

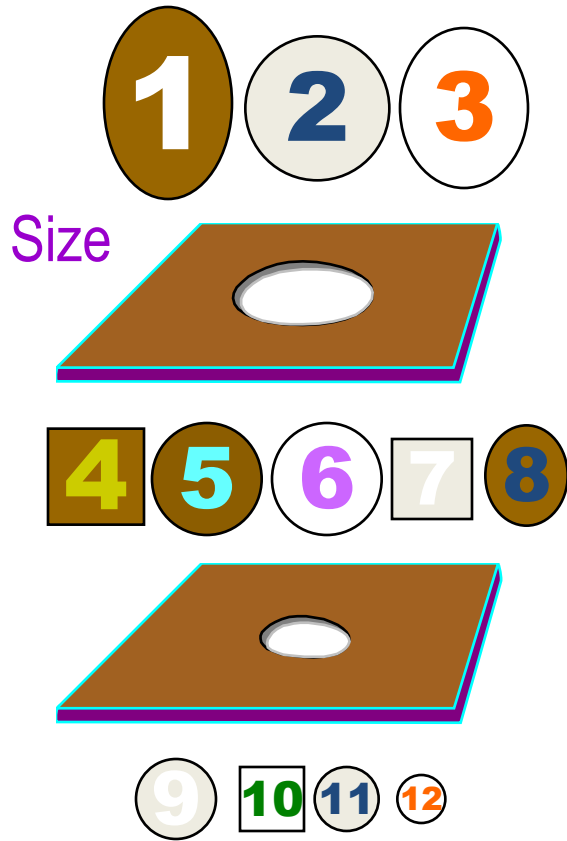
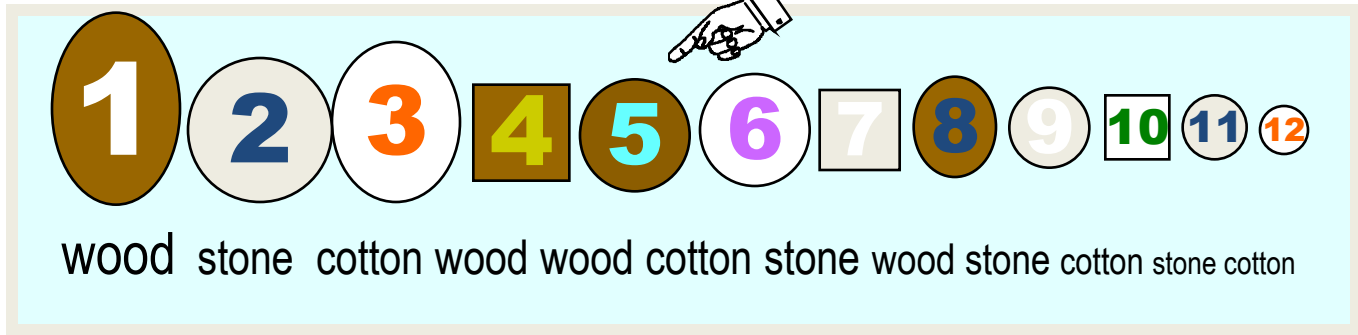
Protein Purification

Why purify a protein?

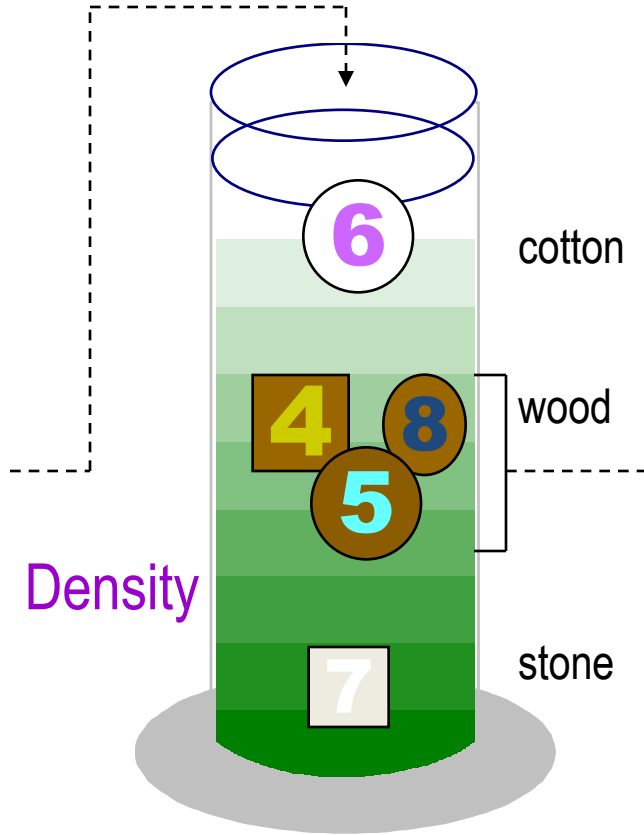
- To study its function
- To analyze its physical properties
- To determine its sequence
- For industrial or therapeutic applications

How to Separate These Objects

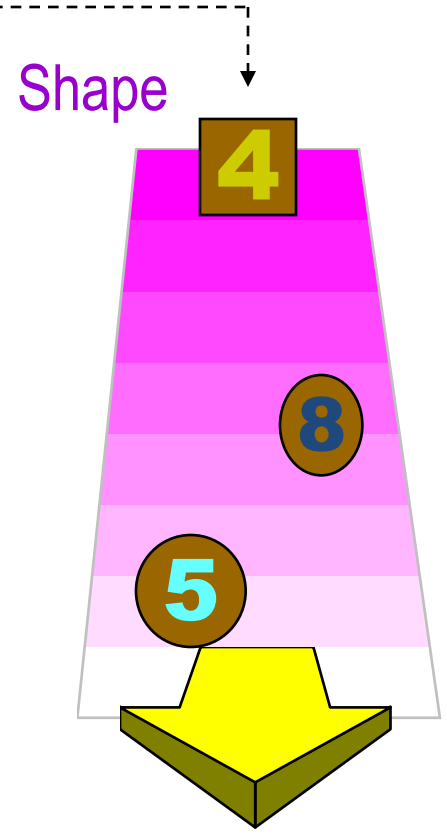
Shape
Size
Density



Sieving different sizes



Different sedimentation



Different rolling speed

Protein Purification

*“To Study Proteins and their Functions,
you must first **Produce**, **Extract**, and **Purify** Them.”*

Produce:

1. Culturing Cells under conditions that maximize *expression*.
2. Harvest cells by centrifugation or filtration.

Extract:

3. Resuspend cells in buffer – cell lysis: disruption, reagent, sonication.
4. Obtain extract by centrifugation or filtration – remove debris.

Purify:

5. Differential solubility (Ammonium sulfate / Acetone / PEG)
6. “Column” chromatography
 - gel exclusion chromatography (size)
 - dextran (Pharmacia - Sephadex)
 - polyacrylamide (Bio-Rad - Bio-Gel P))
 - agarose (Pharmacia -Sephrose; Bio-Rad – Bio-Gel A)
 - ion exchange chromatography (charge: **cation** ; **anion**)
 - (+) CM Sephadex carboxymethyl –
 - P-Cellulose phosphate –
 - AG-50 sulfonic acid (Polystyrene)
 - (-) DEAE-Sephadex diethylaminoethyl –
 - QAE-Sephadex diethyl-(2-hydroxypropyl)-aminoethyl
 - AG 1 tetramethylammonium (Polystyrene)
 - affinity chromatography (ligand)
 - NAD⁺ / ATP / PLP / 6 x His
 - IMAC = Immobilized Metal Affinity Chromatography
 - **TALON** Sepharose bead with tetradentate to Co²⁺
 - **Ni-NTA** (Ni – nitrilotriacetic acid) resins (QIAGEN)
7. HPLC (High-Performance Liquid Chromatography)
 - stationary support with small particles of large surface area
 - improved speed with high pressure to improve solvent flow

A Systematic Approach to Purification Development - Summary

- Develop assay methods
- Set the aims (purity and quantity)
- Characterize the target protein
- Use different separation principles
- Use few steps
- Limit sample handling between purification steps
- Start with high selectivity - increase efficiency
- Remove proteases quickly
- Reduce volume in early step
- Keep it simple!

Protein Expression, Purification and Analysis

In **olden days**, protein purification used to start with a source (organ tissue, plant source, bacteria line) known to be rich in the protein of interest. Often this would involve obtaining many pounds of organ tissue directly from a slaughter house or growing large (30 or 60L) batches of bacterial culture in order to isolate sufficient material for further studies. Purification based on solubility and charge.

*Typical protocol for isolation of a mammalian protein (after procedure for **chicken heart LDH – H4**, Nathan Kaplan et al., JBC, 239: 1753-1761 (1964):*

Step 1: Obtain 25 lbs of fresh chicken hearts on ice from local slaughter-house.

Step 2: Cut tissue in small sections, grind tissue in a commercial meat grinder, extract with 14L cold distilled water.

Step 3: Filter cell and connective tissue debris from extract by passing thru double layer of cheese cloth.

Step 4: Centrifuge using 1L containers and large bucket rotor International centrifuge at 1300 x g for 30 minutes. Pool supernatant. Assay.

Step 5: Adjust pH to 7.0, add solid ammonium sulfate to give 70% saturation of AS, maintain pH ~ 7 by adding dilute ammonium hydroxide as needed. Let stand for 2-4 hours, filter overnight on fluted filter paper. Scrape ppt. off filter paper and suspend in 1 L of cold distilled water. Centrifuge. Assay.

- Step 5:** Adjust pH to 7.0, add solid ammonium sulfate to give 70% saturation of AS, maintain pH ~ 7 by adding dilute ammonium hydroxide as needed. Let stand for 2-4 hours, filter overnight on fluted filter paper. Scrape ppt. off filter paper and suspend in 1 L of cold distilled water. Centrifuge. Assay.
- Step 6:** Dialyze soluble fraction against two 20 L changes of 0.005M Tris buffer, pH 7.0 for a total of 12 hours. To the solution, add solid AS to 25% saturation. Centrifuge, remove ppt. Add more AS to 60% saturation, leave standing for 1 hour, spin at 18,000 x g for in a Sorvall refrig. Centrifuge, save pellet. Dissolve pellet in 300 mL of 0.005 M Tris buffer, pH 7.6. Dialyze against two 20 L changes of 5 mM Tris, pH 7.0 for 8 hours each, centrifuge.
- Step 7:** Add precooled (-15 deg) acetone slowly to dialysate to ppt protein at 25%/(v/v). After 10 minutes, cent. – discard ppt. Add more acetone to 50% (v/v), leave for 20 minutes at 0°C. Collect ppt. At 18,000 x g for 30 min., then extract with 200 mL cold Tris buffer, pH 7.6, cent., discard ppt.
- Step 7:** Add supernatant to DEAE cellulose column. Elute with a gradient of 2 L each of 0.005 M Tris, pH 7.0 and same buffer with 0.20 M NaCl. Combine fractions with LDH activity. Assay. Expected yield 50-60 %.
- Step 8:** Re-crystallization - Ppt. with saturated AS, redissolve in a minimum of buffer, centrifuge, add AS slowly with stirring to 30% saturation. Wait about 1 day, centrifuge to harvest enzyme. Assay. Yield ~ 30%, 100X purification.

Enzyme Assay / Enzyme purification

Enzyme Activity (units)

1 International Unit = amt. of E which cat. $\frac{1 \mu\text{mol S} \rightarrow \text{P}}{\text{min}}$

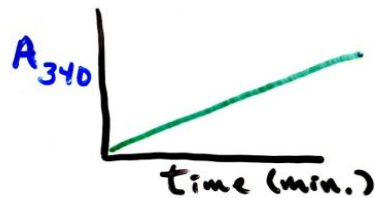
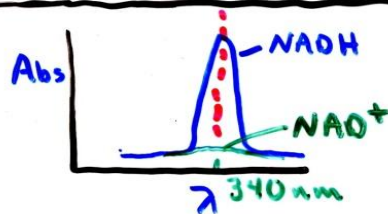
Specific Activity = $\frac{\mu\text{mol S} \rightarrow \text{P}}{\text{min}} / \text{mg protein}$ at 25°C
or IU/mg
(measure of Enzyme purity)

Turnover number = $V_{\text{max}} / [E]_{\text{tot}} = k_{\text{cat}} (\text{min}^{-1})$

Purification of Hypothetical Enzyme



≡ assay



<u>Step</u>	<u>Vol.</u>	<u>Protein</u>	<u>E Act.</u>	<u>Spec. Activity</u>	<u>% Y</u>
1. Cell extract	1400 mL	10,000 mg	100,000	10	100
2. (NH ₄) ₂ SO ₄ ppt.	280	3,000	96,000	32	96
3. DEAE col.	90	400	80,000	200	80
4. Gel "Filtration" (size-exclusion)	80	100	60,000	600	60
5. Affinity chrom.	6	3	45,000	<u>15,000</u>	45%
				1500x	

Protein Purification Schemes

Also, each step of the purification should be monitored by gel electrophoresis.

- In the initial stages of purification we will probably see a variety of bands, of various molecular weights, on our gel.
- After the different purification steps, we should see the disappearance of certain bands concomitant with the increasing concentration of a certain band (or bands) representing our protein.
- If we have successfully purified our protein (and if it is a single polypeptide) we should arrive at a constant specific activity and a *single band on a gel*.
- Analytical methods like HPLC or densitometer scanning of a stained gel can give us a quantitative idea of the purity of our final sample.

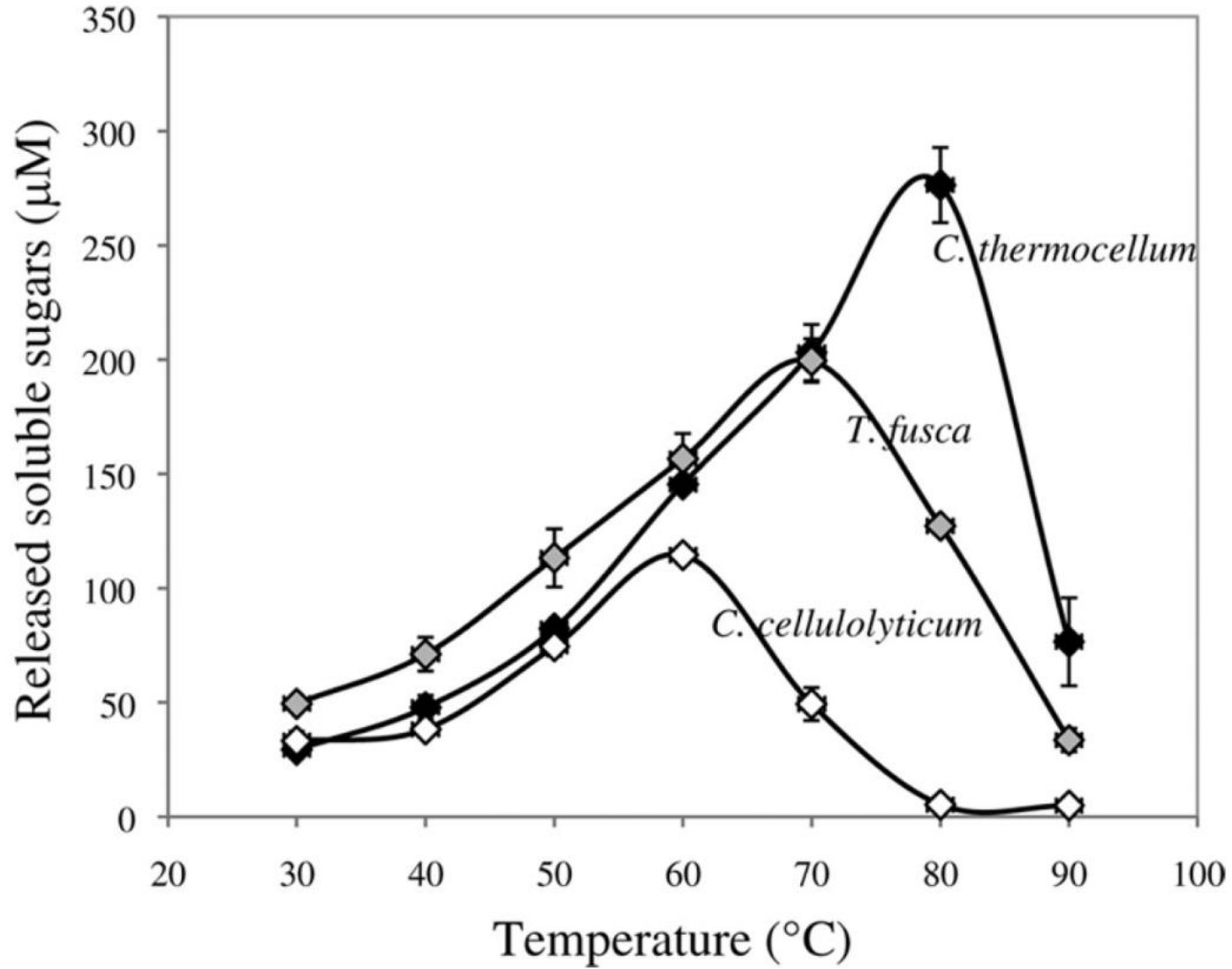
The following chart represents the typical data one would monitor during a purification:

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	% Yield
Crude cell lysate	5500	6600	1.2		
30-70% Ammonium sulfate cut	1020	5910	5.8	4.8	89.5
DEAE Sephadex pool	187	5070	27.1	4.7	85.8
CM Sephadex pool	102	4420	43.3	1.6	87.2
Phenyl Sepharose pool	56	3930	70.2	1.6	88.9
Gel Filtration pool	32	2970	92.8	1.3	75.6
Affinity resin type #1 pool	5.8	2520	434.5	4.7	84.8
Affinity resin type #2 pool	5.3	2390	450.9	1.0	94.8
Total purification	376				
Total yield (%)	36				

Properties exploited to purify a protein

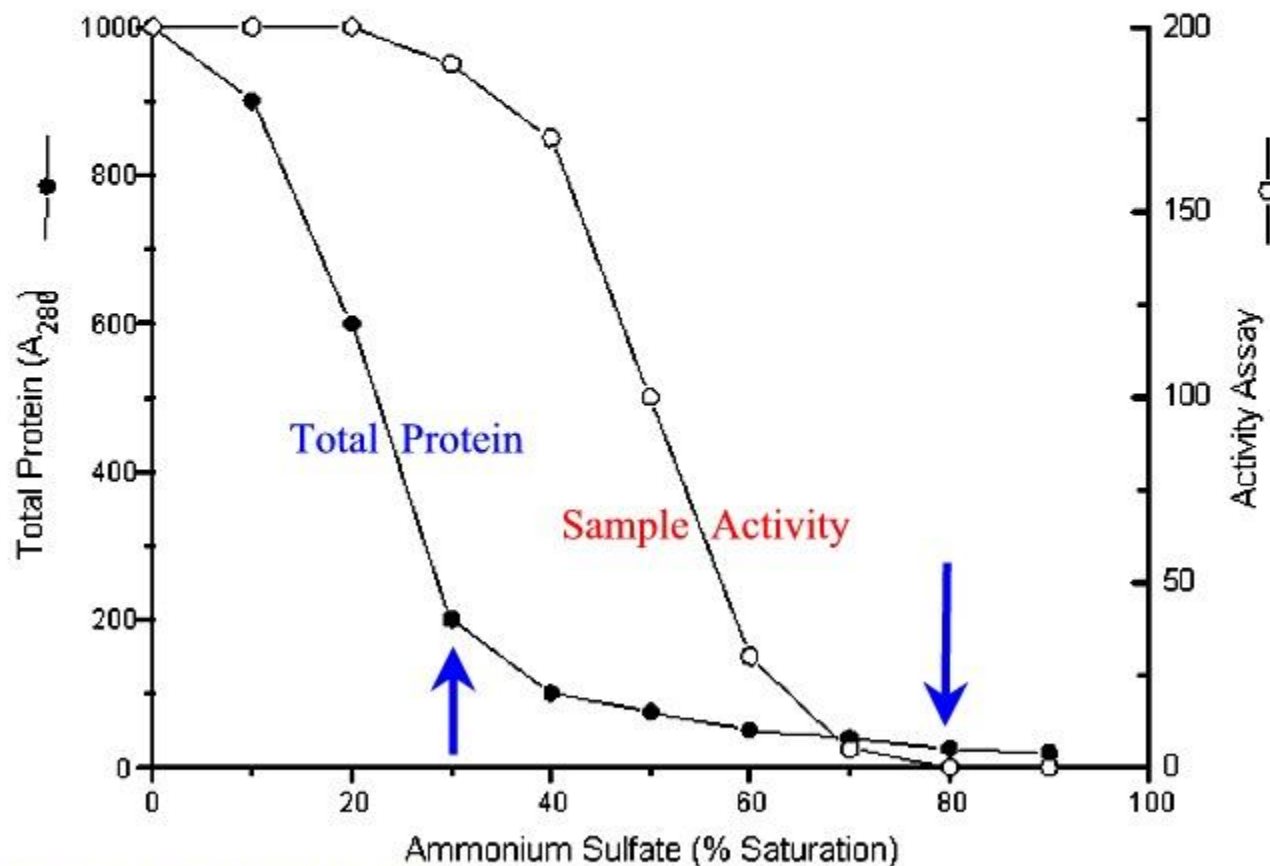
- **Thermostability**
 - Boiling experiment
- **Solubility**
 - Salt-in/salt-out
- **Size**
 - size exclusion (gel filtration) chromatography
- **Charge**
 - Ion exchange chromatography
- **Affinity**
 - Affinity chromatography
- **Hydrophobicity**
 - Hydrophobic chromatography

Optimum temperatures of bacterial endoglucanases



Ammonium Sulfate (% saturated)	0	10	20	30	40	50	60	70	80	90
Sample A ₂₈₀	1000	900	600	200	100	75	50	40	25	20
Activity assay(units)	200	200	200	190	170	100	30	5	0	0

Solubility and Protein Purification - AS Fractionation



Basic Principles of Protein Purification



Small molecule	Macromolecule				Cell Debris
Amino acid, Sugar, Nucleotides, etc	Nucleic Acid	Protein	Carbohydrate	Lipid	



Ammonium sulfate fractionation

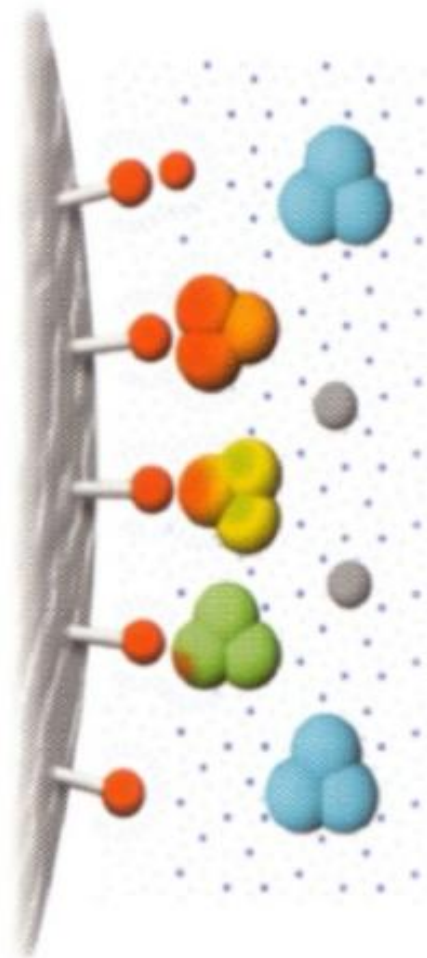
The following techniques listed in each box can be used to separate proteins based upon the following characteristics:

Size (GF)	Charge (IEX)	Polarity (HIC)	Affinity (AC)
Gel filtration, SDS-PAGE, Ultrafiltration	Ion exchange, Chromatofocusing, Disc-PAGE, Isoelectric focusing	Reverse phase chromatography, HIC, Salting-out	Affinity chromatography, Hydroxyapatite

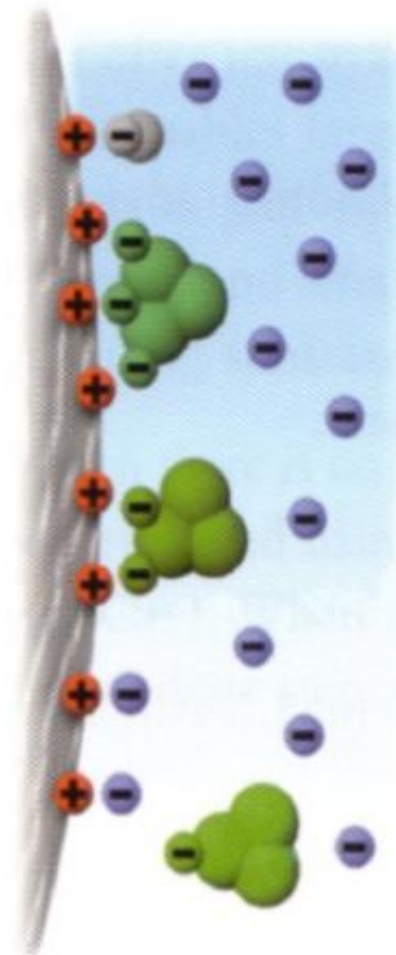
Chromatography



Gel filtration
(GF)



Hydrophobic interaction
(HIC)



Ion exchange
(IEX)

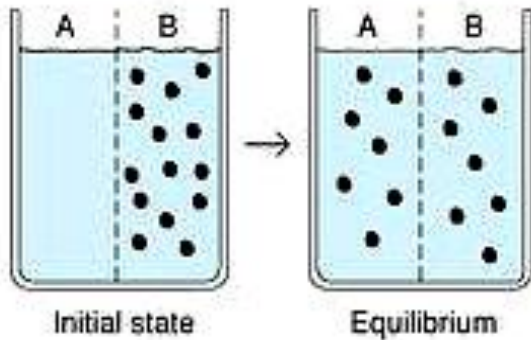


Affinity
(AC)

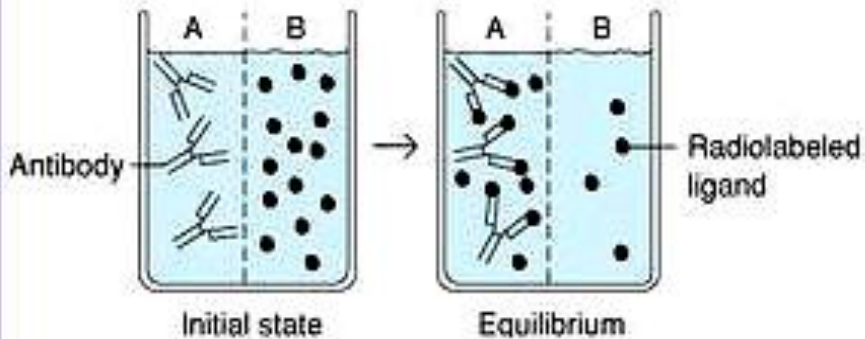
Separation by Size: Dialysis

(a)

Control: No antibody present
(ligand equilibrates on both sides equally)

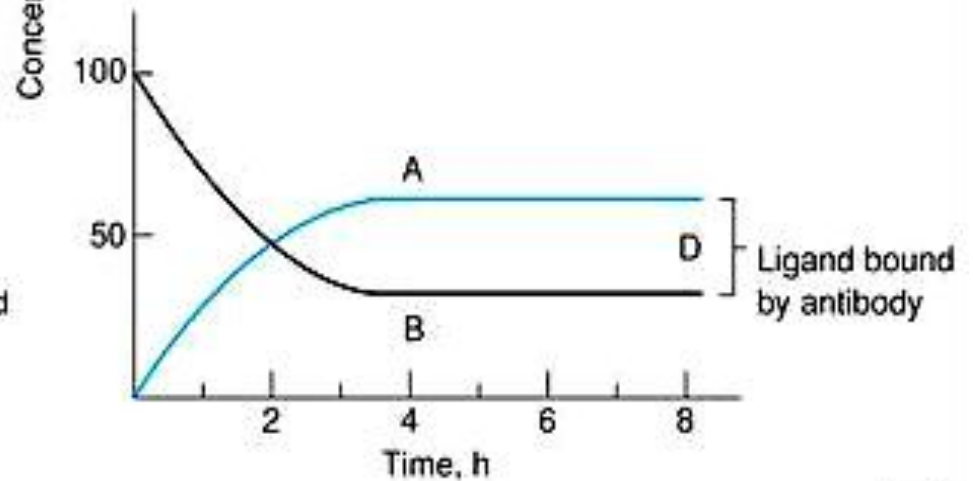
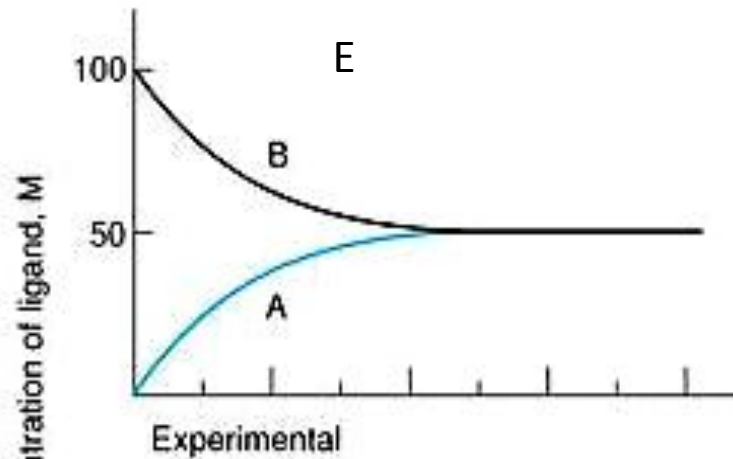


Experimental: Antibody in A
(at equilibrium more ligand in A due to Ab binding)



(b)

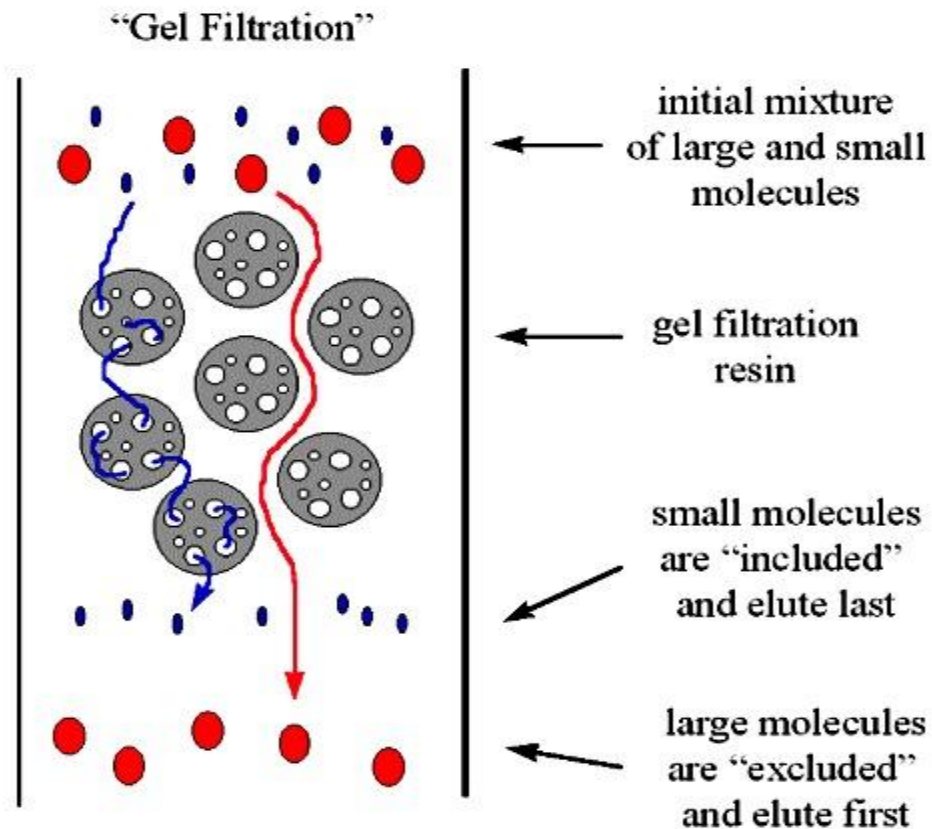
Control



Separation by Size: GF

GEL FILTRATION / SIZE EXCLUSION CHROMATOGRAPHY

- Gel filtration resin can be thought of as beads which contain pores of a defined size range.
- Large proteins which cannot enter these pores pass around the *outside* of the beads.
- Smaller proteins which can enter the pores of the beads have a longer, tortuous path before they exit the bead.
- Thus, a sample of proteins passing through a gel filtration column will *separate based on molecular size*: the big ones will elute first and the smallest ones will elute last (and "middle" sized proteins will elute in the middle).

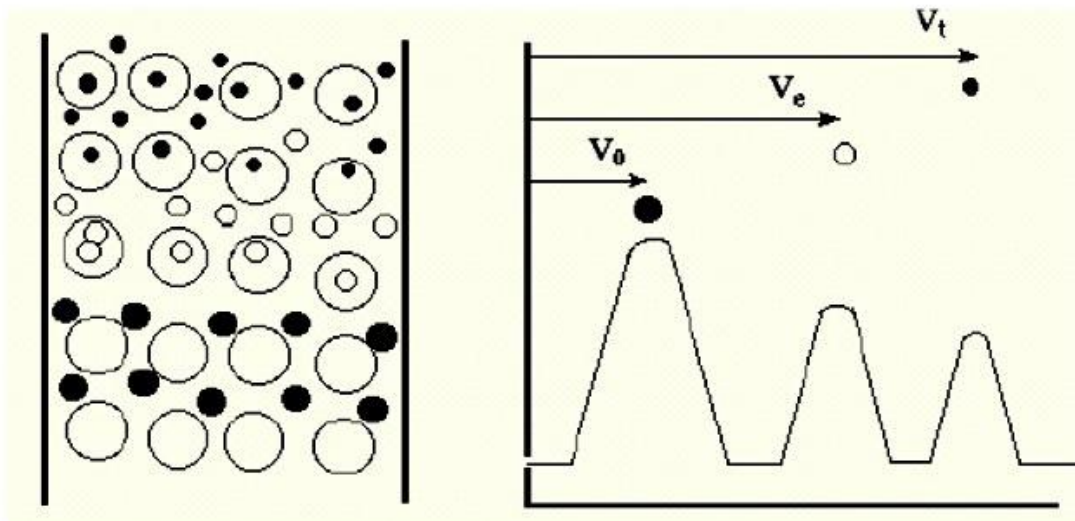


Gel Filtration Chromatography

V_0 = Void Vol; V_e = Elution Vol; V_t = Total Vol

The sample is applied in a narrow band at the top of the column and then it is washed through the column by the mobile phase. Large molecules in the sample that cannot pass through the pores of the beads are excluded from the beads and are restricted to the outer voids. They elute from the column after an amount of the mobile phase equal to V_0 has passed through the column. Molecules that are much smaller than the pores equilibrate with the entire liquid volume and elute at a volume equal to V_t , which is the sum of V_0 and V_i . Molecules that are small enough to pass through some of the pores of the beads, however, elute at various volumes (V_e), depending on how small they are and what fraction of the pores of the beads are accessible to them. Such molecules are referred to as partially included. Molecules in the sample can be separated in order of their size by collecting fractions as the mobile phase is eluted through the column, with the largest molecules eluting first and the smallest last.

The figure below represents the principle of gel-filtration chromatography.

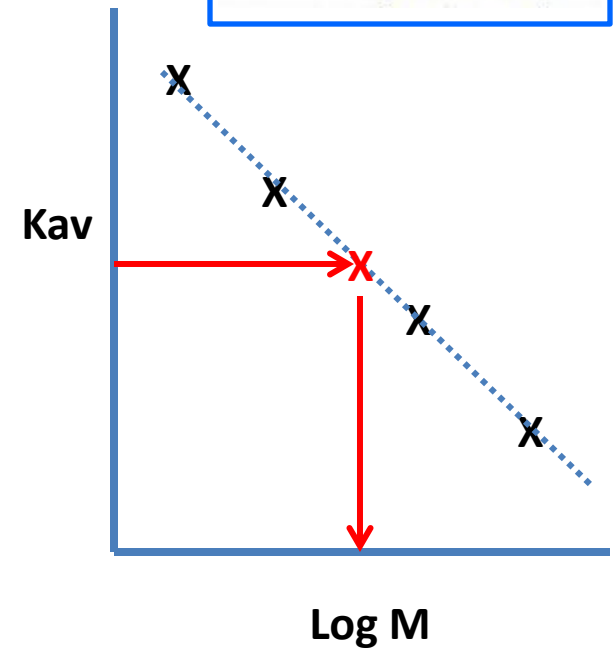


A partition coefficient can be calculated from the values above using the equation $K_{av} =$

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}$$

A semilogarithmic plot of the relationship of K_{av} to molecular weight can elucidate the efficiency of separation of molecules. Assuming that all molecules are of similar shape, the separation of molecules on the basis of molecular weight will be greatest in the linear range of the curve.

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}$$



Separation by Affinity: AC

Ni-NTA for the highest purity proteins

Purification of His-tagged proteins with Ni-NTA follows — as usual for QIAGEN purification products — a simple bind-wash-elute procedure (Figure 5). Due to the structure of NTA and its tight binding of nickel ions (Figure 6) proteins can be eluted in high yields with high purity (Figure 7). You can run several cycles without stripping and reloading the resin, which reduces also Ni²⁺ waste. The purification process itself is very robust and allows the use of a broad range of reagents, meaning you can use the specific conditions your proteins need to stay active to purify them (Table 5).

Ni-NTA — the world's most cited affinity purification resin

- Highest purity of protein after a single purification step (Figure 7)
- Minimal nickel leaching resulting in highly pure proteins and avoiding recharging and toxic waste (Figure 6)
- High yields of up to 50 mg/ml Ni-NTA resin or up to 2 µg/µl magnetic bead suspension (Figure 8)
- Broad reagent compatibility with the only resin that is compatible to 10 mM DTT (Table 5)

Cell lysate or cell-free
expression reaction

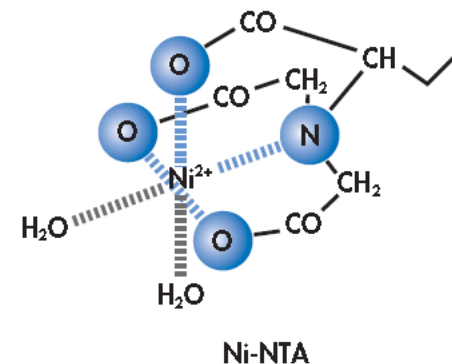


Figure 6. Ni-NTA gives you highly pure proteins. In Ni-NTA resins nickel ions are bound via four coordination sites. Nickel ions bound to IDA-based resins are only bound via three coordination sites and can be lost more easily. Empty charged coordination sites will function like ion-exchange matrices that can bind untagged contaminating proteins.

Purification of 6xHis tagged Proteins

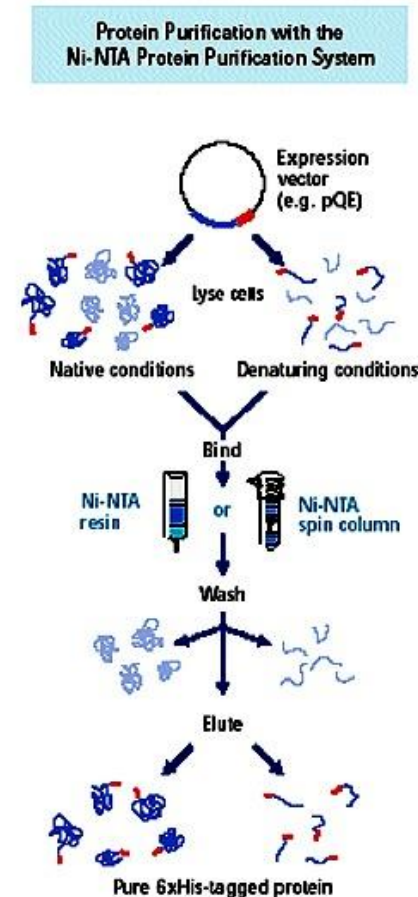
Ni-NTA Protein Purification System is based on the selectivity of patented Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive histidine residues — the 6xHis tag.

Procedure (from - www.qiagen.com)

The purification of 6xHis-tagged proteins consists of 4 steps: cell lysis, binding, washing, and elution ([see flowchart](#)). Purification of recombinant proteins using the QIAexpress system does not depend on the 3-dimensional structure of the protein or 6xHis tag. This allows one-step protein purification under either native or denaturing conditions, from dilute solutions and crude lysates. Strong denaturants and detergents can be used for efficient solubilization and purification of receptors, membrane proteins, and proteins that form inclusion bodies. Reagents that allow efficient removal of nonspecifically binding contaminants can be included in wash buffers ([see table "Reagents compatible with the Ni-NTA-6xHis interaction"](#)). Purified proteins are eluted under mild conditions by adding 100–250 mM imidazole as competitor or by a reduction in pH.

Reagents compatible with the Ni-NTA-6xHis interaction*

- 6 M guanidine HCl
- 8 M urea
- 2% Triton X-100
- 2% Tween 20
- 1% CHAPS
- 20 mM β -ME
- 50% glycerol
- 20% ethanol
- 2 M NaCl
- 4 M MgCl₂
- 5 mM CaCl₂
- \leq 20 mM imidazole



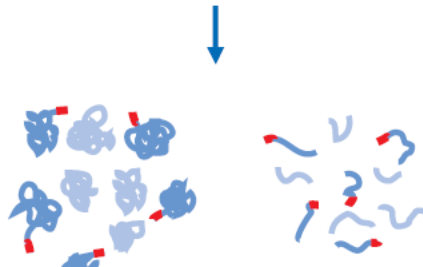
Cell lysate or cell-free expression reaction

Native conditions

Phosphate buffer, pH 8
300 mM NaCl
10–20 mM imidazole

Denaturing conditions

Phosphate buffer, pH 8
8 M urea or 6 M GuHCl
(imidazole optional)



Ni-NTA resin Bind

Wash

20 mM imidazole

pH 6.3

Elute

100–500 mM imidazole

pH 5.9 or pH 4.5

Pure 6xHis-tagged protein

- **His tag**

- Most commonly used.
- Small
- Fast and tight binding
- Resin cost effective and reusable
- **But doesn't help folding**

One-Step Purification



1. 100 kDa
2. 75 kDa
3. 50 kDa
4. 25 kDa
5. 12.5 kDa
6. 6.25 kDa
7. 3.125 kDa
8. 1.5625 kDa
9. 781.25 Da
10. 390.625 Da
11. 195.3125 Da
12. 97.65625 Da

Comparison of affinity tags for protein purification

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Table 1
Comparison of affinity tag technologies

Tag	Size (aa)	Resin	Eluting agent	Source	Capacity	Cost	Cost/10 mg
MBP	396	Amylose	Maltose	Biolabs	3 mg/ml	\$105/10 ml	\$12
HIS	6	Talon	Imidazole	Clontech	5–14 mg/ml	\$220/25 ml	\$18
		Ni-NTA	Imidazole	Qiagen	5–10 mg/ml	\$257/25 ml	\$21
GST	218	GSH-Sepharose	Glutathione	Amersham	10 mg/ml	\$396/25 ml	\$36
CBP	28	Calmodulin affinity	EGTA	Stratagene	2 mg/ml	\$227/10 ml	\$114
STR (Strep II)	8	Strep-Tactin-Sepharose	Desthiobiotin	IBA	50–100 nmol/ml	\$1100/25 ml	\$293
FLAG	8	Anti-FLAG M2 MAb agarose	FLAG peptide	Sigma	0.6 mg/ml	\$1568/25 ml	\$1045
HPC	12	Anti-Protein C MAb matrix	EDTA	Roche	2–10 nmol/ml	\$299/1 ml	\$4983
CYD	5	InaD	DTT	N/A	>0.2 mg/ml	N/A	N/A

Capacity for the fusion protein and retail cost information were obtained from manufacturer's catalogs and websites, except for the InaD resin for which the capacity was estimated from our experiments. The unit cost was calculated for purification of 10 mg of a 30 kDa protein fused to the appropriate tag, and the values for the MBP and GST fusions take into account the large size of those affinity tag. Since the affinity resins listed are reusable, actual costs may be less.

Proteases (*recognize specific sequence*) used to remove tags

- **Thrombin**

- Thrombin is a serine protease (uses serine cleave the peptide).
- Designed to perform the specific cleavage needed to activate fibrinogen, without digesting all the other important proteins in the blood.
- Highly specific.
- Cleavage site (Leu-Val-Pro-Arg-Gly-Ser)



- **Tev protease**

- **PreScission** - the new fashion, 3C protease

- A genetically engineered fusion protein of glutathione S-transferase (GST) and human rhinovirus (HRV) type 14 3C protease (1) with a molecular weight of approx 46kD.
- Enables the low-temperature cleavage of fusion proteins containing the PreScission™ Protease recognition sequence.

- Recognition site:

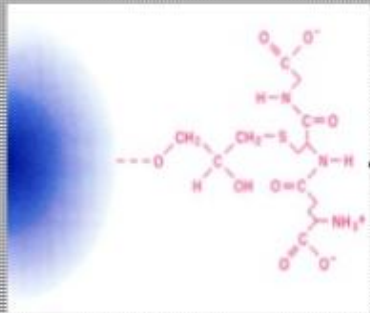


Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro.

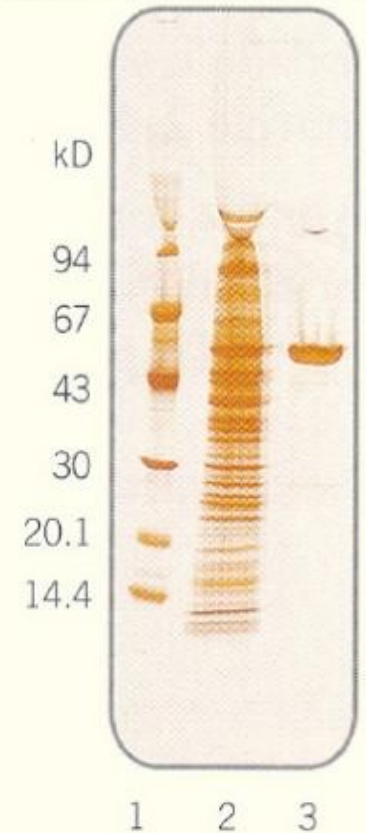
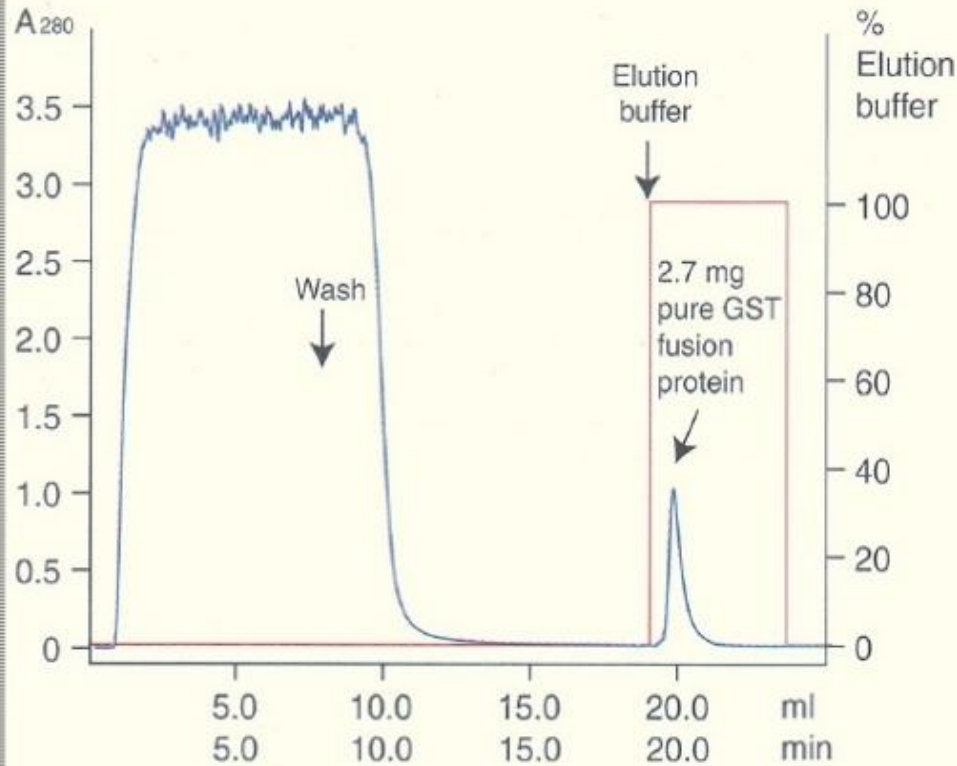
Cleavage Sites Table for Fusion Proteins

PROTEASE	COMPANY	Protease Capture
Thrombin Leu-Val-Pro-Arg ▼ Gly-Ser	Amersham-Biosciences, Novagen, SIGMA, Roche	Benzamidine-Agarose
Factor Xa Ile-Glu/Asp-Gly-Arg ▼	Amersham-Biosciences, New England Biolabs, Roche	Benzamidine-Agarose
Enterokinase Asp-Asp-Asp-Asp-Lys ▼	New England Biolabs, Novagen, Roche	Trypsin Inhibitor-Agarose
TEV protease Glu-Asn-Leu-Tyr-Phe-Gln ▼ Gly	Invitrogen – Life Technologies	Ni-NTA (6His recomb. TEV)
PreScission Leu-Glu-Val-Leu-Phe-Gln ▼ Gly-Pro	Amersham-Biosciences	GSTrap for GST fusion enzyme
TAGzyme His-tag removal by Exoproteolytic Digestion	Quiagen	Ni-NTA (6His recomb. enzyme)
Intein Site dithiothreitol cleavage	New England Biolabs	DTT elimination by dialysis
HRV 3C Protease Leu-Glu-Val-Leu-Phe-Gln ▼ Gly-Pro	Novagen	Ni-NTA (6His recomb. enzyme)
SUMO Protease recognize the tertiary structure of the ubiquitin-like (UBL) protein, SUMO	Invitrogen – Life Technologies, LifeSensors	Ni-NTA (6His recomb. enzyme)

GST fusion protein purification



glutathione sepharose



Column: GSTrap 1ml

Sample: 8 ml cytosolic extract from *E. coli* expressing a GST fusion protein

Binding buffer (BB): PBS, pH 7.3

Elution buffer (EB): 50 mM Tris-HCl, pH 8.0 with elution

Lane 1: MW Stds

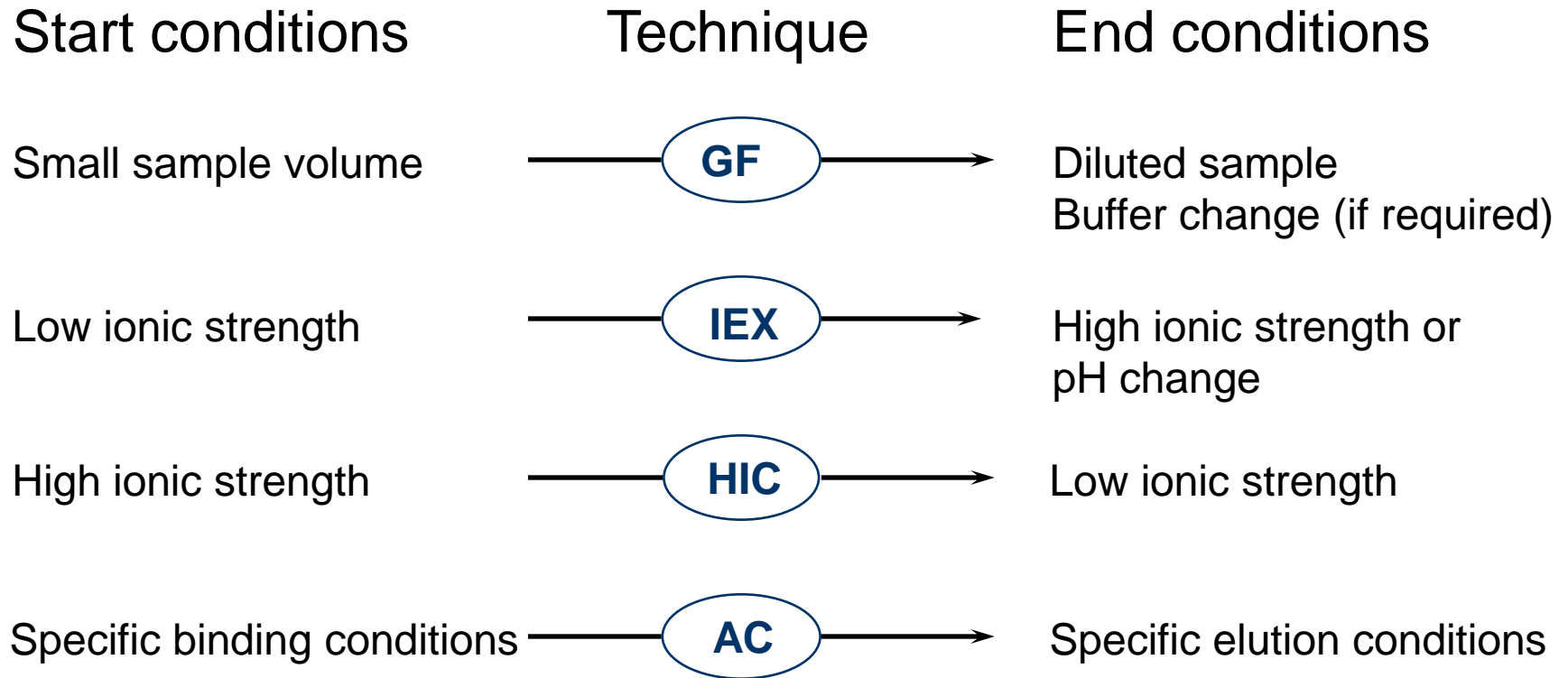
Lane 2: *E. coli* cytosolic extract

Lane 3: GSTrap

Linking Chromatography Techniques into a Purification Protocol - General Rules

- Combine techniques with complementary selectivities (e.g. IEX, HIC and GF).
- Minimize sample handling between purification steps (e.g. concentration, buffer exchange).

Linking Chromatography Techniques



G Protein Receptor Kinase Purification

A. Tobin et al. (1996)
 J. Biol. Chem. 271, 3907-3916

Technique	Porcine cerebella homogenate	Purification factor	Comment
Ppt	Ammonium sulfate precipitation	7	<ul style="list-style-type: none"> All buffers contain protease inhibitors
HIC	Butyl Sepharose Fast Flow	20	<ul style="list-style-type: none"> All purifications done at +4° C
AIEX	RESOURCE Q	2408	<ul style="list-style-type: none"> Removal step, main contaminant is bound
CIEX	RESOURCE s		<ul style="list-style-type: none"> Elution buffer is used as starting buffer for next column
AC	HiTrap Heparin	18647	<ul style="list-style-type: none"> 10 mg homogenous protein obtained