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C8116

Antibodies as immunochemical tools

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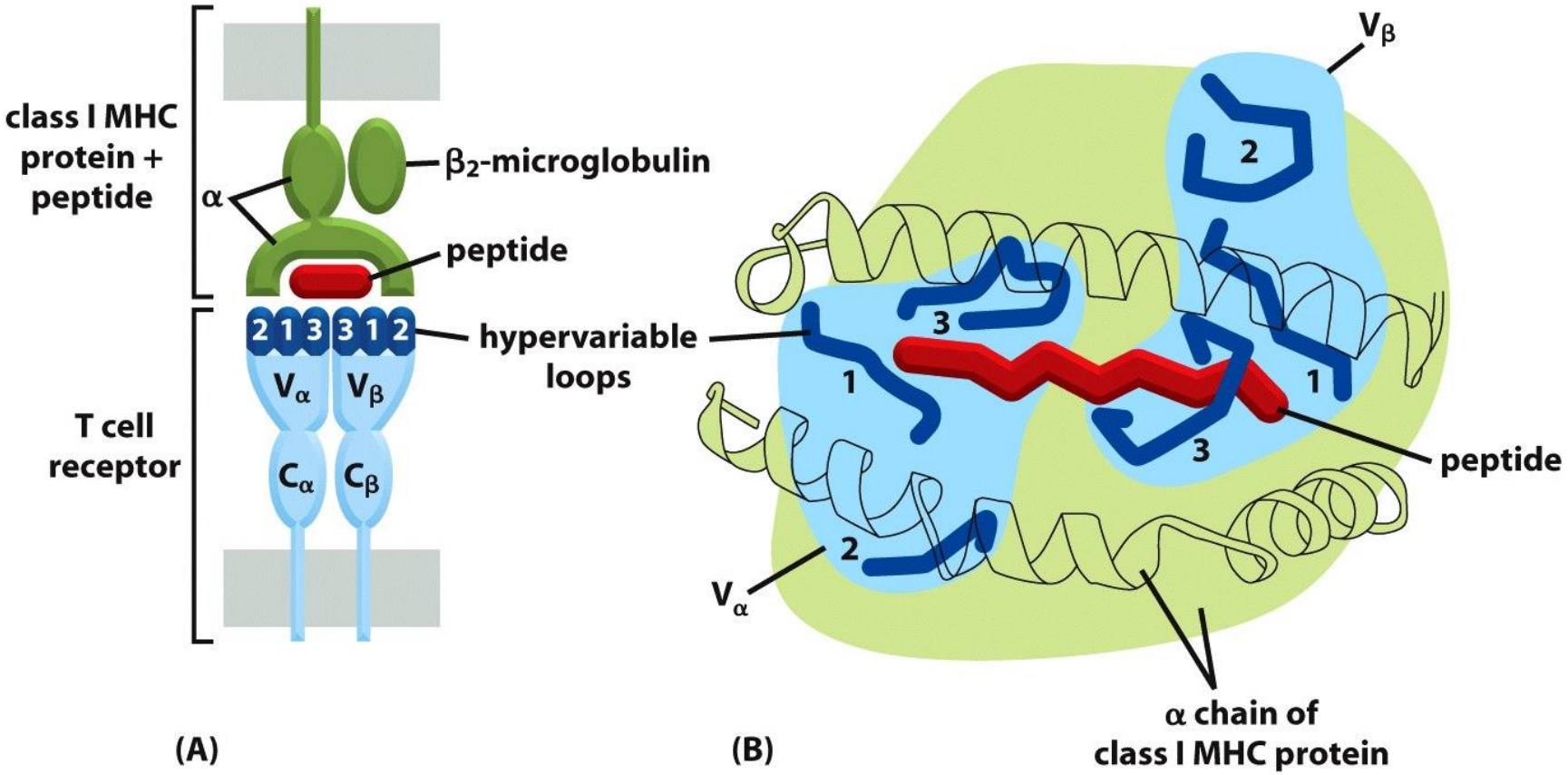
Large diversity in the recognition of antigens

BCR and antibodies: gene rearrangement + somatic hypermutation
=> Each individual can recognize any hapten/epitop
(linear *and* conformational epitopes)

TCR: gene rearrangement
=> Each individual can recognize any linear peptide in context with MHC molecule

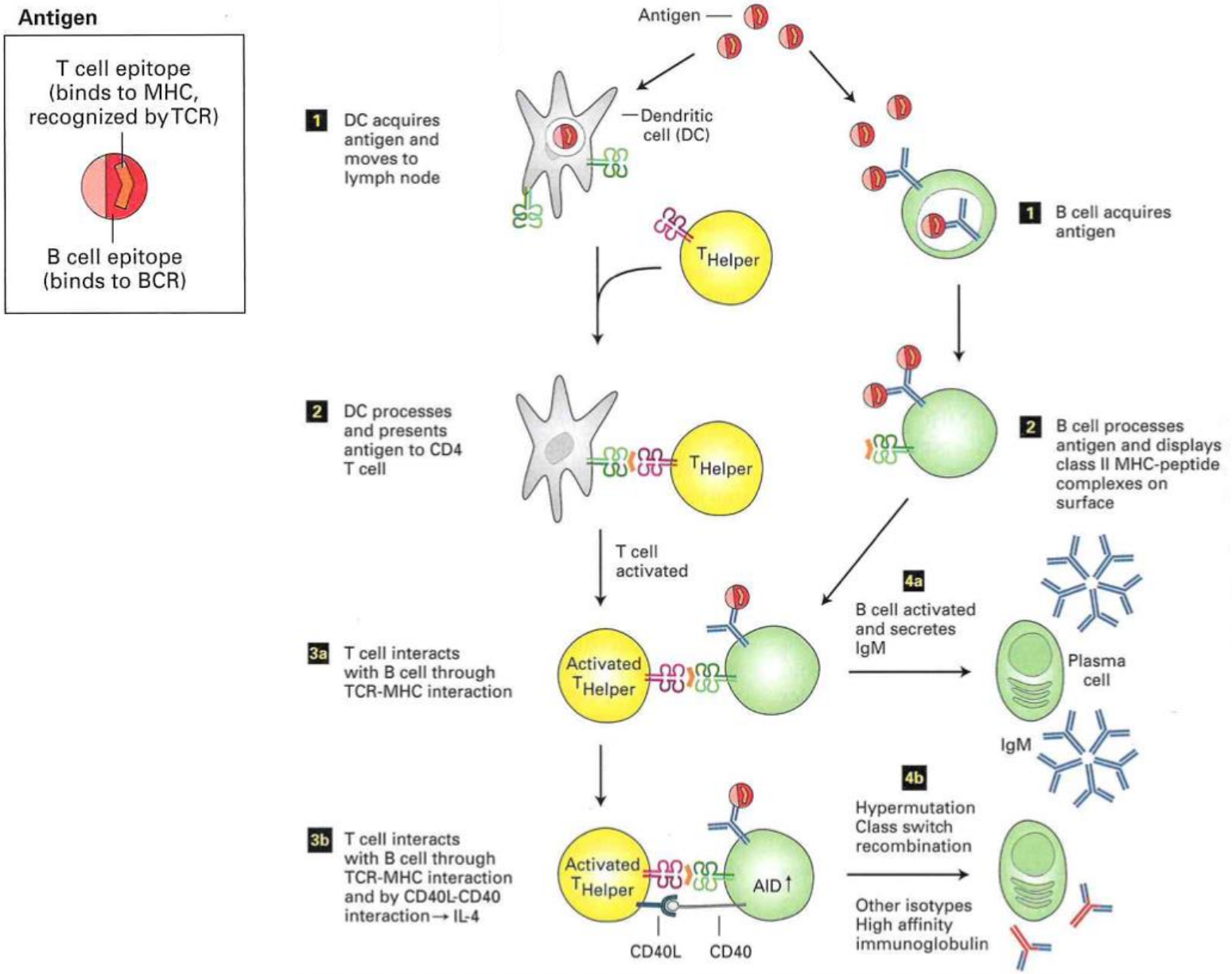
MHC: no gene rearrangement but 3 genes and several thousand alleles in a population
=> Can bind a large variety of peptides (but not all)
=> a whole population is well protected but there is an individual risk of missing some pathogenic peptides
=> populations with a large gene pool are more resistant to an epidemic

Interaction of TCR with a peptide on MHC class I



=> Only linear peptide epitopes

Summary of interplay between T_H and B cells

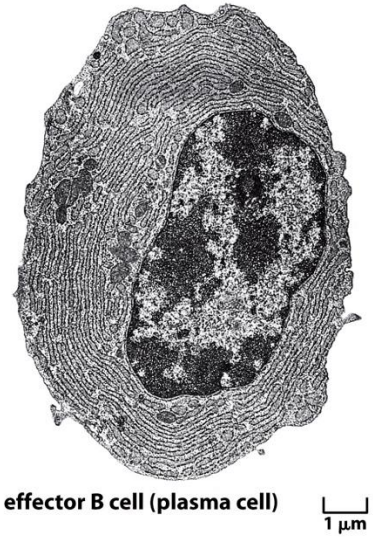


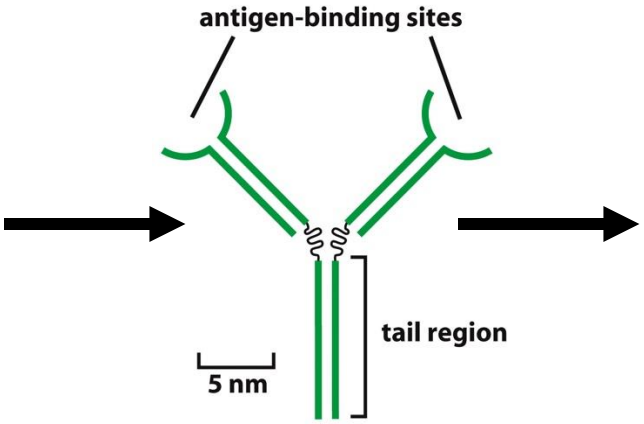
Antibodies as immunochemical tools

Immunology

The “tools“: antibodies

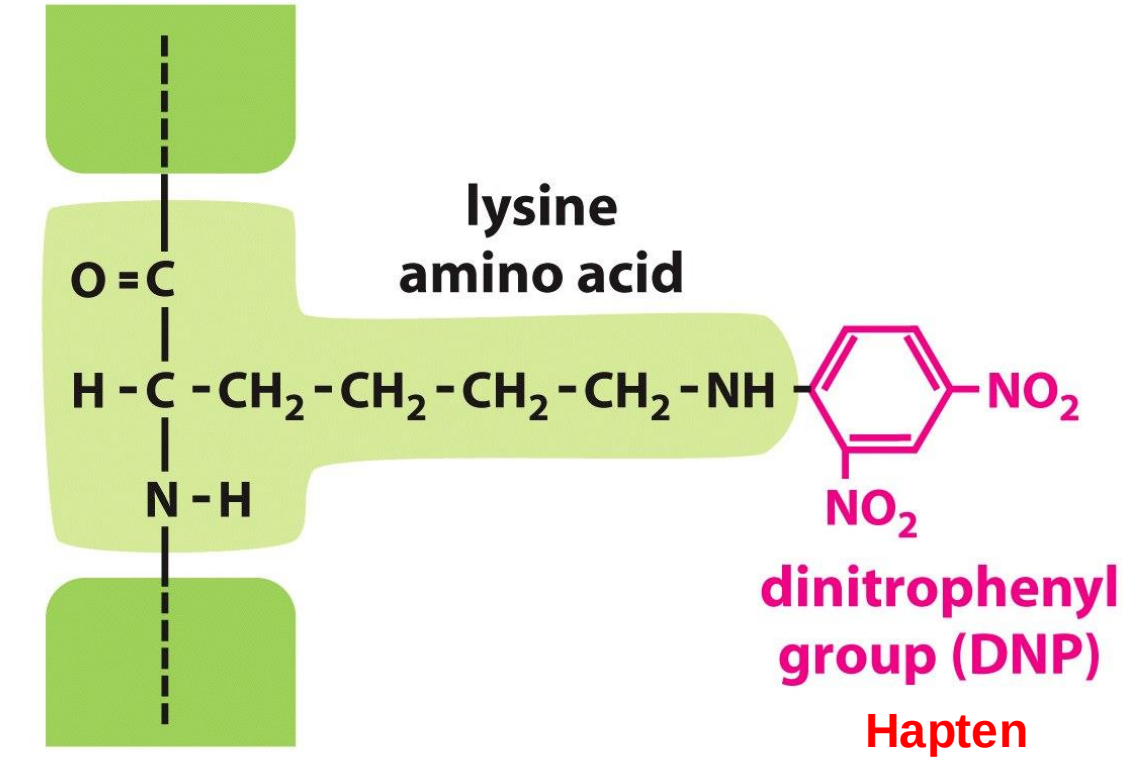
Immunoassay





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**.



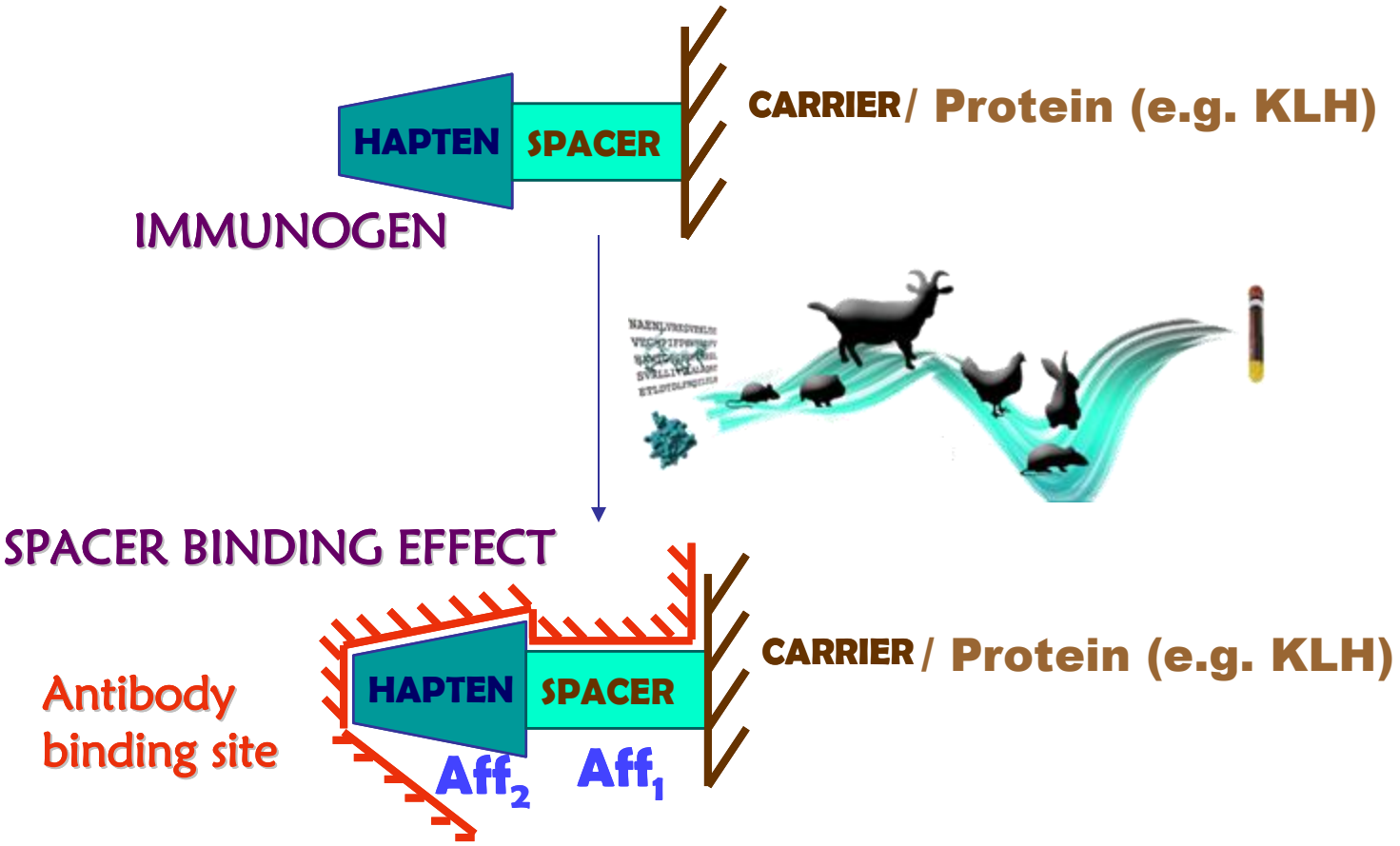
Definition of hapten:
A low-molecular weight molecule which contains an antigenic determinant but which is not itself antigenic unless bound to an antigenic carrier

Other examples:
toxins, pharmaceticals, hormones…

=> Why do we need a carrier protein to launch an immune response against DNP?

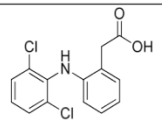
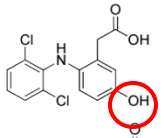
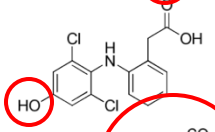
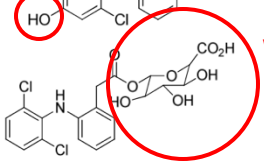
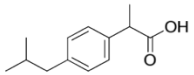
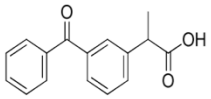
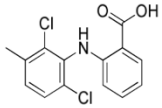
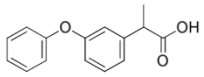
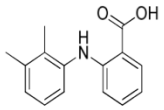
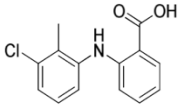
Antigenic determinants: hapten

Immunogens are always antigens but not all antigens are immunogens



Cross reactivity (CR) in competitive assay

depends on antibody clone

Compound		6D8	8B1	10C9	12G5
		CR (in %)			
DCF		100	100	100	100
5-OH-DCF		3.5	9.6	10	13
4'-OH-DCF		6.2	1.7	5.3	11
DCF-GLU		24	14	8.8	8.5
Ibuprofen		< 0.42	< 0.10	< 0.43	< 0.0069
Ketoprofen		< 0.42	< 0.10	< 0.43	< 0.069
Meclofenamic acid		< 0.42	< 0.10	< 0.43	0.35
Fenoprofen		< 0.25	< 0.06	< 0.43	< 0.25
Mefenamic acid		2.4	0.74	< 0.43	0.55
Tolfenamic acid		3.5	4.0	17	0.85

why?

Similar chemical structures

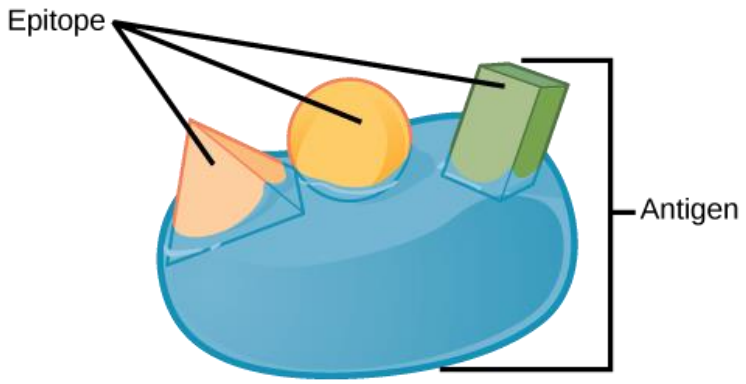
Other painkillers

Some definitions

Antibodies, or immunoglobulins (Igs), are γ-globulin proteins folded into well defined three-dimensional structures synthesized by living organisms, e.g. mice, rabbits or goats, or by living cells, in response to the presence of a foreign substance known as the antigen.

Immunogen: Molecule that is capable of eliciting an immune response by the immune system of an organism.

Antigen: Molecule that is able to bind to the product of that immune response: the antibody.



Epitope: An epitope is a specific location on the surface of an antigen that has a particular molecular structure and that is recognized by a particular antibody or a set of specific antibodies that the epitope elicits during the immune response.

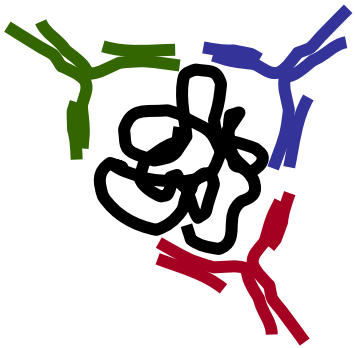
Hapten: Small molecules (< 5000 Dalton) that need to be conjugated to a carrier protein (e.g. bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) or ovalbumin) to elicit the immune response.

Polyclonal vs. monoclonal antibodies

polyclonal

Antibodies that are collected from sera of exposed animal

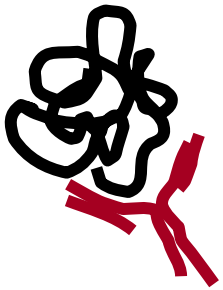
recognize
multiple antigenic sites
of injected substance



monoclonal

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

recognize
ONE antigenic site
of injected substance



Polyclonal antibodies (Pab, antiserum)

- **Antibody production:**

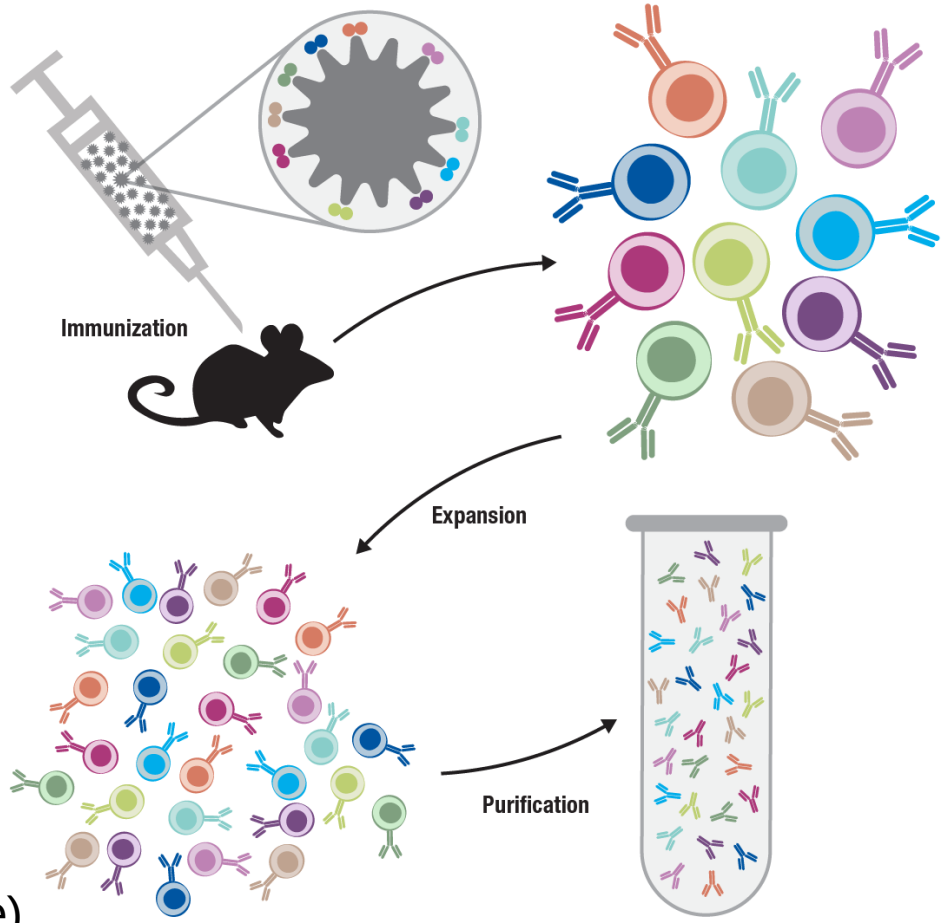
  quick and inexpensive

- **Immunization procedure:**

- animal is immunized with pure **antigen** (immunogen) and with **adjuvant** (substance that strengthens the immune response)
- the immunization is repeated (boost)
- the animal's blood is collected

- **Antibody host species:**

- typically mouse/rabbit
- sheep/goat in case large antibody amounts are needed (for commercial use)



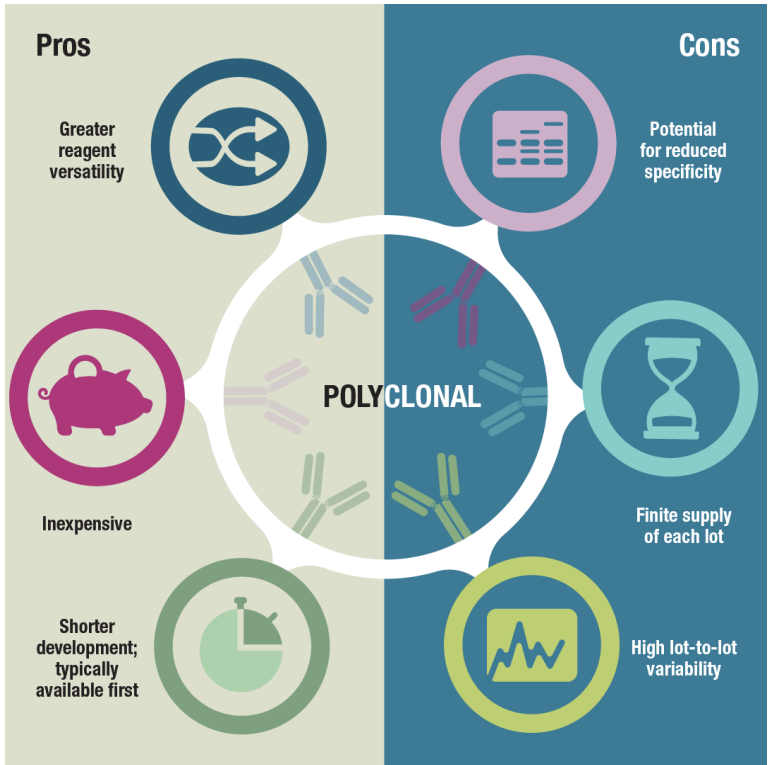
Polyclonal antibodies + and -

+

- Fast preparation
- Inexpensive
- Sometimes very high affinity which is difficult to obtain with monoclonal antibodies (e.g. anti-steroid antibodies)
- May be advantageous for the detection of very heterogenous antigens

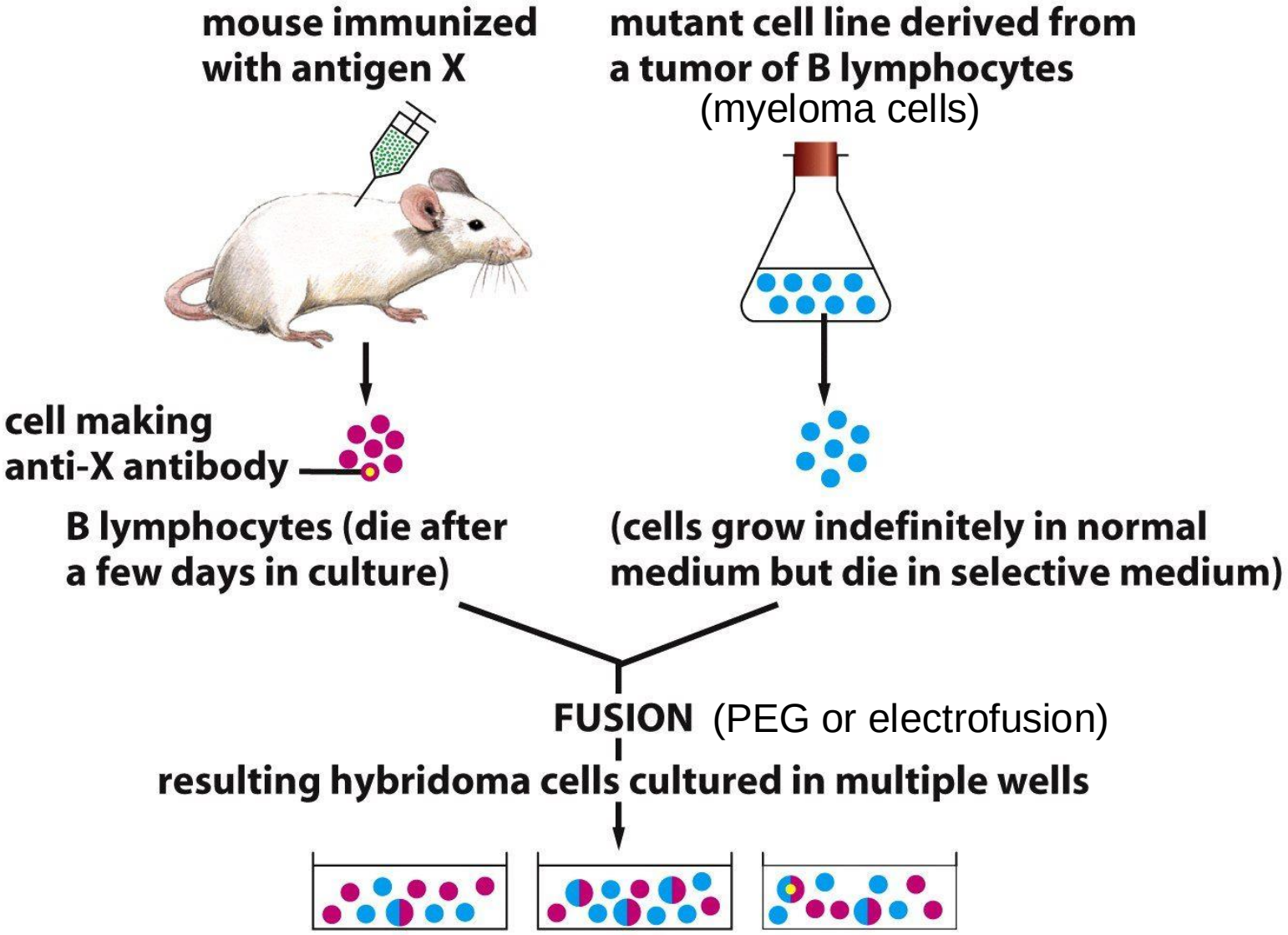
-

- Limited amounts (typically not sufficient for in excess reagent systems)
- Variations between batches
- Often lack full antigen specificity
- Cannot discriminate between closely related antigens
- Pure antigen required for immunization

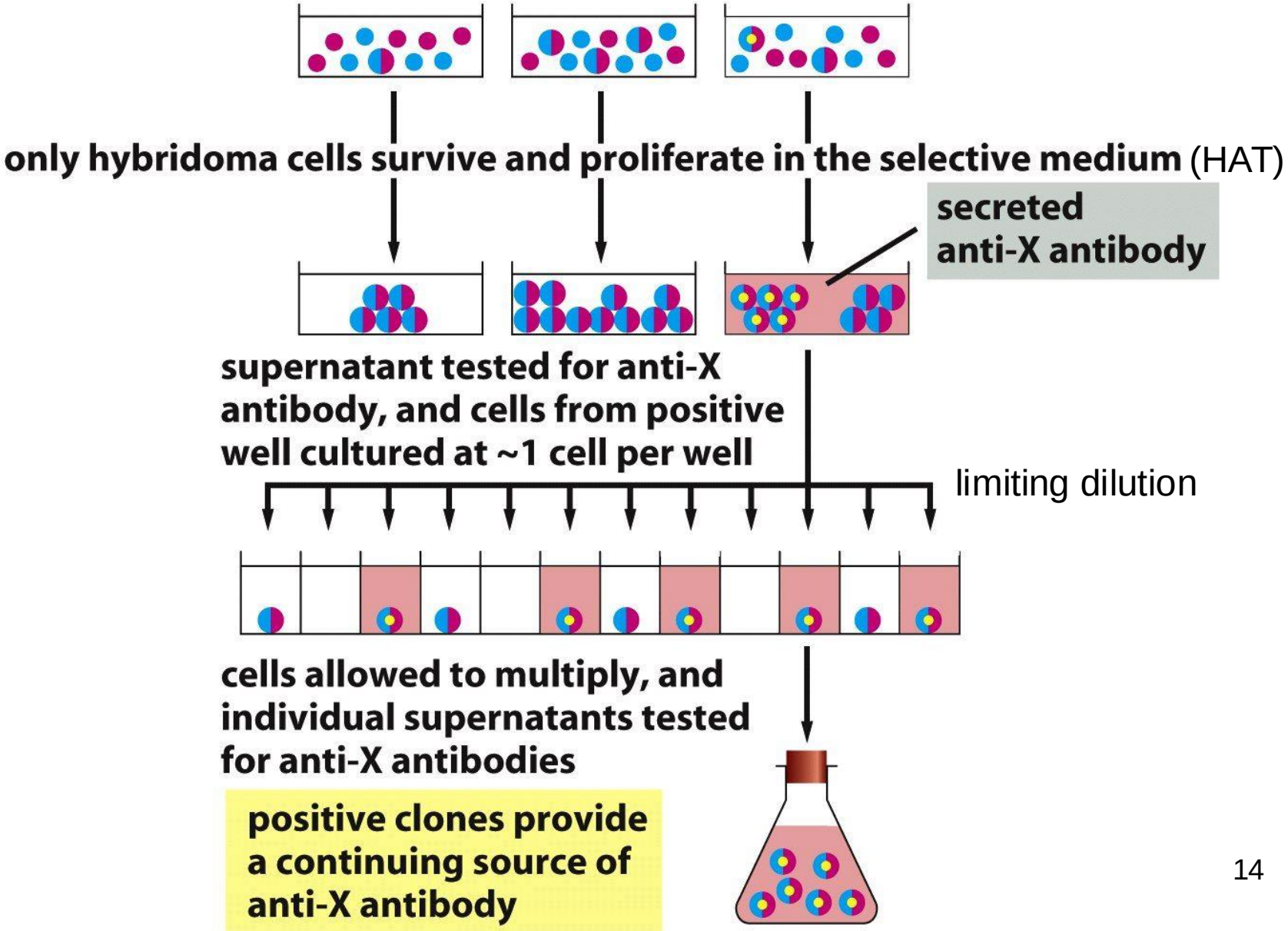


Generation of monoclonal antibodies

=> first described by Köhler/Milstein (1975)



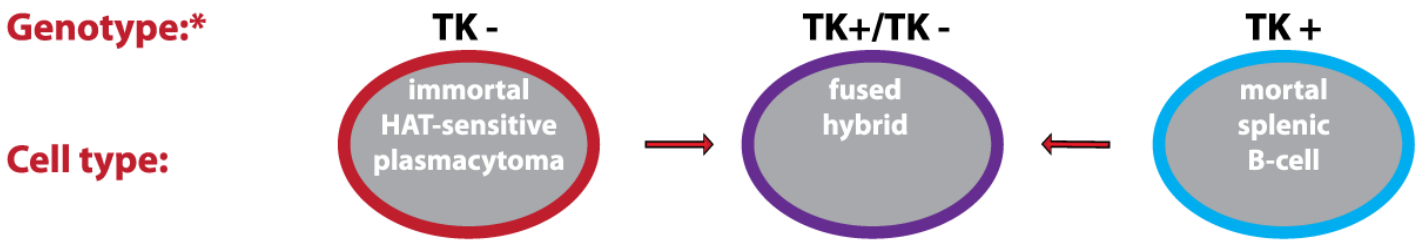
Generation of monoclonal antibodies



Generation of monoclonal antibodies

1. Hyperimmunize mouse with **antigen** and **adjuvans** (immunostimulant)
2. Fuse B cells with tumor (*myeloma*) cell line in PEG (*polyethylene glycol*) or by electrofusion
3. Limiting dilution in 96 well MTPs to fractionate fused cells in **HAT** medium (**h**ypoxanthine, **a**minopterin, **t**hymidine)

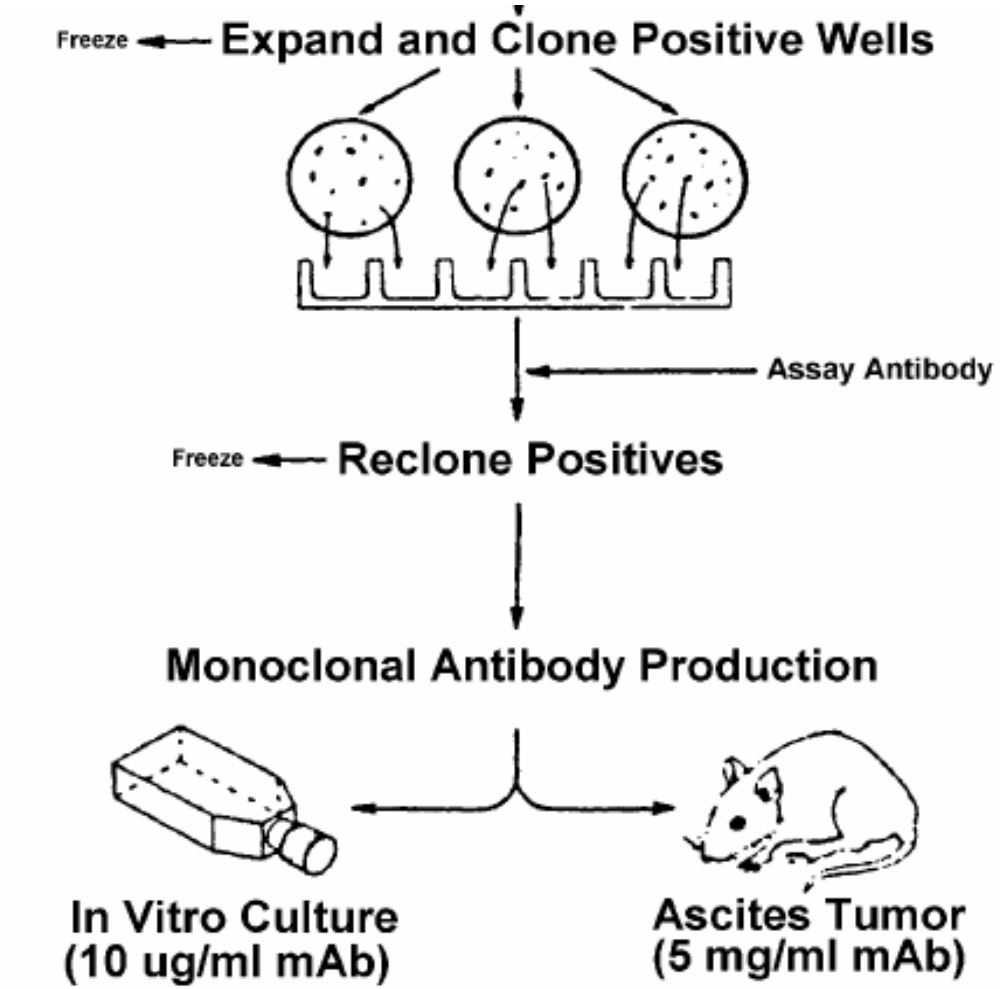
HAT Selection



HAT fate:	**DIES**	**SURVIVES**	**DIES**
Explanation:	**Unable to synthesize DNA:** (1) Thymidine kinase* mutation causes a loss-of-function in the "salvage" pathway and (2) Aminopterin blocks "De novo" pathway.	**Immortal and restored DNA synthesis:** (1) Immortality from plasmacytoma and (2) rescued ability to synthesize DNA due to restored thymidine kinase* function.	**Mortal:** (1) Functional DNA synthesis, but (2) eventually dies because of limited number of replication cycles

**HGPRT (hypoxanthine-guanine phosphoribosyltransferase) mutants can be used in place of TK (thymidine kinase) mutants*

Expand in mice or *in vitro*



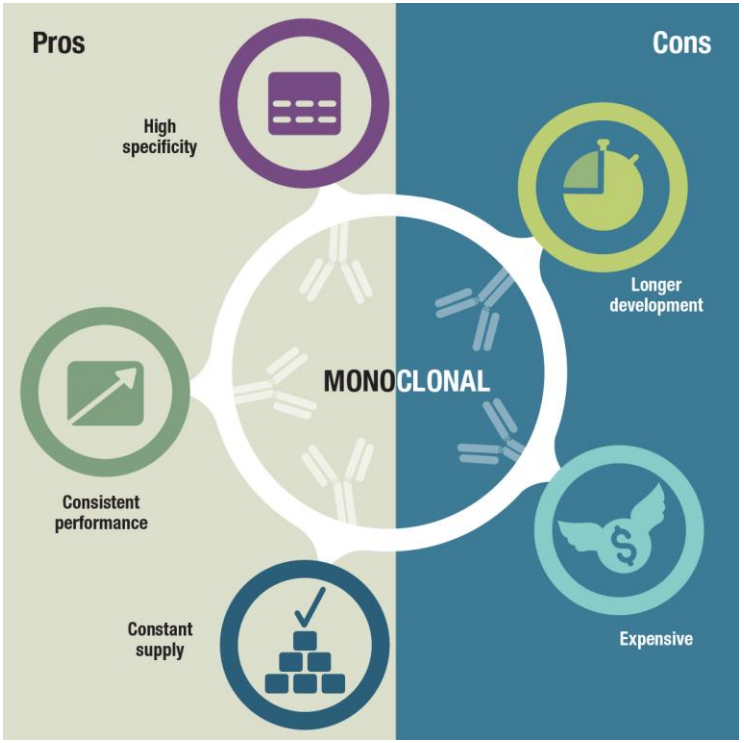
***in vitro* material** is less concentrated and contains bovine serum

ascites fluid contains high [mAb] and has some contamination with the mouse natural Ig

Monoclonal antibodies + and -

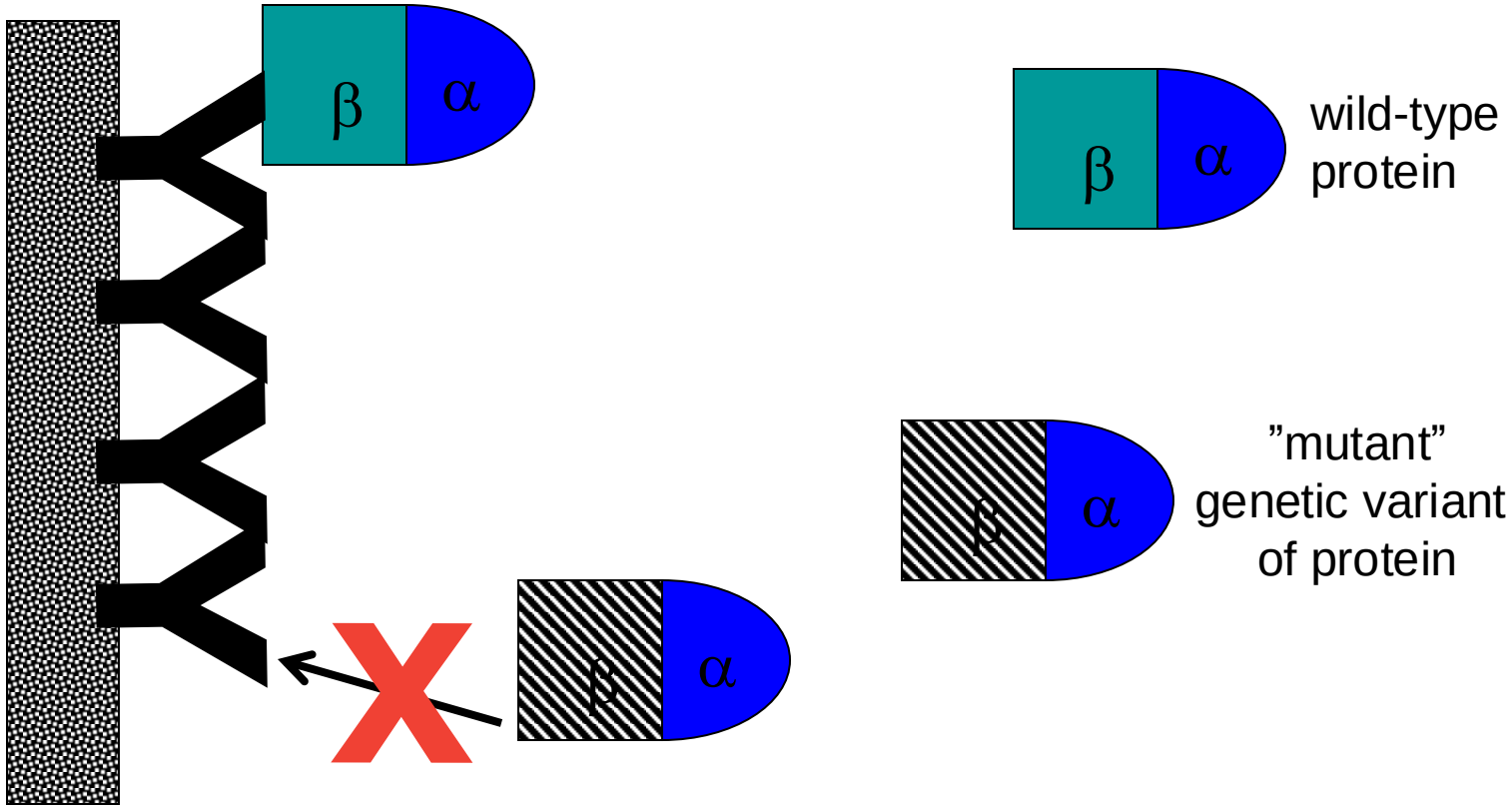
Constant supply of same antibody (from *in vitro* culture)
Constant affinity and specificity
IgG fraction yields in practice a **≈ 100 % active preparation**
100 percent epitope specificity
=> possible to design very specific assays for closely related antigens, and posttranslational variants (fragments, cleaved forms, sugar variants etc.)
No need for 100 % purity of Ag for immunization

Sometimes too specific (does not recognize a genetic or other variant)
Often of lower affinity than polyclonals
=> especially important if used in a competitive assay
(in sandwich assay excess compensate the lower affinity)



Monoclonal antibodies can be too specific

if there is a common genetic variant of protein



-> false negative result !

Using antibodies as immunochemical tools

Polyclonal vs. monoclonal antibodies as a reagent

An antibody reagent differs in the way **how it is produced against the analyte**.

The production determines the **recognition specificity for analyte epitopes*.**

Polyclonal and monoclonal antibodies are very similar protein reagents **except for the amino acids in the paratope region.**

*In the context of antibodies, we only talk about **B cell epitopes!**

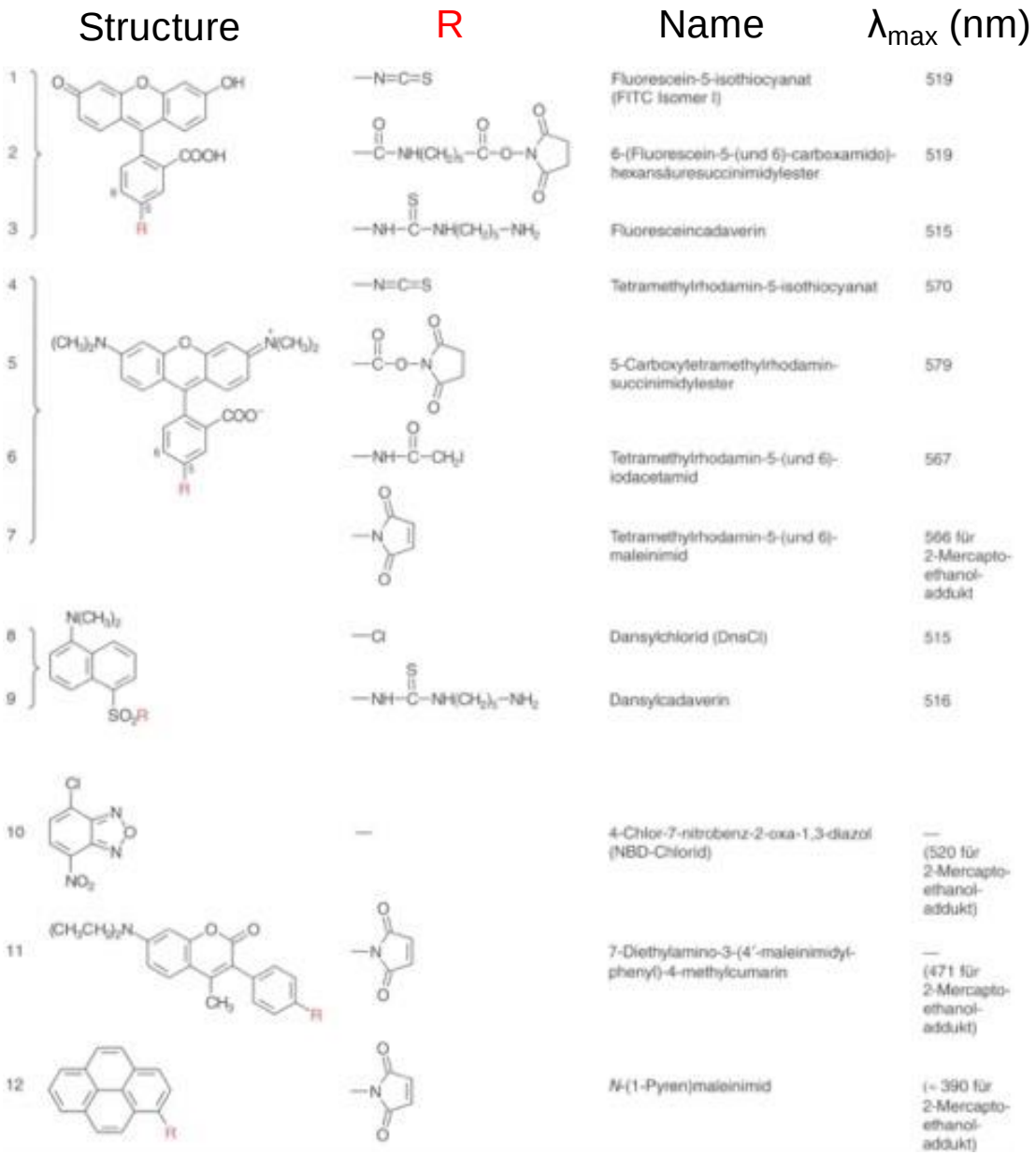
Handling of antibodies (IgG)

Advantages as chemical reagents:
- well soluble (unlike IgM)
- also active with low salt content
- binding over wide pH range (pH 4-9.5)

Storage:
- can be stored in sterile serum for several months up to a few years at 4 ° C
- long-time storage after snap freezing in liquid nitrogen at -20° C or better at -80° C
- freeze drying (lyophilization): mainly from commercial suppliers

Problems:
- damage through bacterial growth
  => add 0.02% (final concentration) of sodium azide (NaN_3) or 0.01% thimerosal
- isolated, purified antibodies are prone to aggregation after freezing and at low concentrations lead to losses by attachment to plastic surfaces
  => add 1% bovine serum albumin (BSA)
- the freezer **should not have a de-frosting cycle**!
- avoid repeated thawing / freezing; better prepare small aliquots
- some antibody-enzyme conjugates (e.g. horseradish peroxidase) lose activity after freezing
  => dilute with glycerol (50%) and store at -20° C (sample does not freeze)

Labeling of antibodies with fluorescent dyes



Reversible unbinding of antibody-antigen complexes

Acidic conditions
Optimal: pH 2.6; with very high affinity antibodies harsher conditions are required pH 1.8 at 4° C for a short time, but leads to some damage.

Alkaline conditions
Optimal: pH 11.2; harsher conditions damage the antibody even more strongly than acidic conditions.

Chaotropic ions
Cl^-, I^-, Br^-, SCN^-, typical eluents: 3 M $MgCl_2$, 1-3 M NaSCN.

Epitopes
Higher concentration of competing free antigen, hapten, synthetic peptides

Elevated temperatures
Not in use any more

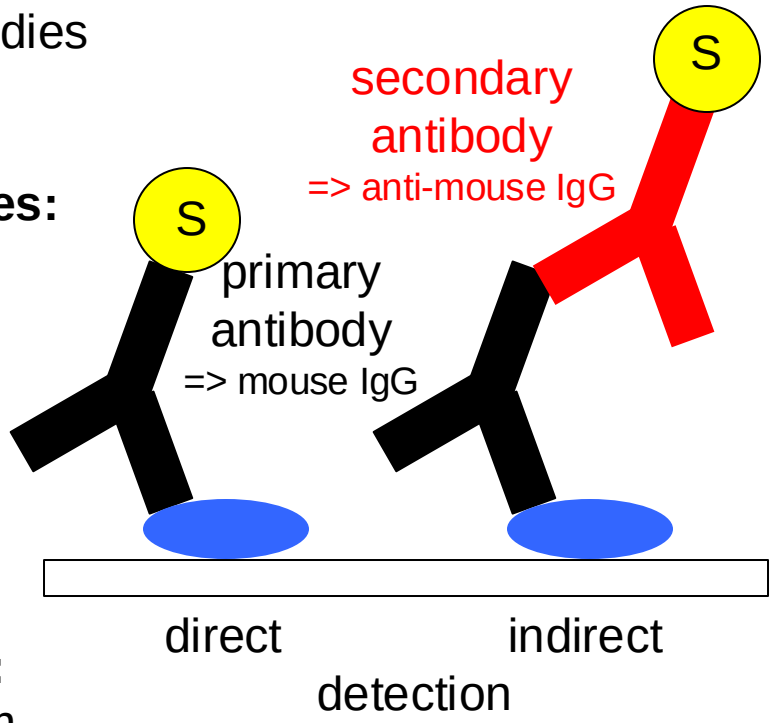
Choosing your antibody host species

We discussed the following animals for obtaining antibodies:

- Mice: easy breeding, but only small amounts
- Rabbits: larger amounts => for polyclonal antibodies
- Goats: large amounts => for polyclonal antibodies

Other consideration for using different species:

- it is not possible to obtain anti-mouse IgG by immunizing mice (=> immunol. tolerance)
- if a mouse sample is to be investigated, mouse-antibodies may show cross-reactivity.

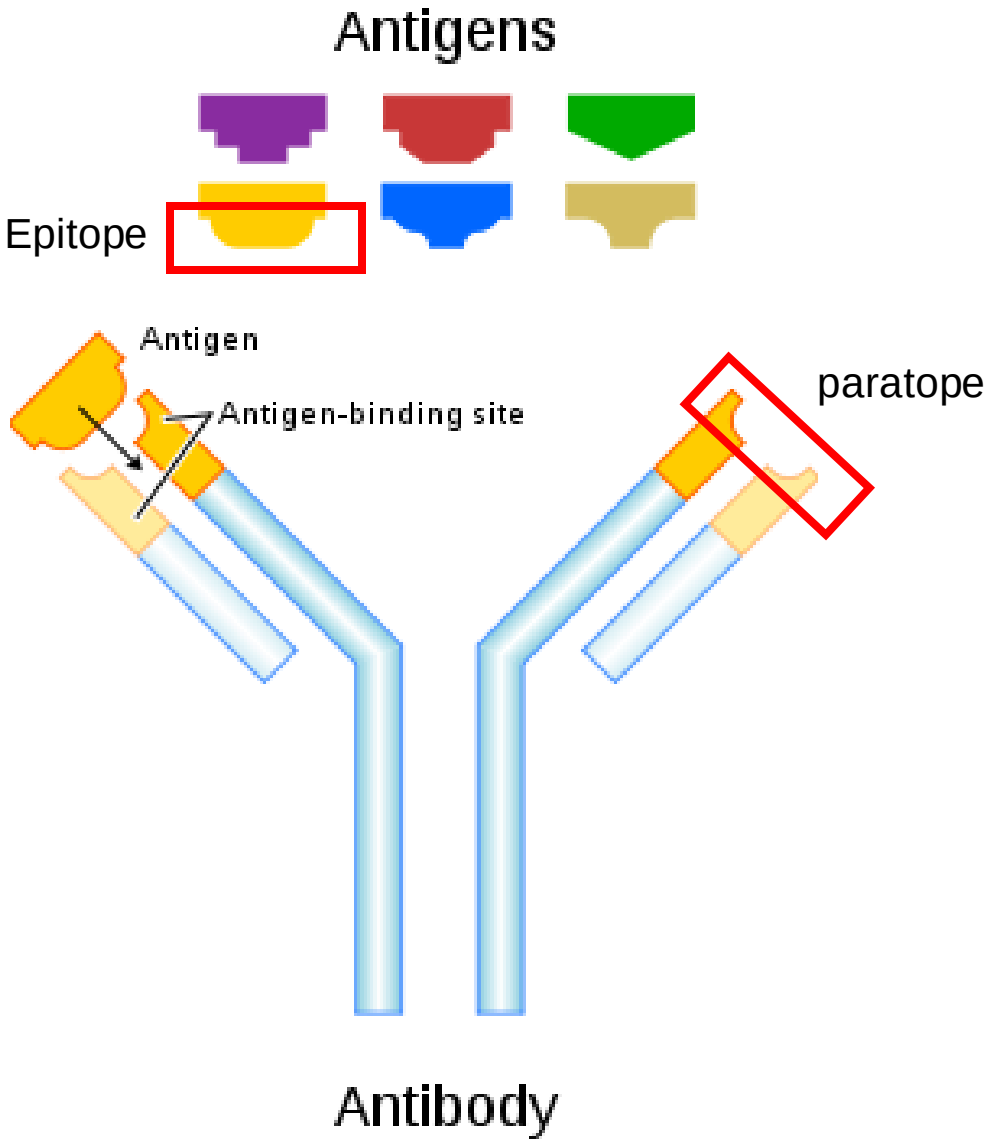


Similar considerations for blocking reagents:

- blocking reagents should not be obtained from species of the primary antibody because then all non-specifically bound primary abs would also be detected (in addition of non-specific binding of secondary ab)

Antibodies as immunochemical reagents

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



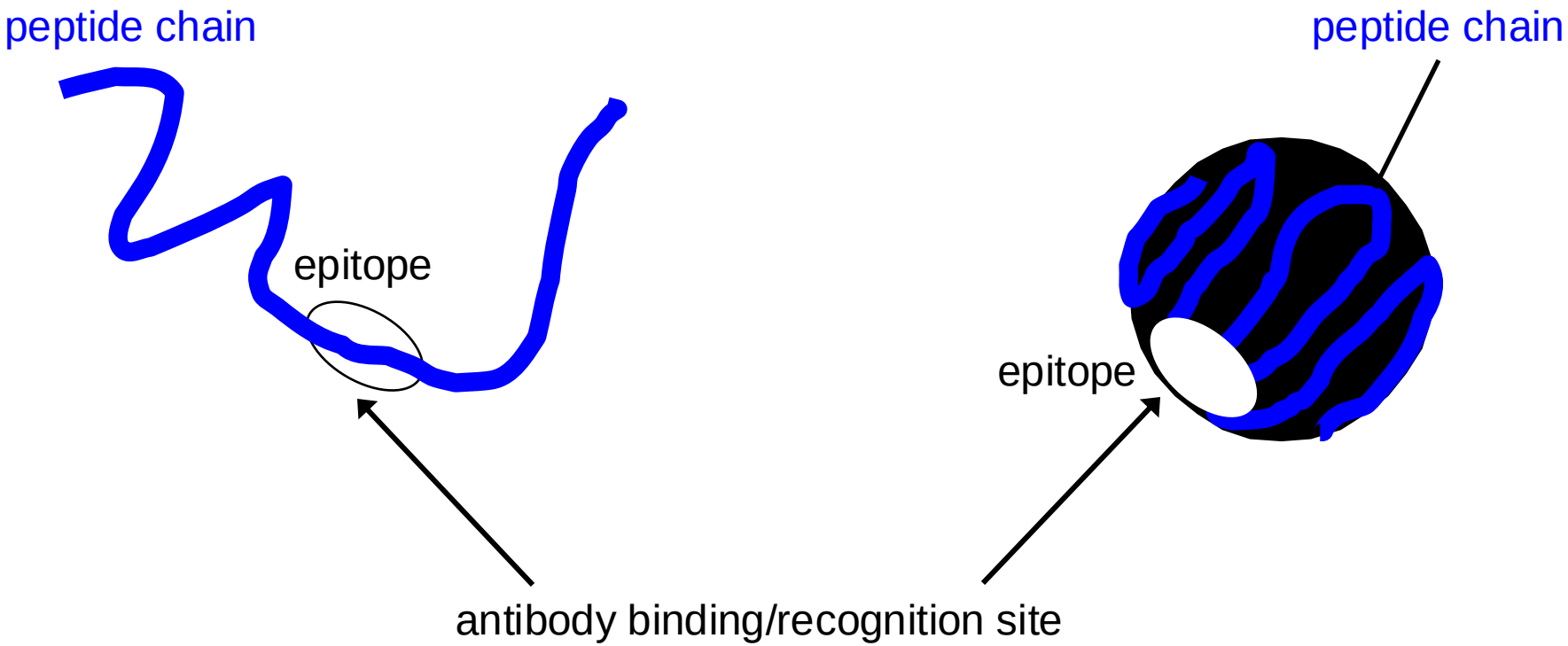
Continuous vs. discontinuous eptiopes

Continuous epitope:

short peptide or denatured protein structure, epitope consist of **sequential** amino acids

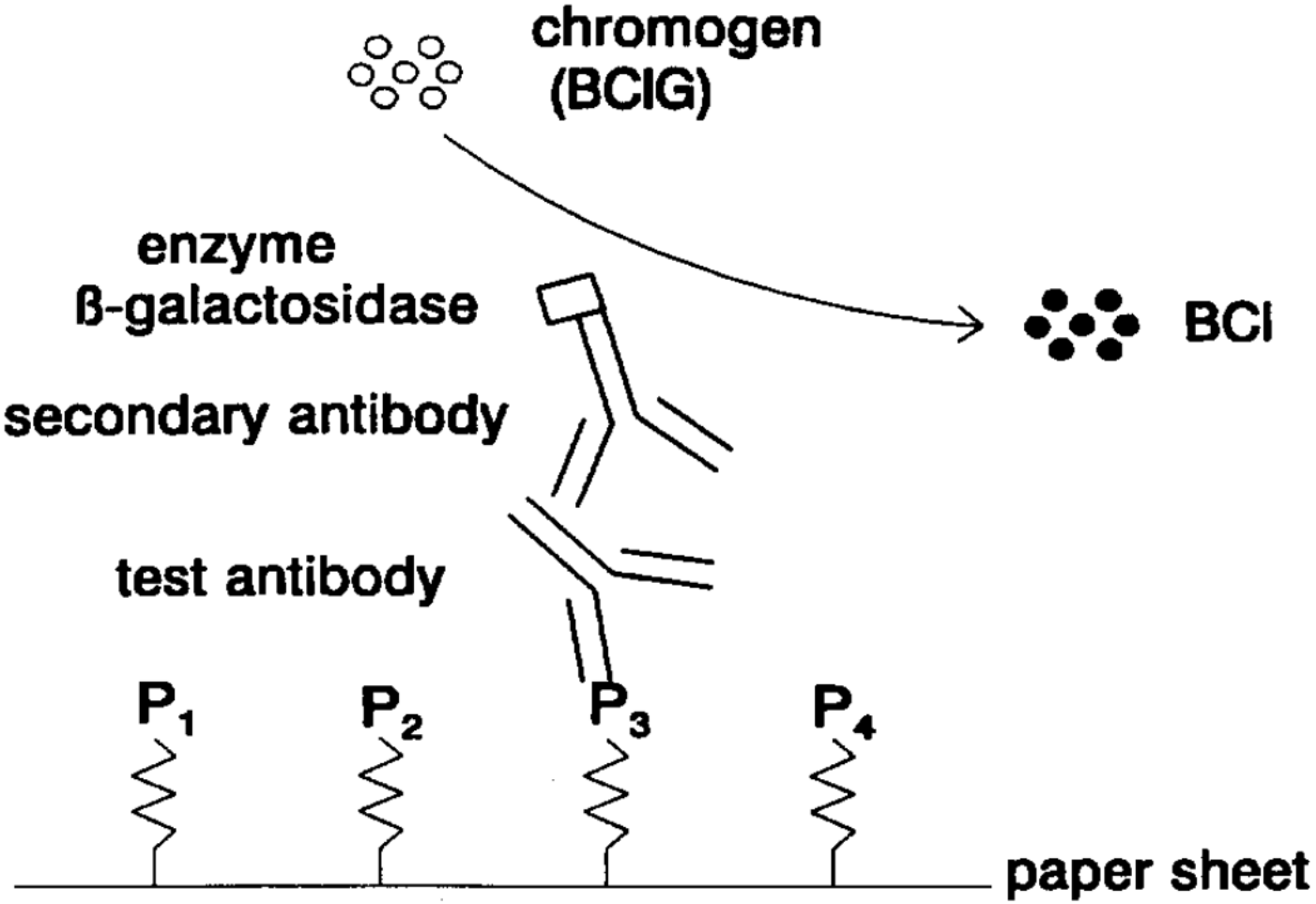
Discontinuous epitope:

present only in 3-dimensional protein structure, epitope comprises **non-sequential** amino acids

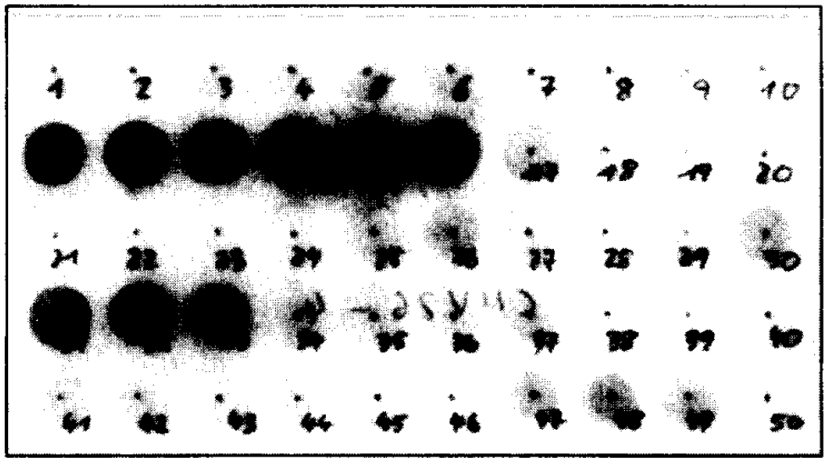


Excursion: Epitope mapping

How do we know to what epitope an antibody binds?



Epitope mapping



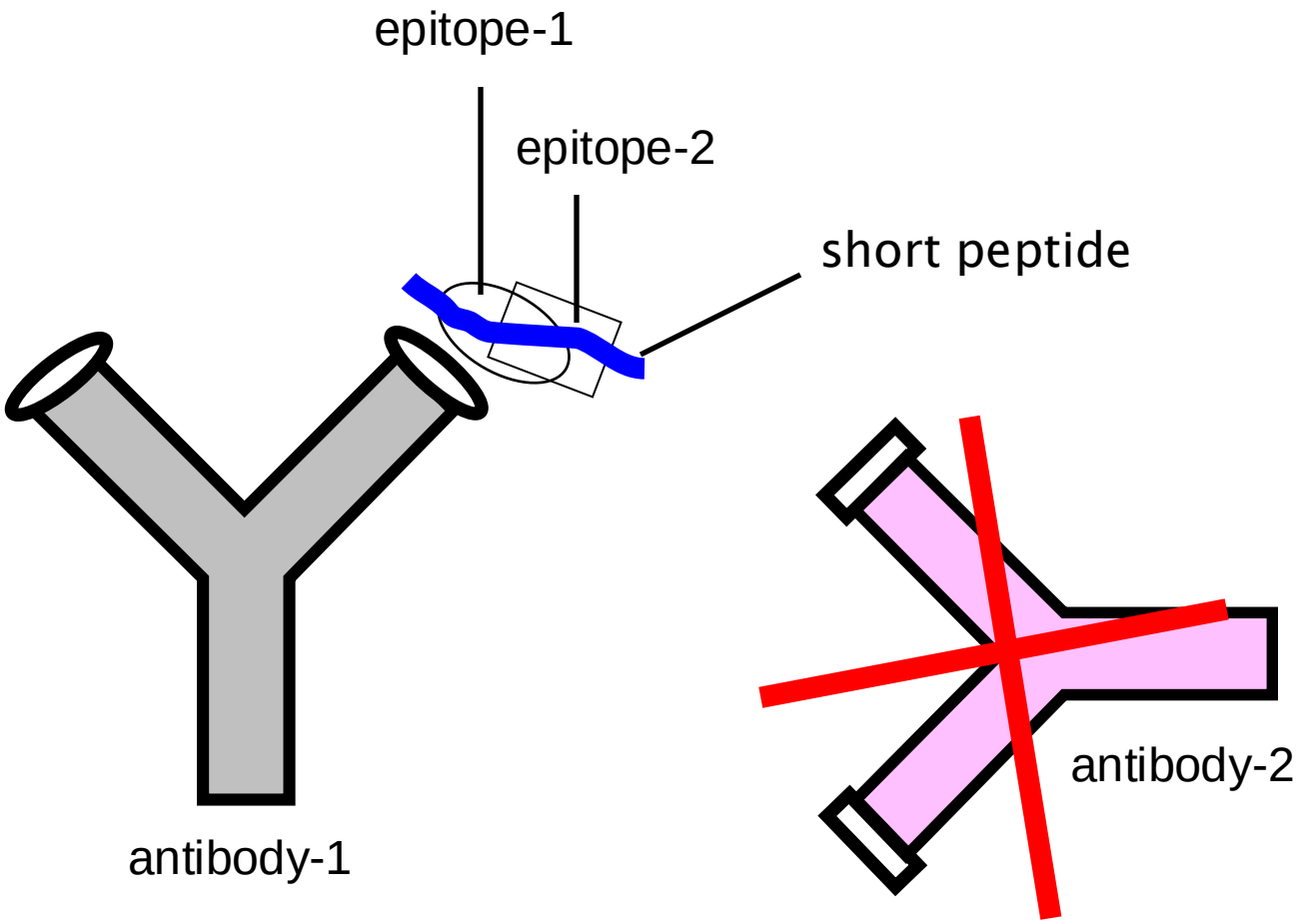
CMV26-decapeptide scan

```
           10         20         30         40         50
  IEGRGKSRGGGGGGGSLSSLANAGGLHDDGPGLDNDLMNEPMGLGGLGGGGGGGGKKH
1.IEGRGKSRGG        21.ANAGGLHDDG        41.PMGLGGLGGG
 2.EGRGKSRGGG        22.NAGGLHDDGP        42.MGLGGLGGGG
  3.GRGKSRGGGG        23.AGGLHDDGPG        43.GLGGLGGGGG
   4.RGKSRGGGGG        24.GGLHDDGPGL        44.LGGLGGGGGG
    5.GKSRGGGGGG        25.GLHDDGPGLD        45.GGLGGGGGGG
     6.KSRGGGGGGG        26.LHDDGPGLDN        46.GLGGGGGGGG
      7.SRGGGGGGGS        27.HDDGPGLDND        47.LGGGGGGGGK
       8.RGGGGGGGSL        28.DDGPGLDNDL        48.GGGGGGGGKK
        9.GGGGGGGSLS        29.DGPGLDNDLM        49.GGGGGGGKKH
        10.GGGGGGSLSS        30.GPGLDNDLMN
         11.GGGGGSLSSL        31.PGLDNDLMNE
          12.GGGGSLSSLA        32.GLDNDLMNEP
           13.GGGSLSSLAN        33.LDNDLMNEPM
            14.GGSLSSLANA        34.DNDLMNEPMG
             15.GSLSSLANAG        35.NDLMNEPMGL
              16.SLSSLANAGG        36.DLMNEPMGLG
               17.LSSLANAGGL        37.LMNEPMGLGG
                18.SSLANAGGLH        38.MNEPMGLGGL
                 19.SLANAGGLHD        39.NEPMGLGGLG
                  20.LANAGGLHDD        40.EPMGLGGLGG
```

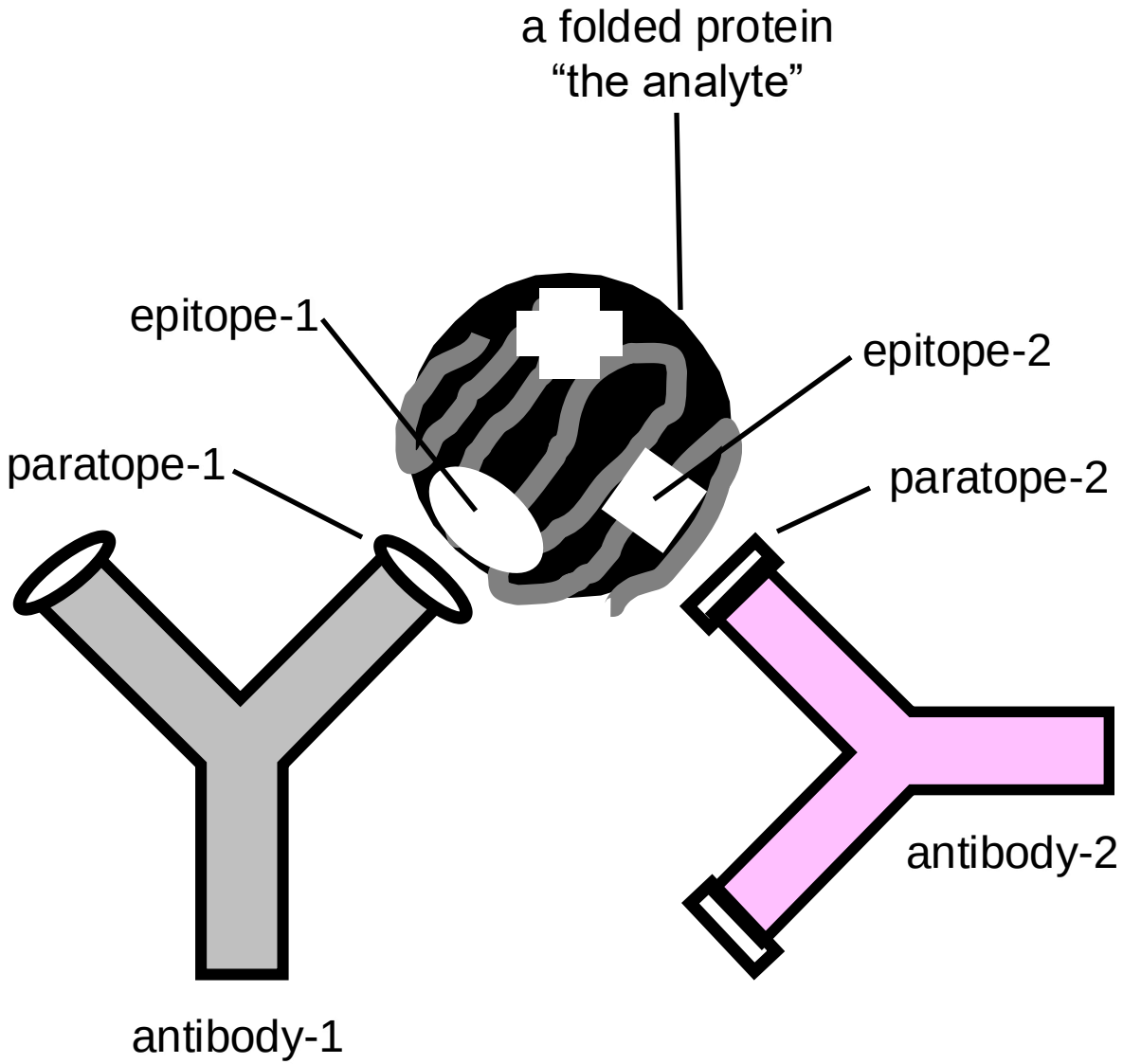
=> But continuous epitopes only

Overlapping eptiopes

Even small analytes can have multiple epitopes, but antibody binding to one epitope **blocks** another epitope, i.e. these epitopes are **overlapping**



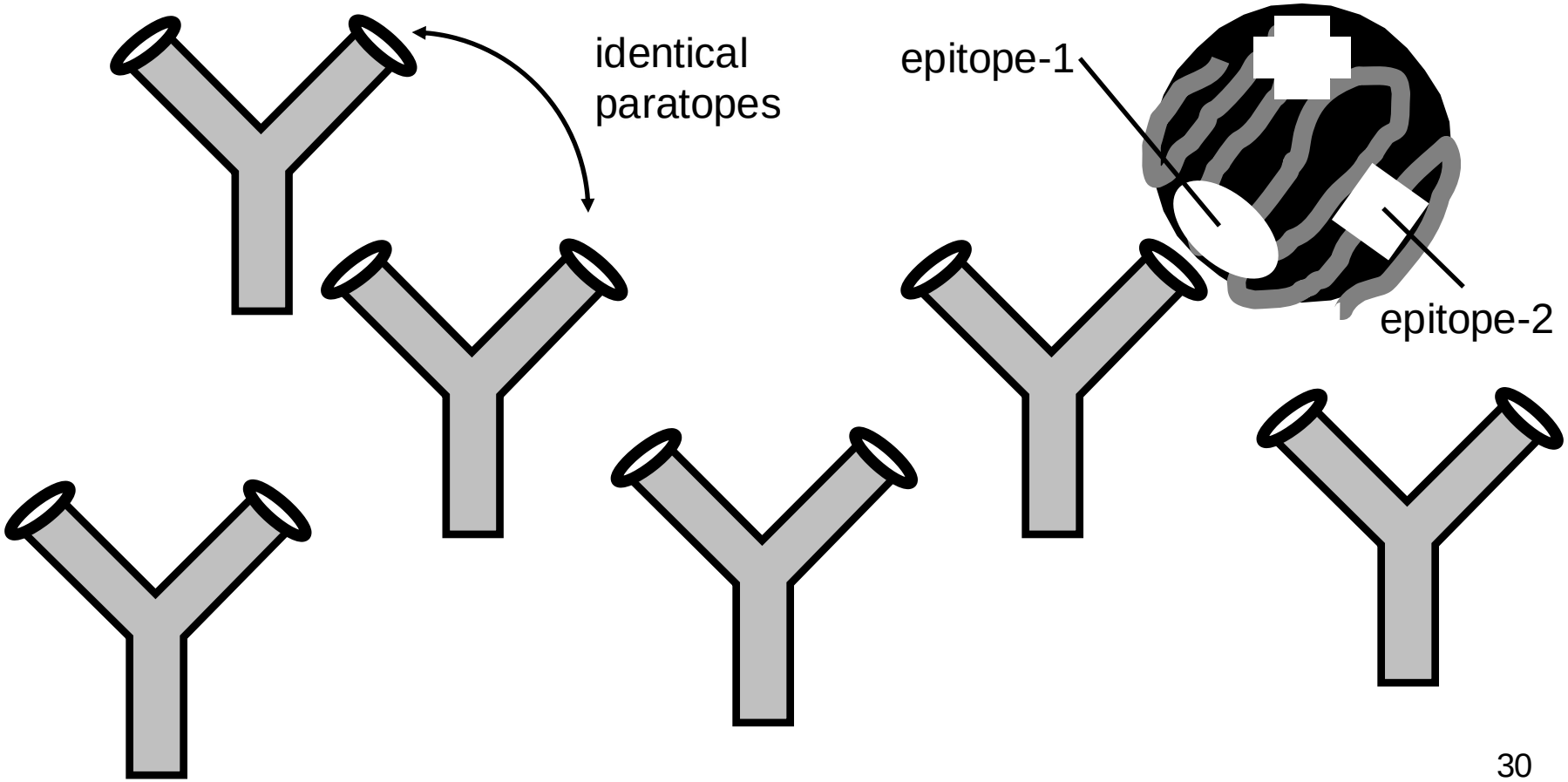
Non-overlapping eptiopes



Monoclonal antibody reagent

all antibodies are from the **same B cell clone**
=> reagent consist of identical antibodies,
and all recognize and are specific
for only one identical epitope

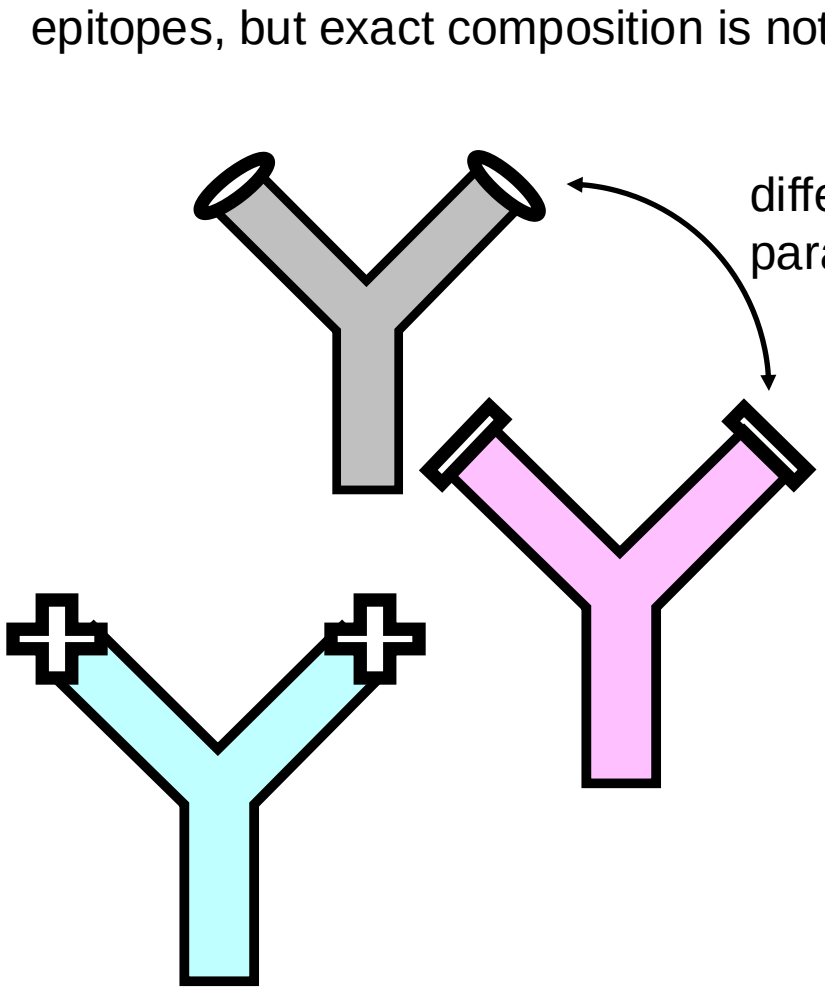
.. will bind only to one specific epitope in the analyte - unless there are multiple identical epitopes in the same analyte

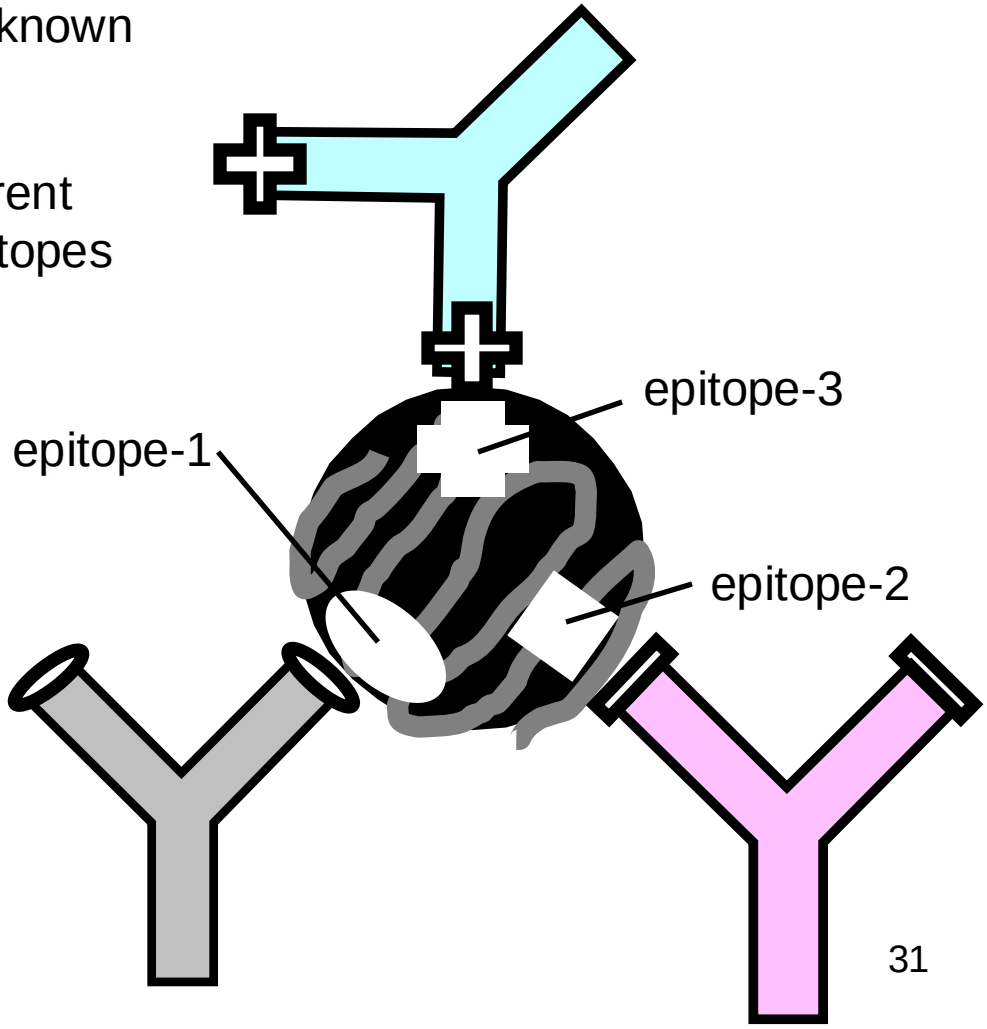


Polyclonal antibody reagent

Mix of **different B cell clones**
=> reagent consist of antibodies that have different paratopes and recognize different epitopes, but exact composition is not known

… will bind simultaneously to one or several non-overlapping epitopes in the analyte





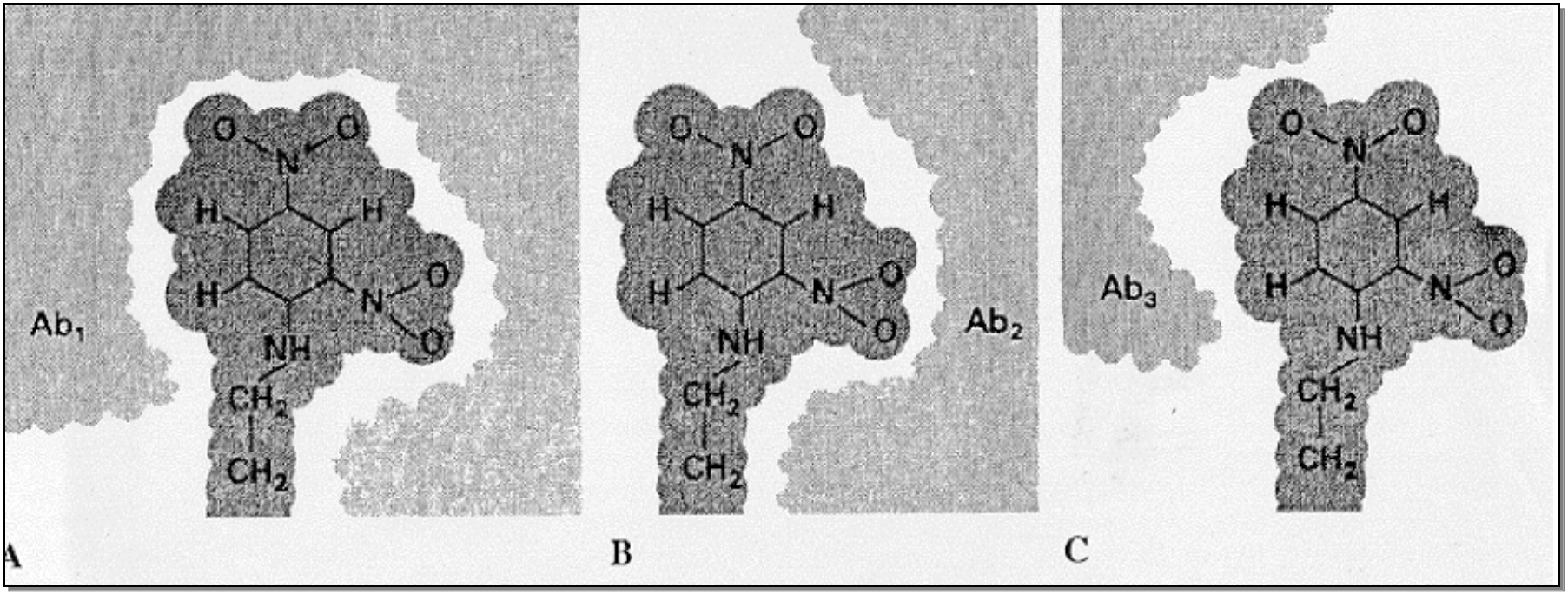
Antibody affinity

Affinity of an antibody

Tight fit / high affinity

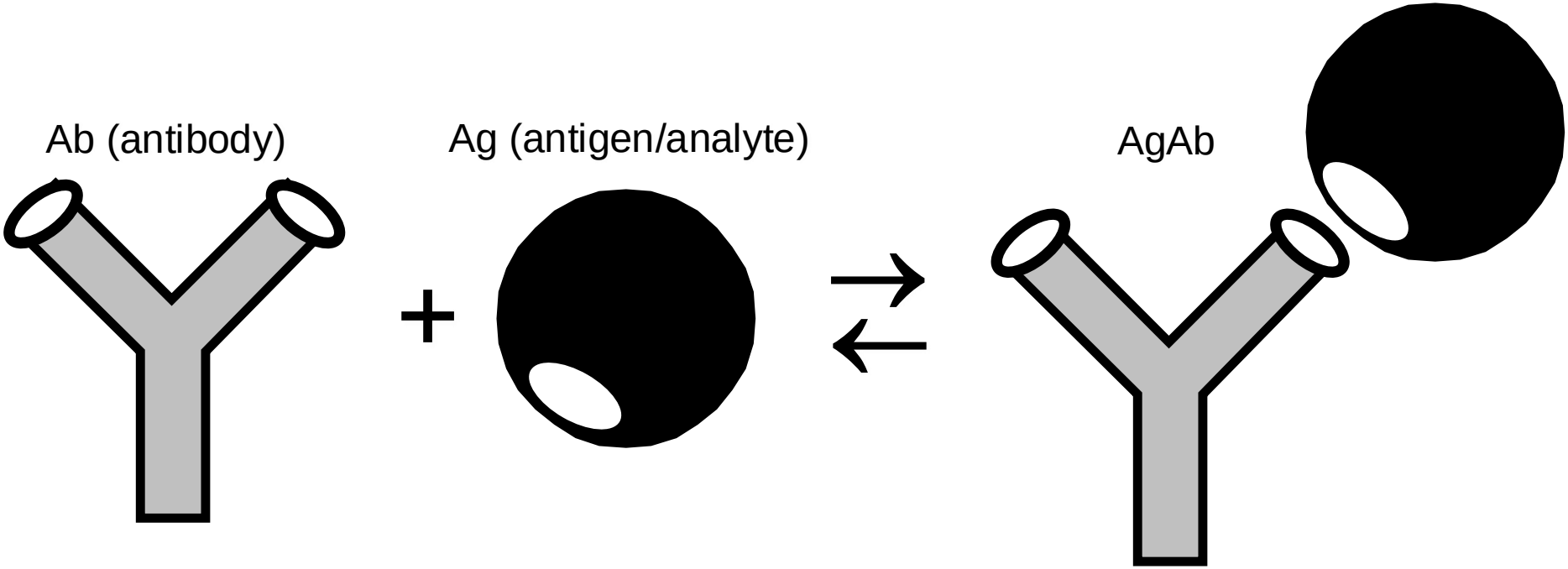
Less interaction / lower affinity

Little interaction / very low affinity



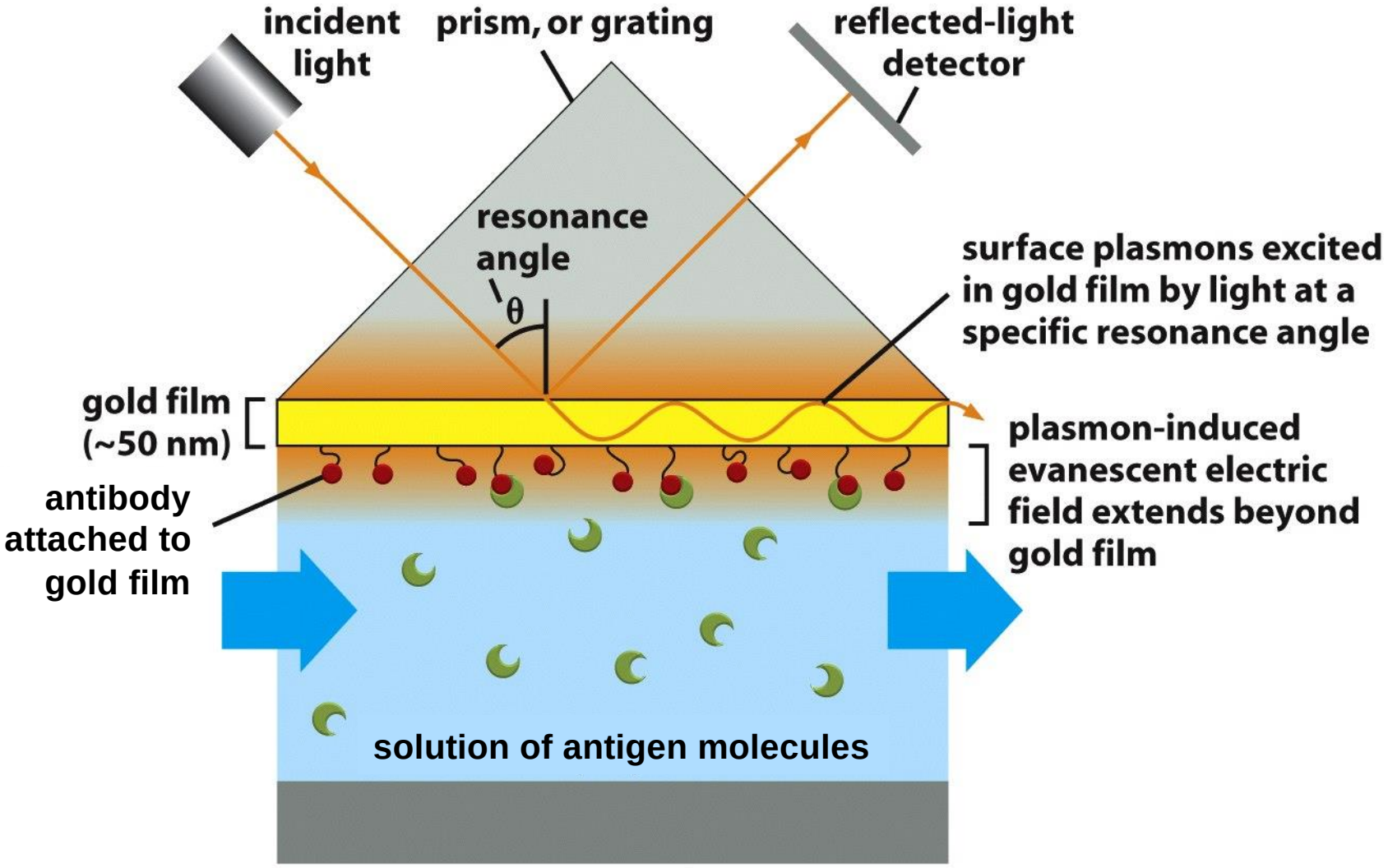
good epitope specificity → decreasing epitope specificity

Antibody-antigen binding reaction



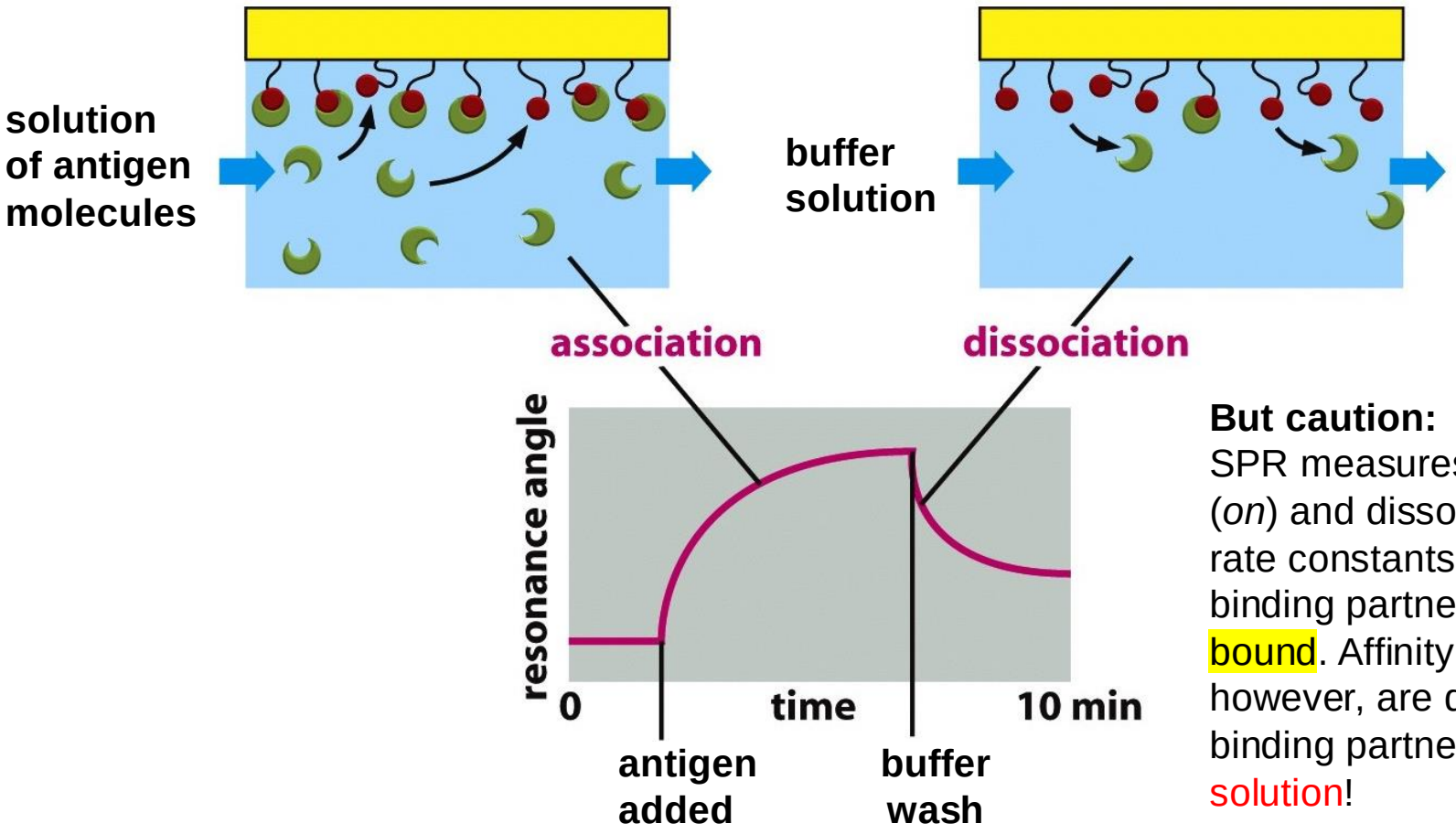
$$\mathrm{Ag} + \mathrm{Ab} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{AgAb}$$

Surface plasmon resonance (SPR)



Determining the affinity of antibodies by SPR

(1) Binding of antigen to surface immobilized antibodies increases the refractive index of the surface layer.
(2) The resulting change of the resonance angel for plamson induction can be measured by a photodetector.



But caution:
SPR measures association (*on*) and dissociation (*off*) rate constants when one binding partner is surface-bound. Affinity constants, however, are defined for both binding partners free in solution!

Affinity of an antibody

$$Ag + Ab \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AgAb$$

$$k_a[Ag][Ab] = k_d[AgAb]$$

reaction velocities at equilibrium:

$$K = \frac{k_a}{k_d} = \frac{[AgAb]}{[Ag][Ab]}$$

Bound antibody and antigen

free antigen

free antibody

k_a: association rate constant (on rate)

k_d: dissociation rate constant (off rate)

K: affinity constant

Affinity of an antibody

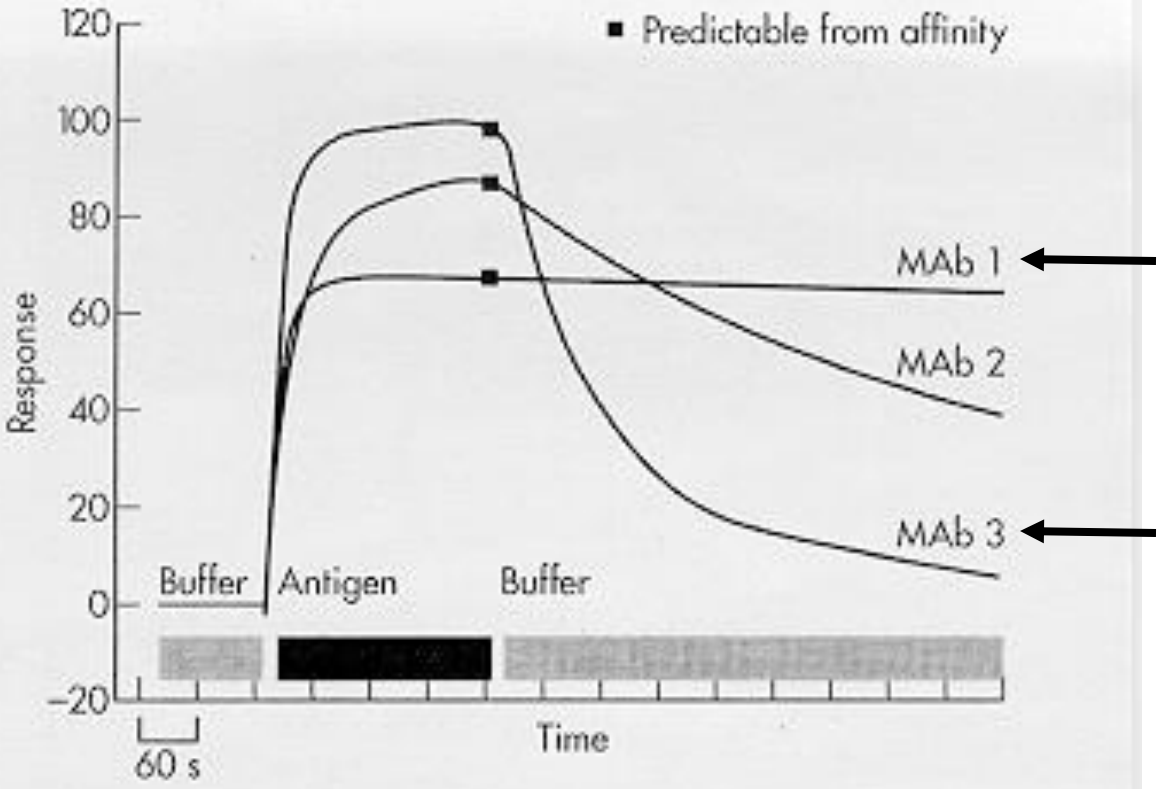


Fig. 1.6 Response curves illustrating the interaction of P24 antigen (125 nM) with three different monoclonal antibodies (MAbs).

extremely stable binding: off-rate is very slow

poorly suited for immuno-assay due to dissociation problems, e.g. during wash or during 2nd step if used as capture antibody

Sensorgrams for 3 moncolonal antibodies against HIV p24 surface Ag

Affinity of an antibody

$$K = \frac{k_a}{k_d} = \frac{[AgAb]}{[Ag][Ab]}$$

Bound antibody and antigen

free antigen

free antibody

approximate calculation of concentrations in equilibrium:

if $[Ag]_{tot} << [Ab]_{tot}$, only a very small antibody fraction is present in the complex $[AgAb]$

=> $[Ab] \approx [Ab]_{tot}$

$$[Ag]_{tot} = [Ag] + [AgAb]$$
$$[Ab]_{tot} = [Ab] + [AgAb]$$

free (unbound) concentrations

$$[AgAb] = \frac{[Ab]_{tot}[Ag]_{tot}\,K}{([Ab]_{tot}\,K) + 1}$$

Affinity of an antibody

Calculating the equilibrium concentration

$$[AgAb] = \frac{[Ab]_{tot}[Ag]_{tot}\ K}{([Ab]_{tot}\ K) + 1}$$

$[Ab]_{tot} = 1 * 10^{-9}$ M
$[Ag]_{tot} = 1 * 10^{-12}$ M (i.e. much smaller)

$K = 1 * 10^{9}$ M^{-1}

by calculating we get $[AgAb] = 0.5 * 10^{-12}$ M (i.e. 50%)

"rule of thumb": when $[Ab]_{tot} = 1/K$ then $[AgAb] = 50\%\ [Ag]_{tot}$
$[Ab]_{tot} = 10/K$ then $[AgAb] = 90\%\ [Ag]_{tot}$
$[Ab]_{tot} = 0.1/K$ then $[AgAb] = 10\%\ [Ag]_{tot}$

Affinity of an antibody

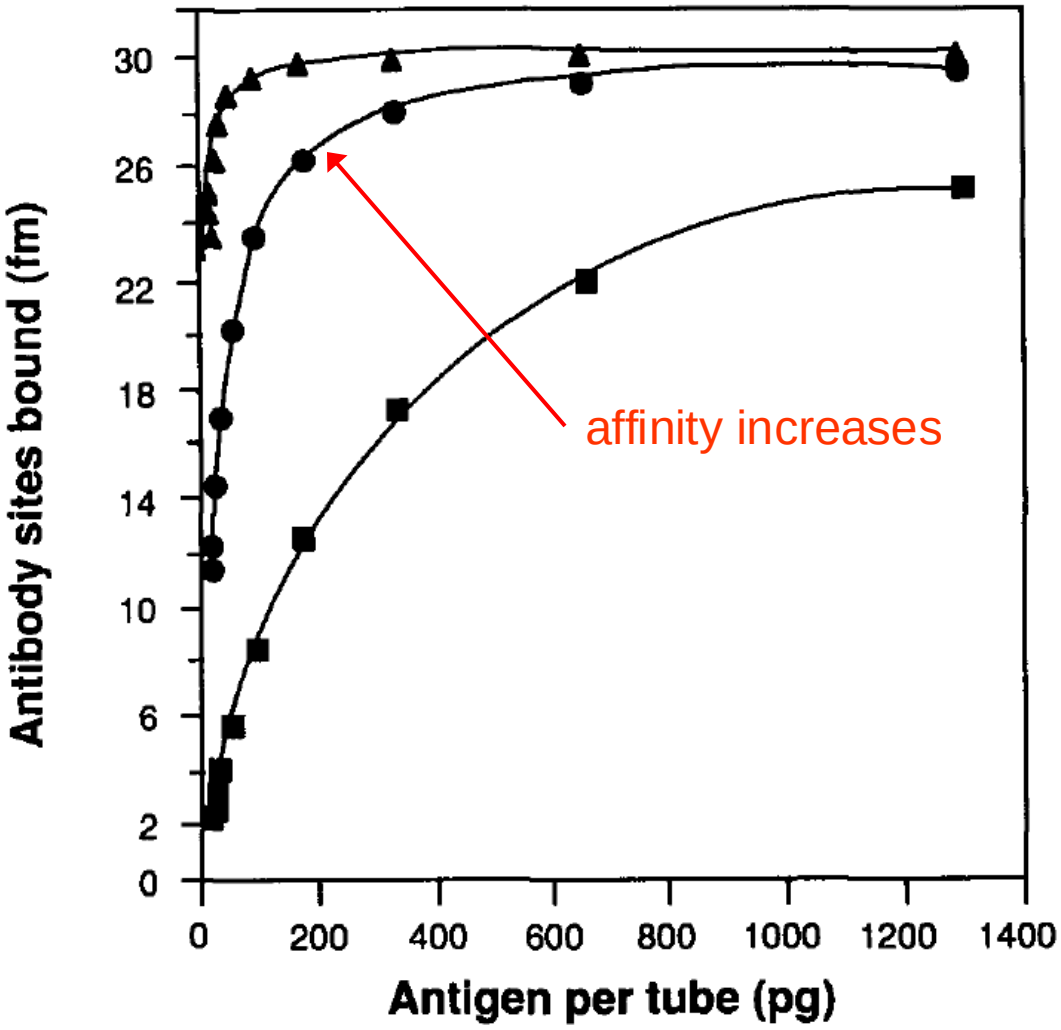
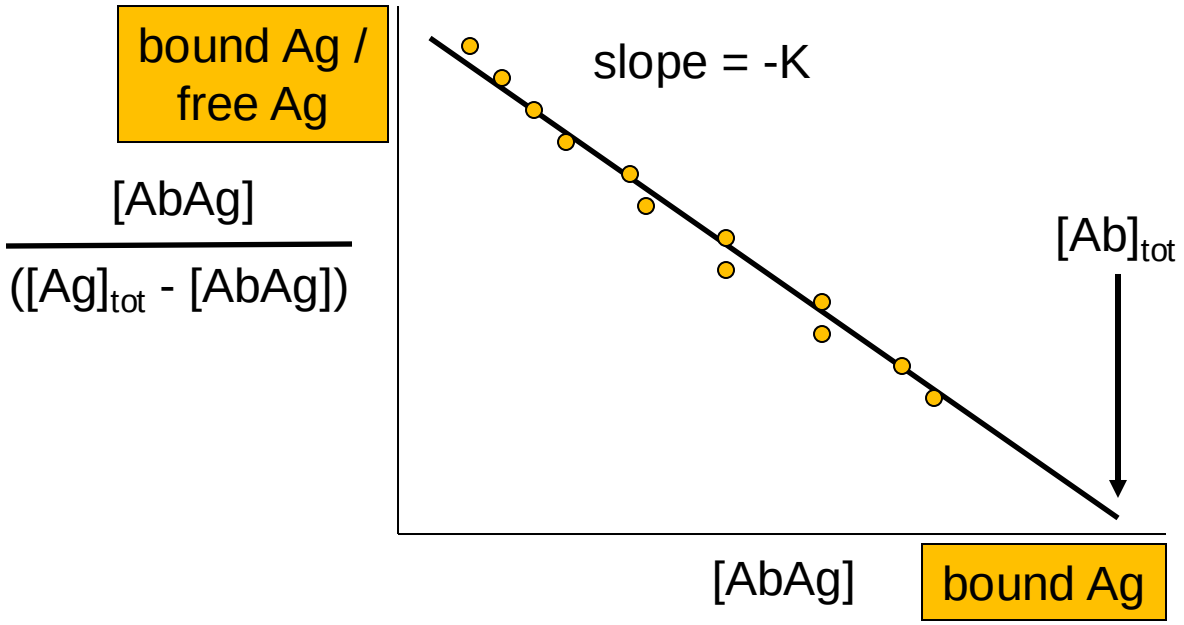


Figure 8.4 Estimation of filled antibody sites, at different concentrations of antigen, for three antibodies with different affinity constant (l/mol). ■ = 1×10^9, ● = 1×10^{10}, ▲ = 1×10^{11}.

Affinity of an antibody: Scatchard plot

linearization:

$$K\,([Ab]_{tot} - [AbAg]) = \frac{[AbAg]}{([Ag]_{tot} - [AbAg])} = \frac{\text{bound Ag}}{\text{free Ag}}$$

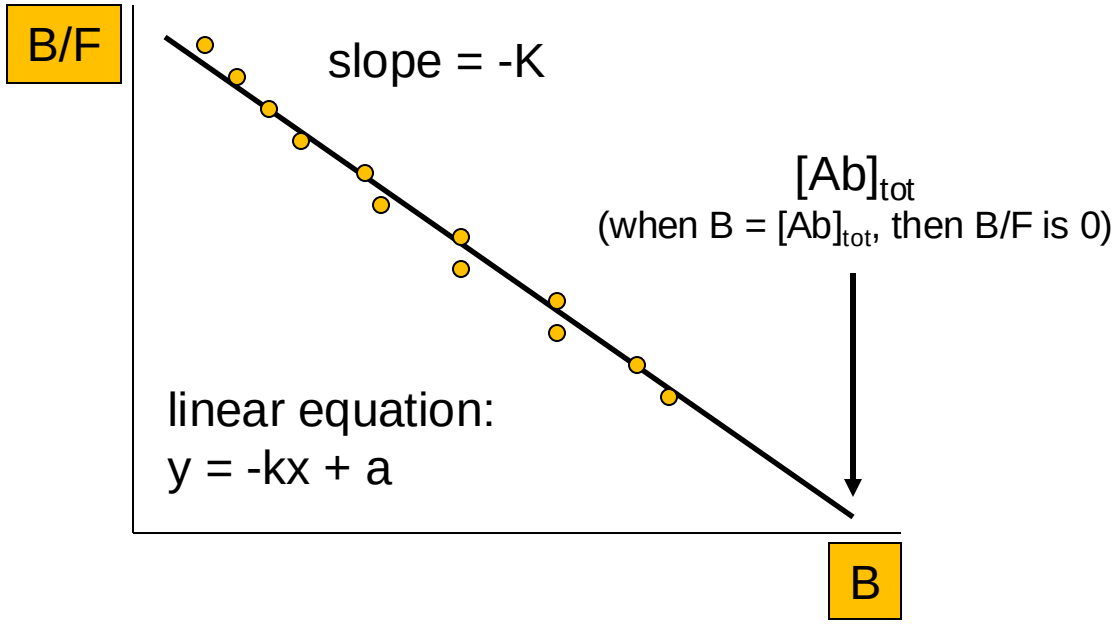


Affinity of an antibody: Scatchard plot

B = [Ag-Ab] F = [Ag] = $[Ag]_{tot}$ – [AgAb]

K ($[Ab]_{tot}$ – B) = B/F => B/F = – K B + K $[Ab]_{tot}$

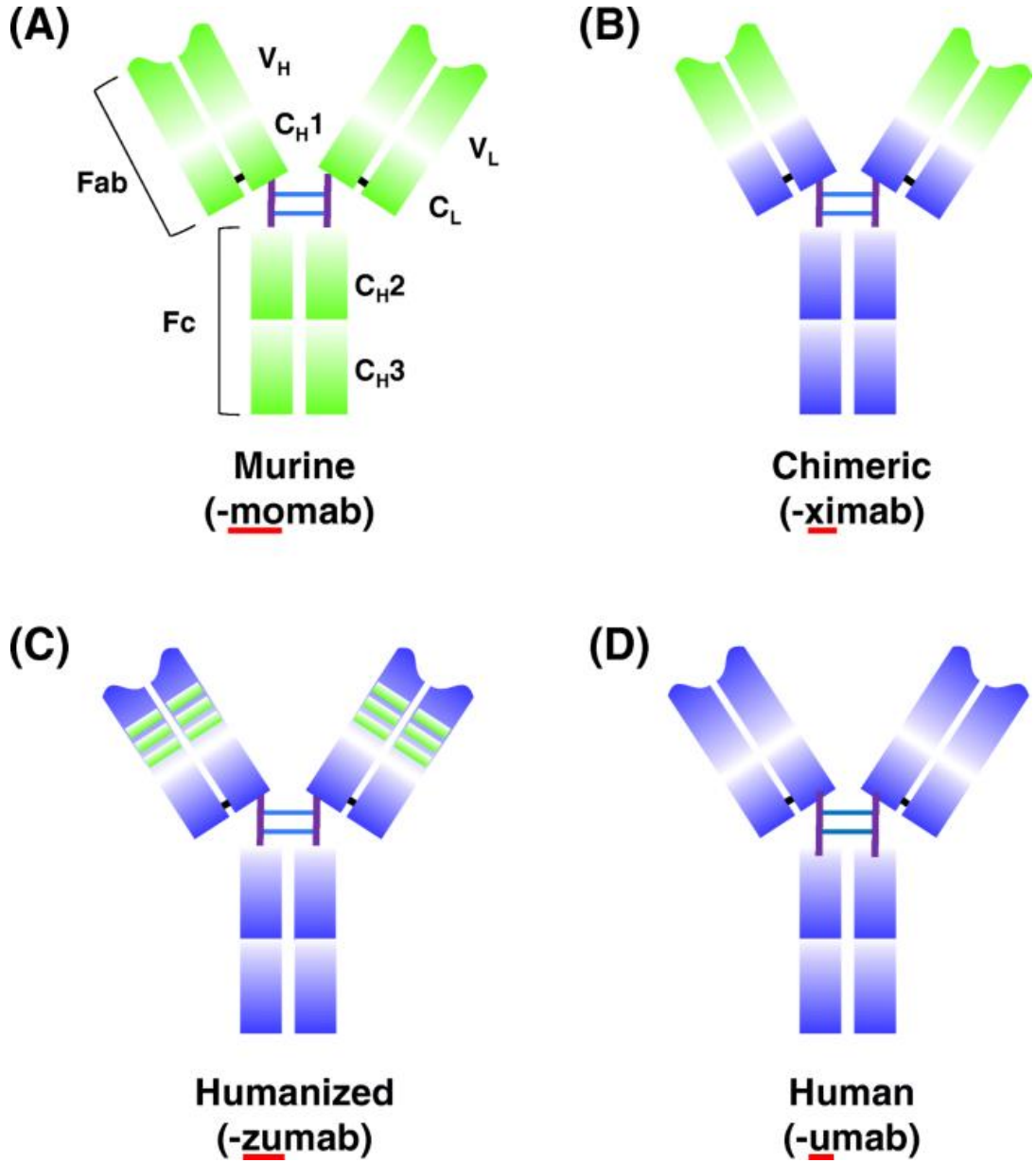
constant



=> Typcially replaced by non-linear fitting using computer programs

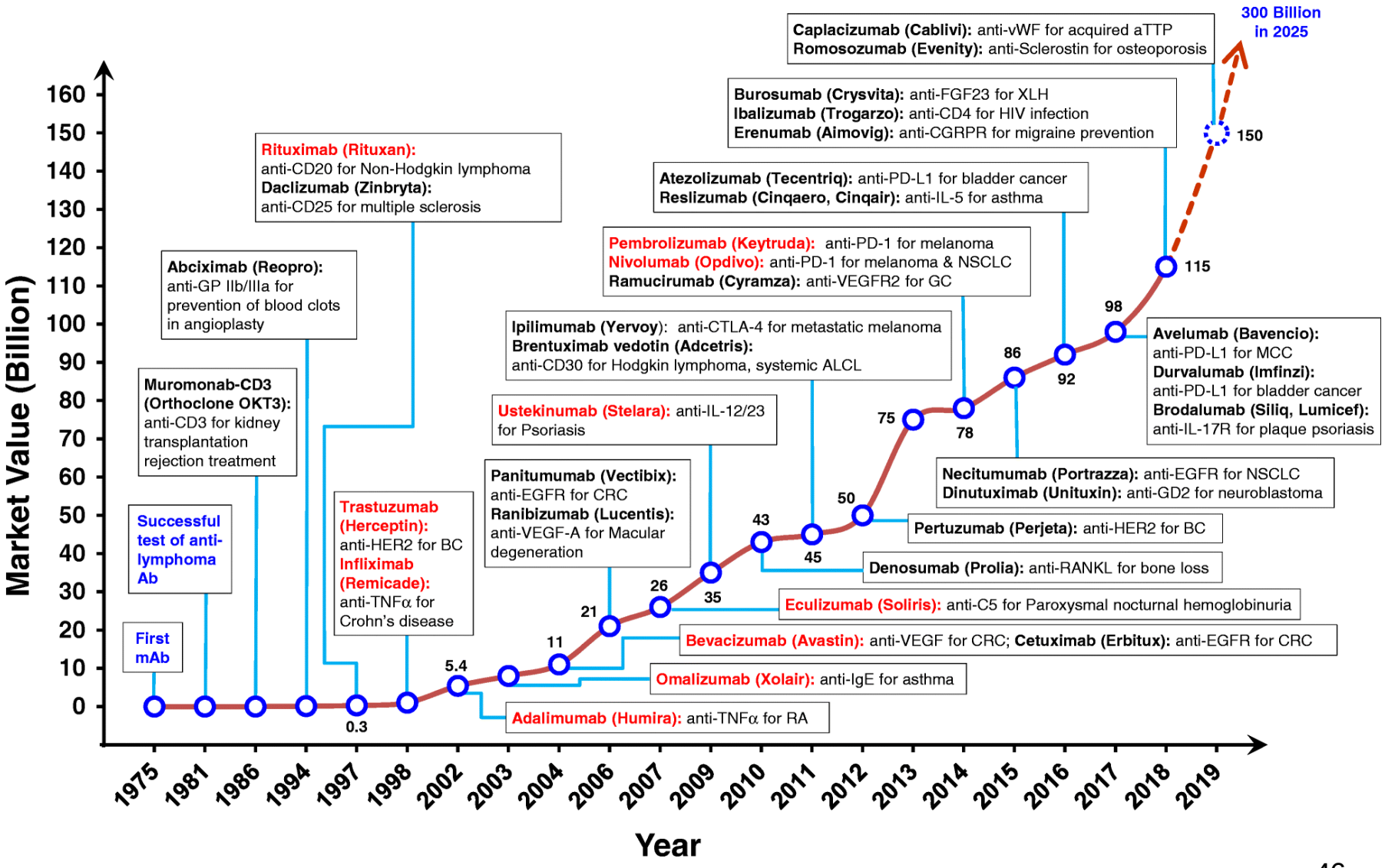
Antibody engineering

Excursion: Antibody enginering for therapy



Natural antibodies (raised in mice) are potentially immunogenic => Potential side effects

Therapeutic antibodies (market value)

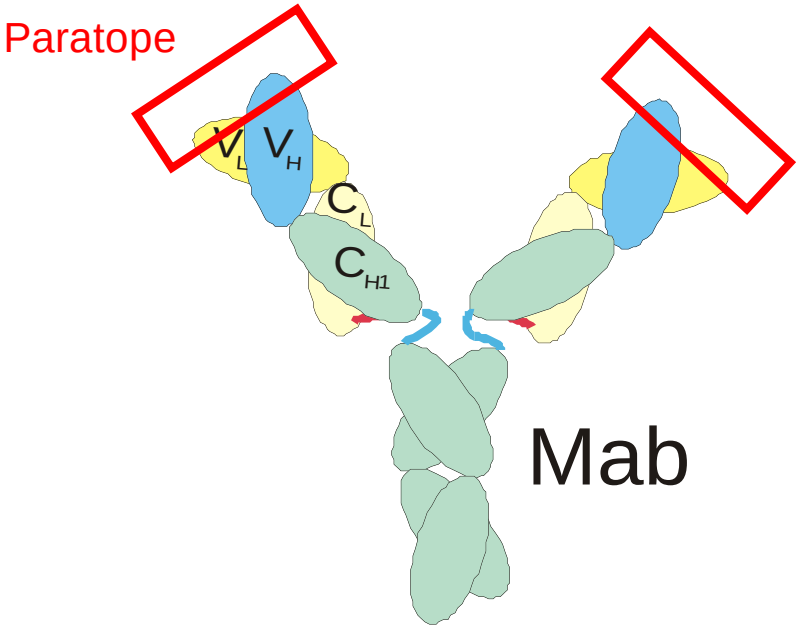


Humanized antibodies

No.	Drug	Indication (1st US FDA Approval Year)	Company	2018 Revenue (USD, billion)
1	Adalimumab (Humira)	Rheumatoid arthritis (2002)	AbbVie	$19.9 bn
		Psoriatic arthritis (2005)		
		Ankylosing spondylitis (2006)		
		Juvenile Idiopathic Arthitis (2008)		
		Psoriasis (2008)		
		Crohn's disease (2010)		
		Ulcerative colitis (2012)		
		Hidradenitis suppurativa (2015)		
		Uveitis (2018)		
2	Nivolumab (Opdivo)	Melanoma (2015)	Bristol-Myers Squibb	$7.6 bn
		Non-small cell lung cancer (2015)		
		Renal cell carcinoma (2015)		
		Head and neck squamous cell (2016)		
3	Pembrolizumab (Keytruda)	Melanoma (2014)	Merck & Co	$7.2 bn
		Head and neck cancer (2016)		
		Non-small cell lung caccer (2015)		
		Lymphoma (2018)		
		Cervical cancer (2018)		
		Microsatellite instability-high cancer (2018)		
4	Trastuzumab (Herceptin)	Breast cancer (1998)	Roche	$7.0 bn
		Gastric cancer (2010)		
5	Bevacizumab (Avastin)	Colorectal cancer (2004)	Roche	$6.8 bn
		Non-small cell lung caccer (2006)		
		Breast ERB2 negative cancer (2008)		
		Renal cell carcinoma (2009)		
		Glioblastoma (2011)		
6	Rituximab,	Non-Hodgkin's lymphoma (1997)	Roche	$6.8 bn

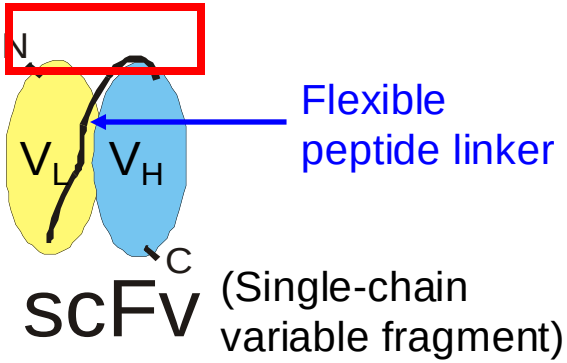
Recombinant antibody fragments

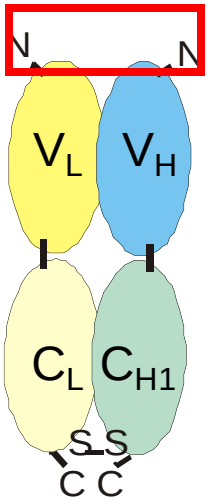
Natural IgG antibody



Disadvantage of antibodies as a reagent: relatively large size

Artificial smaller constructs





=> same paratope, but much smaller

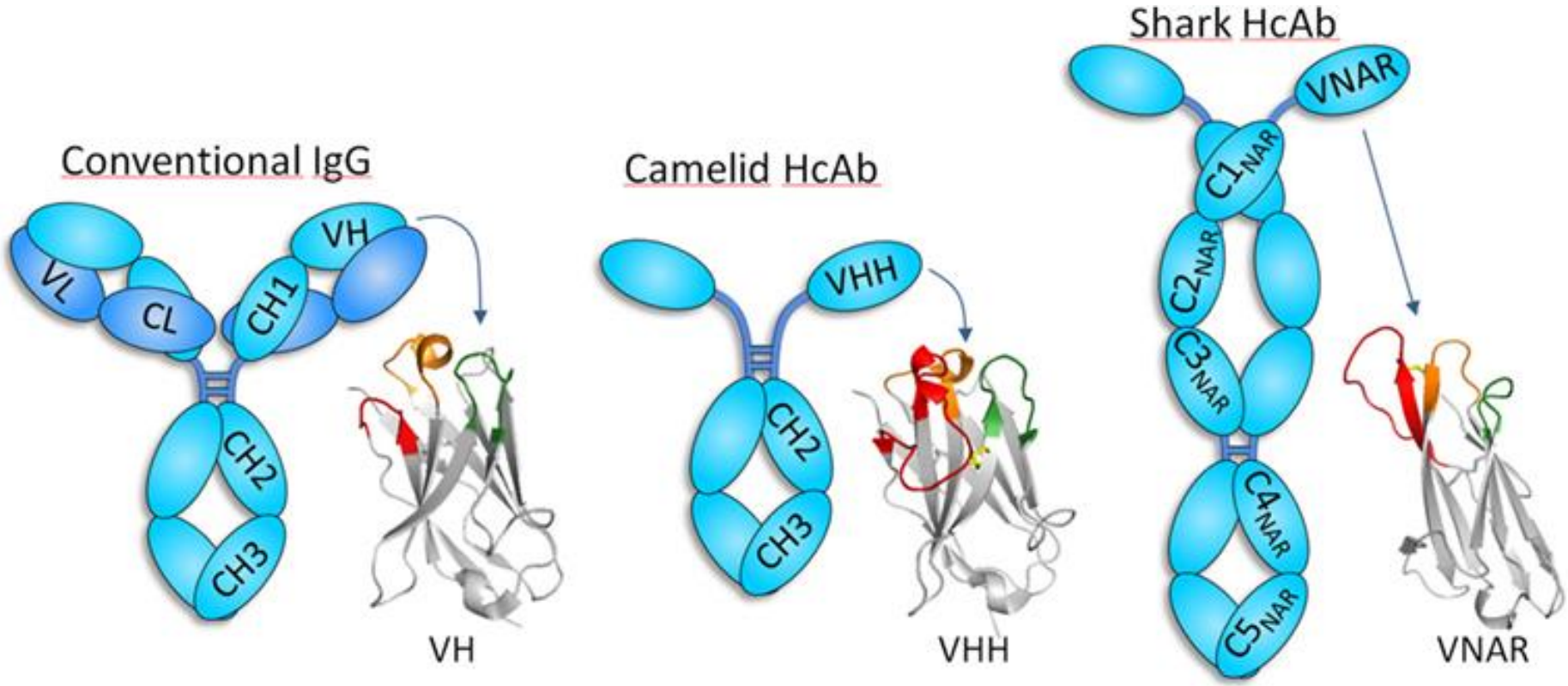
Recombinant antibody fragments

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

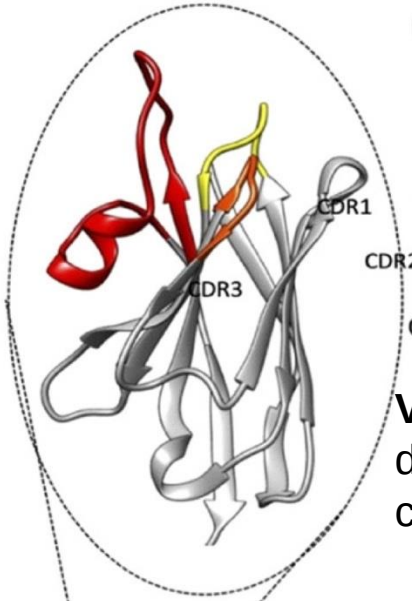
heavy chain antibodies

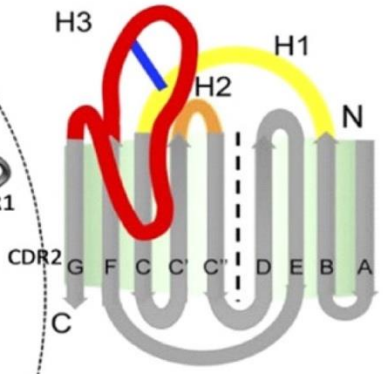
(velbloud, dromedár, lama)

(žralok)

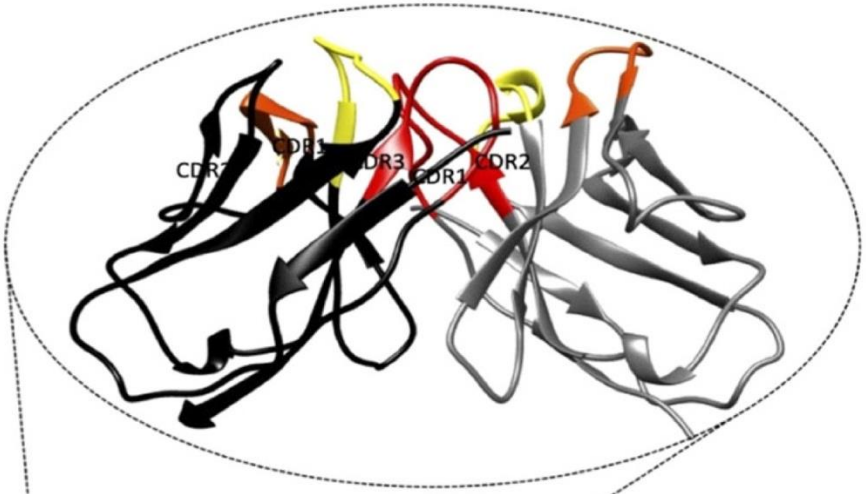
Front. Immunol., 2017 https://doi.org/10.3389/fimmu.2017.00977

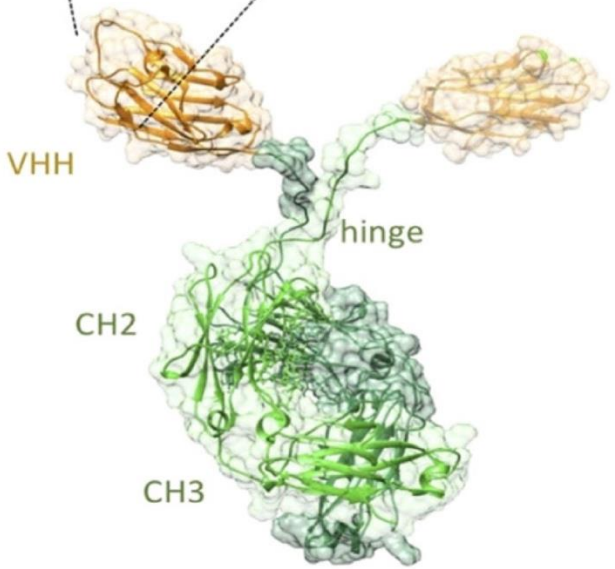
From heavy chain antibodies to nanobodies



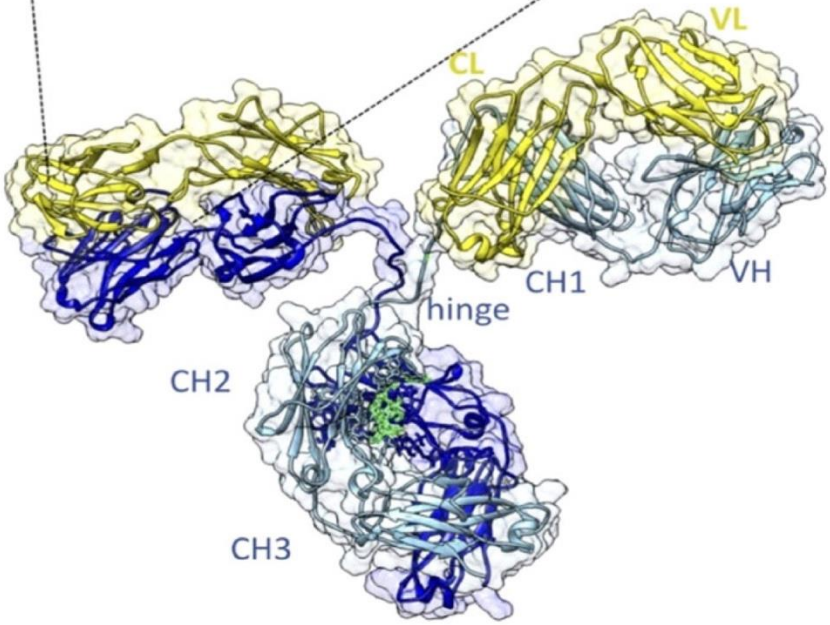


VHH: Single variable domain on a heavy chain (=> nanobody)



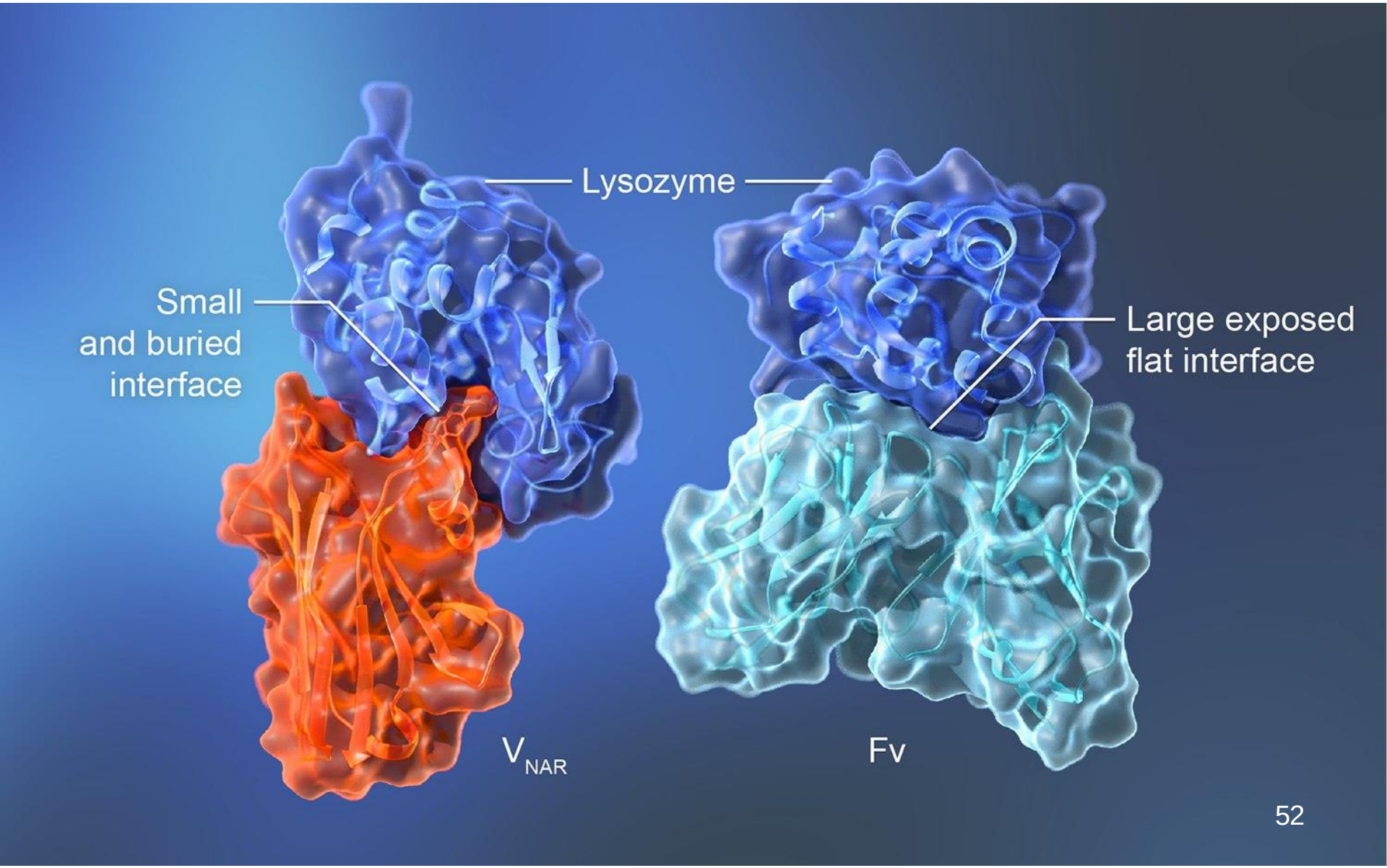


heavy-chain antibody



IgG antibody

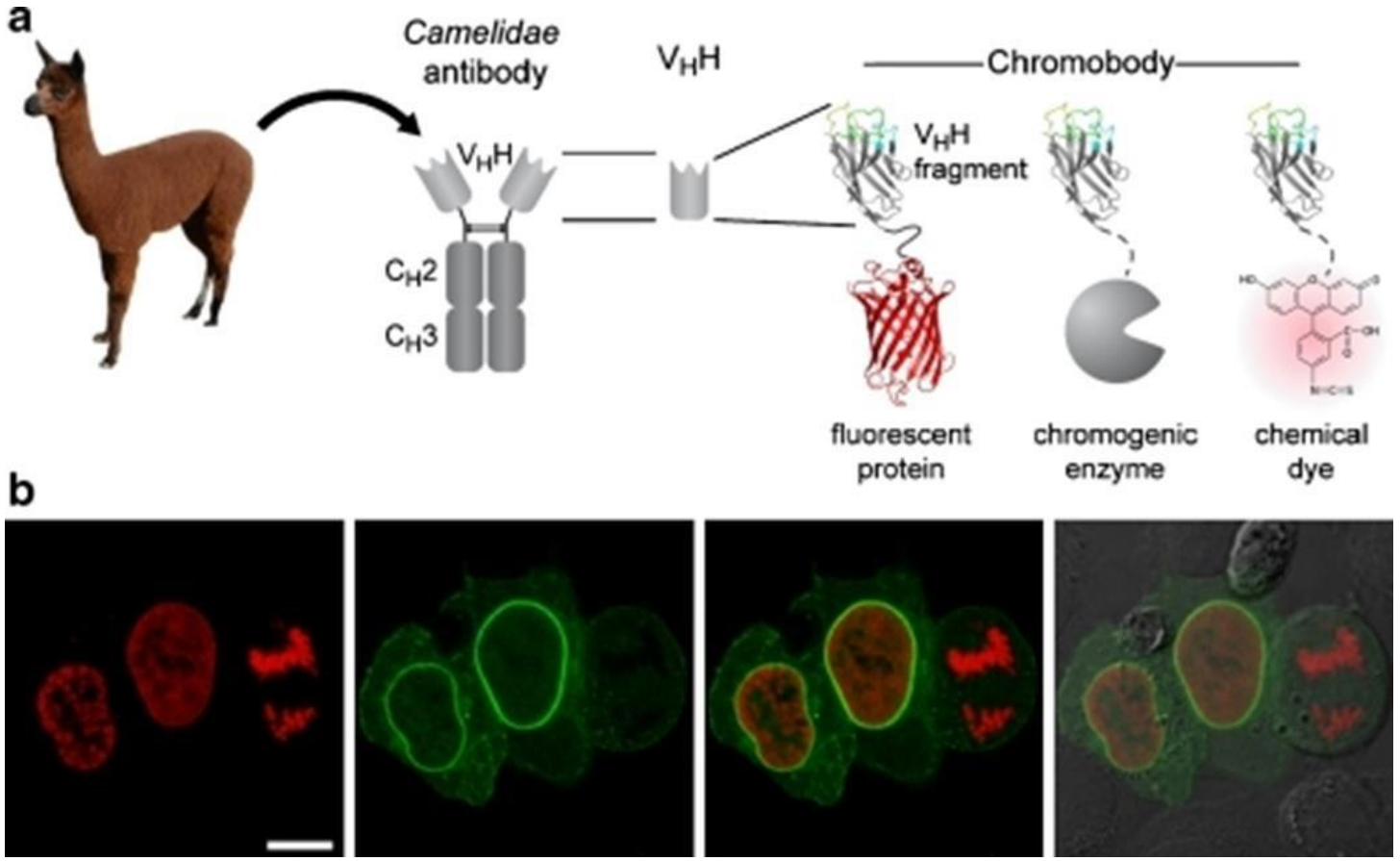
Nanobodies: Detection of hidden epitopes



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

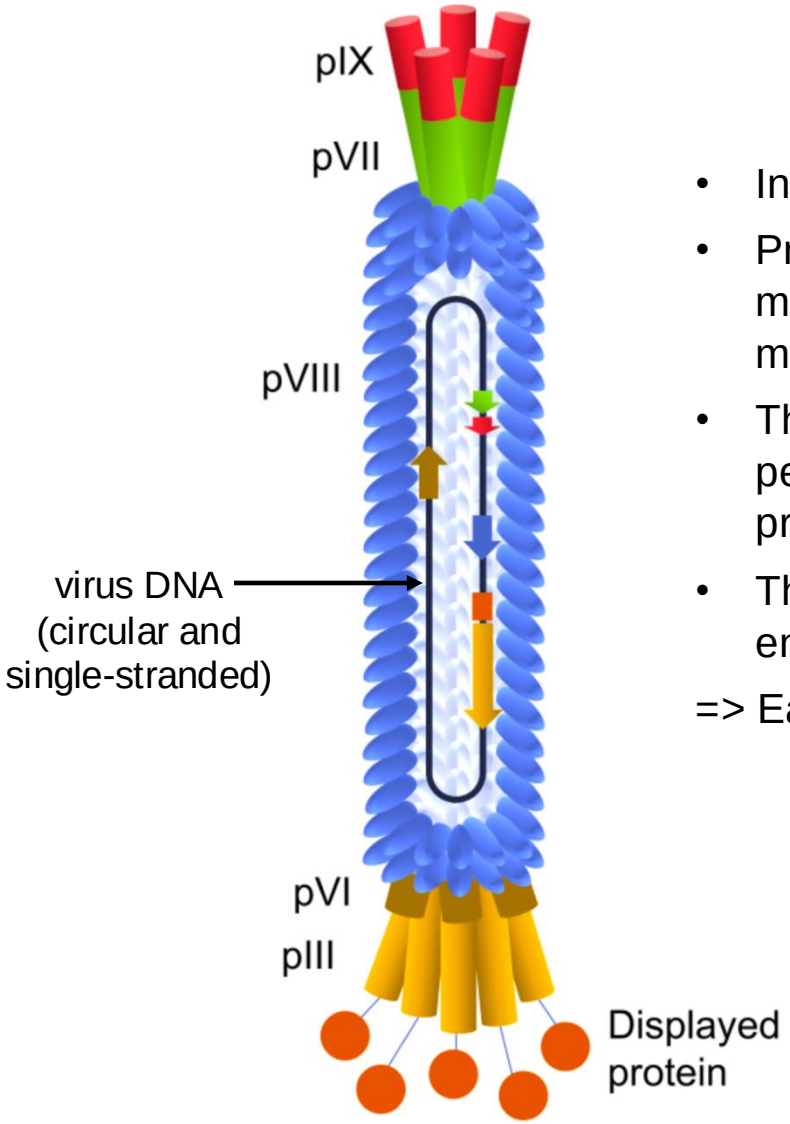
Recombinant nanobodies



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

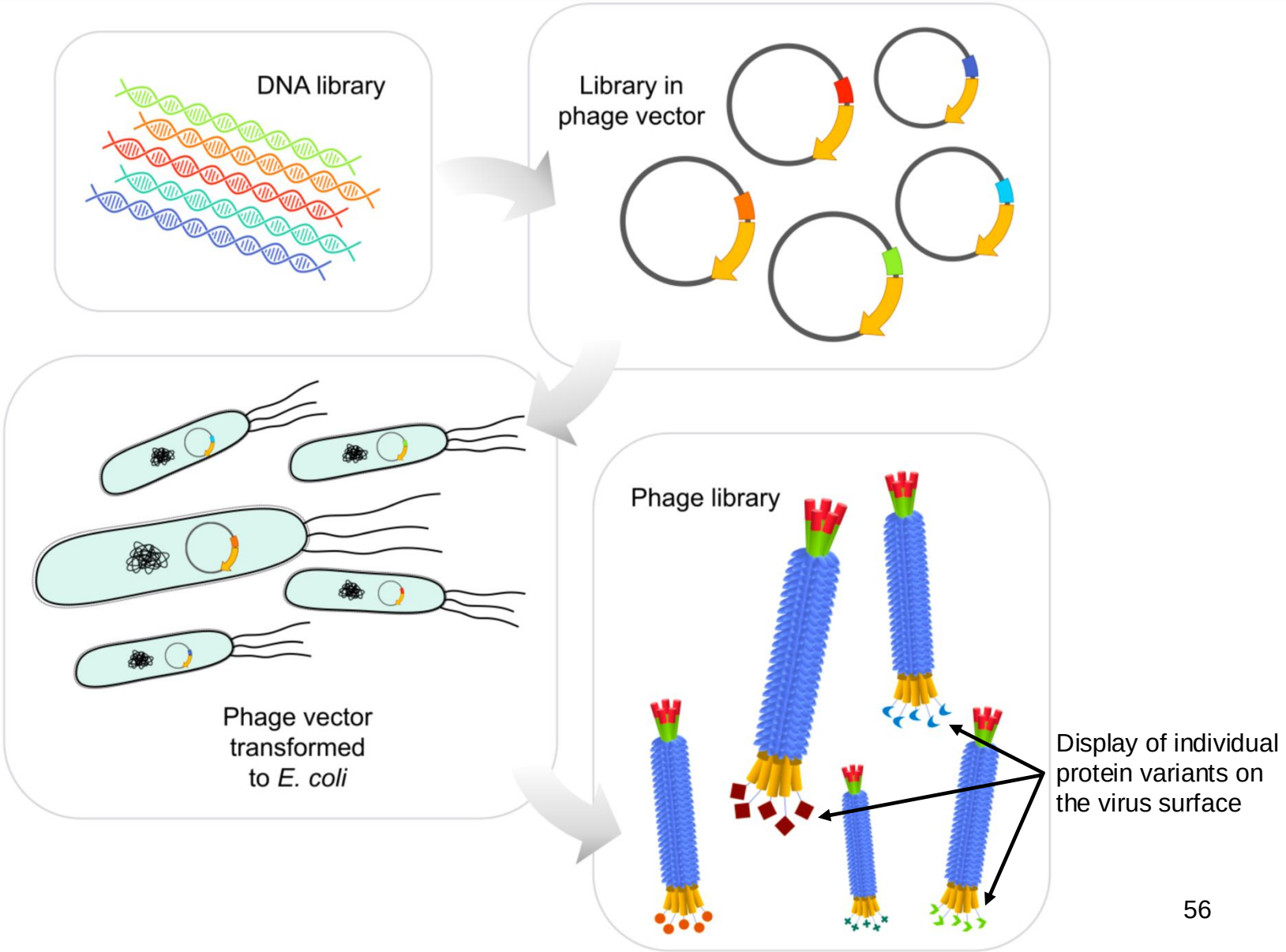


- Infects / replicates in *E. coli*
- Protein coat:
  major coat protein: pVIII
  minor coat proteins: pIII, pVI, pVII, pIX
- The phage can be engineered to display foreign peptides or proteins as a fusion with one of the coat proteins, most commonly pIII.
- The genomic DNA encoding for the coat proteins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

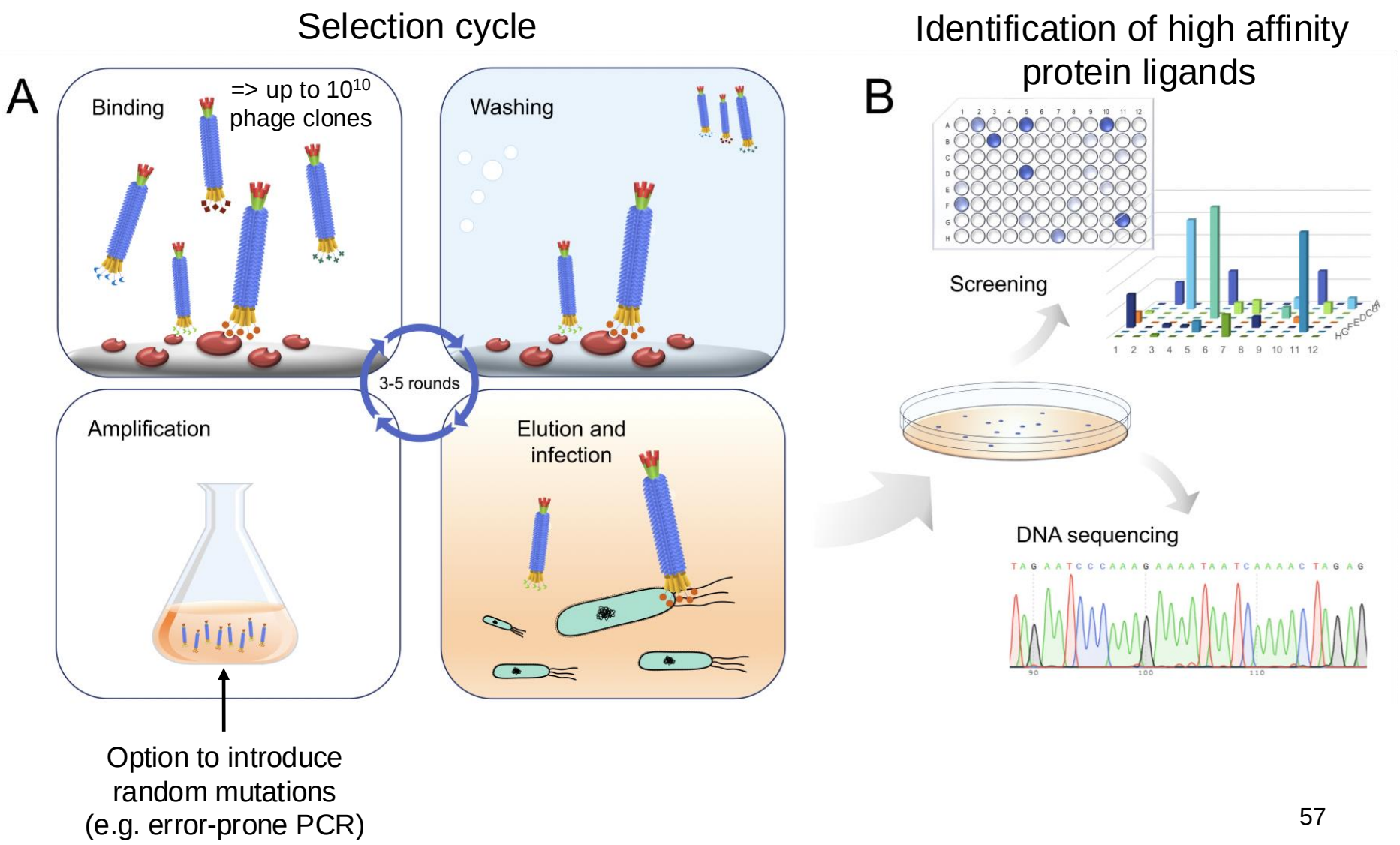
=> Each protein remains connected to its encoding DNA

George Smith / Greg Winter:
Nobel prize in chemistry 2018

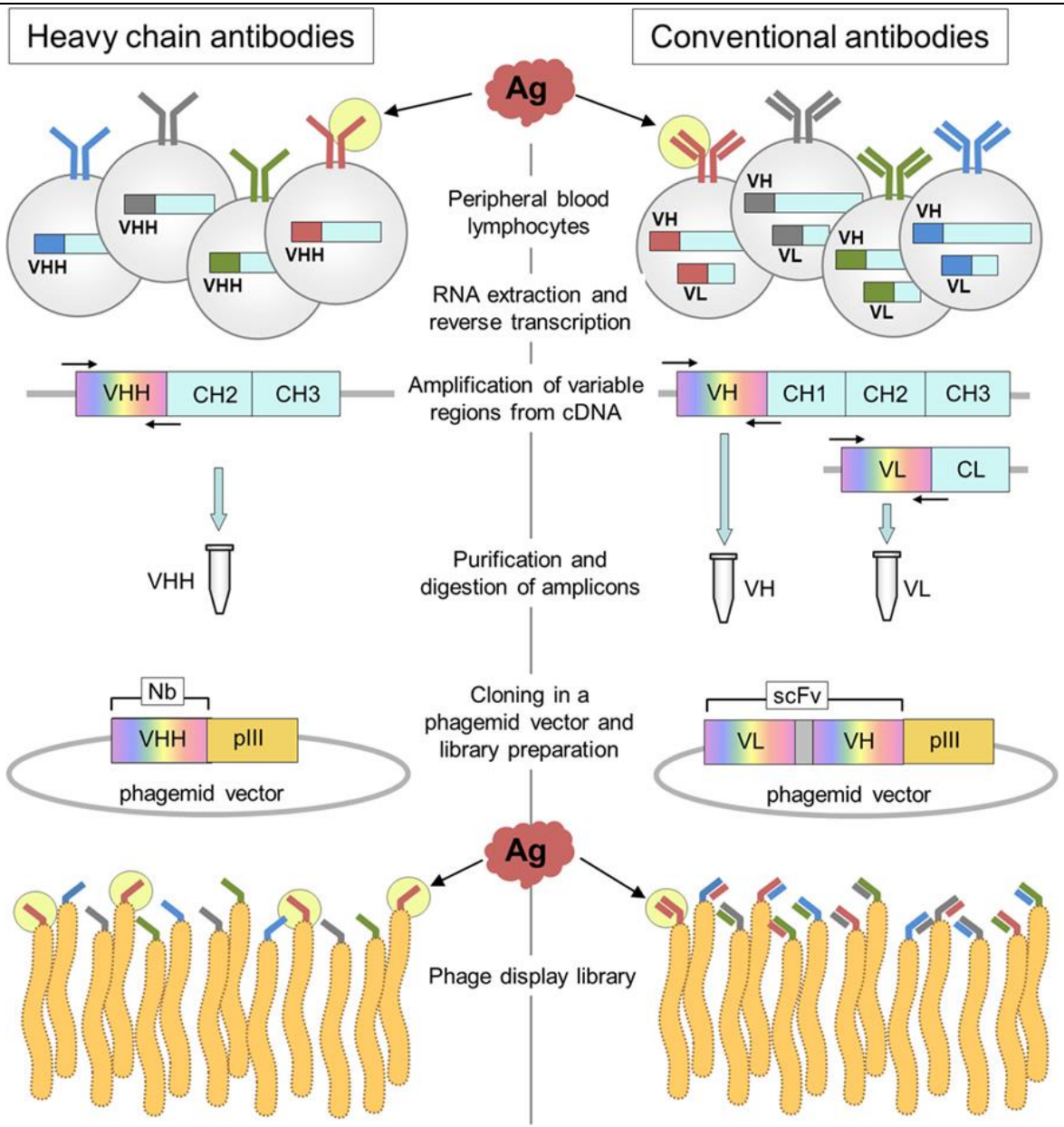
Construction of phage displayed protein libraries



Protein engineering by *in vitro* evolution

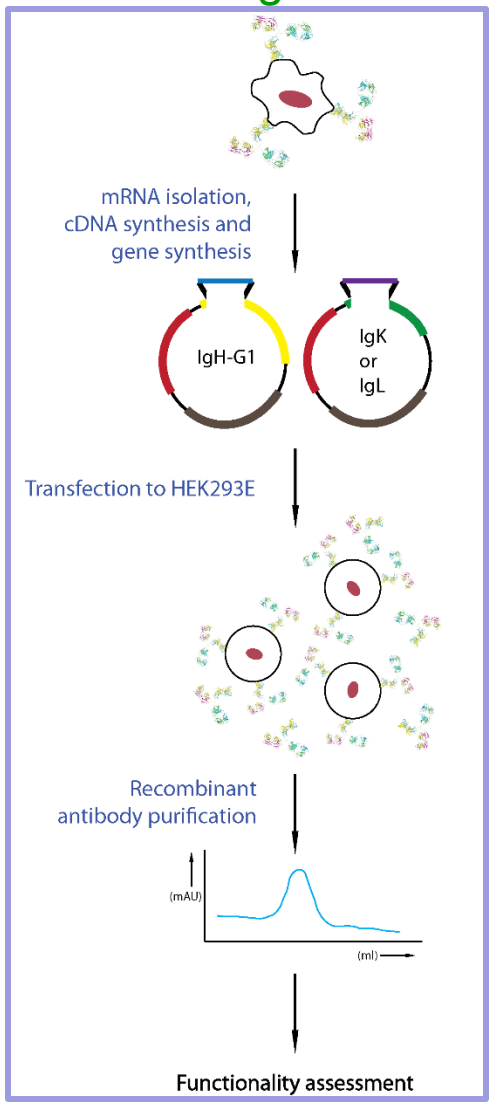


Single-domain antibody (nanobody)

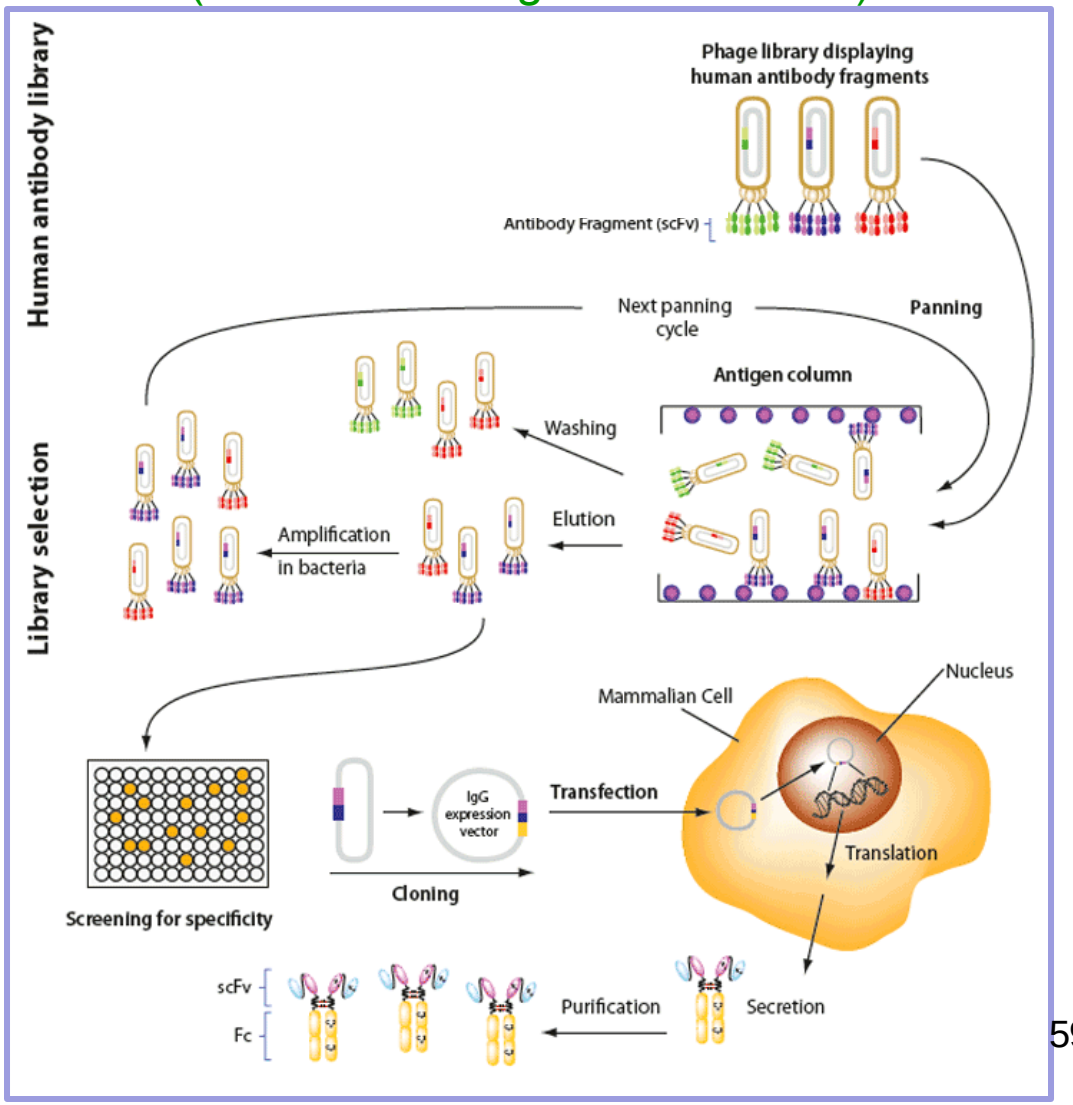


Production of recombinant antibodies

Expression system
(Gene of Ab fragment known)



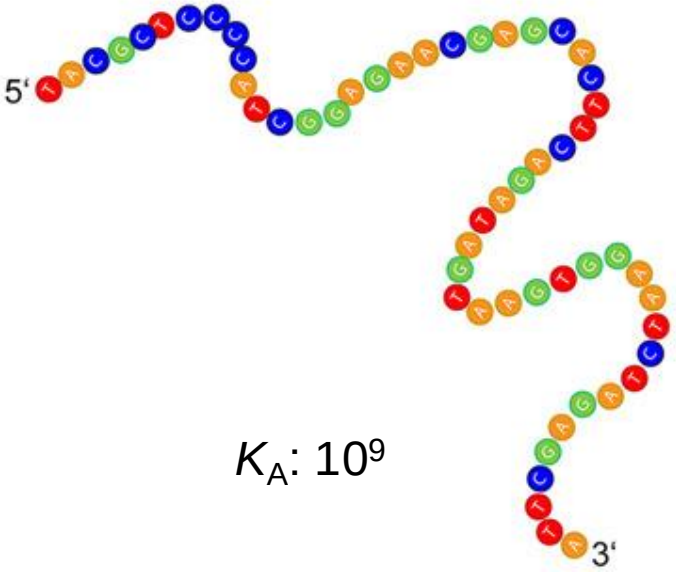
Phage display
(Gene of Ab fragment unknown)



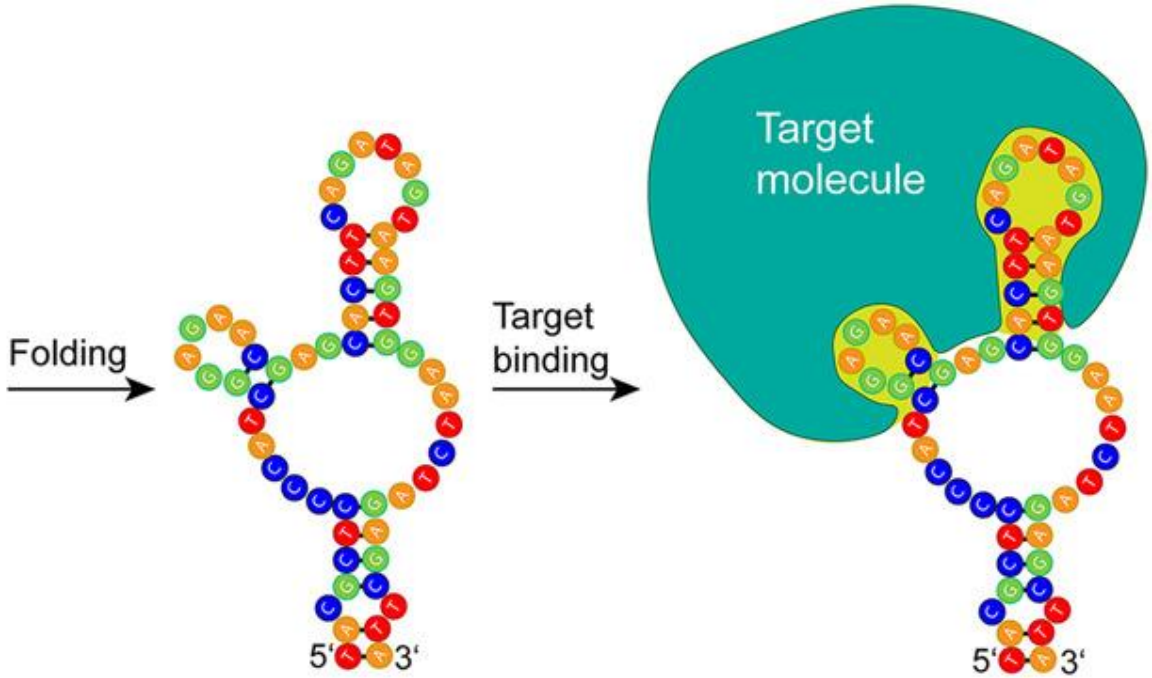
Alternatives for antibodies

Aptamers

RNA or DNA aptamer



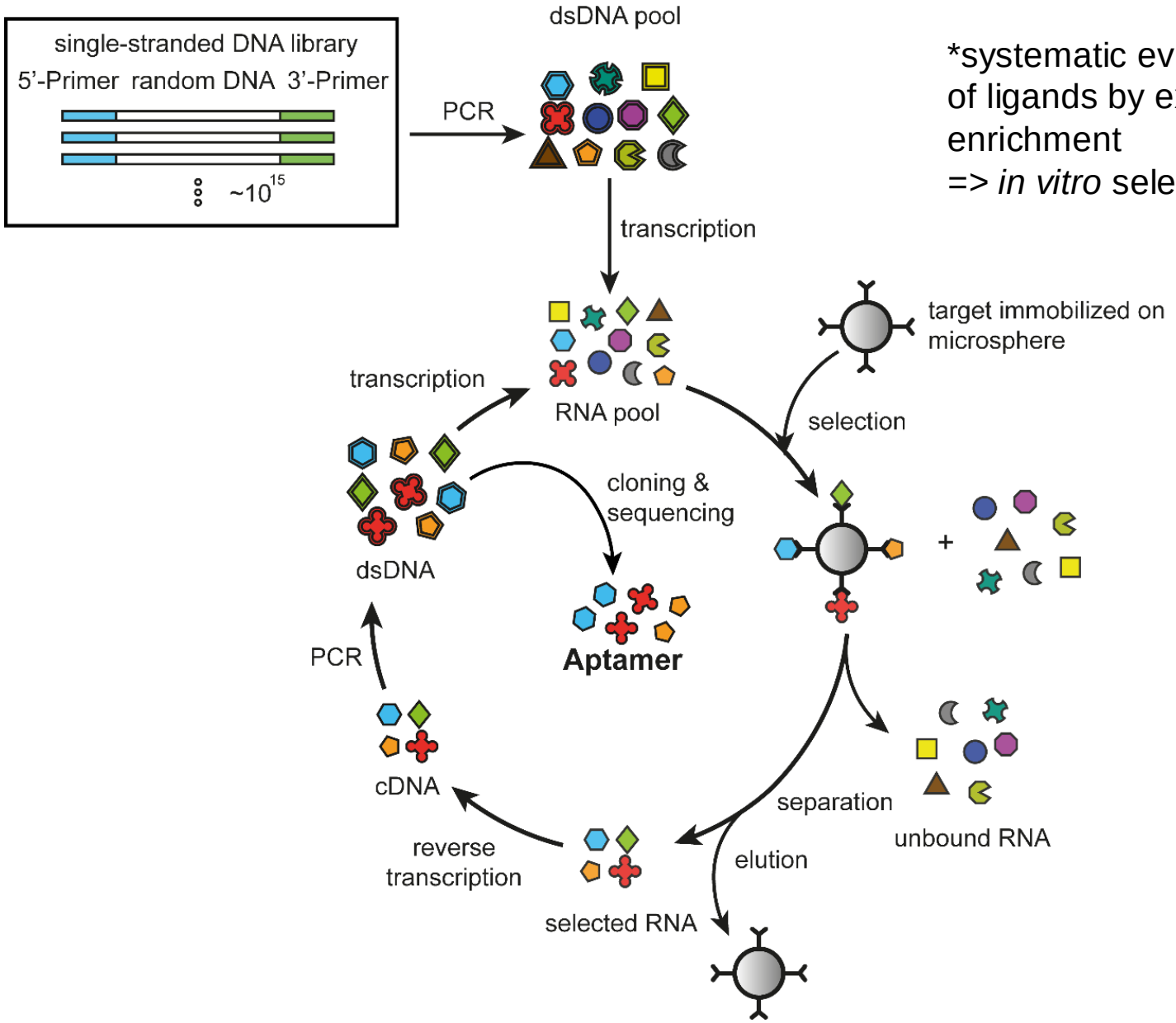
K_A: 10^9



Complementatary base pairing

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

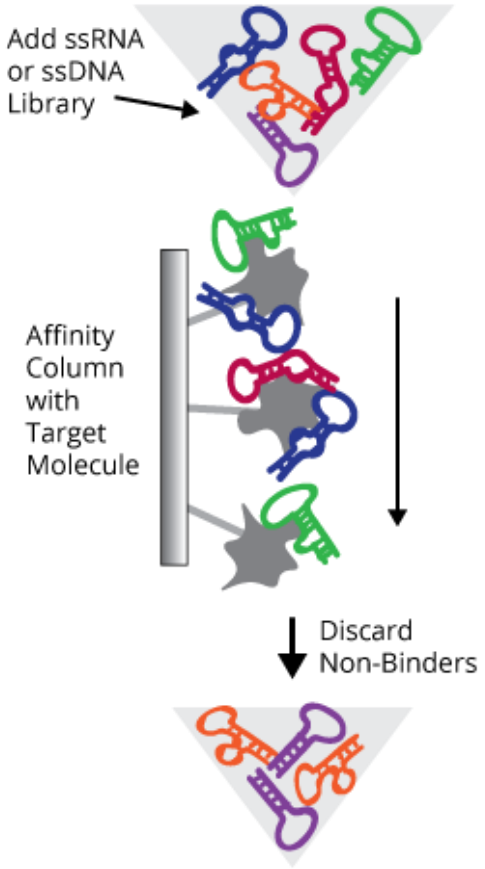
SELEX*



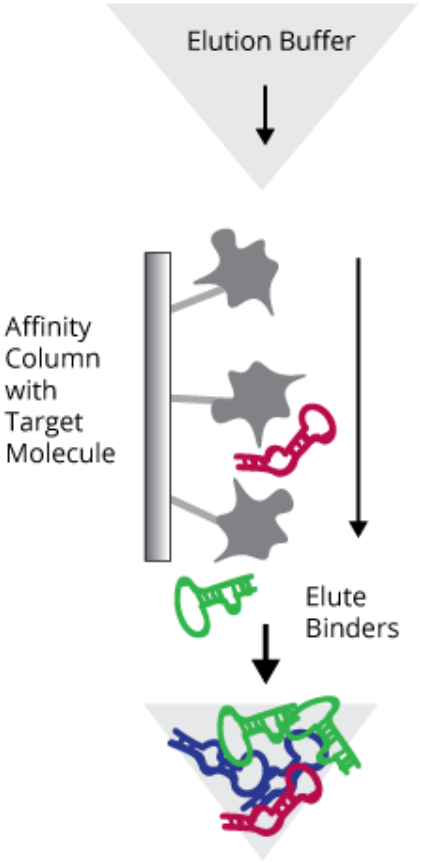
*systematic evolution of ligands by exponential enrichment
=> *in vitro* selection

SELEX*

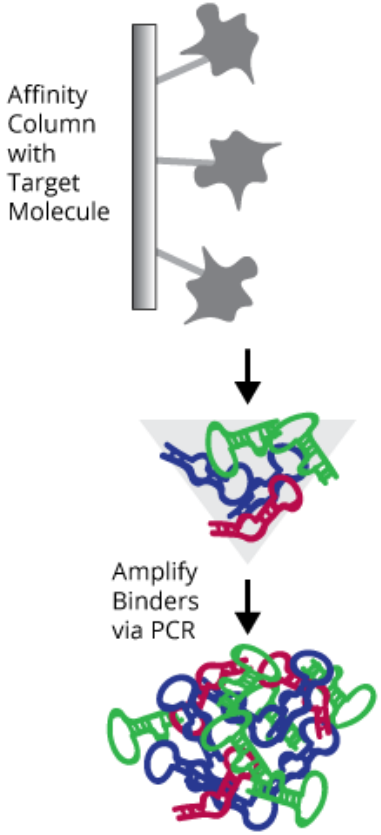
Step 1: Bind oligonucleotide library and discard non-binder



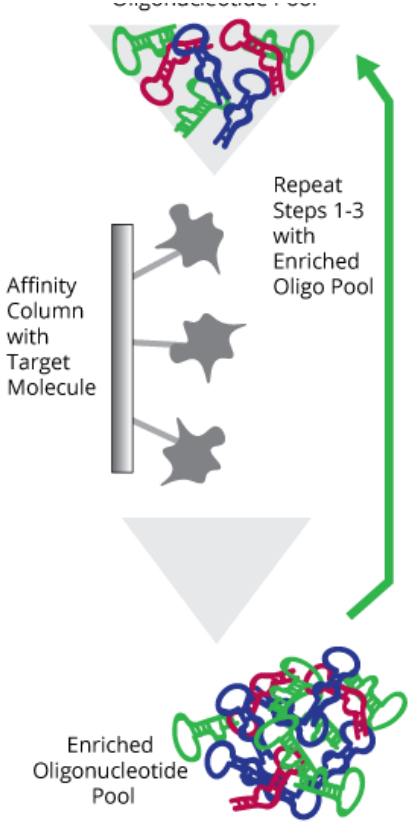
Step 2: Elute oligonucleotides that bind desired targets



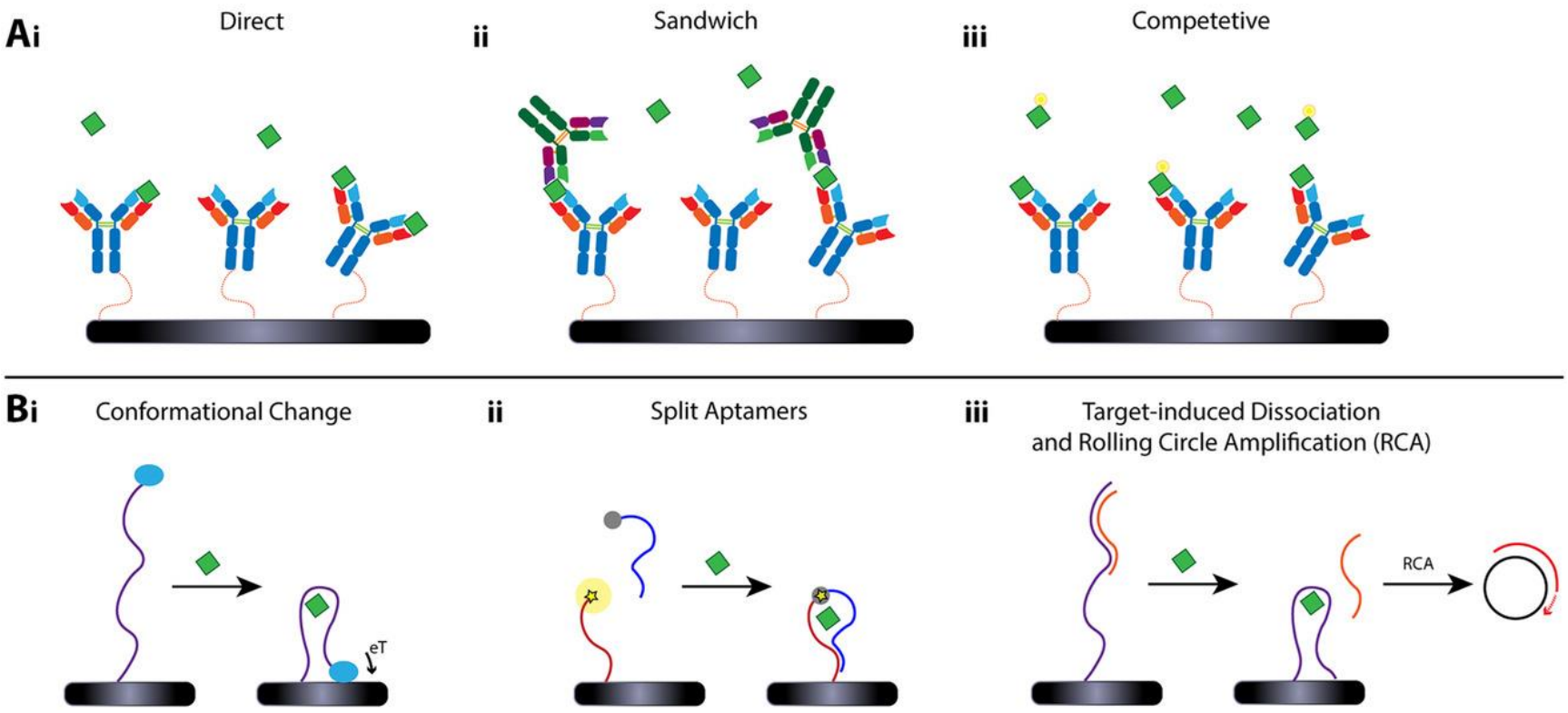
Step 3: Perform PCR to amplify eluted binders



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



Aptamers: Assay designs



Molecularly imprinted polymer (MIP)

"Plastic antibodies"