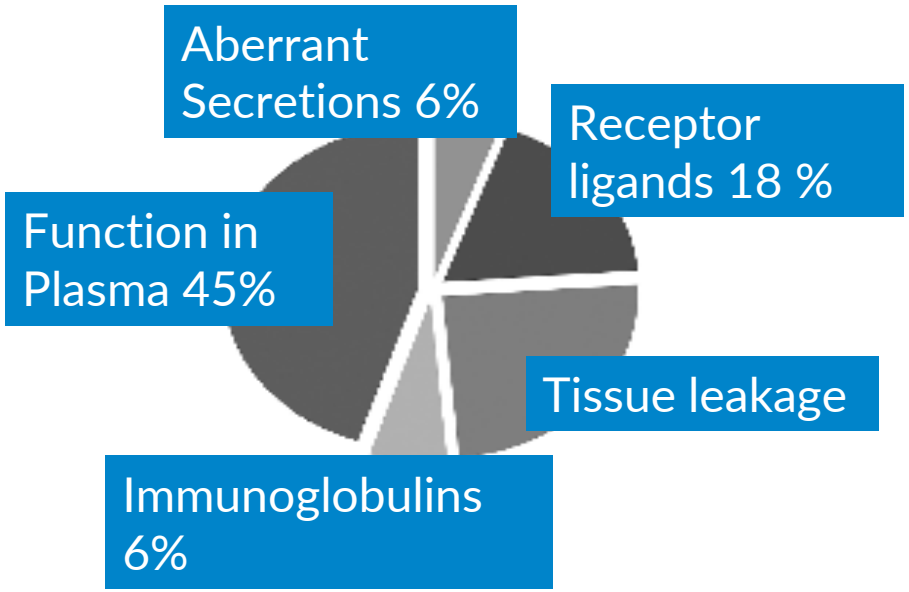
Plasma Biomarker – The Challenge



N. L. Anderson, N. G. Anderson, *Mol Cell Proteomics 2002, 1. 845-67*

Protein Assays in Diagnostics – Status

- Total Number of FDA approved non-redundant protein assays 109
- Novel approvals 1.5 per year
- Additional 98 protein assays on the menu of large clinical labs
- About 1 % of the human proteome



Anderson, N.L. et al. , *Clinical Chemistry* 2010

A brief history of immunoassays

1960 Radioimmunoassay
R. S. Yalow, S. A. Berson, J Clin Invest 1960, 39. 1157-75.

1971 Enzyme-linked immunosorbent assay
E. Engvall, P. Perlman, Immunochemistry 1971, 8. 871-4.

1977 Nobel Prize for RIA

1979 Western blot
H. Towbin, T. Staehelin, J. Gordon, *Proc Natl Acad Sci U S A* 1979, *76*. 4350-4.

1981 Sandwich immunoassay
M. Uotila, E. Ruoslahti, E. Engvall, *J Immunol Methods* 1981, *42*. 11-5.

1989 Microspot concept, first “Protein Array”
R. P. Ekins, *J Pharm Biomed Anal* 1989, 7. 155-68.

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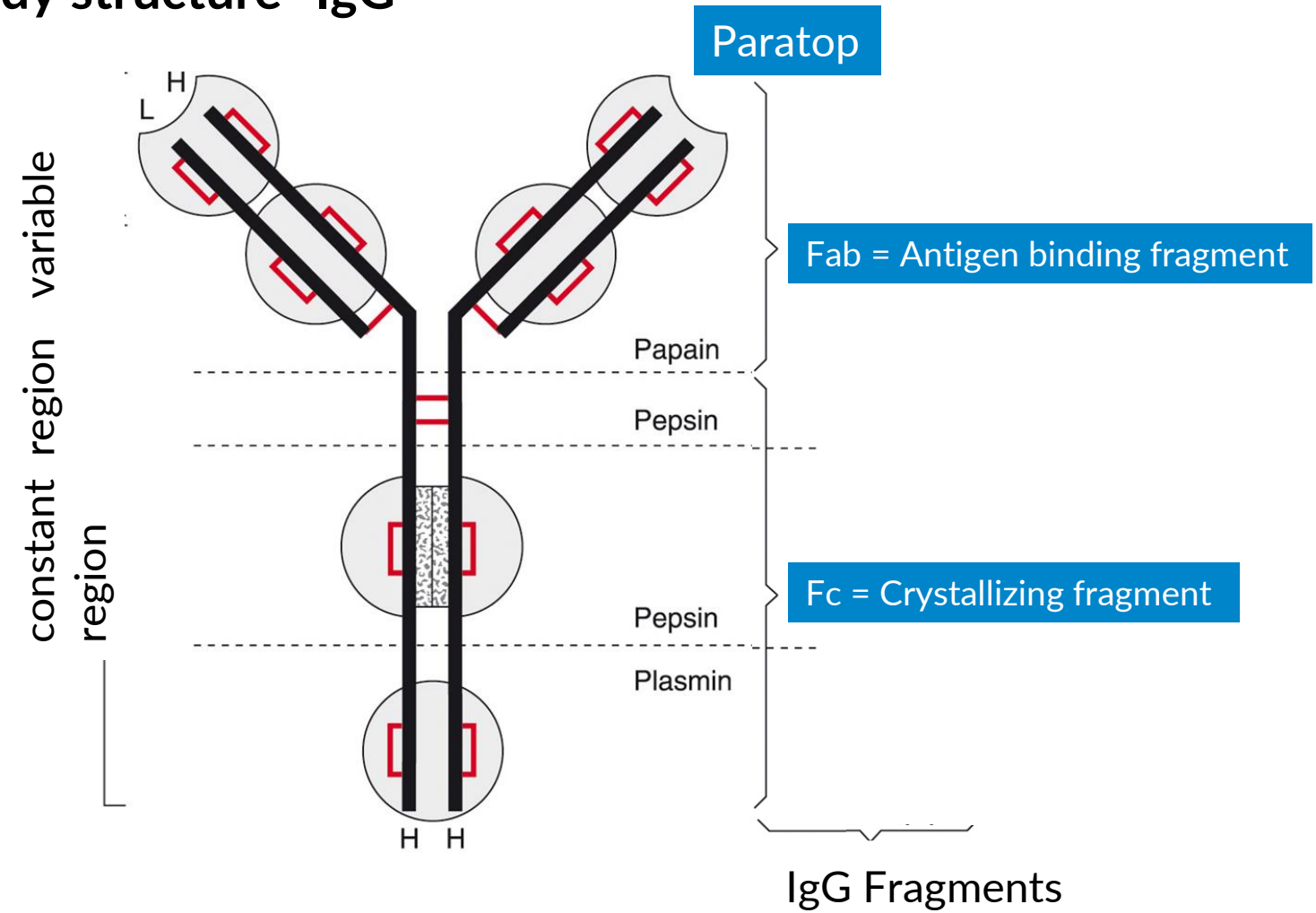
Antibody structure- IgG



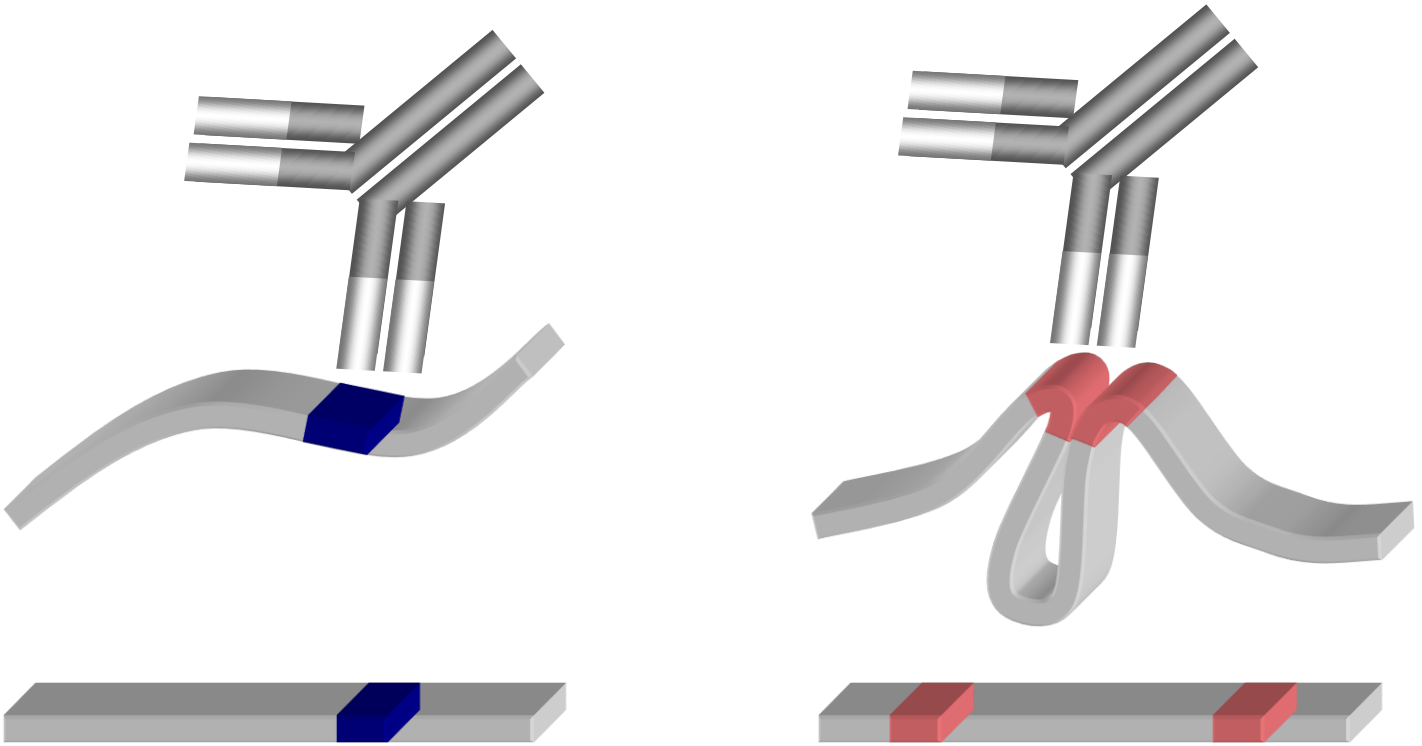
Internal disulfid bonds

L-and H chain joining disulifid binds

Glycosyl residues

Lottspeich/ Engels, Bioanalytics, 2nd ed., 2006 Elsevier GmbH

Linear vs. conformational epitope

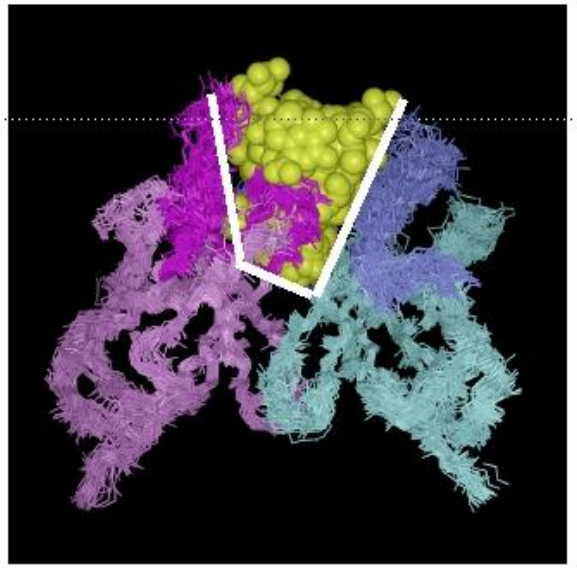


Linear

conformational/ discontinous

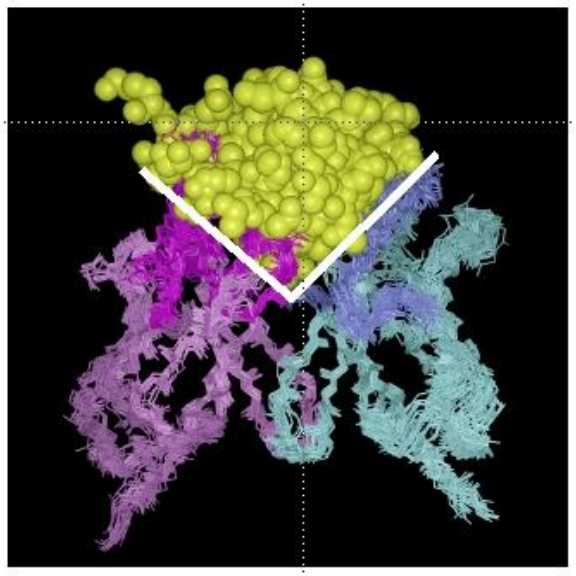
Antibody-Antigen Interaction
Different Antigens - Different Binding Modes

Hapten Binders



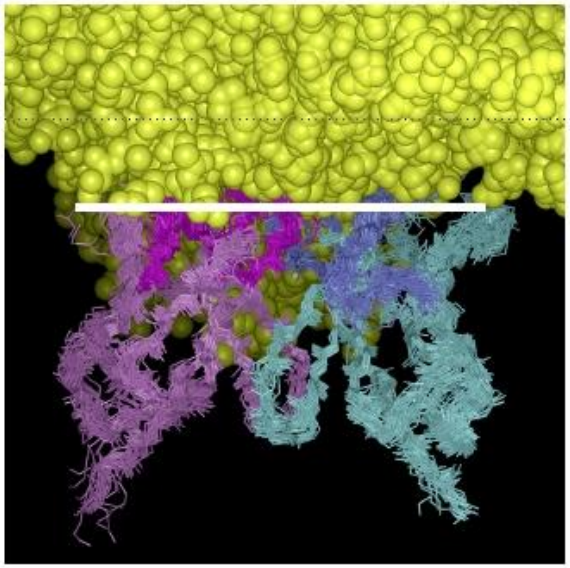
small molecules
deep pocket

Oligomer Binders



peptides and DNA
cleft

Protein Binders



proteins
large contact area

Andreas Plueckthun, Zuerich, PepART Meeting 2005

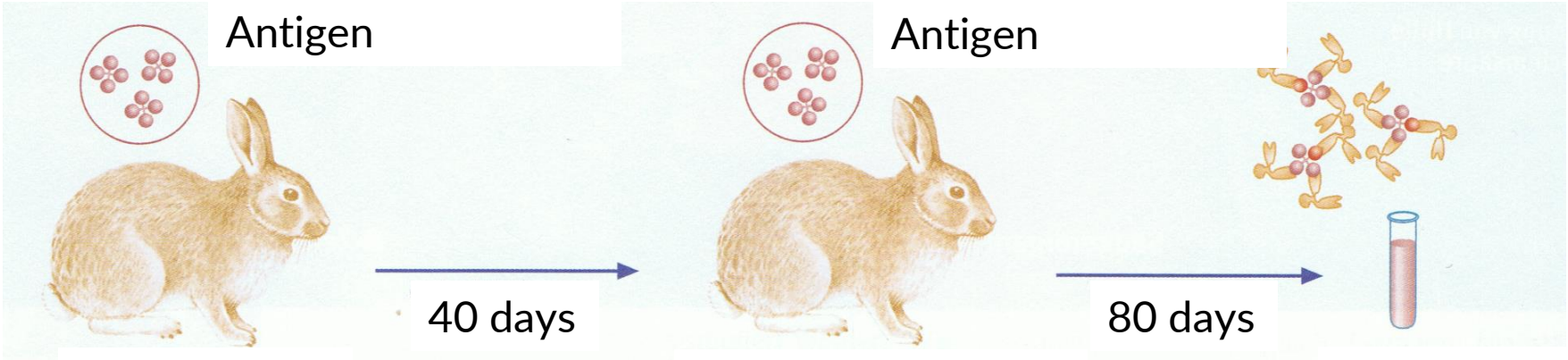
Affinity: Formation of antibody-antigen complex

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

$$K_D = \frac{[A] \cdot [B]}{[AB]} = \frac{k_d}{k_a} = \frac{1}{K_A}$$

K_A= Association constant
K_D= Dissociation constant

Classical immunization



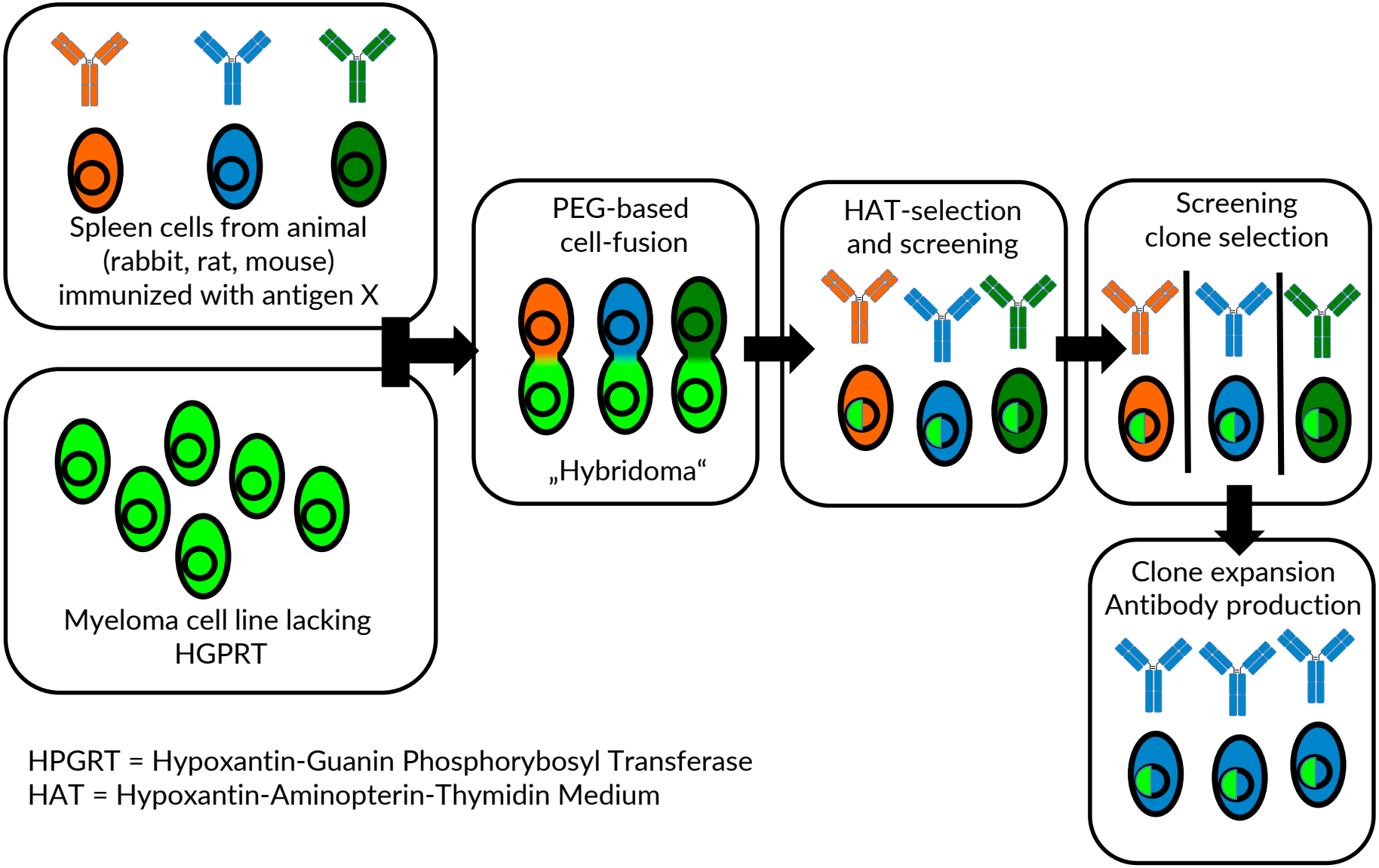
Animals
Mono & polyclonal
- rabbit
- mouse
- rat

polyclonal
- donkey
- guinea pig
- chicken
- goat

IgG Purification Assay Development

Bildquelle: Renneberg, Biotechnologie für Einsteiger, Spektrum Verlag

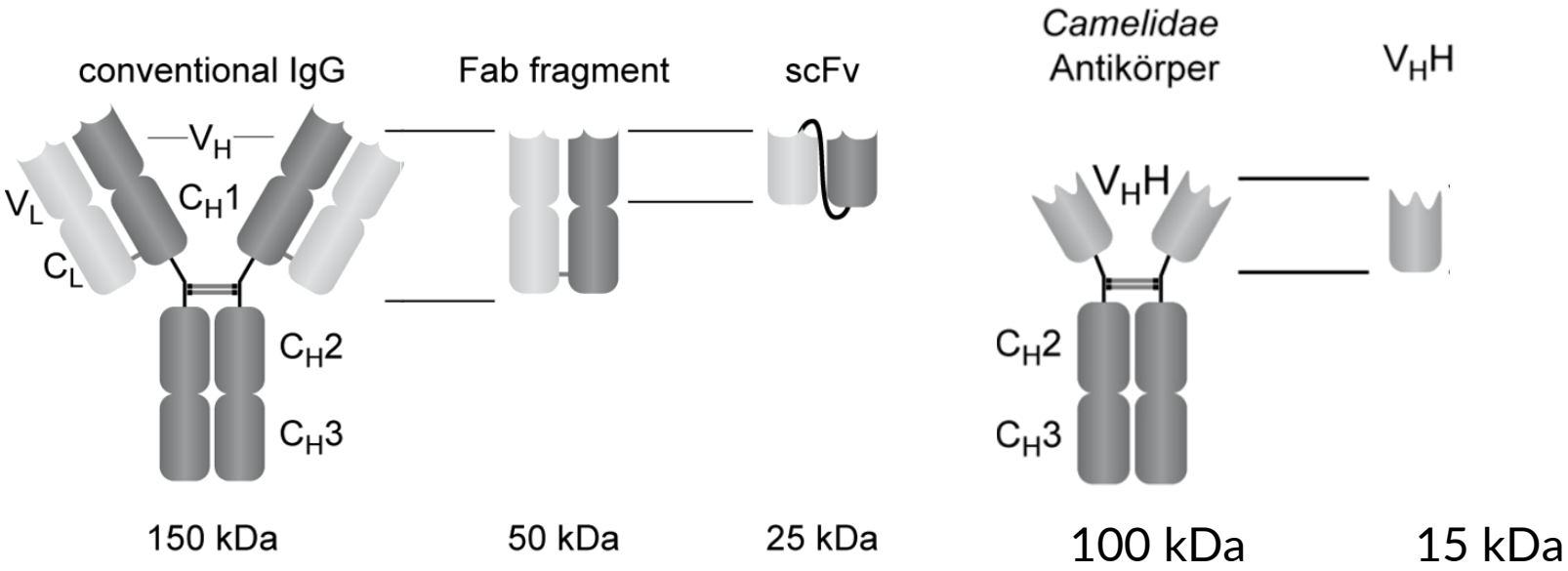
Antibody Generation - Mab



HPGRT = Hypoxantin-Guanin Phosphorybosyl Transferase
HAT = Hypoxantin-Aminopterin-Thymidin Medium

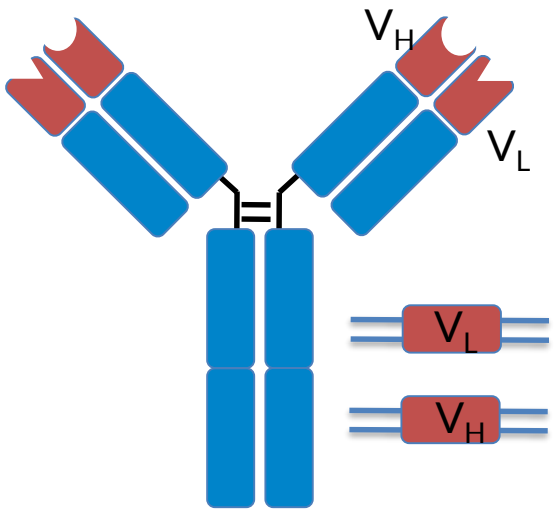
Modified after Janeway, Immunology 2008

Recombinant antibodies …



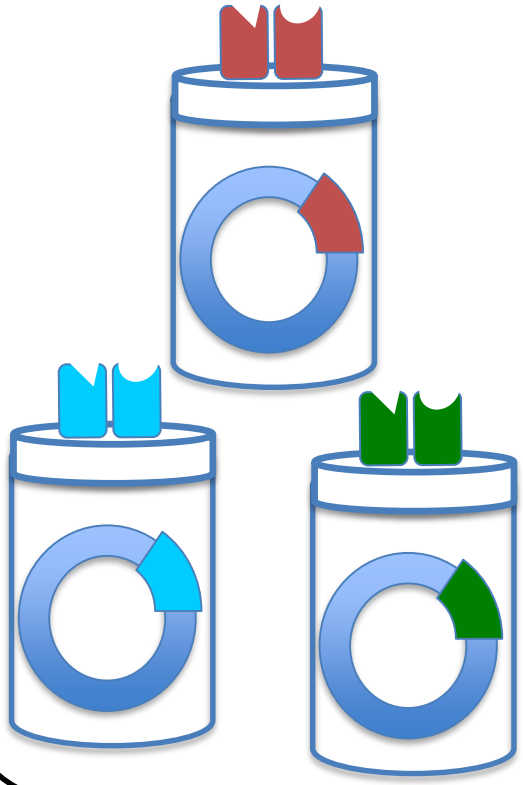
Antibody Generation – recombinant fab, scfv

Isolate DNA encoding V_L and V_H domains

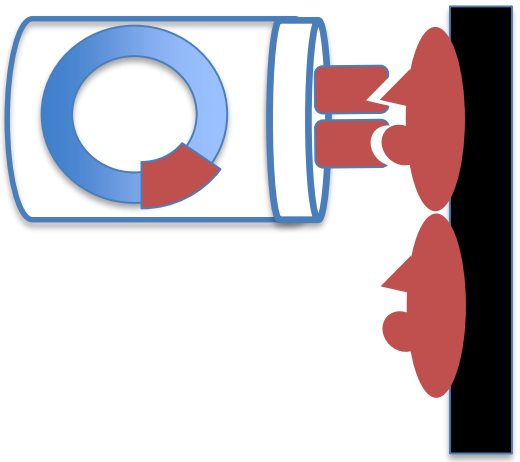


Protein DNA

Construct fusion of V regions and phage coat protein



Enrich antigen-specific phages (several rounds)



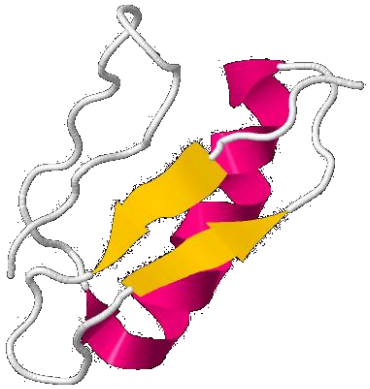
+ Maturation process

Modified after Janeway, Immunology 2008

…and other capture molecules



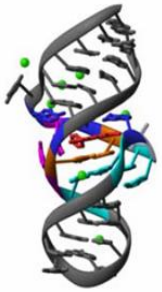
Ankyrin
(DARPins)



Protein A
(Affibody)



Lipocalin
(Anticalin)



Aptamer
(SOMAmer)

Antibodies vs. other capture molecules

Molecule	Development time	Antigens	yield	Work load	Companies
Pab	3-4 months	Peptides, haptens, proteins	10-20 mg	low	...many, differ in quality
Mab	12-24 months	Peptides, haptens, proteins	unlimited	medium	...many, differ in quality
Rec Fabs	2-24 months	Peptides, haptens, proteins	unlimited	high	AbD (Biorad)
V_HH (nanobody)	12-24 months	Proteins	unlimited	high	Chromotek
Aptamers	3-4 months	Proteins	unlimited	high	Somalogic

Antibodies and other capture molecules

Antibodies

- monoclonal

Kohler, G., and Milstein, C. (1975) Nature 256, 495-497

- polyclonal

Behring, EA., Anti-diphterietoxin, (1890)

recombinant

other formats: scfv, fab, nanobody

fabs (HuCal Gold Library)

Knappik, A., et al. (2000) J Mol Biol 296, 57-86

VHH (nanobody)

Muyldermans, S., (1994) Protein Eng 7, 1129-1135

Scaffolds

Affibodies (Protein G)

Nord, K., et al. (1997) Nature Biotechnol. 15: 772-777.

Ankyrins

Binz, H. K., et al. (2003). J. Mol. Biol. 332: 489-503.

Lipocalins

Beste, G., et al. (1999) Proc Natl Acad Sci U S A 96, 1898-1903

Aptamers/ Somamers

Tuerk, C., & L. Gold (1990). Science, 249: 505-510.
Gold, L., et al. (2010). PLoSONE, 5(12), e15004

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Competitive and non-competitive immunoassay

Competititve

- Radioimmunoassay (RIA)
- Competitive ELISA
- Lateral Flow Assay

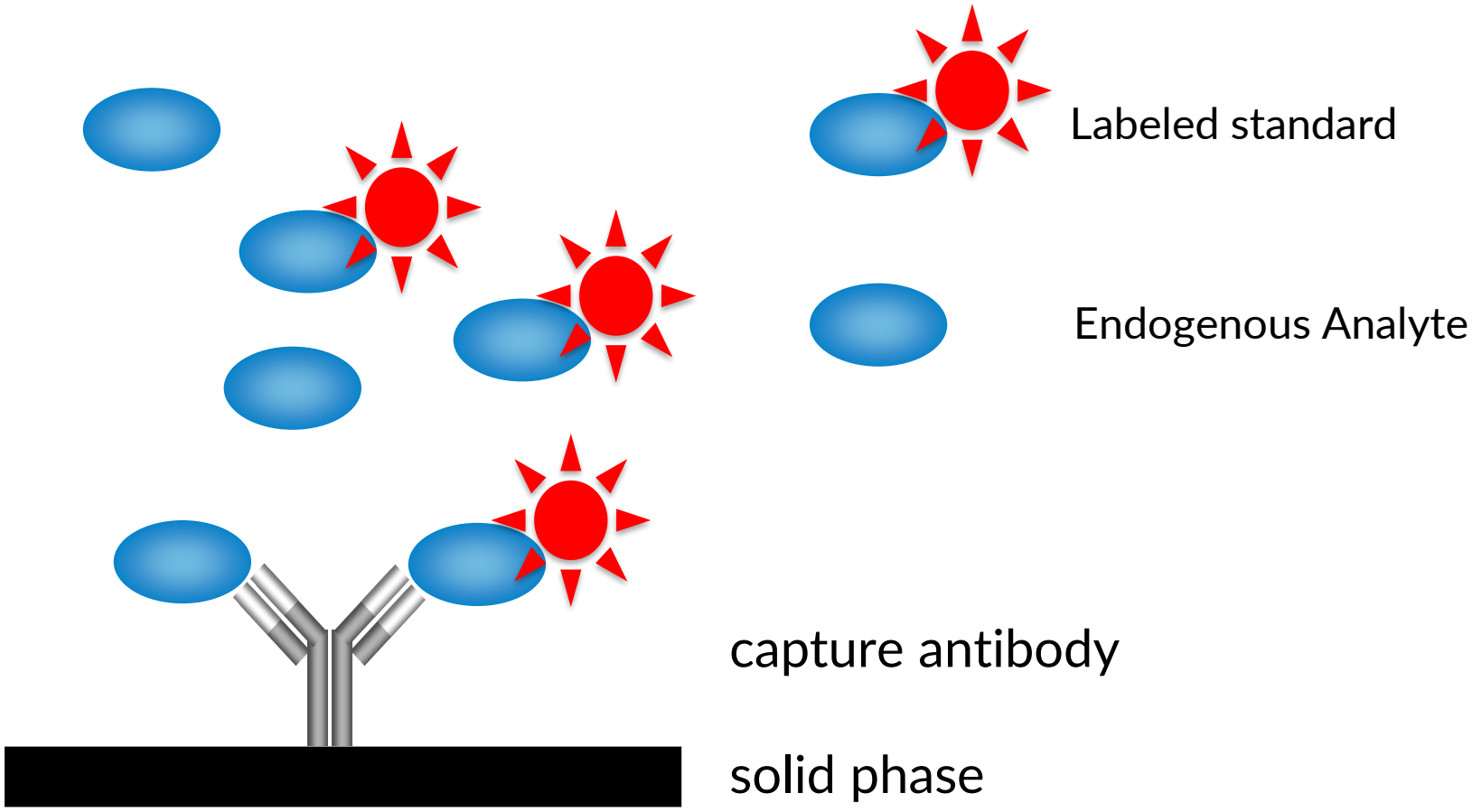
Non-competitive

- Sandwich immunoassay

Direct immunoassay

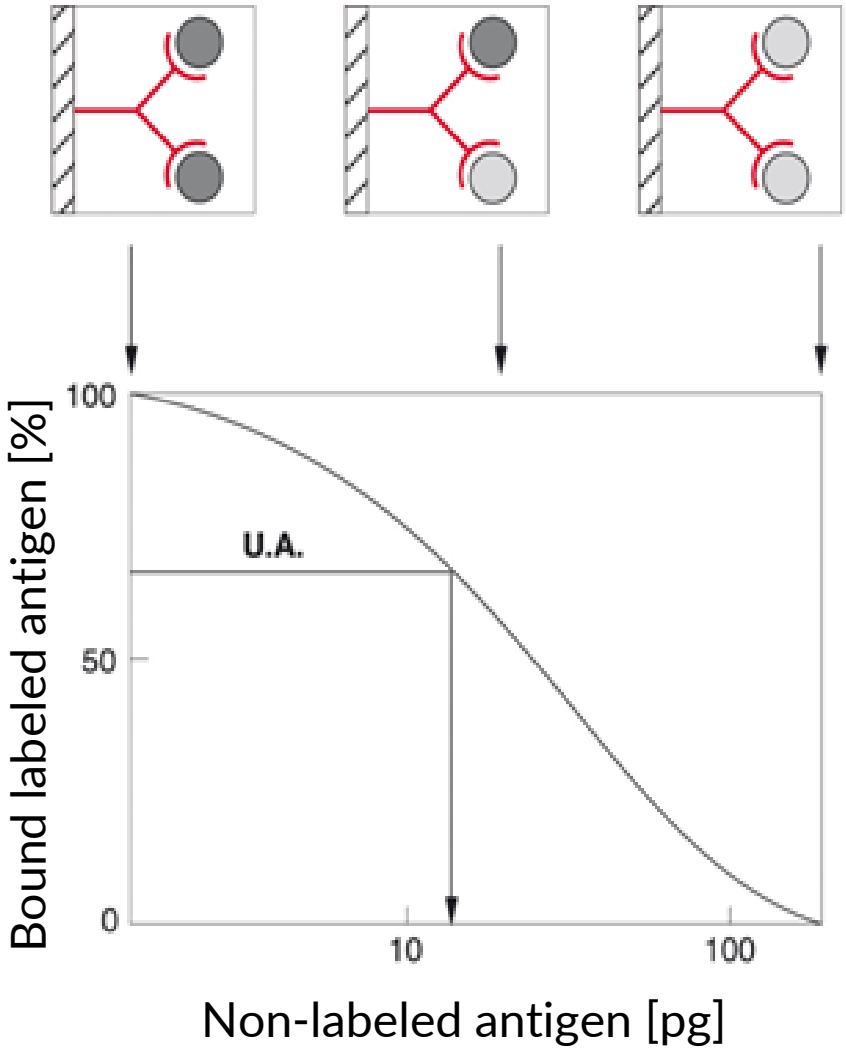
- Immunohistochemistry (IHC)
- Serological assays
- Auto immundiagnostics

Competitiver assay - Radioimmunoassay



Yalow, R.S. Berson S.A., *J Clin Invest*, 1960.

Competitiver assay - Radioimmunoassay



Labeled standard

Endogenous analyte

Antibody specific for and

U.A. Unknown conc. can be back-calculated

Solid support

Modified after Lottspeich/ Engels, Bioanalytics, 2nd ed., 2006 Elsevier GmbH

Competitive assay - Radioimmunoassay

Prerequisite:

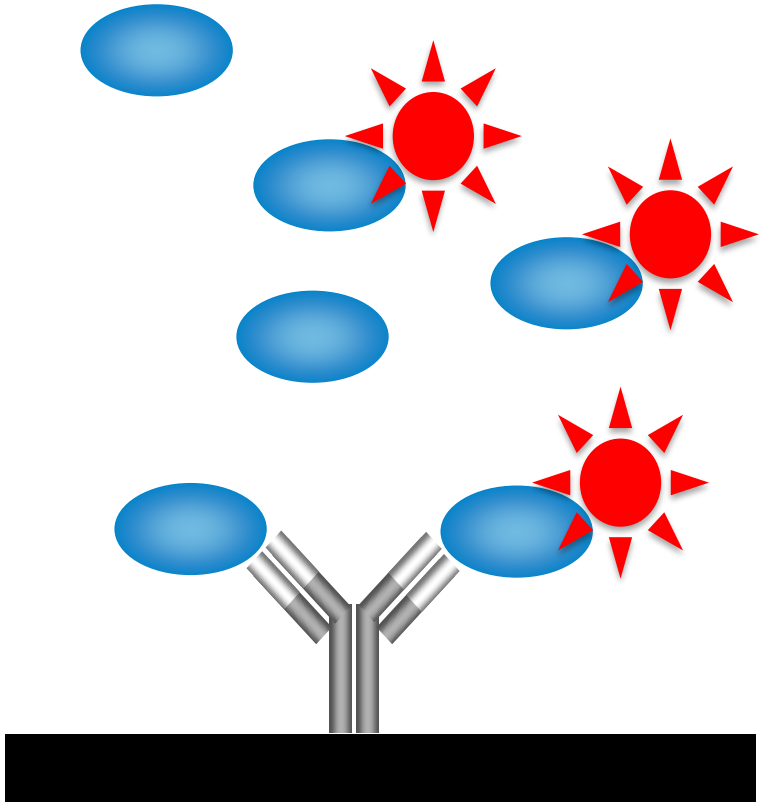
- Radioactively-, fluorescently or enzyme-labeled analyte

Pros:

- One antibody
- Quantitative
- **Suitable for small molecules, e.g. pesticides, hormones, neurotransmitter,...**

Cons:

- Radioactive reagents
- Specificity
- Narrow dynamic range



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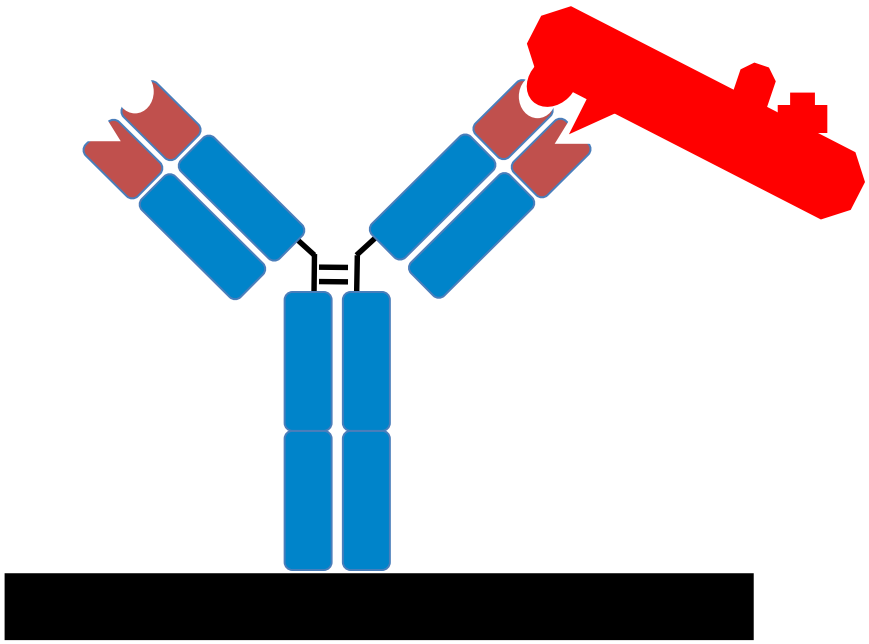
Non-competitive assay – Sandwich immunoassay

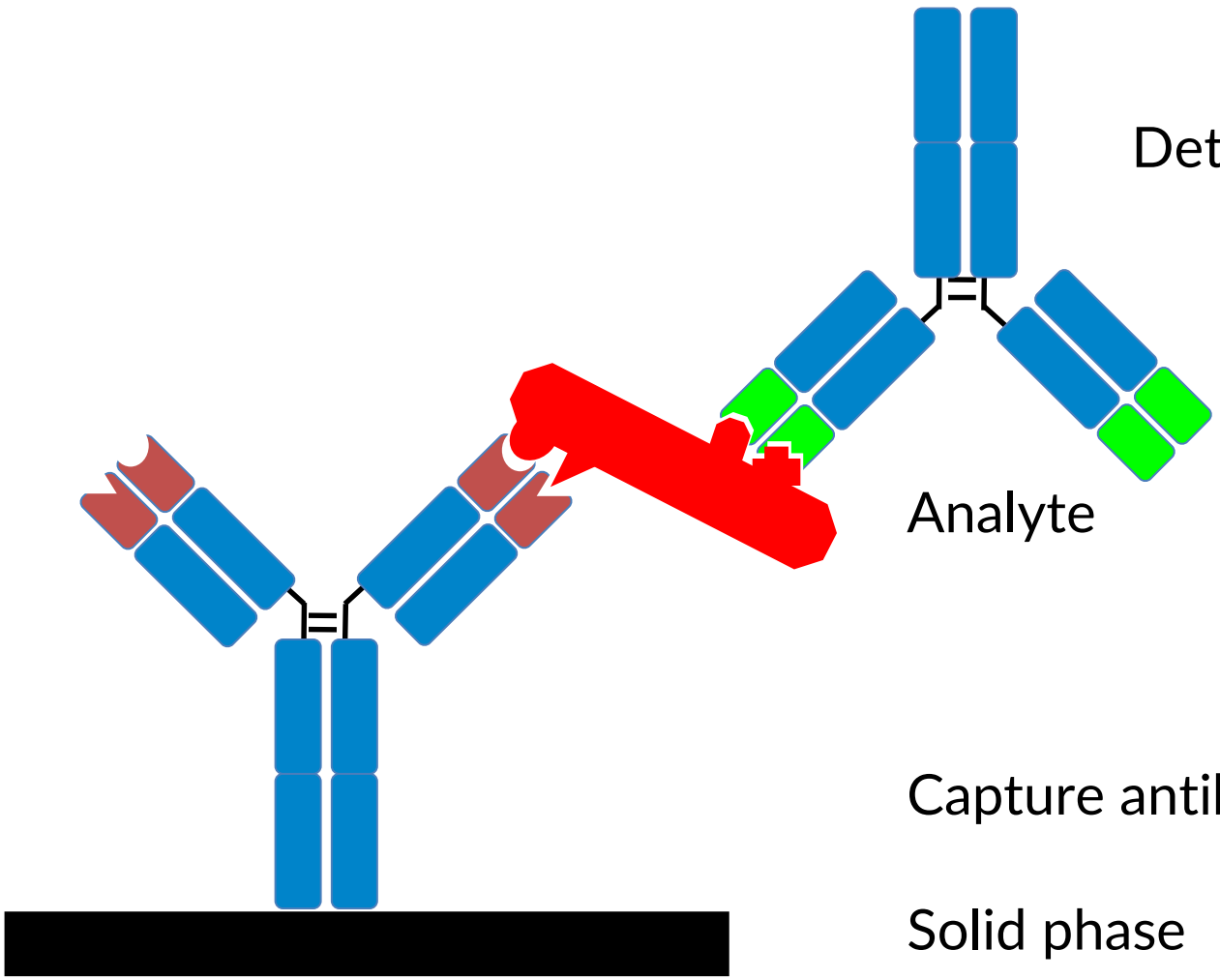


Analyte

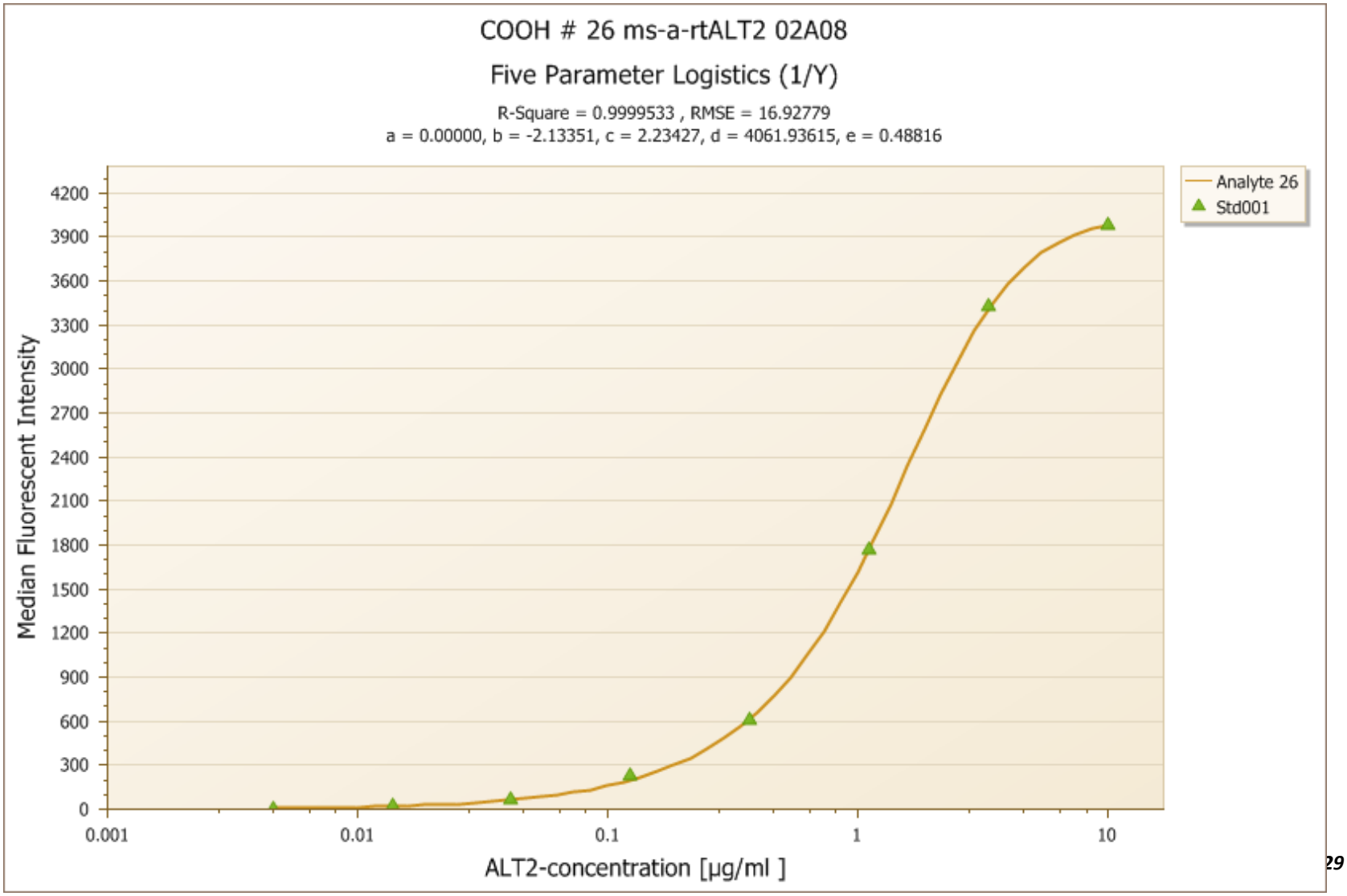
Capture antibody

Solid phase

Non-competitive assay – Sandwich immunoassay



Non-competitive assay – Sandwich immunoassay



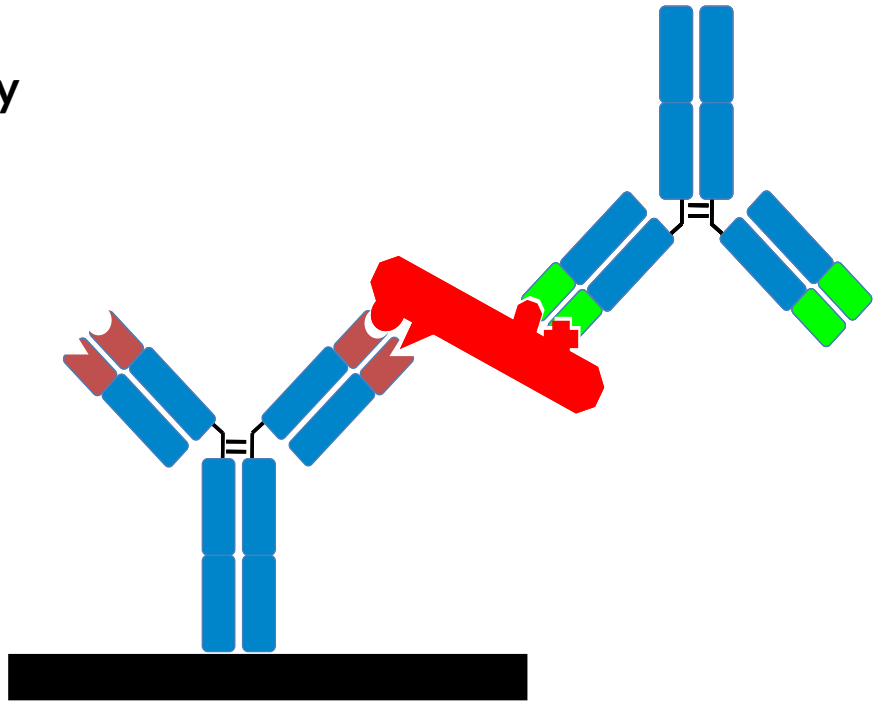
Non-competitive assay – Sandwich immunoassay

Pros:

- quantitative
- fast, robust
- **two independent binding events – high specificity**
- **signal amplification – high sensitivity**

Cons:

- two antibodies required



Uotila, M., Ruoslahti, E., and Engvall, E. *J Immunol Methods* 1981

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Direct immunoassay –
Serology or autoimmune diagnostic

Serology

z.B. anti-HIV antibodies, anti-HCV antibodies

…

virus- or bacteria-specific antibodies are detected

Autoimmune diagnostic

z.B. *Lupus erythematodes* (anti nuclear antibodies (ANA))

antibodies towards “own“ proteins/ DNA are detected

Direct immunoassay –
Serology or autoimmune diagnostic

Prerequisite:

- Immobilized antigen

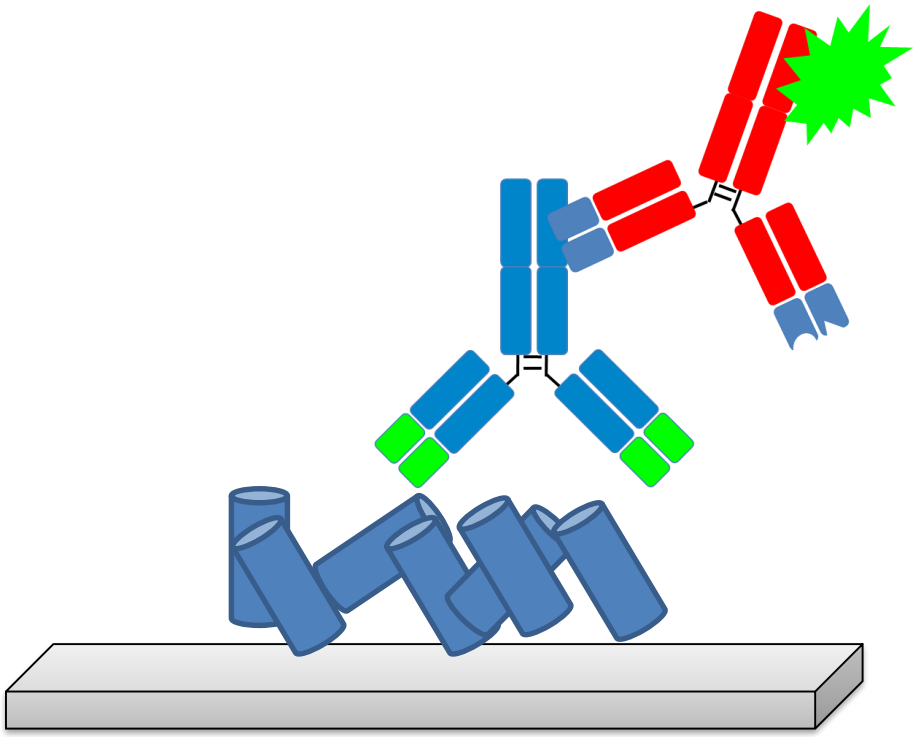
Pros:

- No capture antibody
- Simple
- Fast
- Robust

Cons:

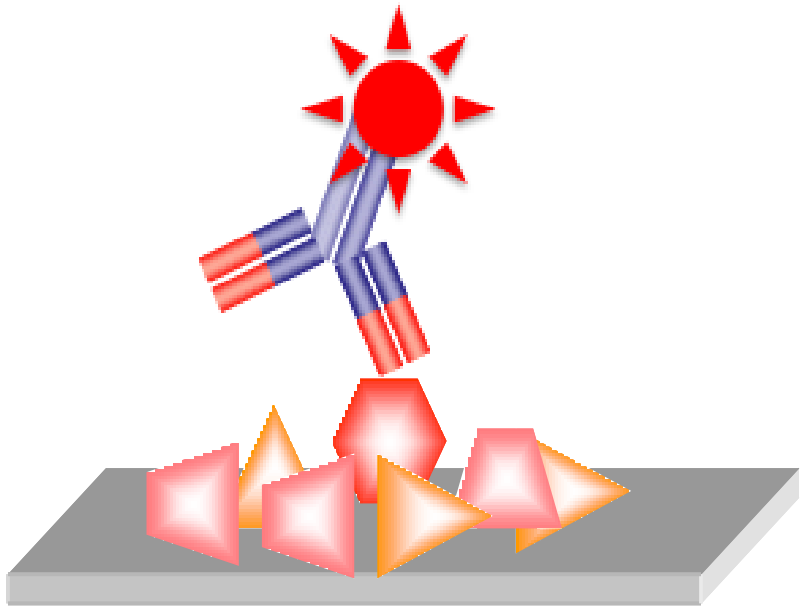
Limited applications

e.g. serology or autoimmune diagnostic



Direct immunoassay – Immunohistochemistry

Tissue section immobilized on a slide

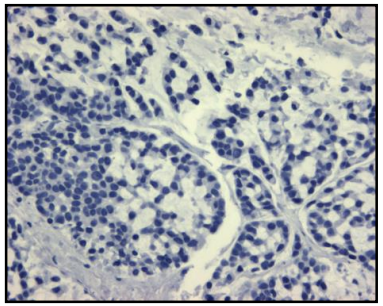


Direct immunoassay – Immunohistochemistry

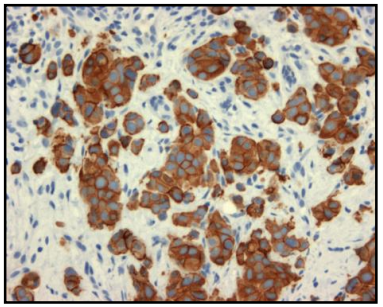
drug therapy yes or no?

e.g. - HER2

- HER2 present in 20 % of all breast tumours
- Companion Diagnostics
- Anti-HER2 (Herceptin ®) is drug and diagnostic antibody
- Pathologist envaluates staining (0 bis 3+)
- Stage 3+ → Herceptin-treatment



IHC 0



IHC 3+

this patient should get the antibody drug

Direct immunoassay – Immunohistochemistry

Prerequisite

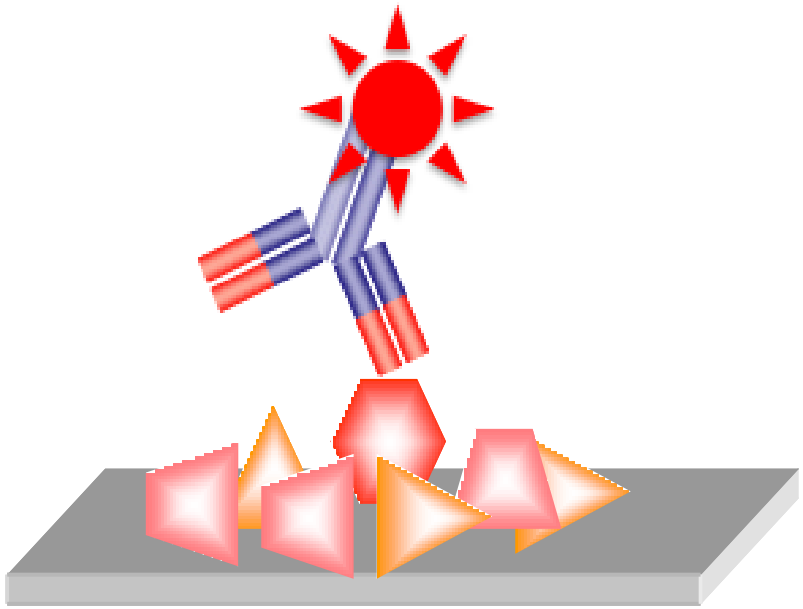
- Antibody binds to fixed antigen

Pros:

- Only one antibody

Cons:

- Labour-intensive
- Subjective – results depend on pathologist



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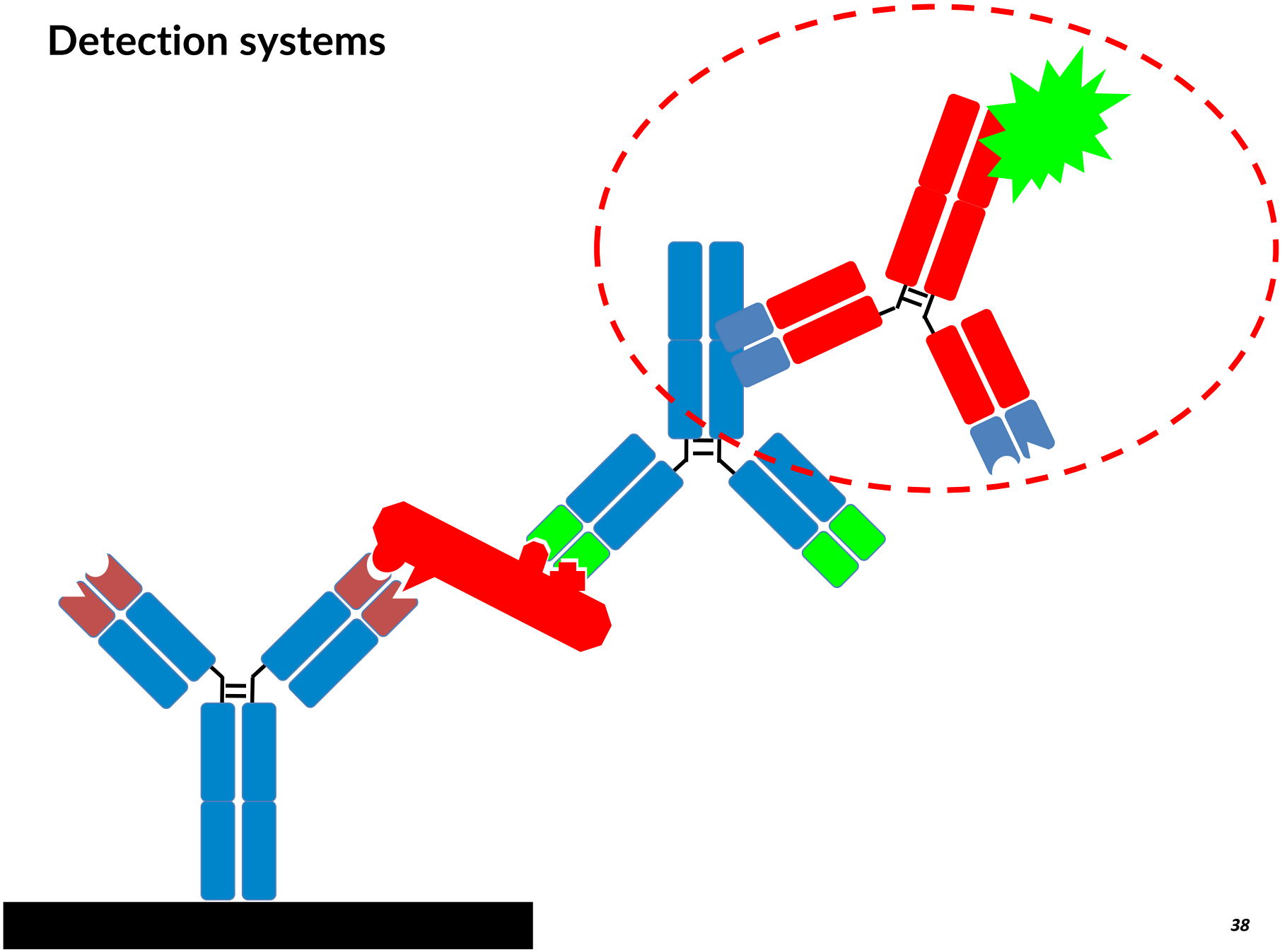
Detection systems



Detection systems

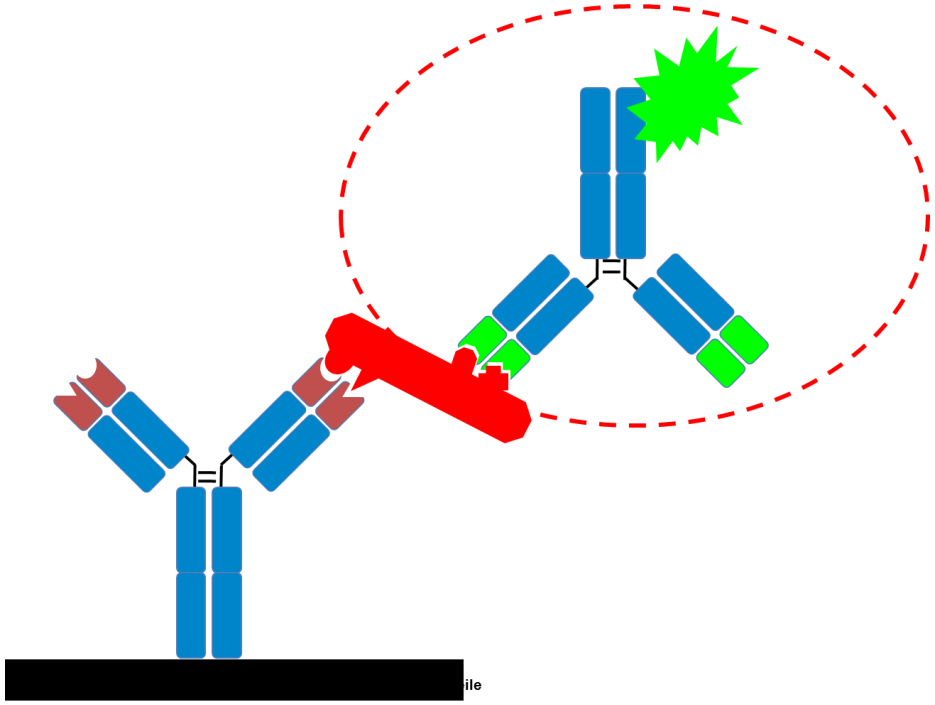
Direct labeling detection antibody:

- enzyme
  e.g. horseradish peroxidase (HRP), alkaline phosphatase
- fluorescent dyes
  e.g. FITC, TAMRA, polymethin dyes (Cy3, Cy5), phycoerythrin

Indirect labeling detection antibody:

- biotin-avidin system
- enzyme-conjugated secondary antibody
- fluorescently labelled secondary antibody

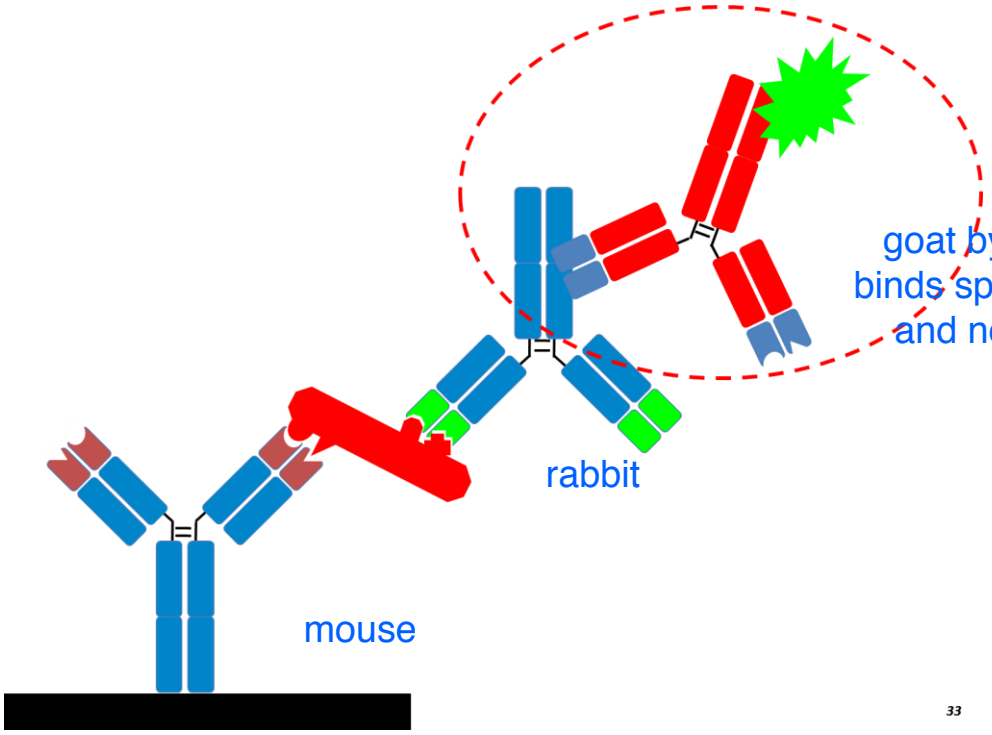
Direct labeling detection antibody



Fluorophores:
e.g. FITC, TAMRA,
Polymethin dyes (Cy3, Cy5),
Phycoerythrin

- one conjugate for one assay → no flexibility
- rapid assay: only 2 incubation times!

Indirect labeling detection antibody I

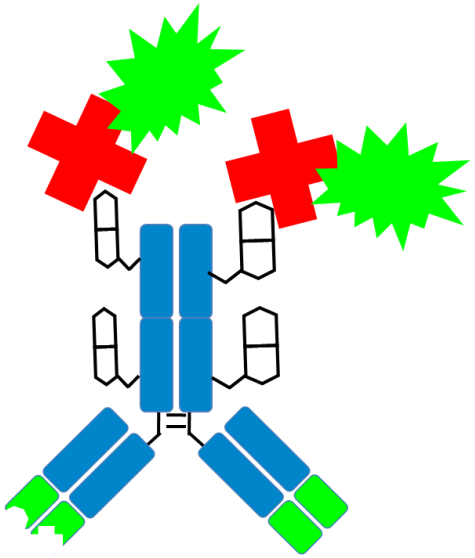


Fluorophores:
e.g. FITC, TAMRA,
Polymethin dyes (Cy3, Cy5),
Phycoerythrin

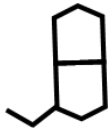
goat by injecting rabbit
binds specific to the rabbit
and not to the mouse

- one conjugate for many assays → flexibility
- signal amplification, up to 20 fluorophore per IgG
- > 1 secondary antibody binds to detection antibody

indirect labeling detection antibody II



System: biotinylated detection antibody
+
Avidin conjugate



biotin
Vitamin H



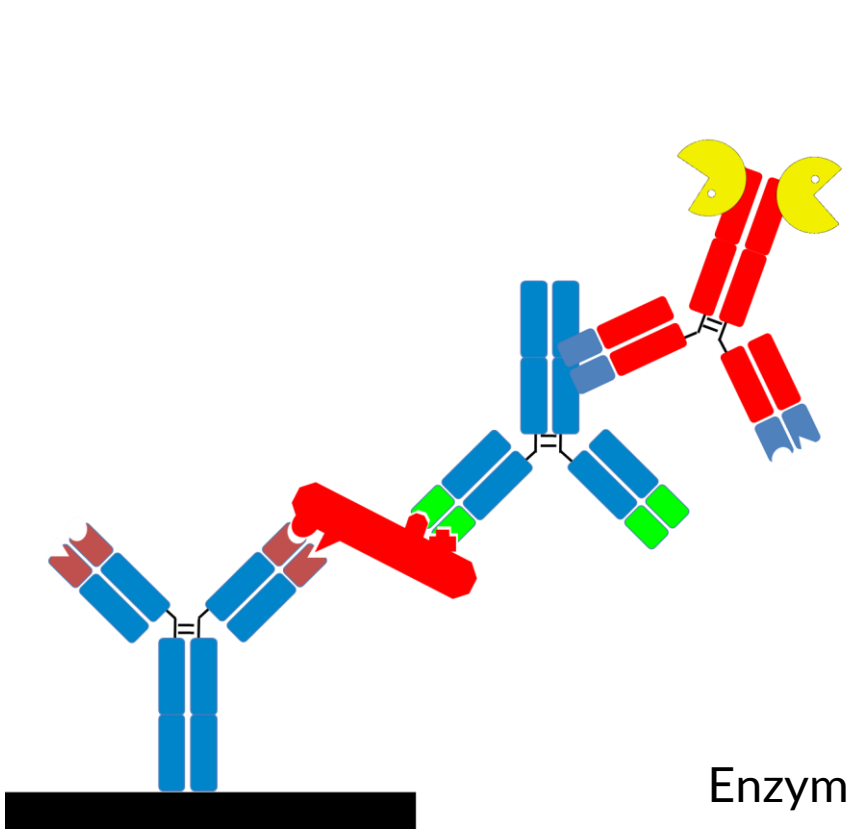
fluorophor



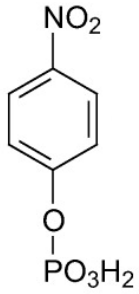
avidin (protein with 4 biotin binding sites)

- one conjugate for many assays (avidin conjugate) → flexibility
- signal amplification, up to 20 biotin per IgG
- > 1 Conjugate binds to detection antibody
- Capture and detector from one species in one assay
- but, detection antibody has to be chemically modified

indirect labeling detection antibody III

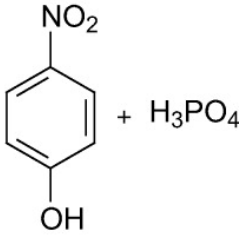


substrate
(colorless)



e.g. alkaline phosphatase

product
(yellow, 405 nm



+ H_3PO_4

Enzyme Linked Immunosorbent Assay!!! (ELISA)

33

- one conjugate for many assays (avidin conjugate) → flexibility
- signal amplification, enzymatic reaction
- > 1 secondary antibodies per detection antibody

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Specificity and sensitivity of a test
biological aspects

Sensitivity

Rate of correctly classified positive “sick” patients (true positives, tp)
below the positive patient group (tp + fn)

$$\frac{tp}{tp + fn}$$

tp = true positive
fn = false negative
tn = true negative
fp = false positive

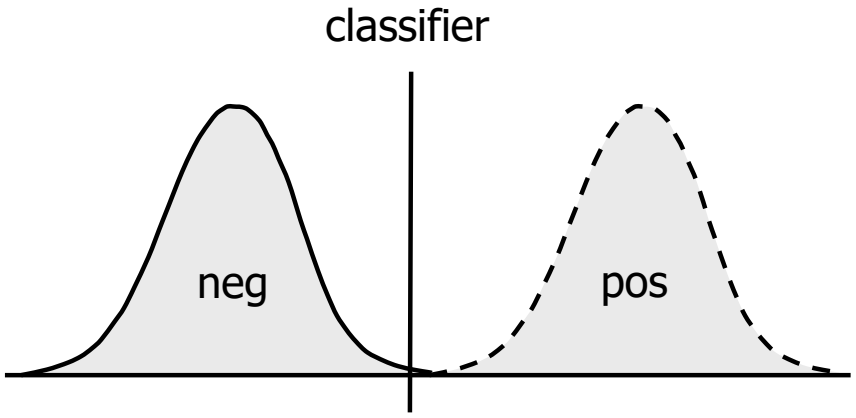
Specificity

Rate of correctly classified negative “healthy” individuals (true negatives, tn)
below the negative group (tn + fp)

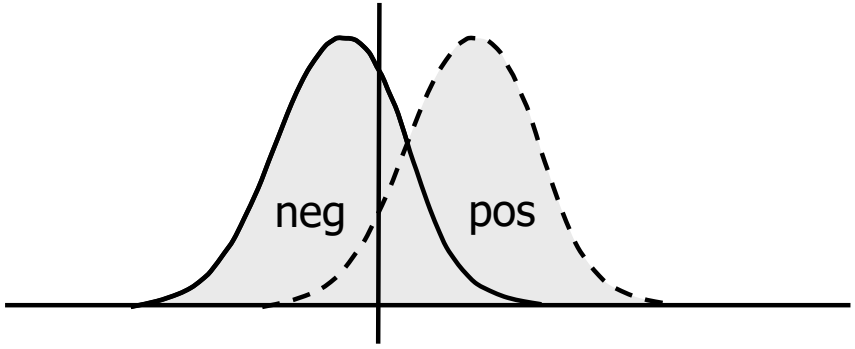
$$\frac{tn}{tn + fp}$$

Richter, *Der Internist*, 1997, 38: 325-336

Sensitivity and *specificity*



ideal



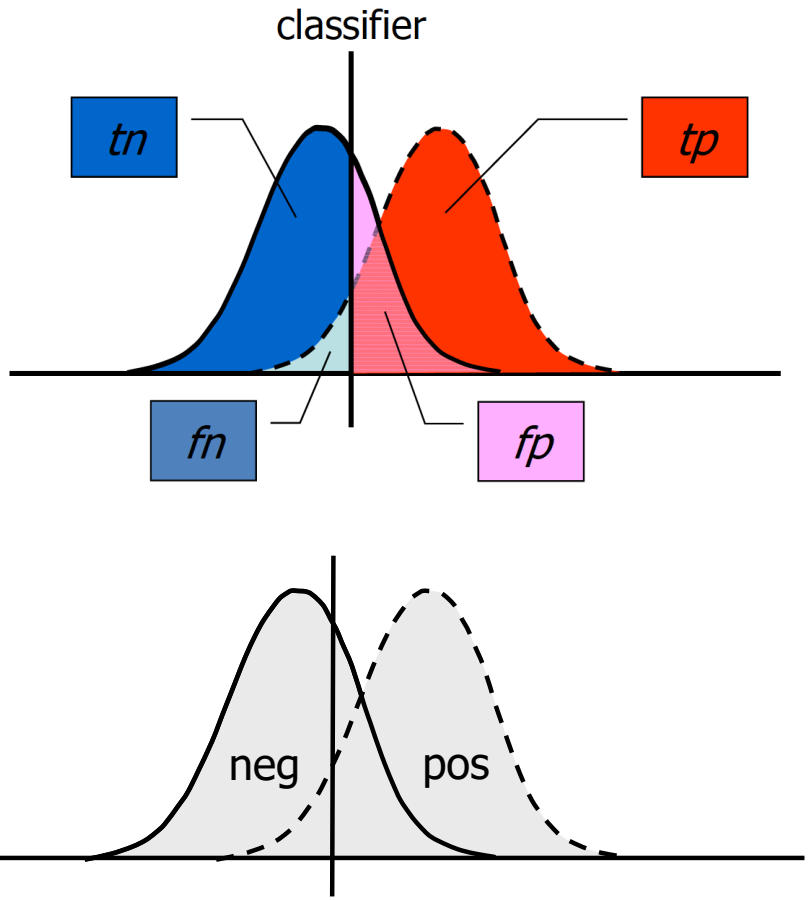
reality

Diagnostic test: distribution of *positive* and *negative* candidates

How to choose the classifier for overlapping populations of *positive* and *negative* candidates?

Richter, *Der Internist*, 1997, 38: 325-336

Sensitivity and *specificity* of a diagnostic test



		Actual value	
		pos	**neg**
Test	**pos'**	*true positive*	*false positive*
	neg'	*false negative*	*true negative*

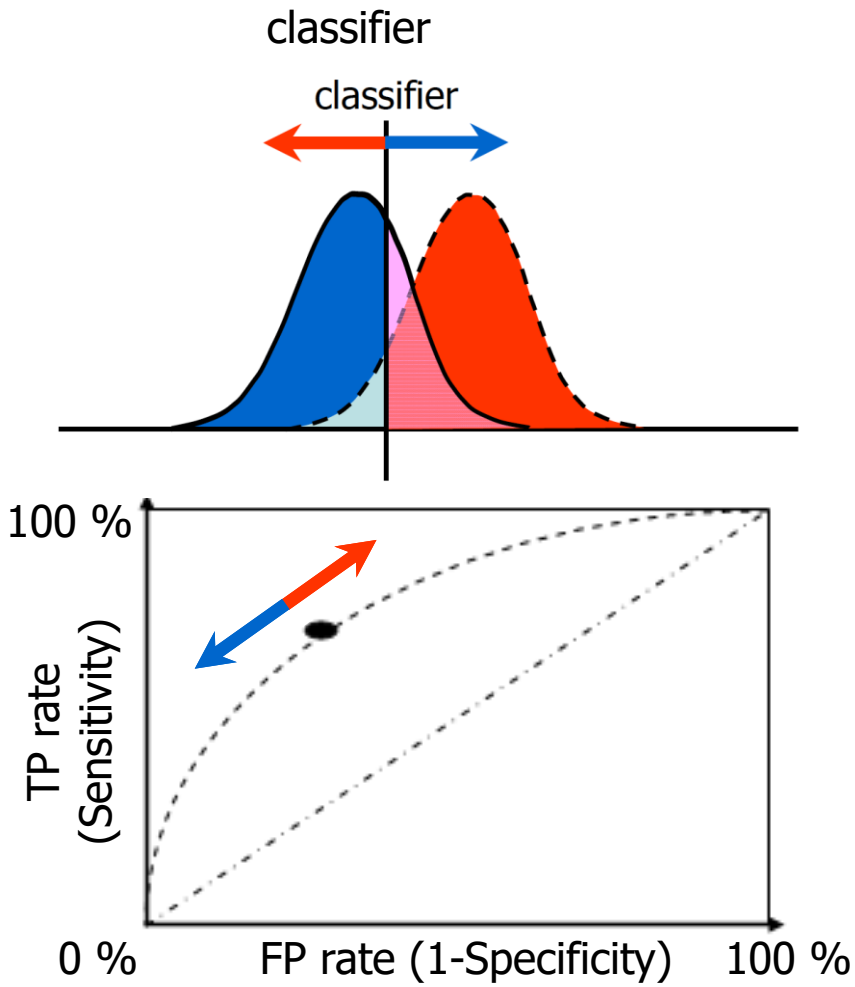
sensitivity
= tp / (tp + fn) = tp / pos

specificity
= tn / (tn + fp) = tn / neg

→ depends on classifier

Richter, *Der Internist*, 1997, 38: 325-336

Receiver-Operating-Characteristics (ROC)-Analysis



		Actual value	
		pos	**neg**
Test	**pos'**	*true positive*	*false positive*
	neg'	*false negative*	*true negative*

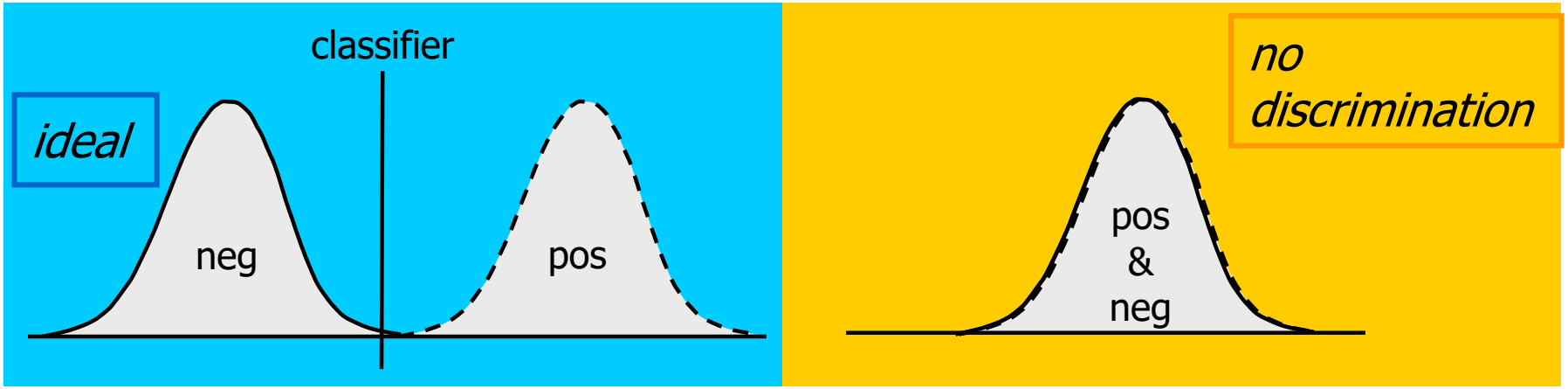
sensitivity
= tp / (tp + fn) = tp / pos

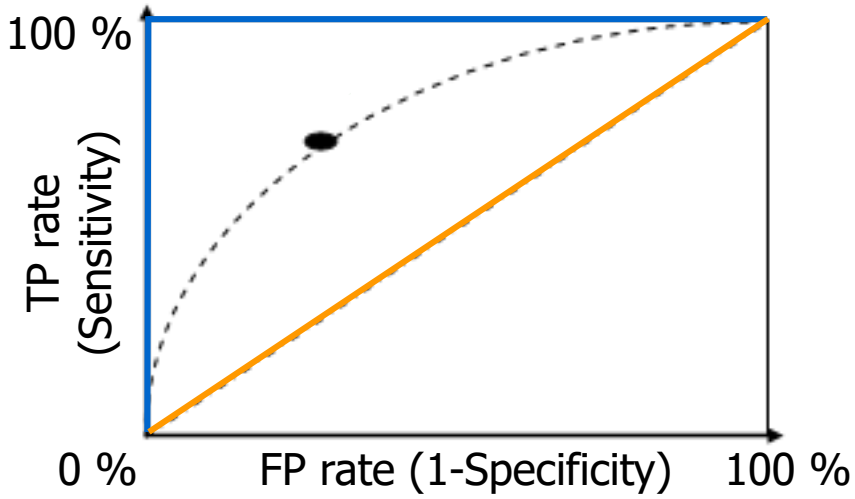
specificity
= tn / (tn + fp) = tn / neg

→ depends on classifier

Richter, *Der Internist*, 1997, 38: 325-336

Receiver-Operating-Characteristics (ROC)-Analysis





close to blue line good, close to yellow line not so good

AUC (Area under the curve)

AUC = 1, 100% discrimination

AUC = 0.5, *no discrimination*

Definition biomarker

...A characteristic that can be measured and evaluated as an indicator of normal biologic processes, pathologic processes or pharmacologic responses to therapeutic intervention....

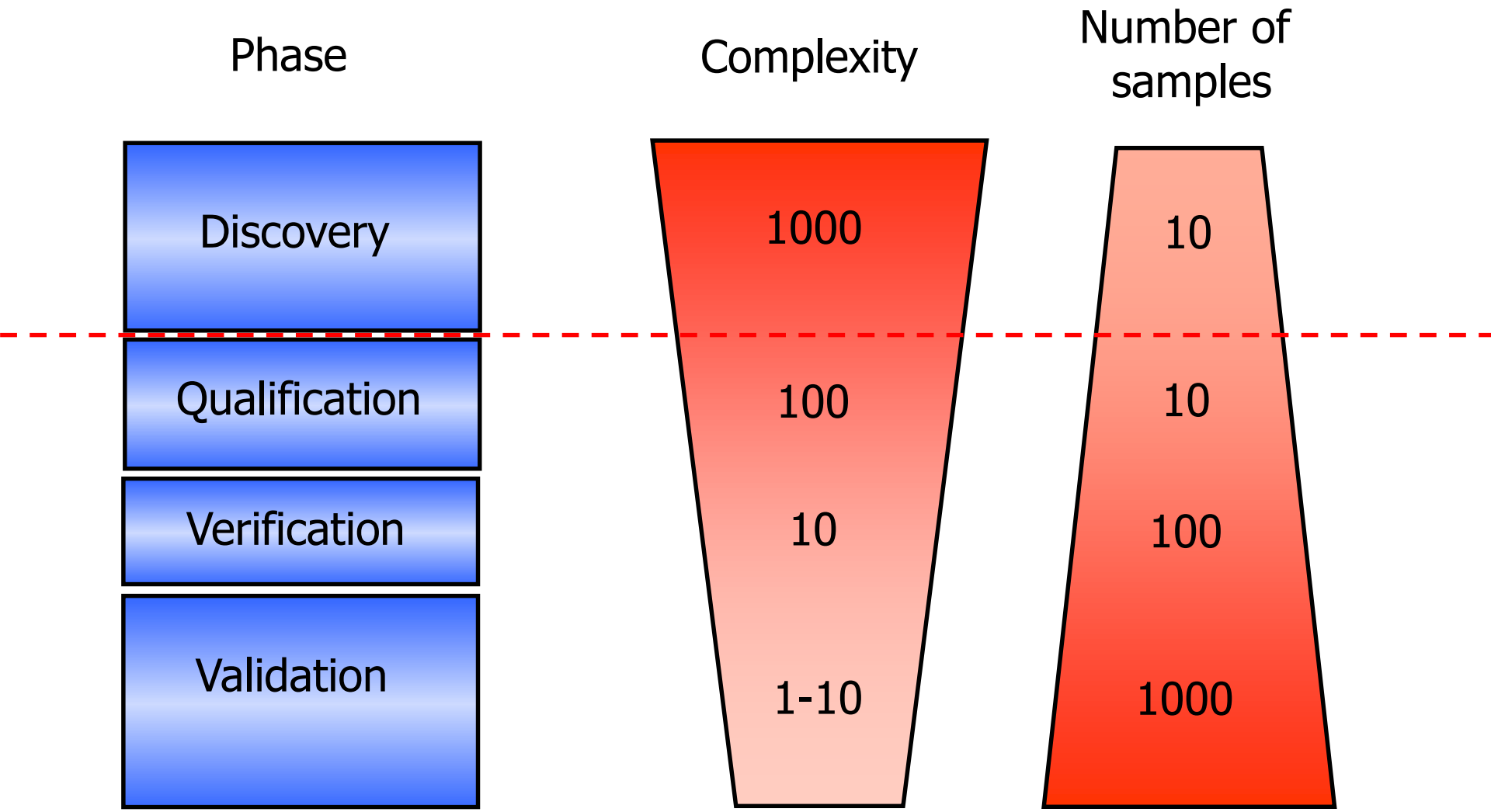
NIH *Biomarker Definitions Working Group*, Clinical and pharmacological Therapeutics, 2001

...any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease....

WHO *Biomarkers in risk assesment Validity and Validation*, International Programm on Chemical Safety, 2001

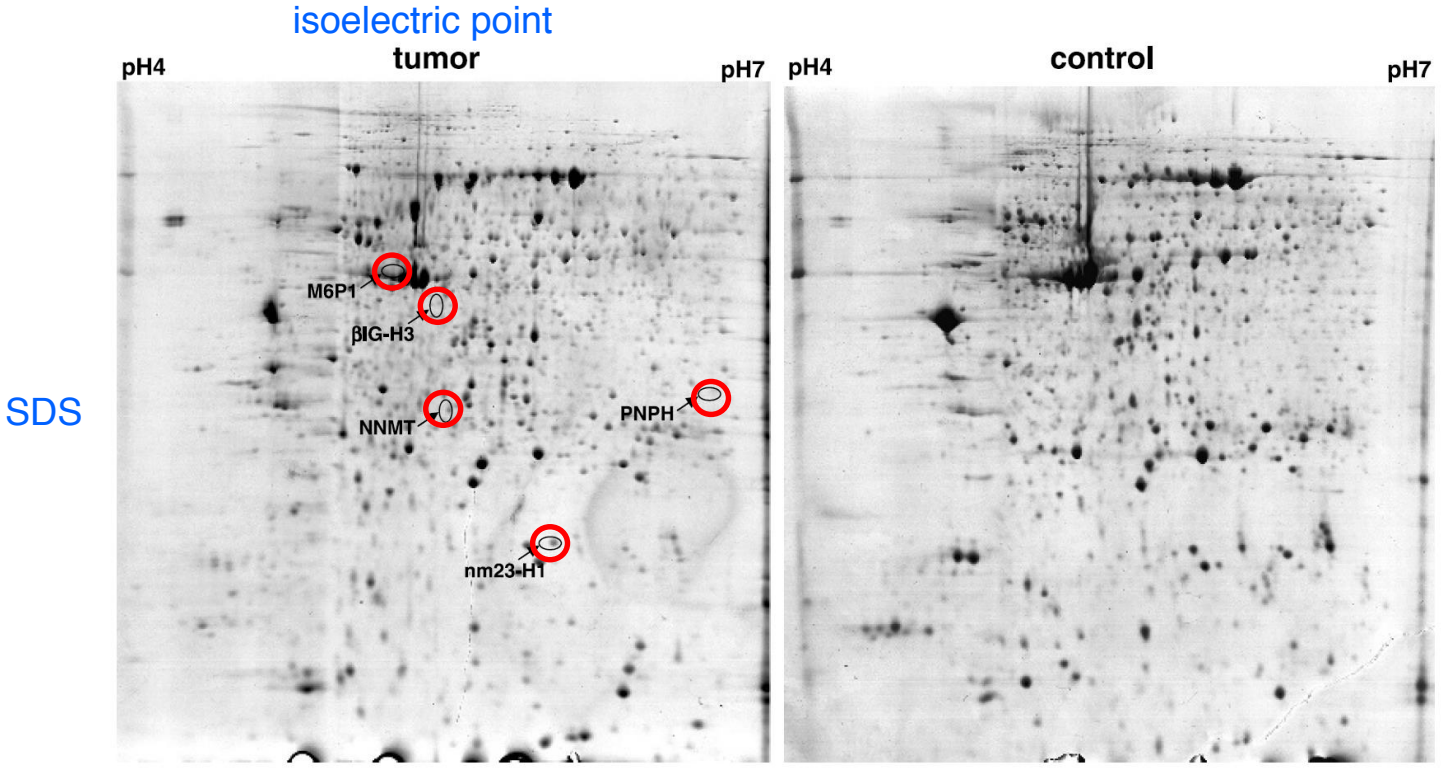
Biomarker discovery: an example

Protein Biomarker Identification



Modified after Rifai et al. 2006

Biomarker Discovery MS- Approach

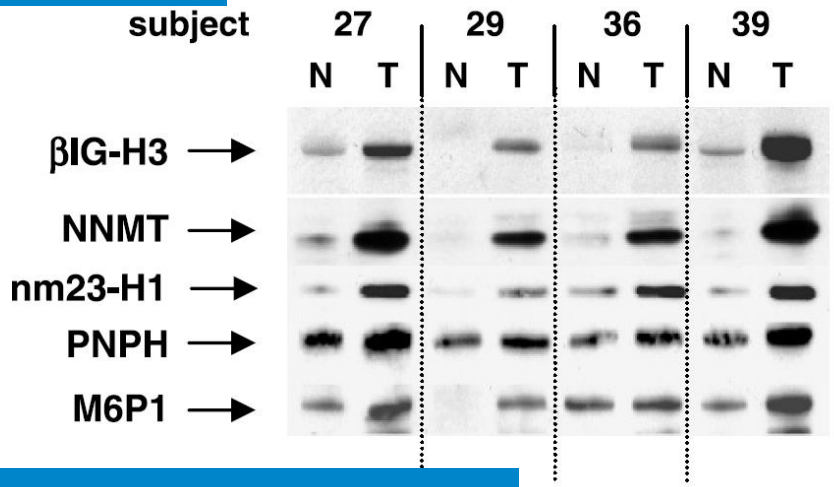


16 Colorectal cancer tissues
16 corresponding normal tissue

Roessler M. et al.,*Clin. Cancer Res*. 11, 6550-6557

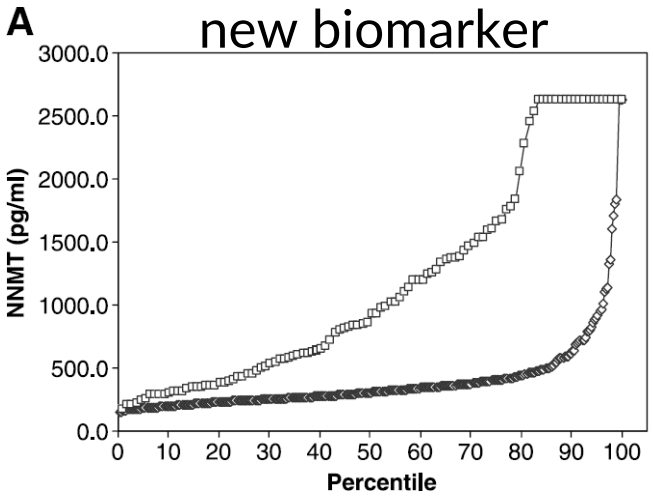
Biomarker validation

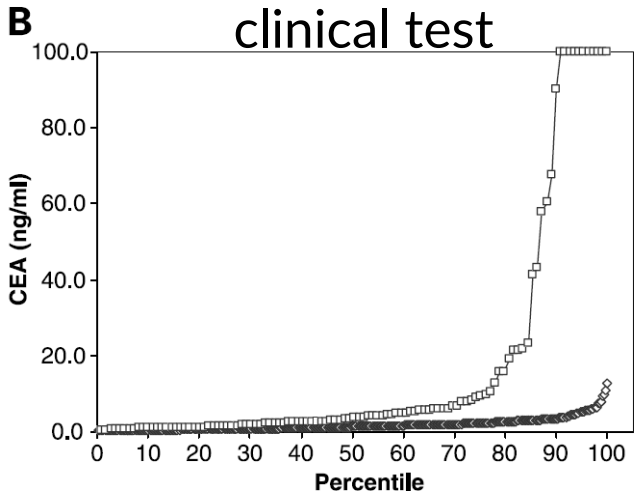
Western Blot



Tissue samples of cancer versus healthy tissue

Sandwich Immunoassay

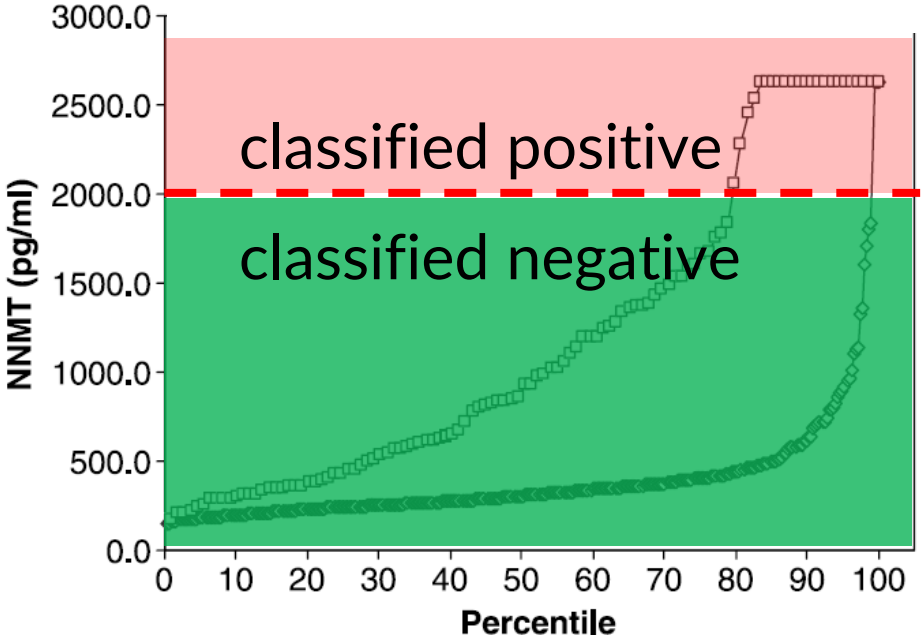




Serum screen of 109 patients versus vs. 317 healthy donors

Roessler et al.,*Clin. Cancer Res.* 11, 6550-6557

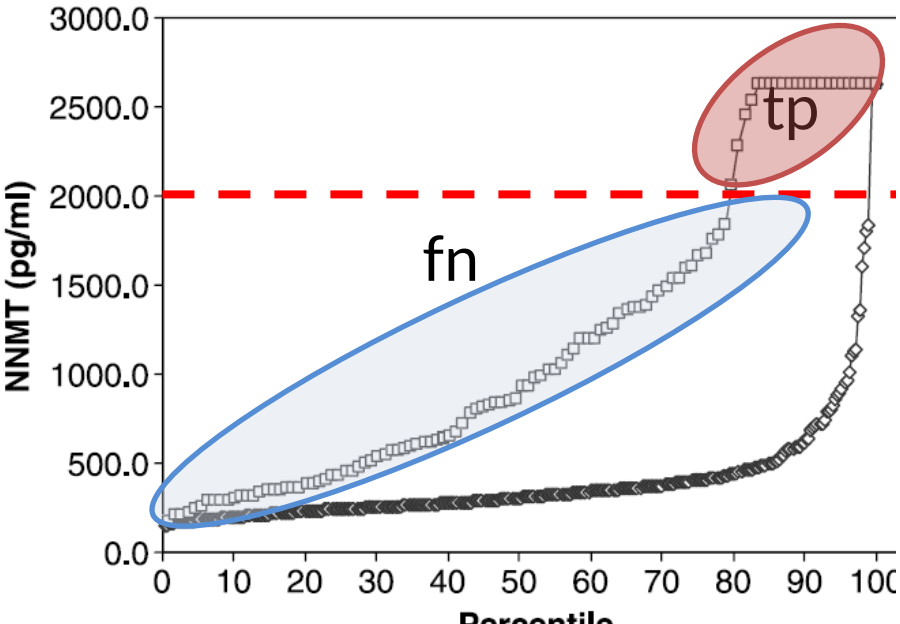
Biomarker validation

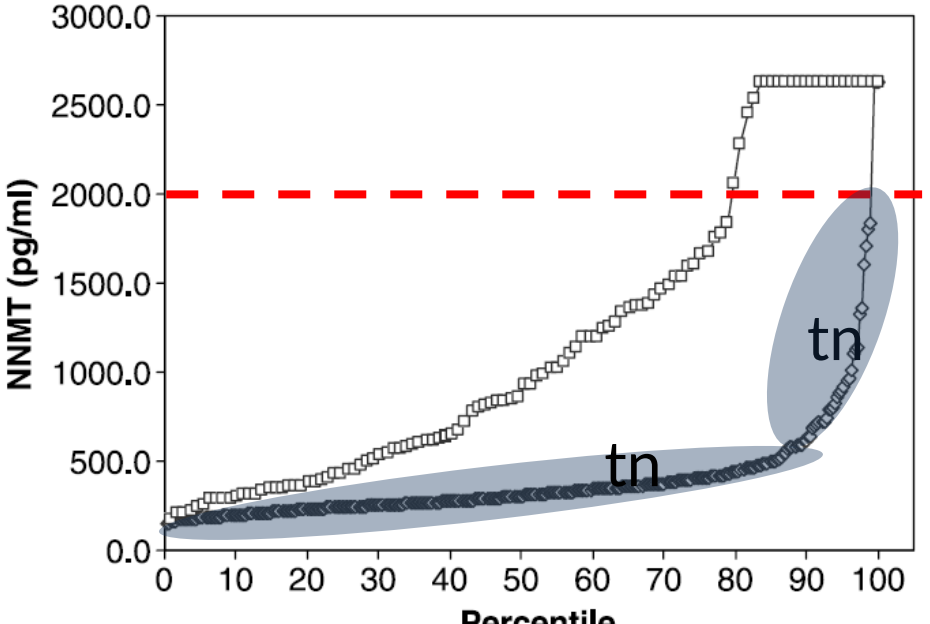


Classifier: 2000 pg/ml

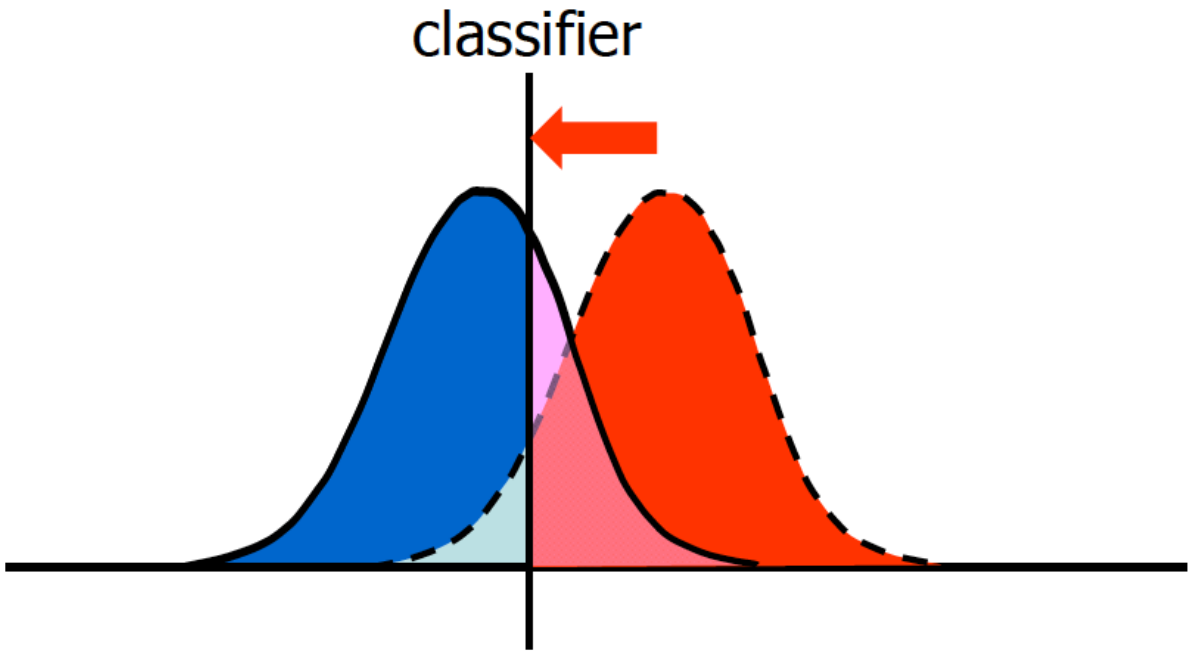
sensitivity = tp / (tp + fn)
= 20/ (20 + 80) = 0.2

specificity = tn / (tn + fp)
= 100 / (100+0)= 1

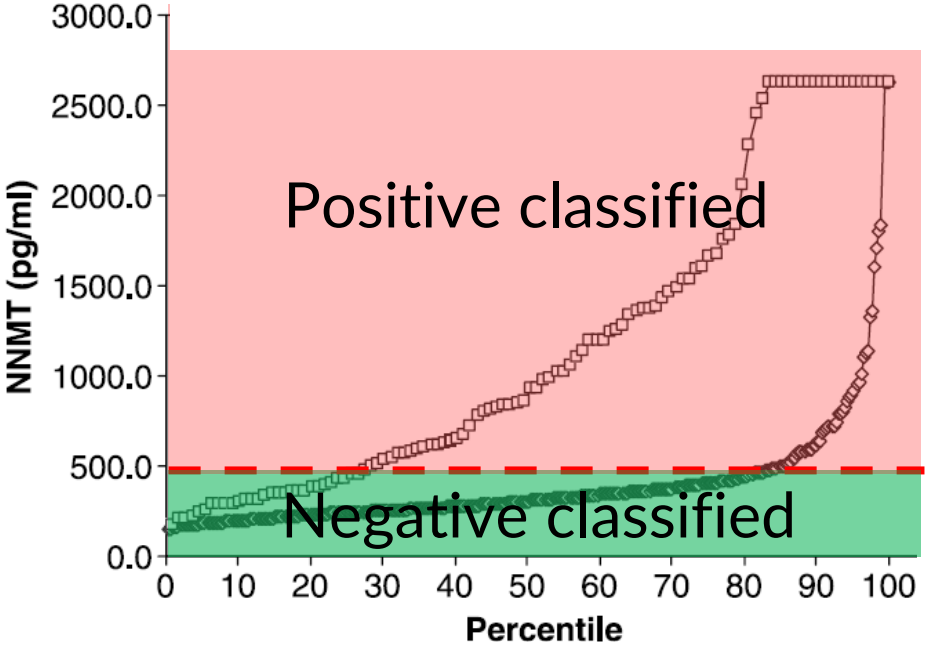




Biomarker validation



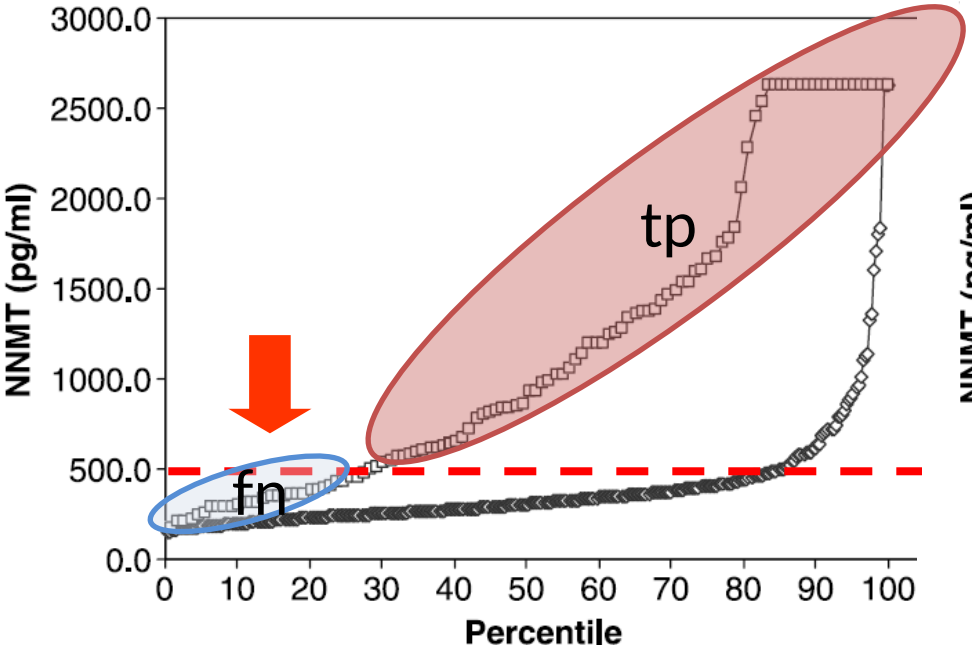
Biomarker validation

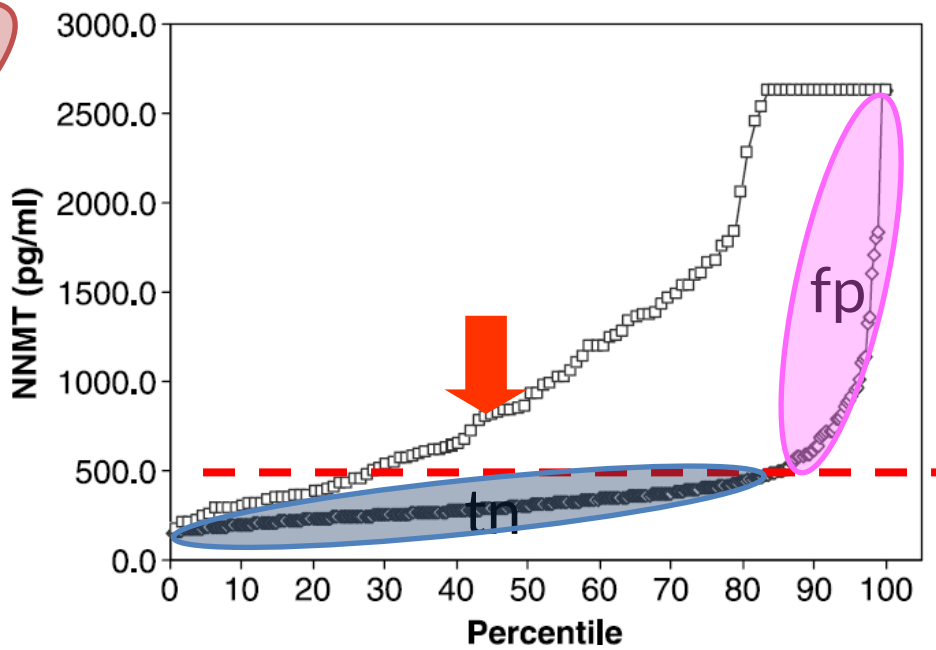


Classifier: 500 pg/ml

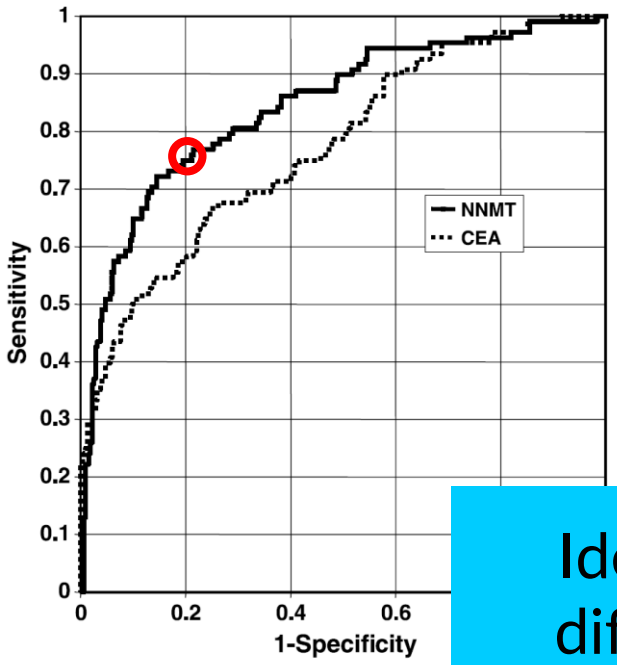
sensitivity = tp / (tp + fn)
= 75/ (75+ 25) = 0.75

specificity = tn / (tn + fp)
= 80 / (80 + 20)= 0.8

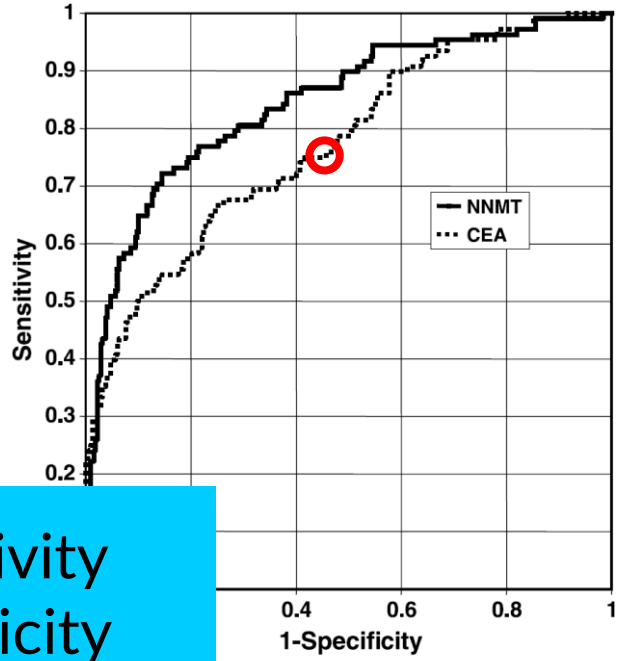




new biomarker



clinical test



Identical sensitivity
different specificity

NNMT

sensitivity = tp / (tp + fn)
= 80/ (80 + 20) = 0.75

specificity = tn / (tn + fp)
= 80 / (80 + 20)= 0.8

CEA

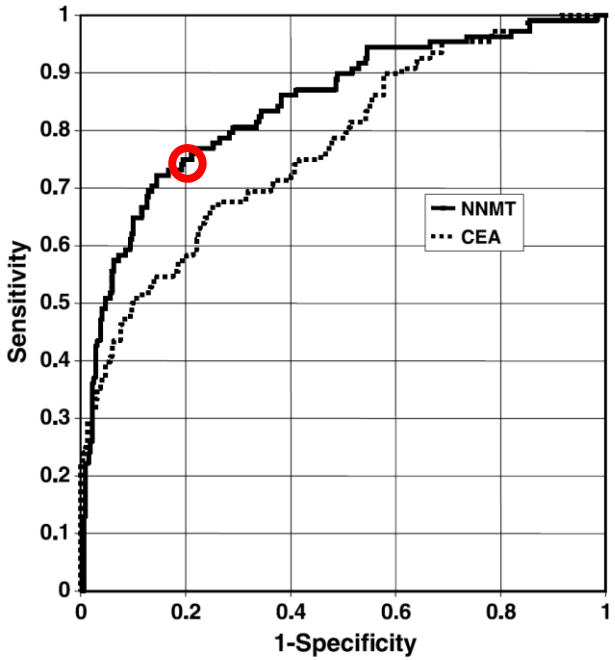
sensitivity = tp / (tp + fn)
= 75/ (75 + 25) = 0.75

specificity = tn / (tn + fp)
= 50 / (50 + 50)= 0.5

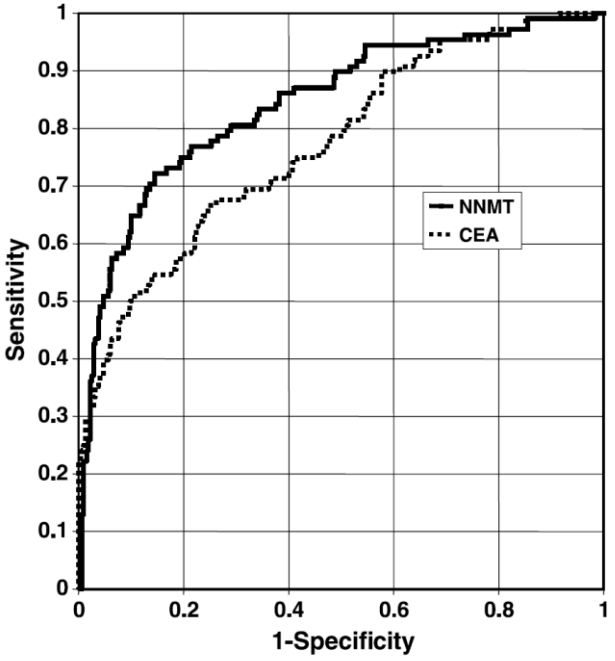
Roessler et al.,*Clin. Cancer Res.* 11, 6550-6557

What does this mean for a patient?

new biomarker



clinical test



NNMT

sensitivity = tp / (tp + fn)
= 75/ (75 + 25) = 0.75

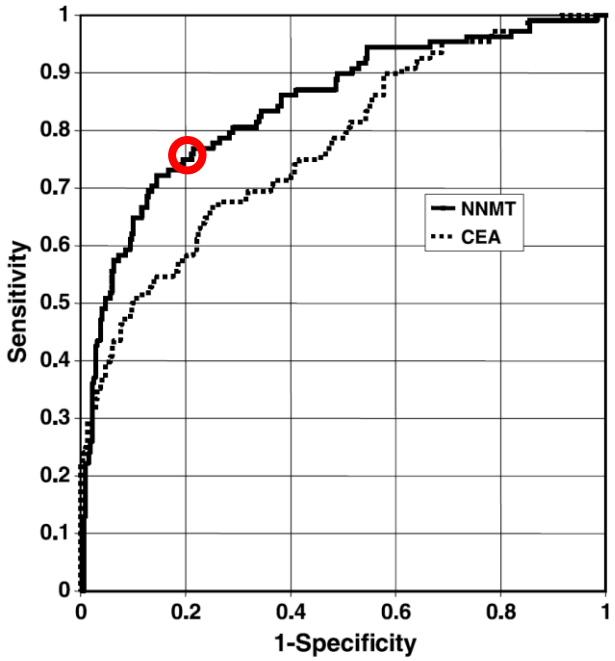
CEA

sensitivity = tp / (tp + fn)
= 75/ (75 + 25) = 0.75

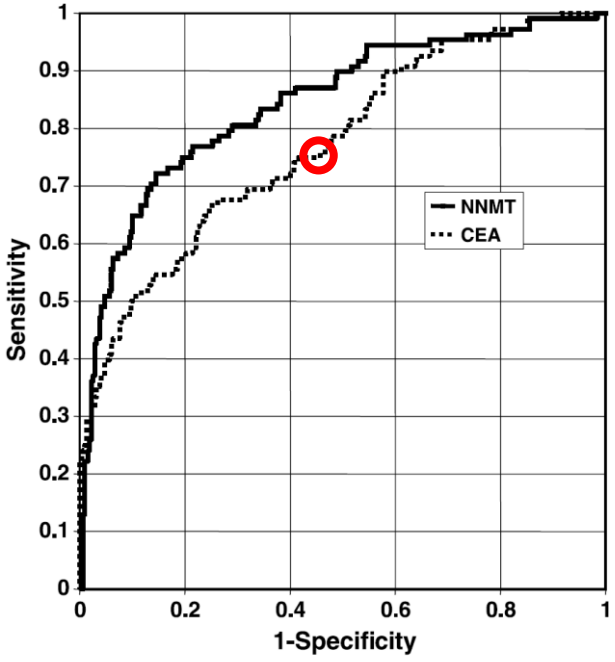
75% of tumors patients are correctly classified

with both methods

new biomarker



clinical test



Every 5th

Every 2nd

”healthy” individual is classified as “sick”

specificity = tn / (tn + fp)
= 80 / (80 + 20)= 0.8

specificity = tn / (tn + fp)
= 50 / (50 + 50)= 0.5

Roessler et al.,*Clin. Cancer Res.* 11, 6550-6557

EXAM - Multiple Choice

- Define the term “Epitope“ or “Paratope“?
- Explain the terms “continous” and “discontinous epitope”?
- Which reagents are required for a sandwich immunoassay?

capture antigen …

- Describe the difference between competitive and non-competitive assay concept?
- What is detected in a serological assay?
- What is clinical specificity?
- What is clinical sensitivity?