Antibody Specificity: What's the problem?

The Antibody Society Webcast series – Antibody Validation #1

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Antibodies are known to be specific. So how can there be a problem?

The main reason:

- They have not been checked for specificity
- Specificity cannot be assumed, but must be experimentally verified

What causes non-specific binding, and absence of specific binding?

1. Protein surfaces always bind several things

2. Antigens can be in various conformations, which present different surfaces

3. Composition of an antibody solution may not be what you think it is

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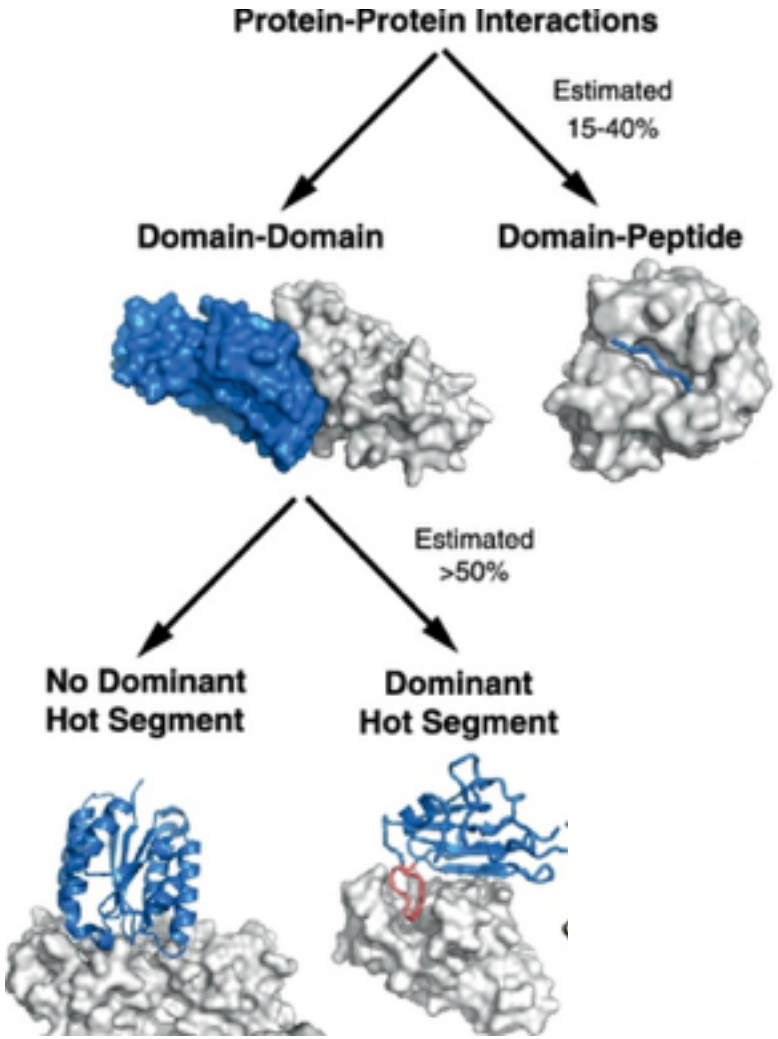
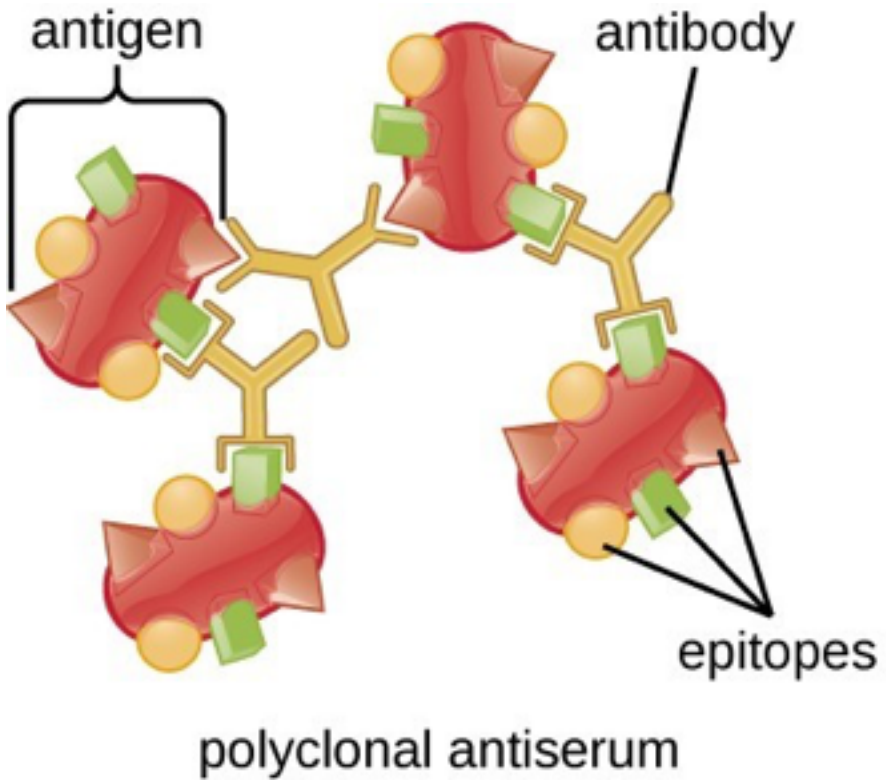
Fundamental properties of proteins: They bind one another!



- Proteins (antigens, antibodies) have the intrinsic property of interacting with other proteins
  - through adventitious hydrophobic patches
  - though adventitious residues that can make hydrogen bonds
- Since antibodies are proteins, they cross-react with proteins (antigens) unrelated to their antigens – albeit at very different affinities

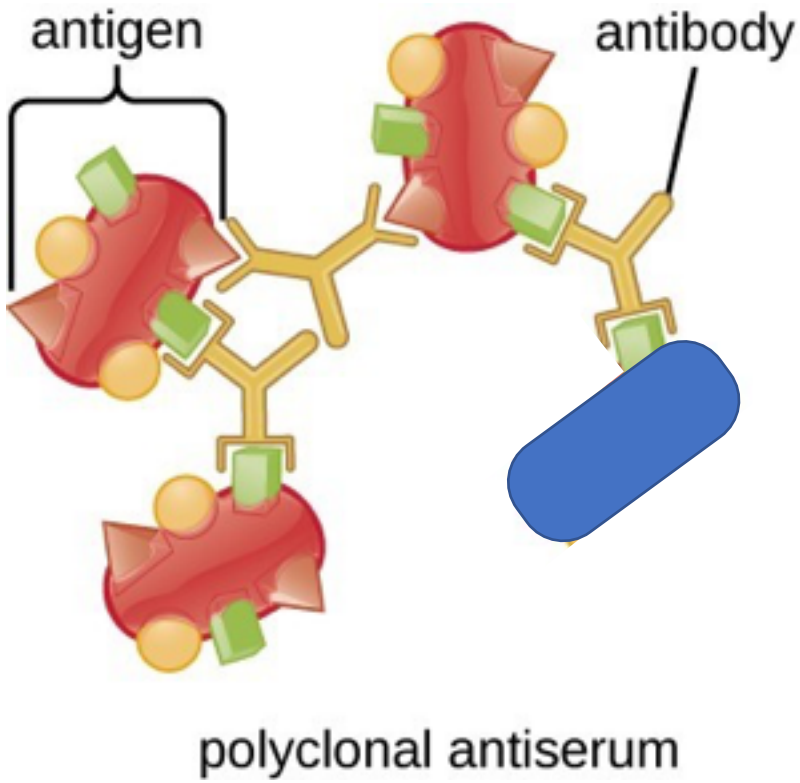
Types of antibodies



Polyclonal antibodies:

- Popular, because they are cheap
  - taken directly from serum
- And can give strong signals
  - they take advantage of many epitopes
  - they can bind bivalently in many orientations

Types of antibodies

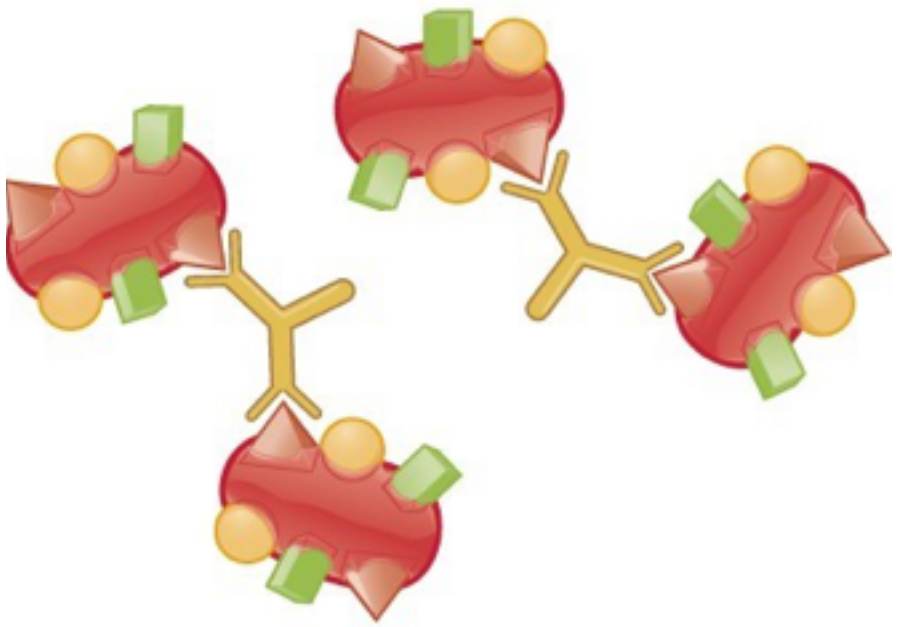


Polyclonal antibodies

But:

- **There are always antibodies in the antisera that crossreact with other components**
- **The composition of two antisera will never be the same**
- **It is impossible to reproduce results from polyclonal sera**

Types of antibodies

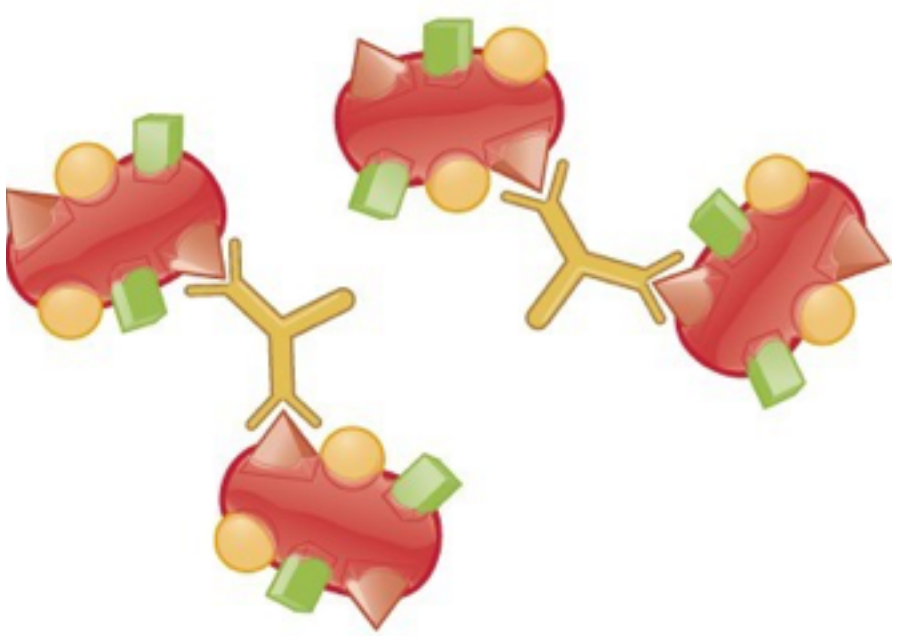


monoclonal antibodies

Monoclonal antibodies

Popular, because they are believed to be automatically super-specific

Types of antibodies



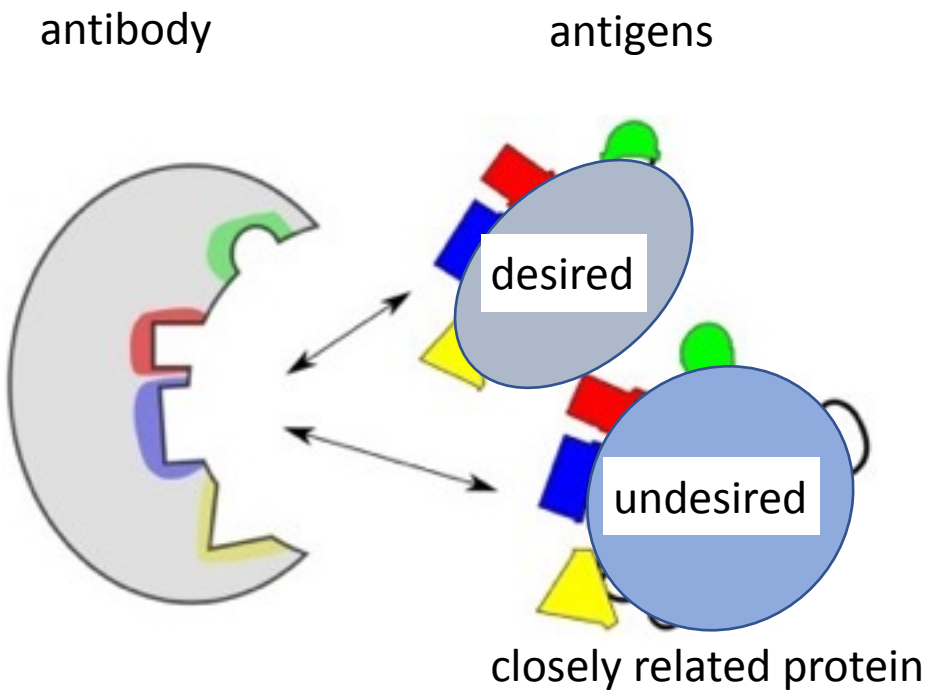
monoclonal antibodies

Monoclonal antibodies

But:

- **They can also crossreact with other proteins**
- **They may detect other proteins <u>better</u> than the desired target**
- **A “monoclonal antibody” is not necessarily monoclonal !**

Types of antibodies



The expected case: related proteins
"Legitimate crossreactivity"

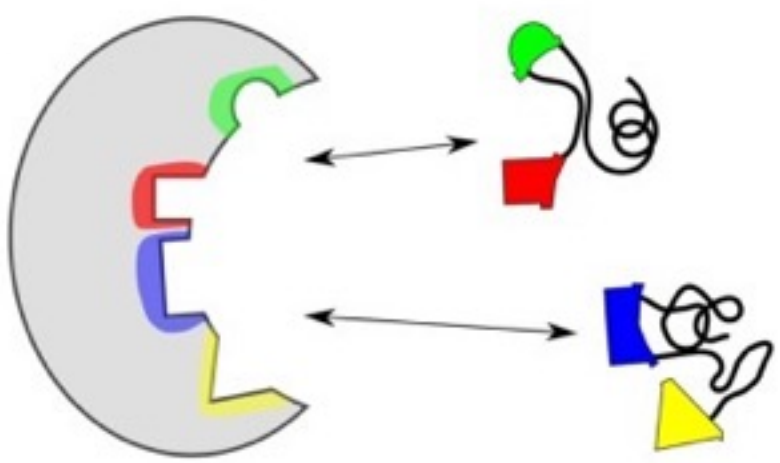
Monoclonal antibodies

But

- **A monoclonal antibody can also crossreact with other proteins**

Types of antibodies

antibody antigens



The (perhaps) unexpected case:
unrelated proteins
- may be unfolded, "sticky"
- may have only few epitopes
"Illegitimate crossreactivity"

Monoclonal antibodies

But

- **A monoclonal antibody can also crossreact with other proteins**
- **If not checked properly, it may detect other proteins better than the desired one**

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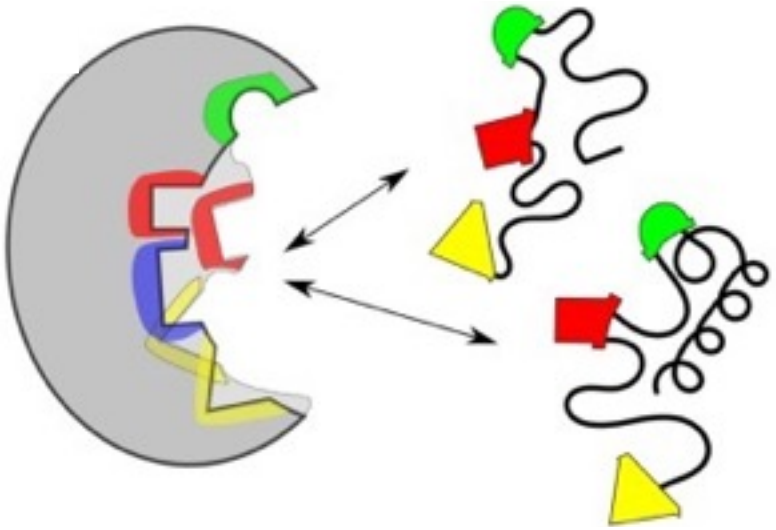
Types of antibodies

antibody antigens



The antibody may even adapt to other targets!
"Illegitimate crossreactivity"

Monoclonal antibodies

But

- **A monoclonal antibody can also crossreact with other proteins**
- **If not checked properly, it may detect other proteins better than the desired one**

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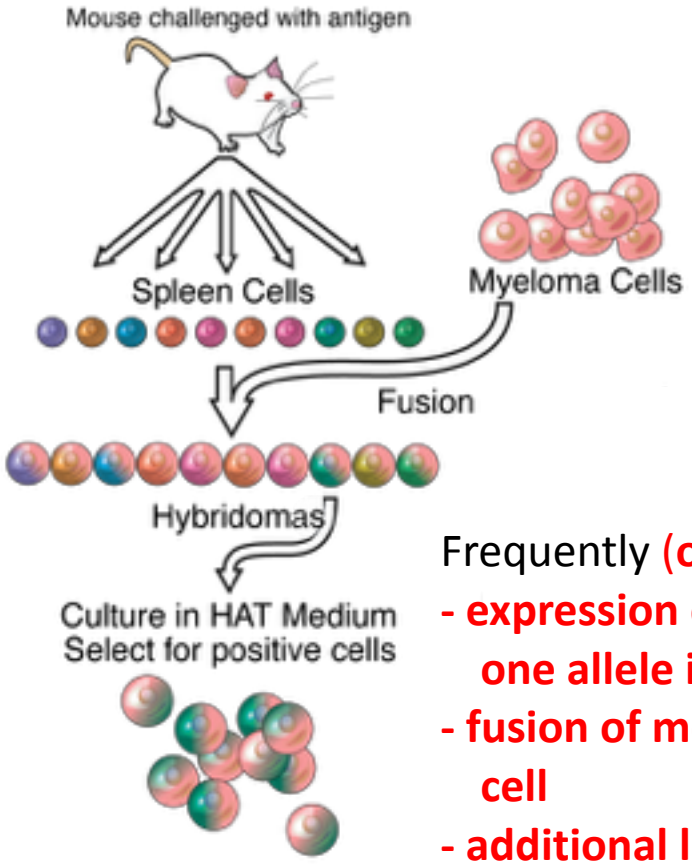
Types of antibodies



Frequently **(one third!):**

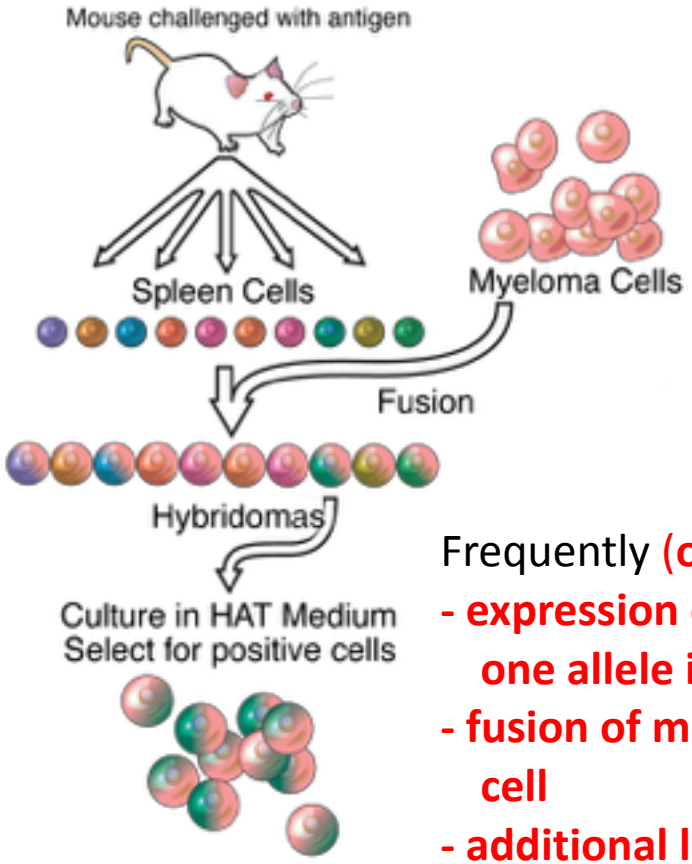
- **expression of more than one allele in B-cell**
- **fusion of more than one B-cell**
- **additional light chains from myeloma fusion partner**

Monoclonal antibodies

But

- **A monoclonal antibody can also crossreact with other proteins**
- **If not checked properly, it may detect other proteins <u>better</u> than the desired one**
- **A monoclonal antibody may not even be monoclonal**

Types of antibodies



Frequently (**one third!**):

- **expression of more than one allele in B-cell**
- **fusion of more than one B-cell**
- **additional light chains from myeloma fusion partner**

Monoclonal antibodies

Yet another problem:

- **As long as the sequence of the antibody has not be determined, you cannot know whether two antibodies are the same**
  - **Manufacturers sell to each other (same antibody, different label)**
  - **Manufacturers produce a new lot, maybe different composition**
- **It may be impossible to reproduce an experiment**

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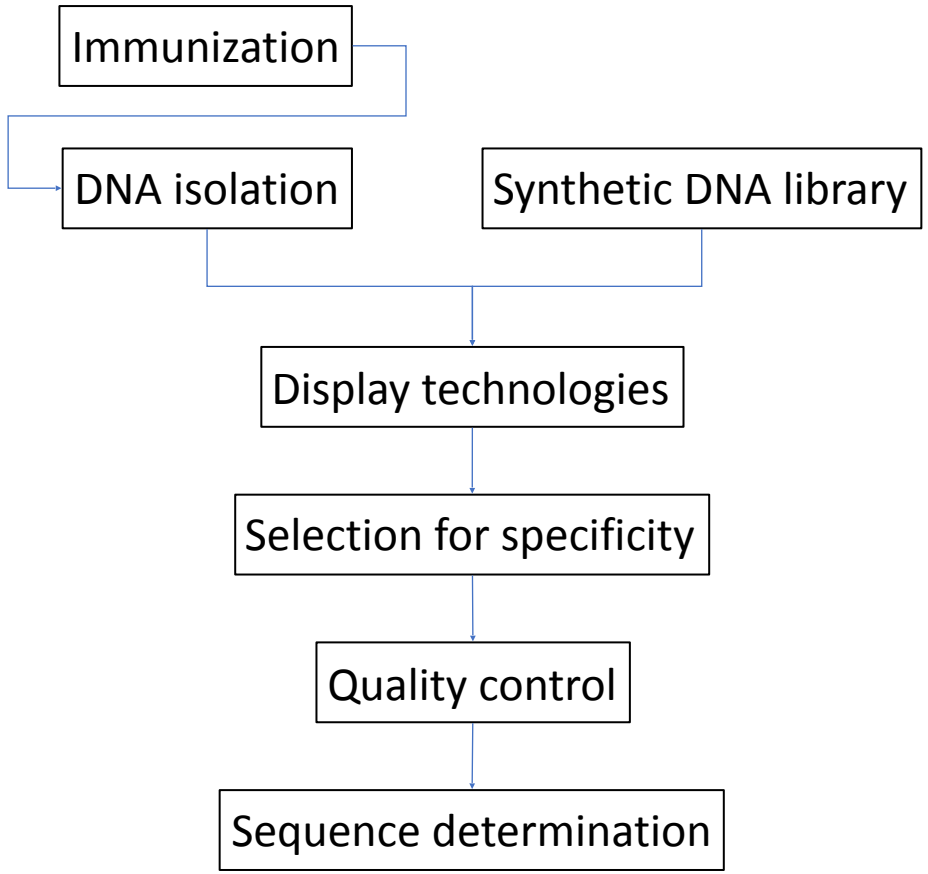
Types of antibodies



Recombinant antibodies

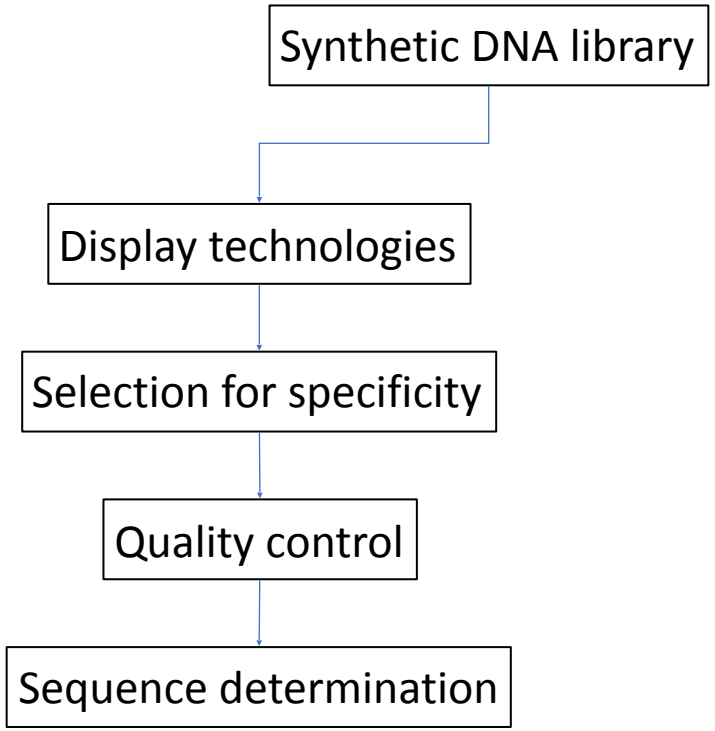
The sequence is known.

It can be reproduced forever, the antibody is "immortal"

Of course, quality control still has to be done as for every antibody!

→ **By most experts, recombinant technologies are seen as the future**

Types of "antibodies"



Recombinant affinity reagents

The antibody itself has become dispensable

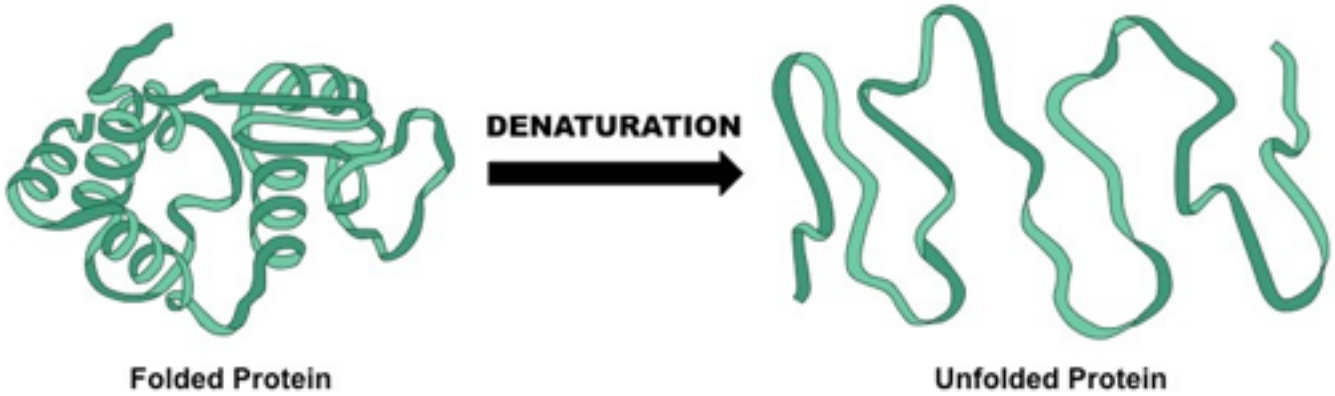
Affinity reagents can be used that are much more stable than antibodies

➔ Other non-antibody scaffolds

These can be produced much more cheaply

➔ **By most experts, recombinant technologies are seen as the future**

Types of antigens



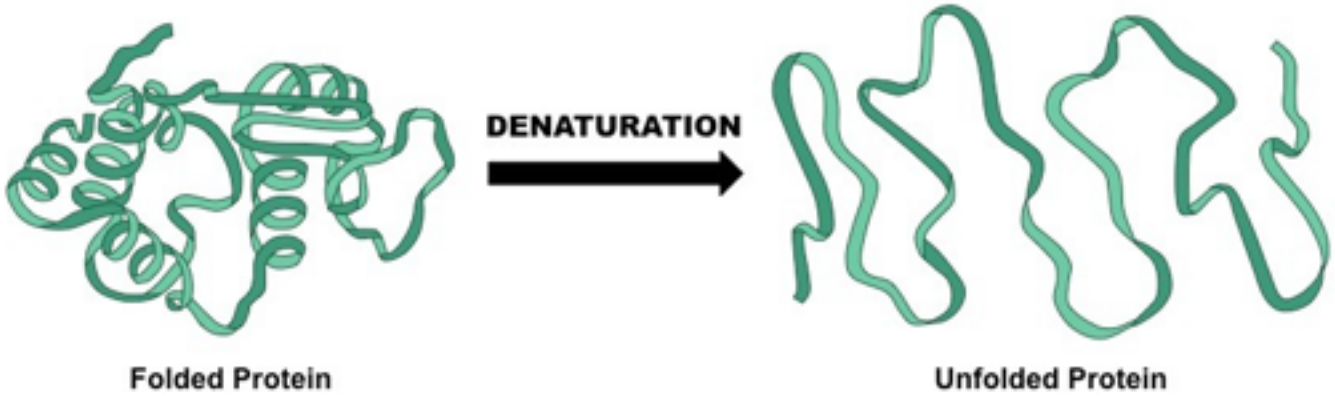
Folded proteins

- usually the cellular state
- usually more soluble

Denatured (unfolded proteins)

- usually expose hydrophobic residues, become more "sticky"
- usually need to be kept in solution by detergent (SDS), or denaturant (urea, GdnHCl)

Types of antigens



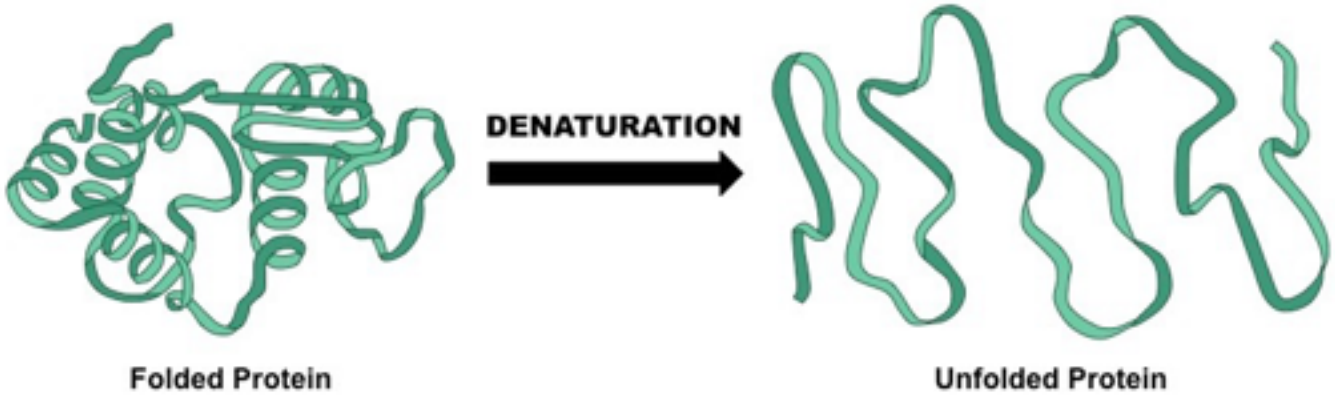
Folded proteins

- in cell extracts (pull-down assays)
- on the cell surface (FACS experiments)

Denatured (unfolded proteins)

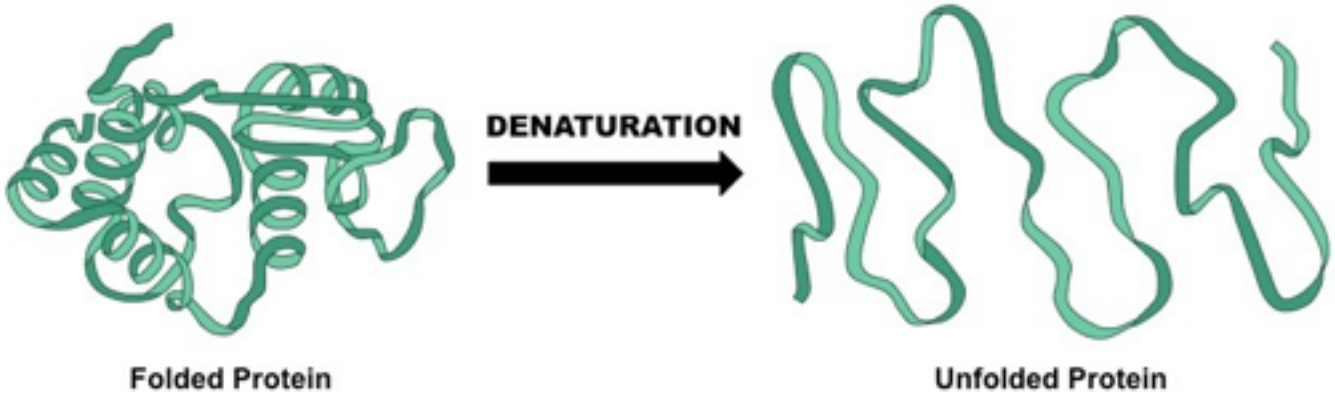
- after SDS-Gel electrophoresis (Western blots)
- after proteolytic digestion
- after tissue fixation (antigen "retrieval" with a microwave oven!)

Types of antigens



- Many conditions can denature a protein:
  - antibodies that recognize the native state no longer bind
- Heat, shaking (=foam), loss of ligands, loss of metals, loss of subunits,...

Types of antigens



Antibodies can recognize

<u>conformational epitopes</u>, which will only be accessible in the folded protein

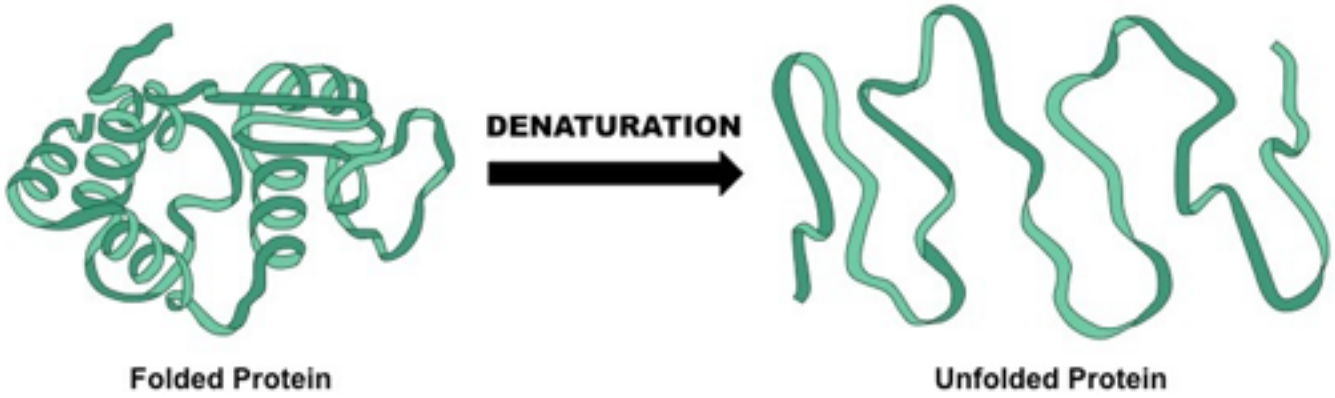
- residues that are typically on the surface, but far apart in sequence

Antibodies can recognize

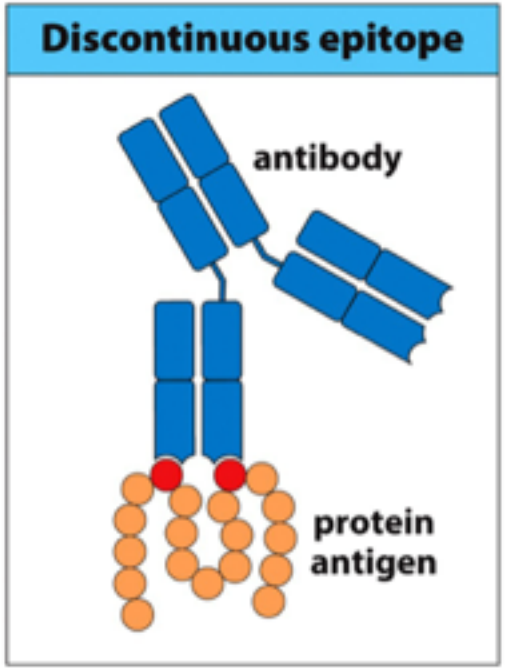
<u>linear epitopes</u>, which will only be accessible in a denatured protein, or in peptide digest

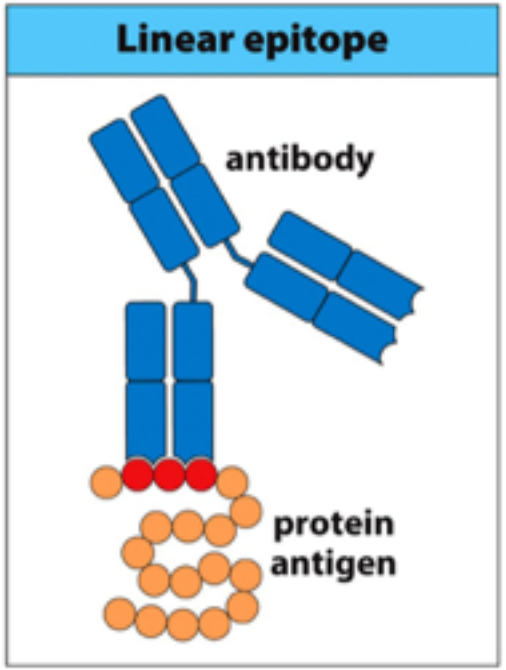
- residues that are close in sequence but may be hidden in interior

Types of antigens

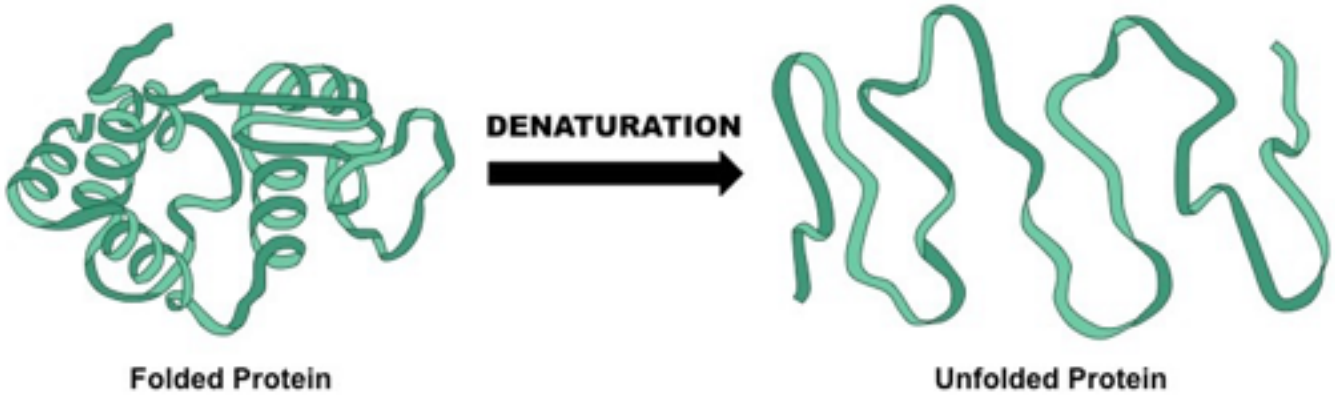


Most antibodies can only recognize either the folded or the unfolded state!





Types of antigens

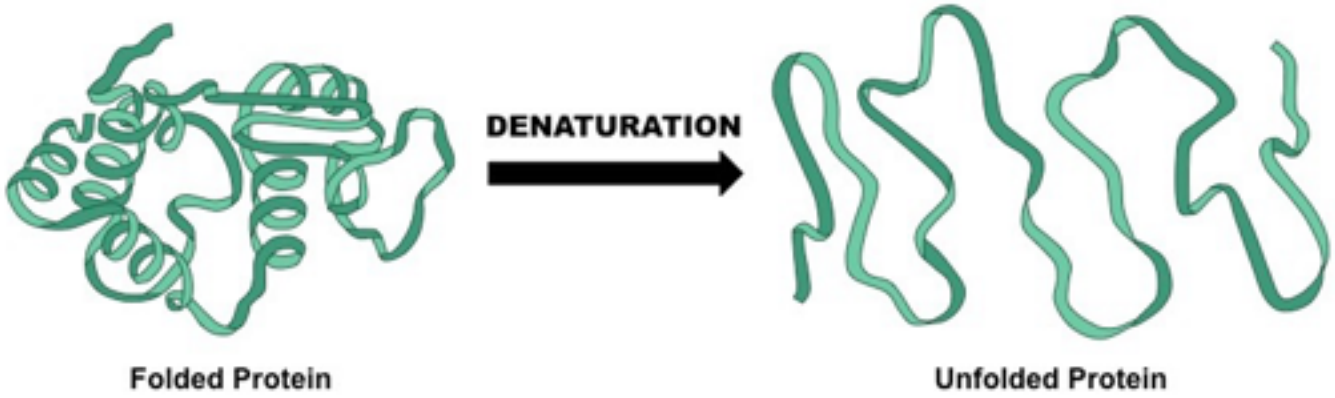


Most antibodies can only recognize either the folded or the unfolded state!

Most antibodies can thus only be used only for

- either Western blots, IHC (unfolded state recognition)
- or FACS, pull-downs (native state recognition)

Types of antigens



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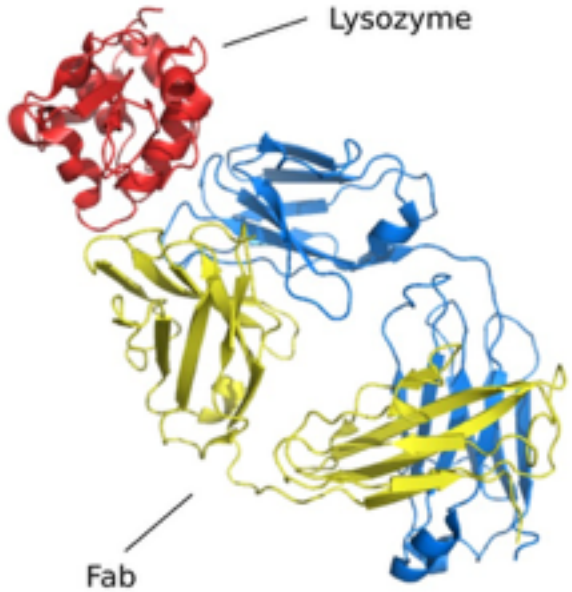
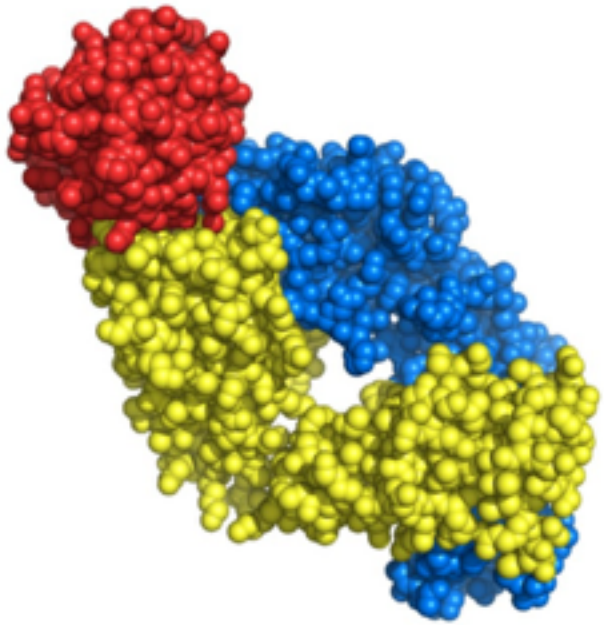
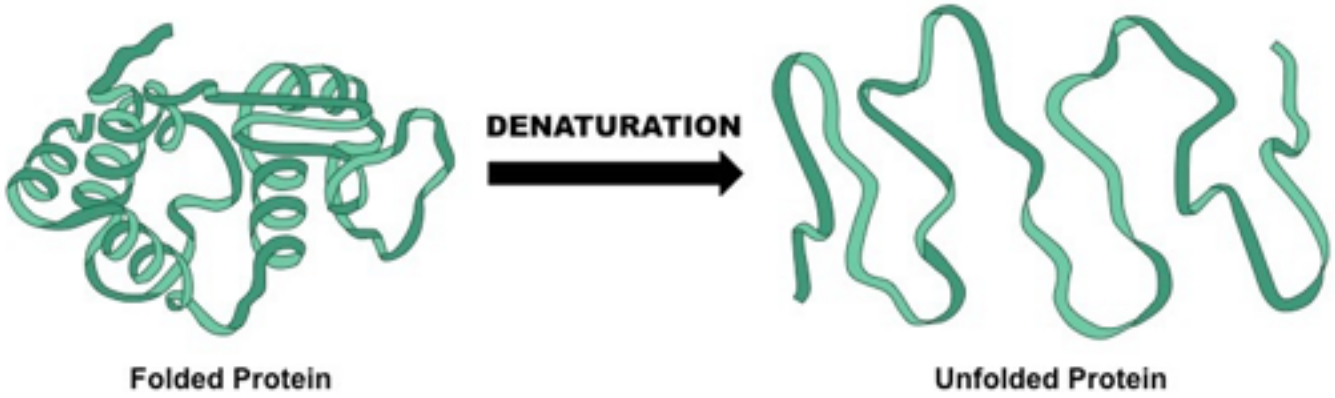
folded





Types of antigens



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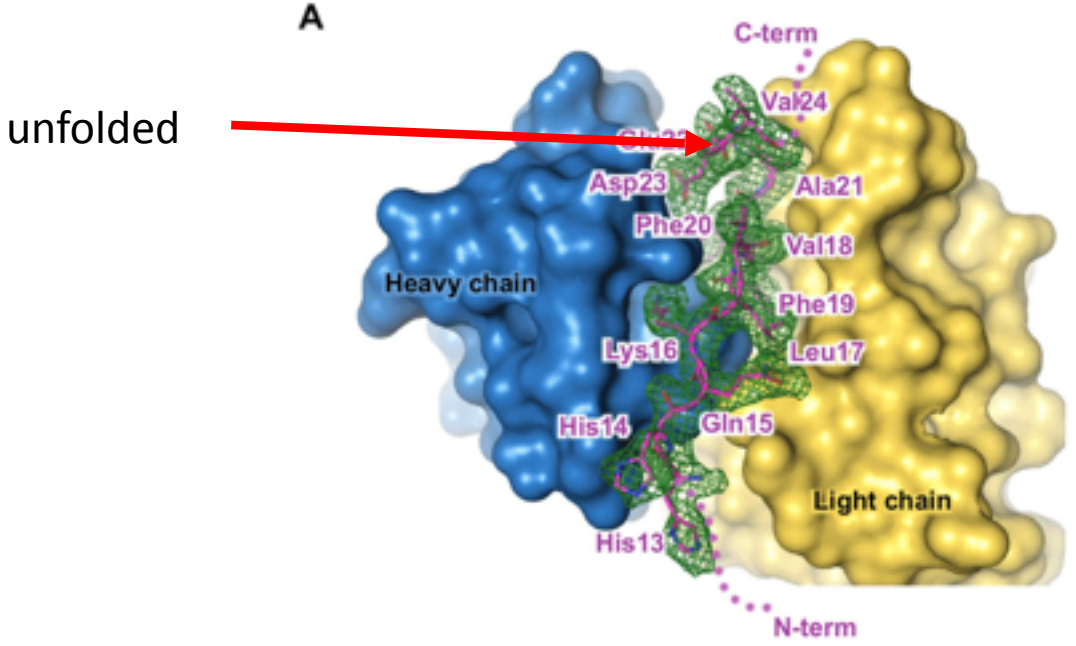
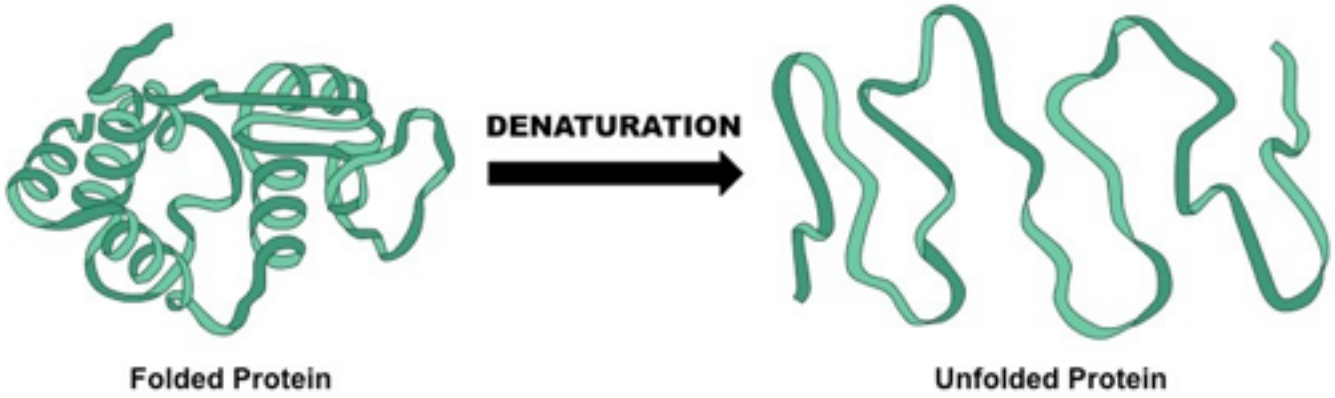


Types of antigens



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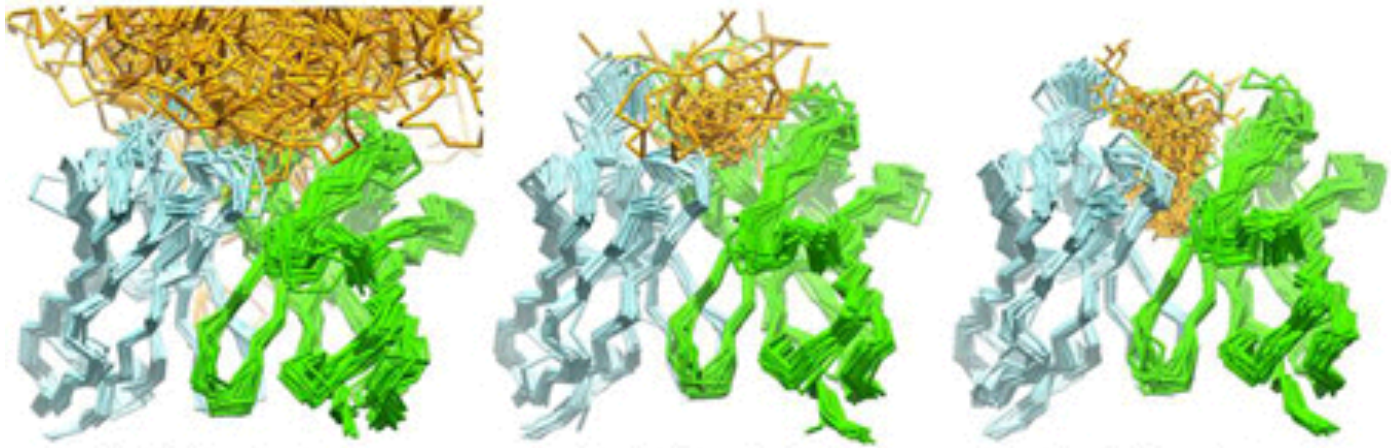

(or peptide side chain)

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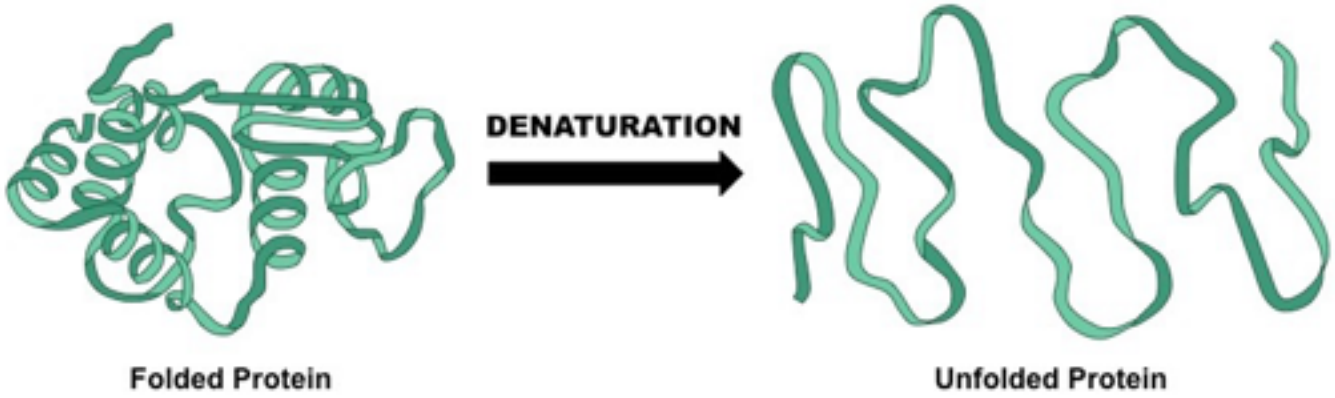
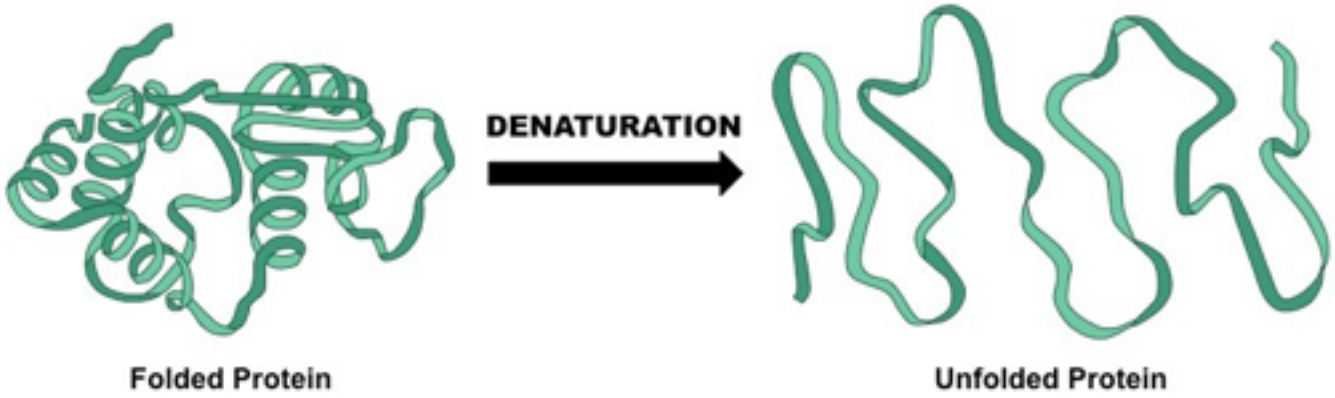
Types of antigens



Quality-controlling antibodies is by definition application specific!

- You must check antigen recognition in the state of the antigen that will be used later
- Cross-reactivity will also depend on context:
  - other denatured proteins
  - other cell components?
  - non-proteins contaminants?

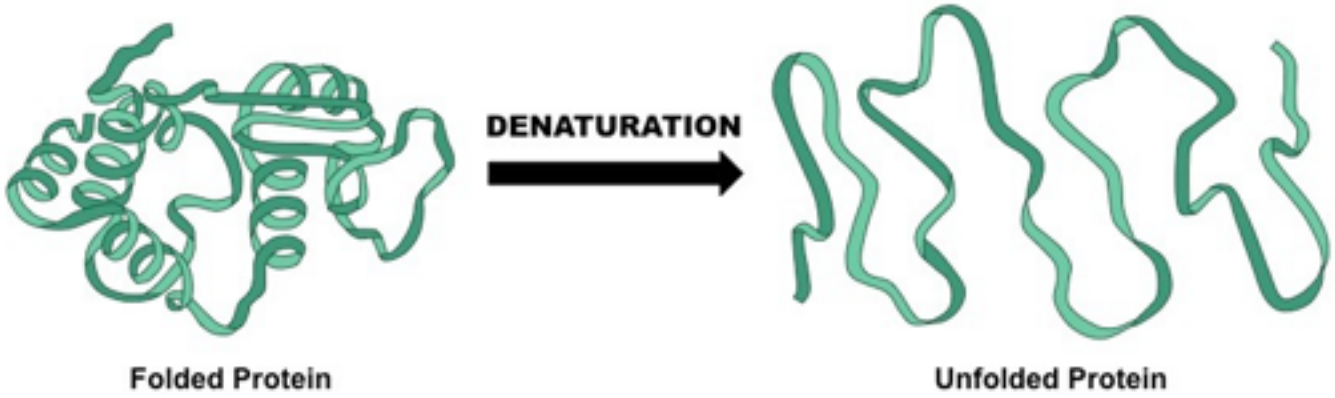
Types of antigens



What about ELISA:

- In order to bind to polystyrene, at least part of the protein **must** denature!
- Small proteins will almost certainly denature
- Most peptides will not even bind (can be biotinylated)
- Large proteins: often, only one domain denatures, the rest remains folded. (Also true for many IgGs themselves)

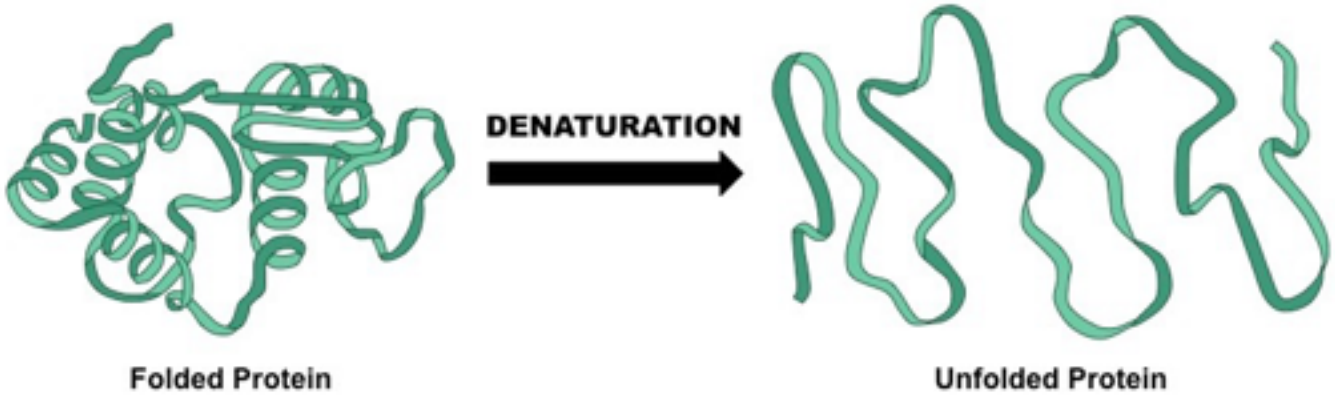
Types of antigens



What about Immunohistochemistry:

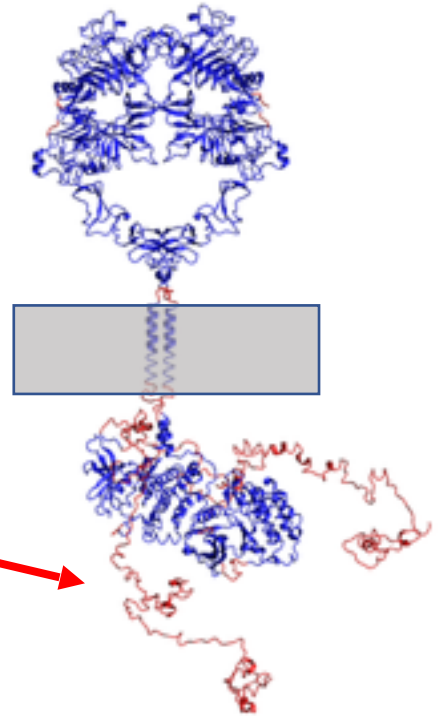
- Antigens are typically crosslinked, epitopes are blocked
- Antigen "retrieval" (heat) denatures the antigen
- Only a small subset of epitopes is suitable for IHC
- It is still very difficult to mimic the "IHC conformation" in vitro, and thus to test it outside an IHC experiment.

Types of antigens

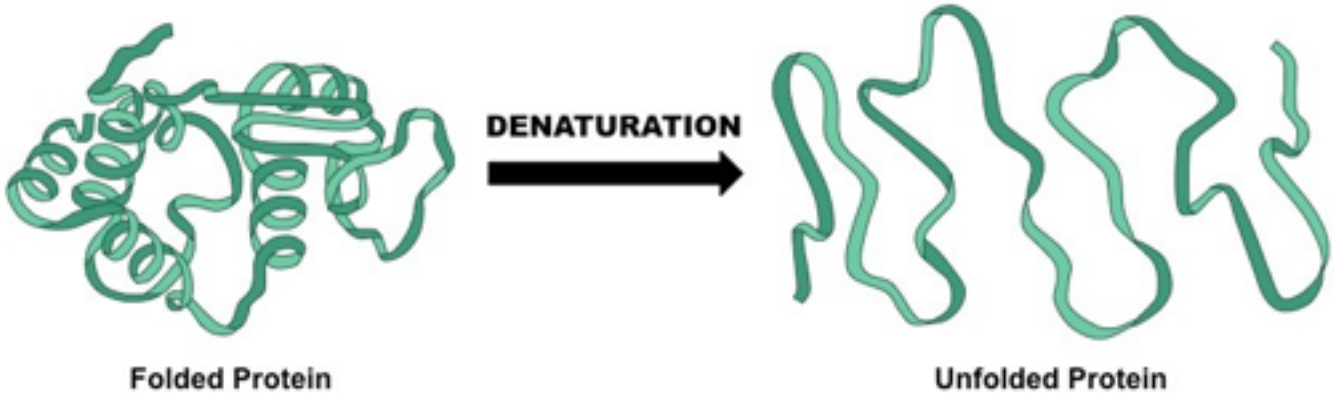


Are there antibodies can work both in several applications?

- Yes, but only if they are made against a piece of the protein which is always unfolded, e.g. termini (tails) receptors



Types of antigens



Are there antibodies can work both in several applications?

- Yes, polyclonal antibodies
- **BUT: they come with the very high price of cross-reactivities almost impossible to control.**

Summary

1. Cross-reactivity of antibodies is to be expected. Therefore, it must be checked
2. Monoclonal antibodies are **not** specific by definition. They must be checked
3. Cross-reactivity is application-specific
4. Recombinant antibodies are defined, identifiable and distinguishable by their sequence – unlike conventional monoclonal antibodies, whose sequence is **not** known.

   But recombinant antibodies must undergo the **same** checks for cross-reactivity

Antibody Specificity: What's the problem?

The Antibody Society Webcast series – Antibody Validation #1

Andreas Plückthun
University of Zürich

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Next Webcast in Antibody Validation: a 9-part series

1.	Andreas Pluckthun	:	Antibody Specificity: What's the problem?
2.	**Glenn Begley**	**:**	**Antibodies and the reproducibility crisis in biological science**
	Cecilia Williams	**:**	**The Erß story – is your antibody like this?**
3.	Jan Voskuil	:	Beware the supplier OEM
	Andy Chalmers	:	Finding antibodies in the Antibody Databases
4.	Anita Bardowski	:	Which antibody are you looking for? The RRID
	Jan Voskuil	:	Points to note on the supplier datasheets
5.	Giovanna Roncador:	:	Correct positive and negative controls in validation
6.	Aldrin Gomes	:	Standard technology: "even" Western blots are non-trivial
	Jim Trimmer	:	IHC issues in brain sciences
7.	Travis Hardcastle	:	Cell KO technology
	Alejandra Solache	:	Validating Antibodies with KO technology
8.	Mike Taussig	:	Validating antibodies using array technologies
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9.	Andrew Bradbury	:	Why publish sequences?
	Andreas Pluckthun	:	What are the coming alternatives ?

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Validation of Commercial tool Antibodies

Antibody Specificity: What's the problem?

The Antibody Society Webcast series – Antibody Validation #1

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Produced and Directed by Simon L. Goodman

Production Manager Fran Breden

Writen by Simon Goodman

https://www.antibodysociety.org/

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Validation of Commercial Tool Antibodies

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