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C8116

Antibodies as immunochemical tools

Spring semester 2025

Hans Gorris
Department of Biochemistry
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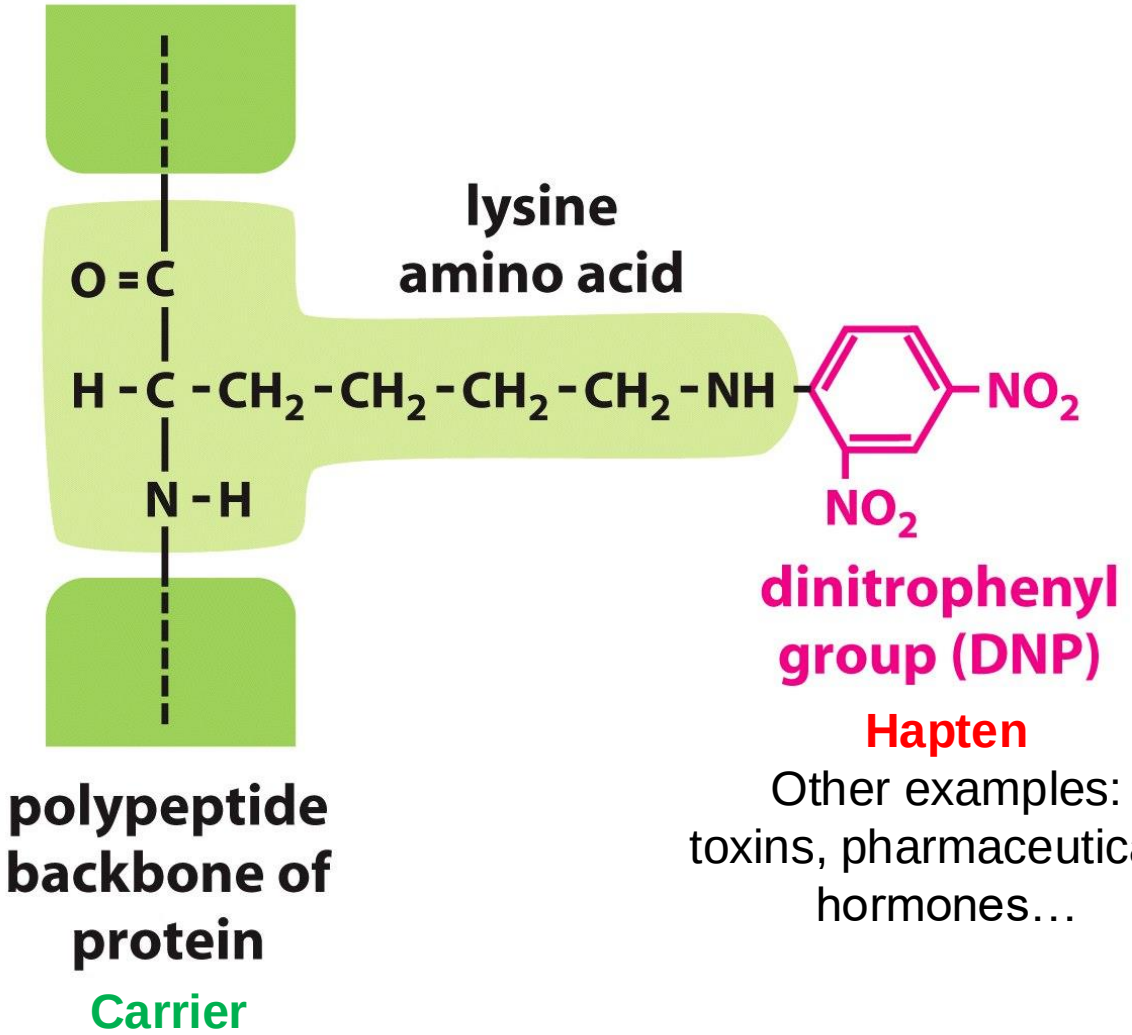
Antigenic determinants: hapten

- Immunization generates antibodies only against large molecules, e.g. proteins
- Antibodies against small molecules (**haptens**) must be produced by coupling (typically derivatized) small molecule **onto the surface a large carrier protein**.



Definition of hapten:
A low-molecular weight molecule which contains an antigenic determinant but which is not itself antigenic unless bound to an antigenic carrier

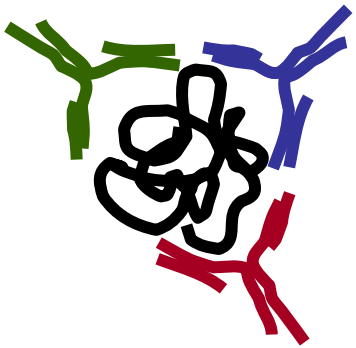
Other examples:
toxins, pharmaceuticals, hormones…

Polyclonal vs. monoclonal antibodies

polyclonal

Antibodies that are collected from sera of exposed animal

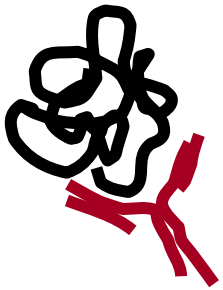
recognize
multiple antigenic sites
of injected substance



monoclonal

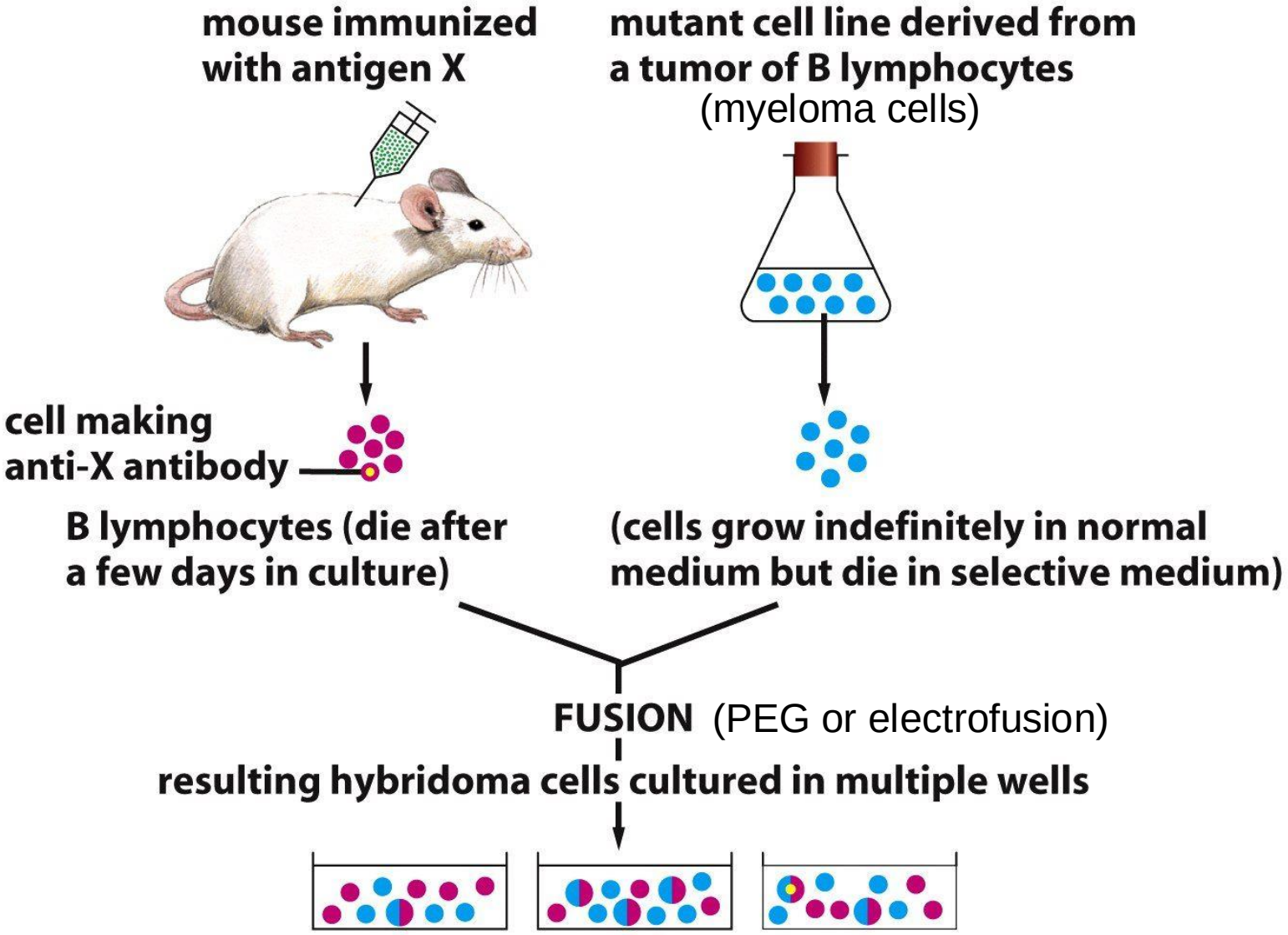
Individual B cell hybridoma is cloned and cultured.
Secreted antibodies are collected from culture media

recognize
ONE antigenic site
of injected substance

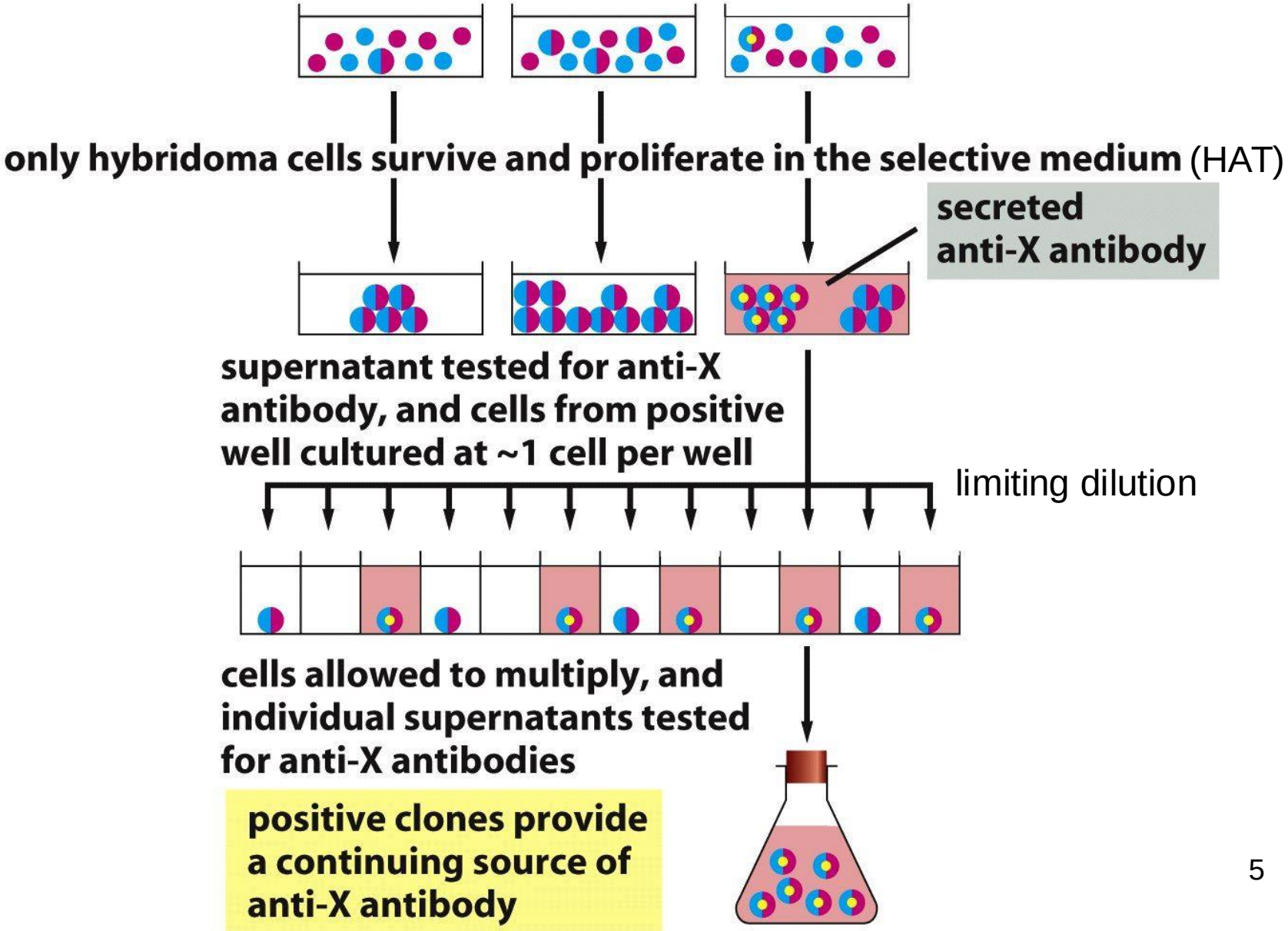


Generation of monoclonal antibodies

=> first described by Köhler/Milstein (1975)

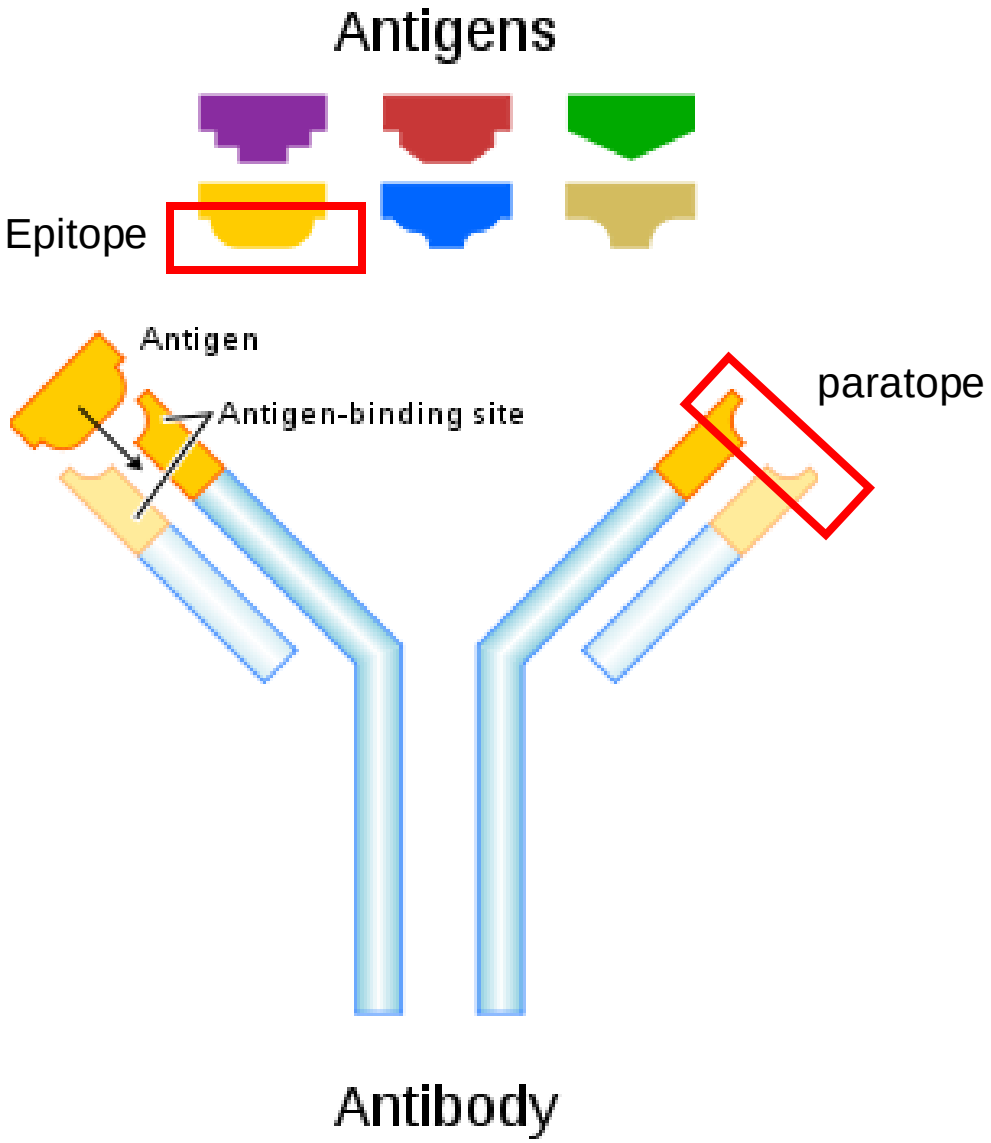


Generation of monoclonal antibodies



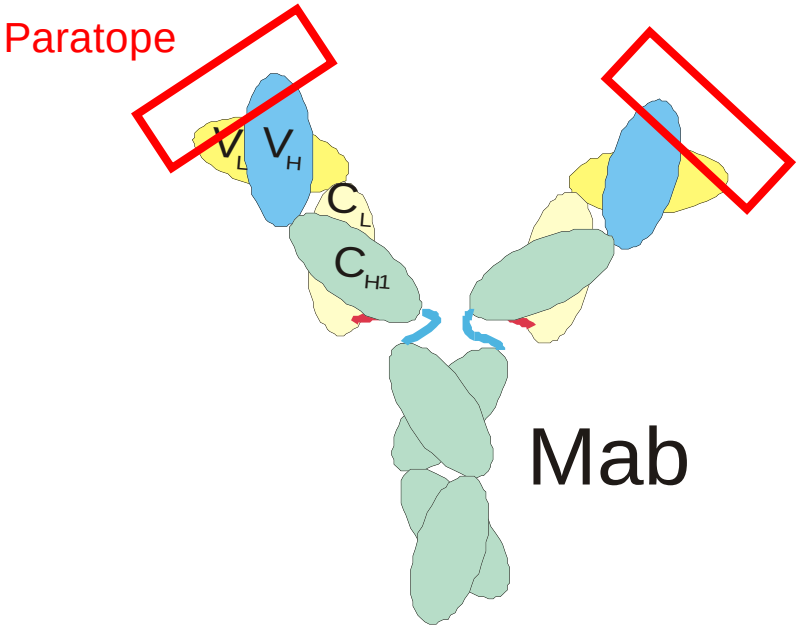
Antibodies as immunochemical reagents

=> Antibodies are used as bioanalytical reagents to specifically detect and quantify other molecules



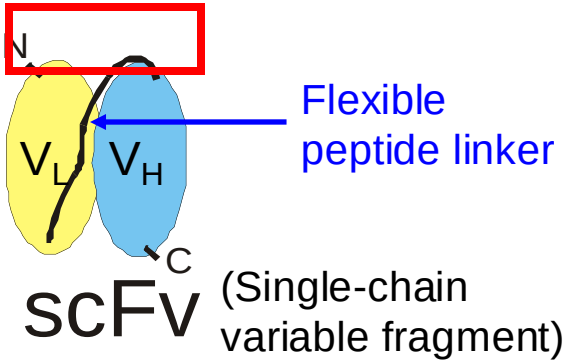
Recombinant antibody fragments

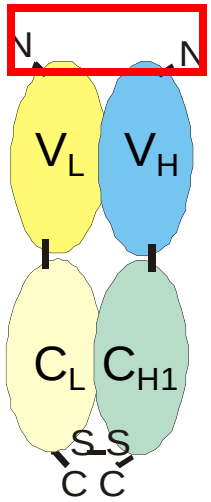
Natural IgG antibody



Disadvantage of antibodies as a reagent: relatively large size

Artificial smaller constructs





=> same paratope, but much smaller

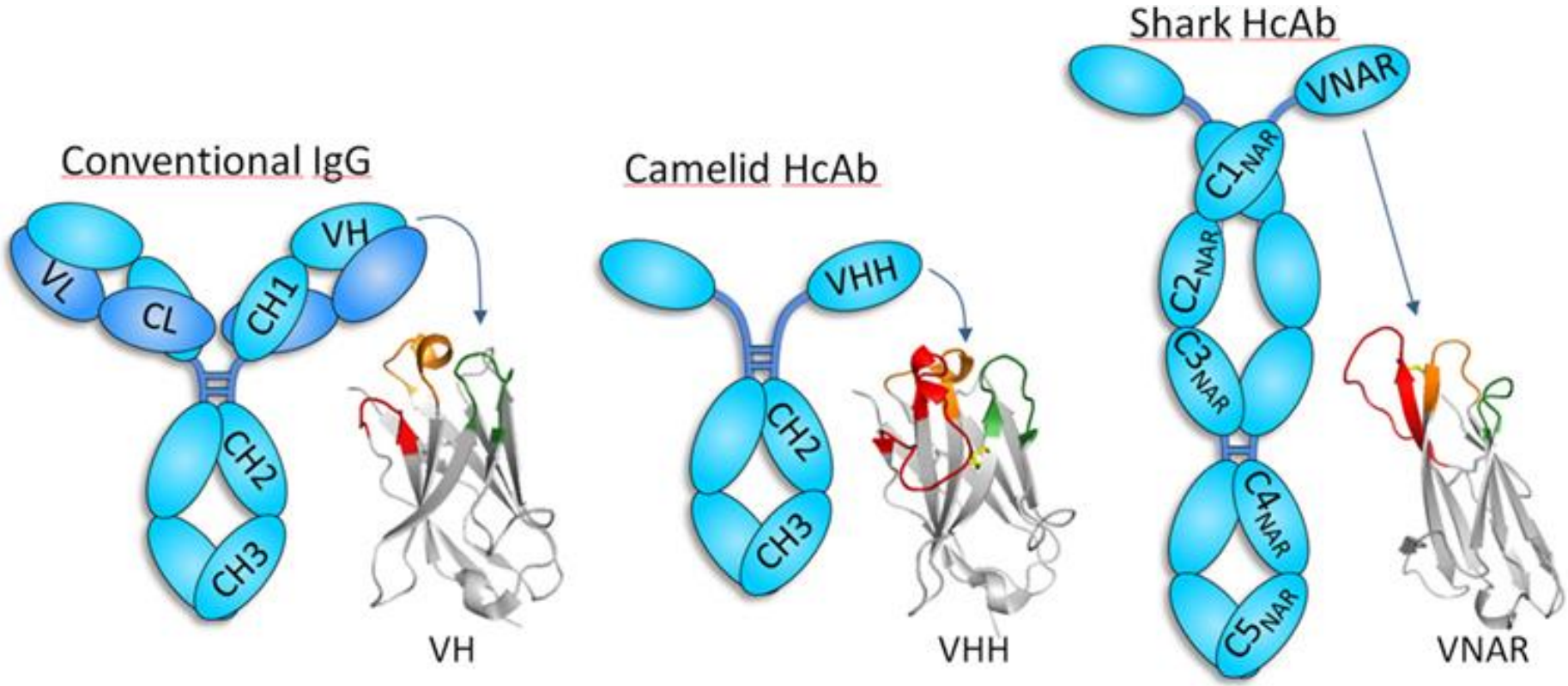
Recombinant antibody fragments

Immortalization of hybridomas through cloning
or
generation of new **antibodies without immunization**

- Greater speed of **production** *(E. coli batch fermentation)*
- New specificities especially for **poor immunogens**
- Possibility to **fine-tune** antibody specificity and affinity
- Possibility to **tailor make** the antibody to perform special tasks
    - tags, handles (for conjugation, immobilization)
    - fusing to other protein (e.g. enzymes)

Likely to be increasingly used in **miniaturised systems** to enable full control of antibody performance.

Heavy chain antibodies



our own most common antibody

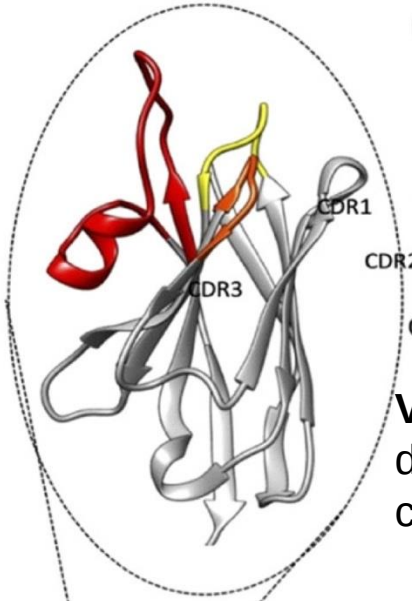
heavy chain antibodies

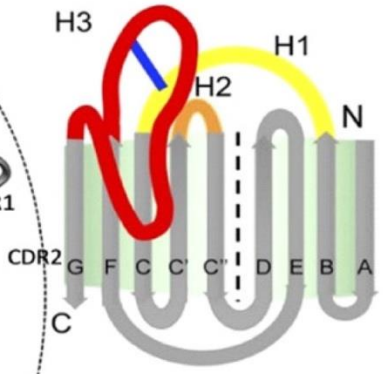
(velbloud, dromedár, lama)

(žralok)

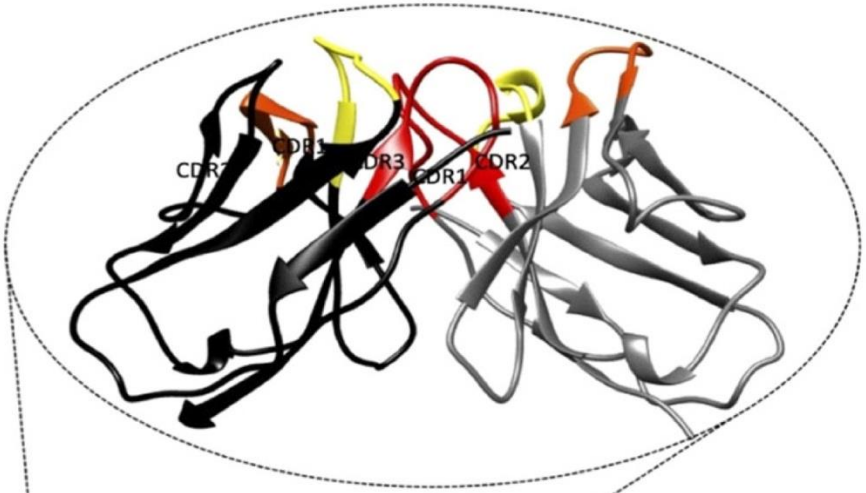
Front. Immunol., 2017 https://doi.org/10.3389/fimmu.2017.00977

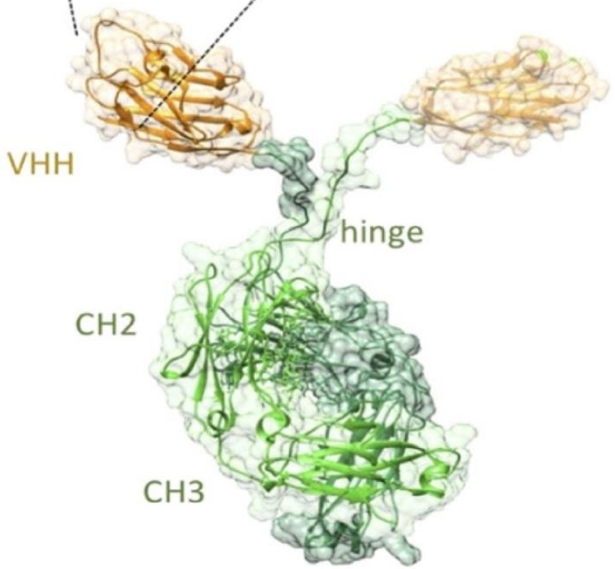
From heavy chain antibodies to nanobodies



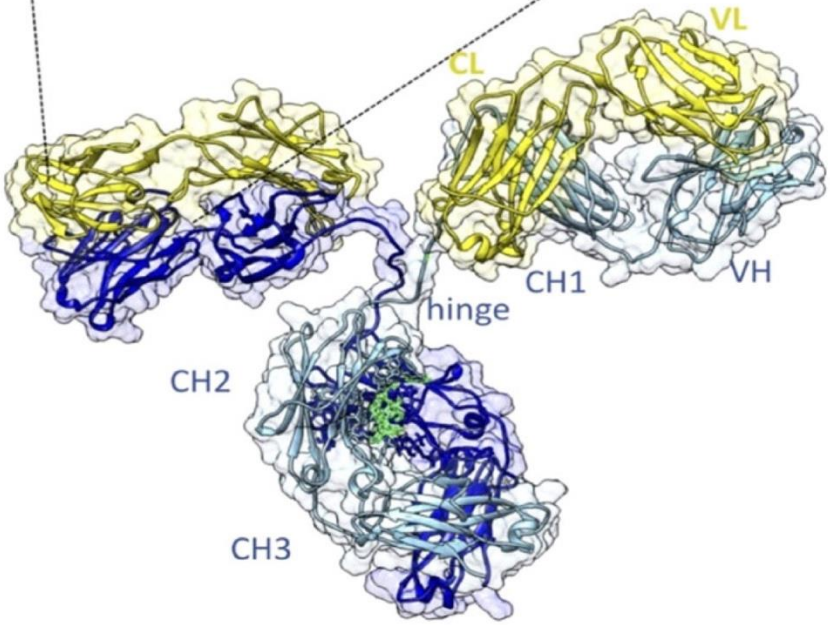


VHH: Single variable domain on a heavy chain (=> nanobody)



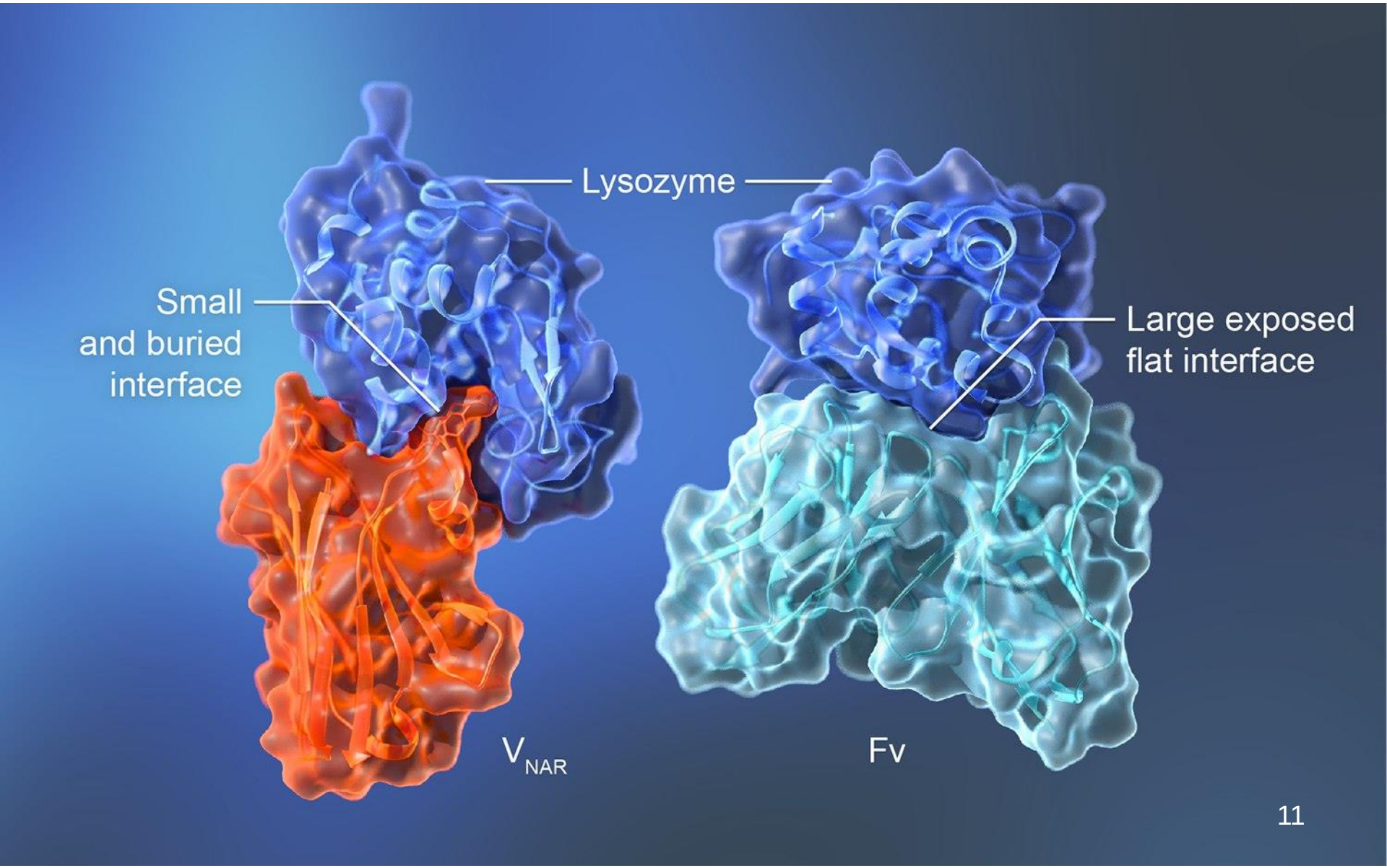


heavy-chain antibody



IgG antibody

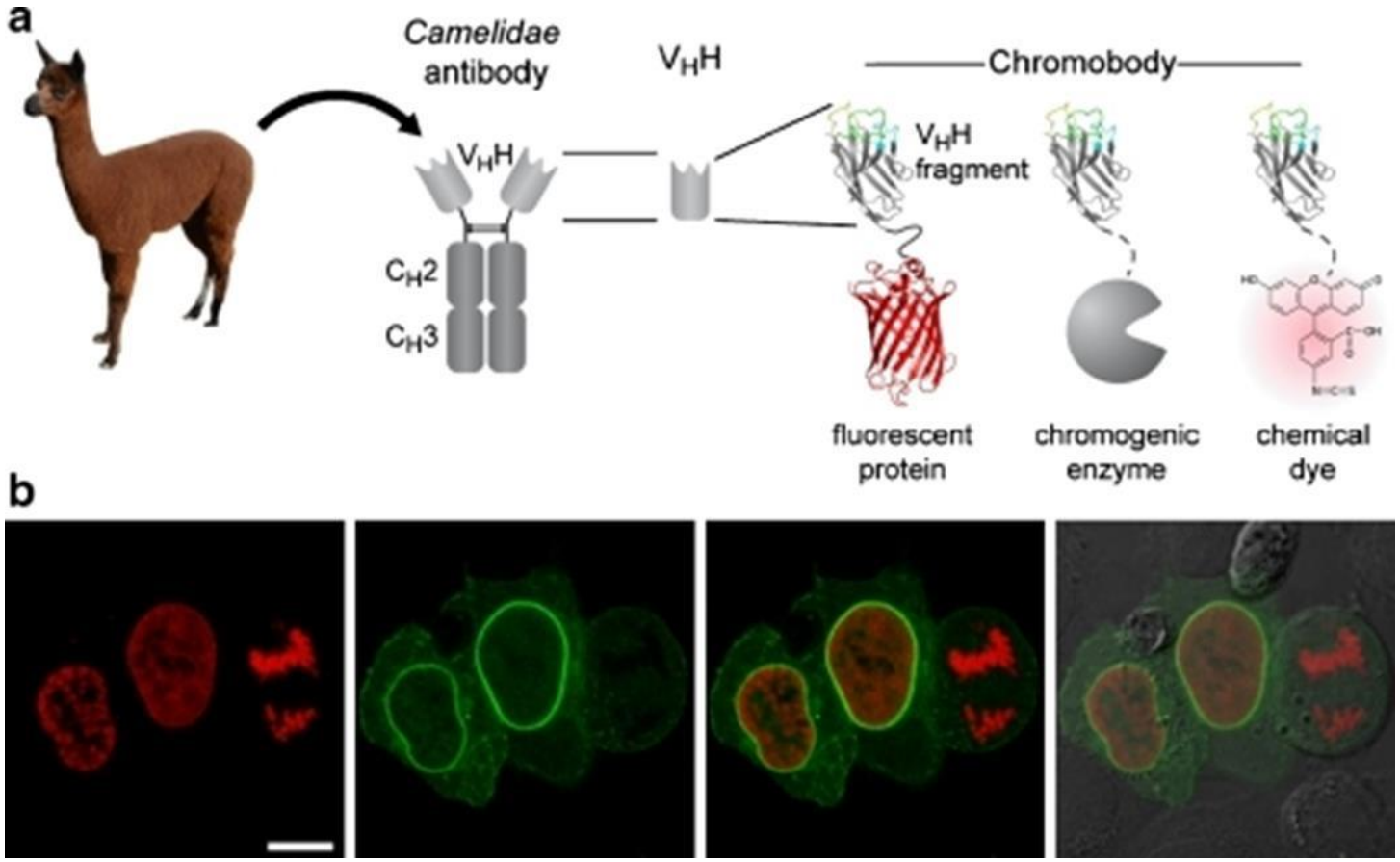
Nanobodies: Detection of hidden epitopes



Advantages of nanobodies

- Mass: ca. 15 kDa (IgG: 150 kDa), 2.5 nm diameter (IgG 15 nm)
- High solubility
- Rapid targeting and fast blood clearance
- Detection of “hidden“ epitopes
- Easy cloning: Recombinant engineering and protein expression *in vitro* in bacterial production systems are much simpler
- Very stable and heat resistant (no cold storage required)
- Simple genetic structure allows easy re-engineering of nanobodies to introduce new antigen-binding characteristics or attach labels

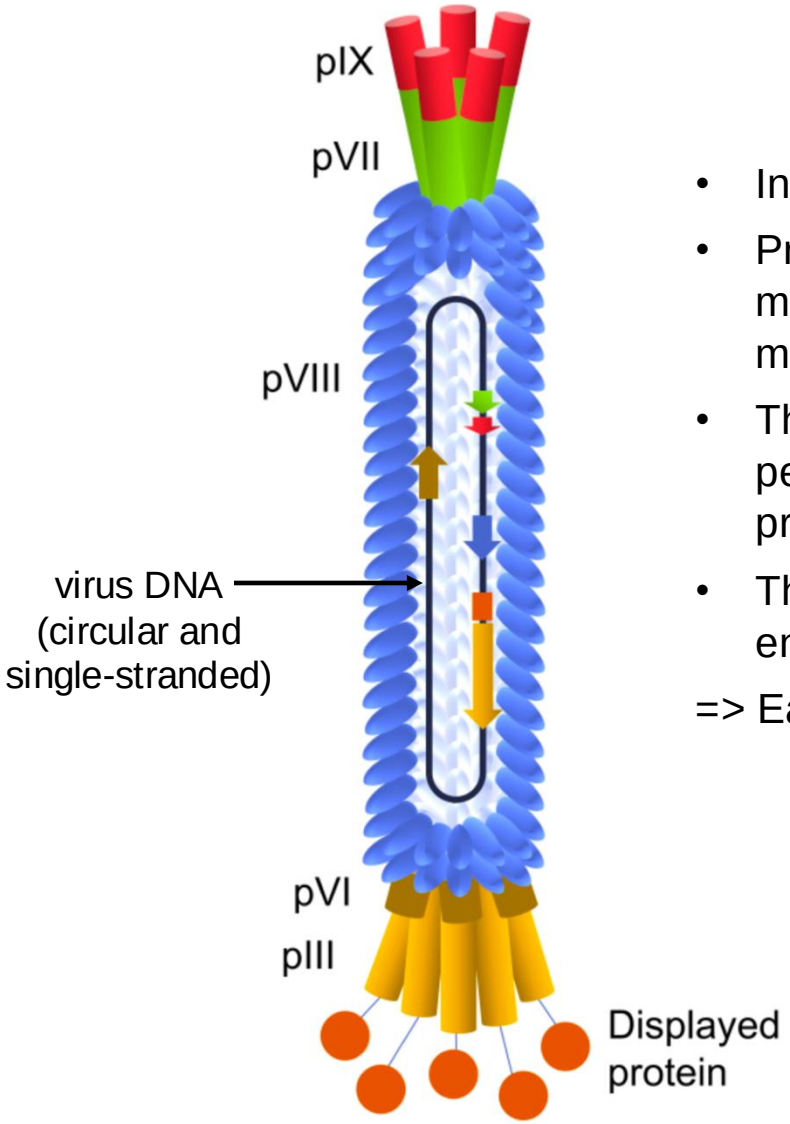
Recombinant nanobodies



a Chromobodies.
b Detection of the nuclear lamina with lamin chromobody in living cells. Confocal images of HeLa cells coexpressing lamin chromobody (*green*) and red fluorescent histone H2B as a mitosis marker. Scale bar: 10 μm

Anal. Bioanal. Chem. (2010) 397: 3203–3208

Phage display using filamentous phage M13

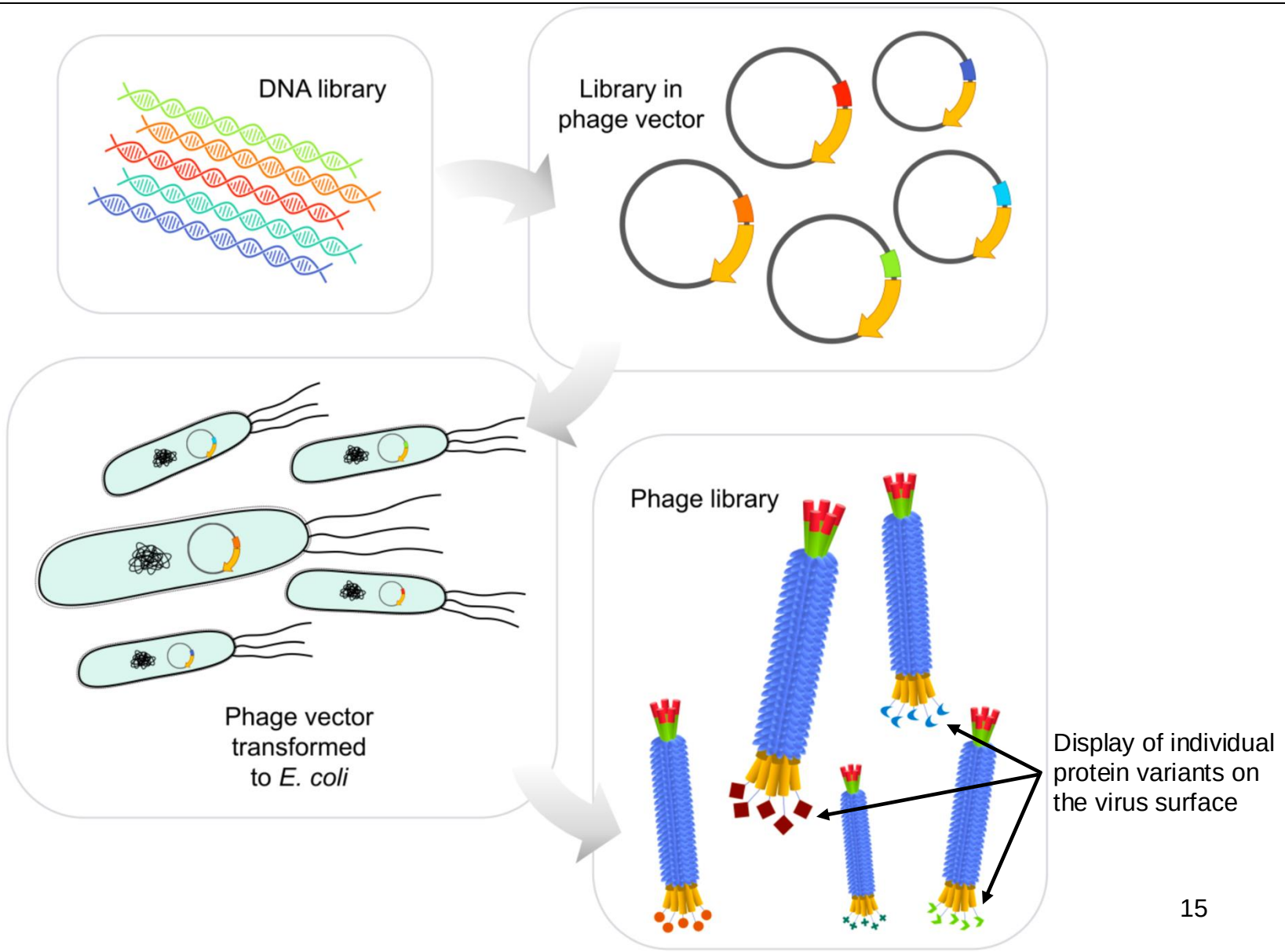


- Infects / replicates in *E. coli*
- Protein coat:
  major coat protein: pVIII
  minor coat proteins: pIII, pVI, pVII, pIX
- The phage can be engineered to display foreign peptides or proteins as a fusion with one of the coat proteins, most commonly pIII.
- The genomic DNA encoding for the coat proteins is enclosed within the protein coat.

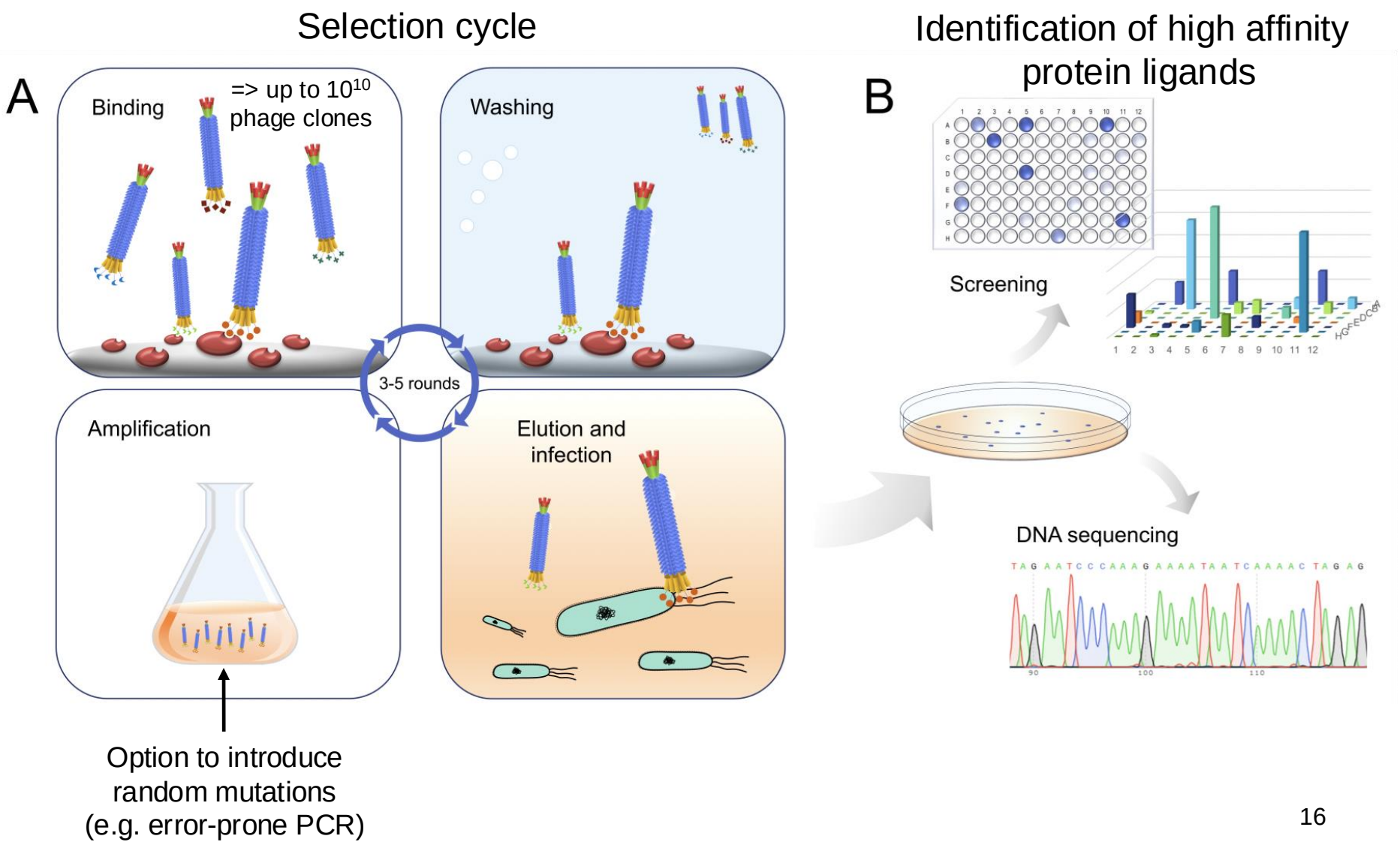
=> Each protein remains connected to its encoding DNA

George Smith / Greg Winter:
Nobel prize in chemistry 2018

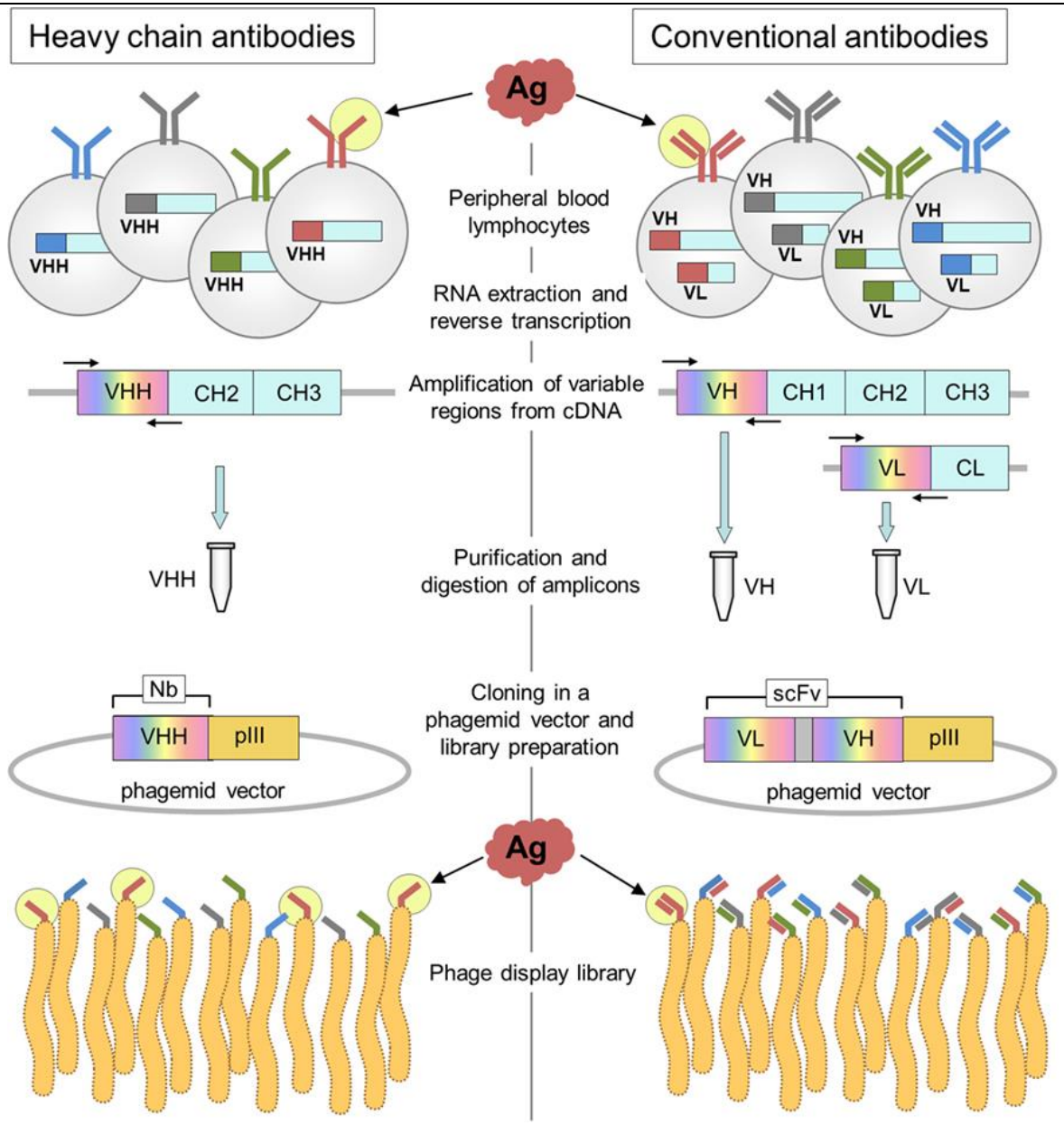
Construction of phage displayed protein libraries



Protein engineering by *in vitro* evolution



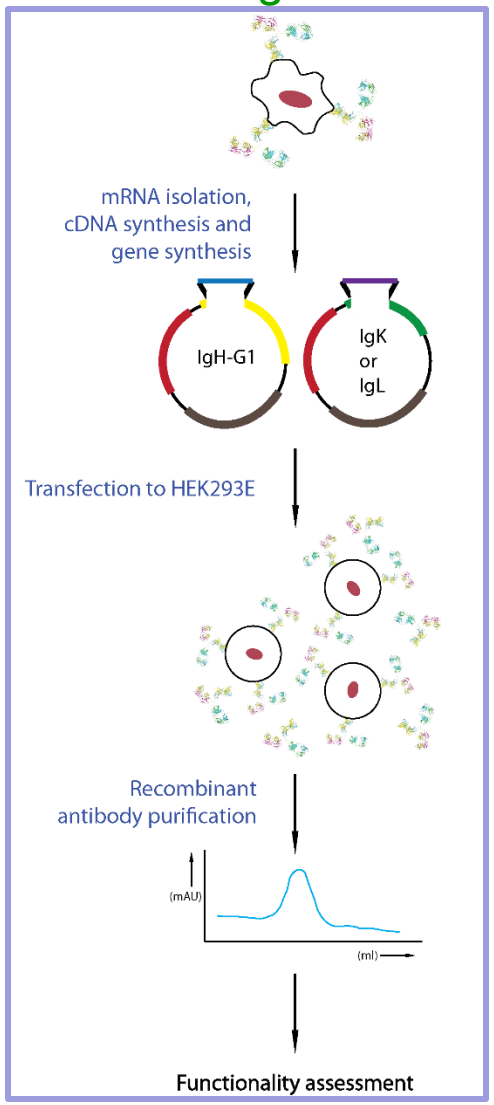
Single-domain antibody (nanobody)



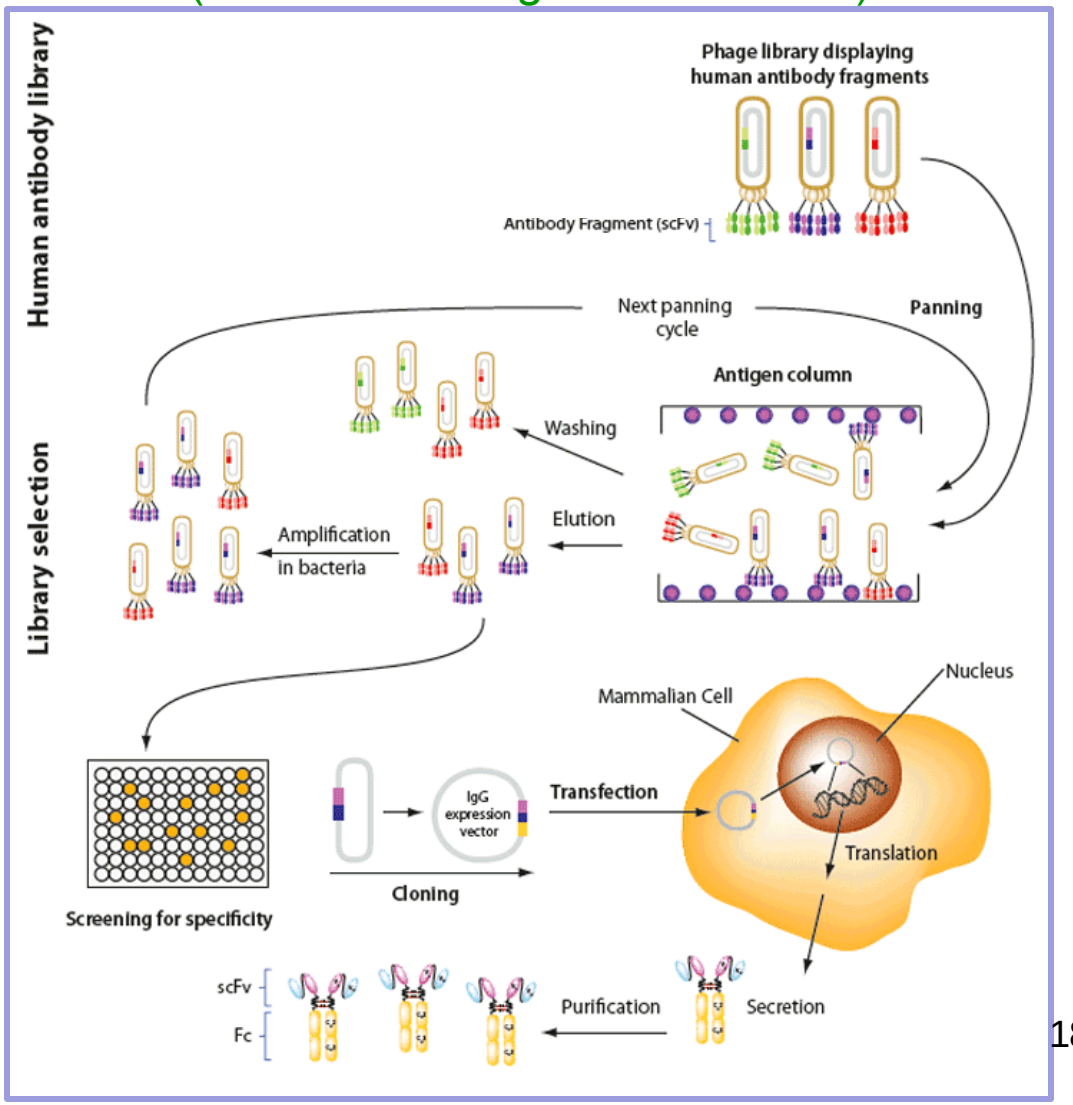
Problem here:
the genetic information for Vh and VL need to be fused to get functional paratops

Production of recombinant antibodies

Expression system
(Gene of Ab fragment known)



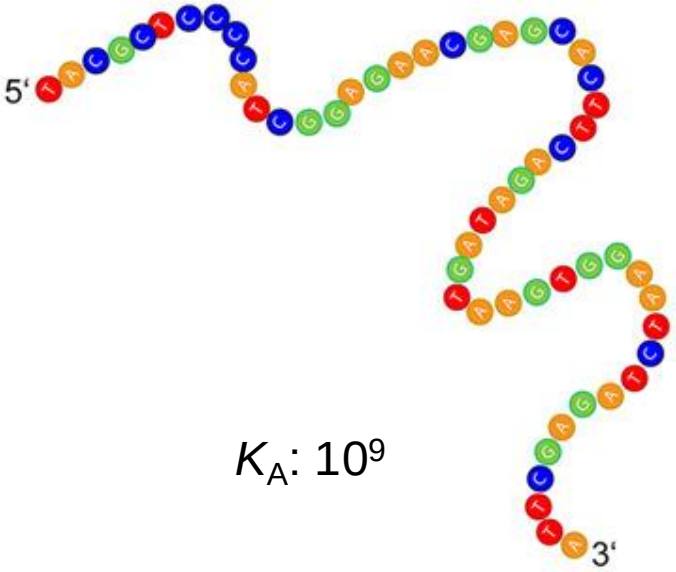
Phage display
(Gene of Ab fragment unknown)



Alternatives for antibodies

Aptamers

RNA or DNA aptamer

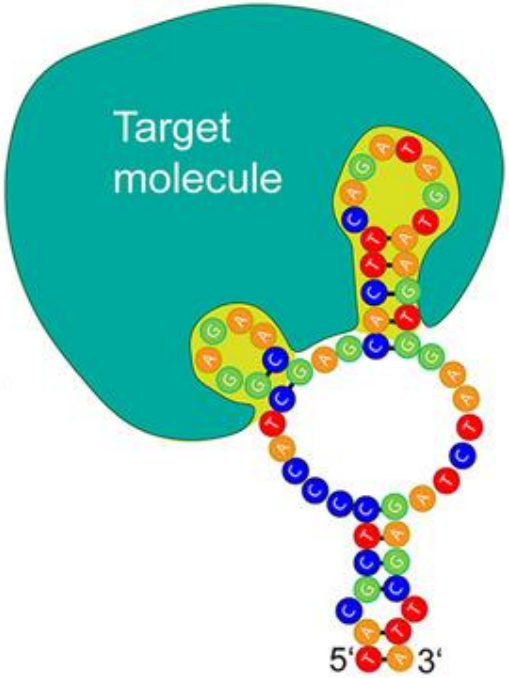


K_A: 10^9

Folding

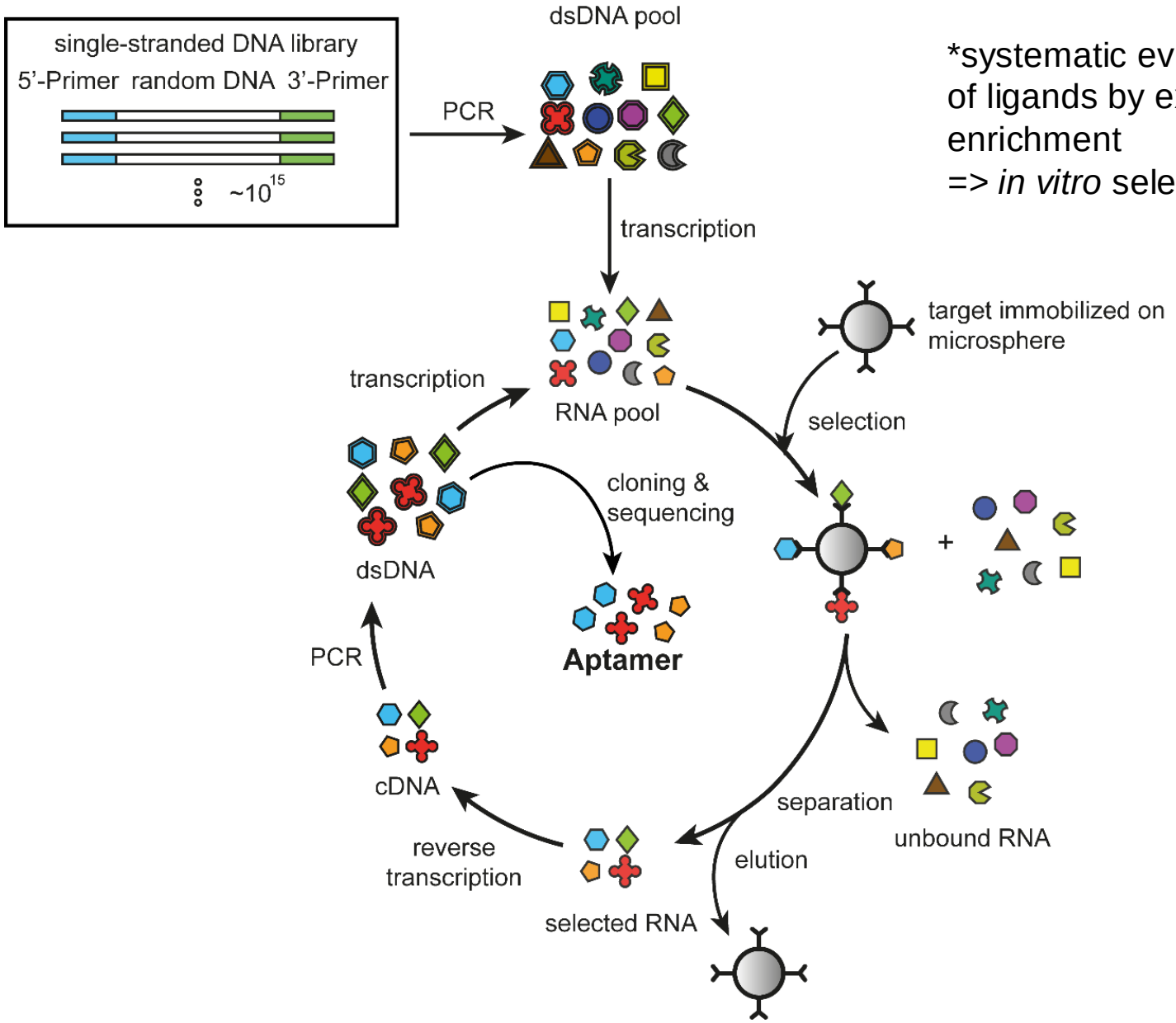
Complementatary
base pairing

Target
binding



Binding through:
(1) 3-dimensional, shape-dependent interactions
(2) hydrophobic interactions, base-stacking, intercalation

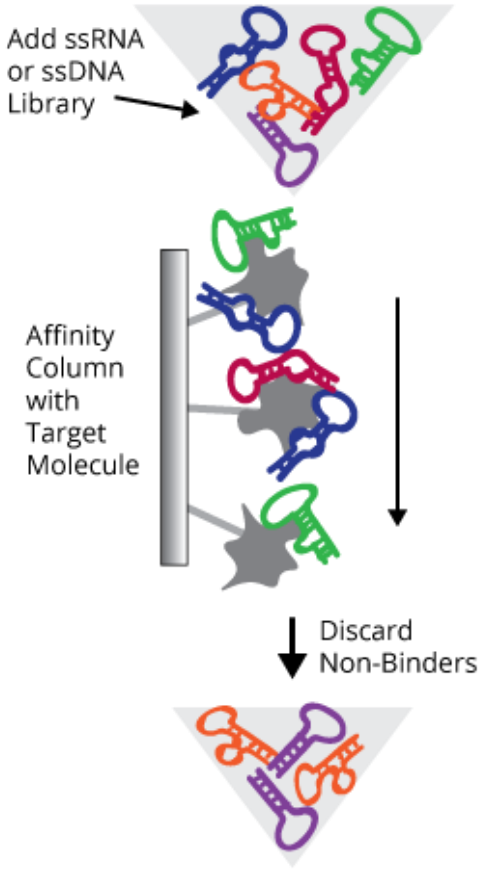
SELEX*



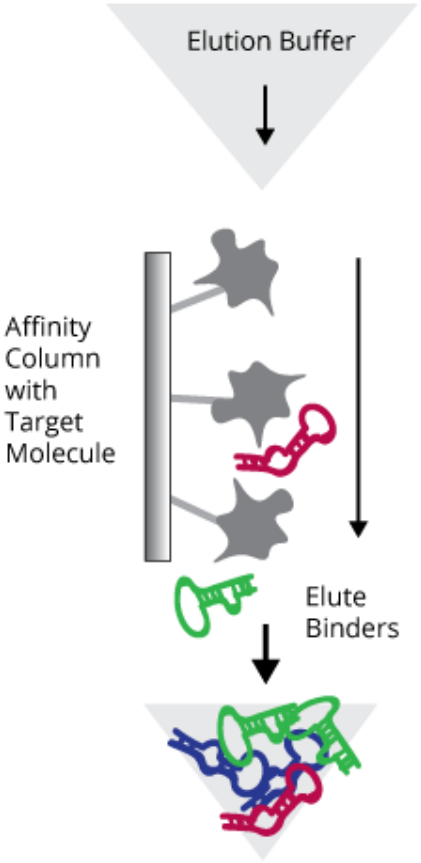
*systematic evolution of ligands by exponential enrichment
=> *in vitro* selection

SELEX*

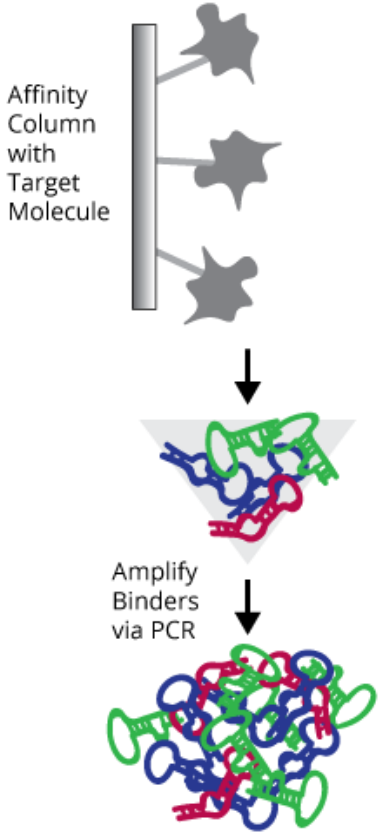
Step 1: Bind oligonucleotide library and discard non-binder



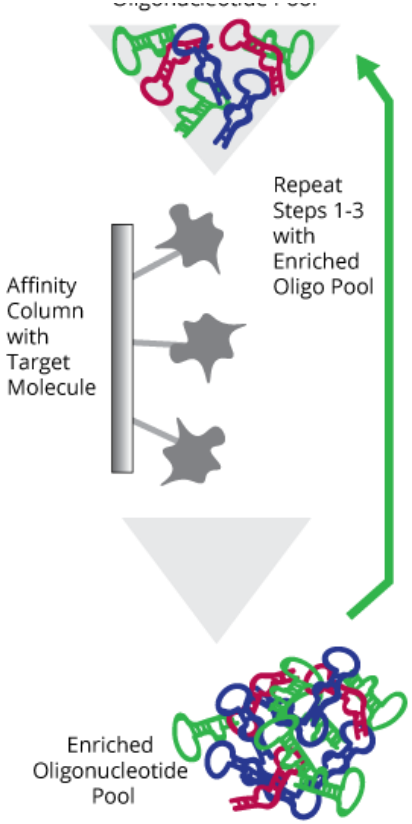
Step 2: Elute oligonucleotides that bind desired targets



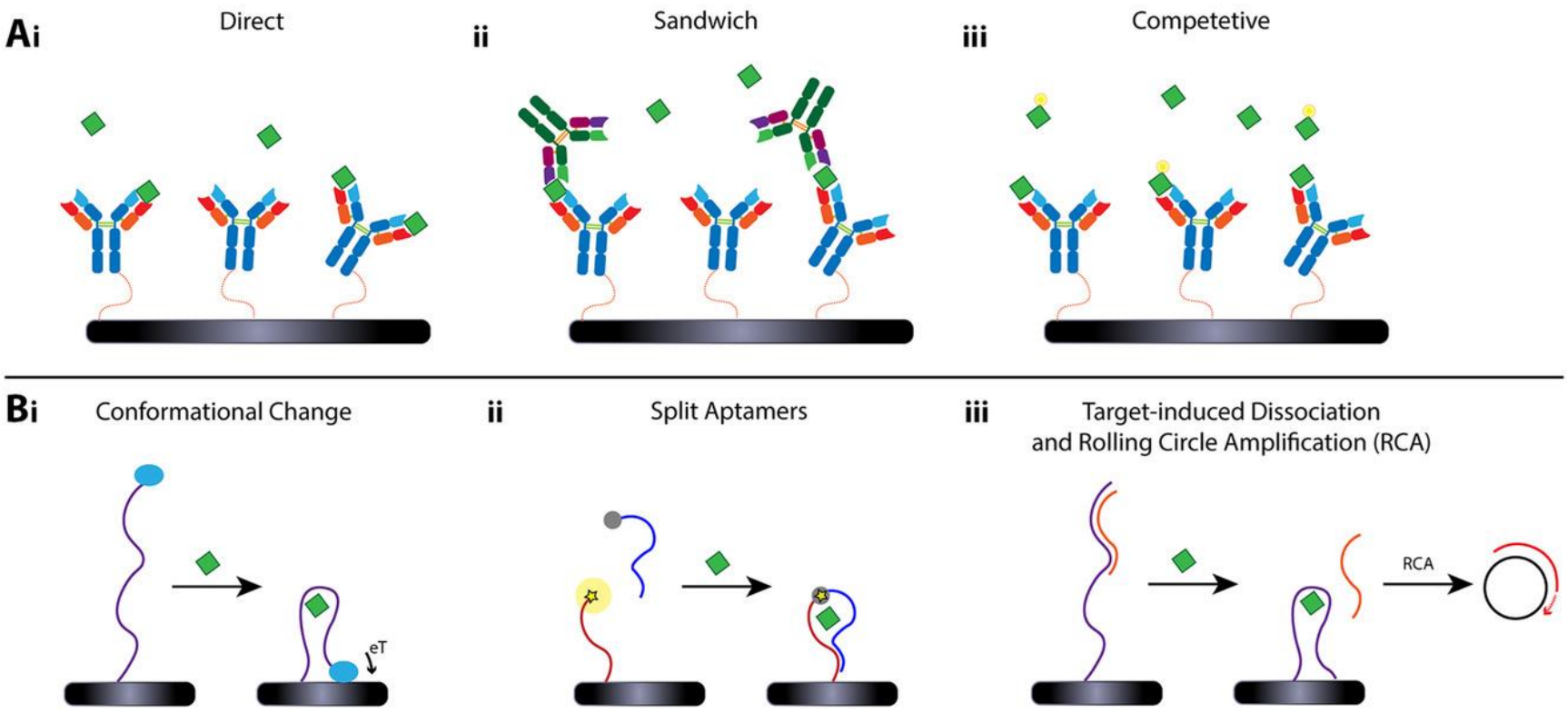
Step 3: Perform PCR to amplify eluted binders



Step 4: Repeat steps 1 through 3 using enriched oligonucleotide pool

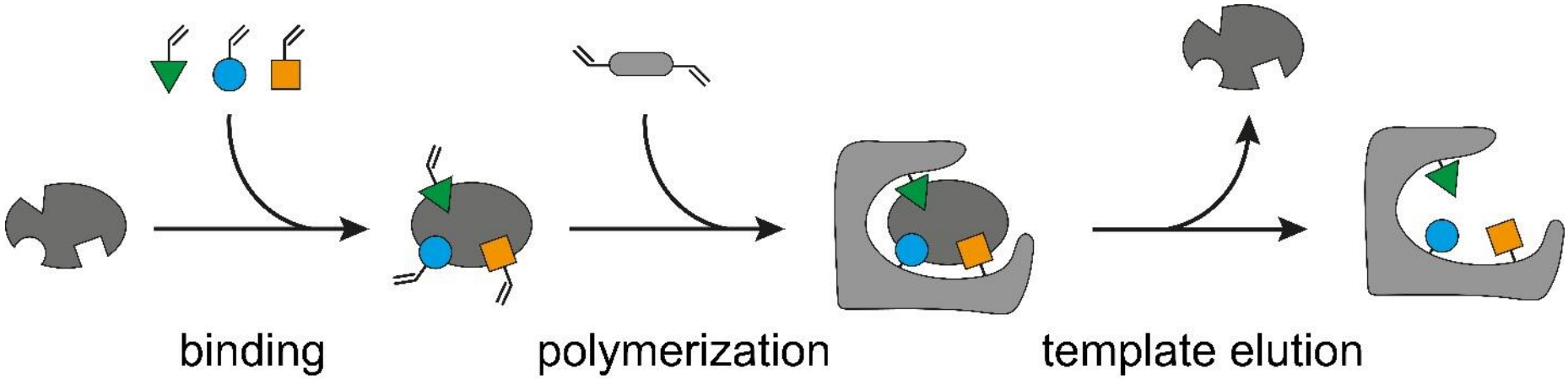


Aptamers: Assay designs



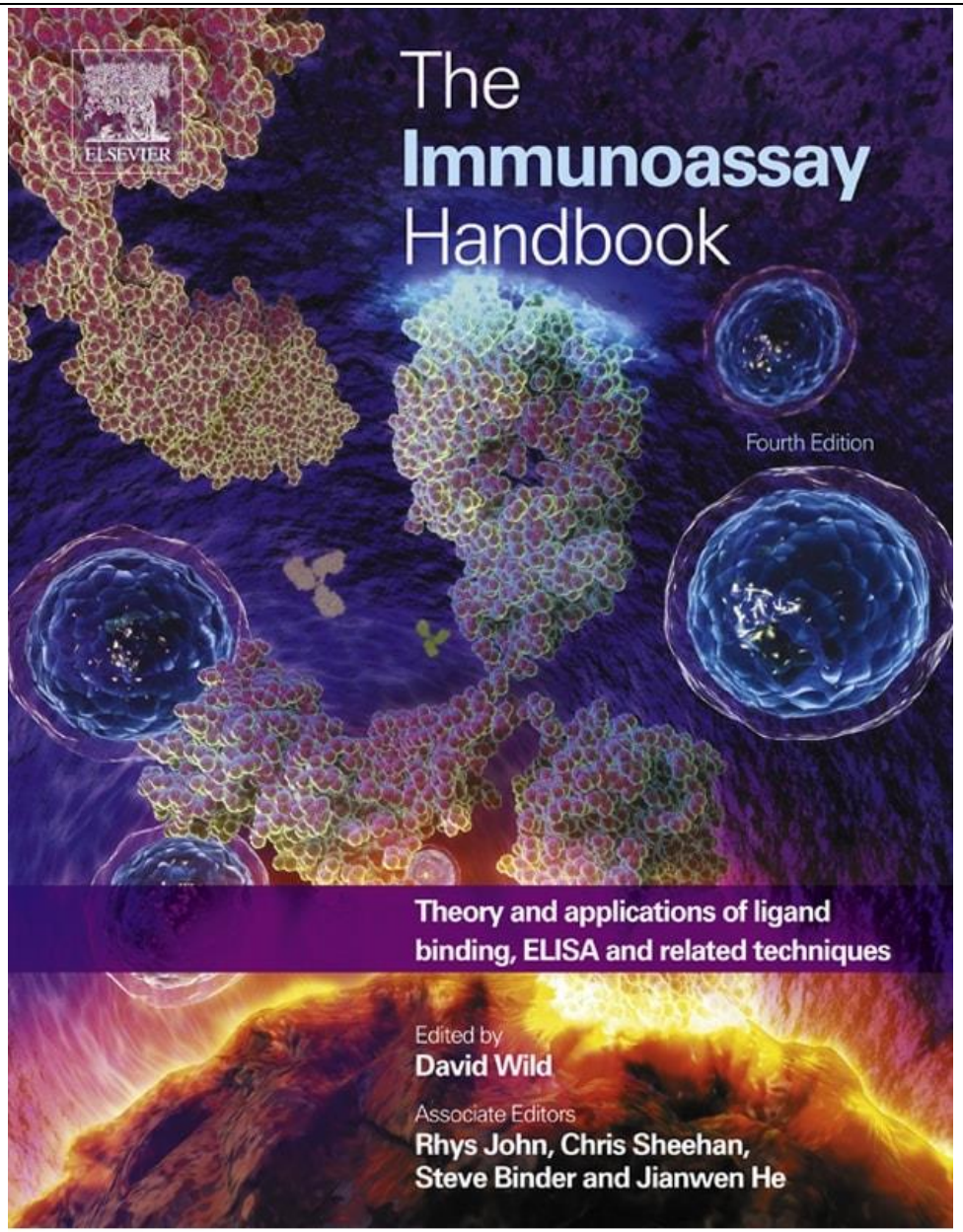
Molecularly imprinted polymer (MIP)

“Plastic antibodies“

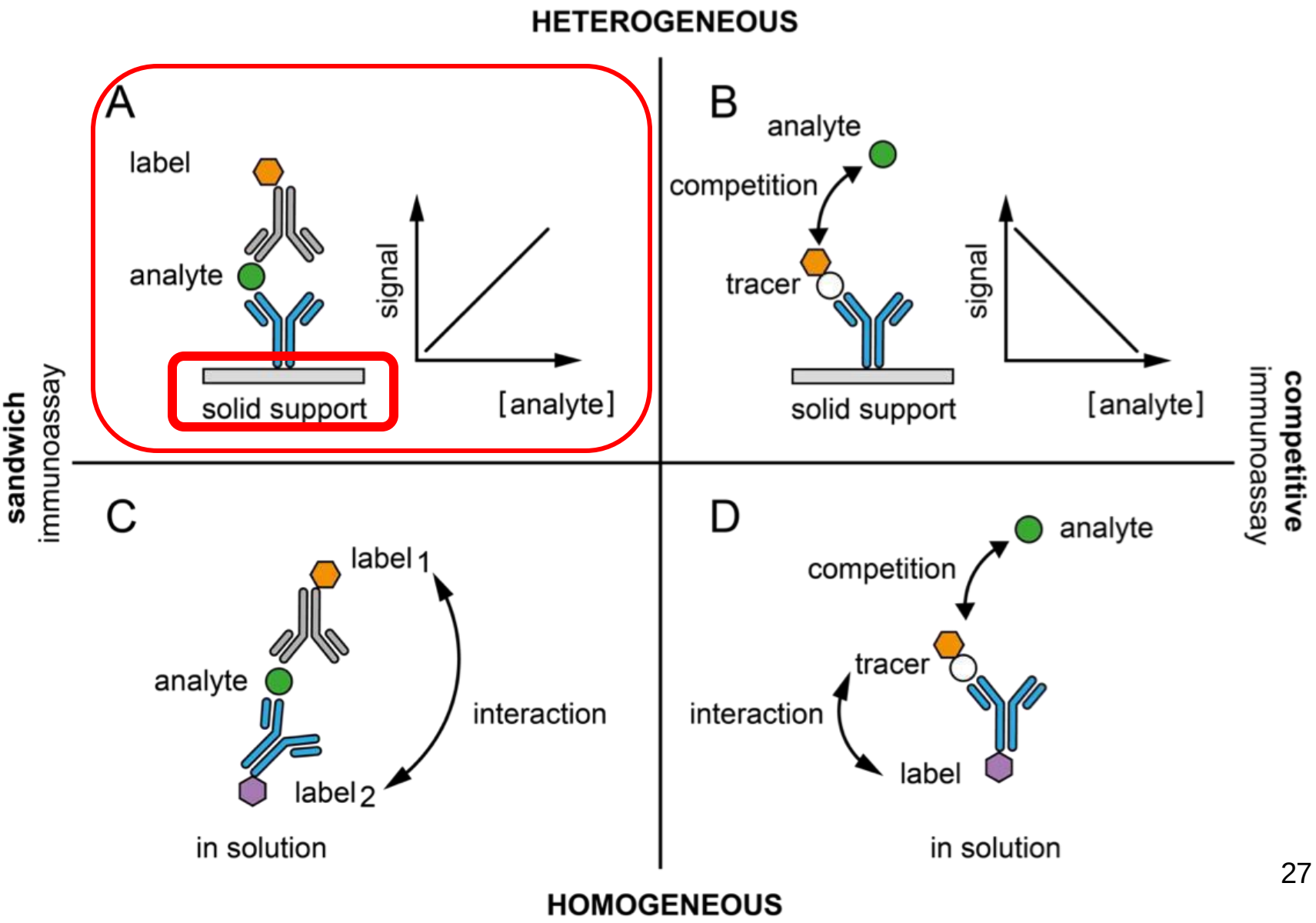


Immunoassays

Literature for in-depth reading



A rough categorization of immunoassays



Solid phase matrix

in heterogeneous non-competitive sandwich immunoassays

Performance-related issues:

1) low background in detection system
2) immobilization qualities:
   - high capacity
   - suitable and easy coupling chemistries
   - large surface
   - maintained reactivity of capture protein
   - no leakage
3) easy handling
4) inert in binding the labelled antibody/analyte => low background
5) effectively washed => low background
6) antibody excess through **high density** - surface measurement
7) antibody excess through **large surface** - integrating measurement

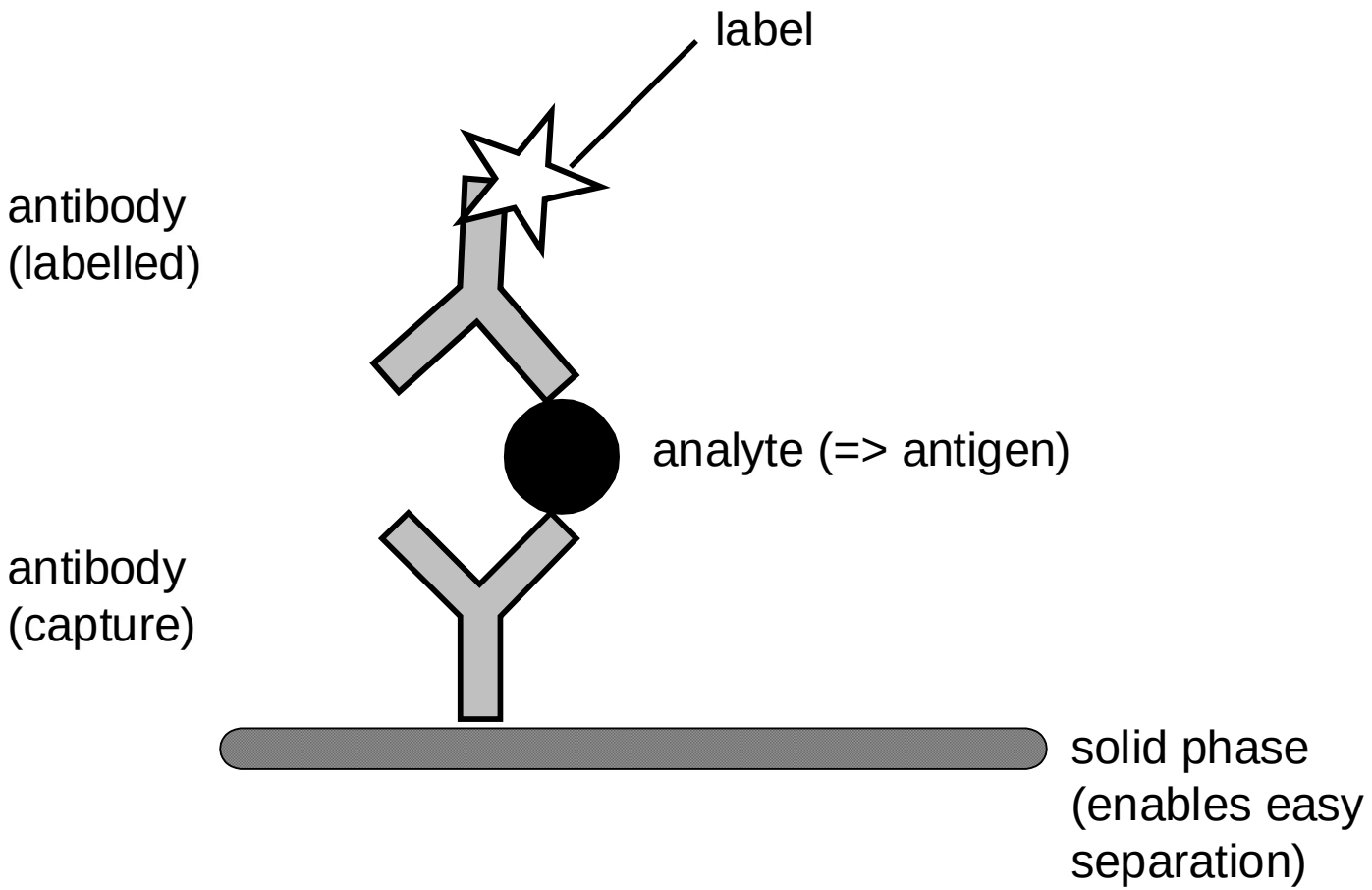
Solid phase matrices

Size	Examples	Advantages	Disadvantages
Small particle / "beads" (< 20 µm)	Latex Microcrystalline cellulose Fine porous glass Magnetic beads Liposomes Starburst™ dendrimers	Dispensing as for liquids Agitation not required High antibody binding capacity	Centrifugation required (unless used with a membrane capture) Long magnetic precipitation
Medium particle (< 1 mm)	Sepharose beads Sephacryl beads Sephadex beads	Centrifugation not required Short magnetic separation	Agitation required Slower binding kinetics than above Moderate antibody binding capacity
Single particle	Polystyrene	Centrifugation not required Agitation not required	Some variability in antibody coupling Lower antibody binding capacity Difficulty in dispensing Poor binding kinetics
Fibers	Membranes Glass fibers Nylon Silicon rubber	Centrifugation not required Agitation not required No dispensing of reagent Simple to use	Medium antibody binding capacity Can be fast binding kinetics
Solid surface	Coated tubes Dipsticks Microtiter plates (MTP)	Centrifugation not required Agitation rare No dispensing of reagent Simple to use	Variability in antibody coupling Lowest antibody binding capacity Slowest binding kinetics

Most frequently used solid phase matrices

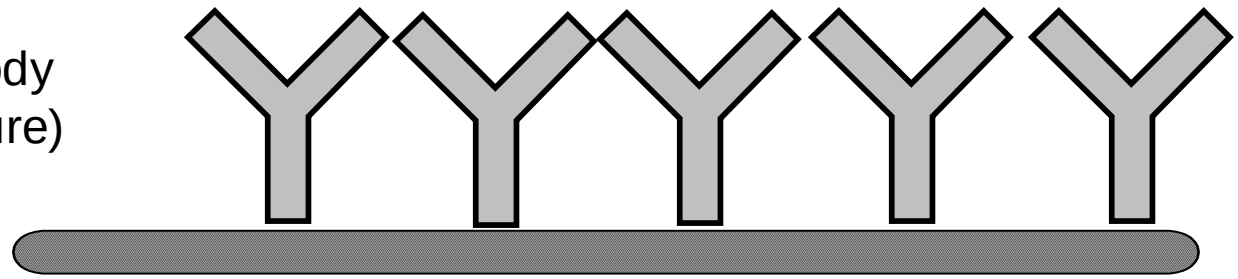
Non-competitive immunoassay

("sandwich" immunoassay)



Non-competitive immunoassay

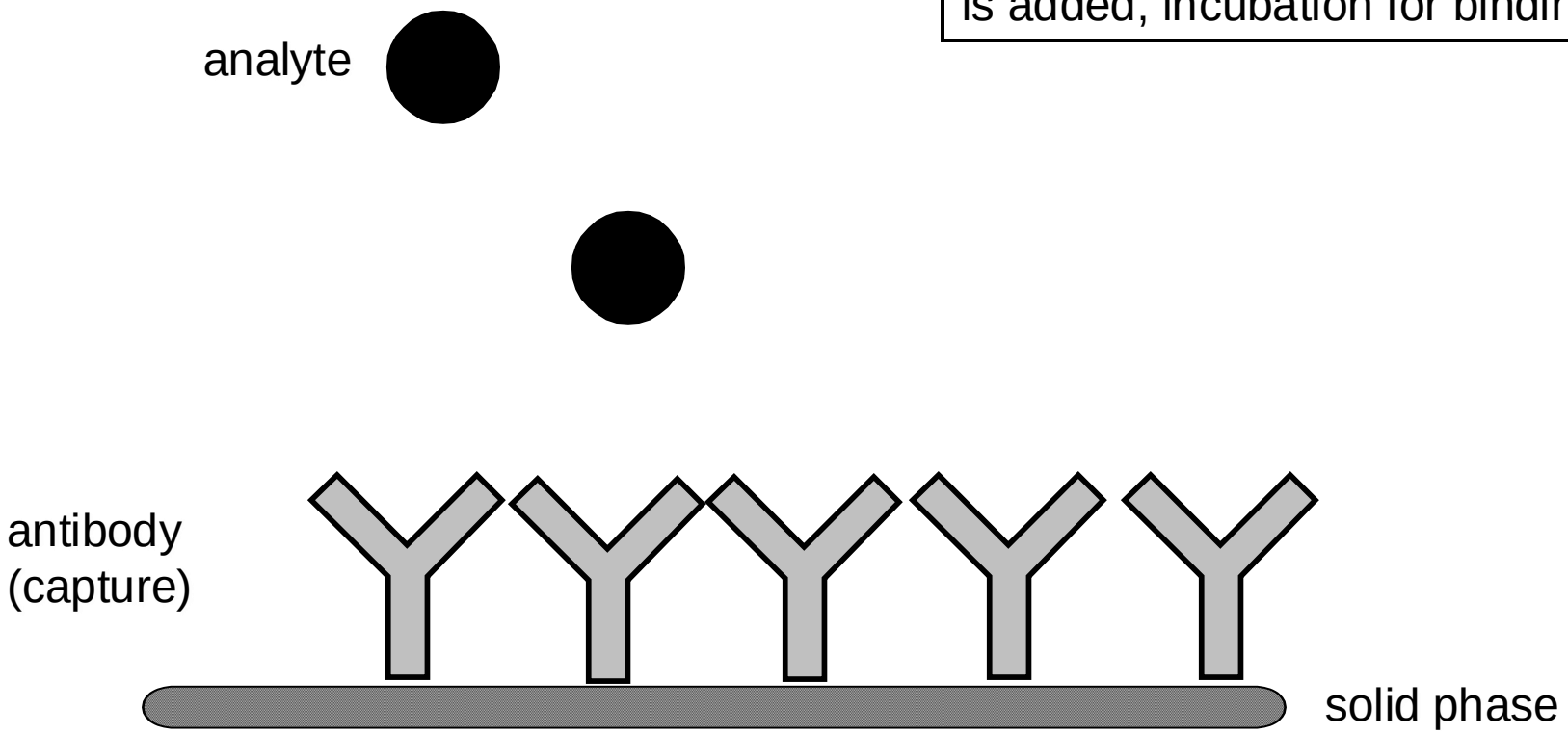
a capture antibody specific for a single epitope of the analyte is coated on a solid phase
(e.g. on a microtiter plate)
(=> monoclonal antibody preferred)



excess of binding sites

Non-competitive immunoassay

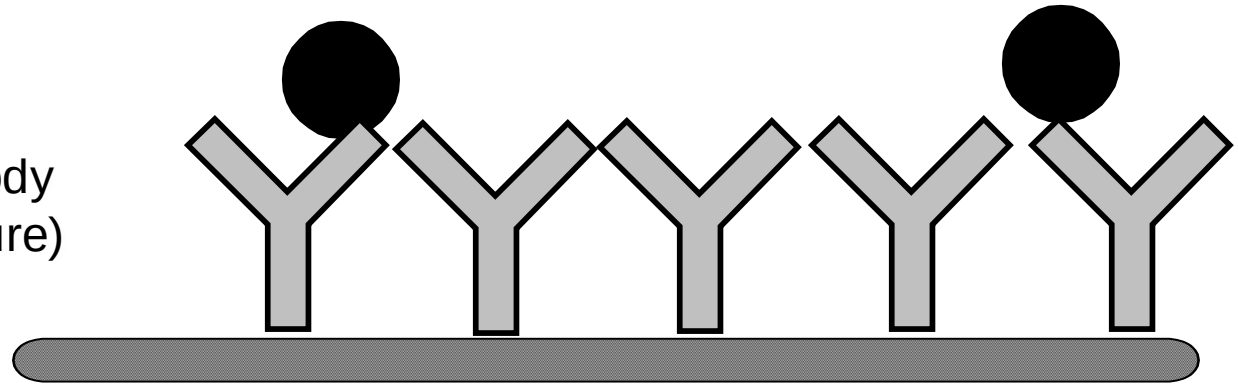
sample containing the analyte (at least two non-overlapping epitopes) is added; incubation for binding



excess of binding sites

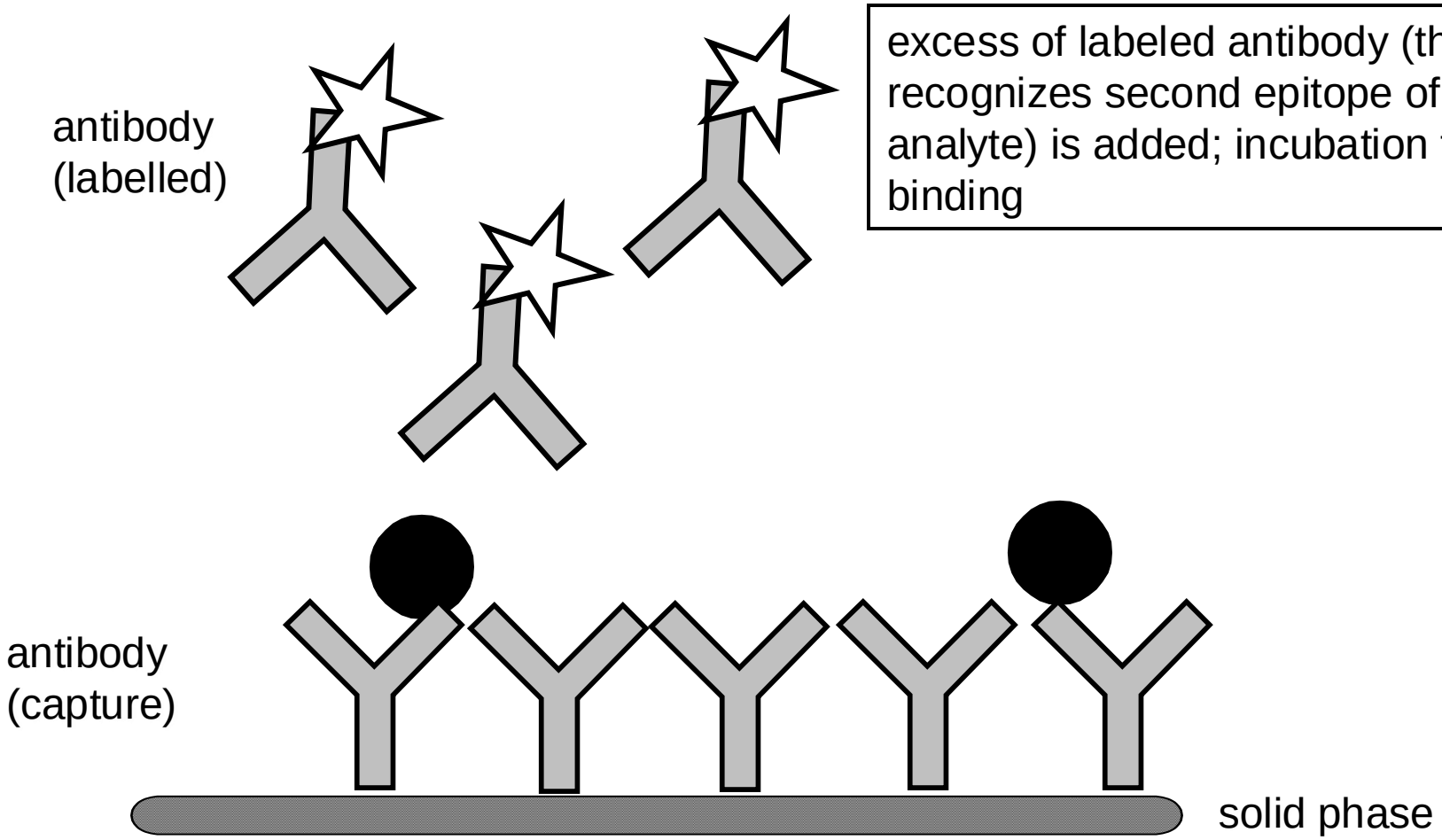
Non-competitive immunoassay

analyte is bound; in two-step assay: sample is washed away with excess of analyte



excess of binding sites

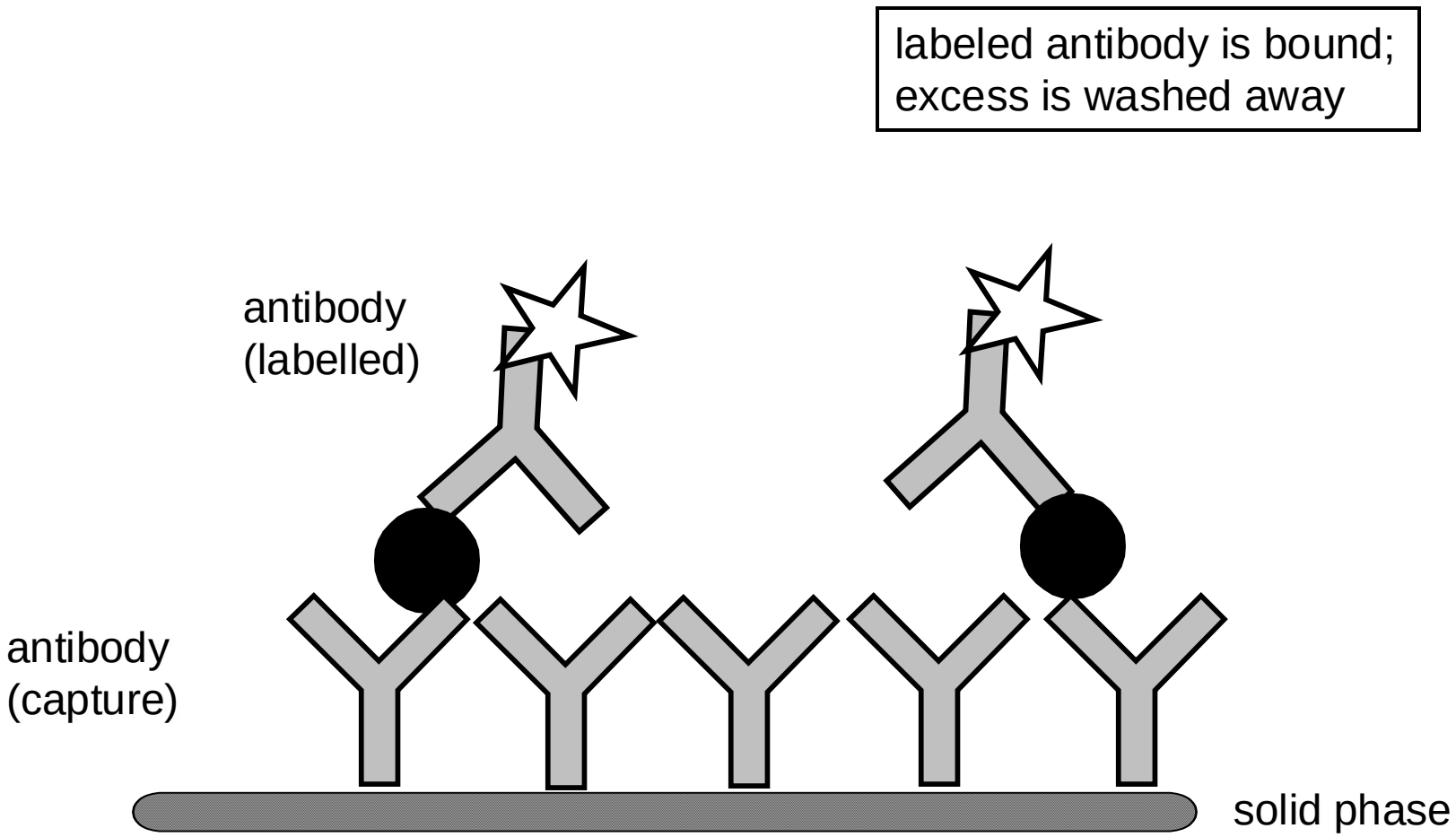
Non-competitive immunoassay



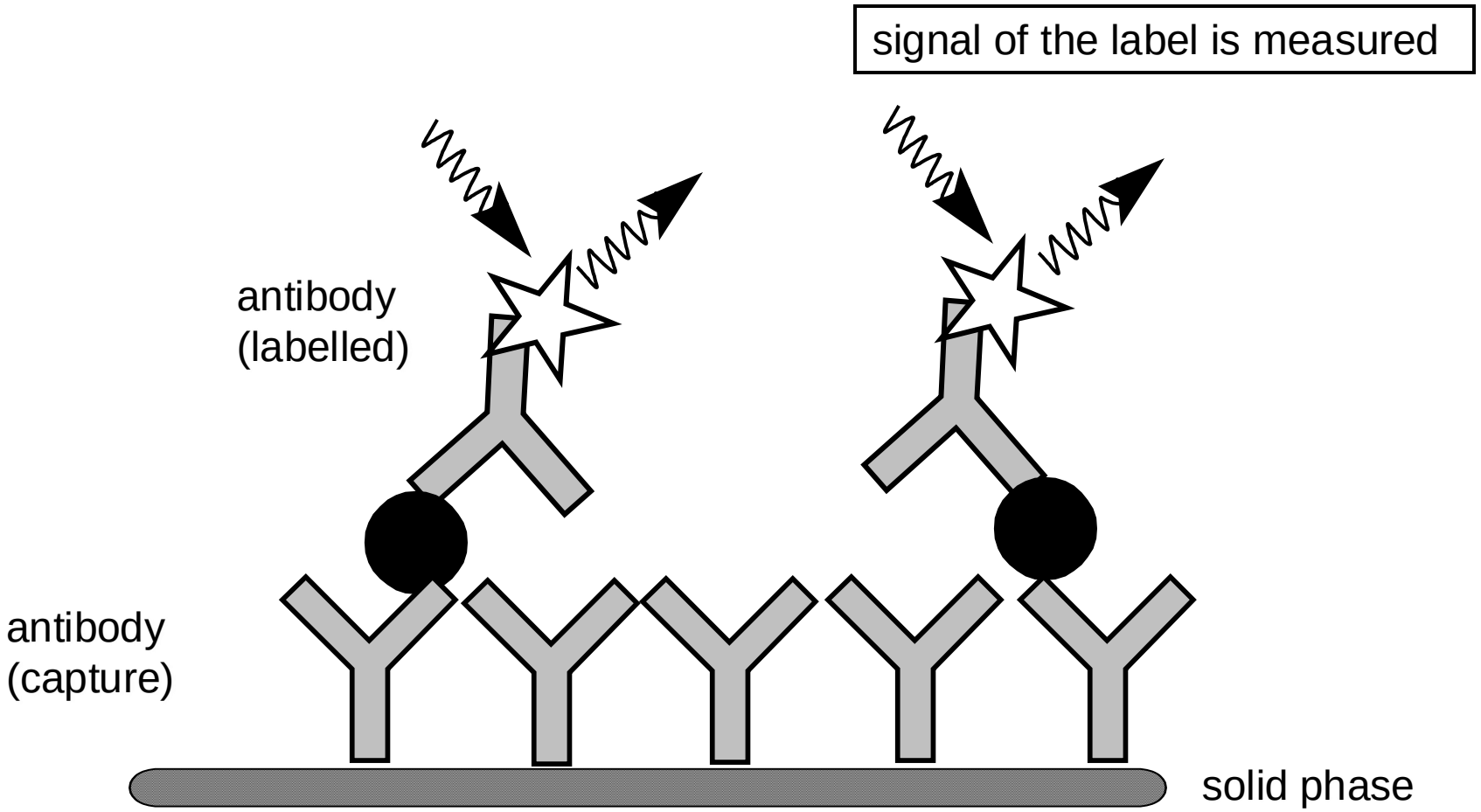
excess of labeled antibody (that recognizes second epitope of the analyte) is added; incubation for binding

excess of binding sites

Non-competitive immunoassay

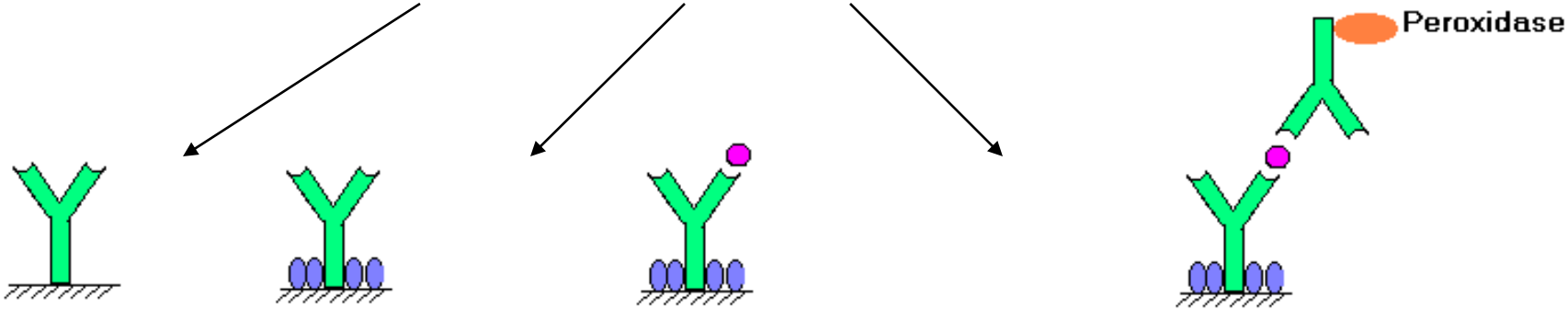


Non-competitive immunoassay



Enzyme-linked immunosorbant assay (ELISA)

Thorough washing steps required



A. Coating anti-IgG

Non-covalent absorption of capture antibody to polystyrene surface (microtiter plate)

B. Blocking

Block surface with BSA or detergents to prevent non-specific binding of other proteins

C. React sample antigen

Add sample that contains the antigen (the analyte), e.g. tumor markers, viruses, or antibodies in serum.

D. React POx labelled secondary antibody

1. Add enzyme-labeled detection antibody (e.g. horseradish peroxidase); wash
2. Add chromogenic reagent (e.g. TMB)
3. Add "stop solution" (e.g. H_2SO_4)

Blocking is essential to avoid non-spec. binding

Normal serum
Normal serum (1-5% w/v) carries antibodies that bind to reactive sites and prevent non-specific binding of the secondary antibody. Serum is rich in albumin and other proteins that readily bind to non-specific protein binding sites of the sample.

Protein solutions
Blocking buffers often contain proteins such as bovine serum albumin (BSA), gelatin or nonfat dry milk (1-5% w/v). These inexpensive and readily available proteins are present in large excess compared to the antibody, so they compete with the latter for binding to nonspecific sites in the sample. Many labs developed homemade blocking buffers. It is important that blocking buffers are free of precipitates and other contaminants that can interfere with the detection.

Commercial buffers
Ready-made blocking buffers can contain highly purified single proteins or proprietary protein-free compounds. Many options are available that perform better than gelatin, casein or other proteins used alone, and they have improved shelf lives compared to homemade preparations.

Blocking tips

- Monitor both background (negative control) and signal strength (positive control) with various blocking reagents.
- Choose the blocking buffer that yields the highest signal-to-noise ratio.
- Ensure that there are no substances in the blocking buffer that interfere with a particular assay. Non-fat dry milk, for example, contains biotin and is inappropriate for use with any detection system that includes a biotin-binding protein.
- For optimal assay conditions, use the same blocking buffer for diluting the antibody that is used for the blocking step.

Enzyme-linked immunosorbant assay (ELISA)

Enzyme	Properties
peroxidase galactosidase	rarely found in biosamples, high activity
phosphatase glucose oxidase	rarely found in biosamples, moderate activity
catalase	high activity but often present in samples
protease	low activity

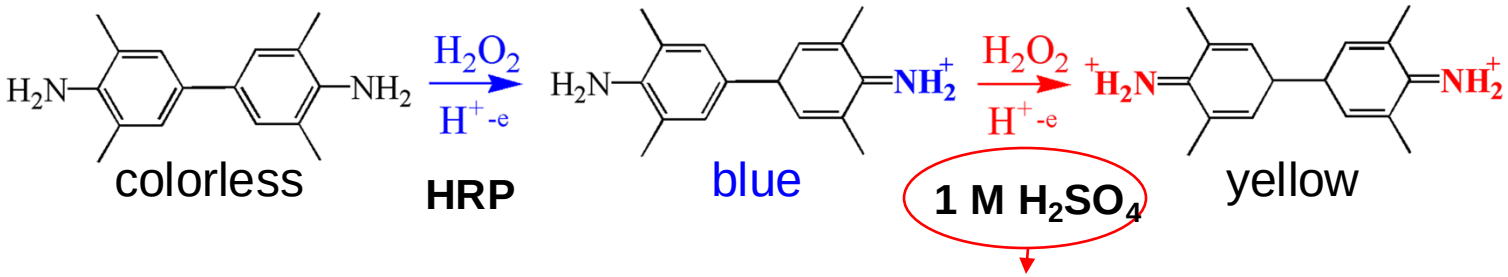
very common

less suitable

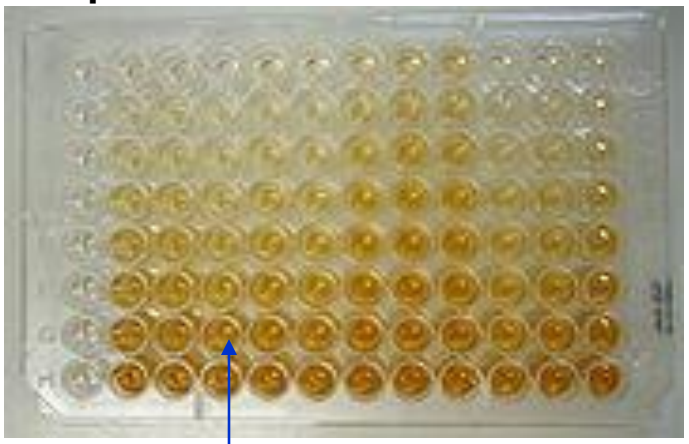
=> Effect: strong signal amplification
(one enzyme label generates 100 - 1000 chromophores / fluorophores per second!)

Enzyme-mediated signal generation

(a) a chromogenic substrate (3,3‘,5,5‘-Tetramethyl-benzidine (TMB))**:**

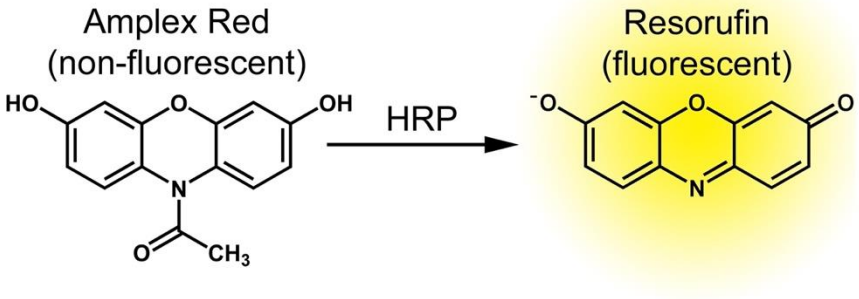


Stops the enzyme reaction:
Endpoint measurement

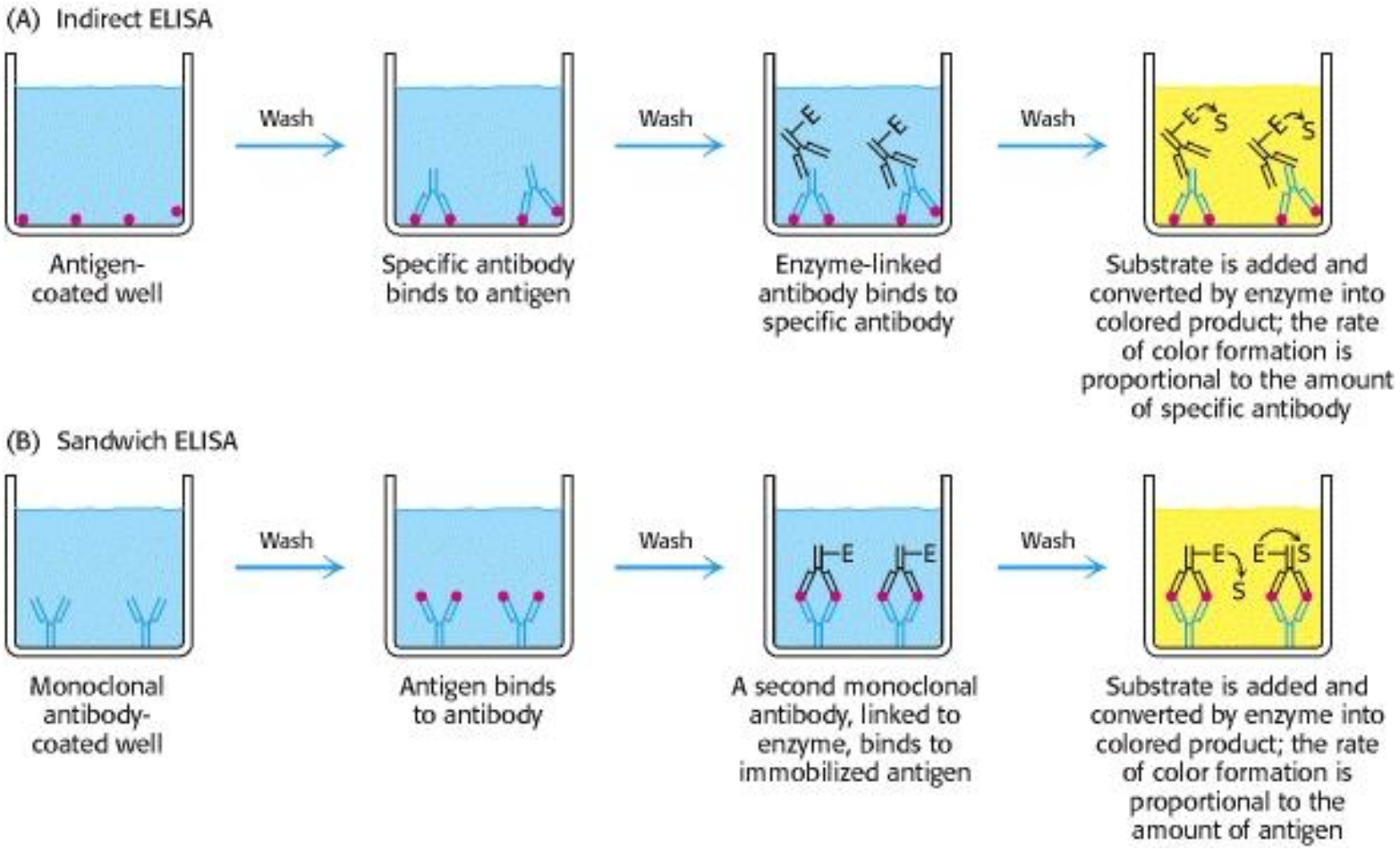


- Coloration depends on the amount of enzyme-labeled secondary Ab;
- microtiter plate reader; absorbance at 450 nm expressed as Optical Density (OD)

(b) a fluorogenic substrate:

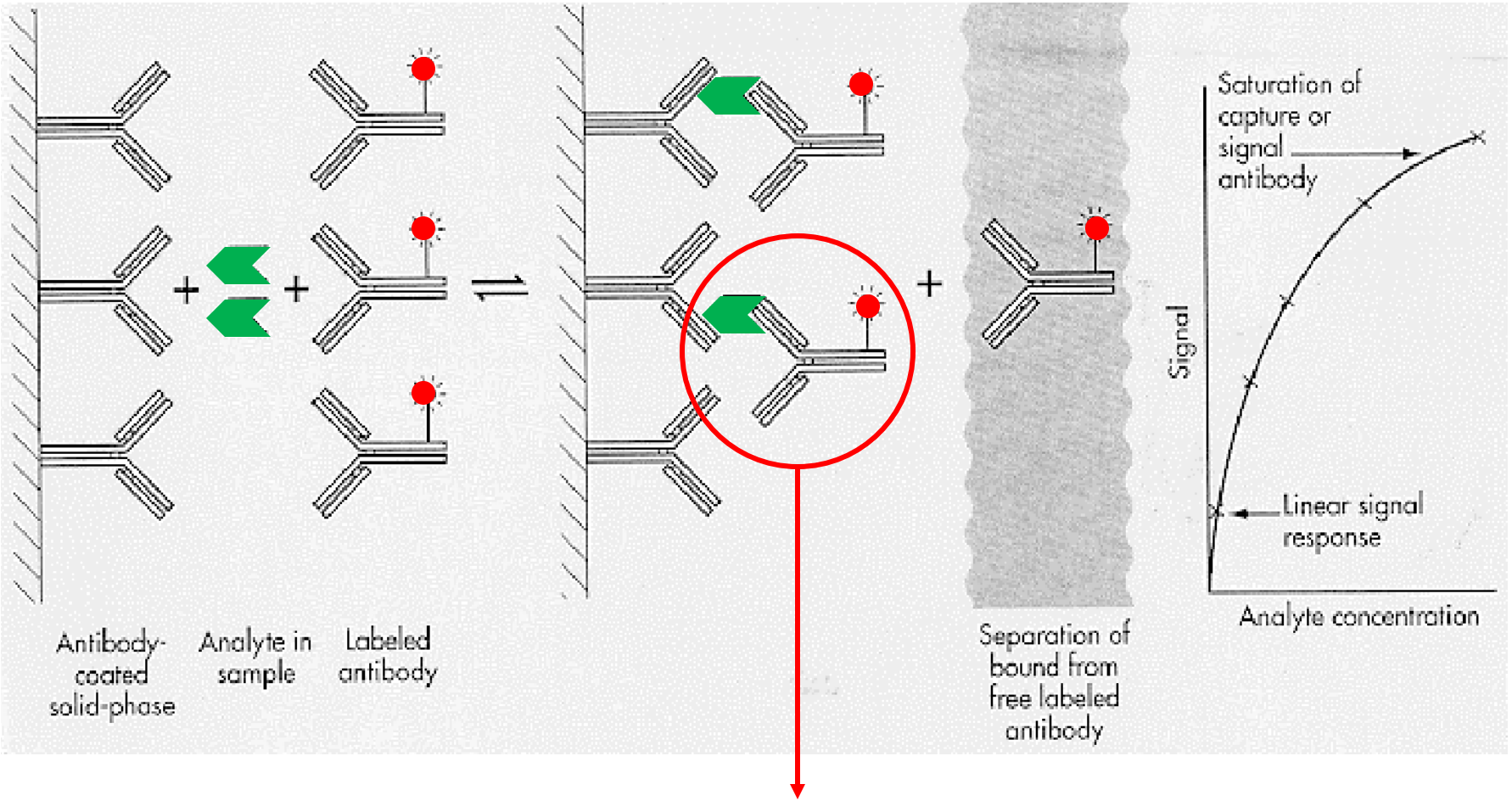


Alternative non-competitive ELISA formats



Immunometric assay

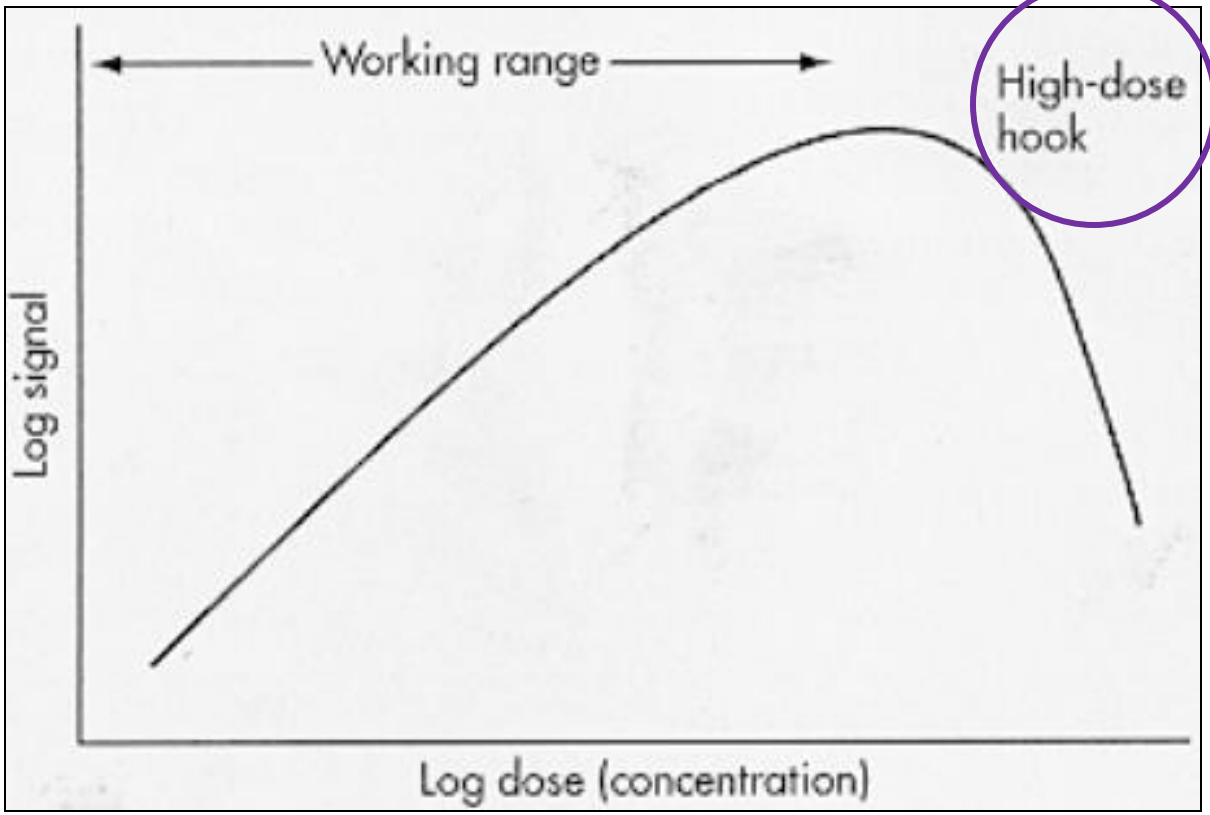
one-step assay



=> analyte is detected directly,
i.e. signal from immune complexes containing analyte

Immunometric assay

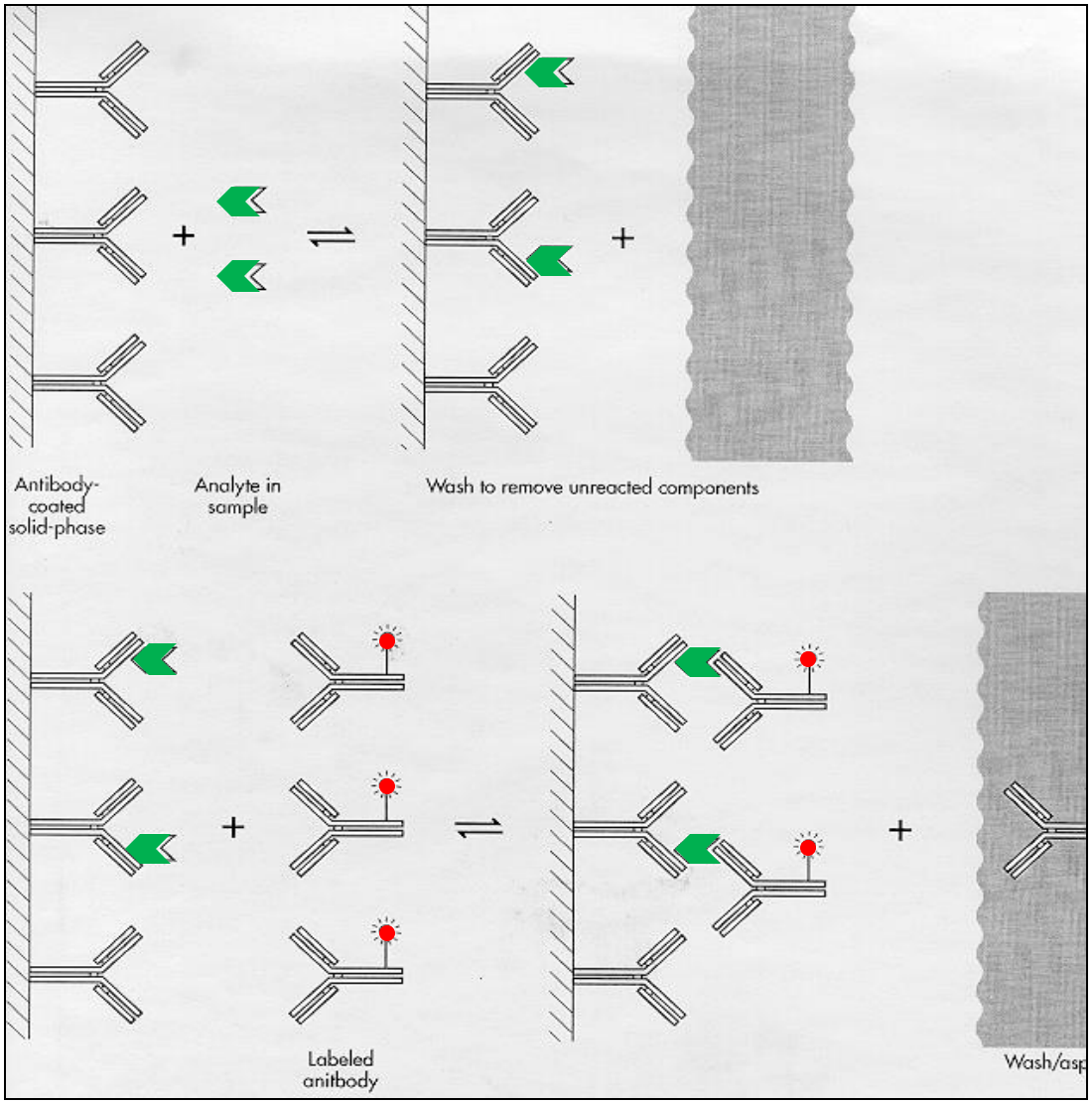
one-step assay



When capture antibody becomes saturated, free analyte in solution binds to the detection antibody and prevents it from binding to the antigen on the solid phase

Immunometric assay

two-step assay



=> avoids **high dose hook effect**

ELISA: data analysis

An-Ab binding:

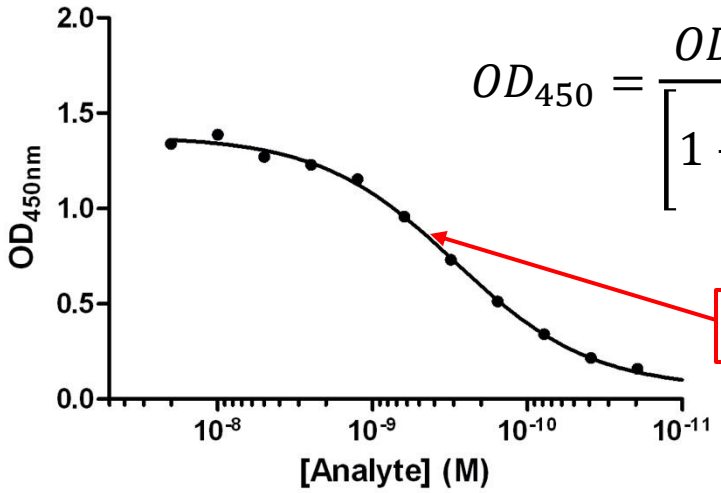
$$[An] + [Ab] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [AnAb]$$

$$K = \frac{[AnAb]}{[An][Ab]}$$

Surface-bound immune complex

4-parameter logistic function

$$OD_{450} = \frac{OD_{max} - bg}{\left[1 + \left(\frac{[An]}{C_{50}}\right)^{s}\right]}$$

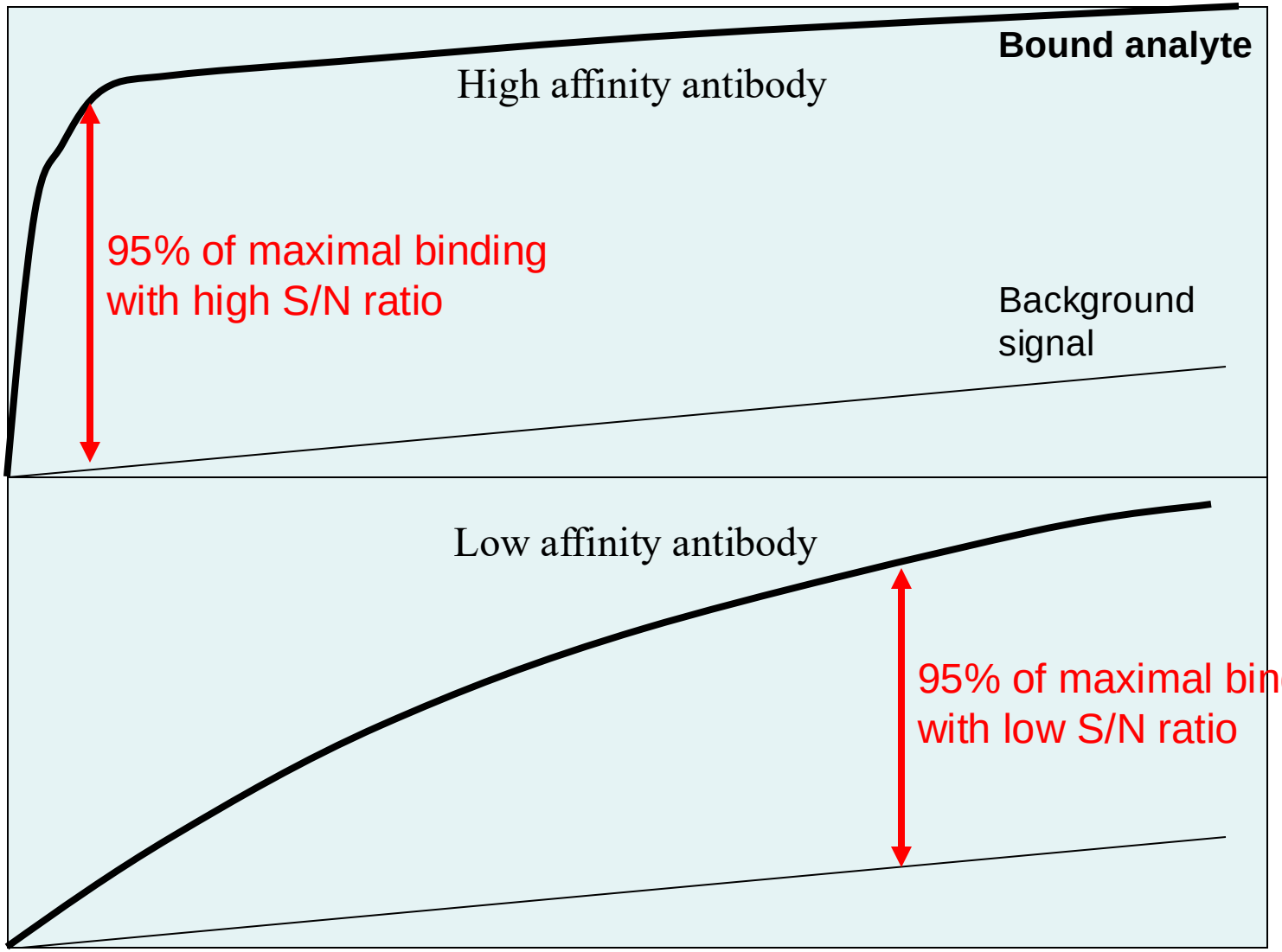


Variables: "optical density" (*OD = absorbance)* and [*An*]

Fitted parameters:

- OD_{max} (signal at saturation)
- *bg* (background signal)
- C_{50} (midrange concentration)
- *s* (slope)

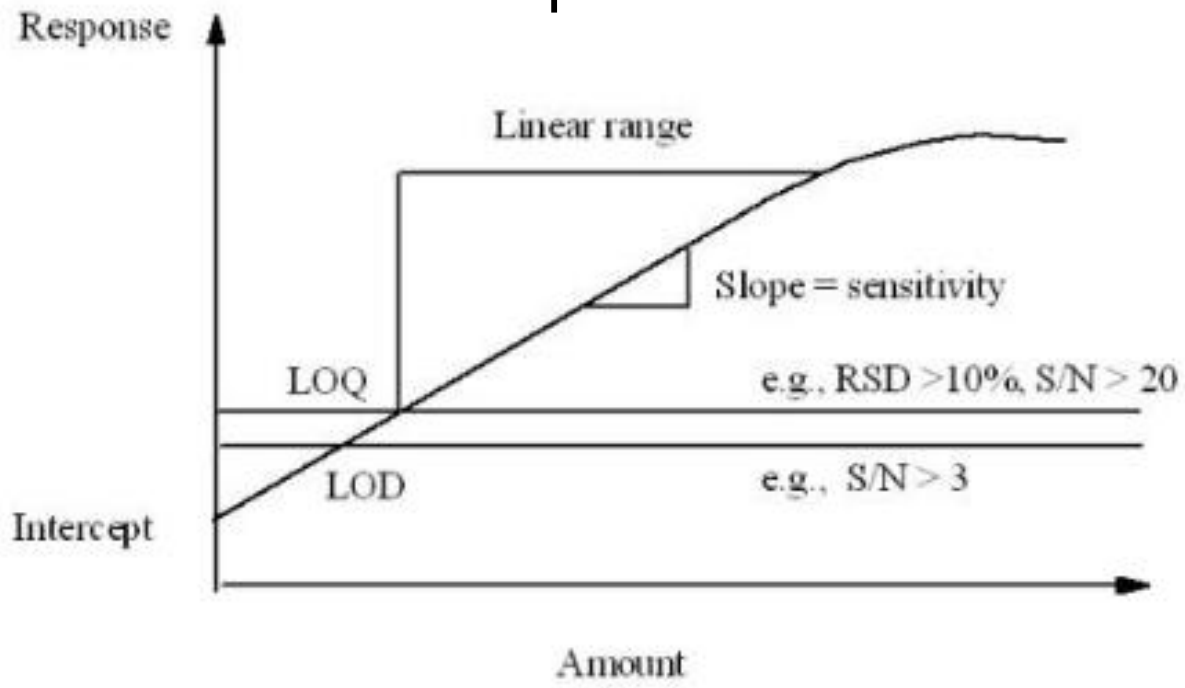
Detection limit of non-competitive assay



[labeled detection Ab]

Immunometric assay

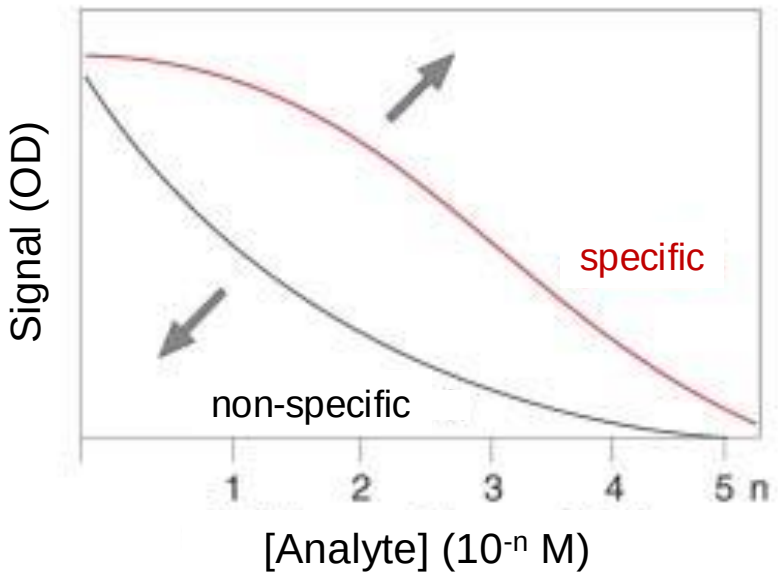
dose-response curve



Limit of detection (LoD) vs. limit of quantification (LoQ)	
LoD	**LoQ**
The smallest concentration of an analyte in a test sample that we can easily distinguish from zero	The smallest concentration of an analyte in a test sample that we can determine with acceptable repeatability and accuracy

Optimization of immunoassays

Non-optimized assay



Optimized assay