The Immunoassay Guide to Successful Mass Spectrometry

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SUMS User Meeting
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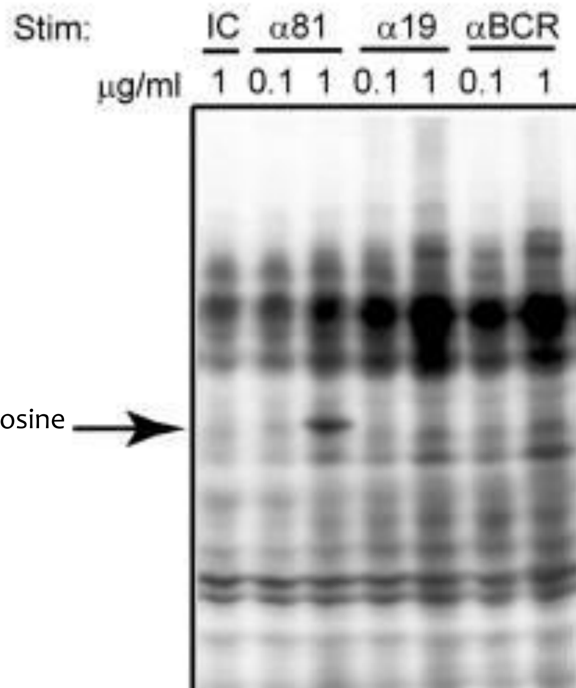
What is it ?



Hey! Look at that! Something is reacting in here! I just wish I knew what it is!

Maybe we should mass spec it!

Coffey GP et.al. 2009 JCS 22(3137-44)

True or false

1. A big western blot band means I have a LOT of protein
2. One band = 1 protein

Big band on Western blot

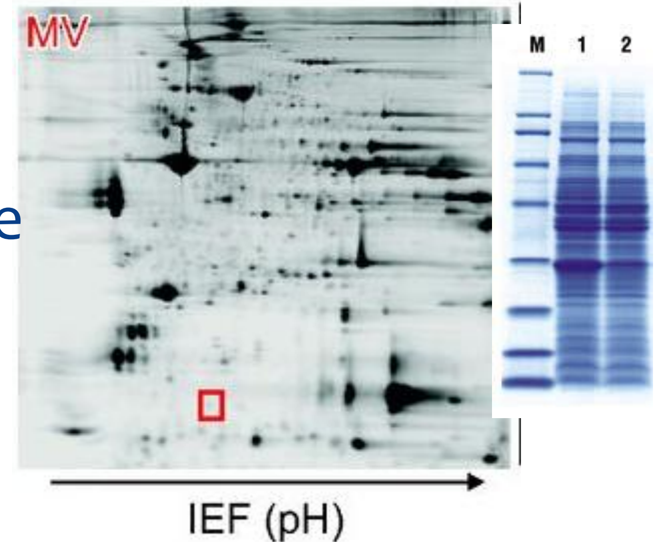
Bands are affected mainly by:

* Antibody affinity to the antigen
* Number of available epitopes

Remember: After the Ag-Ab interaction, you are **amplifying** the signal by using an enzyme linked to a secondary antibody.

How many proteins are in a band?

* Human genome: 20,000 genes=100,000 proteins
* There are about 5000 different proteins, not including PTMs, in a given cell at a single time point.
* Huge dynamic range
* 2D-PAGE: about 1000 spots are visible.
* 1D-PAGE: about 60 -100 bands are visible
    - So, how many proteins are in my band?



Separation is the key!

Can you IP your protein of interest?

Can you find other way to help with the separation?

- -Organelle enrichment
- -PTMs enrichment
- -Size enrichment

Have you optimized your running conditions?

Choose the right gel and the right running conditions!

Thermo Scientific PageRuler Plus Prestained Protein Ladder

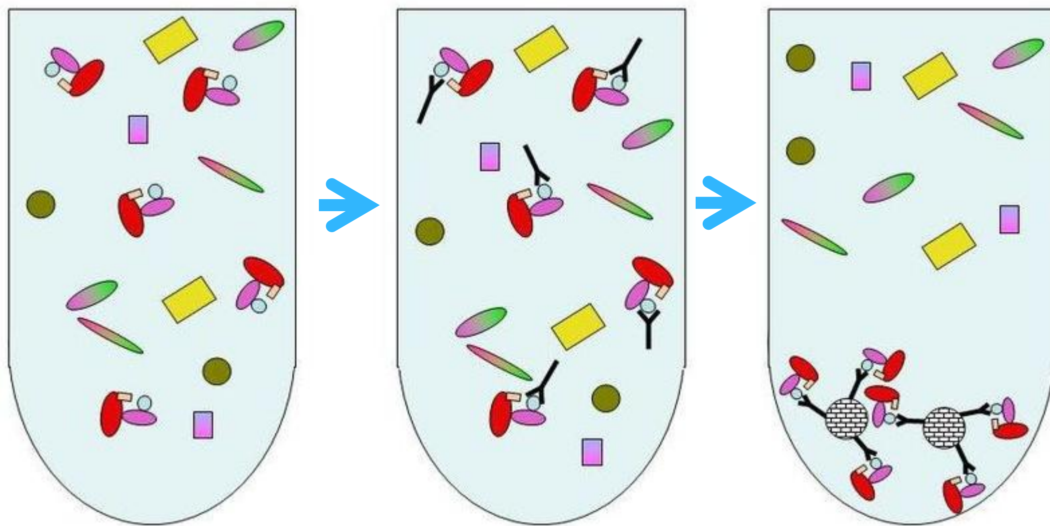
Gel type	Tris-Glycine						
Gel concentration	4-20%	8-16%	10-20%	8%	10%	12%	15%
Running buffer	Tris-Glycine						
Apparent Molecular Sizes (kDa)	250, 130, 100, 70, 55, 35, 25, 15, 10	250, 130, 100, 70, 55, 35, 25, 15, 10	250, 130, 100, 70, 55, 35, 25, 15, 10	250, 130, 100, 70, 55, 35, 25, 15	250, 130, 100, 70, 55, 35, 25, 15	250, 130, 100, 70, 55, 35, 25, 15, 10	250, 130, 100, 70, 55, 35, 25, 15, 10

% length of gel: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100

Gel type	Tris-Acetate		Bis-Tris					
Gel concentration	3-8%	7%	4-12%		10%		12%	
Running buffer	Tris-Acetate		MOPS	MES	MOPS	MES	MOPS	MES
Apparent Molecular Sizes (kDa)	205, 120, 85, 65, 50, 30, 25	205, 120, 85, 65, 50, 30, 25	185, 115, 80, 65, 50, 30, 25, 15, 10	190, 115, 80, 70, 50, 30, 25, 15, 10	185, 115, 80, 65, 50, 30, 25, 15	190, 115, 80, 70, 50, 30, 25, 15, 10	185, 115, 80, 65, 50, 30, 25, 15	190, 115, 80, 70, 50, 30, 25, 15, 10

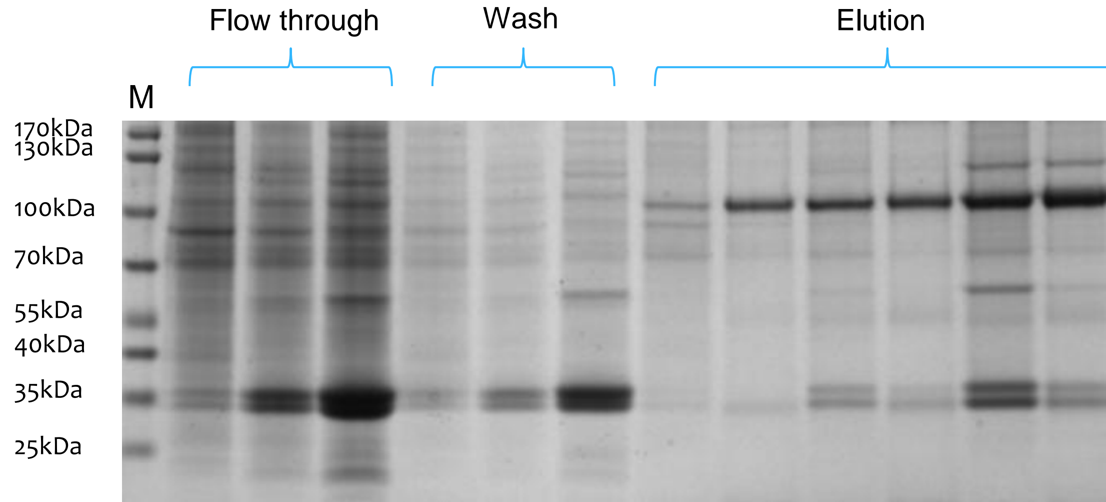
% length of gel: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100

Immunoprecipitation, in theory



Step 1: Create a complex between a desired protein (Antigen) and an Antibody
Step 2: Pull down the complex and remove the unbound proteins
Step 3: Elute your antigen and analyze

Immunoprecipitation, in real life



Lung tissue lysate, IP with patient sera , Coomassie stain
Rabinovitch and Robinson labs, unpublished data

Optimizing immunoprecipitation

- You need:
  - A good antibody that can IP
  - The right beads:
    1. Protein A
    2. Protein G
    3. Protein L
- Each protein binds with a different affinity to different antibodies

Choosing the right bead: affinity!

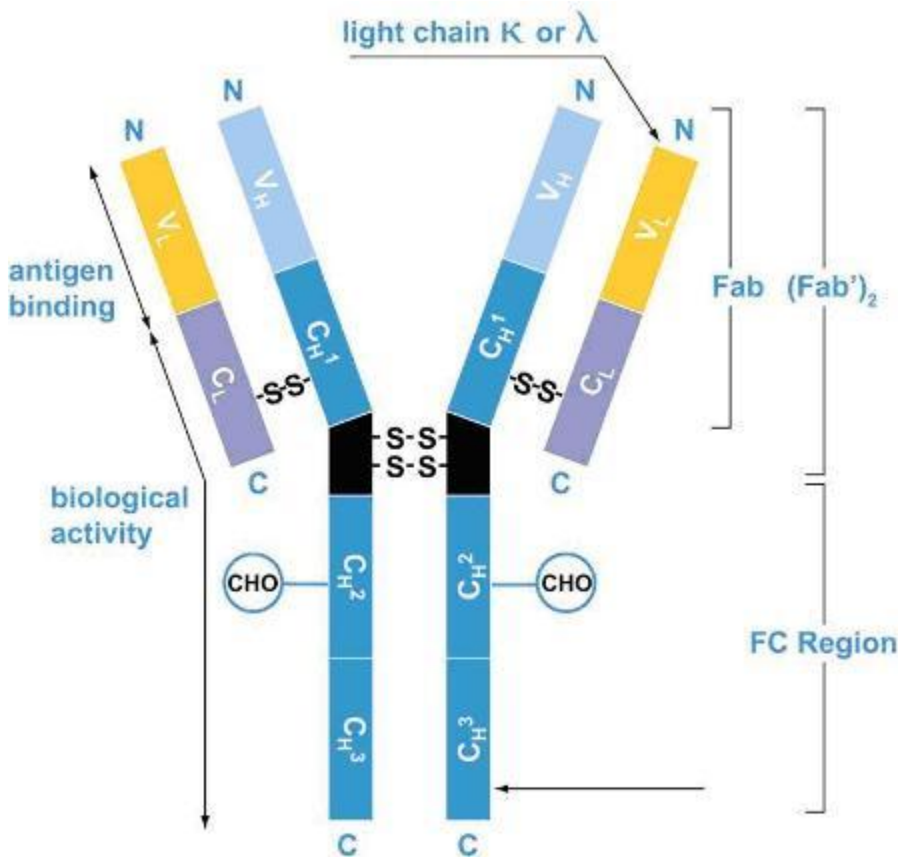
Species	*Antibody Class*	*Protein A*	*Protein G*	*Protein L*
Human	Total IgG	+++	+++	+++
	IgG_1	+++	+++	+++
	IgG_2	+++	+++	+++
	IgG_3	+/-	+++	+++
	IgG_4	+++	+++	+++
	IgM	+/-	-	+++
	IgD	-	-	+++
	IgA	+/-	-	+++
	Fab	+/-	+/-	+++
	ScFV	+/-	-	+++
Mouse	Total IgG	+++	+++	+++
	IgM	-	-	+++
	IgG_1	+/-	+/-	+++
	IgG_{2a}	+++	+++	+++
	IgG_{2b}	+++	+++	+++
	IgG_3	+++	+++	+++
Rat	Total IgG	+/-	+/-	+++
	IgG_1	+/-	+/-	+++
	IgG_{2a}	-	+++	+++
	IgG_{2b}	-	+/-	+++
	IgG_{2c}	+++	+++	+++

Magnetic or Sepharose

* Magnetic beads:
  * Can help reduce background
  * Require a magnet
* Sepharose beads:
  * Can work in a column format
  * Easier to cross-link
  * Require centrifugation or gravity

Magnetic or Seprhaose

Antibodies 101



Each antibody contain two identical copies of a heavy chain (~50Kda) and two identical copies of a light chain (~25kDa).

Each heavy and light chain is covalently joined by a disulfide (-S-S-) bond.

By adding a reducing agent, we are breaking the S-S bonds between two cysteine residues, generating 4 separate protein chains and 2 distinct bands on the gel.

Abcam

Crosslinking

* Crosslinking is the process of chemically joining two or more molecules by a covalent bond. This enables us to :
  * **Capture** and identify unknown protein interactors or interaction domains.
  * **Conjugate** an enzyme or tag to an antibody or other purified protein.
  * **Immobilize** antibodies or other proteins for assays or affinity-purification.

To crosslink or not to crosslink?

* Pro:
  - Can stabilize the Ab-Ag interaction
  - Can be used to immobilize the Ab to beads, thus removing it completely from the elution product. This can result in a cleaner result.

* Cons:
  - Can bind an innocent bystander, thus creating either a false positive or increase the background

Choose your crosslinker wisely!

Elution

* Elute in 1x loading buffer, then boil.

  - Point of attention: choose your loading buffer with or without reducing agent.

  - Only suitable for gel loading downstream applications.

Elution

Summary of elution conditions commonly used for immunoaffinity and protein-protein affinity purification:

Condition	**Examples**
Low pH	100 mM glycine•HCl, pH 2.5-3.0 100 mM citric acid, pH 3.0
High pH	50-100 mM triethylamine or triethanolamine, pH 11.5 150 mM ammonium hydroxide, pH 10.5 0.1 M glycine•NaOH, pH 10.0
Ionic strength (and chaotropic effects)	5 M lithium chloride 3.5 M magnesium or potassium chloride 3.0 M potassium chloride 2.5 M sodium or potassium iodide 0.2-3.0 M sodium thiocyanate 0.1 M Tris-acetate with 2.0 M NaCl, pH 7.7 100 mM sodium phenyl phosphate
Denaturing	2-6 M guanidine•HCl (also counts as chaotropic) 2-8 M urea (also counts as chaotropic) 1.0 M ammonium thiocyanate 1% sodium deoxycholate 1% SDS
Organic	10% dioxane 50% ethylene glycol, pH 8-11.5 (also counts as chaotropic)
Competitor	> 0.1 M counter ligand or analog

In summary….

- The key to successful MS discovery is having a clean, enriched protein
- Know your friends and your enemies
- Try to enrich and separate your protein of interest
- As proteins are very different from one to another, it might take time to really work out all the fine tuning, so be ***patient*** ….