Ion exchange and affinity chromatography

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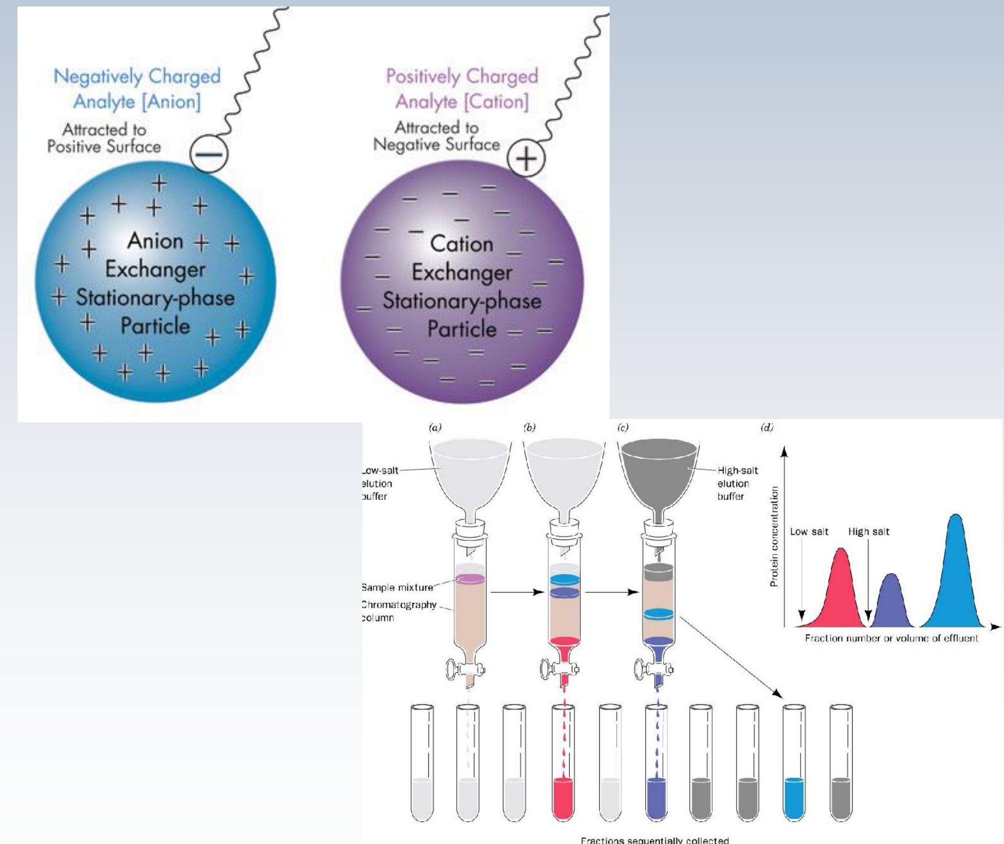


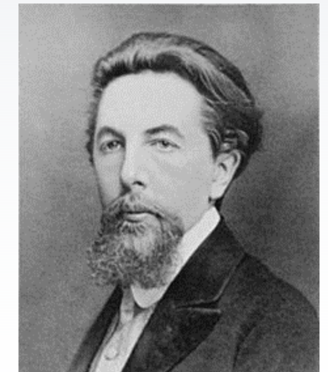
Introduction to Basic Protein Chemistry and Proteomics with Clinical Applications: 30.09.2014

Chromatography

- Separation of biomolecules which is based on their physicochemical characteristics:
  - Polarity (solubility, volatility, adsorption) HIC, RP
  - Size/mass (diffusion, sedimentation) Size exclusion Chr.
  - **Ionic characteristics** (charge) **Ion Exchange Chromatography**
  - **Shape** (ligand binding, affinity) **Affinity Chromatography**
- Based on these properties the molecules can be differentially separated between the stationary phase and mobile phases
- Introduced in 1901 by Mikhail Tsvet,

  for separation of plant pigments

  (Proceedings of the Warsaw Society of Naturalists, biology section, 1905)

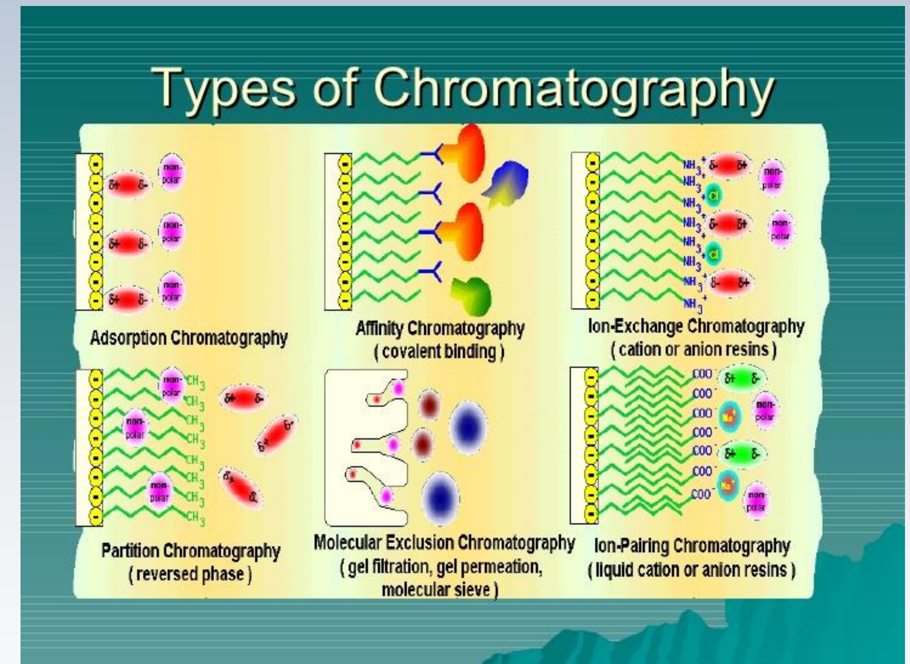


Liquid chromatography (LC)

- IEX ➡ anion exchange
  ➡ cation exchange
  ➡ chromatofocusing
- Affinity chromatography

group separations vs. specific interactions

- **HIC** (Hydrophobic Interaction chromatography)
- **RP** (Reverse-Phase) **chromatography**



- Size exclusion/Gel filtration

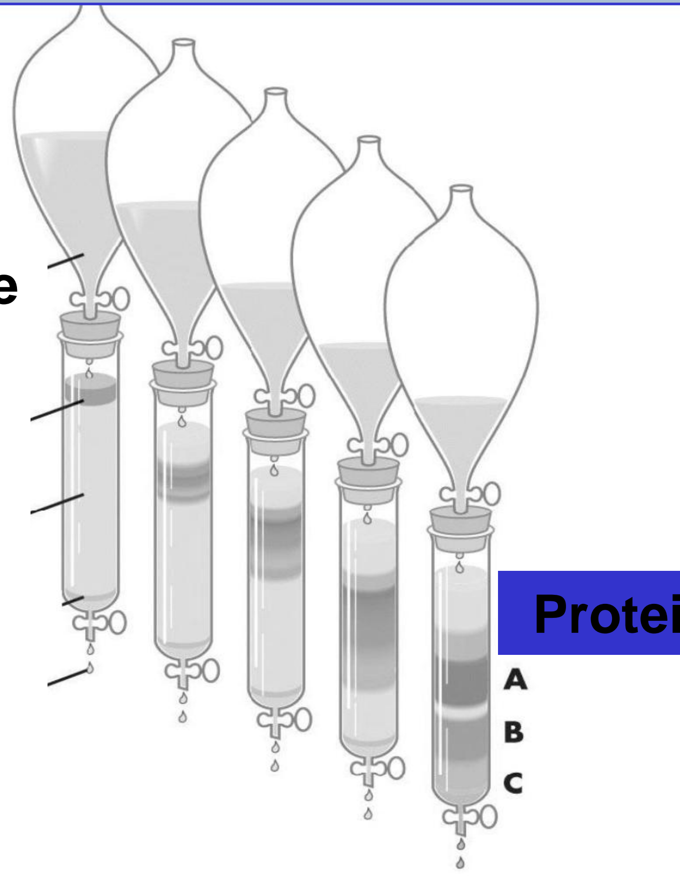
  non-interacting & medium resolution

Adsorptive chromatography

- **Biomolecule adsorps to the matrix (stationary phase) reversibly**
- **Adsorption is controlled by the mobile phase - elution**

IEC: proteins in a low-salt mobile phase may be bound to the matrix, but when the composition of the mobile phase is changed to high-salt, the interaction is reversed and the proteins elute

Liquid chromatography: basics

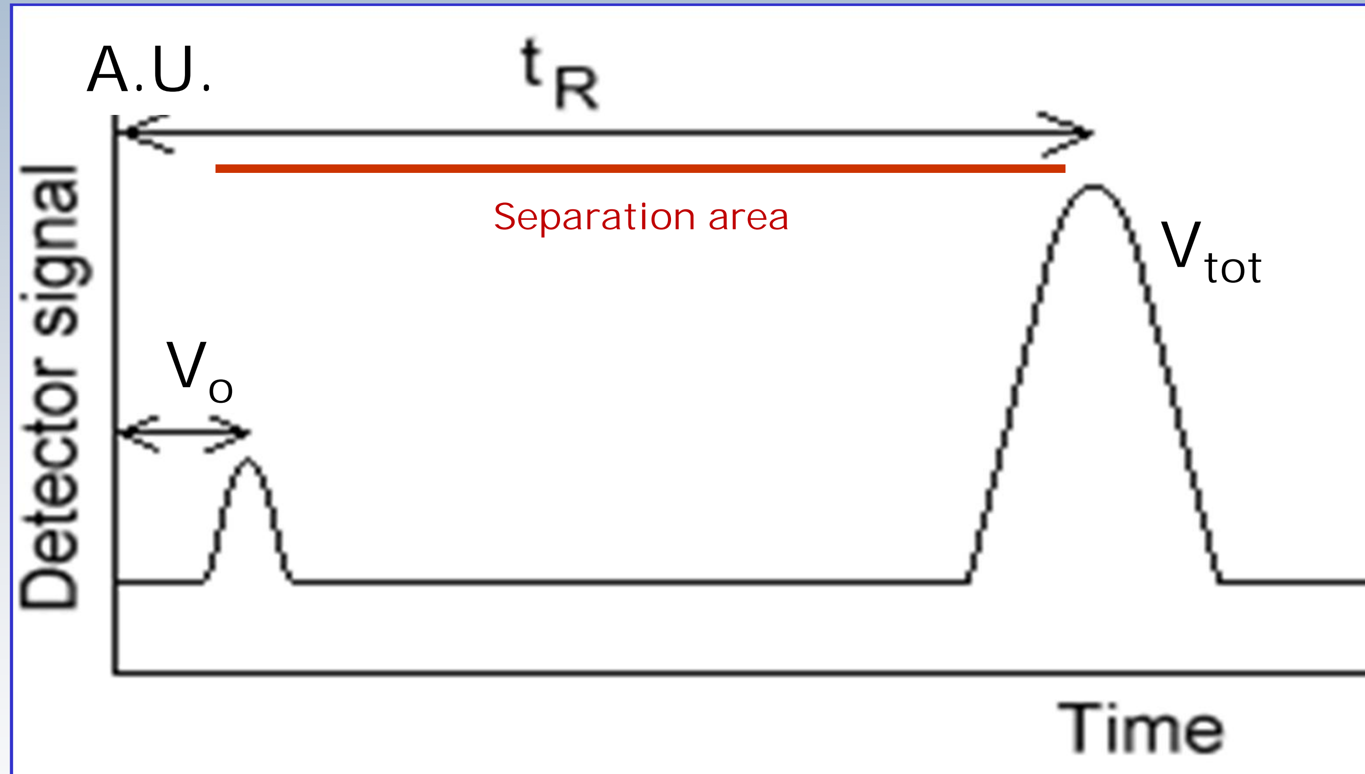


Chromatography steps:

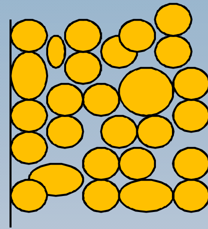
1. Equilibration
2. Injection
3. Elution
4. Washing

http://www.youtube.com/watch?v=Z54ec_G12QE

Chromatogram: basics



Matrix

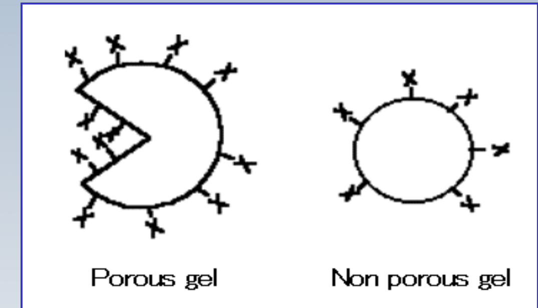


Porous

Cellulose
sugar polymers (agarose, dextran)
polymers (acrylamide, styrene)
silica coated with polymers

Nonporous

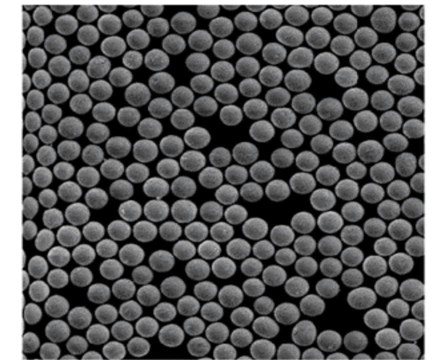
Styrene
Acrylates
Zirconia
Monolith
(*ProSwift*, *Dionex*)



- Particle size, ~5 µm — 2 µm
- Pore size i.e. 100-200 Å

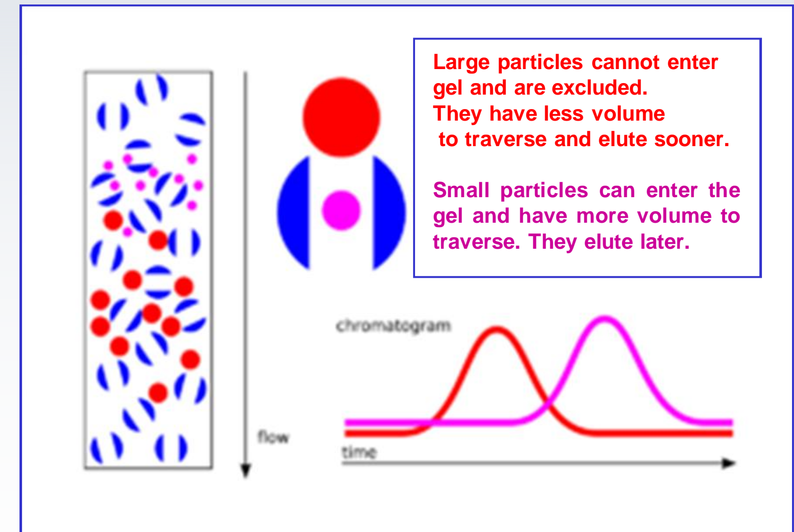
- Chemical and Physical stability
- Temperature <80°C vs >200°C)

- Pressure — Porous < Nonporous
- pH — Porous < Nonporous
- Capacity — Porous > Nonporous



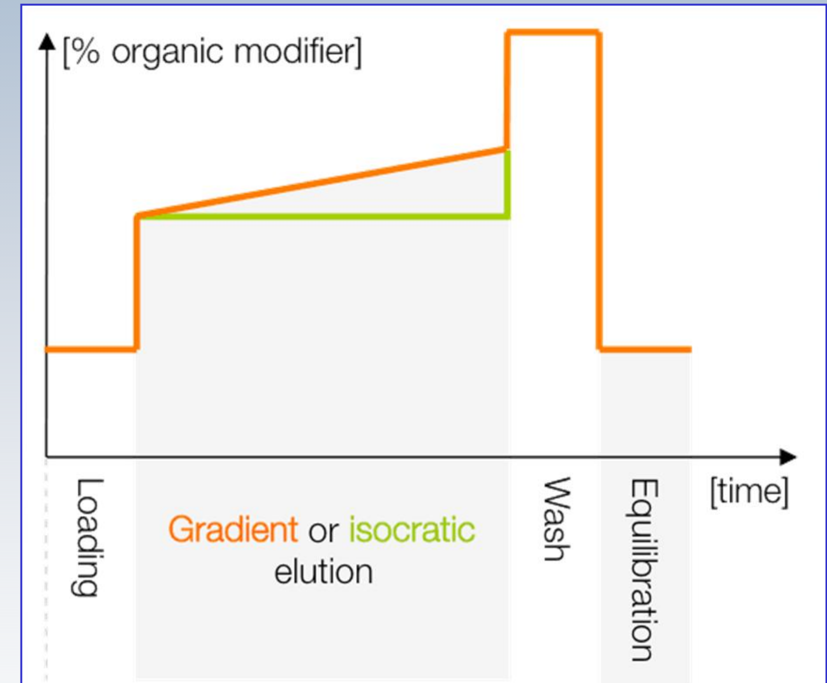
Diffusion and porous matrices

- **Size exclusion chromatography is based on *diffusion* and molecules in solution are separated by their size** (molecular weight)
- -Small molecules diffuse into smaller pores and travel slowly, therefore elute last, **close** to V_{total}
- Large molecules do not fit into pores and elute first, **close** to V_0
- **In *affinity* and *IE chromatography* the functional groups are attached to surface of particles inside the pores**



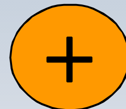
Mobile phase

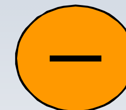
- **Composition**
- **Type of elution**
  - **isocratic** vs **gradient elution (gradient shape)**
- **Flow rate**
  - **Depends on the type of matrix**
    ***Affects* resolution**
  - in **porous** matrixes should be **slow** enough to allow **diffusion** to pore cavities
  - in **nonporous** matrixes **higher** flow rates may be used

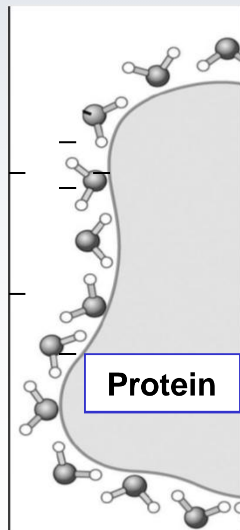


Ion exchange chromatography

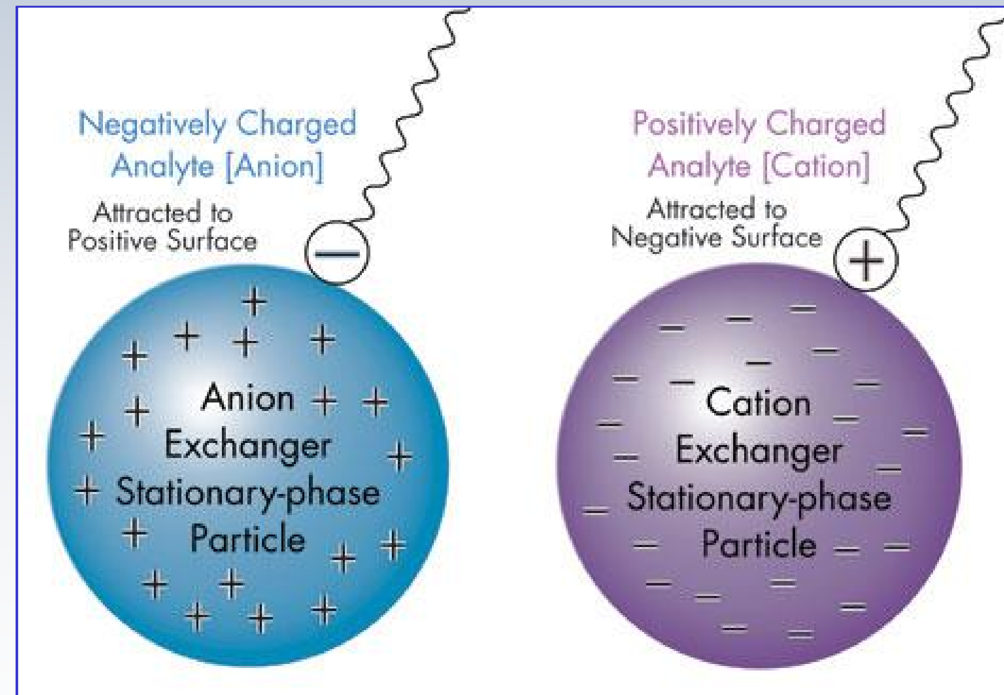
- Based on ionic interactions

Anion exchange 

Cation exchange 



local versus net charge



IEC in practise

1. Choose the **matrix type** according to your target protein
2. Equilibrate (**low salt**, 20 mM)
3. Inject protein sample (in **low salt**), balance
4. Apply gradient (**increasing salt**) to elute proteins

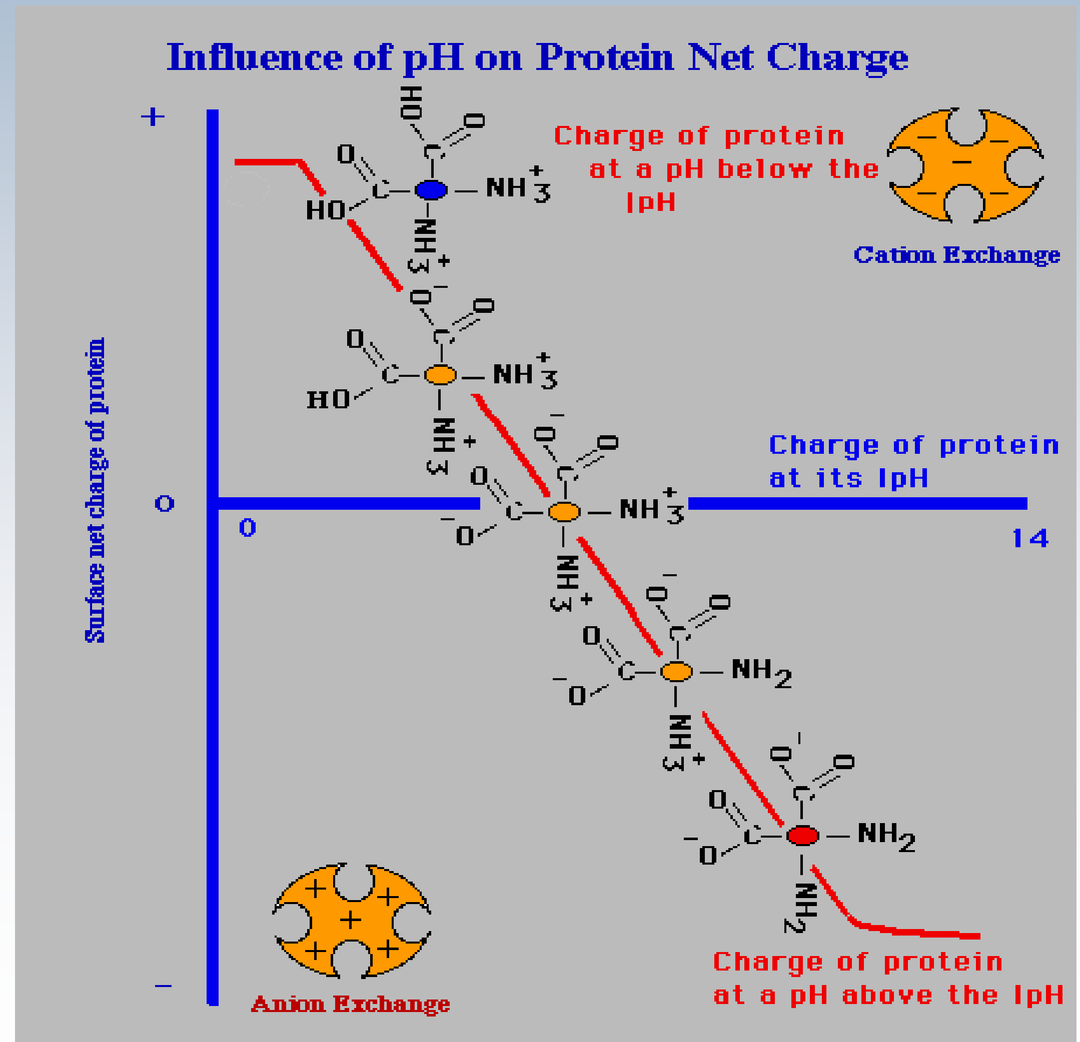
- Obey buffer instructions:
  AEC- cationic buffers, CEC- anionic buffers
- Non-ionic detergents (!)
- Elution:

1. *Increasing* salt gradient (0 ➡ 1M NaCl in 20 mM buffer)
2. *pH gradient* (ampholytes in chromatofocusing)
3. *Type of gradient*: linear gradient /step wise/shape of gradient

Titration curve of a protein

- **Charge of a protein is pH dependent !**

  Amino acids with **ionizable** side chains:
  Arg, His, Lys
  Asp, Glu
  Cys, Tyr
- pI =isoelectric point

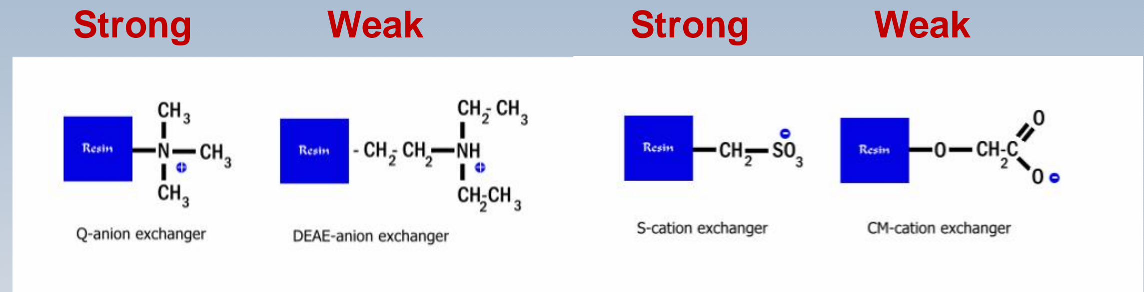


if pH > pI use anion EC

if pH < pI use cation EC

IEC matrices

- **Strong** versus **weak ion exchangers**

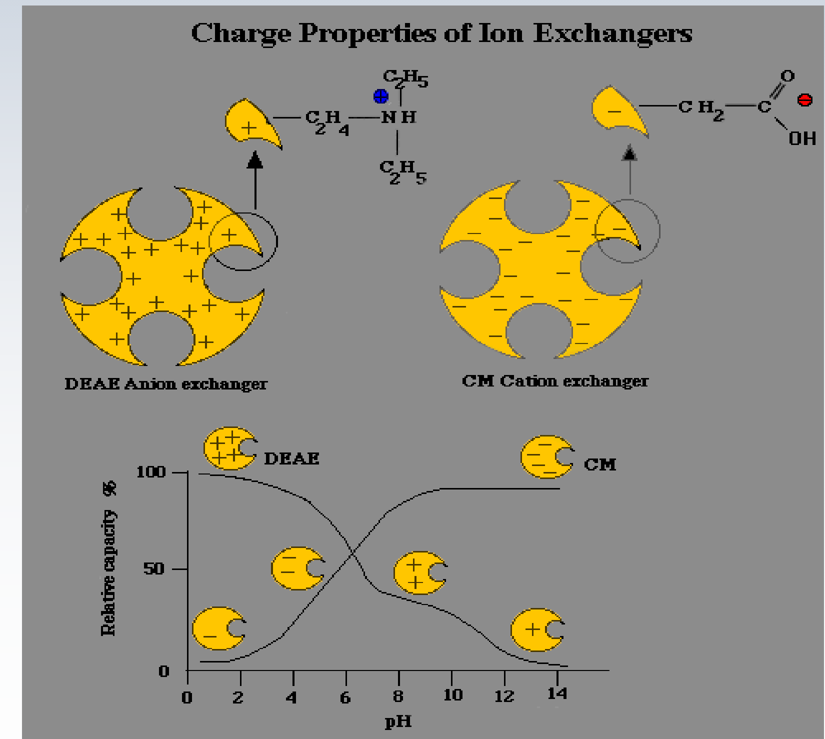


- **anion EC**: positive matrix

DEAE	diethyl aminoethyl (W)
QAE	quaternary aminoethyl
Q	quaternary amine

- **cation EC**: negative matrix

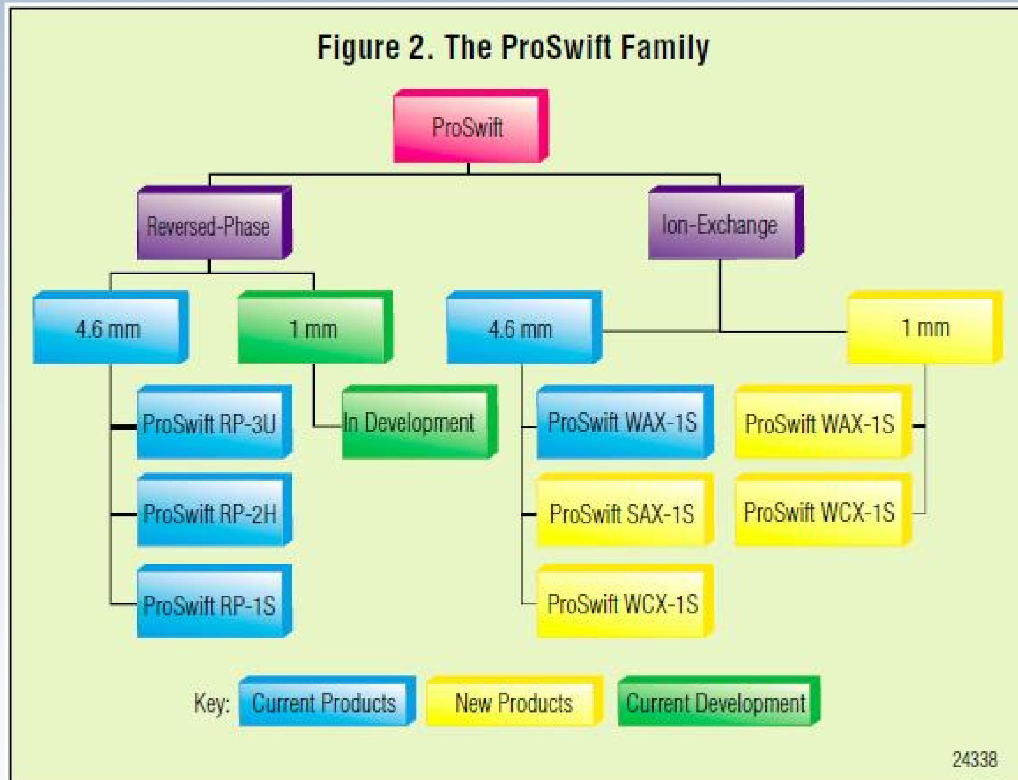
CM	carboxymethyl (W)
SP	sulphopropyl
S	sulphonate



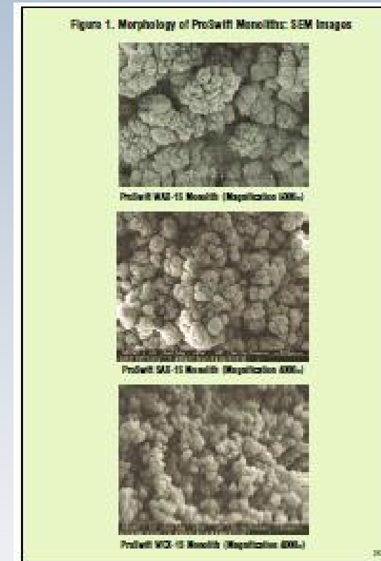
Buffers for exchange chromatography

Molecule (AEC)	pKa	dpKa/degree C.	Counter ion	Molecule (CAC)	pKa	dpKa/degree C.	Counter ion
N-methyl piperazine	4.75	-0.015	chloride	Maleic acid	2.00		sodium
piperazine	5.68	-0.015	chloride or formate	Malonic acid	2.88		sodium
L-histidine	5.96		chloride	citric acid	3.13	-0.0024	sodium
bis-Tris	6.46	-0.017	chloride	lactic acid	3.81		sodium
bis-Tris propane	6.80		chloride	formic acid	3.75	0.0002	sodium or lithium
triethanolamine	7.76	-0.020	chloride or acetate	butaneandioic acid	4.21	-0.0018	sodium
Tris	8.06	-0.028	chloride	acetic acid	4.76	0.0002	sodium or lithium
N-methyl-diethanolamine	8.52	-0.028	chloride	malonic acid	5.68		sodium or lithium
diethanolamine	8.88	-0.025	chloride	phosphate	7.20	-0.0028	sodium
1,3-diaminopropane	8.64	-0.031	chloride	HEPES	7.55	-0.0140	sodium or lithium
ethanolamine	9.50	-0.029	chloride	BICINE	8.35	-0.0180	sodium
piperazine	9.73	-0.026	chloride				
1,3-diaminopropane	10.47	-0.026	chloride				
piperidine	11.12	-0.031	chloride				
phosphate	12.33	-0.026	chloride				

Examples: ProSwift matrices (www.dionex.com)



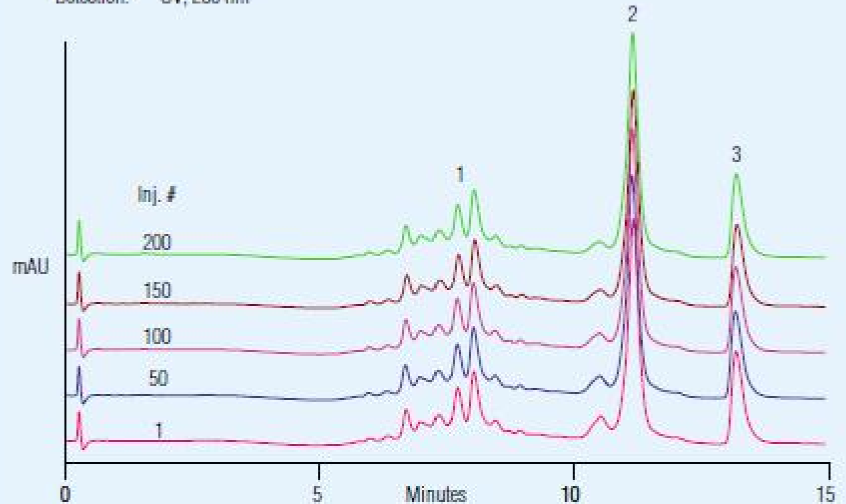
- **Monolith** matrix

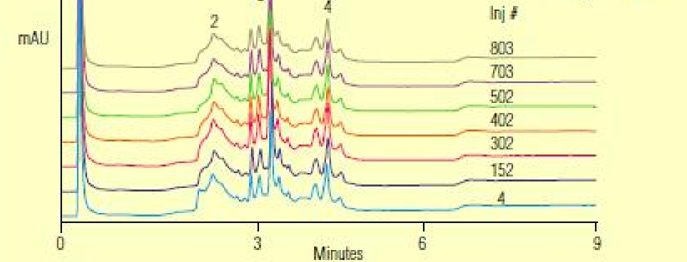


- Combines the **stability** of nonporous and **capacity** of porous matrices
- Optimal mass transfer ➧ minimal diffusion…
- High loading capacity and reproducibility!
- Can be used as 1st dimension in multidimensional chromatography

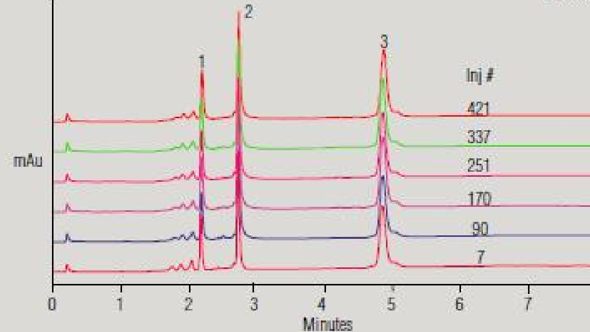
ProSwift: IEC columns

Weak anion exchanger





Strong Anion exchanger



Strong cation exchanger

IEC: summary

PROS:

- **Concentrates** the sample
- Sample volume **is not** a restricting factor
- **Gentle**
- **High resolution**

 (strong vs weak)

CONS:

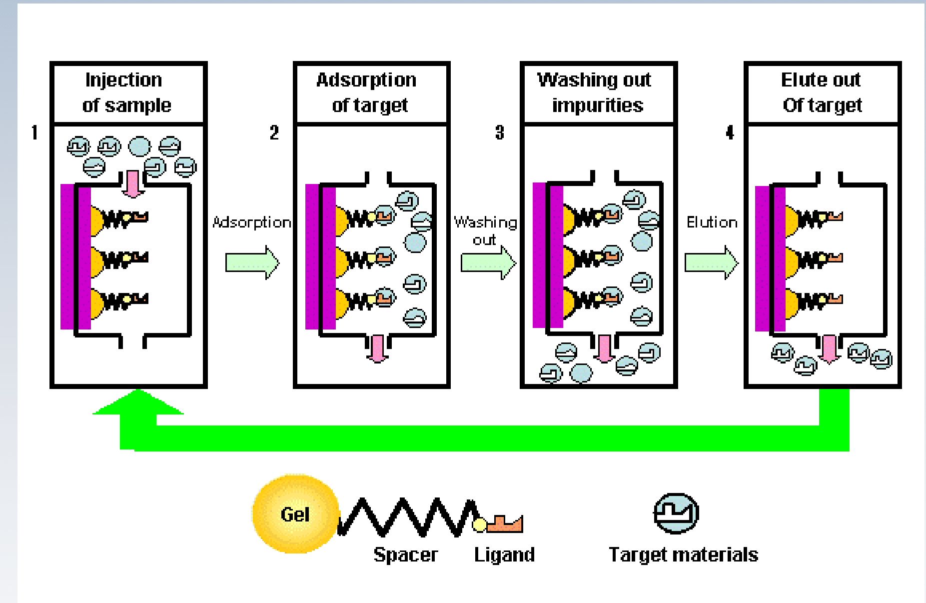
- **No salt** in binding phase
- Sample eluted in **high salt**

http://www.youtube.com/watch?v=q3fMqgT1do8

Affinity chromatography

- Discovered in 1968 by

  P. Cuatrecasas and M. Wilcheck
- Based on specific interactions
- Group affinity vs specific affinity



- Elution nonspecific vs specific
- Effective purification in one step
- Concentrating

- Various types of affinity chromatographies are used

Specificity of affinity chromatography

Specificity of Affinity Chromatography

- Specificity is based on three aspect of affinity

Matrix: for ligand attachment.

Spacer arm: used to bind ligand to matrix

Ligand: molecule that binds reversibly to a specific target molecule(site of interaction)

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Matrices in affinity chromatography

- The matrix simply provides a structure to increase the surface area to which the molecule can bind
- The matrix must be activated for the ligand to bind to it but still able to retain it's own activation towards the target molecule

- Amino, hydroxyl, carbonyl and thiol groups located with the matrix serve as ligand binding sites
- Matrices are made up of agarose and other polysaccharides
- The matrix also must be able to withstand the decontamination process of rinsing with sodium hydroxide or urea

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Examples of interactions in affinity chromatography

Ligand : Target

- **Antigen** : antibody
- **Enzyme** : substrate analogue
- **Binding protein**: Ligand
- **Receptor** : ligand
- **Lectin** : polysaccharide, glycoprotein
- **Nucleic acid** : complementary base sequence
- **Hormone, vitamin** : receptor, carrier protein.
- **Glutathione : glutathione-S-transferase** or GST fusion proteins.
- **Metal ions** : Poly (His) fusion proteins, native proteins with histidine or cysteine on their surfaces.

- **Other tags: tag affinity chromatography**

Ligands

- The ligand binds only to the desired molecule within the solution
- The ligand attaches to the matrix which is made up of an inert substance
- The ligand should only interact with the desired molecule and form a temporary bond
- The ligand/molecule complex remains in the column, while the contaminants are eluted
- The ligand/molecule complex dissociates by changing the pH

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Applications

- **Purify** and concentrate a substance from a mixture into a buffering solution
- **Reduce** the amount of a substance in a mixture
- **Discern** what biological compounds bind to a particular substance, such as drugs
- **Purify** and concentrate an enzyme solution

- **Genetic Engineering** - nucleic acid purification
- **Production of Vaccines** - antibodies purification from blood serum
- **Basic Metabolic Research** - protein or enzyme purification from cell free extracts

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Definitions

- Avidin (or Streptavidin) -biotin interaction is used to purify proteins
- Avidin: protein from egg white (birds, reptiles…)
- Streptavidin is a tetrameric protein purified from the bacterium *Streptomyces avidinii*
- Biotin: (vitamin H or B7) cofactor in the metabolism of fatty acids and leucine, and in gluconeogenesis

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- The non-covalent bond formed between biotin and avidin or streptavidin has a binding affinity >most antigen and antibody bonds ~ strength of a covalent bond
- Affinity chromatography using immobilized avidin or streptavidin to separate the biotinylated protein from a mixture of other proteins and biochemicals

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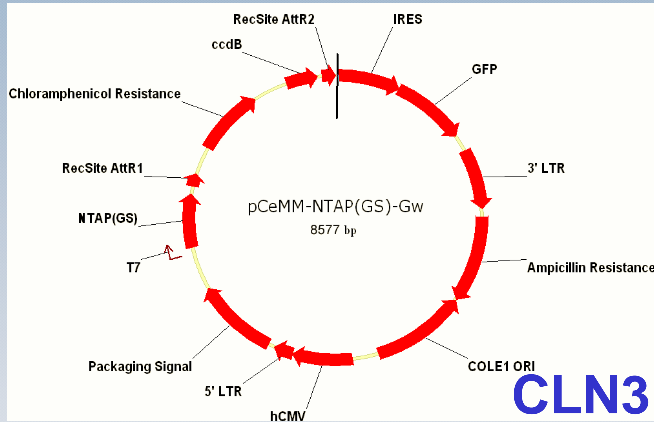
Peptide and protein affinity tags commonly used in purification of fusion proteins

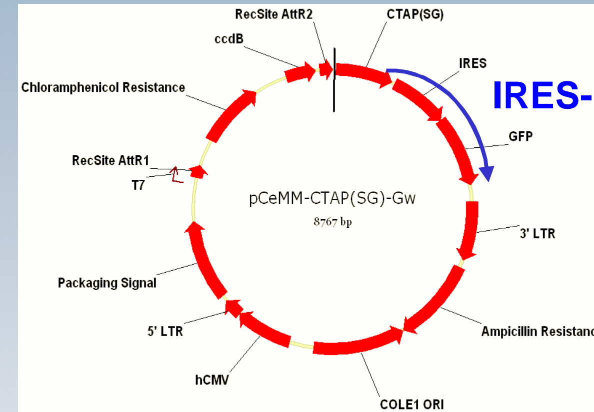
Tag name	Sequence	Origin	Size	Binding partner	Comment	Reference
Peptide tags						
Calmodulin-binding peptide	KRRWKKNFIAVSAANRFKKISSSGAL	Rabbit skeletal muscle	26 aa – 2960 Da	Calmodulin	Binding in presence of calcium chloride	[41]
c-myc	EQKLISEEDL	Human oncogene c-myc	10 aa – 1202 Da	Anti-c-myc antibody (9E10)		[42]
FLAG	DYKDDDDK	Bacteriophage T7	8 aa – 1012 Da	Anti-FLAG antibody (M1, M2, M5)	Improved 3XFLAG system	[43]
HA	YPYDVPDYA	Hemaglutinin from human influenza virus	9 aa – 1102 Da	Anti-HA antibody (12CA5)		[44]
Hexa-Histidine (His-Tag)	HHHHHH	Artificial	6 aa – 840 Da	Ni^{2+}-NTA, Co^{2+}-CMA		[45]
Isopeptag	TDKDMTITFTNKKDAE	*Streptococcus pyogenes*	16 aa	Pilin-C	Forms covalent bond	[46]
Strep-tag II	WSHPQFEK	*Streptomyces avidinii* (Streptavidin)	8 aa – 1060 Da	Streptavidin		[47]
Protein tags						
BioEase™/ AviTag™	http://www.tools.invitrogen.com/content/sfs/manuals/pmtbioeasedest_man.pdf GLNDIFEAQKIEWHE	*K. pneumonia*/*E. coli*	72/15 aa	Biotinylation by birA (*E. coli*) binding by Streptavidin/ Avidin	Variation of the BCCP approach	[48]
Biotin Carboxyl Carrier Protein (BCCP)		*Sulfolobus tokodaii*	169 aa	Biotin Protein-ligase OR Streptavidin/Avidin	kD = 1.2 nm	[49]
Green fluorescent protein (GFP)	http://www.colorado.edu/mcdb/MCDB1151/indproj/gfpseq.html	*Aequorea victoria*	238 aa – 26.9 kDa	Anti-GFP Antibody	Numerous derivates	[50,51]
Glutathione S-transferase (GST)	http://www.ncbi.nlm.nih.gov/nuccore/U58012.1	*Schistosoma japonicum*	26 kDa	Glutathione	Forms dimers	[52,53]
Maltose-binding protein (MPB)	http://www.ncbi.nlm.nih.gov/nuccore/J01648.1	*Escherichia coli*	40 kDa	Amylose		[54]
Tandem-Affinity-Purification (TAP)	http://www.embl.de/ExternalInfo/seraphin/TAPdescription.html	Rabbit + *Staphylococcus aureus*	~20 kDa	1st step: IgG matrix 2nd step: Calmodulin	ProteinA + TEV cleavage site + CBP	[9]

Hosp and Selbach, Methods 2011

TAP purification of protein complexes from mammalian cells

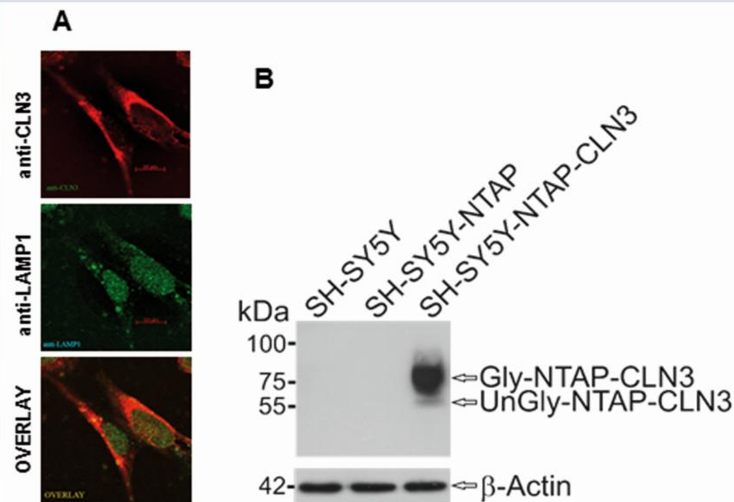


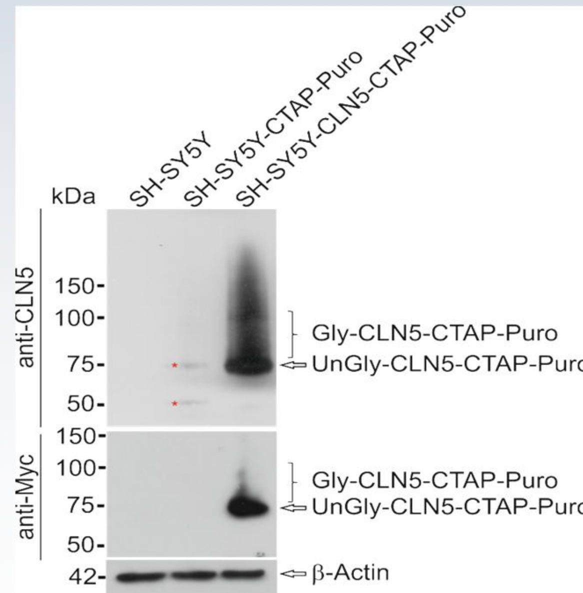
CLN3

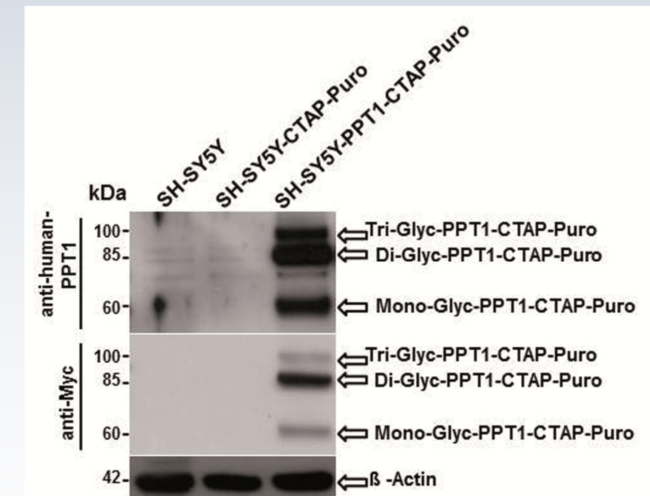


IRES-Puro

PPT1 and CLN5







Protein G	Protein G	TEV	SBP	-GS

SG-	Myc	SBP	TEV	TEV	Protein G	Protein G

Scifo et al. J Proteome Res 2013 and Scifo et al., 2014, submitted

TAP purification of protein complexes from mammalian cells

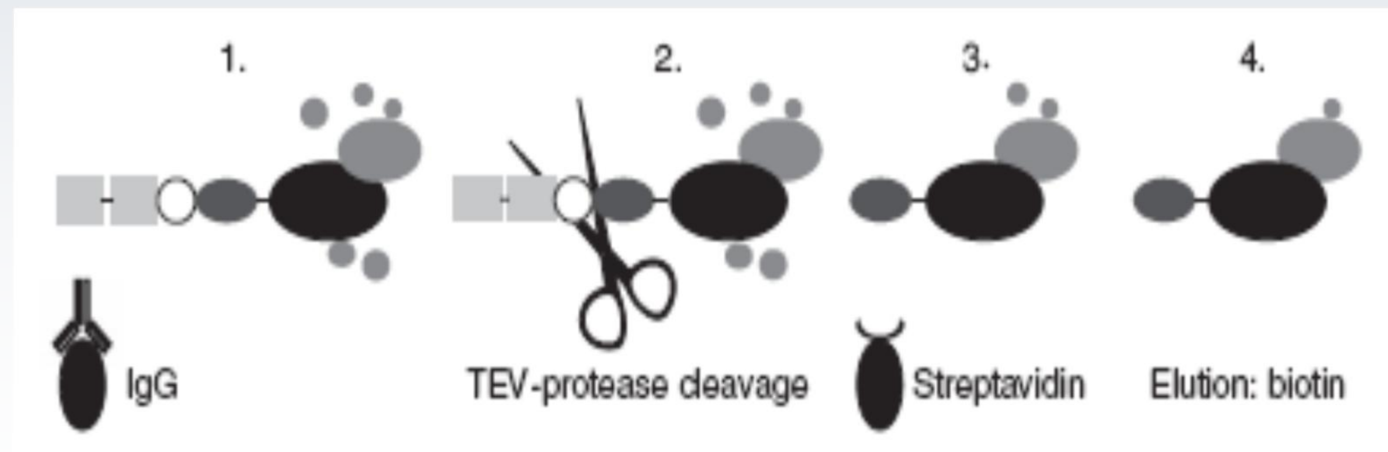
Benefits:

- Several members of a complex can be tagged, giving an internal check for consistency
- Detects real complexes in physiological settings

Drawbacks:

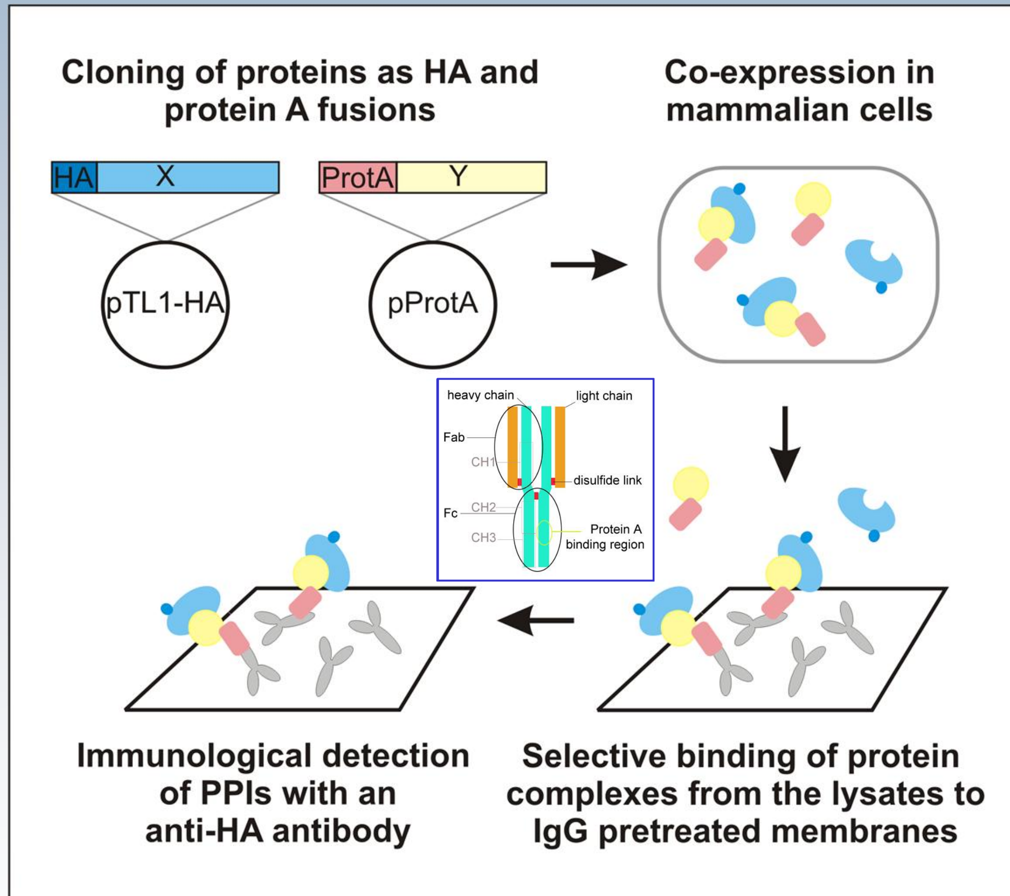
- Might miss some complexes not present under the given conditions
- Tagging may disturb complex formation
- Loosely associated components may be washed off during purification

TAP Complexes purification

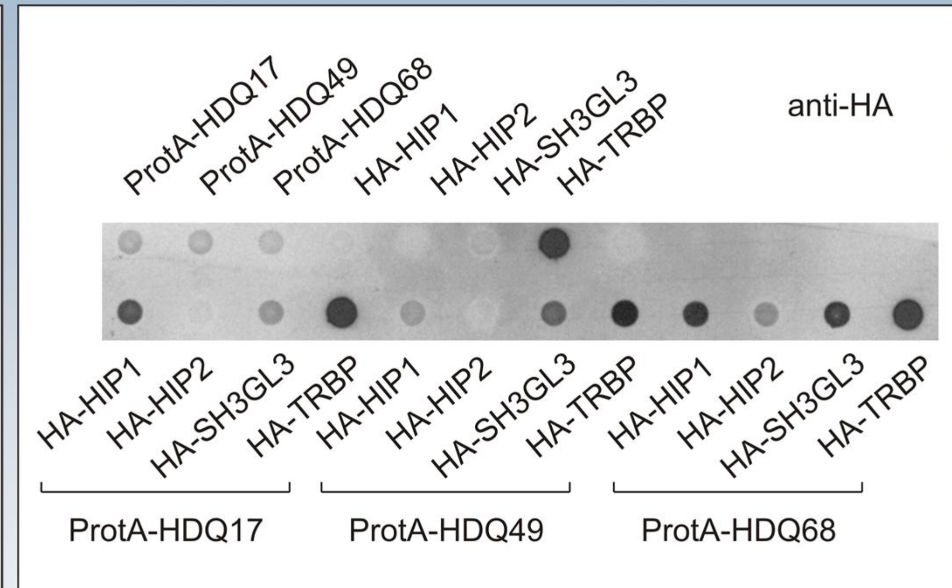


Buerckstummer et al. 2006

Validation of interactions using affinity-membrane technology

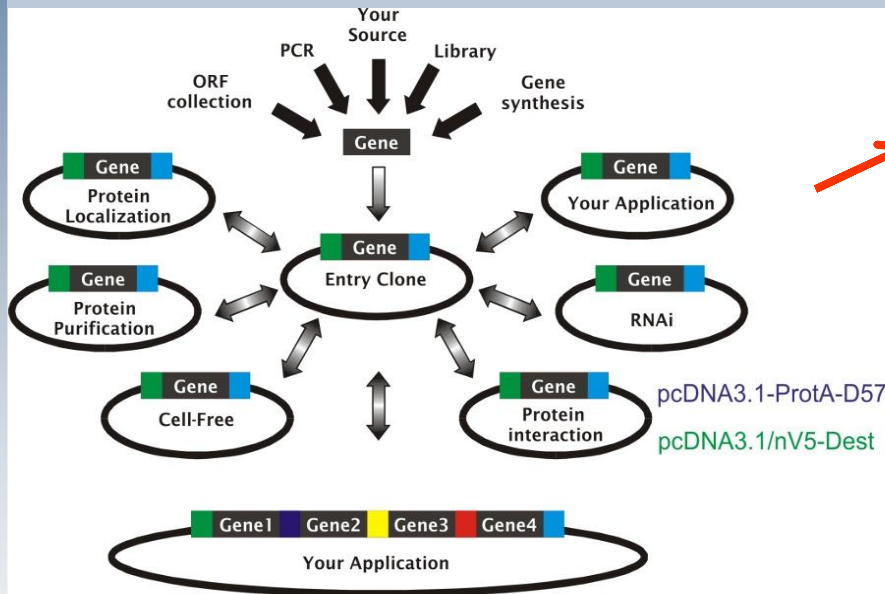


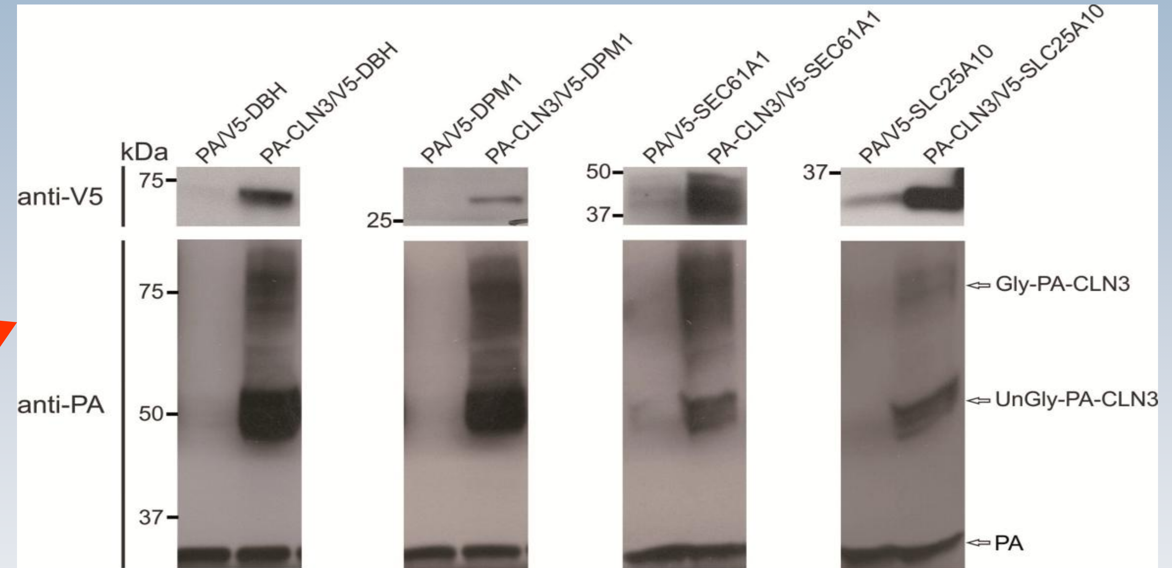
The principle

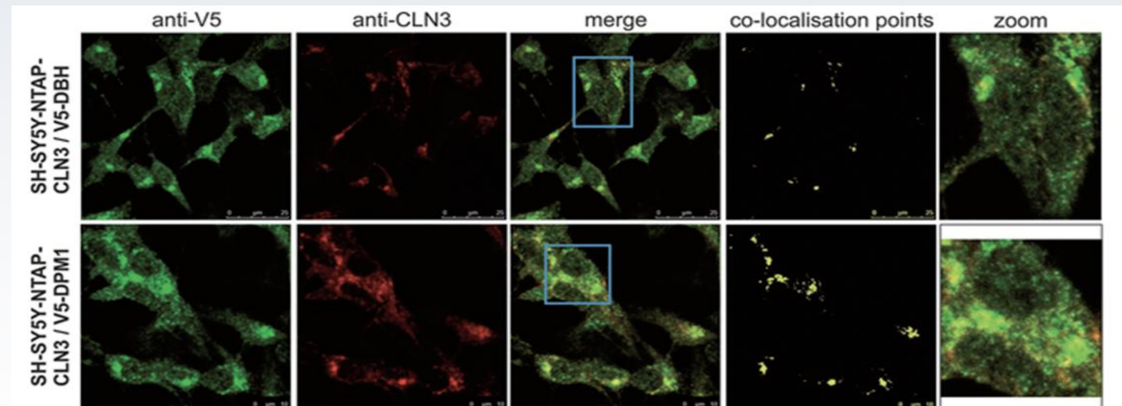


Interactions between huntingtin and known interaction partners

Systematic validation of TAP-MS based CLN3 complex interactions



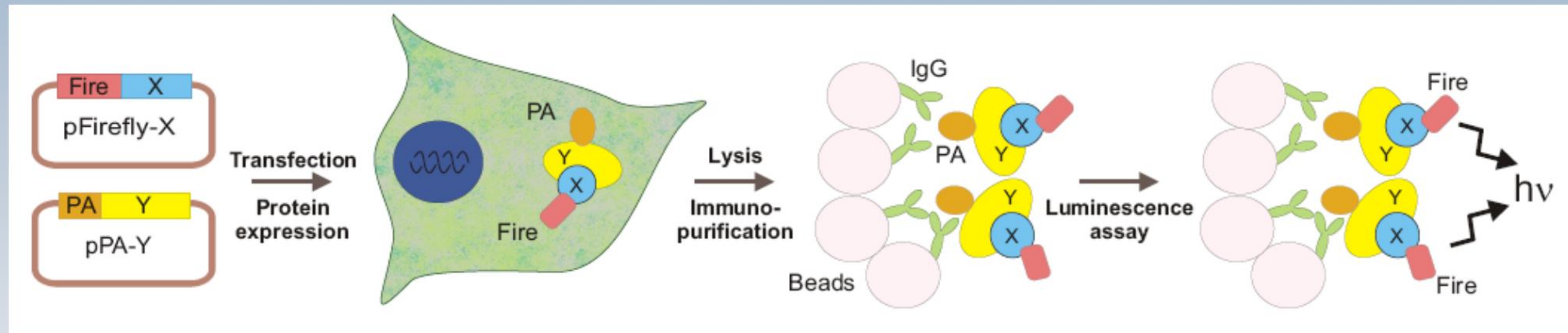




DBH- dopamine β-hydroxylase
DPM1- dolichol P-mannosyl transferase 1
Lysosomal colocalization

Scifo et al. J Proteome Res 2013

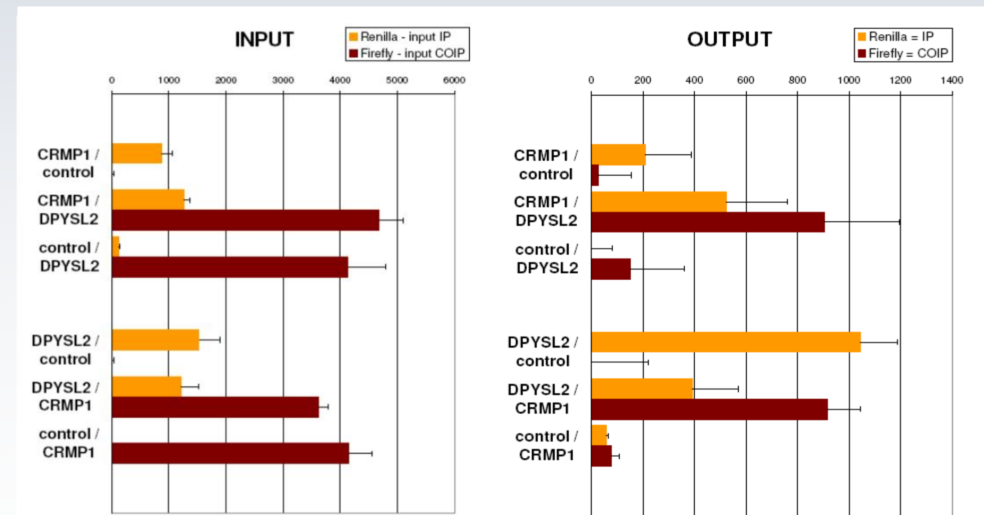
Validation of interactions using affinity-LUMIER technology



Adaptation of LUMIER (Barrios-Rodilles et al., 2005)

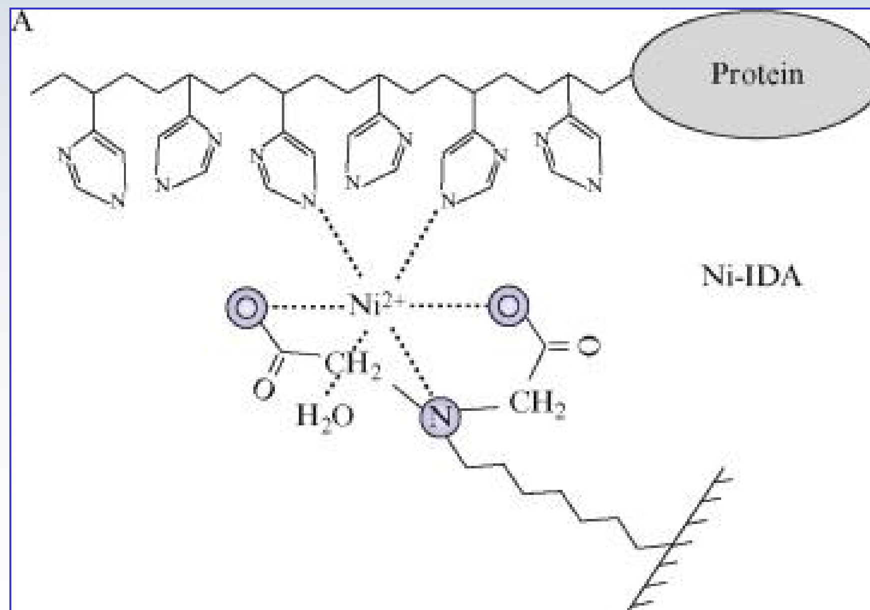
Reporter assay format:

- Readouts for IP and Co-IP
- Determination of binding affinities
- Validating and quantification of binary interactions



IMAC: Immobilized metal ion affinity chromatography

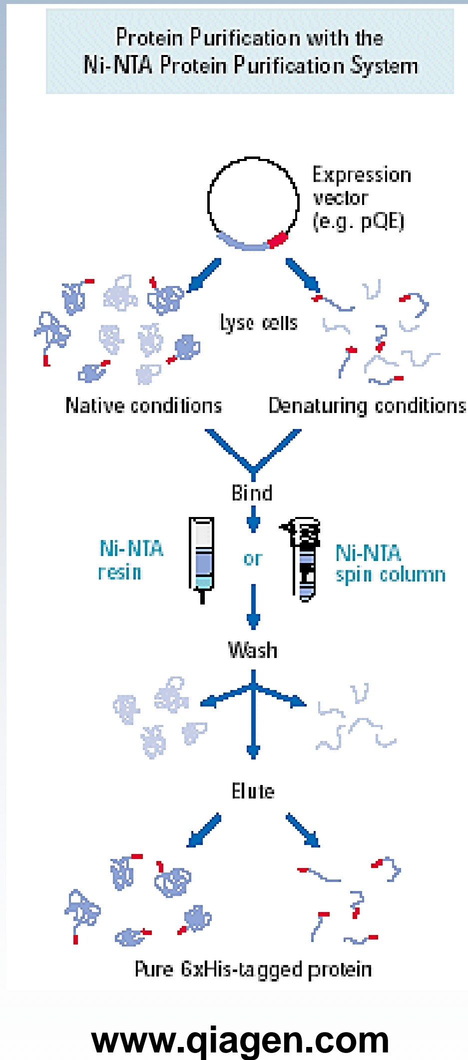
- Single step chromatography
- It is based on the known affinity of transition metal ions such as Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} to histidine and cysteine in aqueous solutions – First described by Hearon, 1948 and later Porath et al., 1975
- For Zn-finger proteins and recombinant proteins with ≥6x His-tag



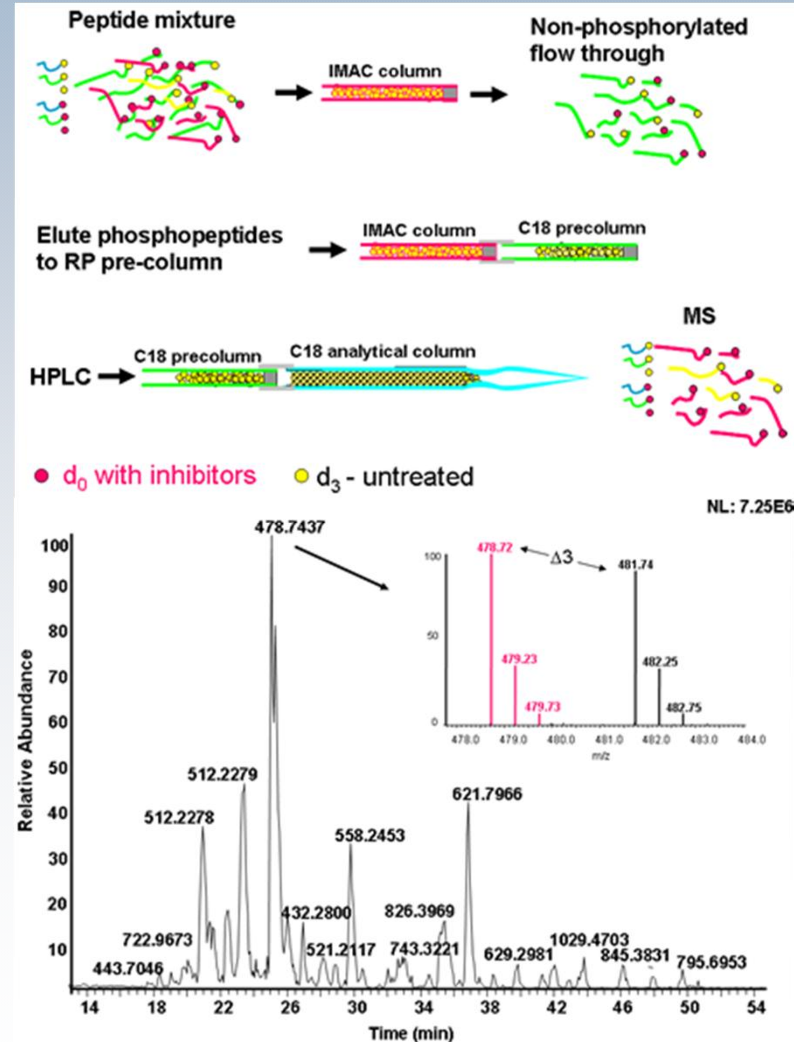
Methods in Enzymology Volume 463 2009 439 - 473

Model of the interaction between residues in the His tag and the metal ion in tri- (IDA), tetra- (NTA), and pentadentate IMAC ligands (TED)

Examples of purifications with IMAC



www.qiagen.com



Chemical compatibility of purification of His-tagged protein using agarose-based IMAC (Ni-NTA) resins and its limitations

IMAC chemical compatibility

Component	Limitation (up to)	Component	Limitation (up to)
Buffers		**Salts**	
Na-phosphate	Recommended, limit not known	NaCl	4 *M*
Phosphate citrate	Limit not known	$MgCl_2$	4 *M*
Tris–HCl, HEPES, MOPS	100 m*M*	$CaCl_2$	5 m*M*[c]
Citrate	60 m*M*	$NaHCO_3$	Not recommended
Detergents (in 300 m*M* NaCl)		Ammonium salts	Not recommended
n-Hexadecyl-*β*-D-maltoside	0.0003% (w/v)	**Protease inhibitors**	
n-Tetradecyl-*β*-D-maltopyranoside	0.005% (w/v)	EDTA	1 m*M*[a]
n-Tridecyl-*β*-D-maltopyranoside	0.016% (w/v)	Commonly used protease inhibitors[d]	Compatible in effective concentrations
Brij 35	0.1% (v/v)	Complete cocktail (EDTA-free)	1× concentrated
Digitonin	0.6% (w/v)	**Denaturants**	
Cymal 6	1% (w/v)	Urea	8 *M*
n-Nonyl-*β*-D-glucopyranoside (NG)	1% (w/v)	Gu–HCl	6 *M*
n-Decyl-*β*-D-maltopyranoside (DM)	2% (w/v)	**Amino acids**	
n-Dodecyl-*β*-D-maltoside (DDM)	2% (w/v)	Histidine	1–2 m*M*[b]
C12–E9	1% (w/v)	Glycine	Not recommended
n-Octyl-*β*-D-glucopyranoside (OG)	1.5% (w/v)	Cysteine	Not recommended
Triton X-100, Tween, NP-40	2% (v/v)	Glutamate	Not recommended
Triton X-114	2% (v/v)	Aspartate	Not recommended
Fos-Cholines	0.05% (w/v)	Arginine	500 m*M*
Dodecyldimethyl-phosphineoxide	0.15% (w/v)	**Organic solvents**	
N,N-Dimethyldodecylamine-*N*-oxide (LDAO)	0.7% (w/v)	Isopropanol	60% (v/v)[e]
CHAPS	1% (w/v)	Ethanol	20% (v/v)
Laurosyl-sarcosine	1% (w/v)	**Reducing reagents**	
SDS	0.3% (w/v)[a]	*β*-ME	20 m*M*
Other		TCEP	20 m*M*
EGTA	1 m*M*[a]	DTT	10 m*M*
Imidazole	10–20 m*M*[b]	DTE	10 m*M*
Hemoglobin	Not recommended		
Glycerol	50% (v/v)		

Affinity chromatography: summary

PROS:

- Extremely high specificity
- High degrees of purity can be obtained
- The process is very reproducible
- The binding sites of biological molecules can be simply investigated

CONS:

- Expensive ligands
- Leakage of ligand
- Degradation of the solid support
- Limited lifetime
- Non-specific adsorption
- Relatively low productivity

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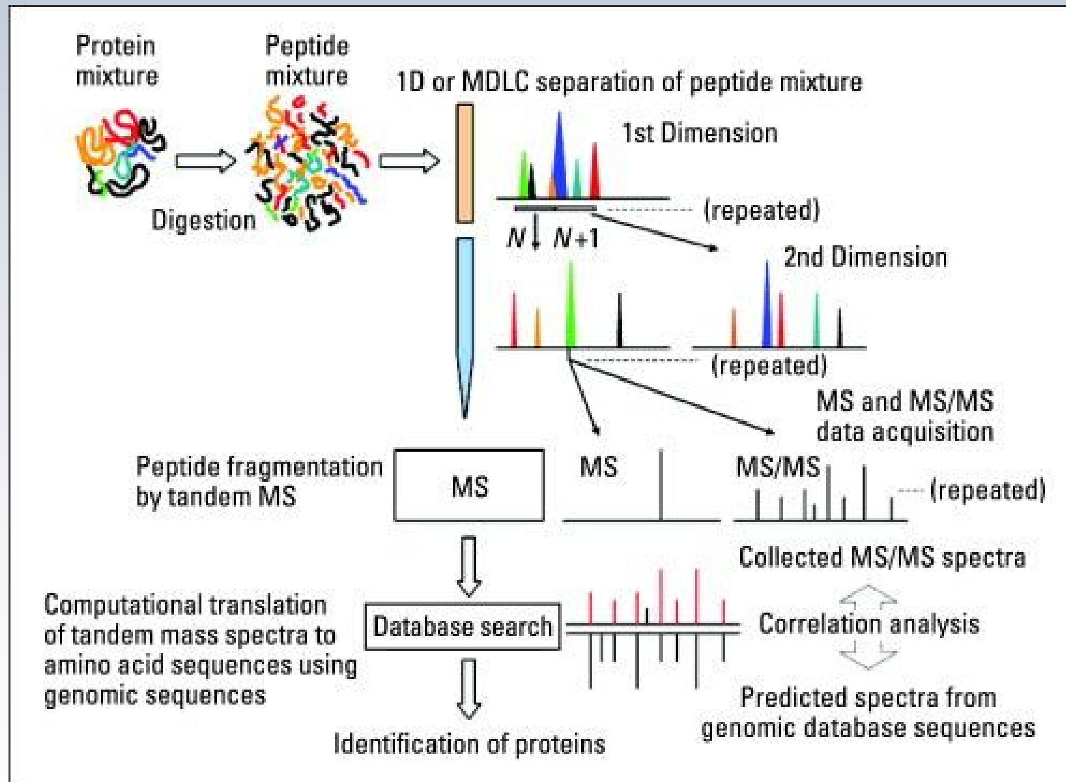
MudPIT proteomics

- **MudPIT = Multidimensional Protein Identification Technology**
- Used for separation and identification of complex protein/peptide mixtures- alternative to 2D-electrophoresis



MudPIT

Mud Pit

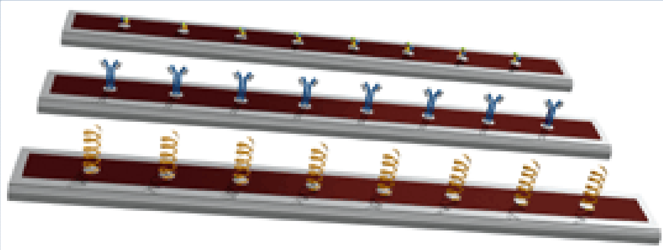
SELDI-TOF Mass Spectrometry

- Surface-enhanced laser desorption/ionization-TOF Mass spectrometry (Biorad Inc.)

  - Mass spectrometric technology on a chromatographic chip surface
  - Used to analyze complex biological mixtures such as serum, urine, milk, blood
  - Biomarker discovery, protein profiling, immunoassay applications, protein-protein interactions
  - Differentially expressed proteins are determined by comparing protein peak intensities between mass spectra

Proteomics using SELDI-TOF

1



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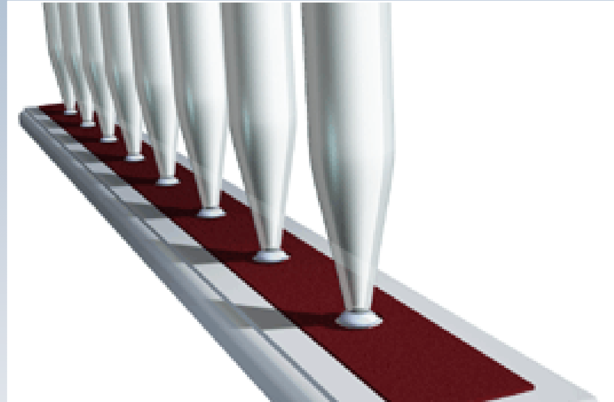

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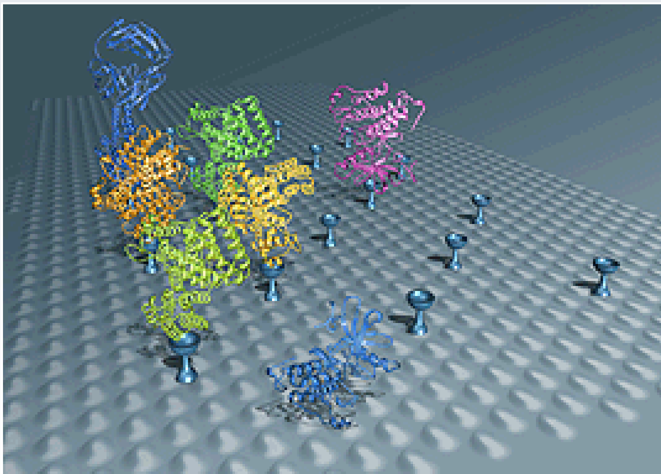

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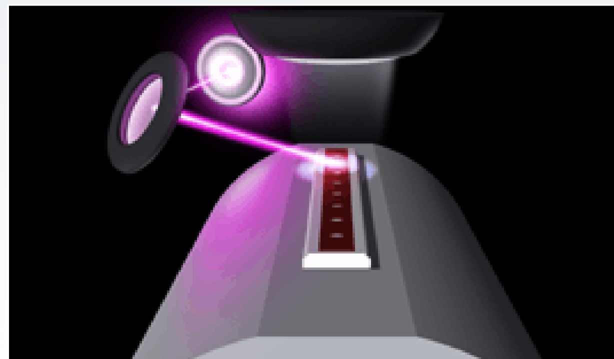

1. Choosing an array (hydrophobic, hydrophilic, anion exchange, cation exchange, and metal affinity surfaces)
2. Sample application (serum, cell and tissue lysates, urine, cerebrospinal fluid, or other protein homogenates (including those with high salt or detergent concentrations); 96-well format)
3. Removal of Unbound Components and Application of Energy Absorbing Molecules (EAMs) selective washes create on-chip protein (retentate) maps. Afterwards, a solution containing EAMs in an organic solvent is applied, uniform crystalization
4. Analysis in the ProteinChip SELDI Reader (compund detection by Time-of-Flight mass spectrometry, nitrogen laser