Affinity Chromatography

Overview and applications to antibody production

ligand interactions

- Affinity Chromatography is based upon ligand Interactions
- Based upon “lock and key principal” or “
- “Fit and attraction”
- These Pairings can be specific protein to protein
- Or chance generic fitting of a small molecule into a pocket

proteins can have specific interaction partners

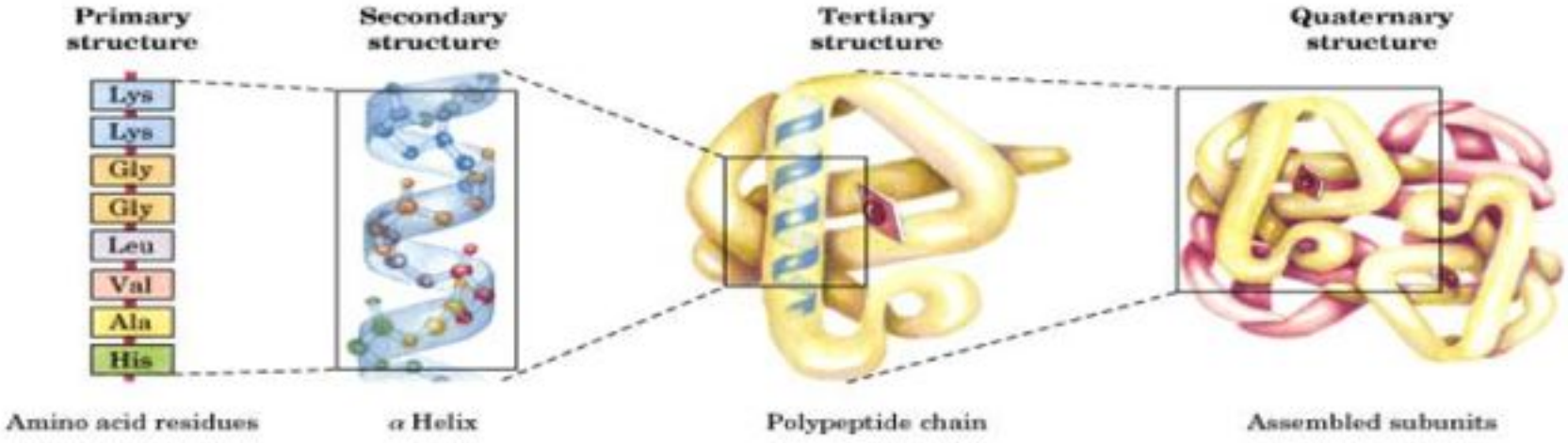
Examples :

- ICAM LFA (Lymphocyte adhesion molecules)
- Lectins- Glycoconjugates (Carbohydrate)
- Enzymes- inhibitors– Alpha I PI
- **Protein A- Antibodies**
- **Antibodies- Antigens**

For Chromatography purposes these interactions have to be ***reversible***

First things First: Protein structure

The levels of protein structure:

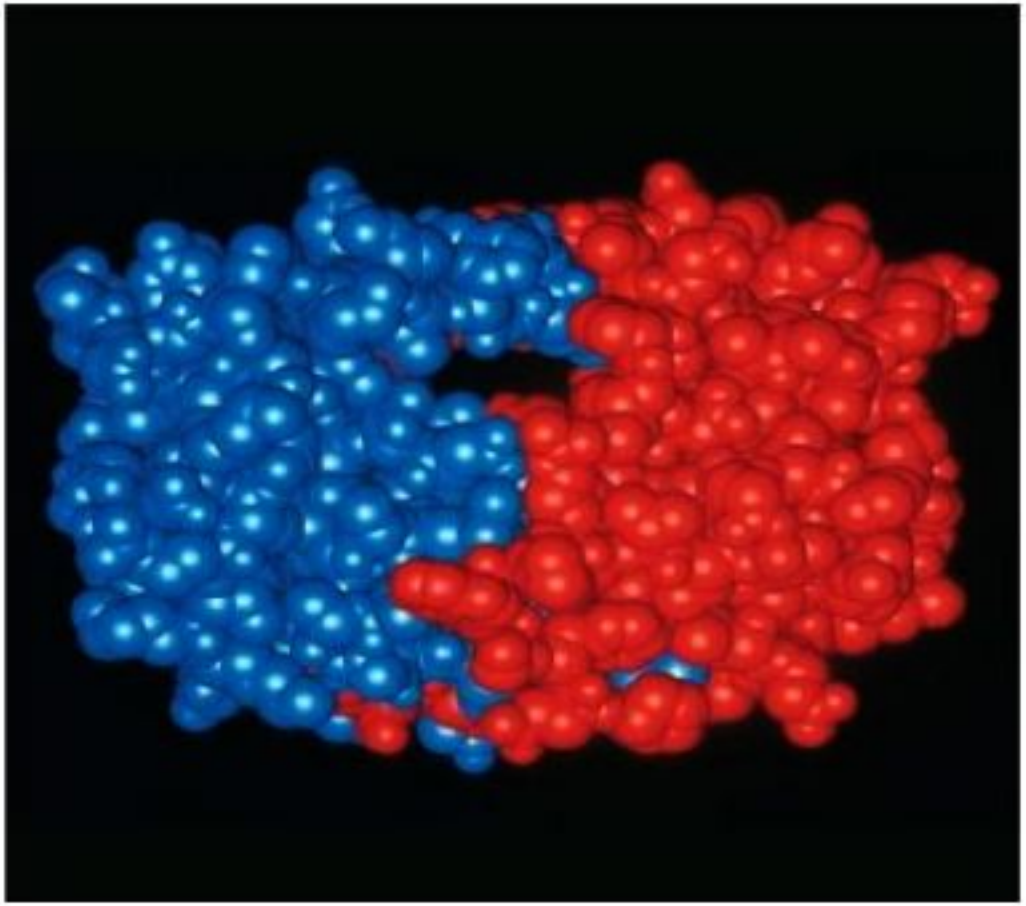


Assembled proteins have grooves and folds and bumps

Epitopes

Epitopes are unique three dimensional structures that an antibody will recognize

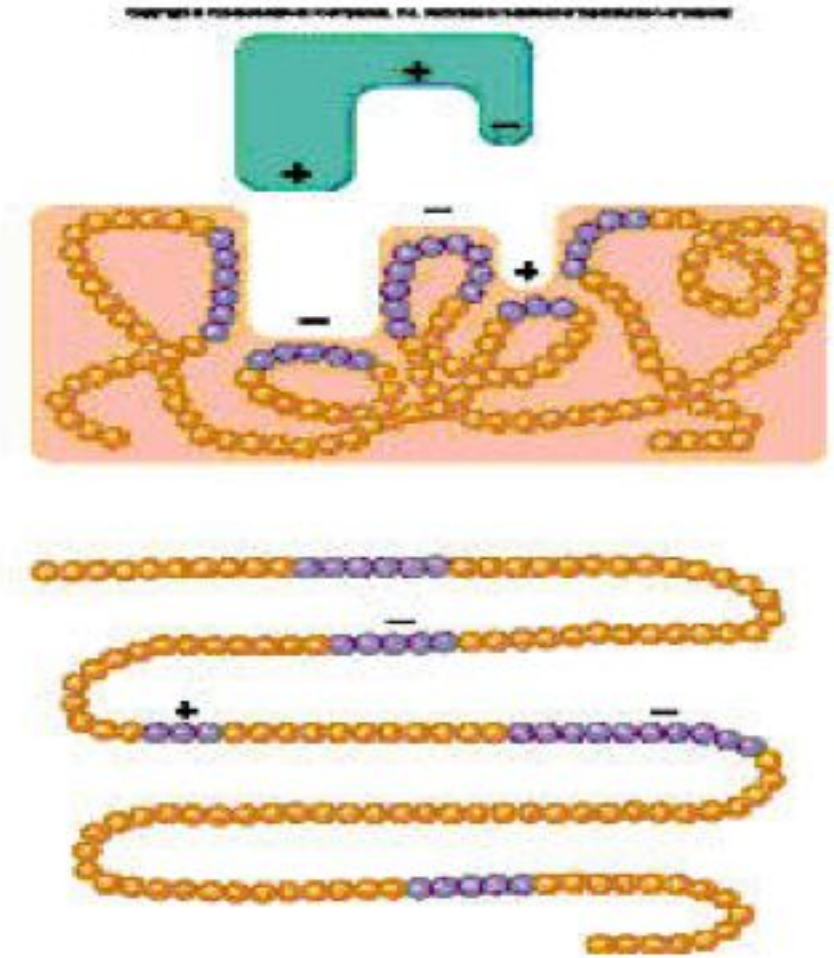
The final folded proteins



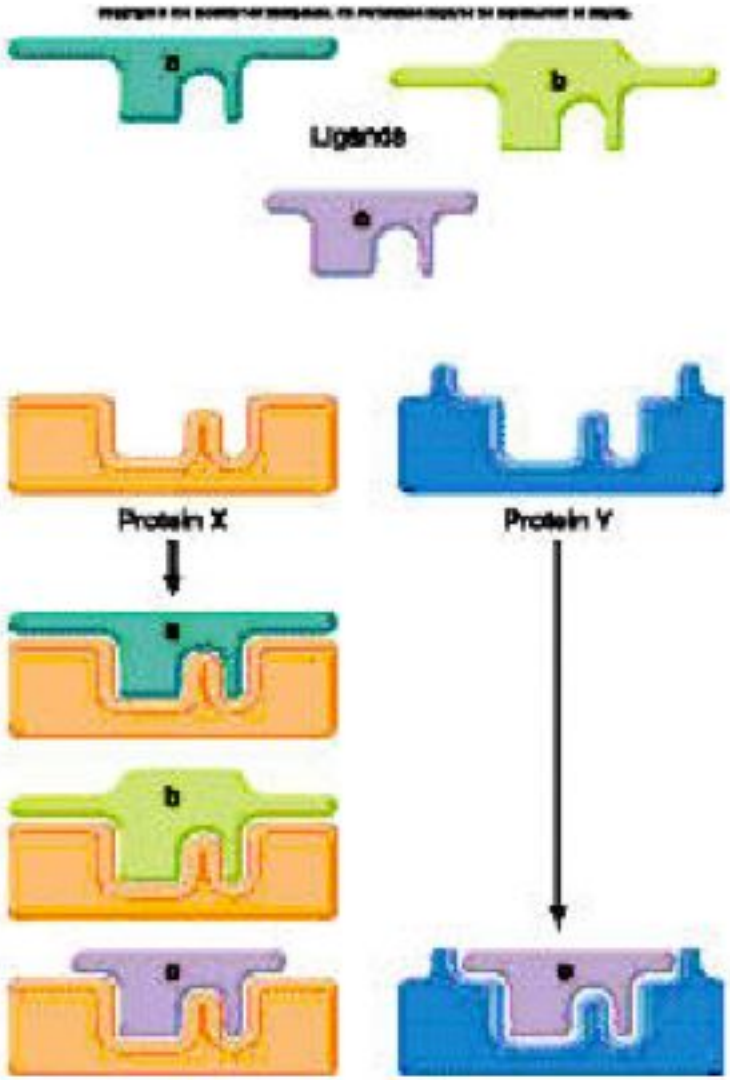
The folded protein takes on a globular form with folds & crevices to allow it to perform its specific function.

The red & blue show how two proteins can fit together.

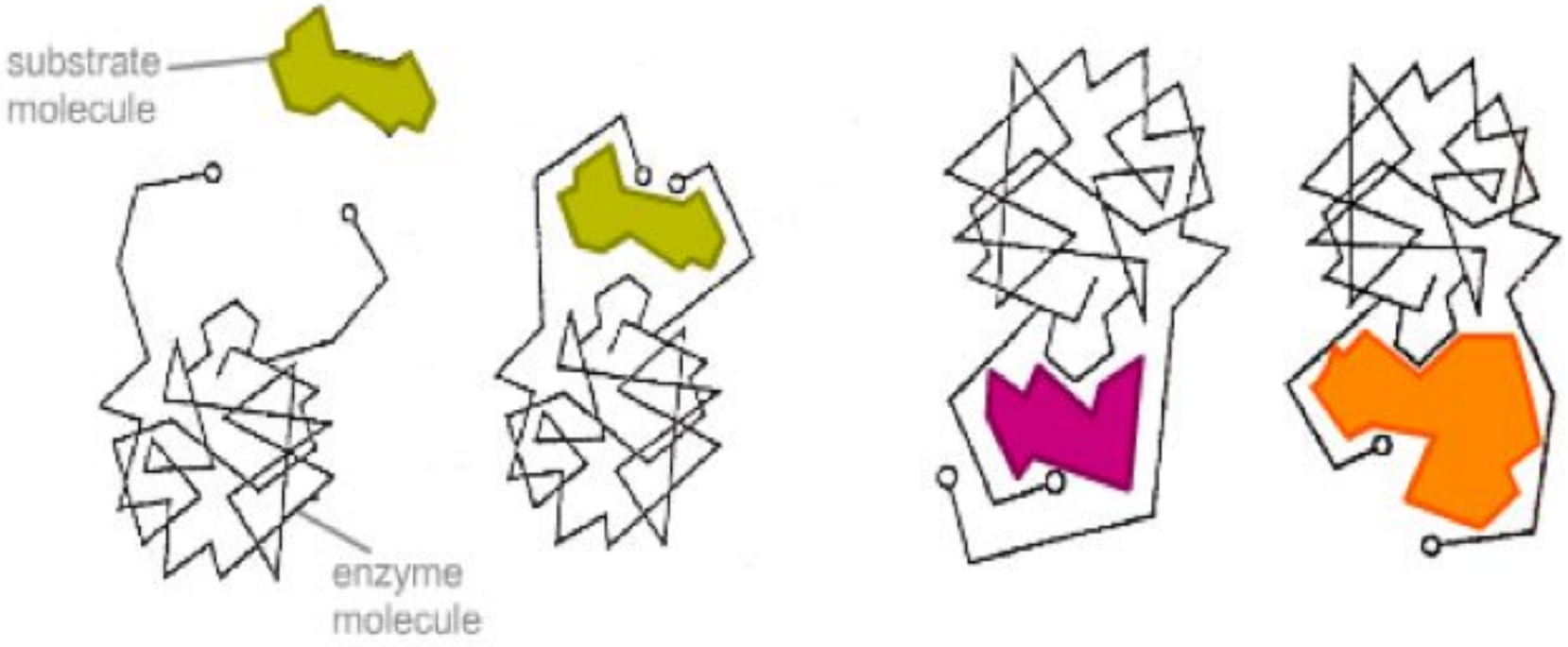
Lock and Key Concept



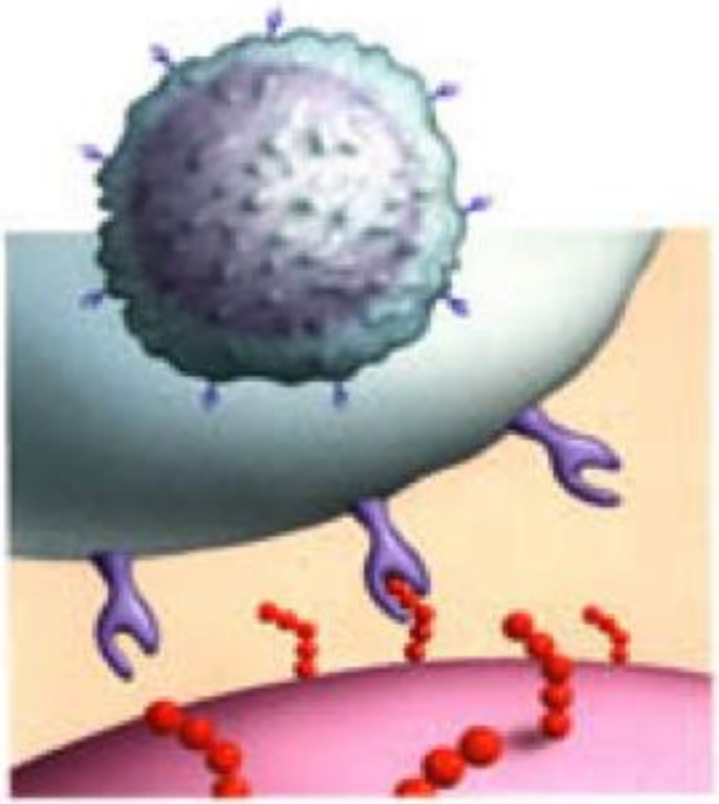
Specificity can Vary



A diagram showing a more realistic situation for **induced fit hypothesis**. Incorrect substrates, either too big or too small in size, do not fit with the active site. Ex: The Rhino virus induces a fit with our cell surface protein "ICAM" as its mechanism of infection.



ICAM- LFA interaction



- T- Cell and B-Cell communication requires several proteins to dock between cells

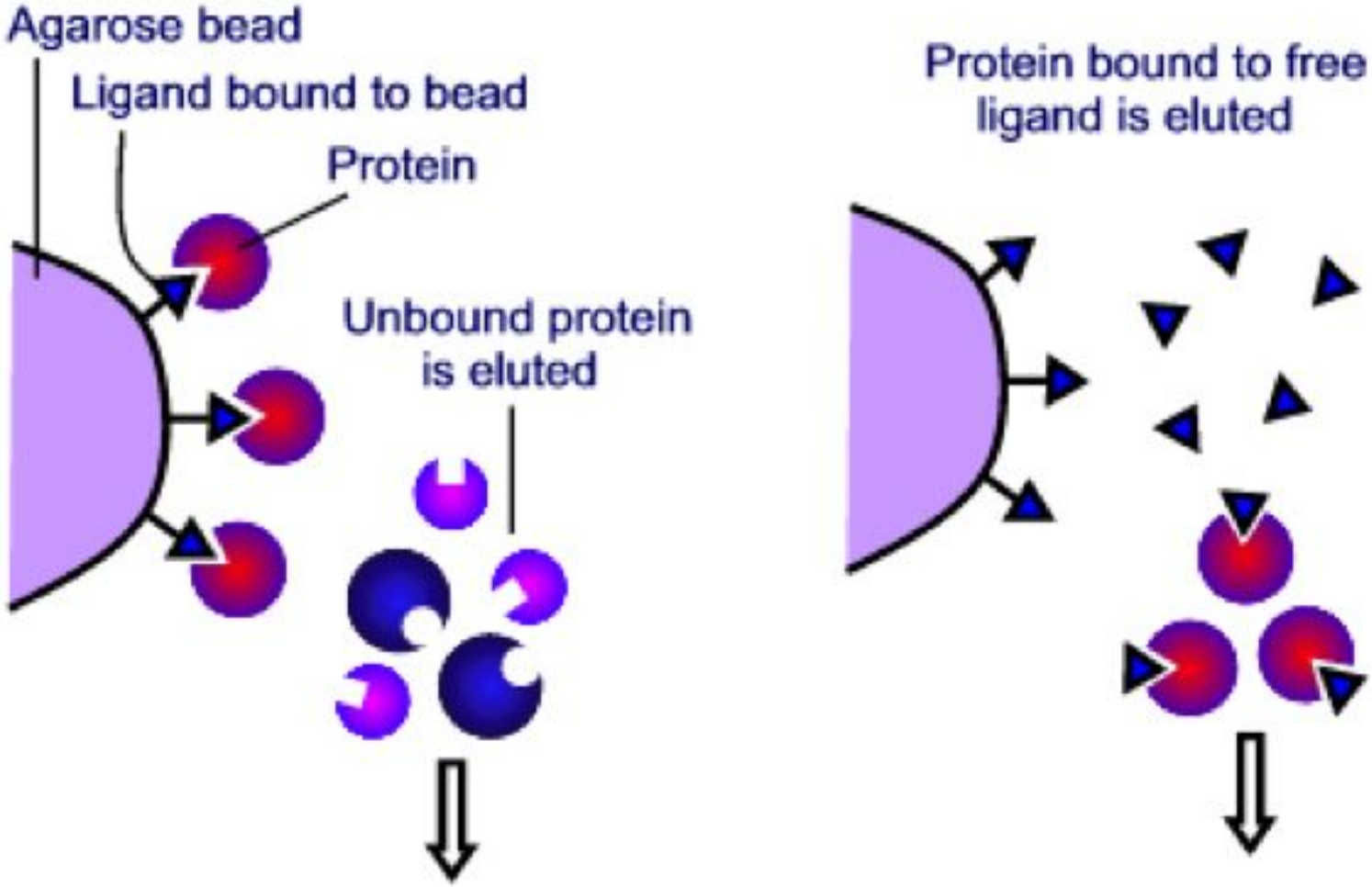
- Rod like domains of ICAM fit into folds of LFA molecule

Why Affinity Chromatography?

- Because it is specific it enables purifying a target from a very complex matrix (ie fermentation broth with serum

- It provides a means of producing high purity in a single step with reasonable recovery

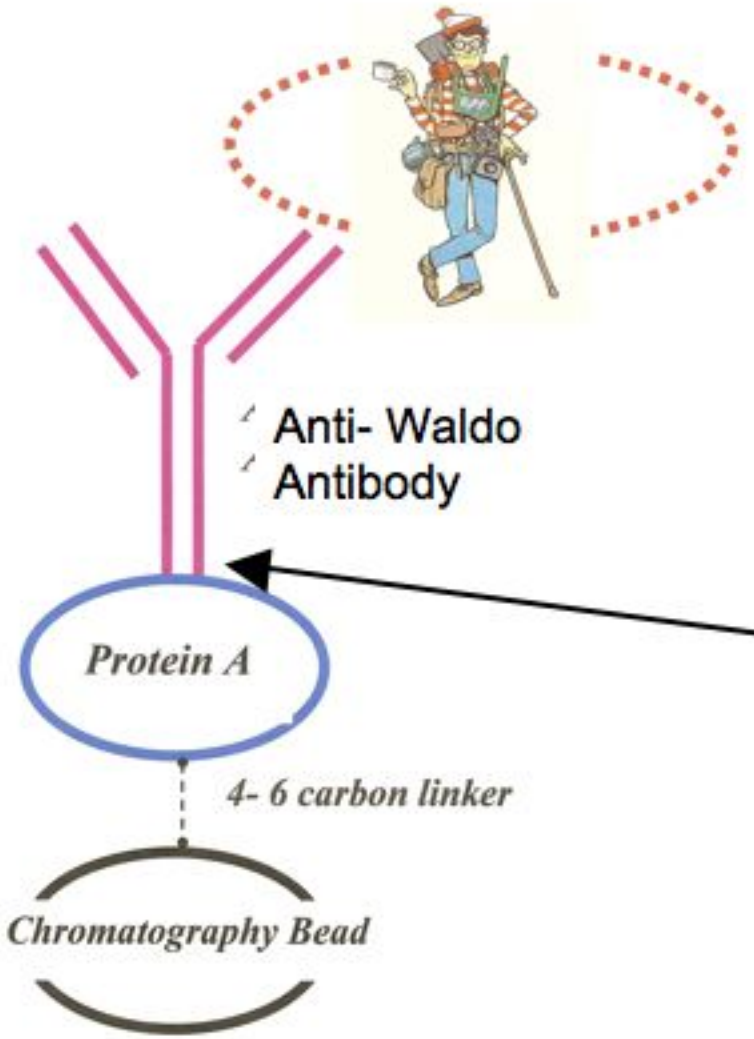
Affinity Chromatography is based upon a ligand recognizing a unique epitope



Various shapes of protein crevices, but only pie-shaped crevices fit pie-shaped proteins.

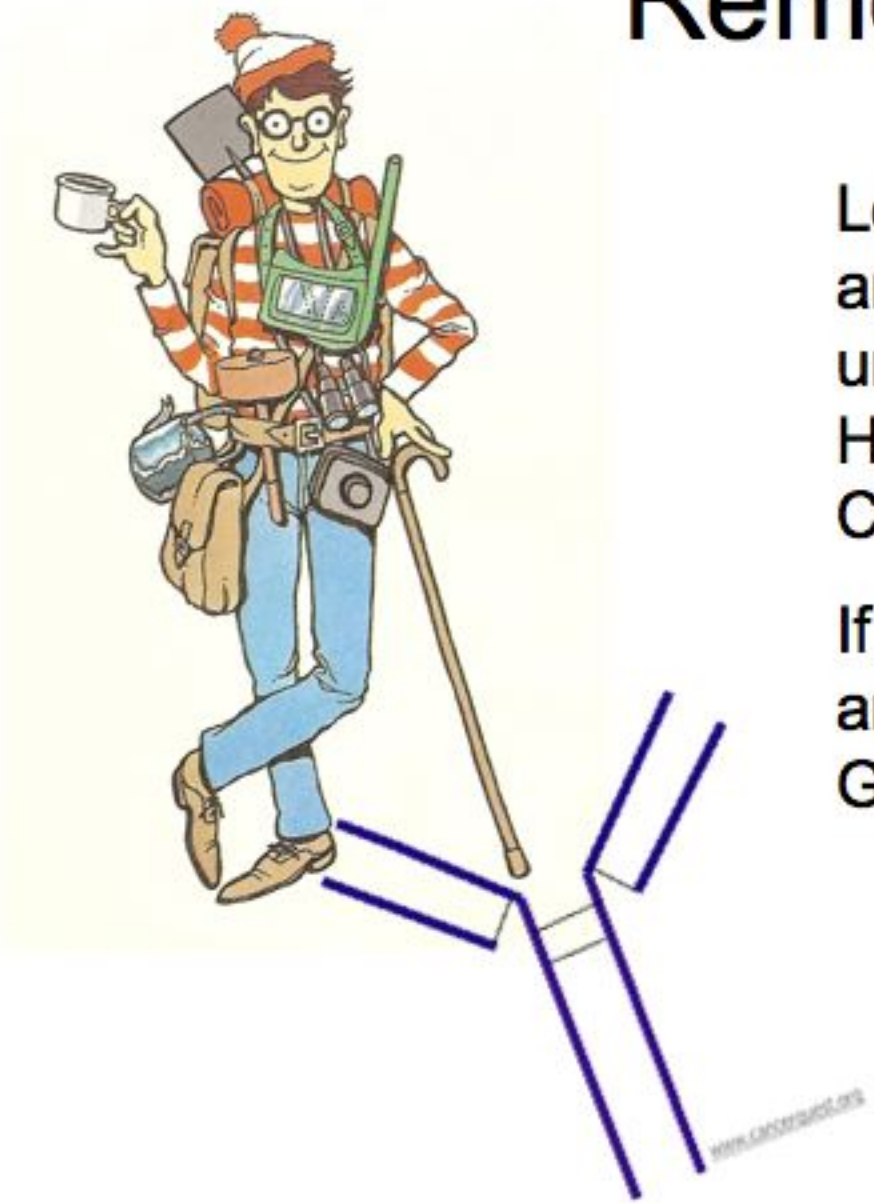
Analytical and Purification Work is a lot like finding Waldo
Let's purify Waldo!

Monoclonal Abs as an Immunoaffinity Ligand



- **Immobilize Protein A to particle**
- **Allow Protein A to bind Immunospecific Ab**
  - **Covalently cross- linked**
- **Column now is specific for the Waldo !**

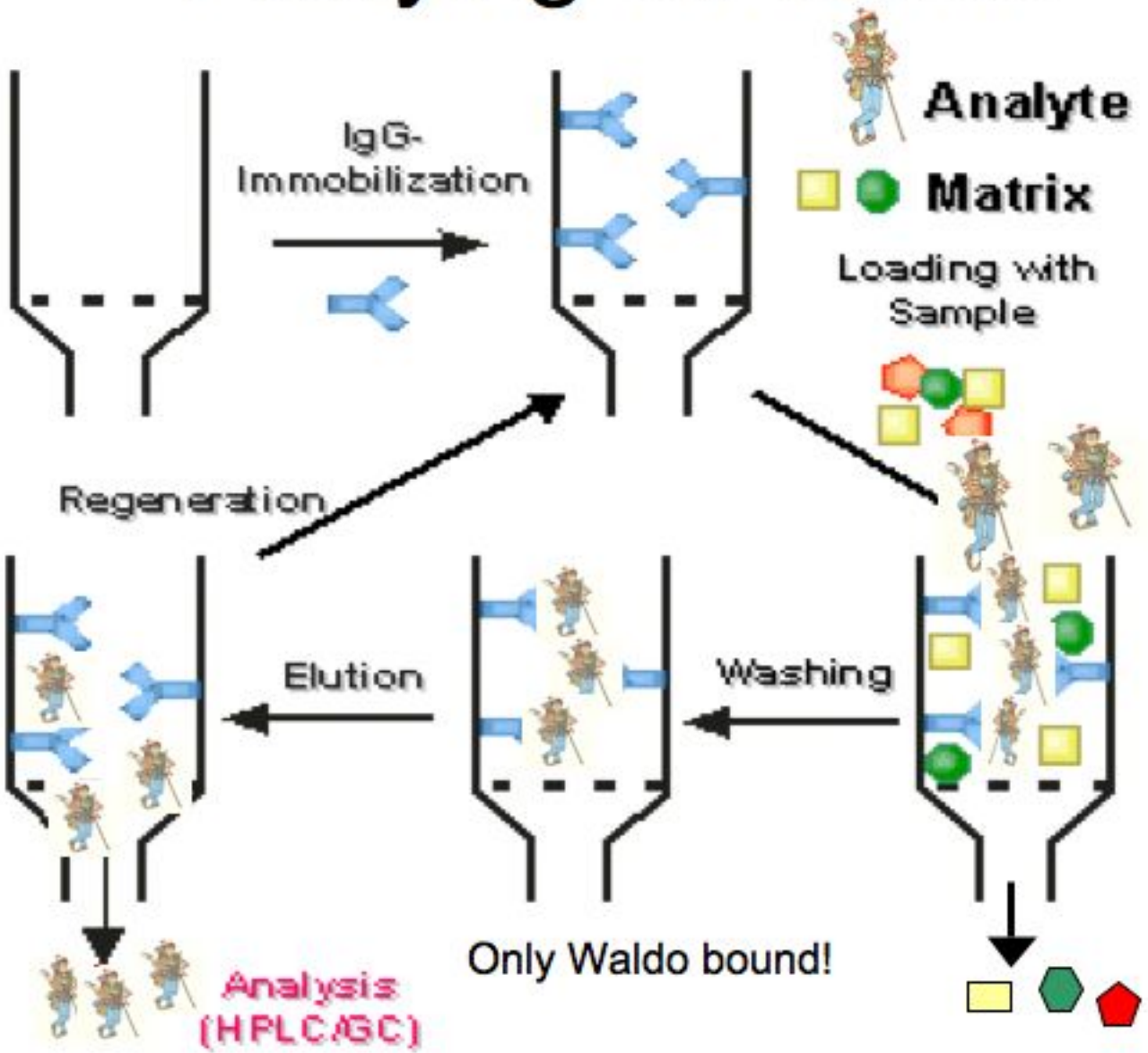
Remember Waldo?



Let's say we have an antibody that recognizes a unique epitope on Waldo : His size 16 Bruno Maglis Custom Shoe!

If we had a column full of this antibody it should be able to Grab Waldo by the Shoe!

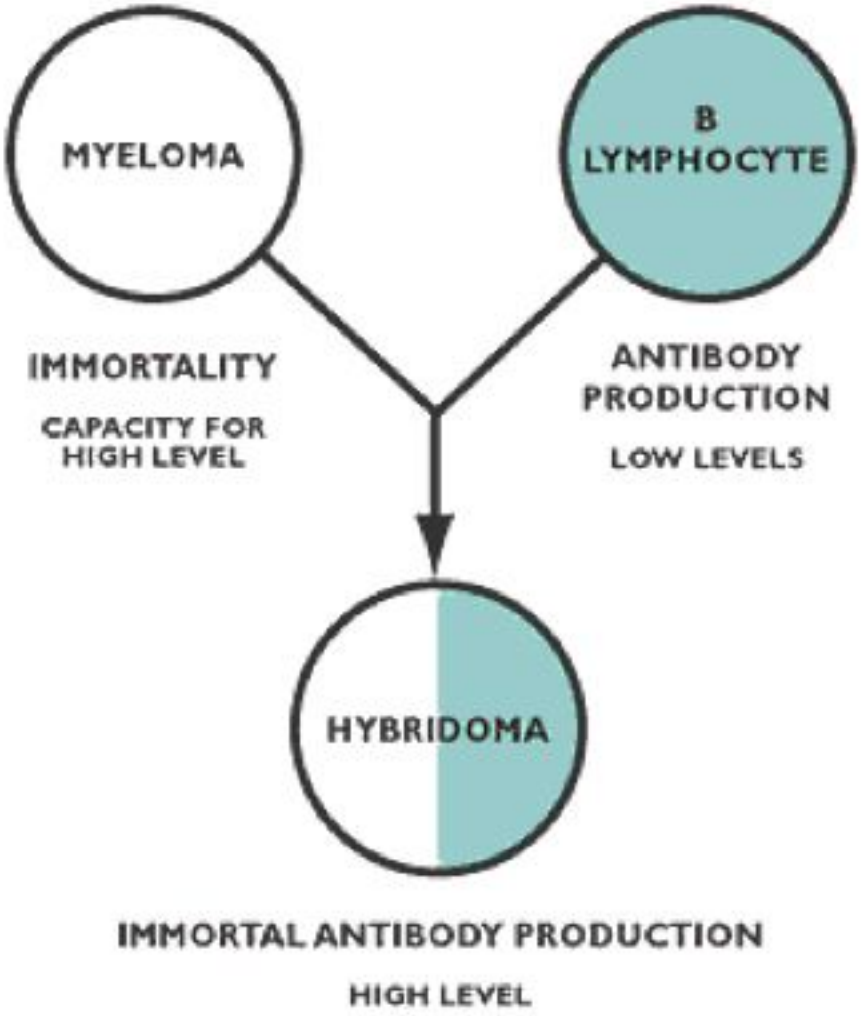
Purifying for Waldo



Waldo gently denat-ured to let go of IgG Ab


In the End: Pure Waldo!

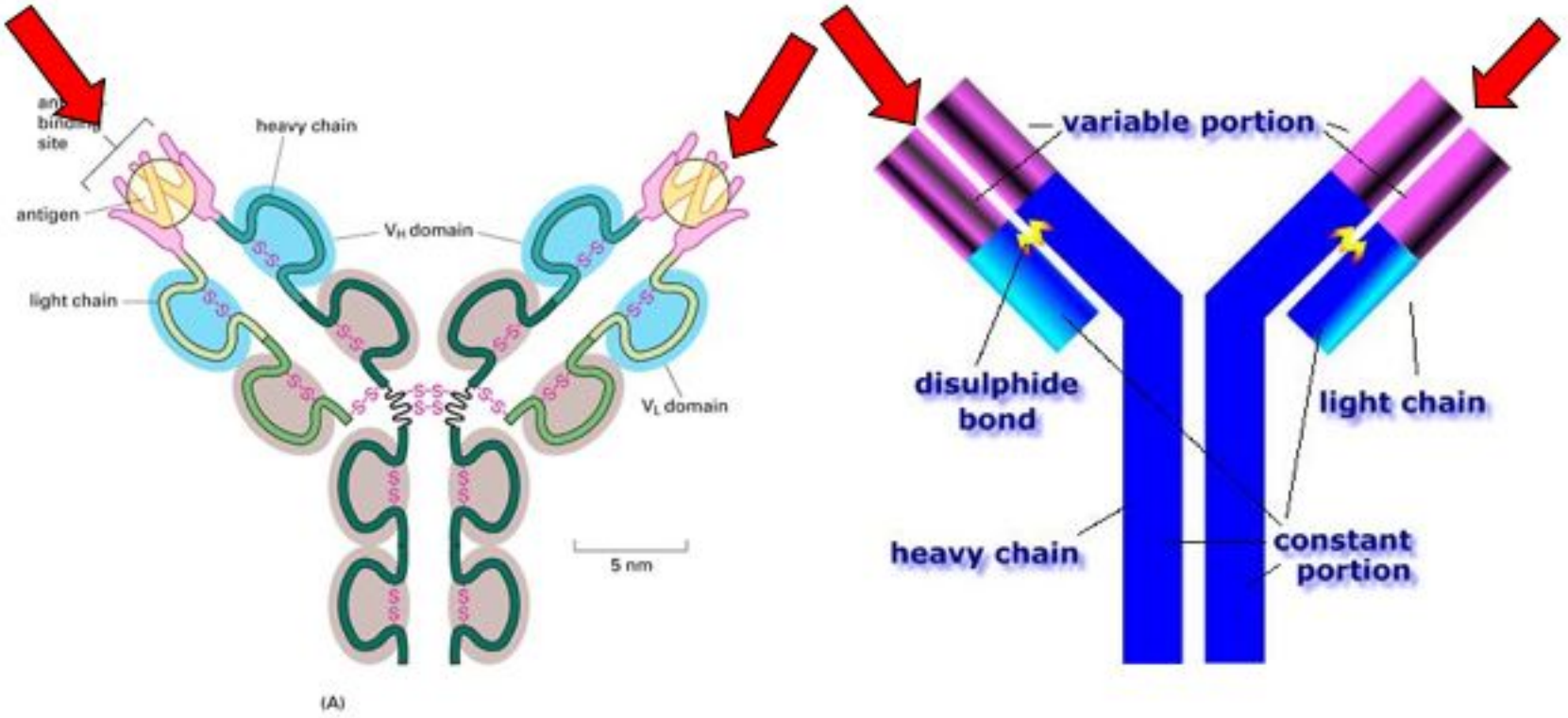
Producing Monoclonals (Then)



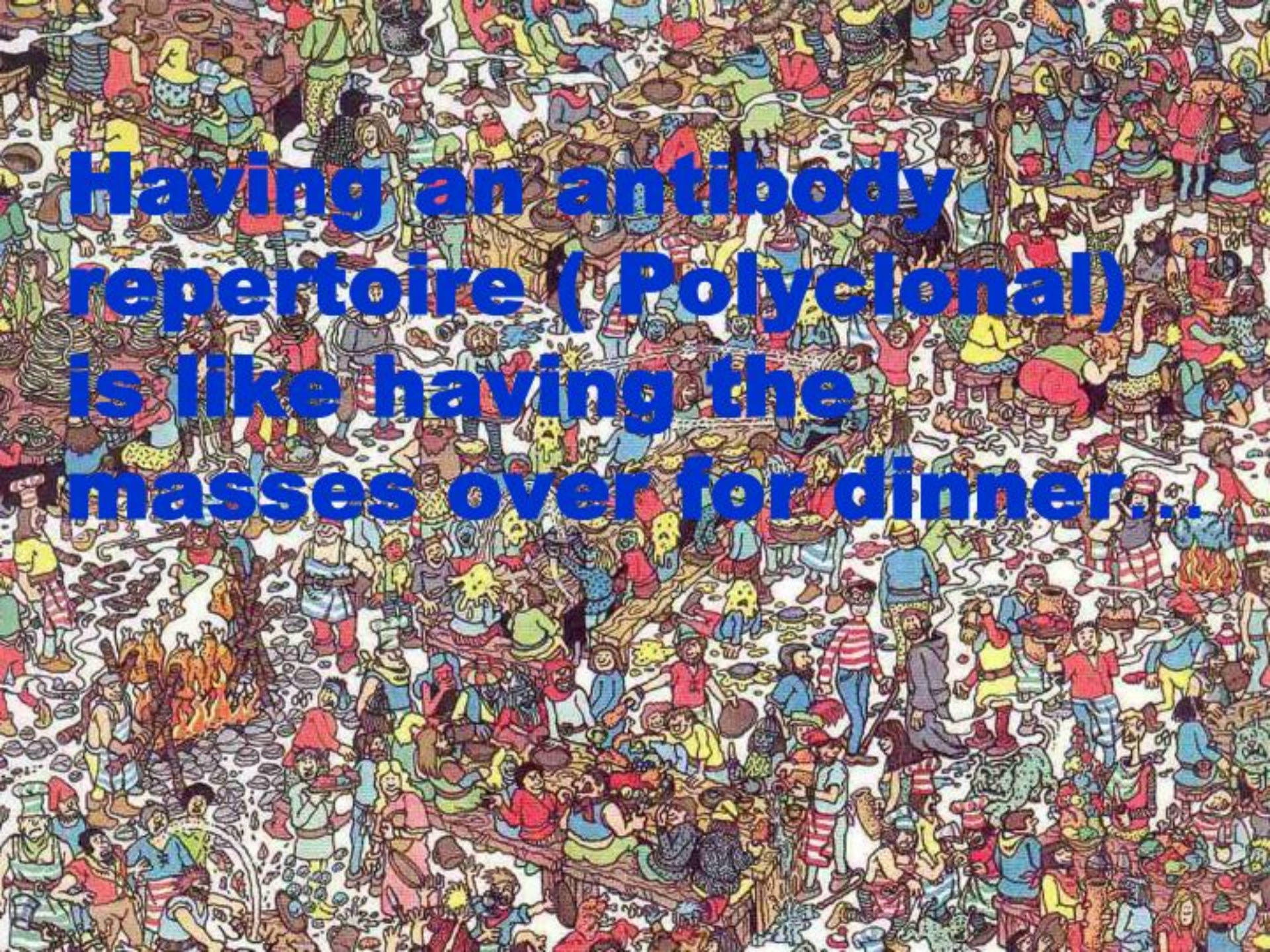
Monoclonal means to be derived from a **single cell** that **is cloned**

A single unique clone implies that the binding properties of that clone are **specific** and **unique**

Antibody Structure



- The binding sites are unique for each antibody generated from a unique b-cell

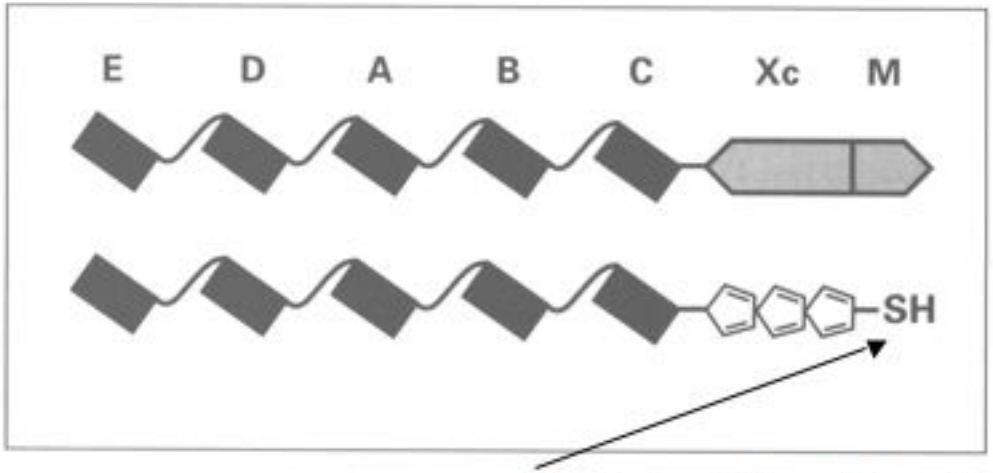

Having an antibody repertoire (Polyclonal) is like having the masses over for dinner...


Monoclonals are like having Waldo and his twins at your house

Protein A

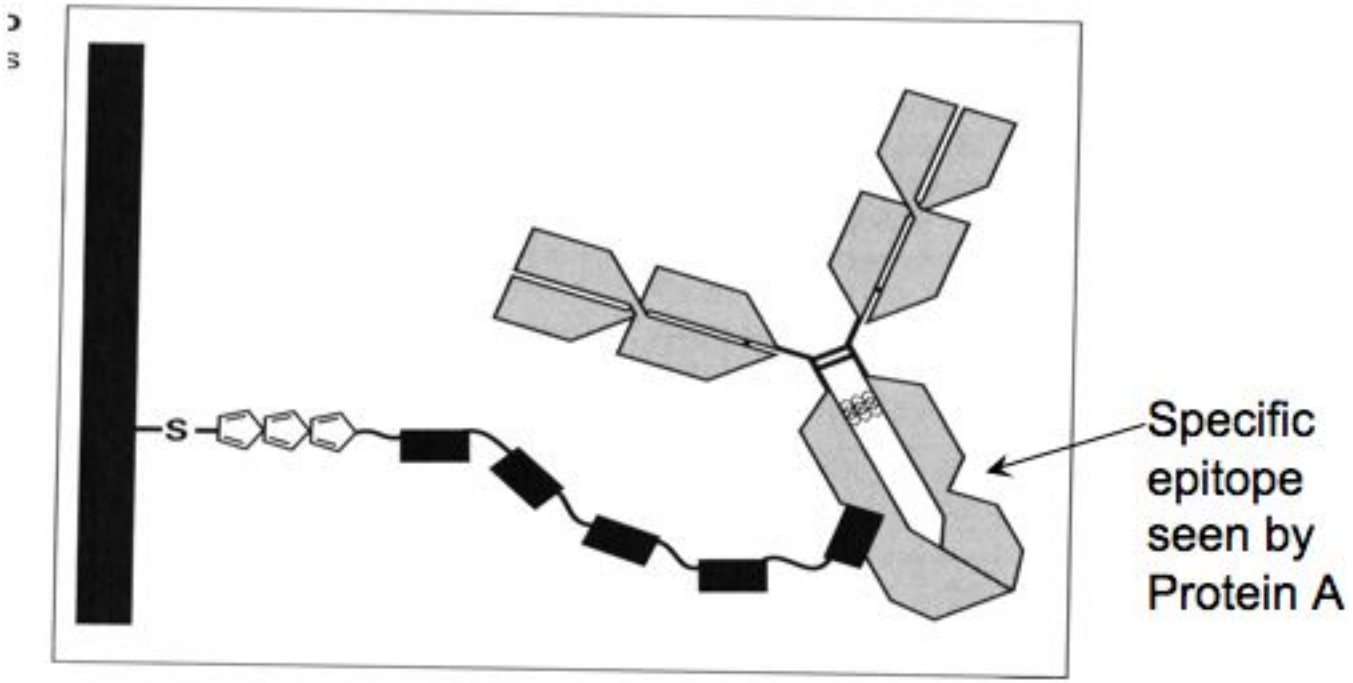
- Protein A is a cell wall component derived from Staphylococcus aureus (Cowain strain 1)
- Native protein (top) consists of a 5 domain polypeptide with Xc M transmembrane region

Figure 9.1. Domain structure of wild-type and recombinant protein A. Black rectangles indicate IgG-binding domains. Xc and M indicate the transmembrane and trans cell wall domains of Cowan strain. Pentagons indicate histidyl residues on recombinant protein A. The sulfhydryl indicates a terminal cysteine for gel anchorage



Binds to SH on bead forming a strong disulfide bond.

Protein A has unique binding to IgG

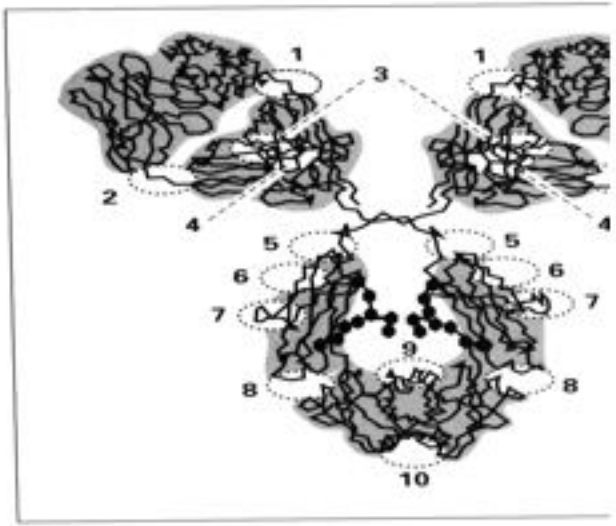


- Protein A binds to a specific epitope on the "fc" region of Antibody molecules

Binding Mechanism

rophobic Interaction Chromatography

ydrophobic sites on . Black lines indicate skeleton as revealed ystallography. Black dicate carbohydrate ashed ovals indicate ıcation of hydropho- Table 6.2 for legend linated and redrawn ɔm references 63-65.



- Binding is primarily hydrophobic
- Binding is enhanced by addition of salts which absorb water (1M NaCl, 3M Na_2SO4)
- Water displacement (addition of Polyethylene Glycol 6000 or higher absorbs water like a sponge)
- High pH enhances binding

Elution is accomplished by several means:

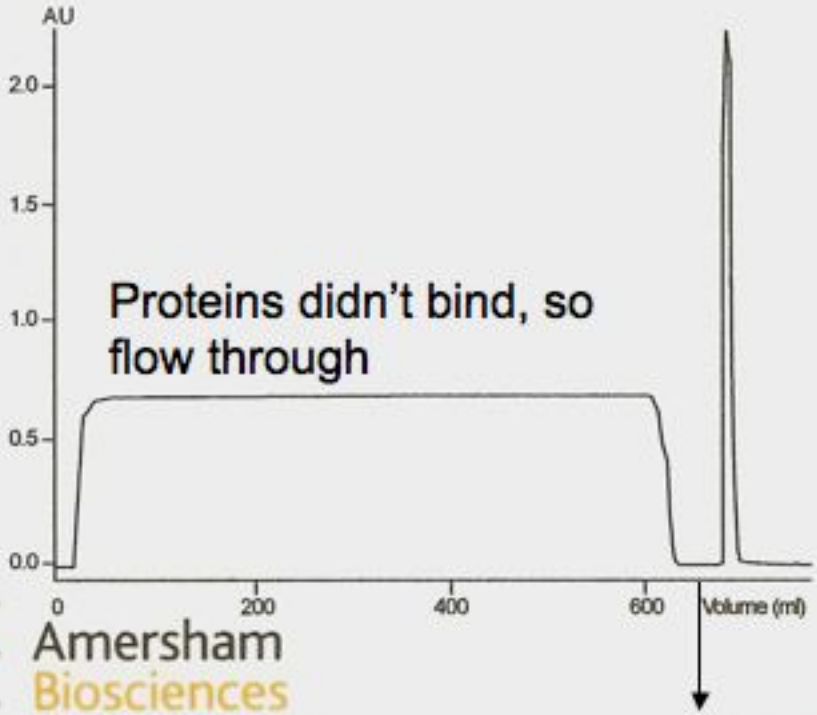
- **Hydrophobic competition** : usually Organic solvents ie Ethylene Glycol (like the K2R spot lifter to remove greasy clothing stains)
- Most common elution is to **lower pH**
- Effects minor denaturation
- Charge repellency : High pH Histdyl residues are uncharged unfettered in interaction
- At low pH Histdyls are charged and become repellant
- **Mild denaturants:** Urea, Guanidine ,problematic in assuring that denaturation is fully reversed.

Merits of Protein A

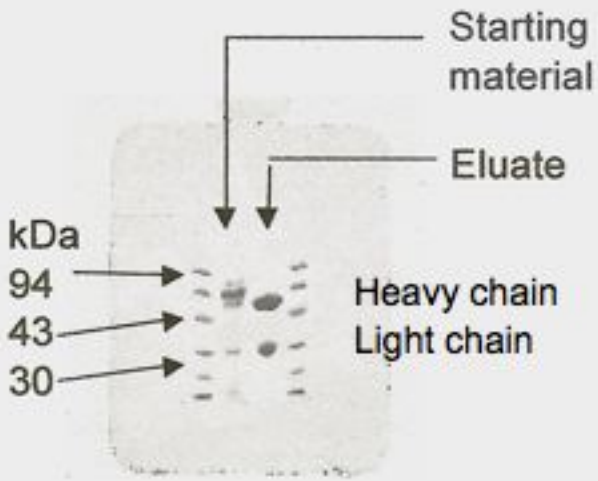
- Highly selective for Antibodies/ provides very high purity product
- Provides 4 to 5 logs DNA removal
- Provides 6 logs of Virus clearance
- Provides some Endotoxin clearance.
- Has a very high capture efficiency

IgG purification on rProtein A Sepharose Fast Flow

Sample:	**600 ml of mouse monoclonal IgG $_{2a}$, 2.5 % FCS, 0.146 mg/ml of MAb**
Column:	XK 16/20, bed height 4.8 cm
Matrix.	rProtein A Sepharose Fast Flow
Equilibration buffer:	20 mM Sodium Phosphate, pH 7.0
Elution buffer:	20 mM Sodium Citrate, pH 4.0
Regeneration buffer:	100 mM Sodium Citrate, pH 3.0
Recovery:	**95%, 82.9 mg**



SDS-PAGE reduced



Amersham Biosciences

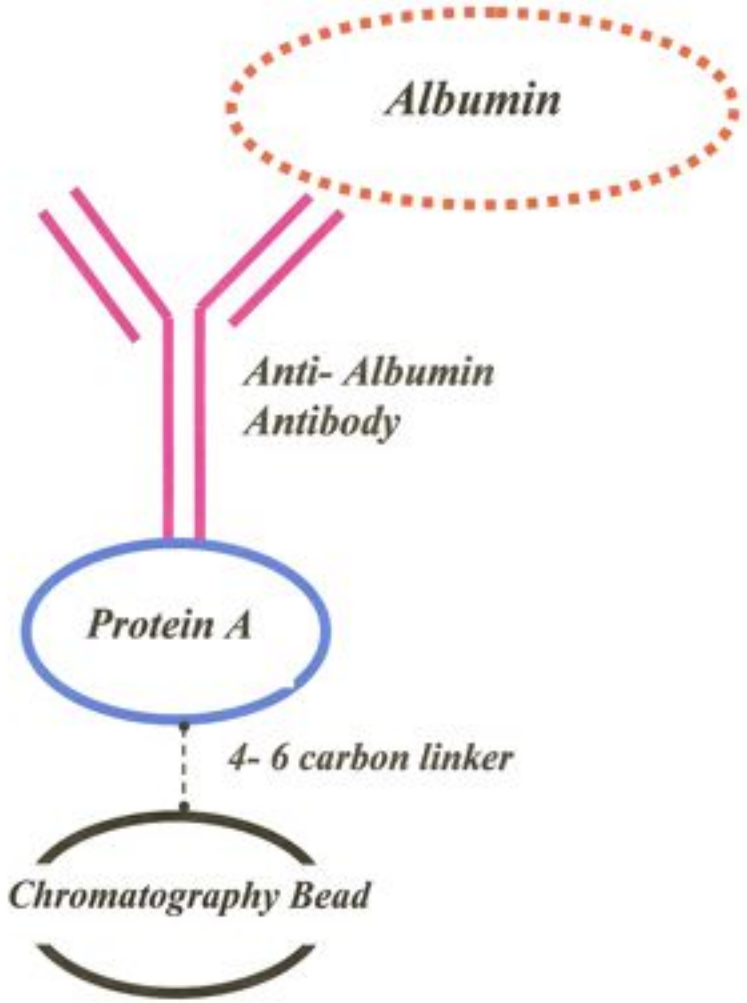
Caveats to using Protein A

- Will co-purify host antibodies : problematic if feed sourced from Milk, Serum, Ascites, or Serum supplemented Cell culture.
- Elution conditions may be denaturing to antibody. Affecting fc Effector functions.
- It's expensive at $8500 liter
- A biological entity that can't be cleaned by traditional NaOH treatment

100(or almost) uses for Protein A

- Analytical tool for determining quantitations, HPLC or Small scale chromatography.
- ELISA reagent
- Western blot reagent
- Process step for production of Antibodies
- Secondary ligand for immuno-affinity Chromatography

Protein A as a Immunoaffinity Ligand



- Immobilize Protein A to particle
- Allow Protein A to bind Immunospecific Ab
  - Covalently cross- link
- Column now is specific for Albumin

Adding tags to purify Recombinant proteins

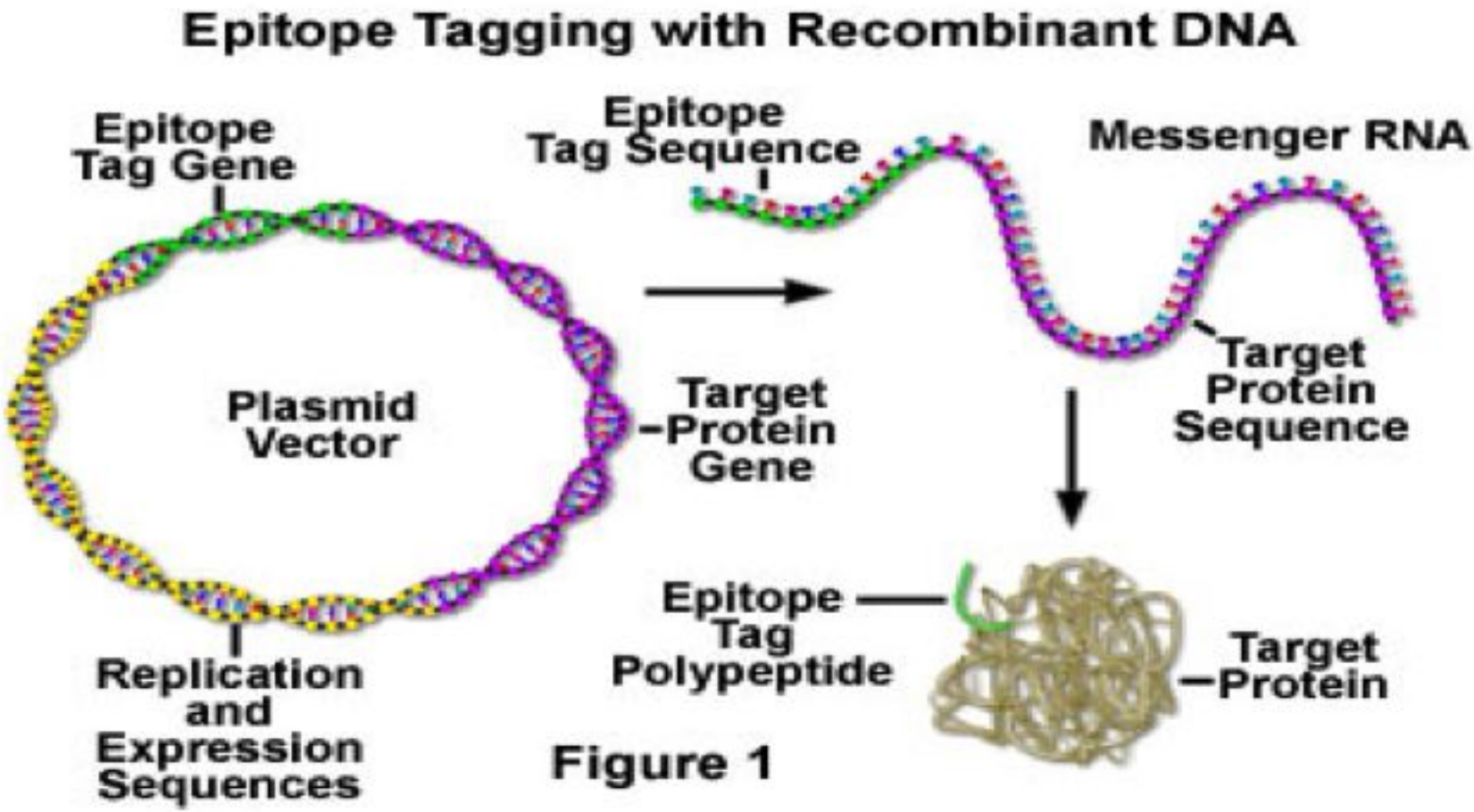
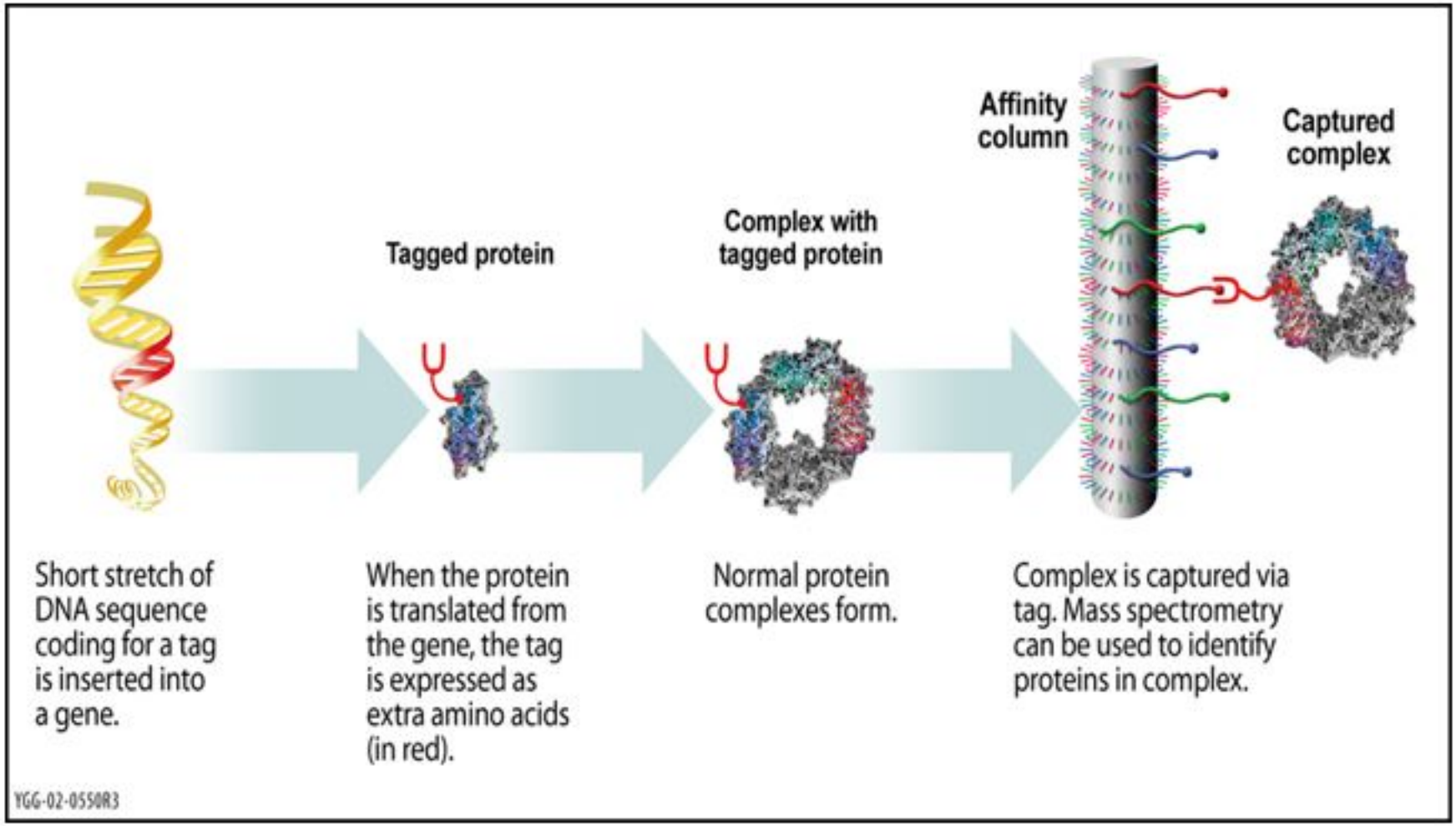


Figure 1


Affinity column
Captured complex
Tagged protein
Complex with tagged protein
Short stretch of DNA sequence coding for a tag is inserted into a gene.
When the protein is translated from the gene, the tag is expressed as extra amino acids (in red).
Normal protein complexes form.
Complex is captured via tag. Mass spectrometry can be used to identify proteins in complex.
YGG-02-0550R3

Large scale Commercial chromatography skid – same concepts as lab, just larger!

- Pump, detector, column & recording device
- Note: grey support in column = beads weighted with stainless steal so they'll sink to the bottom