Genetically engineered Tags

Fusion proteins with solubility-enhancement tags

Specific Proteases

Affinity Chromatography (AC)

- Affinity Tags and Fusion Proteins
- Chelating, Strep-tag, GST, MBP, SUMO , C-terminal, etc
- Cleavage sites – Proteases
- Parameters for development optimization
- Troubleshooting
- Examples

Affinity Tags - I

- Small stretches of amino acids added to the N-terminal or C-terminal end of a protein with a high affinity for a specific biological or chemical ligand
- Allow purification and detection of the expressed protein
- Enables different proteins to be purified using a common method (HTPS)
- Most popular: His-tag, comprised of six histidine that provide specific binding to metal chelate resins
- Polyarginine or polyaspartic acid tags can be used to alter binding of a protein on ion-exchange resins

Affinity Tags - II

- The *Strep*-tag II (8 amino acids, WSHPQFEK), which binds with high selectivity to Strep-Tactin.
- The tag may be placed at either end of the protein or in a region with appropriate surface exposure to allow binding or recognition.
- To enable removal of the tag, a linker region is typically included between the tag and the native protein sequence.
- The linker contributes to increased accessibility of the affinity tag and is often required for effective endoprotease cleavage.

IMAC: Immobilized metal affinity chromatography

Chelating-Chromatography for poly-His fusion proteins

- The most widely used.
- It's small in size. Less immunogenically active
- It does not need to be removed for many downstream applications
- Availability of a large number of expression vectors
- Tag may be placed at either the N or C terminus
- A protease cleavage site allows the tag to be removed after purification
- The interaction of the tag with the Ni^{2+}column does not depend of the tag structure, making it possible to purify otherwise insoluble proteins using denaturating conditions (8 M urea or 6 M GuHCl)

Immobilized metal ion affinity chromatography (IMAC) – for poly-His fusion proteins

- The basis of the purification is the interaction of the imidazole moiety of the poly His with the metal (Nickel in most cases).
- The metal is immobilized to a support through complex formation with a chelate that is covalently attached to the support
- Avoid use of chelating: EDTA, DTT, others
- Cell chelating molecules in lysates. Reduce final yield in low expression lysates
- In some resins we can use Co , Cu , Zi or Fe instead of Ni and obtain different results
- Experts in membrane proteins claim that Co resins (Talon) are better

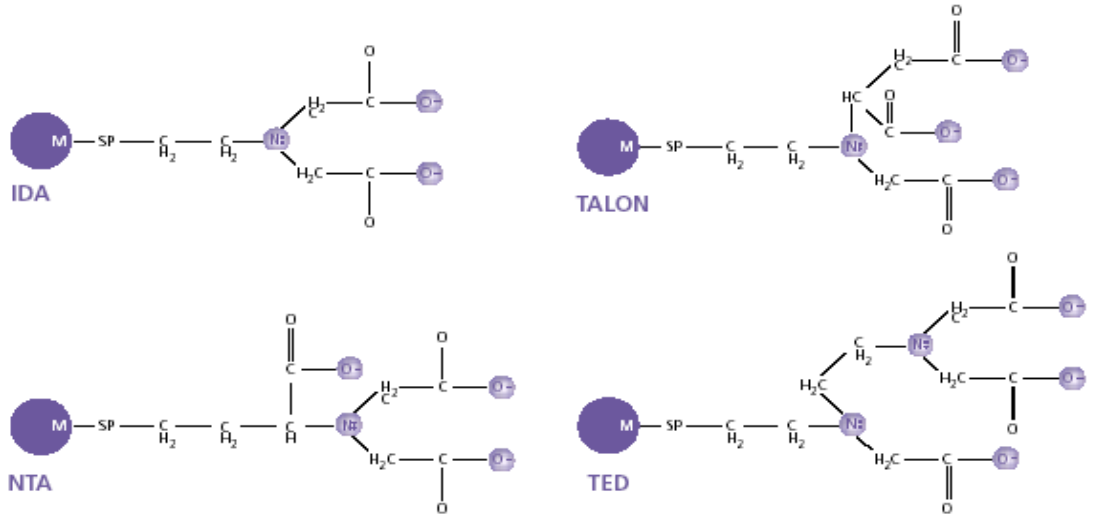


Figure 5. Chemical structures of chelating ligands used in IMAC. Binding groups are colored purple. SP = spacer. M = matrix.

IMAC

Binding Conditions:

- 20 mM phosphate or TrisHCl buffers
- + (0.5 M NaCl) or low imidazol concentrations (10-20mM) to avoid non-specific binding of contaminants to the resin.
- Buffers may include 8 M urea or 6 M GuHCl for inclusion bodies solubilized proteins
- Use max capacity of the column to reduce impurities in the final product

Washing Conditions:

- Wash till very low OD280nm (<0.05 OD280nm/ml) for open columns
- Other strategies to reduce impurities in final product: detergents, high salts, others

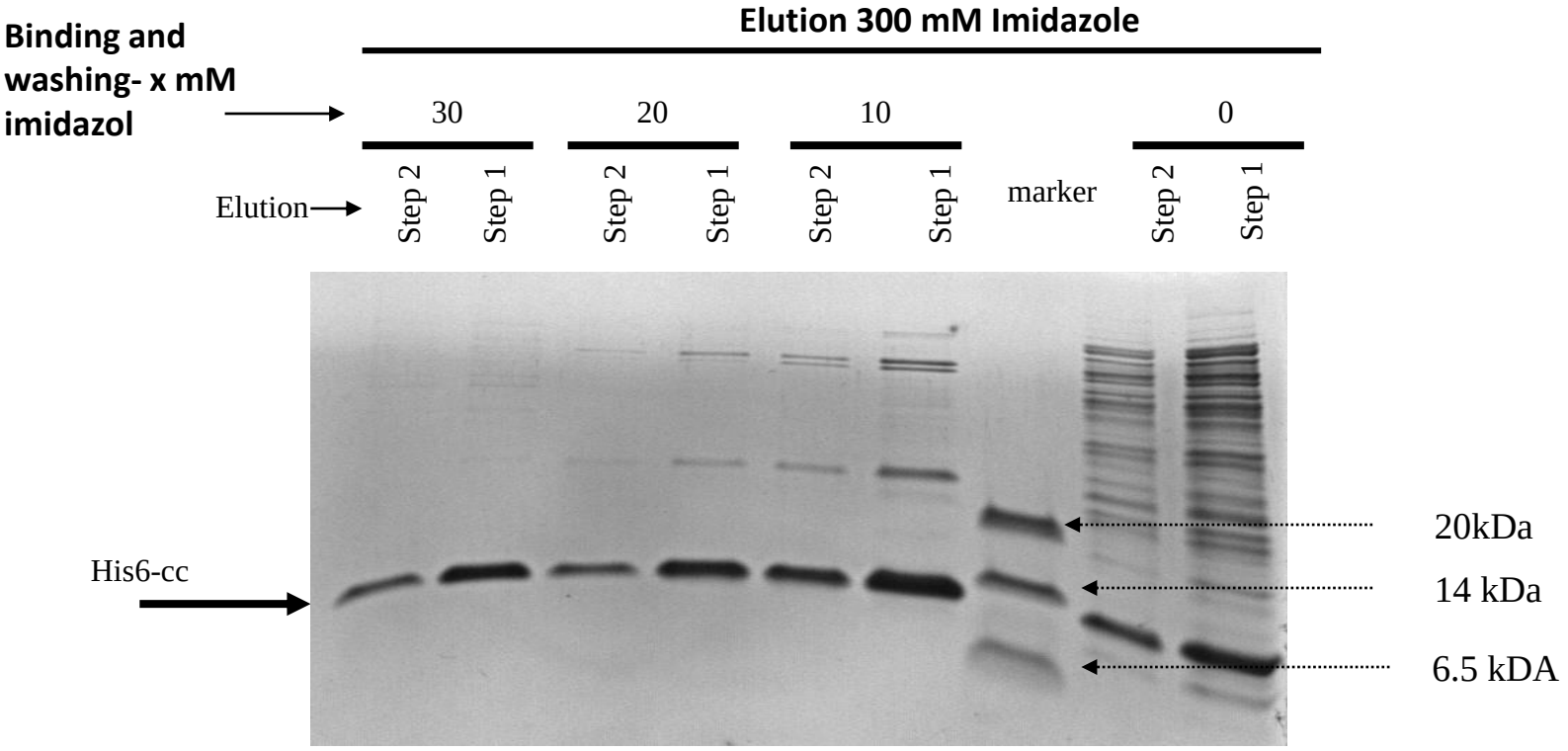
Elution Conditions:

- Usually Imidazol (Histidine analoge) or low pH (~4.5)
- Alternatives: EDTA, Histidine

Purity Optimization by Imidazol competition

pDest 17 (His tag) cc-stop of ARNO *Tamar Shultz , Altschuler lab*

- Lysis of 10ml culture
- Bound and washed (in the presence of 10, 20, 30 mM imidazol) to nickel resin
- Elution was with 300 mM imidazol

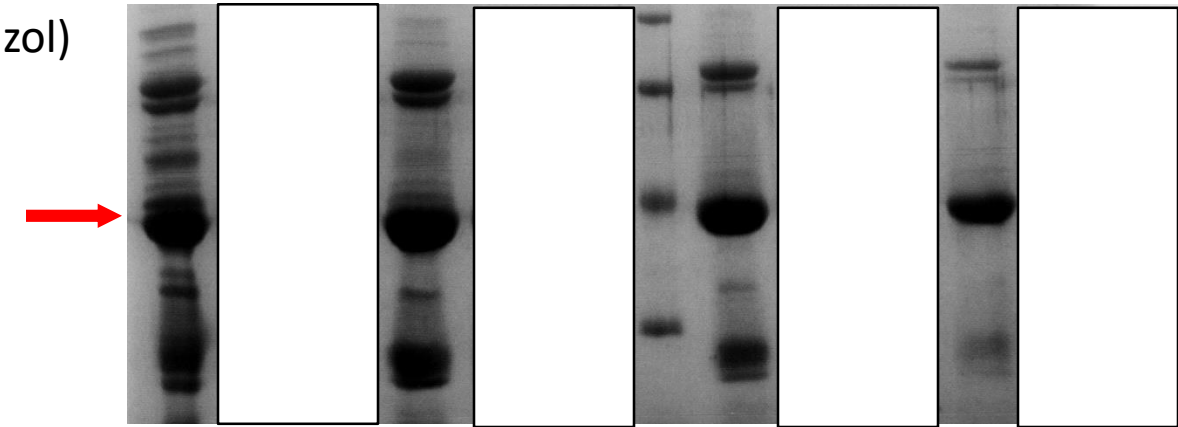


Each well contains 10 micro liter of the desired step (from 40 microliters)+ 5 micro liter sample buffer.

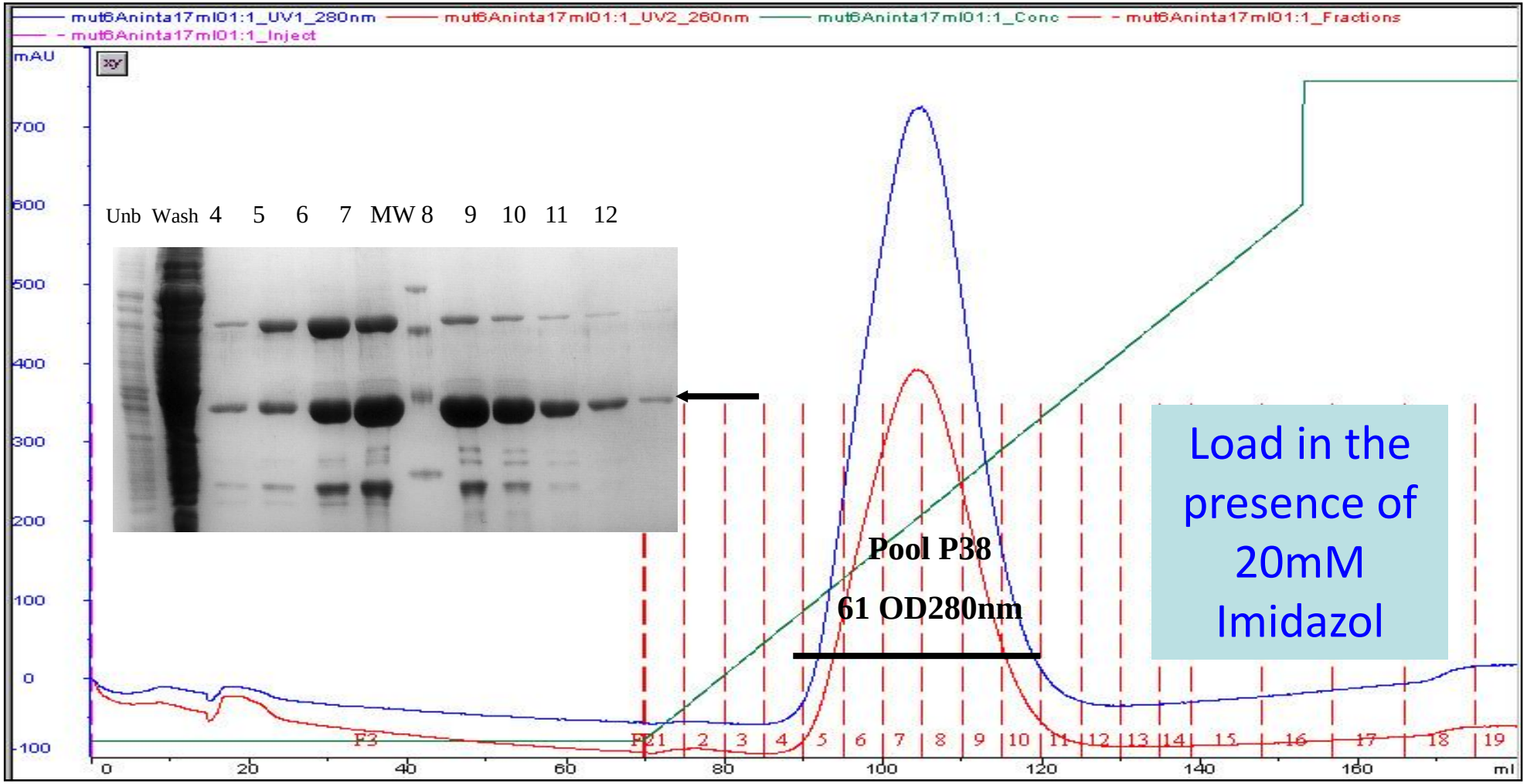
Each of the marker proteins is 2 micro-grams.

P38 CAPTURE -Optimization

- Cells lysis from 250ml culture.
- Spin 30min 20000g 4°C.
- Divide supernatant in 4 fractions, add to each one different [Imidazol]: 10, 20, 30 & 40mM f.c.
- Incubate each fraction (batch binding) 60min 4°C with 150µl Ni-NTA (equilibrated with different [Imidazol]).
- Spin 3min 3000rpm 4°C. Discharge unbound material.
- Wash resin 6x1ml washing buffer + different [Imidazol].
- Elution 600µl elution buffer (250mM Imidazol)
- Run PAGE-SDS of each elution.



P38 CAPTURE - Affinity Chromatography



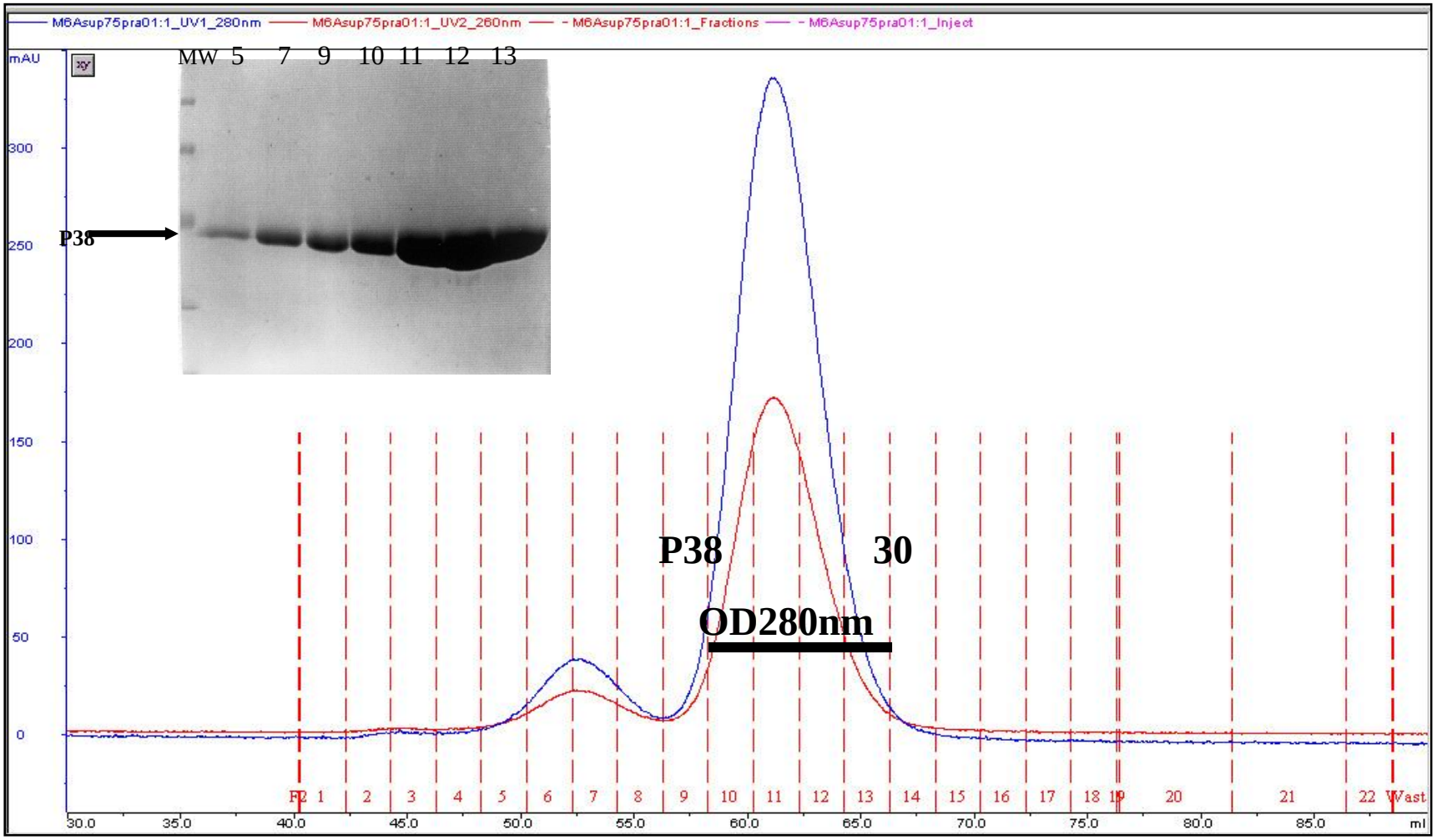
100gr cells (6L culture). Cell disruption with Mountain Goulin in 900ml lysis buffer. Batch binding to 15ml Ni-NTA 90min 4˚C in the presence of 20mM Imidazol. Wash with 20mM Imidazol buffer and elution with 5cv gradient 20-250mM Imidazol.

P38 INTERMEDIATE PURIFICATION: Anion Exchange

Charge heterogeneity

60 OD 280nm P38 after Ni-NTA and dialysis ON vs buffer A. Load on Resource Q-30 6ml column. Wash with 10cv 50mM NaCl buffer. Elute with gradient 5cv 50-300mM + 40cv 300-750mM + 5cv 750-1000mM NaCl

P38 FINAL POLISHING: SEC



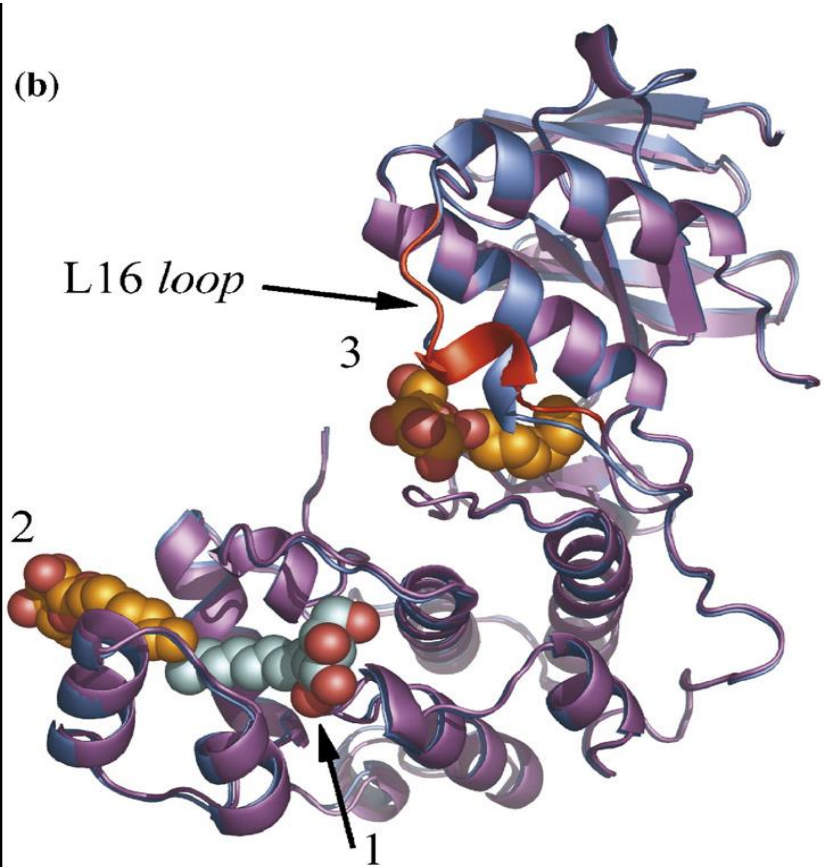
35 OD280nm P38 after Ni-NTA, Res.Q & ultrafiltration cut-off 10000. Load on Superdex 75 60x1.6cm column (2 runnings).

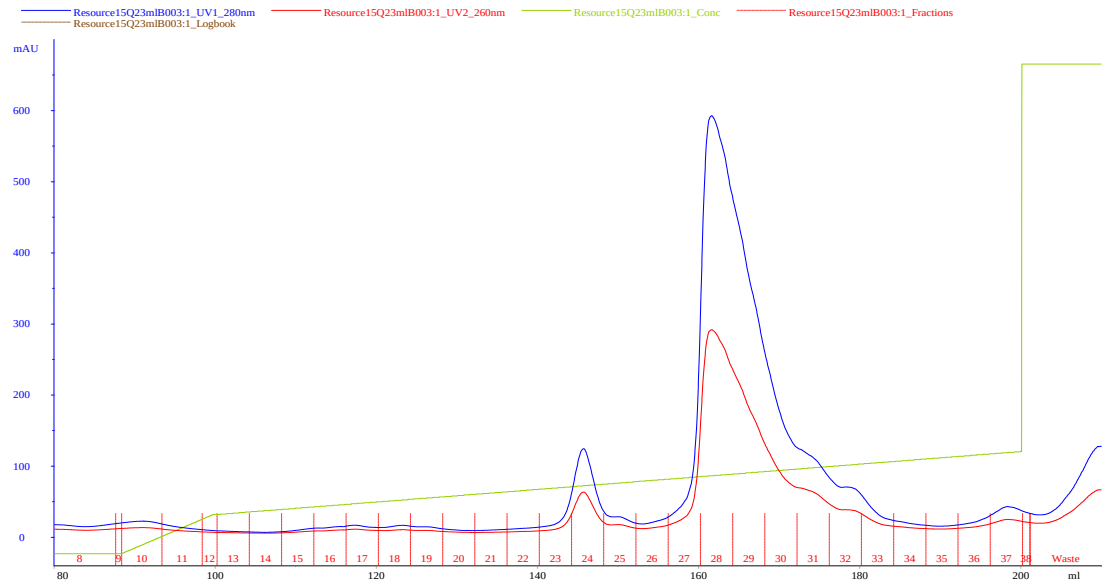
Elution 1ml/min

P38 After Affinity

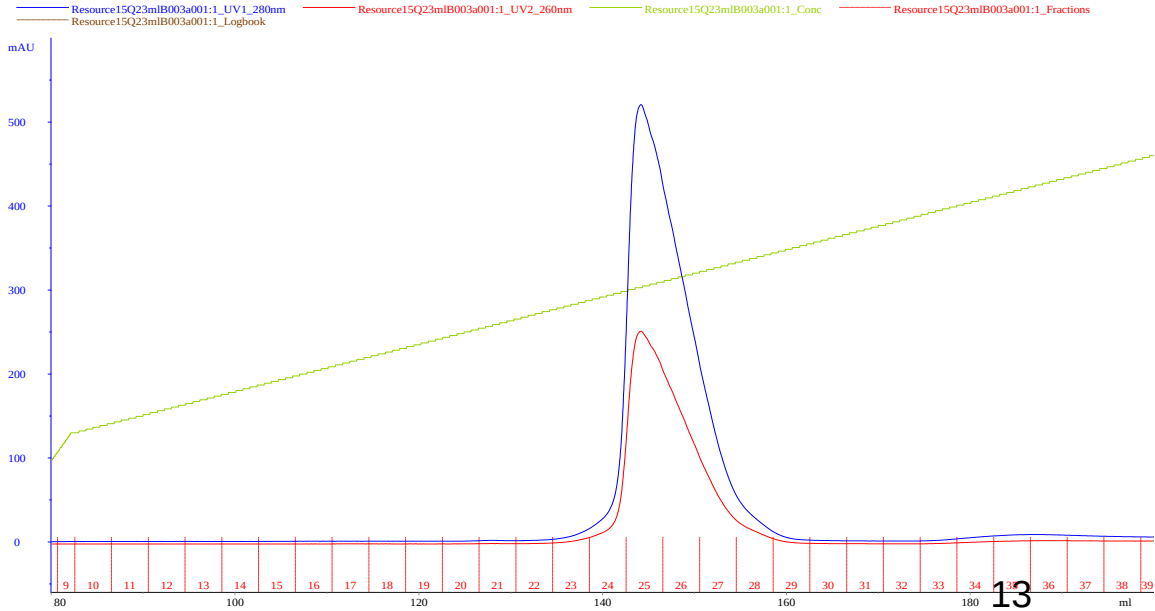
Highly resolutive anion exchange Resource 15Q 29 x 1cm ~23ml column

Ron Diskin et al J. Mol. Biol. (2007) 365, 66–76





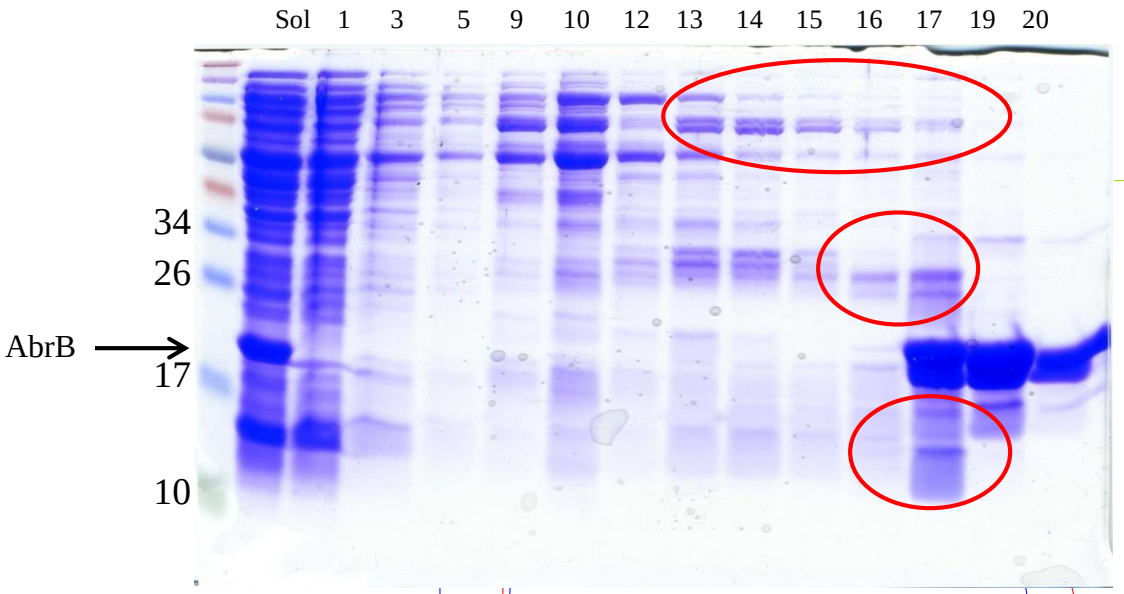
1st run main peak of Affinity column

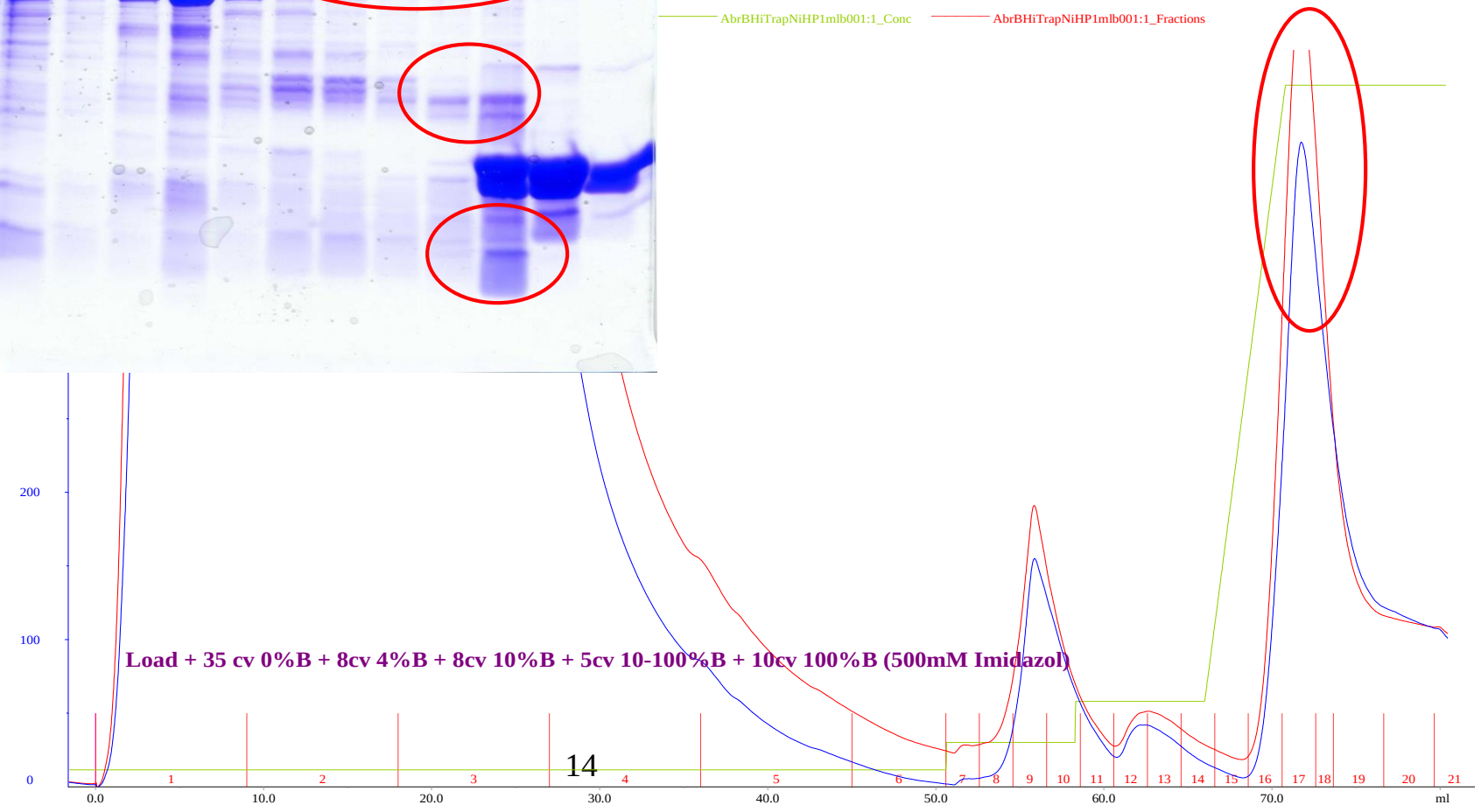


2nd run: main peak of previous run

IMAC purification: Low Imidazol step washing before elution

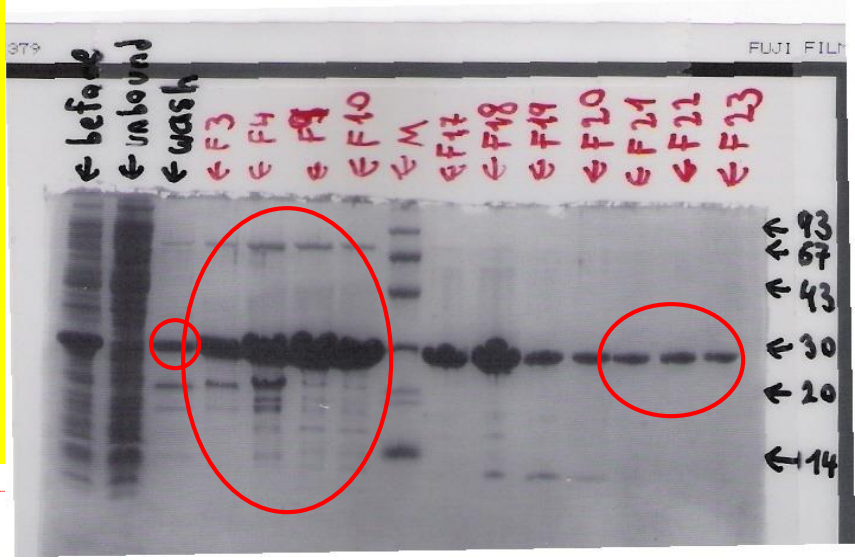
Case study: AbrB- Collaboration with A.Kaplan & D. Schatz

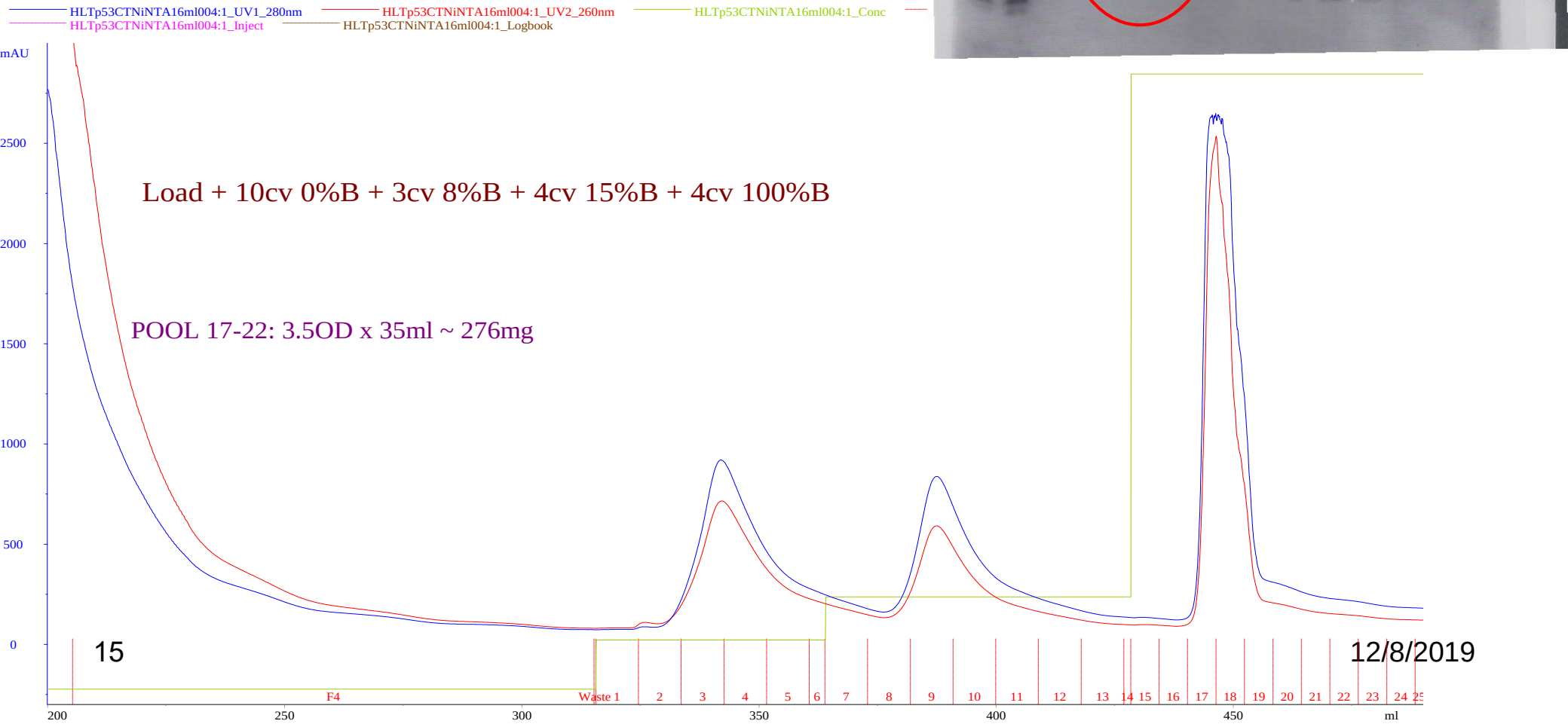




HLT-p53CT- Affinity
start with pellet of 1.5L culture
Ni-Sepharose FF 14ml

Ronen Gabizon – Assaf Friedler Group

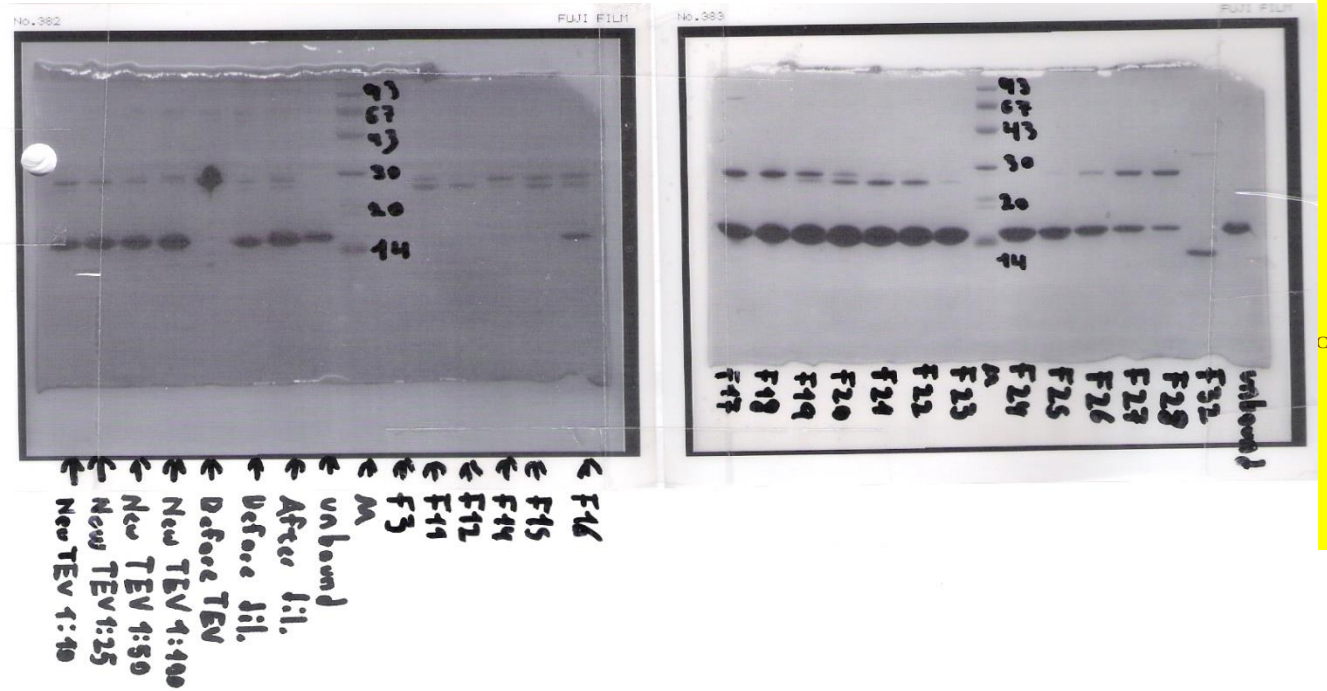


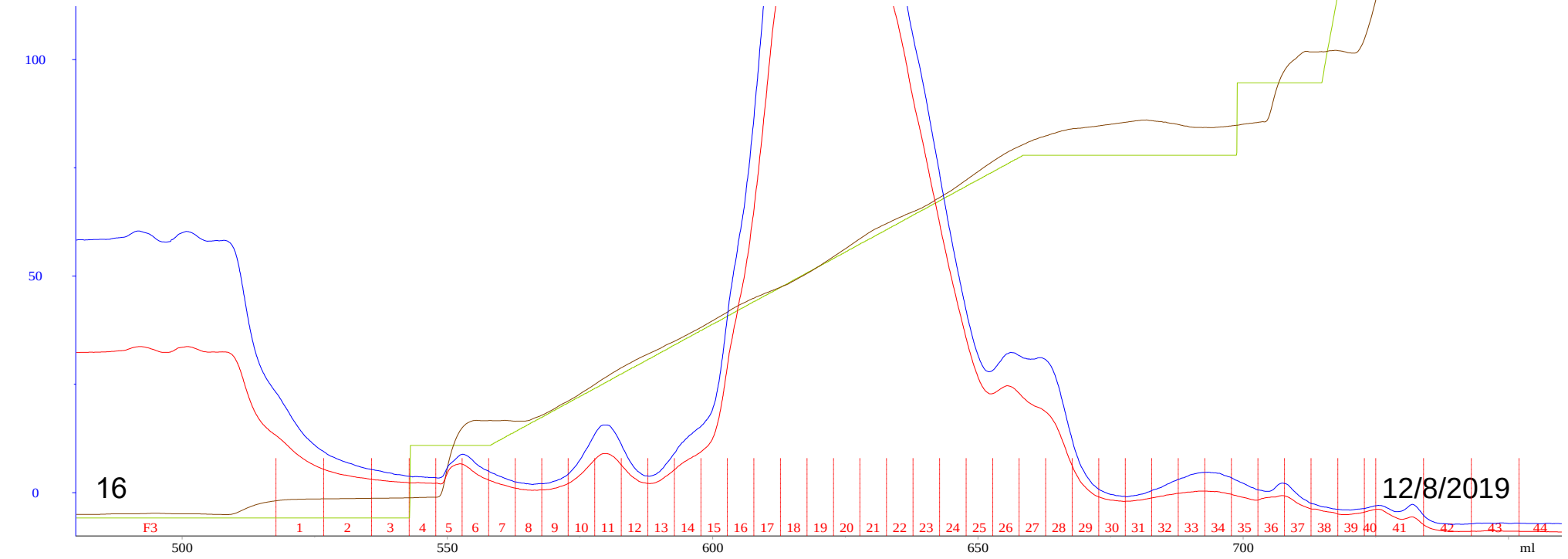


HLT-p53CT- Cation Exchange after TEV protease cleavage

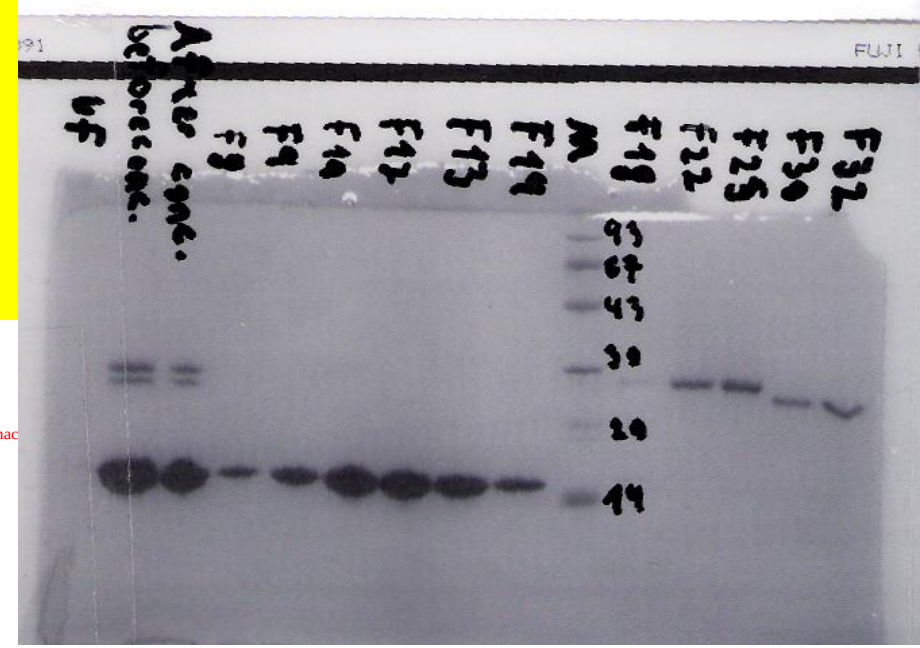
ON 4ºC

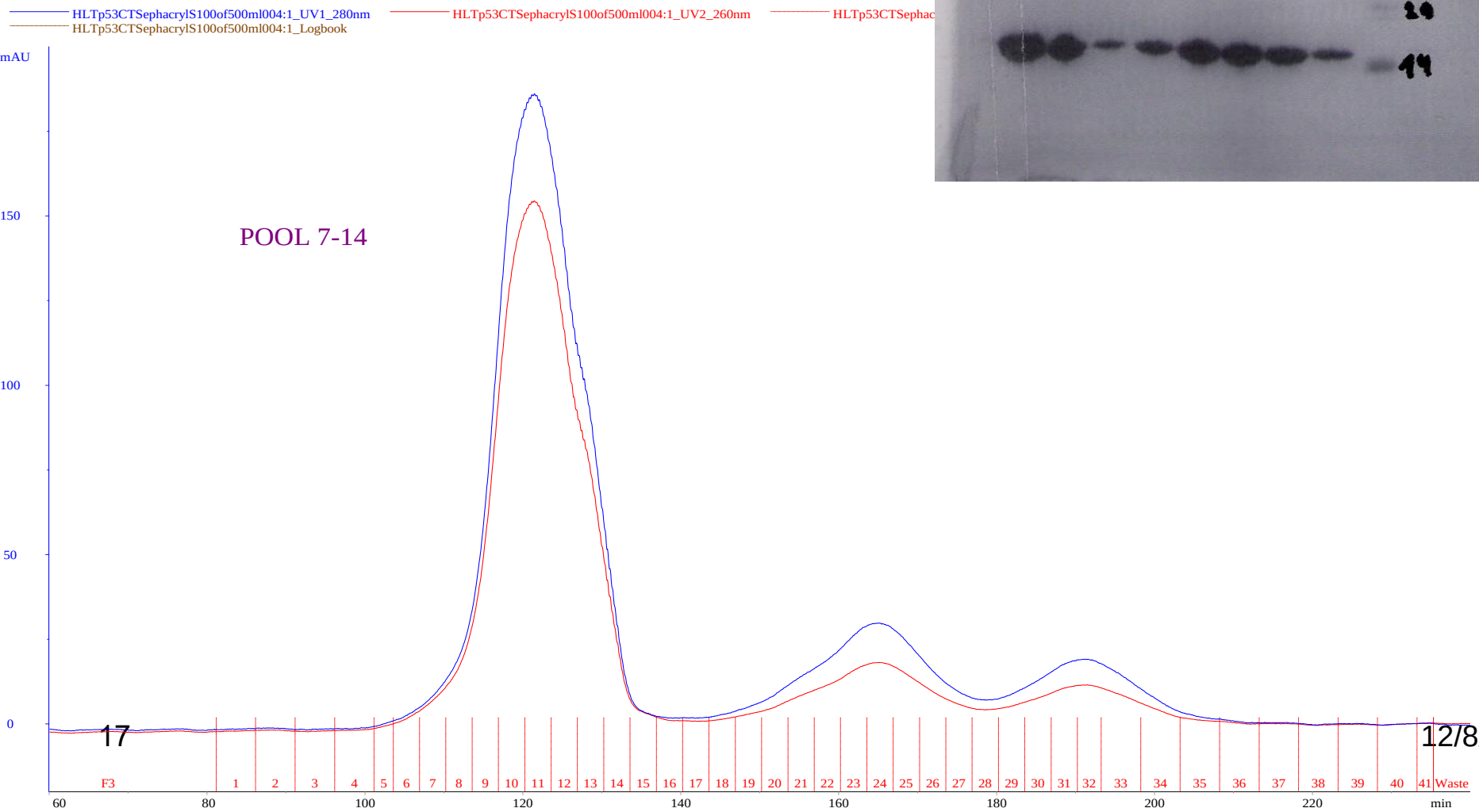
SP-Sepharose FF 5ml





HLT-p53CT- GF after Cation Exchange Sephacryl S100 FF 500ml





Parameters for optimization during binding and washings- I

- **If protein does not bind to the resin, there are several options to choose**

- Check the quality of the resin (use an His-tag control protein) or check for the presence of reagents that avoid binding. Use reliable supplier. Presence of chelants in the lysates??

- Partial binding: use more resin, or bind for longer time (BUT: the longer the duration of purification, the greater the risk of protein degradation). Use unbound material for a new purification.

- Try additives to prevent interaction with other proteins: glycerol, detergents, low [chaotropics]: urea, GuHCl , or more [kosmotropics] as NaCl $AmmSO_4$ (soluble aggregates??)

- Tag is inaccessible: try purification under denaturating conditions (for poly-His fusion proteins). Inaccessible because of folding or because aggregation? Different answers!!!

- Check by western-blot if the tag has been degraded; if this is the case, try to work all the time at 4°C and use more protease inhibitors during lysis

- Construct a new vector with the tag in the opposite end of the protein

- Presence of contaminants (chelants for IMAC or biotin in Streptag)

Parameters for optimization during binding and washings- II

- **If multiple proteins bands are seen in the elution try:**
- Increase the washing step volume.
- Protein degradation (you can check previously with western blot) try to work all the time at 4°C and use protease inhibitors during lysis.
- Removal of impurities associated with the IMAC resin. Use more stringent competitive conditions during binding and washing (example: use low Imidazole concentrations during binding and washings to increase competition for the same sites on the resin)
- Decrease resin volume (allows higher competition between fusion protein and contaminants for the same sites on the resin)
- If contaminants are associated with the tagged protein, try to disrupt the non-specific interaction by adding to the wash buffer before elution additives as ß-ME, glycerol up to 50%, detergents as Triton X-100, NP40 or Tween 20 up to 2% or increase ionic strength up to 1.5M NaCl or KCl.
- Consider an additional purification step before or after purification.
- Consider the use of a pre-column of beads without ligand to adsorb proteins that bound to beads non-specifically

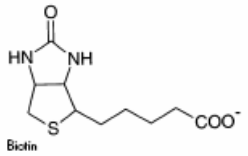
Parameters for optimization during elution

- **Optimize resolution**
- Adjust gradient slope/shape, or step elution with increasing competitor concentration. Change/add different steps before elution
- Include additives in buffers
- Adjust flow rate: lower flow rate to obtain narrow peaks
- **If the protein slightly elutes or does not elute**
- Use higher competitor concentration (Examle: up to 1M Imidazol for chelating columns), or additives if the protein tends to aggregate
- Reduce elution flow-rate
- Change elution conditions, consider elution under more denaturating conditions

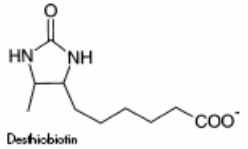
Strep-tag /*Strep*-Tactin system

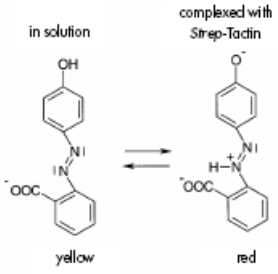
According to *Expression and purification of proteins using Strep-tag and/or 6xHistidine-tag. A comprehensive manual from IBA GmbH*

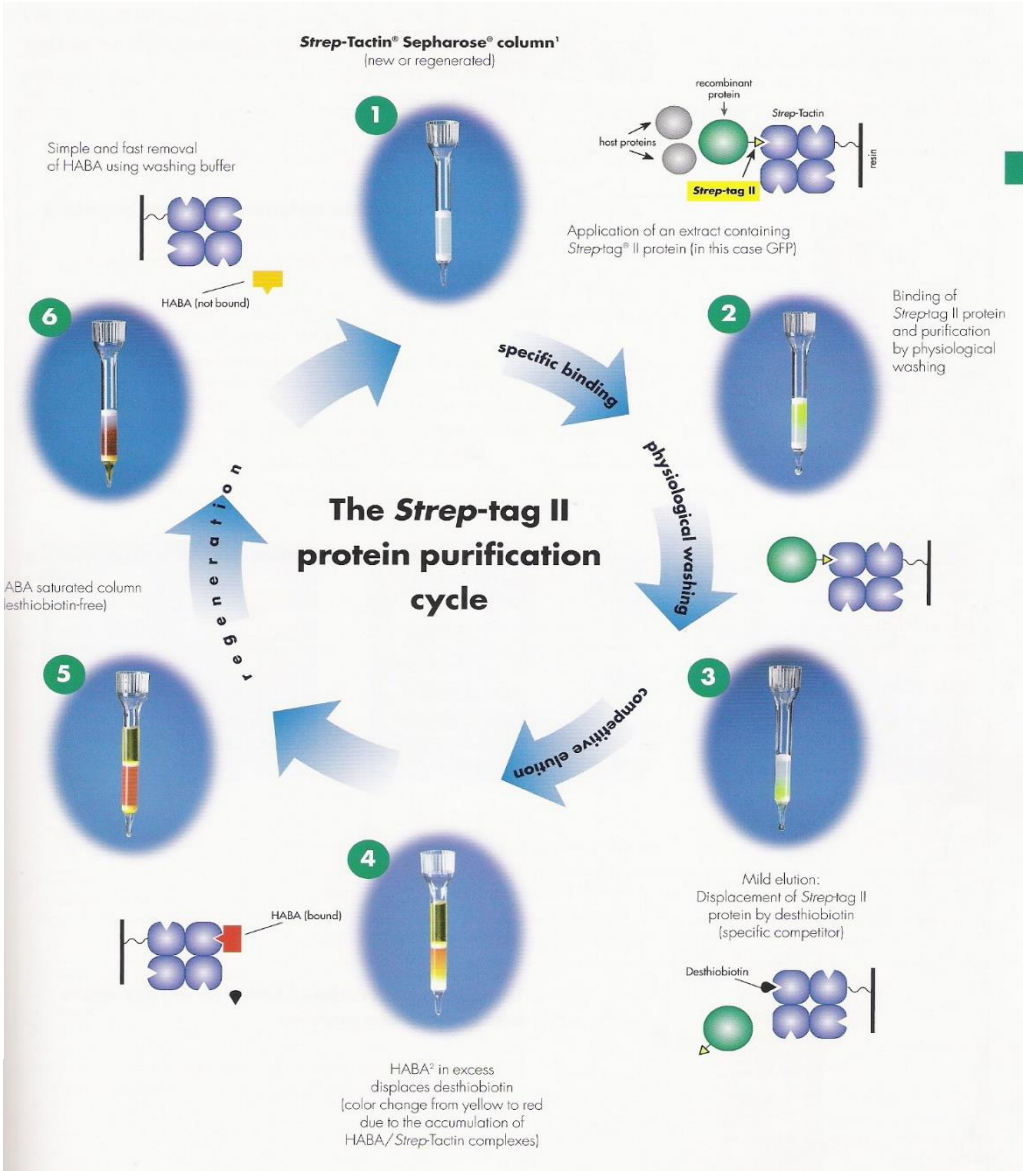
➢The *Strep*-tag II is a short peptide (8 mino acids, WSHPQFEK), which binds with high selectivity to Strep-Tactin, an engineered streptavidin.



➢One-step purification of recombinant protein under physiological conditions, thus preserving its bioactivity: elution with 2.5mM desthiobiotin).







Strep-tag /*Strep*-Tactin system

According to *Expression and purification of proteins using Strep-tag and/or 6xHistidine-tag. A comprehensive manual from IBA GmbH*

- ✓ The *Strep*-tag system can be used to purify functional *Strep*-tag II proteins from any expression system including baculovirus, mammalian cells, yeast, and bacteria.
- ✓ Most popular for extracellular expression in mammalian/insect cells
- ✓ Many labs use as double strep-tag
- ✓ In some culture medium there is biotin contamination that affect target binding
- ✓ Users claims that purity is better than IMAC
- ✓ Time-life of columns is much lower than IMAC columns
- ✓ The *Strep*-tag/Strep-Tactin interaction is compatible with a variety of reagents (detergents, reducing agents, etc.) making the system attractive for purifying metallo- and membrane proteins, large proteins and protein complexes.

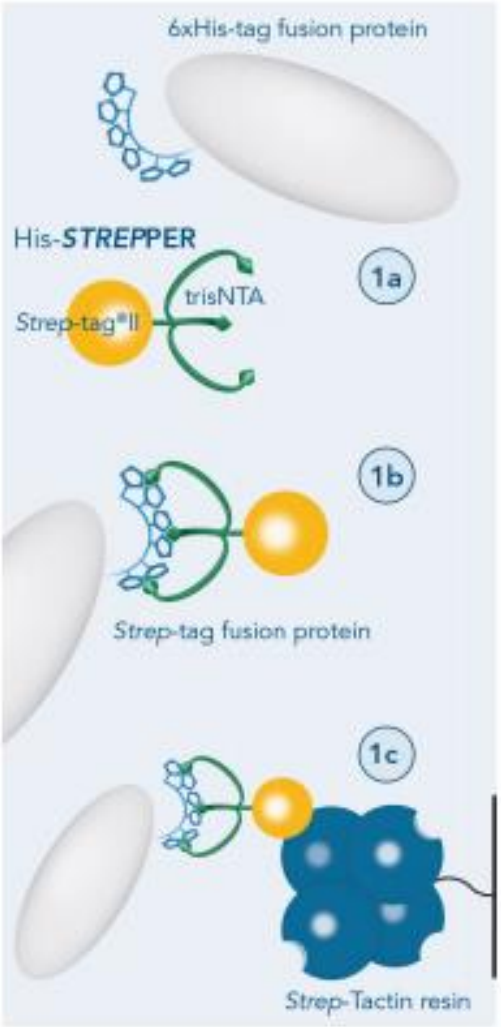
Strep/6 x Histidine system (double-tag)

IBA and QIAGEN

- Useful for full length recombinant proteins purification at high purity under standardized and non-denaturative conditions (Imidazol and desthiobiotin elution)
- Especially useful to eliminate "difficult to resolve" protease cleavage fragments of target protein.
- Recombinant proteins that carry 6 x His-tag at the N-terminus and *Strep* tag II at the C-terminus (or vice versa).
- Efficiently expressed in *E. coli*, yeast, insect, or mammalian cells.
- **Recommendation: use IMAC as first capture step. No buffer exchange is required for the second purification step: *Strep*-Tactin resin**

His-STREPPER - His/Strep-tag®II Adapter

IBA



✓ *Strep*-tag®II (SA-WSHPQFEK) conjugated with a nickel charged trisNTA

✓Tightly binds to 6xHis-tag converting a 6xHis-tag fusion protein to a *Strep*-tag®II fusion protein without the need for cloning

✓Applied to the cell lysate **or** to the His-tag eluate (after complete removal of imidazole)

✓Purification with *Strep*-Tactin® column

Commonly used affinity tags

Preparative Purification of Recombinant Proteins: Current Status and Future Trends

Mayank Saraswat et al. Hindawi Publishing Corporation - BioMed Research International Volume 2013, Article ID 312709, http://dx.doi.org/10.1155/2013/312709

Tag	Size [amino acids or kDa]	Ligand or separation method
Polyhistidine	5–15 a.a.	IMAC
HA-tag	9 a.a.	mAb based
FLAG	8 a.a.	mAb based
Strep tag I	9 a.a.	Streptavidin
Strep tag II	8 a.a.	Streptactin
Softag 1	13 a.a.	mAb based
Softag 3	8 a.a.	mAb based
T7-tag	11–16	mAb based
c-myc	10 a.a.	mAb based
S-peptide	15 a.a.	S-protein
Polyaspartic acid	5–16 a.a.	Ion-exchange or precipitation
VSV tag	11 a.a.	mAb based
Calmodulin binding peptide	26 a.a.	Calmodulin

Flag Tag (SIGMA)

- Highly specific. Very good purity of final product. Use for recognition
- Ligand: Ab anti Tag (short half life).
- Elution highly specific but expensive peptides **or** harsh elution conditions

CaptureSelect C-tag

THERMO

- **A small 4 amino acid peptide tag: E-P-E-A (glutamic acid - proline - glutamic acid - alanine) that binds to a robust 13 kDa Camelid antibody fragment affinity matrix**

- The CaptureSelect C-tag affinity matrix purifies C-terminal tagged proteins with high affinity and selectivity, even in the presence of Urea and Guanidine HCl.

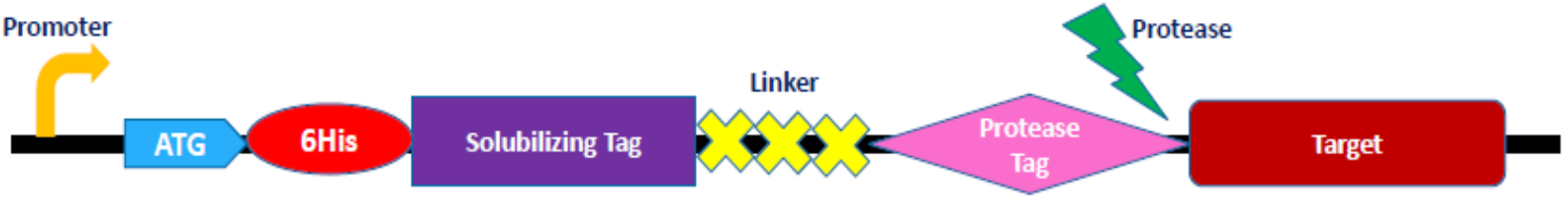
- Mild elution conditions at neutral pH can be applied using 2M magnesium chloride or 50% propylene glycol, which ensures high activity recoveries of pH sensitive target proteins; or more specifically with 2mM S-E-P-E-A peptides.

- The affinity resin recognizes the E-P-E-A tag sequence when fused either directly to the **C-terminus** of a protein or through linkers between the C-terminus and the E-P-E-A tag

Fusion proteins

Solubility-enhancement tags (SETs)

- Use to overcome some of the problems of bacterial expression: protein aggregation, poor expression levels and difficult purification.
- The most popular fusion systems employ maltose-binding protein (MBP), glutathion S-tranferase (GST), thioredoxin (TRX), SUMO and NusA. These genes are well expressed and the proteins are highly soluble and provide specific characteristics to aid purification.
- Typically, the gene of interest is inserted “in frame” at the 3’ end of the carrier protein
- To enable removal of the fusion protein, a linker region is typically included between the fusion protein and the native protein sequence.
- Specific recognition of the linked regions with specific proteases: TEV, Prescission, SUMO protease, Enterokinase



Advantages / disadvantages of fusion proteins

In several cases

- **Facilitate protein refolding / increase solubility**
- Facilitate protein purification
- Improve protein production (high expression) in bacteria, yeast and mammalian cells
- Prevent proteolysis

But in other cases

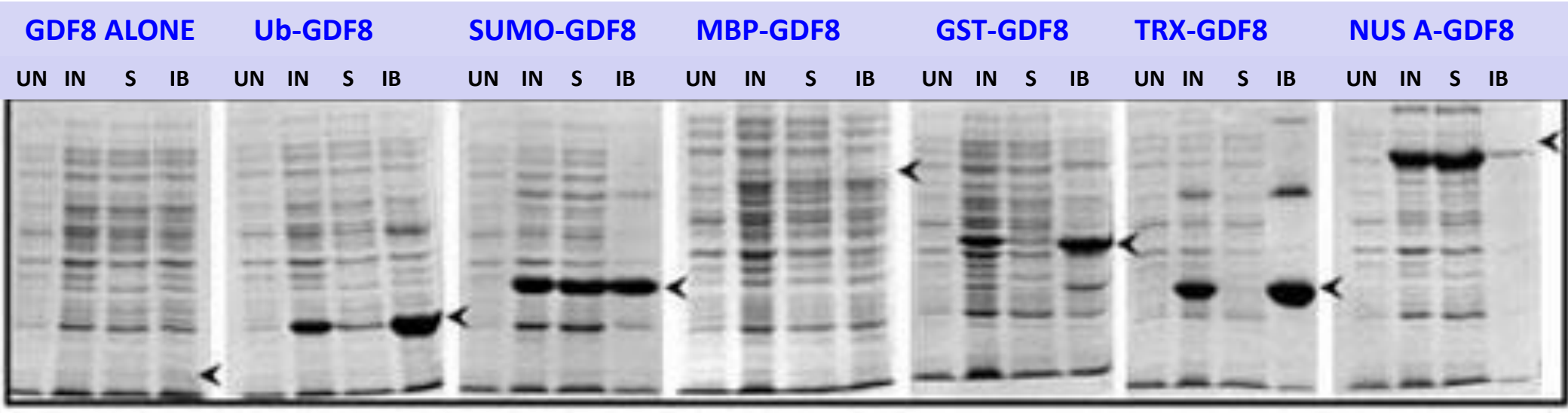
- **Target is not soluble after cleavage**
- **Target bounds to the protein tag by non-covalent forces**
- Extra work if you need to cleave
- Final yield of target protein is gone at expenses of the fusion protein
- Possible alteration in biological activity
- Change in protein conformation and undesired flexibility in structural studies

There is no single fusion tag which is the "best" in every case

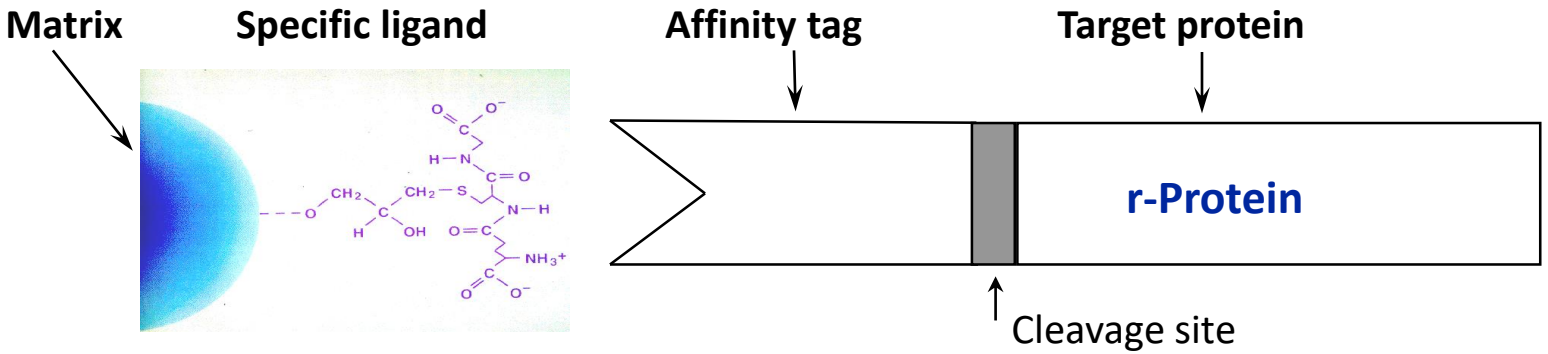
Fusion Tag Comparison Study

LifeSensors (http://www.lifesensors.com/r_and_d/protein_expression.php3)

SUMO (Small Ubiquitin-like MOdifier) and NusA fusion tags dramatically outperform glutathiones transferase (GST), maltose binding protein (MBP), thioredoxin (TRX), and ubiquitin (Ub). The protein target tested in this study is GDF-8, a growth/differentiation factor. UN: un-induced, IN: induced, S: soluble, IB: inclusion body.



Glutathione S-transferase or GST-fusion proteins



- Column: Glutathione Sepharose 4B, pre-packed
- Binding: PBS (+ 1% Triton X-100)
- Elution: 5-10 mM Glutathione, 50 mM Tris.HCl, pH 8.0

➢GST: 26kDa cytoplasmic protein. Binds specifically to glutathione-agarose and elute with Glutathione (mild conditions, cheap elution)

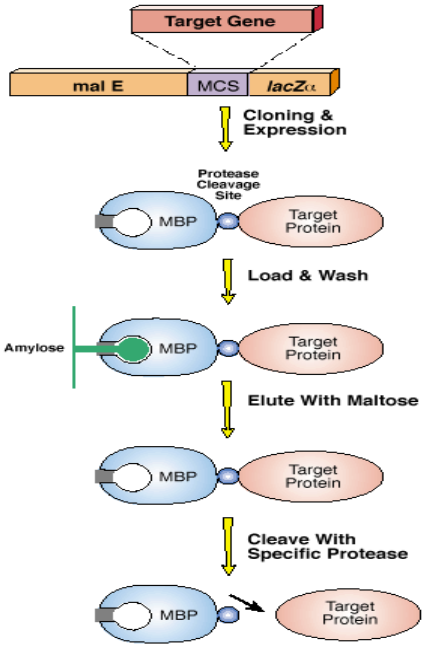
➢Highly use in immunoprecipitation - Less use for increasing solubility

➢Commercial vectors containing either factor Xa, Prescision or a thrombin recognition site to allow cleavage and removal of the GST.

➢Useful for dimerization

Maltose-binding protein pMAL

™



- **MBP:** a 43kDa secreted protein from E. *coli*, binds specifically to maltose
- Purification: Maltose or Amylose agarose columns and Dextrin Seph (most recommended)
- Can be secreted to the periplasm in E.Coli or extracellular in mammalian cells
- Or cytoplasm expression without signal peptide
- **Very effective for solubility enhancement**
- TEV protease cleavage site
- Sometimes additional 6His in N-terminal
- Could be used to aid crystallization of target protein

Good option: widely use in Core Facilities

One of the most widely used fusion protein

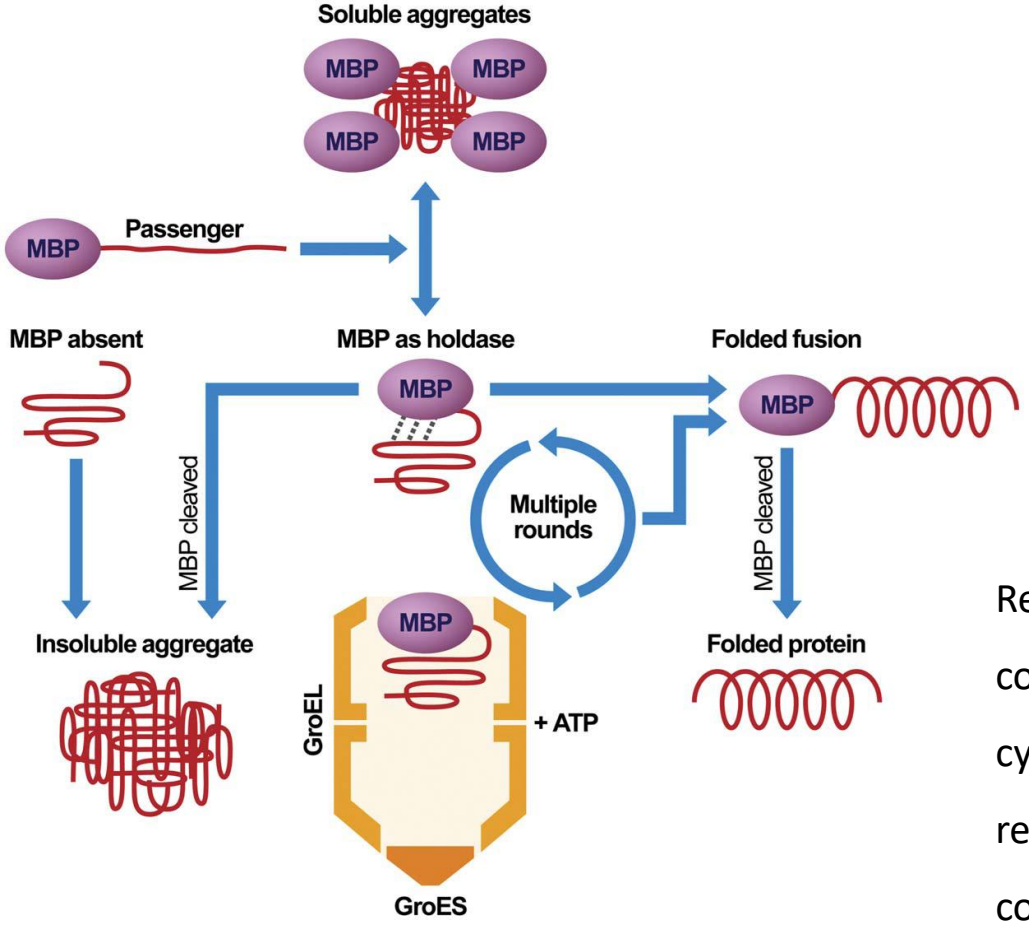
Adding 6His at N-term provides a double affinity

The Mechanism of Solubility Enhancement by MBP

Sreejith Raran-Kurussi and David S. Waugh PLOS ONE (2012) 7 (11): e49589 - doi:10.1371

MBP serves as a passive participant in the folding process; passenger proteins either fold spontaneously or with the assistance of chaperones.

Chaperones and/or chaperonins seem to come into play after a passenger protein has been rendered soluble by MBP.

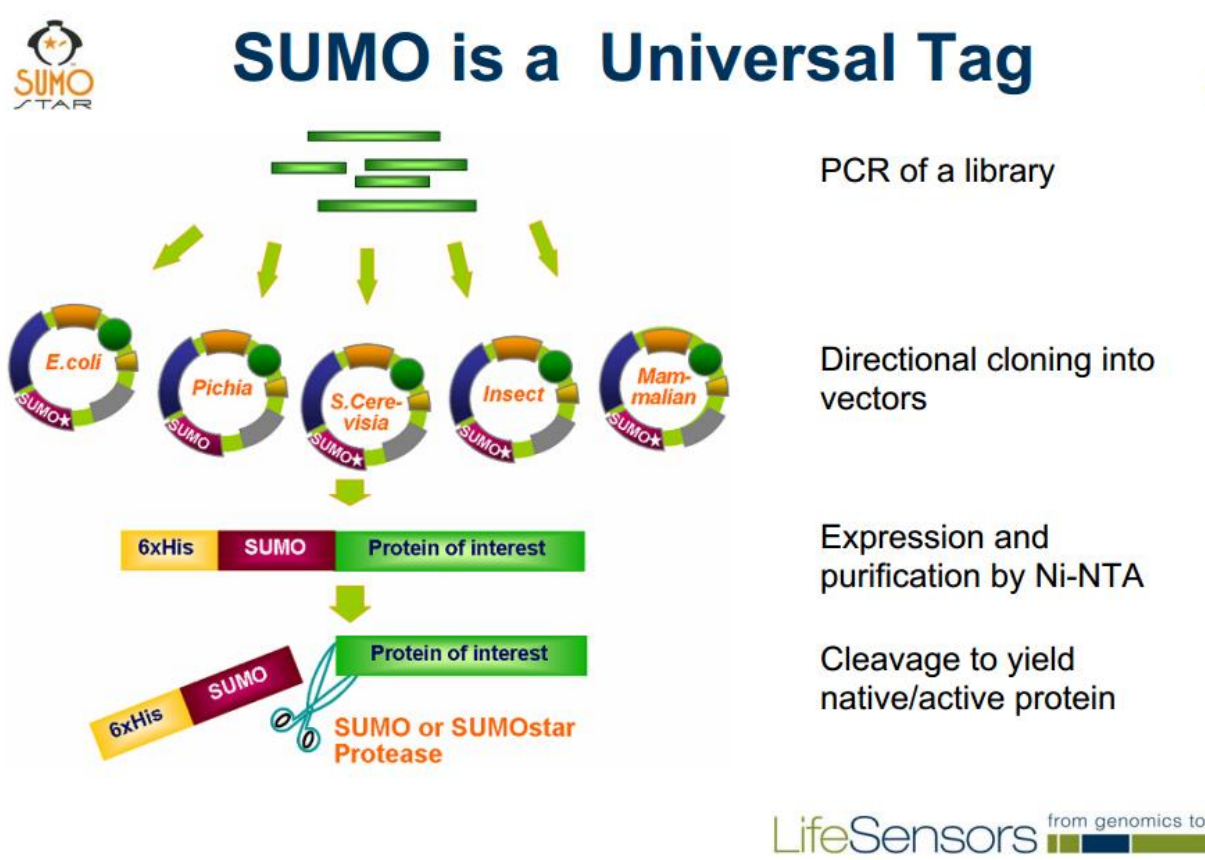


MBP serves primarily as a ‘‘holdase’’, keeping the incompletely folded passenger protein from forming insoluble aggregates until either spontaneous or chaperone-mediated folding can occur.

A third class of passenger proteins is unable to fold and exists in an incompletely folded state and typically precipitate after they are cleaved

Recently, MBP has also been used to maintain proteins that contain disulfide-bonds in a soluble state in the E. coli cytoplasm so that they could be acted upon by appropriate redox enzymes that were co-expressed in the same cellular compartment

SUMO (small ubiquitin-like modifier)



- Increased expression
- Increased solubility
- Acidic protein ~10kDa
- Both the tag and the protease have 6xHis tags
- SUMO Protease extremely efficiently (1:500)
- SUMO Protease leaves no unwanted residues on the N-terminus

Good option: widely use in Core Facilities

Thioredoxin (Trx)

- **Thioredoxin:** 12 kDa intracellular E. *coli* protein.
- Very soluble, and highly over-expressed
- Periplasmic or cytoplasmatic expression
- Robust folding (some chaperone activity)
- **Most popular to increase disulfide bonds**
- 6His Tag can be add to the N terminal of TRX for IMAC purification
- Does not work well with larger MW target proteins
- Since TRX is thermostable, and some heat-stable fusion proteins can be purified by thermal denaturation of contaminants by thermo-osmotic shock (osmotic shock coupled with heat-treatment) *According to Q.-R. Guo et al. / Protein Expression and Purifffication 49 (2006) 32–38*

Good option: widely use in Core Facilities to increase S-S bonds

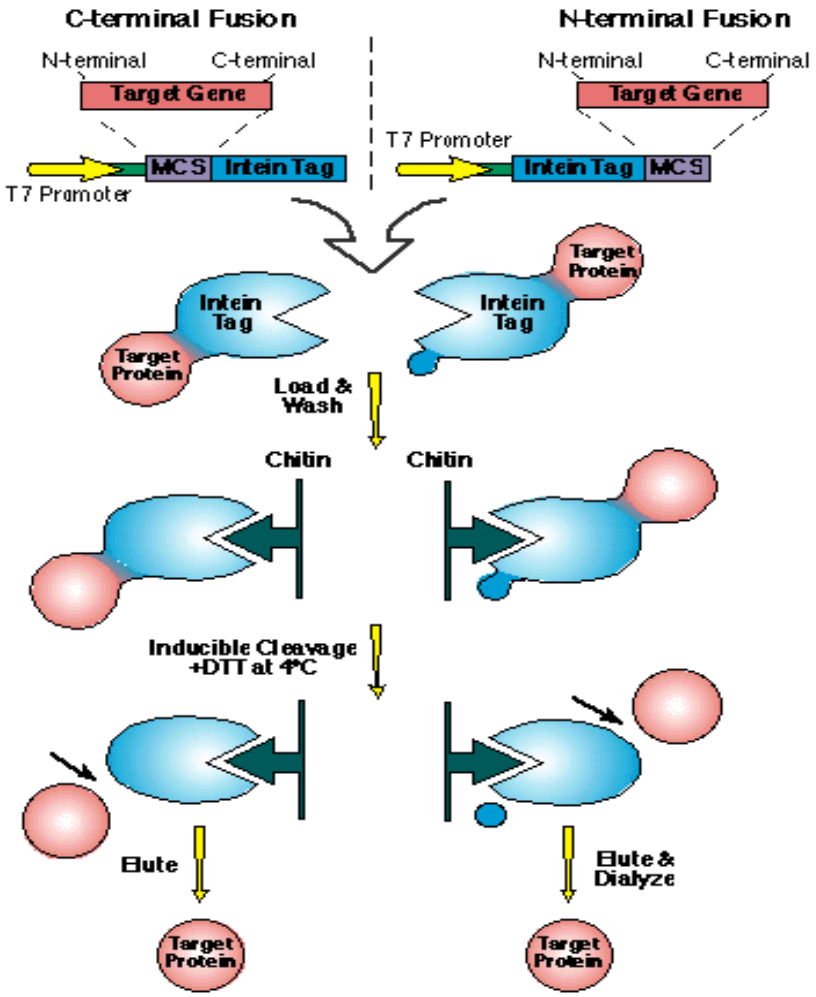
IMPACT™-CN System
(NEB)

➢Intein Mediated Purification with an Affinity Chitin-binding Tag protein purification system.

➢Use inducible self-cleavage activity of a protein splicing element (intein) to separate the target protein from the affinity tag without the use of a protease.

➢A target protein is fused to a tag consisting of the intein and the chitin binding domain.

➢In the presence of thiols such as DTT, b-mercaptoethanol or cysteine, the intein undergoes specific self-cleavage which releases the target protein from the chitin-bound intein tag



Amazing idea, but… Do not work many times

Biotinylation in vivo

PinPoint™ Xa from Promega

- In vivo Biotinylated Affinity Tags: biotinylation reaction in E. coli through biotin ligase holo-enzyme

- Fusion purification tag with a single biotin specifically on one Lys residue

- The system use a monomeric avidin (Soft Release Avidin Resin): allows protein elution with a non-denaturing 5mM biotin buffer.

- Tag cleavage with Factor Xa

Comparison of Affinity tag technologies

According to J.J. Lichty et al. / Protein Expression and Purifffication 41 (2005) 98–105

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

By starting a new project cost is one of the most commonly confronted decisions

Cleavage of Fusion protein

- Chemicals cleavage is effective but often requires extreme conditions (low pH or high temp.), and is often non-specific (cyanogen bromide, hydroxylamine, etc).
- Enzymatic digestion is the method of choice for soluble fusion protein cleavage. Reaction is carried out under relative mild conditions.
- Commonly used endo-proteases : thrombin, factor Xa, enterokinase
- **Best sellers: TEV, SUMO and rhinovirus 3C protease (Prescission)**
- Exoprotease: TAGZyme, carboxypeptidase A (C-terminal tag)
- Intein cleavage by reduction agents such as DTT, or low pH

Cleavage Sites

- **Thrombin** **Amersham-Biosciences, Novagen, SIGMA, Roche**

  Leu-Val-Pro-Arg▼Gly-Ser **Protease Capture: Benzamidine-Agarose**

  Secondary cleavage sites. Biotinylated form available for removal with immobilized streptavidin.

- **Factor Xa** **Amersham-Biosciences, NEB, Roche**

  Ile-Glu/Asp-Gly-Arg▼ **Protease Capture: Benzamidine-Agarose**

  Will not cleave if followed by proline and arginine. Secondary cleavage sites following Gly-Arg sequences.

- **Enterokinase** **NEB, Novagen, Roche**

  Asp-Asp-Asp-Asp-Lys▼ **Protease Capture: Trypsin Inhibitor-Agarose**

  The site will not cleave if followed by a proline residue. Secondary cleavage sites at other basic residues, depending on conformation of protein substrate. Active from pH 4.5 to 9.5 and between 4°C and 45°C

- **TEV protease** **Invitrogen – Life Technologies**

  Glu-Asn-Leu-Tyr-Phe-Gln▼Gly **Protease Capture: Ni-NTA (6His recomb. TEV)**

  Seven-residue recognition site, making it a highly site-specific protease. Active over a wide range of temperatures. Protease available as a His-tag fusion protein, allowing for protease removal after recombinant protein cleavage.

- **PreScission** **Amersham-Biosciences**

  Leu-Glu-Val-Leu-Phe-Gln▼Gly-Pro

  Genetically engineered form of human rhinovirus 3C protease with a GST fusion tag, allowing for facile cleavage and purification of GST-tagged proteins along with protease removal after recombinant protein cleavage. Enables low-temperature cleavage of fusion proteins containing the eight residue recognition sequence.

- **TAGZyme** **Quiagen: His-tag removal by Exoproteolytic Digestion**

  Protease Capture: Ni-NTA (6His recomb. enzyme)

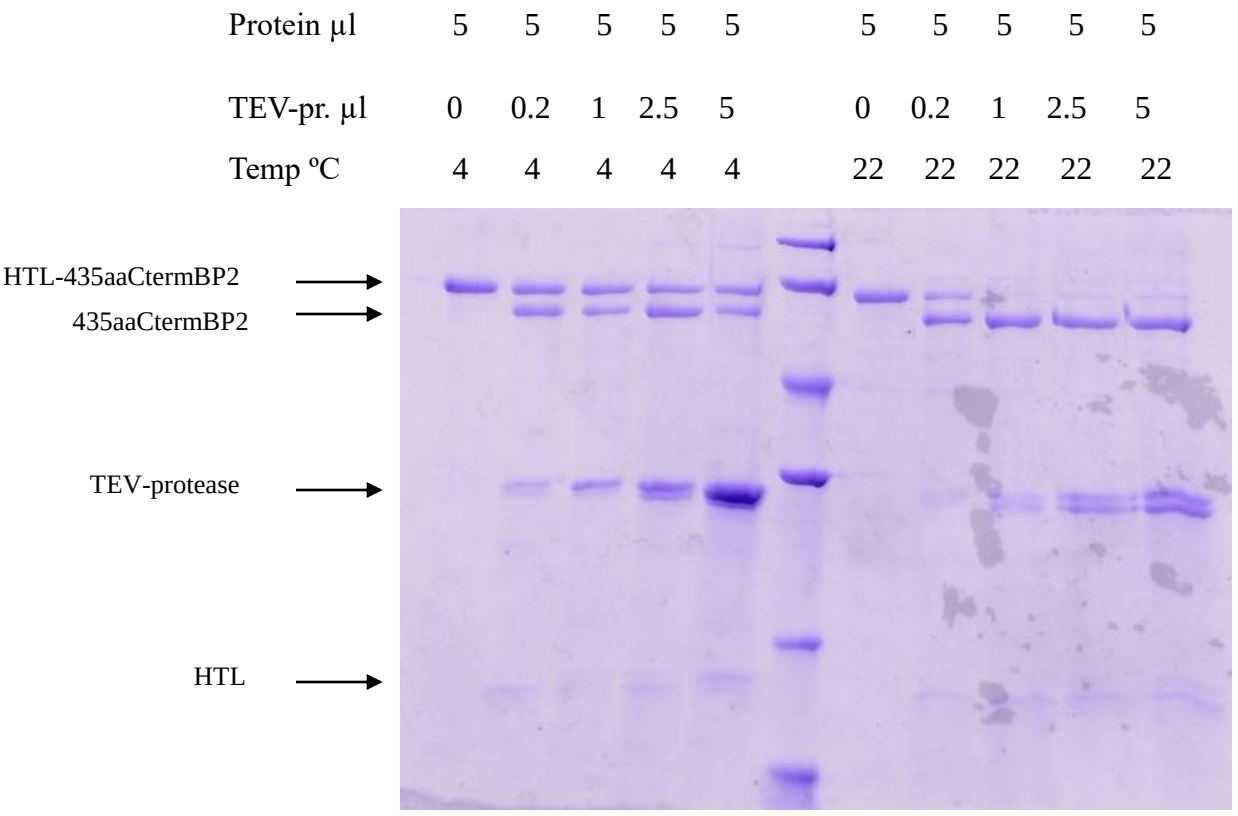
  The TAGZyme System is an efficient and specific solution for the complete removal of small N-terminal His tags and other amino acid tags by the use of exopeptidases. These recombinant enzymes contain a C-terminal His tag and can therefore be bound to Ni-NTA matrices.

- **Intein Site (dithiothreitol cleavage)** **NEB**

  DTT elimination by dialysis

  Uses self-cleavable affinity tags. Even after cleavage un-natural termini are present on the protein of interest.

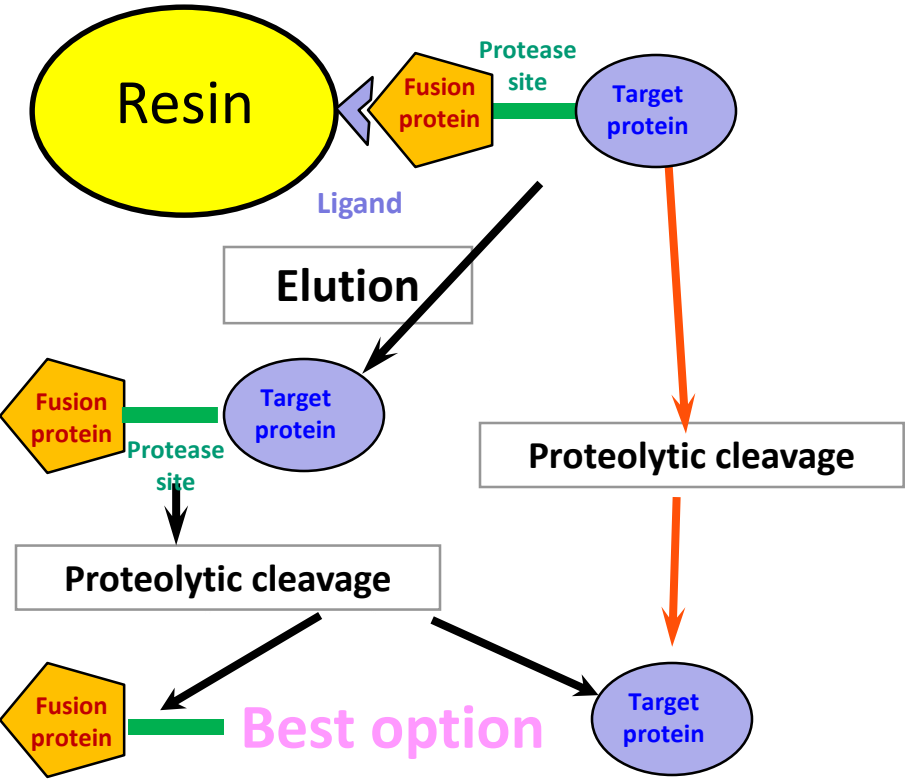
Incubation of HLT-435aaCtermBP2 with TEV-protease



- ✓Protease / protein ratio
- ✓Protease quantity
- ✓Protein concentration (volume)
- ✓Temperature
- ✓Time
- ✓Cleavage site exposure
- ✓Buffer
- ✓Presence of aggregates

Shahar Rotem,
Assaf Friedler

Purification of Fusion Proteins



TROUBLESHOOTING

Protein target and fusion tags are difficult to separate

- Increase competition(Immidazol)
- Reduce non-covalent interaction: detergents, chaotropics, conductivity, etc
- **Separation by charge**
- **Separation by mass/radious**
- **Hydrophobic separation**

Chromatography

- **Negative affinity**
- **IEX, MMC, HAP, etc**
- **SEC, dialysis, buffer exchange, others**
- **RPC for easy to refold low MW proteins or peptides**

Parameters for optimization during protease cleavage and purification

Cleavage depends of:

Target/protease ratio

Reaction volume

Temperature

Time

Buffer

Others

- If possible try to cut at low temperature
- Cleavage can be done inside dialysis bags under dialysis to prepare protein to next step
- If necessary add additives in buffers to avoid aggregation of the target after cleavage
- High aggregation can affect cleavage. So consider SEC before cleavage
- Some targets slightly bound to the IMAC resin in the negative step: increase competition (Imidazol, etc)
- Cleavage is OK, but proteins elute together because of other interactions. Try to reduce these interactions with additives (high salt, detergents, etc)
- Alternatives to negative affinity: IEX, HIC, GF

Advantages / disadvantages of Proteolytic Cleavage

- Spurious, non-specific proteolytic cleavage of the fusion protein
- Extra amino acids in the N-terminal after cleavage for some proteases
- Non-covalent forces maintain proteins connected after cleavage: separation problems
- Enterokinase need elevated temperatures (25-37°C) for efficient cleavage: can denature or cause aggregation of the fusion protein
- Incomplete cleavage, which reduces the yield and/or introduces heterogeneity to the purified protein
- Buffer and additive restriction (mainly detergents)
- $ and time: The need for additional steps to separate the cleaved fusion protein from the fusion tag, remove the protease, and exchange buffer or desalt
- Increase purity by using negative column (retains impurities) or other chromatographic procedure
- Targets are mainly C-terminal of the solubility protein (exoproteases as carboxypeptidase A)

Self-cleaving elastin-like polypeptide tag

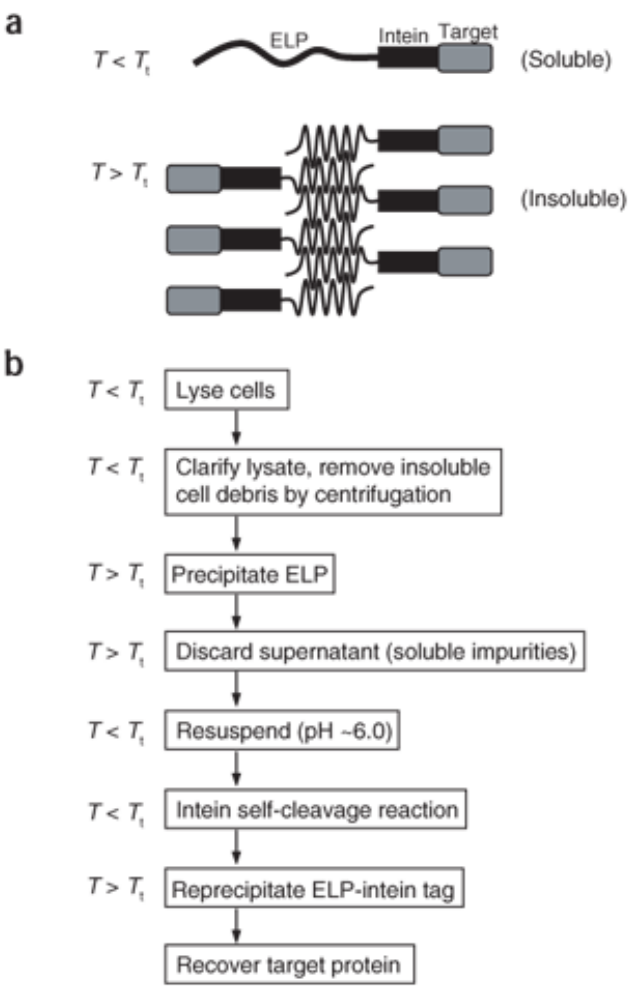
Purification of recombinant protein without the need for affinity chromatography or proteolytic tag removal

Simple bioseparations using self-cleaving elastin-like polypeptide tags. *Banki M. et al. - Nature Methods **2**, 659 - 662 (2005)*

- Self-cleaving ELP tags consisting of repeating pentapeptides of VPGXG (X = any amino acid) fused to a controllable self-cleaving intein
- ELPs can be designed to undergo a reversible transition from soluble to insoluble upon temperature upshift
- The reversibility of the precipitation is distinct from simple irreversible aggregation or misfolding, and can be used by several groups to purify ELPs and protein products fused to ELPs
- Intein-fused affinity tags get inducible self-cleave under mild conditions (room temperature and slightly acidic pH)
- PROBLEMS: Slow growth.

  Expression level

  Optimization will be required for new, uncharacterized products on a case-by-case basis



Self-cleaving elastin-like polypeptide tag

✓**A Cost-Effective ELP-Intein Coupling System for Recombinant Protein Purification from Plant Production Platform**

(2011) Li Tianet al - Tsinghua University, Beijing, *PLoS ONE 6(8): e24183 (2011)*

✓**Single-step purification of recombinant proteins using elastin-like peptide-mediated inverse transition cycling and self-processing module fromNeisseria meningitides FrpC**

Wen-Jun Liu - Yangzhou University *Protein Expr. Purif. (2014),*

✓**Purification of Escherichia coli RNA polymerase using a self-cleaving elastin-like polypeptide tag** .

Baley A. Fong - Princeton University *PROTEIN SCIENCE 2010 VOL 19:1243—1252*

✓**A Cleavable Self-Assembling Tag Strategy for Preparing Proteins and Peptides with an Authentic N-Terminus**.

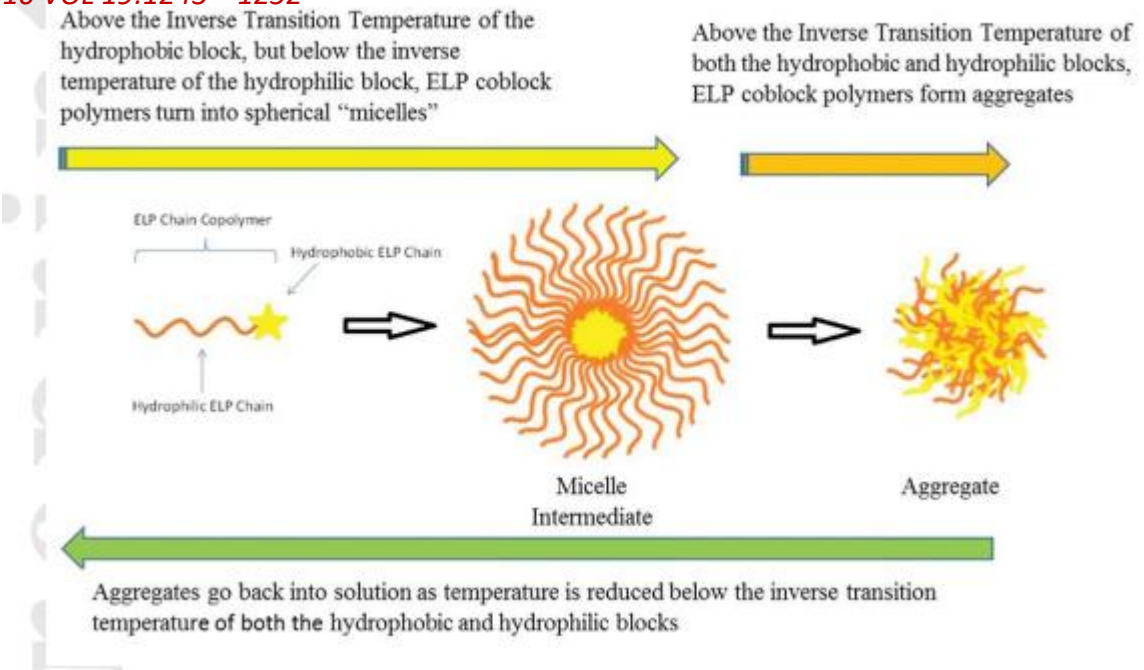
Qing Zhao - Tsinghua University

Biotechnology Journal (2017)

✓**Fusions of Elastin-Like Polypeptides to Pharmaceutical Proteins**

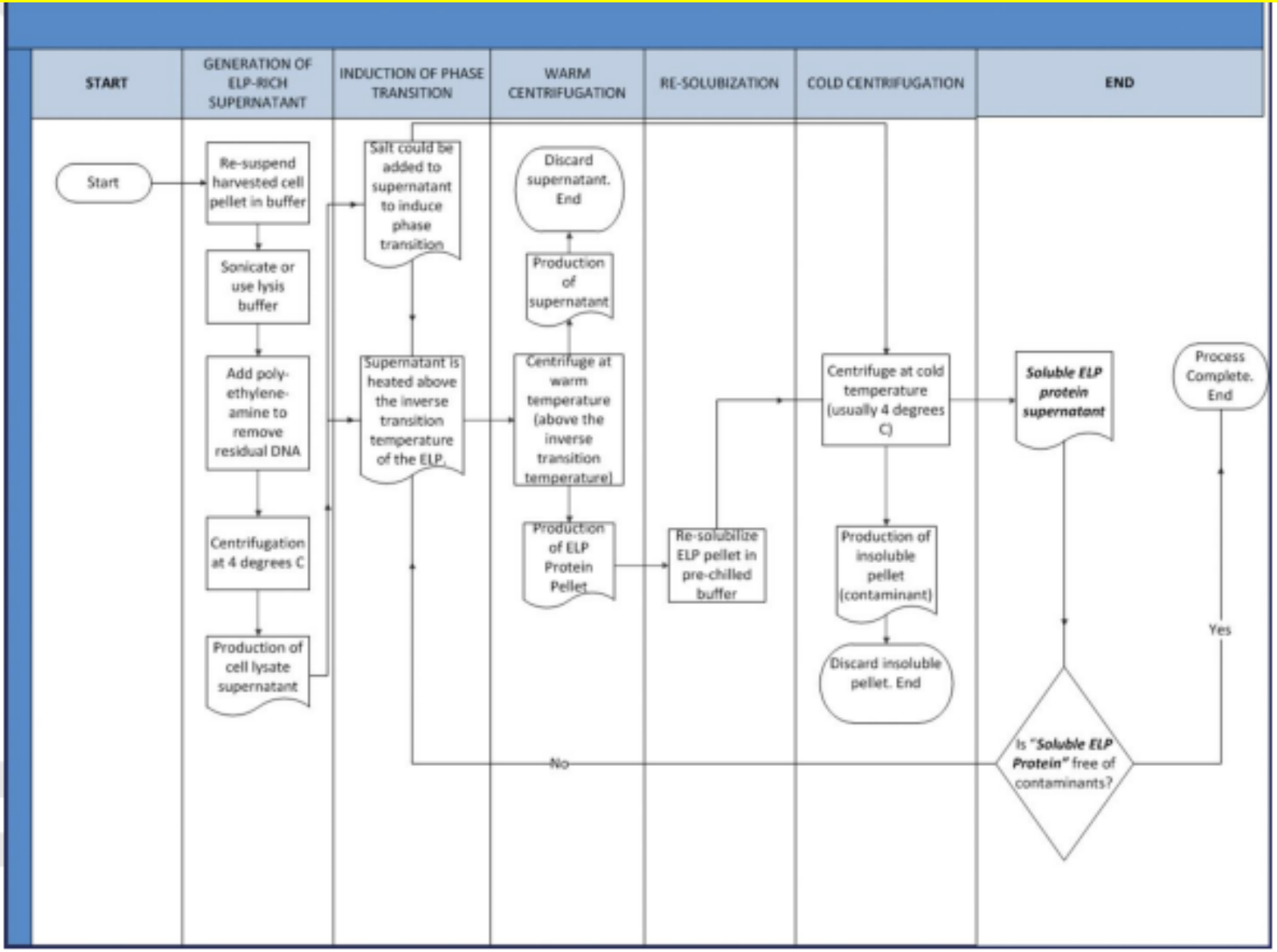
Wafa Hassouneh - Duke University

Methods in Enzymology,Volume 502 (2012)



Elastin-like polypeptides:
A strategic fusion partner for biologics (protein "ELPylation")

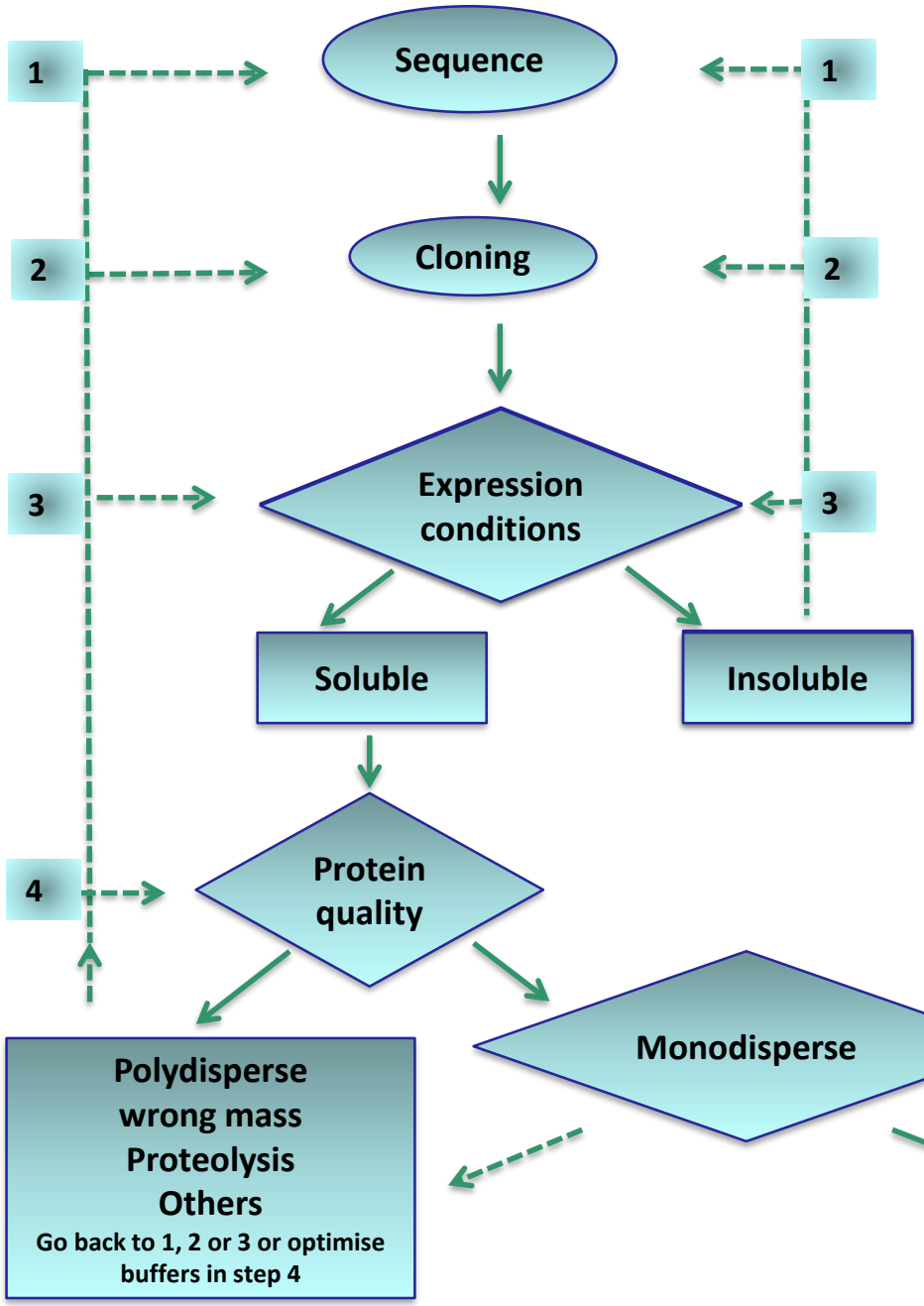
AGNES YEBOAH et al. Rutgers University *Biotechnology and Bioengineering (2016) - DOI 10.1002/bit.25998*

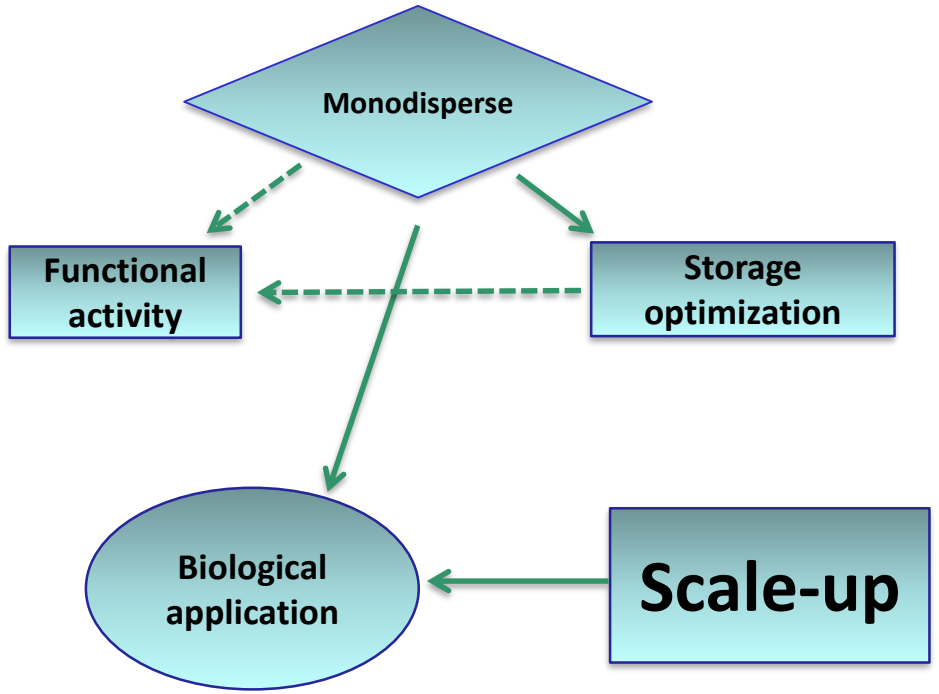


The Trip Adviser guide to the protein science world: a proposal to improve the awareness concerning the quality of recombinant proteins

Mario Lebendiker, Tsafi Danieli and Ario de Marco

*BMC Research Notes 2014, **7**:585 doi:10.1186/1756-0500-7-585*





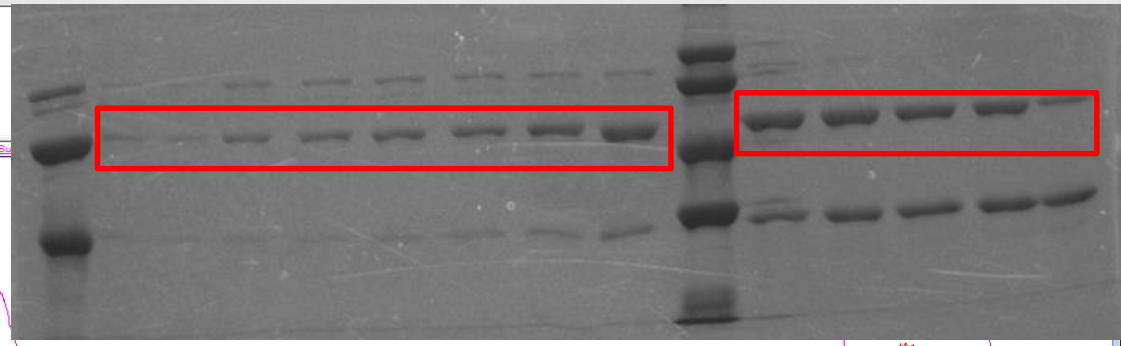
GST-FliD : Glut.Agar, TEV protease ON & split to SEC or AEIX

Aviv Vronen– Assaf Friedler Group

GST-TEV-1-747-FliDPAO1: aa:708, MW:76689.1, pI: 5.92, 4Cys, 4Trp, Ext.55030, Abs:0.718(ox),
1-747-FliDPAO 1(noGST, but including Glycine): aa:474, **MW:**49375.4, **pI:** 6.72, 0Cys,
0 Trp, Ext.coef:10,430, **Abs:0.211** Cys (C) 0
GST Number of amino acids: 23 **Molecular weight:** 27331.7 **Theoretical pI:** 5.7

Purification:

- ✓ GST-Agarose
- ✓ TEV protease + dialysis
- ✓Negative GST-Agarose
- ✓Negative IMAC (eliminate TEV)
- ✓SEC
- ✓AEIX
- ✓Dialysis and concentration

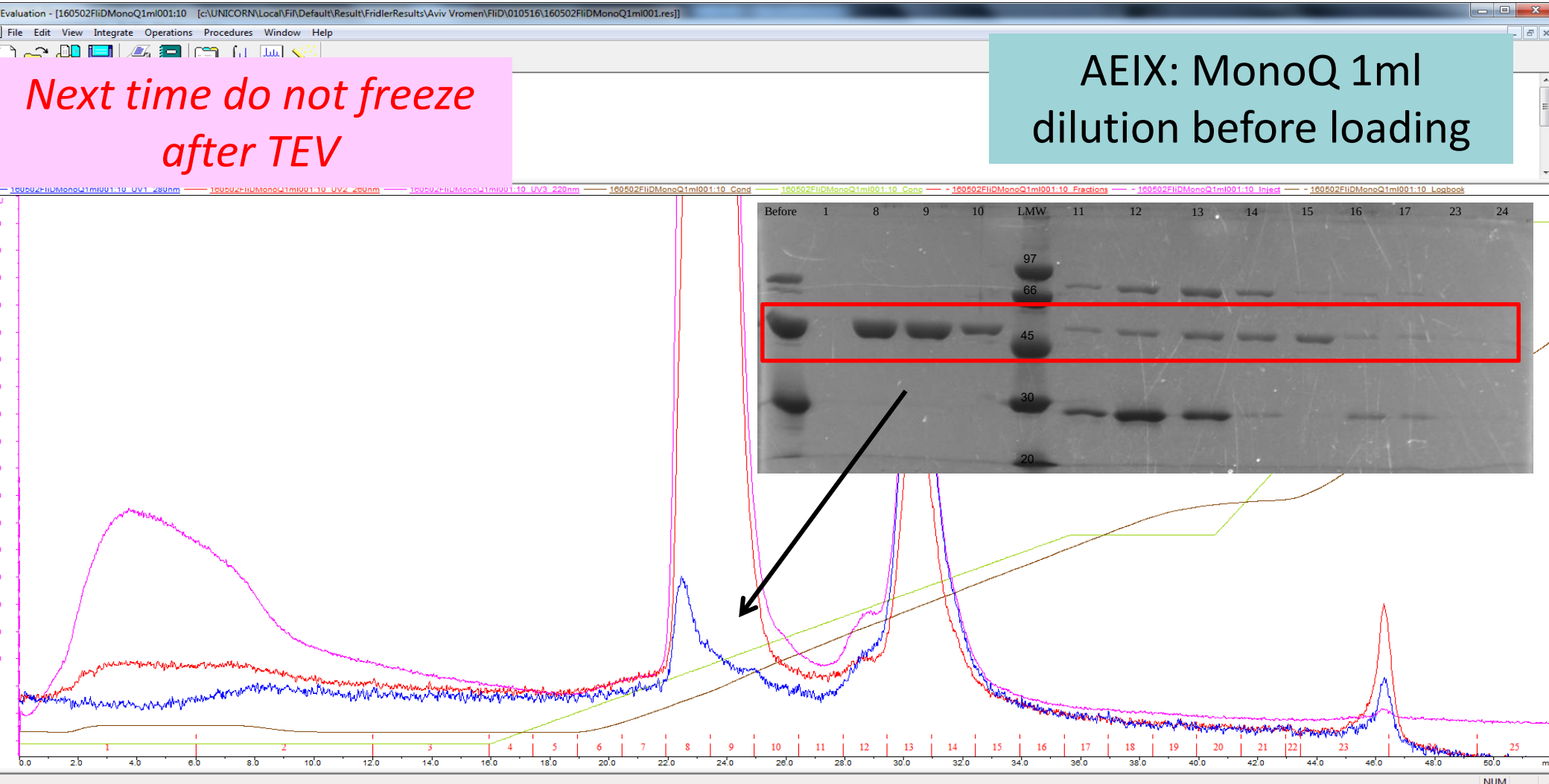


GST-FliD : Glut.Agar, TEV protease ON & split to SEC or AEIX

Aviv Vronen– Assaf Friedler Group

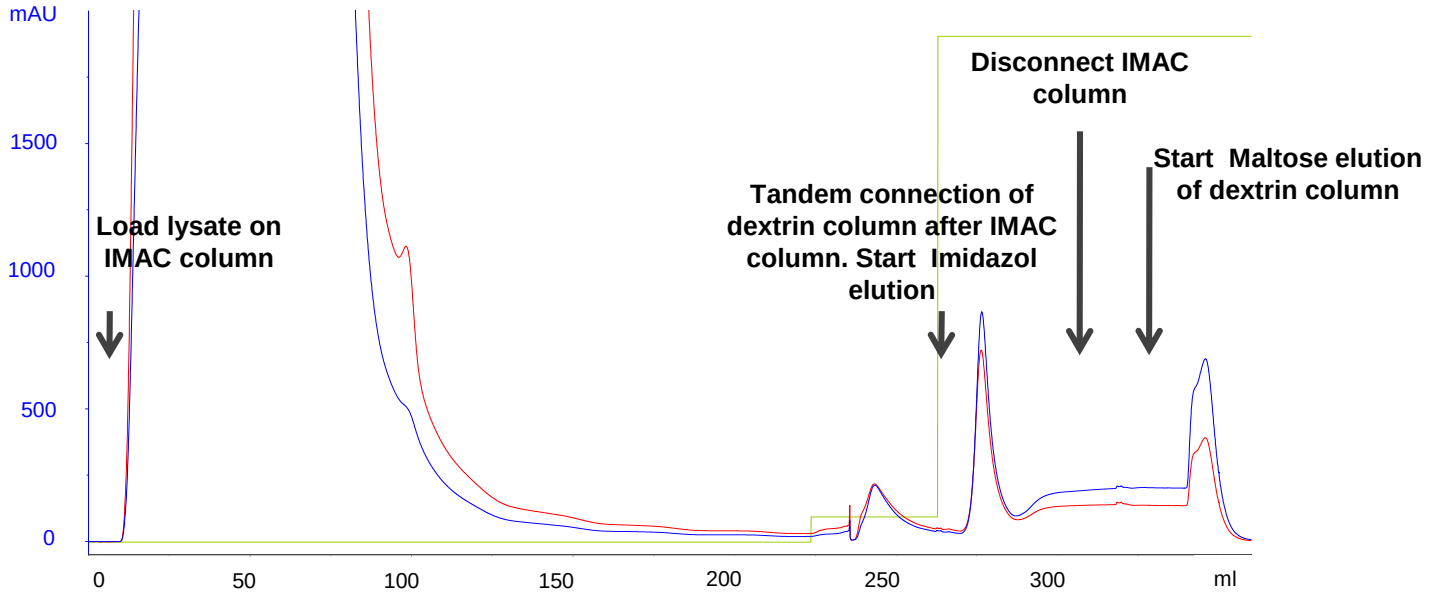
Next time do not freeze after TEV

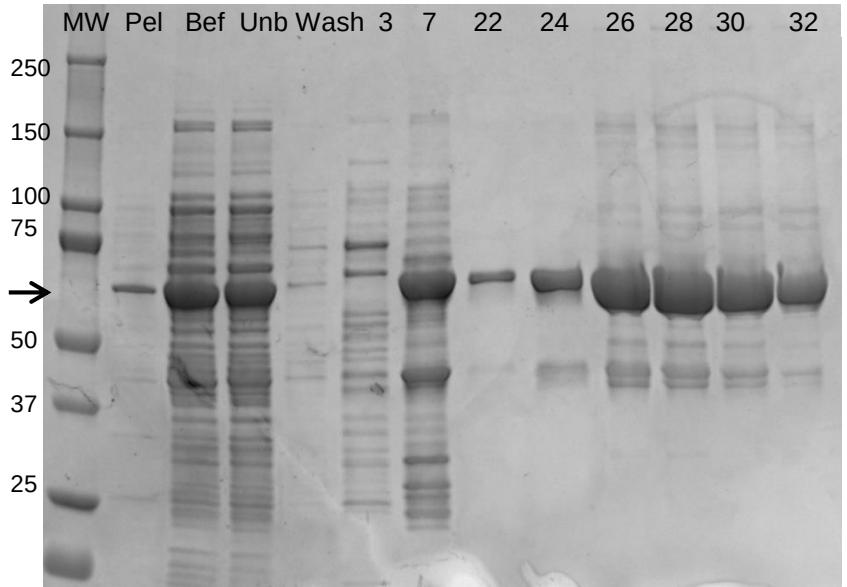
AEIX: MonoQ 1ml dilution before loading

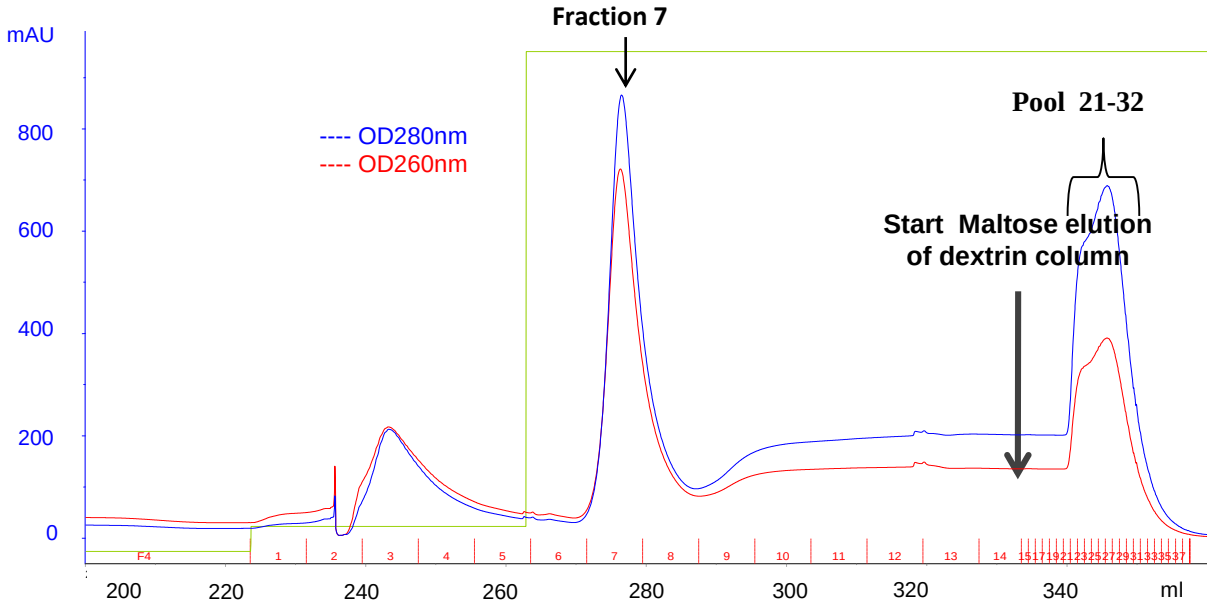


MBP fusion protein

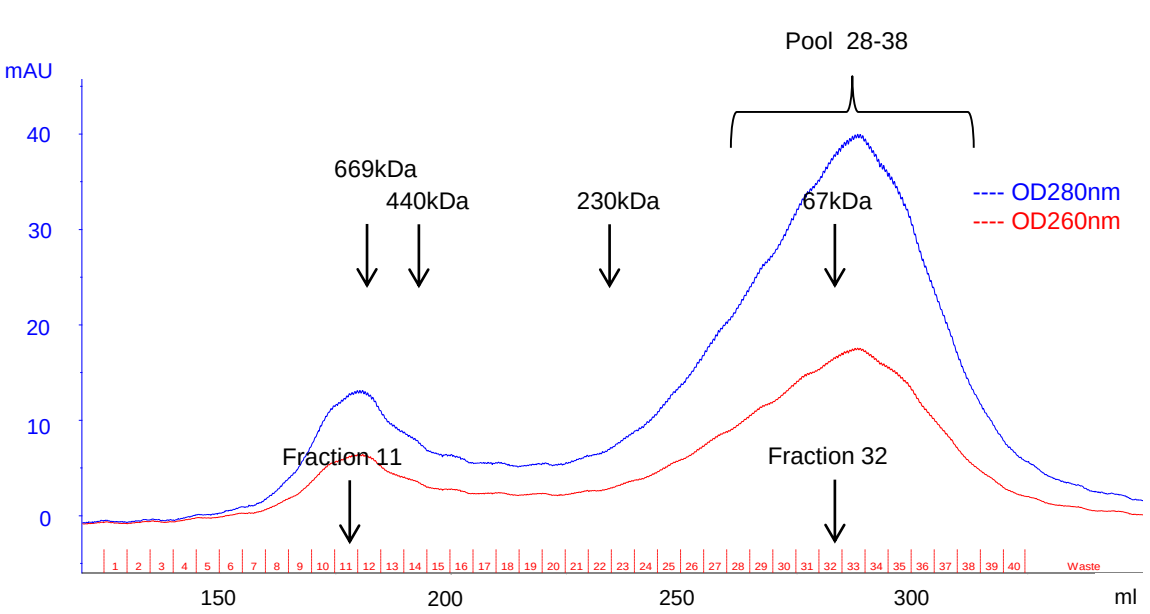
Capture: combined IMAC and Dextrine-Sepharose Affinity Chromatography

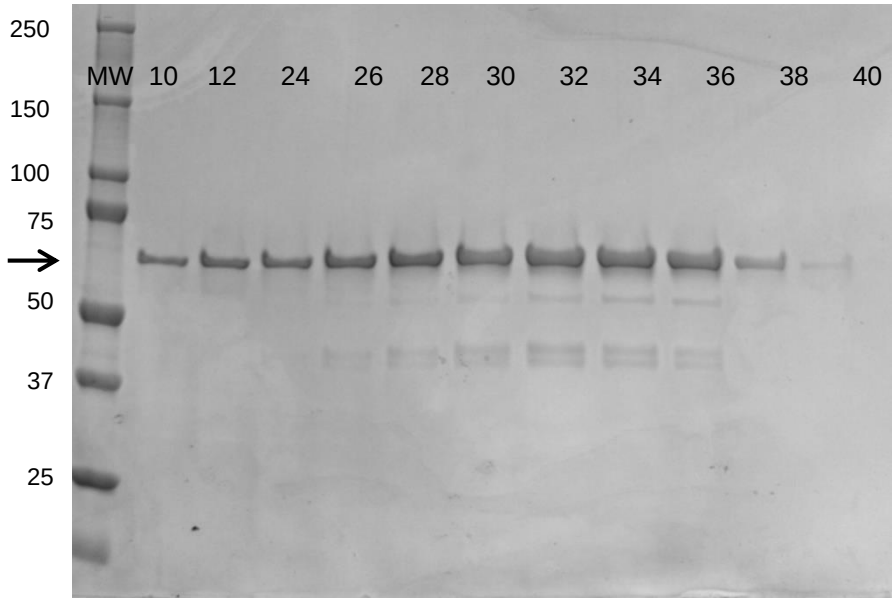




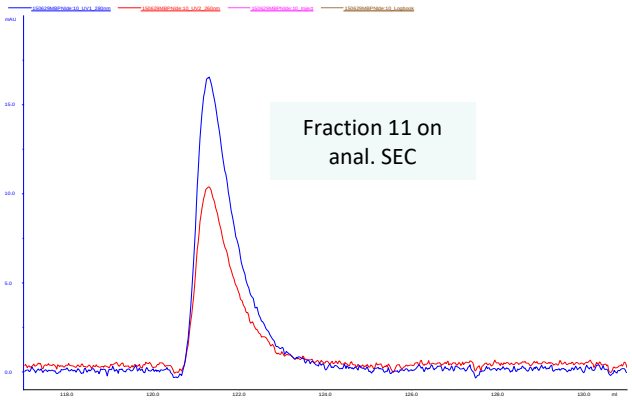


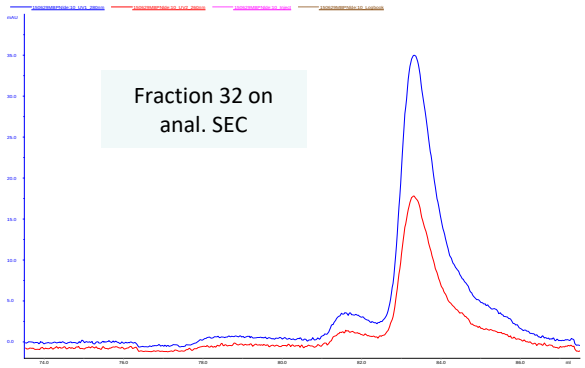
MBP fusion protein
Intermediate purification: Gel Filtration

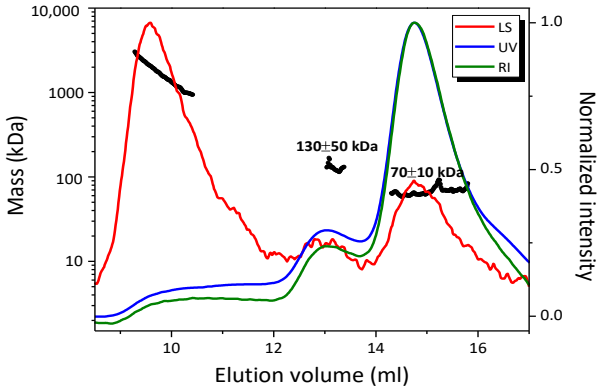




Add 50 m*M* Na glutamate and 50 m*M* L-arginine, **immediately** load on Sephacryl S200 FF column 97 x 2.6 cm ~ 500ml



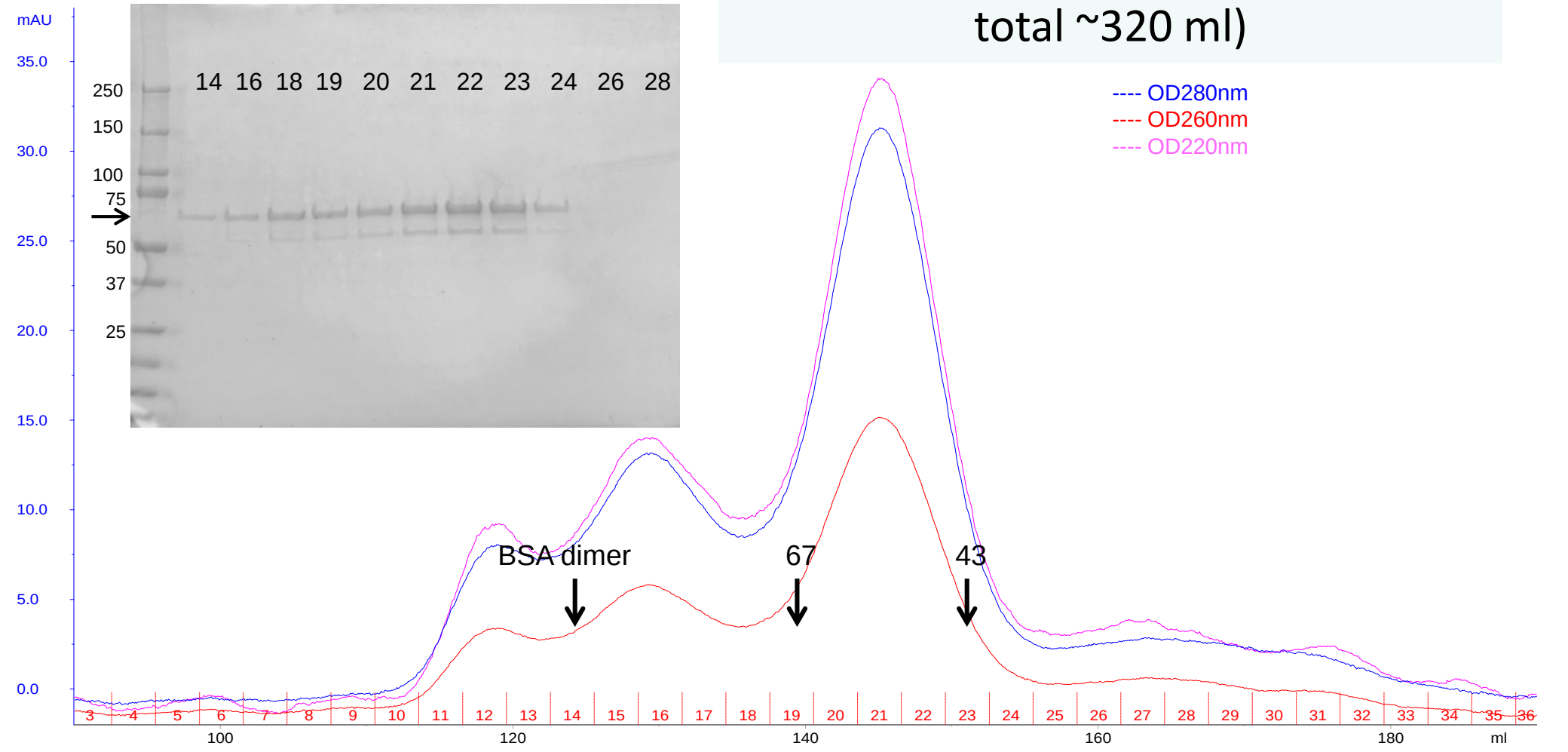




MBP fusion protein

Optimization of Size Exclusion Chromatography

Superdex 75 columns connected in tandem (60 x 1.6 cm and 95 x 1.6 cm, total ~320 ml)



Enhanced Expression of Disulphide-rich Proteins

✓Gene design, fusion technology and TEV cleavage conditions influence the purification of oxidized disulphide-rich venom peptides in Escherichia coli **Renaud Vincentelli** *Sequeira et al. Microb Cell Fact (2017) 16:4*

DsbC was the best fusion tag for venom peptide expression, in particular when the fusion was directed to the bacterial periplasm. While the redox activity of DsbC was not essential to maximize expression of recombinant fusion proteins, redox activity did lead to higher levels of correctly folded target peptides

✓Soluble Expression of Disulfide Bond Containing Proteins FGF15 and FGF19 in the Cytoplasm of Escherichia coli. **Kong B, Guo GL** (2014) *PLoS ONE 9(1): e85890. doi:10.1371/journal.pone.0085890* School of Pharmacy, Rutgers, University of N.Jersey

TRX fusion protein improved FGF19 solubility in strains of thiol-redox system mutants. In addition, DsbC co-expressed in thiol-redox system mutants alone improved and further enhanced FGF19 solubility with combination of TRX fusion tag.

✓Prokaryotic Soluble Overexpression and Purification of Bioactive Human Growth Hormone by Fusion to Thioredoxin, Maltose Binding Protein, and Protein Disulfide Isomerase **Minh Tan Nguyen et al (2014)**

PLoS ONE 9(3): e89038. doi:10.1371/journal.pone.0089038 University of Ulsan College of Medicine, Seoul, Korea

Disulphide-rich Proteins

Production of Recombinant Disulfide-Rich Venom Peptides for Structural and Functional Analysis via Expression in the Periplasm of E. coli

Klint JK et al. (2013) PLoS ONE 8(5): e63865. doi:10.1371/journal.pone.0063865 **The University of Queensland, Australia**

✓*Optimised protocol for the expression of disulfide-rich venom peptides in the periplasm of E. coli.*

Very good for teaching.

✓*Synthetic gene from Geneart cloned into a variant of the pLic-MBP expression vector: 6His-TEV - Peptide*

✓*IPTG concentrations as low as 10 mM are sufficient for high levels of fusion protein expression. Venom peptides generally express better and are more soluble if the temperature is lowered to 16C prior to induction*

✓ *Maximum yield of C^{13} and N^{15} labelled fusion protein: dual media protocol for both isotopes*

✓*Cell disruptor for cell lysis instead of Osmotic Shock*

✓*TEV cleavage: TEV protease in-house, in storage buffer without reducing agent*

✓*RP-HPLC as the most efficient method for the final purification step after cleavage*

Peptide over-expression

✓Rational Design of a Carrier Protein for the Production of Recombinant Toxic Peptides in E. coli

Pane K, et al. (2016) *PLoS ONE 11(1): e0146552. doi:10.1371/journal.pone.0146552* ***Università di Napoli***

Fusion to the C-terminus of Onconase, a small ribonuclease (104 amino acids), which efficiently drove the peptide into IB with very high expression levels (about 200–250 mg/L). Optimize mutation of Onconase to allow acid cleavage <4

✓Enhanced Expression of Cysteine-Rich Antimicrobial Peptide Snakin-1 in Escherichia Coli Using an Aggregation-Prone Protein Coexpression System

Ruhul Kuddus et al. (2016) *DOI 10.1002/btpr.2508* Hokkaido Univers

Cysteine-rich plant antimicrobial peptide, coexpression method using an aggregation-prone partner protein and IB refolding

High throughput screening identifies disulfide isomerase DsbC as a very efficient partner for recombinant expression of small disulfide-rich proteins in E. coli

Microb Cell Fact 2013; 12:37 - **Hervé Nozach** - CEA, iBiTec-S, Service d'Ingénierie Moléculaire des Protéines (SIMOPRO)

One of the big challenges in recombinant protein production in Escherichia coli is the production in sufficient amounts of native and active proteins of the huge family of disulfide-rich proteins (DRPs). As a consequence of the reducing cytoplasm of bacteria, proteins with disulfide bonds are especially prone to aggregation, due to possible mispairing of cysteine residues or undesirable intermolecular disulfide bonds. Partial solutions have been found using commercial strains with an oxidative cytoplasm (SHuffle® T7 Express lysY, Origami B [DE3] pLysS etc.), the relatively low-yield protein exported to the oxidizing periplasm or by using solubilizing fusion partners with redox properties for disulfide bond formation (like thioredoxin, DsbA and DsbC).

In a very convincing publication, three different French groups join efforts and report a high-throughput screening approach for the systematic investigation of the E. coli production of 28 DRPs, using 12 different cytoplasmic partners (looking for their solubilizing and folding influence), and three strains: the popular BL21 (DE3) pLysS, the oxidative strains Origami B (DE3) pLysS, and SHuffle® T7 Express lysY. An impressive work with a total of 1008 conditions checked. Moreover, mass spectrometry (MS) was used to determine the exact masses of the produced DRPs and their redox states.

The authors' conclusions are as follows: a) BL21 (DE3) pLysS, in spite of its reducing cytoplasm, is more efficient than the oxidative strains for producing DRPs in fusion with solubilizing partners. b) Fusion partners with redox activity not only improve the solubility; they can also assist the DRPs to get their native oxidized conformation. c) DsbC as the fusion partner, instead of co-expression as is used by many groups {1}, shows the best results, follow by DsbA and thioredoxin. d) Data are presented suggesting that oxidation of the proteins occurs ex vivo. e) The scale-up of successful high-throughput results shows that DsbC as a fusion partner is able to produce high quantities of a large diversity of native and correctly folded DRPs.

Acylated heptapeptide binds albumin with high affinity and application as tag furnishes long-acting peptides

Zorzi A, Middendorp SJ, Wilbs J, Deyle K, Heinis C. *Nat Commun* 2017 Jul 17; 8:16092 PMID: 28714475 DOI: 10.1038/ncomms16092

Christian Heinis - Institute of Chemical Sciences and Engineering, School of Basic Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne CH-1015, Switzerland

Peptides are extremely attractive for therapeutic development: easy chemical synthesis, low toxicity, and easiness to optimize high affinity and selectivity. But their main obstacle is their rapid clearance from the circulation because of proteolytic degradation and/or fast renal filtration in the kidney. Many strategies were developed to overcome these problems; nevertheless, renal filtration is still the greater challenge.

In this article, this group from the Ecole Polytechnique Fédérale de Lausanne optimize an exciting approach in which peptides bind via a ligand to serum albumin, in this way highly extending the half-life of peptides in the circulation.

They optimize a ligand design of an albumin-recognizing fatty acid conjugated to a short heptapeptide linked to lipidated lysine. **This tag can be easily linked to the therapeutic peptide** with a standard peptide synthesizer.

They applied this strategy to several targets, and show the **half-life increase** in animal studies (25-fold to over seven hours in rats). Although the use of albumin as a carrier is not new, the tag presented here highly improves the possibilities of this strategy for human use.

Lebendiker M: F1000Prime Recommendation of [Zorzi A et al., Nat Commun 2017, 8:16092]. In F1000Prime, 01 Aug 2017; DOI: 10.3410/f.727815153.793534741. F1000Prime.com/727815153#eval793534741

Efficient, ultra-high-affinity chromatography in a one-step purification of complex proteins

Vassylyeva MN et. al *Proc Natl Acad Sci U S A* 2017 Jun 27; 114(26):E5138-E5147

The ultra-high-affinity (CL7/Im7) purification system described in this work allows one-step purification of a wide range of traditionally challenging biological molecules, including eukaryotic, membrane, toxic, and DNA/RNA-binding proteins and complexes. The affinity of the system has very high activity, which is excellent for low-abundance proteins purifications in lysates, as they show in one of the examples.

Moreover, the system produces **highly pure proteins in one single-step purification**, in contrast to the commonly used His tag purification system, where protein elutes are partially purified since many non-specific proteins recognize the affinity resin with low affinity.

Other advantages of the system are as follows:
1.) Loading is not affected by high salt conductivity, reducing agents, chelants such as EDTA, or detergents.
2.) The CL7 tag could be placed at the N or C terminal end of the protein target with a protease site between them.
3.) Elution can occur through specific proteases such as TEVprotease, SUMO, and others.
4.) The **Im7 inhibitor is easy to purify in high quantities** and is covalently bound to iodoacetyl agarose beads (Im7 was engineered to contain an 'immobilization' unit ~26kDa for fast, **stable, and efficient coupling**). The column can be re-used several times.

Since **proteins elute at high purity**, the authors suggest that no further purification steps would be needed. From my point of view, I see size exclusion or other chromatographic methods as being imperative to discard unwanted oligomeric conformations, other post-translational modifications or other kind of molecules that cannot been detected by SDS-PAGE.

MW of CL7 is ~16 kDa (the tag MW may be bigger depending on the size of flexible linkers we normally use for a target fusion). MW of Im7 is ~10 kDa, again for immobilization it contains additional domain to yield the overall MW of the coupling unit of ~26 kD

Vassylyev, Dmitry dmitry@uab.edu
University of Alabama at Birmingham

The Argi system: one-step purification of proteins tagged with arginine-rich cell-penetrating peptides

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The *Argi* system is a new affinity chromatography tool for **one-step purification of arginine-rich cell penetrating peptide (CPP)-tagged proteins**, a very powerful system for the **delivery of proteins into human cells**. This arginine-rich CPP-mediated transport of proteins can be useful in therapeutic applications.

The presented system is based on the **interaction between a DNA aptamer and arginine-rich CPPs**. The aptamer affinity resin can specifically recognize the CPP protein with very high affinity through the Arg rich region, and elution is performed through low and non-denaturative concentration of guanidine HCl. The group claim that the resin can be reused several times.

From my point of view, the **guanidine HCl elution is the Achilles heel** of this new method, since it can affect the conformation/activity of some targets. More mild methods must be checked, in order to avoid this "possible problem". Nevertheless, it looks like an exciting and promising approach.

Efficient protein production inspired by howspiders make silk

Kronqvist Nina et. al *Nat Commun* 2017 May 23; 8:15504
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From my point of view, this study reports on one of the most interesting new findings from the last few years in the field of protein production of prone-aggregation proteins. Protein producing labs are using many solubilizing protein tags like MBP, thioredoxin, SUMO, NusA and others in order to increase over-expression and solubilization of difficult-to-express proteins. The field is full of many interesting ideas and new tags are published all the time. But without a doubt, this time looks to be an amazing finding, inspired by a natural system: **how spiders produce huge concentrations of silk protein by sequestering their aggregation-prone regions in micellar structures, with very soluble N-terminal and hydrophilic domains (NT) in the shell of the micelles**.

The authors mutate and optimize this NT region as an N-terminal fusion soluble tag and they checked their hypothesis with many difficult-to-express proteins. They show the applicability of this new **protein tag to produce aggregation-prone peptides and proteins with relevant pharmaceutical application in heterologous hosts**. They claim that higher yields are obtained, and proteins can be later cleaved in order to get purified protein.

Nevertheless, in order to confirm the applicability of the system in a wider way, benchmarking by other protein producing labs would be needed.

Novel Hydrophobin Fusion Tags for Plant-Produced Fusion Proteins

Reuter Lauri et al. - *PLoS ONE* 2016; 11(10):e0164032 - VTT Technical Research Centre of Finland Ltd., Espoo, Finland

This group from Finland have been working for many years on hydrophobins (HFB), a very interesting technology that has several applications in biotechnology like functional coatings in nanomedicine, immobilization of bioactive proteins on biosensors, etc. HFB fusion technology has been further applied to the purification of recombinant proteins using aqueous two-phase separation (ATPS) {1,2} in plant cell production platforms, and insect and fungal cells. The use of this technology has been reviewed in a previous article {3}.

HFB are small, secretory proteins found in filamentous fungi. When *Trichoderma reesei* hydrophobin (HFBI) is expressed as a fusion protein in plants or plant cell cultures, it induces formation of spherical structures, protein bodies (PB), derived from the endoplasmic reticulum (ER) in an unclear mechanism.

HFB have a hydrophobic patch that is exposed on the surface of the protein and allows assembly in aqueous solutions, interaction with non-ionic surfactants and self-assembly into monolayers at liquid-air interfaces and on hydrophobic surfaces. This property is used in ATPS technology {4} for **extracting the HFBI fusion proteins with Triton X-114 (3% w/v) in a heavy phase**. The detergent is further removed by extraction with isobutanol, while the fusion protein remains in the buffer. In the present article, the group screened several new hydrophobin tags derived from *Trichoderma reesei* and *Fusarium verticillioides*, and present two alternatives for HFBI as functional fusion tags using N-terminal and C-terminal GFP as a fusion partner.

In conclusion, this technology is an interesting breakthrough for plant production and first step purification of recombinant proteins. **Its main limitation are the isobutanol extraction**, which can be used only for isobutanol stable proteins, and the optimization of the detergent extraction.

References1.

1. **Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems.**
2. **The hydrophobins HFBI and HFBII from Trichoderma reesei showing efficient interactions with nonionic surfactants in aqueous two-phase systems.**
3. **Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants.**
4. **Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells.**

N(pro) fusion technology: On-column complementation to improve efficiency in biopharmaceutical production

Protein Expr Purif 2016 Apr; 120:42-50 - **A. Dürauer** - Department of Biotechnology, University of Natural Resources and Life Sciences Vienna

In a previous article {1}, this Austrian group describes an excellent technology used to **overexpress peptides** and proteins in *Escherichia coli* with a **self-cleaving expression tag** that renders **N-terminus without any additional amino acid.**

The technology uses an engineered autoprotease, EDDIE, derived from the N-terminal autoprotease N^{pro} of classical swine fever virus (CSFV) that cleaves itself from the nascent polypeptide chain of CSFV and generates the N terminus of the subsequent viral capsid C-protein. The advantage of EDDIE in fusion with the target, is that the autocleavage remains inactive in the cytoplasm since the construct is only expressed as inclusion bodies (IB). In contrast to other intein systems, cleavage in the N^{pro} system does not have to be induced by thiols, pH shift or temperature shift, but instead is induced during refolding of the autoprotease in kosmotropic conditions. IB expression facilitates the use of very strong expression systems and conventional processing strategies for the efficient production of toxic or challenging proteins or small peptides that are often subject to proteolytic degradation. Moreover, the N terminus of the target is protected from proteolytic processing by proline aminopeptidases.

In this new article, the group improved the original idea by reducing the size of the tag in order to considerably improve the expression yield. They use a complementation strategy where the N-terminal fragment was designed to harbor the protease domain containing all catalytically important residues, while the C-terminal fragment (1/3 of the size) was fused to the target molecule. They demonstrate the complementation activity (target release from the tag) by refolding of both fragments together, or by On-column complementation.

The technology still needs further optimization, but could, in the future, be a smart choice for industrial scale production of short proteins or peptides in *E. coli* for academic or biopharmaceutical production of short proteins or peptides that are able to be refolded.

References: **N(pro) fusion technology to produce proteins with authentic N termini in E. coli.**

Acylated heptapeptide binds albumin with high affinity and application as tag furnishes long-acting peptides

Zorzi A, Middendorp SJ, Wilbs J, Deyle K, Heinis C. *Nat Commun* 2017 Jul 17; 8:16092 PMID: 28714475 DOI: 10.1038/ncomms16092

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Peptides are extremely attractive for therapeutic development: easy chemical synthesis, low toxicity, and easiness to optimize high affinity and selectivity. But their main obstacle is their rapid clearance from the circulation because of proteolytic degradation and/or fast renal filtration in the kidney. Many strategies were developed to overcome these problems; nevertheless, renal filtration is still the greater challenge.

In this article, this group from the Ecole Polytechnique Fédérale de Lausanne optimize an exciting approach in which peptides bind via a ligand to serum albumin, in this way highly extending the half-life of peptides in the circulation.

They optimize a ligand design of an albumin-recognizing fatty acid conjugated to a short heptapeptide linked to lipidated lysine. **This tag can be easily linked to the therapeutic peptide** with a standard peptide synthesizer.

They applied this strategy to several targets, and show the **half-life increase** in animal studies (25-fold to over seven hours in rats). Although the use of albumin as a carrier is not new, the tag presented here highly improves the possibilities of this strategy for human use.

Lebendiker M: F1000Prime Recommendation of [Zorzi A et al., Nat Commun 2017, 8:16092]. In F1000Prime, 01 Aug 2017; DOI: 10.3410/f.727815153.793534741. F1000Prime.com/727815153#eval793534741

Efficient, ultra-high-affinity chromatography in a one-step purification of complex proteins

Vassylyeva MN et. al *Proc Natl Acad Sci U S A* 2017 Jun 27; 114(26):E5138-E5147

The ultra-high-affinity (CL7/Im7) purification system described in this work allows one-step purification of a wide range of traditionally challenging biological molecules, including eukaryotic, membrane, toxic, and DNA/RNA-binding proteins and complexes. The affinity of the system has very high activity, which is excellent for low-abundance proteins purifications in lysates, as they show in one of the examples.

Moreover, the system produces **highly pure proteins in one single-step purification**, in contrast to the commonly used His tag purification system, where protein elutes are partially purified since many non-specific proteins recognize the affinity resin with low affinity.

Other advantages of the system are as follows:
1.) Loading is not affected by high salt conductivity, reducing agents, chelants such as EDTA, or detergents.
2.) The CL7 tag could be placed at the N or C terminal end of the protein target with a protease site between them.
3.) Elution can occur through specific proteases such as TEVprotease, SUMO, and others.
4.) The **Im7 inhibitor is easy to purify in high quantities** and is covalently bound to iodoacetyl agarose beads (Im7 was engineered to contain an 'immobilization' unit ~26kDa for fast, **stable, and efficient coupling**). The column can be re-used several times.

Since **proteins elute at high purity**, the authors suggest that no further purification steps would be needed. From my point of view, I see size exclusion or other chromatographic methods as being imperative to discard unwanted oligomeric conformations, other post-translational modifications or other kind of molecules that cannot been detected by SDS-PAGE.

MW of CL7 is ~16 kDa (the tag MW may be bigger depending on the size of flexible linkers we normally use for a target fusion). MW of Im7 is ~10 kDa, again for immobilization it contains additional domain to yield the overall MW of the coupling unit of ~26 kD

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