# Maximize your purity and yield of Histidine-tagged proteins and the application on FPLC system

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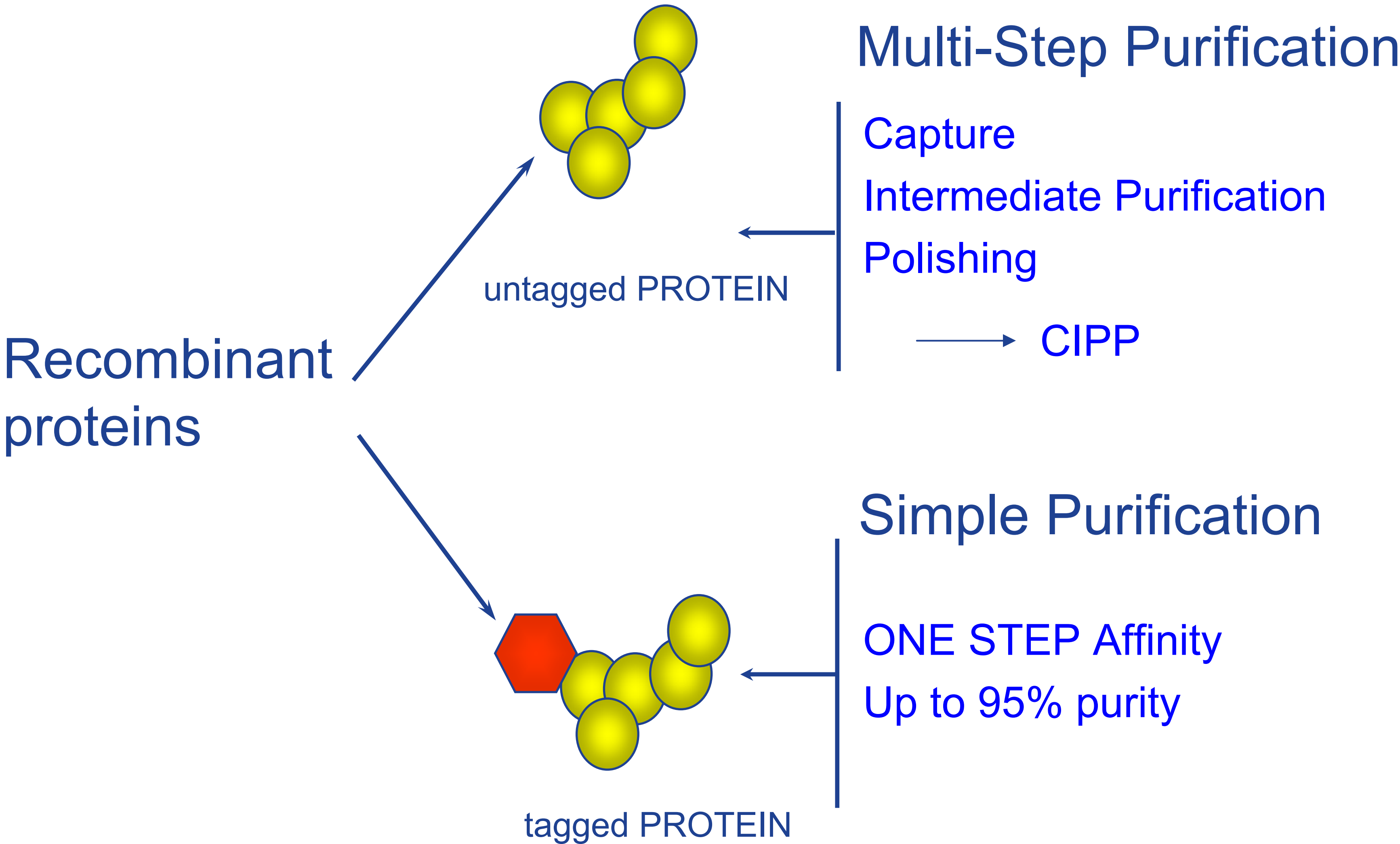
# Protein purification strategy

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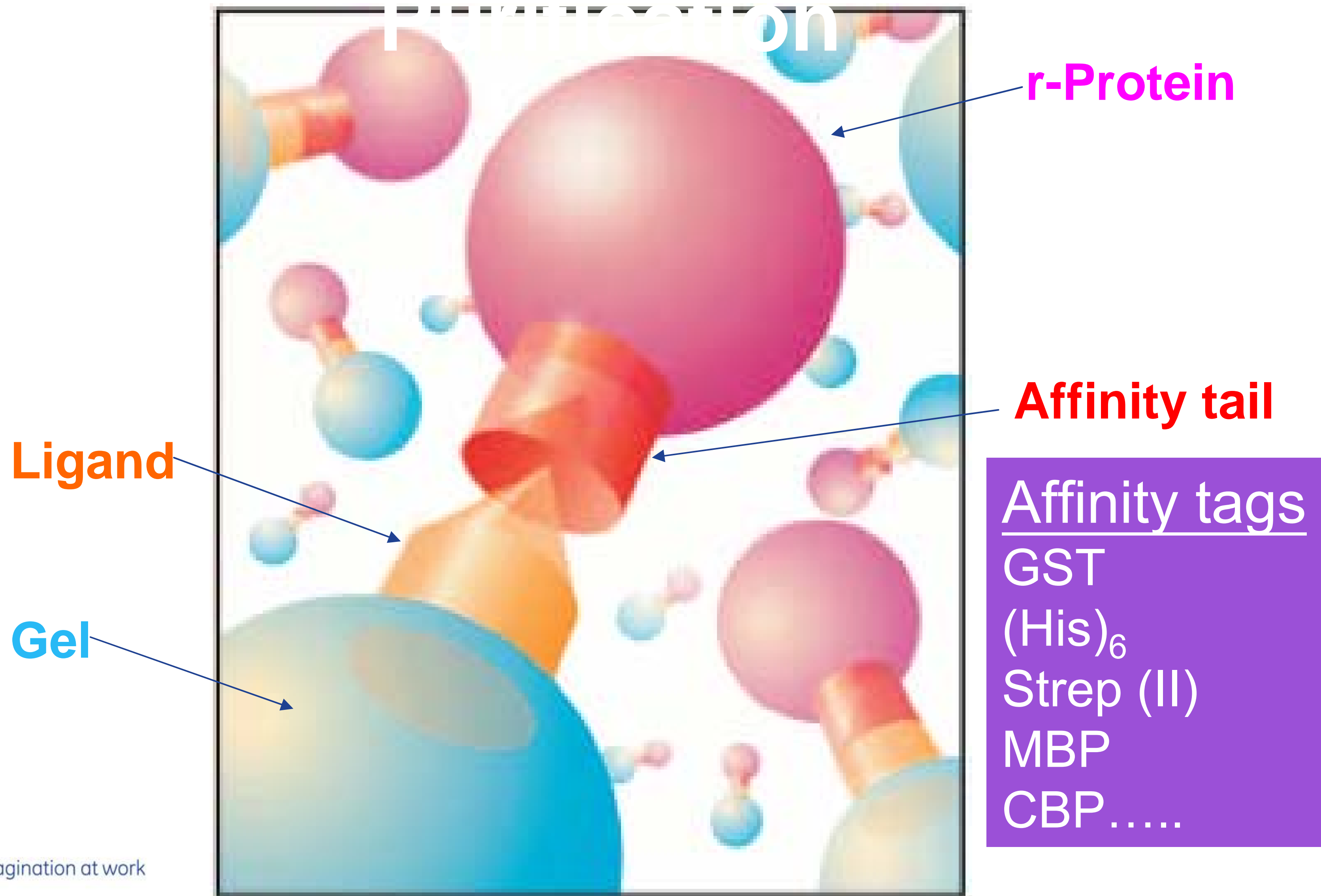
# Recombinant Protein

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# Histidine-tagged protein purification

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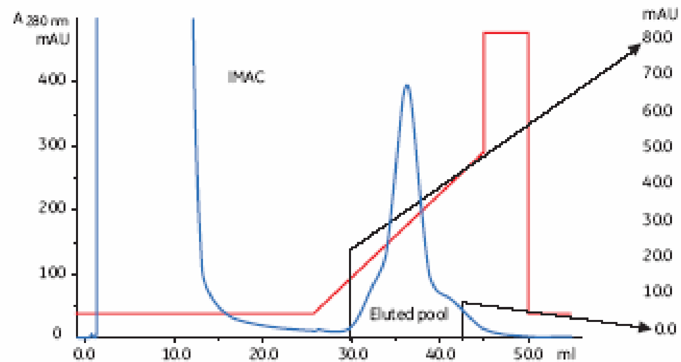
# Two-step purification of a histidine-tagged protein

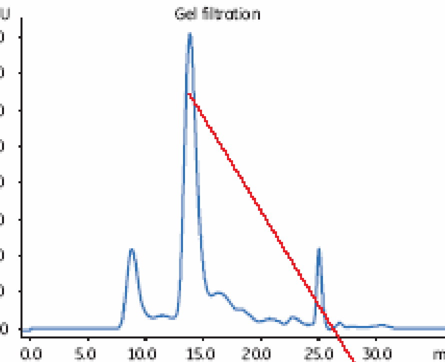
**Fig. 1A: First purification step with IMAC**

Sample: 10 ml *E. coli* extract with low-level expression of a histidine-tagged mannanase, Man 26A, from *Cellulomonas fimi* ($M_r$ ~ 100 000)
Column: HisTrap™ HP 1 ml
Binding buffer: 20 mM sodium phosphate, 30 mM imidazole, 500 mM NaCl, pH 7.4
Elution buffer: 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4
Gradient: 25 ml linear gradient 30–300 mM imidazole
Flow rate: 1 ml/min
System: ÄKTAexplorer™ 100

**Fig. 1B: Second step with gel filtration**

Sample: 0.5 ml concentrated sample from IMAC step
Column: Superdex™ 200 10/300 GL
Buffer: PBS, pH 7.5
Flow rate: 0.5 ml/min
System: ÄKTAexplorer 100




## Conclusions

- The high molecular weight protein histidine-tagged mannanase Man 26A was purified in its enzymatically active form
- Excellent binding properties of Ni Sepharose™ High Performance (HP)
- 60 mg of purified protein in a single run
- A second purification step using gel filtration with Superdex 200 was added for high purity needs of 95 %

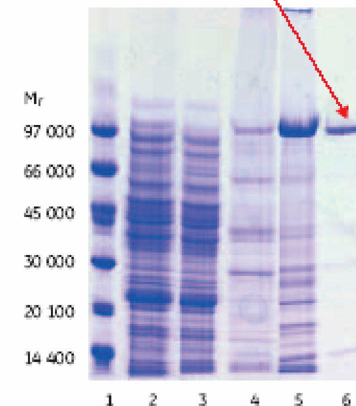



**Fig. 1C: SDS-PAGE**

Lane 1: LMW
Lane 2: *E. coli* extract
Lane 3: IMAC flow-through
Lane 4: Early IMAC fraction
Lane 5: IMAC eluted pool
Lane 6: Gel filtration pool

# Ni Sepharose High Performance (HP)

Ni Sepharose HP delivers narrow peaks and high target protein concentration. It gives:

- High performance purification
- High target protein concentration
- Can be used with a syringe, pump, or system

Ni Sepharose HP is available as bulk media and in expertly prepacked HisTrap HP columns.

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# Scaling up a histidine-tagged protein purification

**Fig. 2A: Scale-up purificaiton of a histidine-tagged protein**

| | |
|---|---|
| Sample: | Histidine-tagged Maltose binding protein in *E. coli* extract (samples loaded contained 8, 40 and 160 mg, respectively) |
| Columns: | HisTrap FF 1 ml, HisTrap FF 5 ml, HisPrep™ FF 16/10 20 ml. All columns are prepacked with Ni Sepharose 6 Fast Flow. |
| Binding buffer: | 20 mM sodium phosphate, 25 mM imidazole, 500 mM NaCl, pH 7.4 |
| Elution buffer: | 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4 |
| Flow rates: | HisTrap FF 1 ml: 1 ml/min; HisTrap FF 5 ml: 5 ml/min; HisPrep FF 16/10: 5 ml/min |

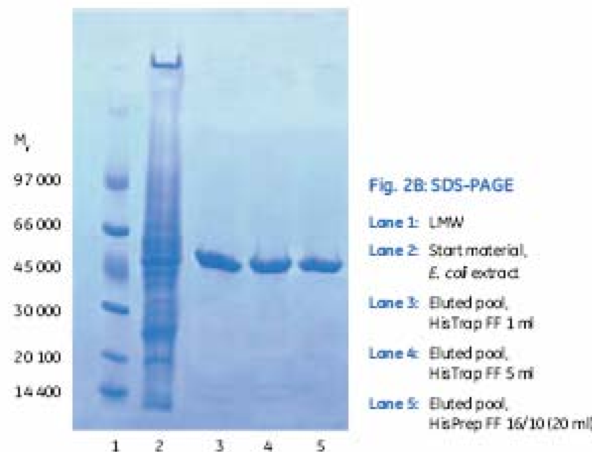
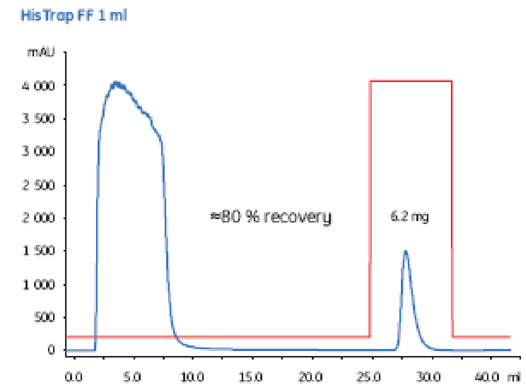
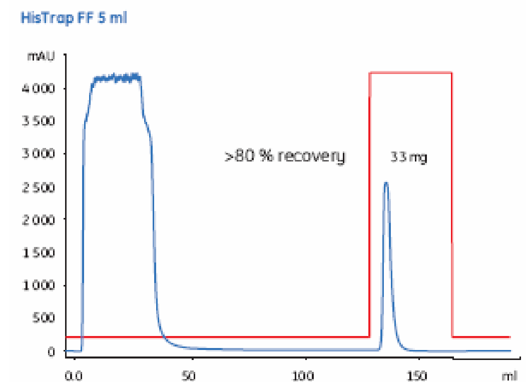
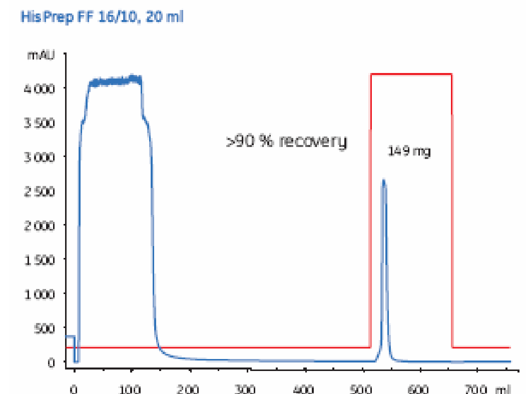



**Fig. 2B: SDS-PAGE**

Lane 1: LMW
Lane 2: Start material, *E. coli* extract
Lane 3: Eluted pool, HisTrap FF 1 ml
Lane 4: Eluted pool, HisTrap FF 5 ml
Lane 5: Eluted pool, HisPrep FF 16/10 (20 ml)




## Conclusions

- Scale-up from HisTrap FF 1 ml via HisTrap FF 5 ml to HisPrep FF 16/10 (20 ml) is easy and efficient
- Scaling up column dimension while running at the same linear flow rate provides highly consistent results
- Pooled fractions analyzed by SDS-PAGE showed almost identical results in terms of purity and recovery
- Consistently high recovery and purity can be obtained in the different scales using the same linear flow rates

# Ni Sepharose 6 Fast Flow (FF)

Ni Sepharose 6 FF delivers fast flow rate purification and easy scale-up.

- Expression screening in multi-well plates
- Available expertly prepacked in convenient HisTrap FF and HisPrep 16/10 FF prepacked columns as well as in bulk
- Manual purification, such as gravity flow and batch purification, and fast flow rate purification on systems

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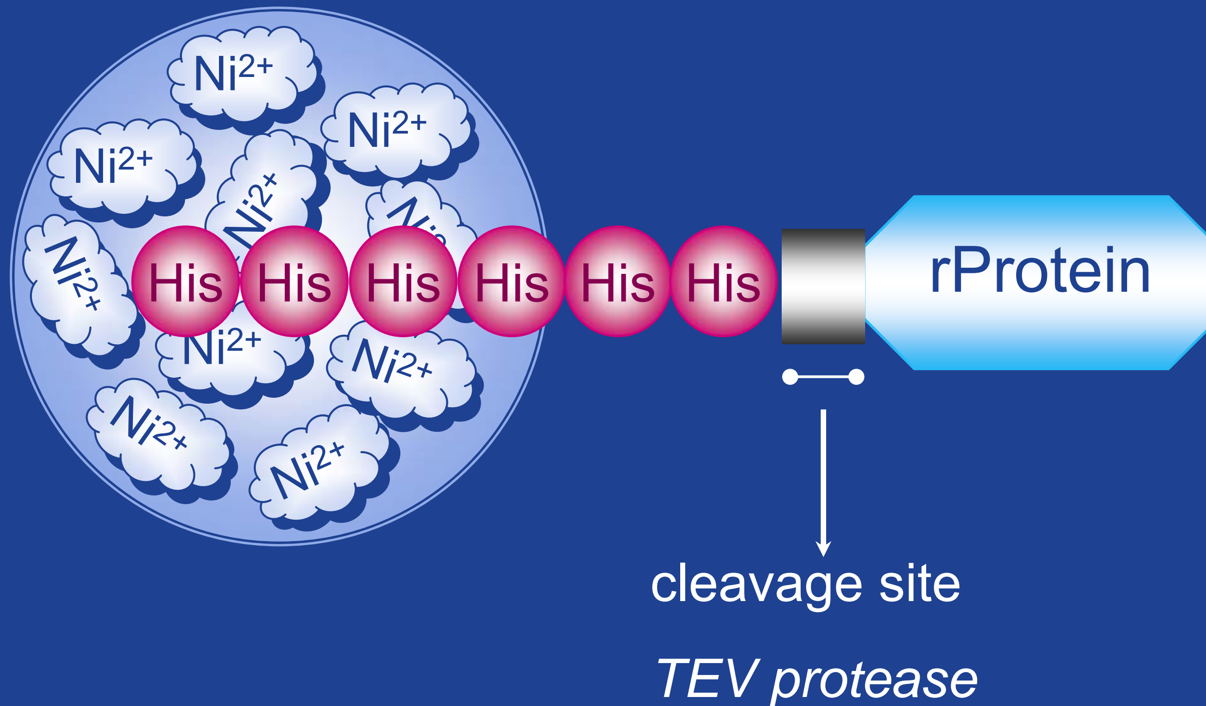
# Purification of histidine-tagged proteins

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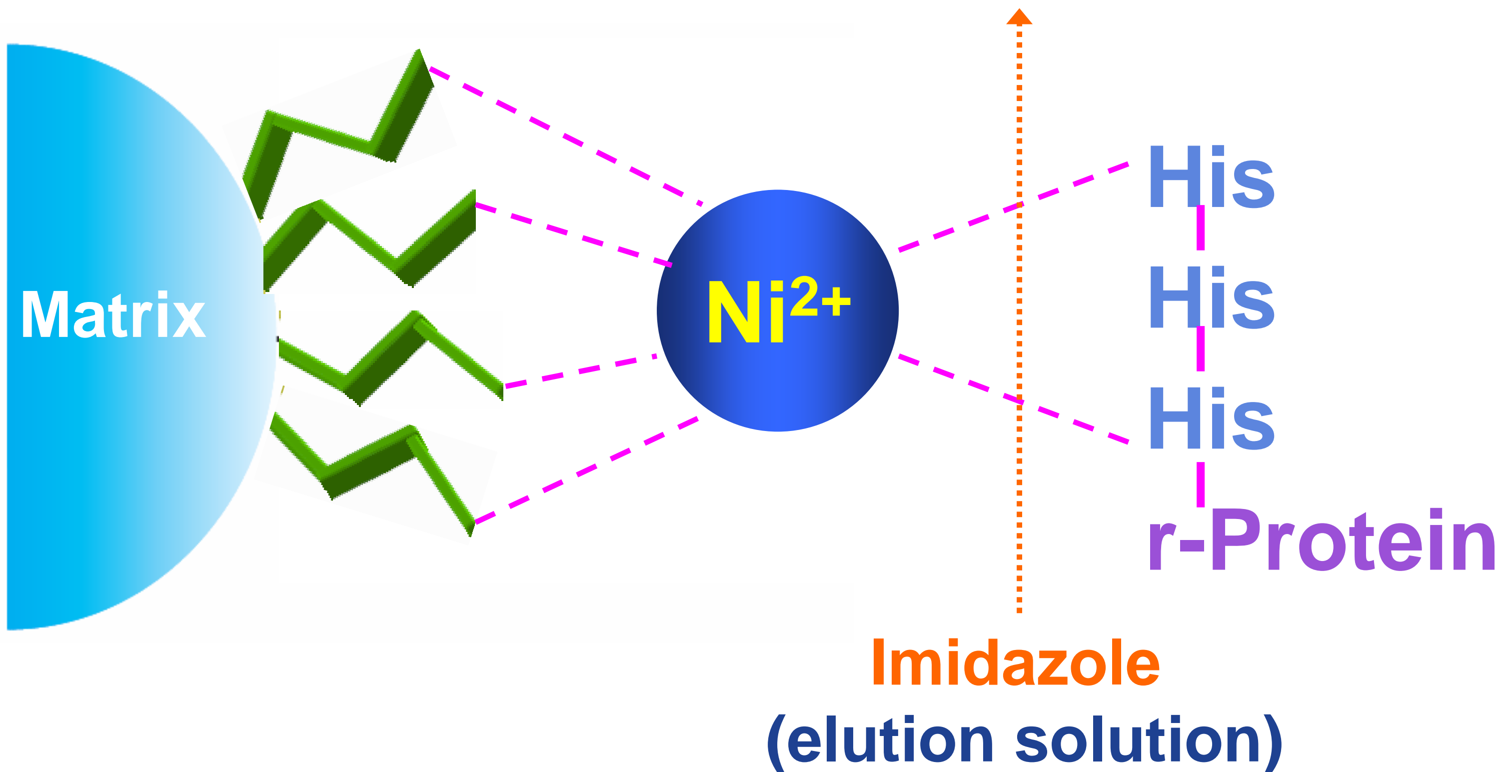
# Histidine-tagged Protein

Transition metals : $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$,$Co^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Cd^{2+}$ and H

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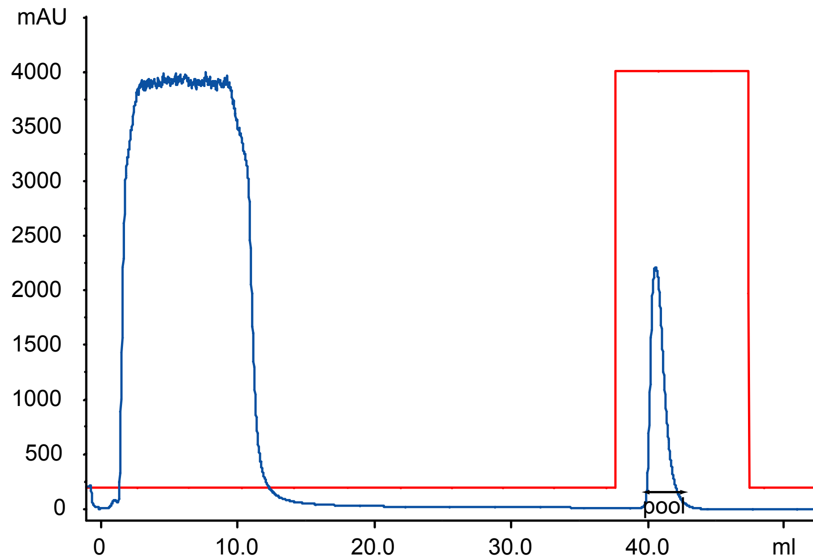
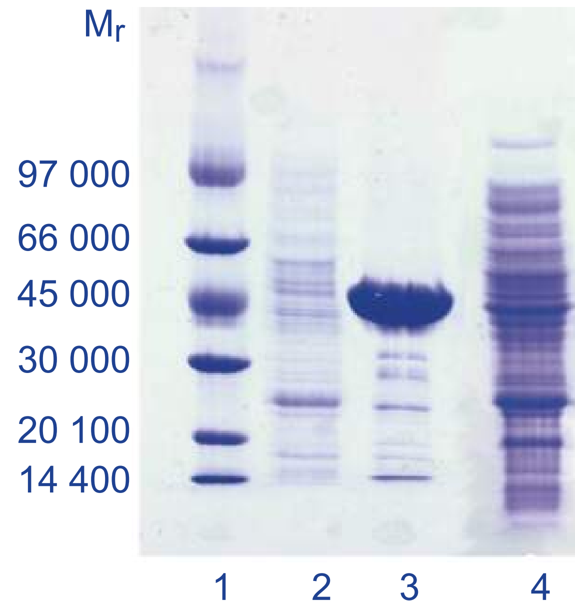
# Purification of MBP-(His)$_6$

## HisTrap™ FF 1 ml




## Non-reduced SDS-PAGE




**Lane 1:** Low Molecular Weight Marker (LMW)
**Lane 2:** Flow through
**Lane 3:** Eluted pool
**Lane 4:** Start material

Precast Phast gels

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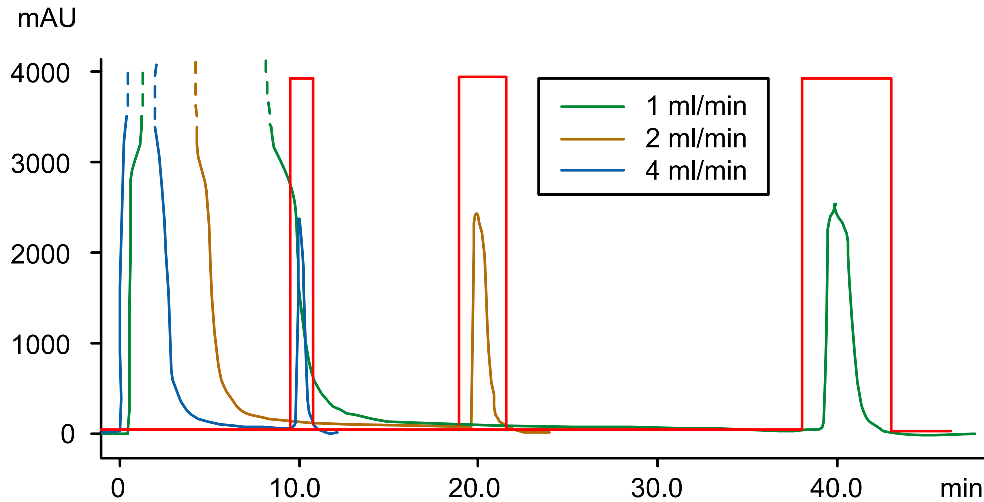
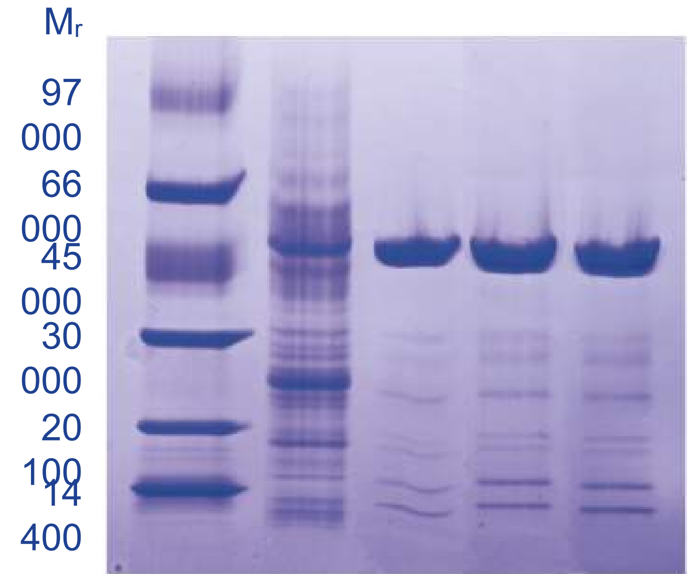
# Effect of flow rate




| Flow (ml/min) | Time (min) | Eluted protein (mg) |
|---|---|---|
| 1 | 48 | 5.4 |
| 2 | 24 | 5.3 |
| 4 | 12 | 5.2 |




**Lane 1:** Low Molecular Weight markers (LMW Marker)
**Lane 2:** Start material
**Lane 3:** Eluted pool, 1 ml/min
**Lane 4:** Eluted pool, 2 ml/min
**Lane 5:** Eluted pool, 4 ml/min

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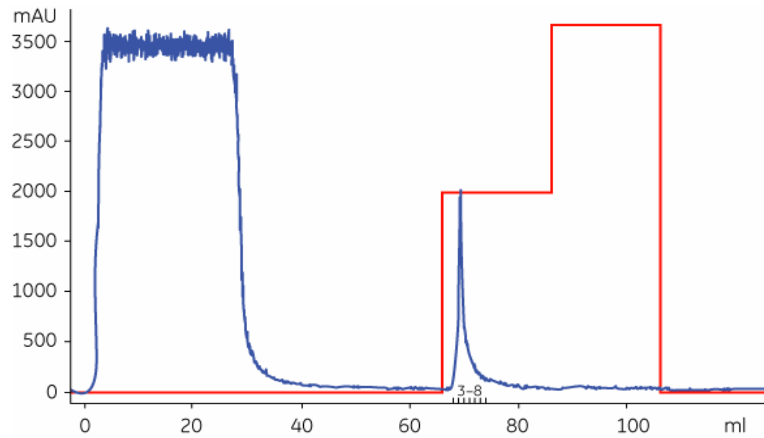
# Optimize your histidine-tagged protein purity

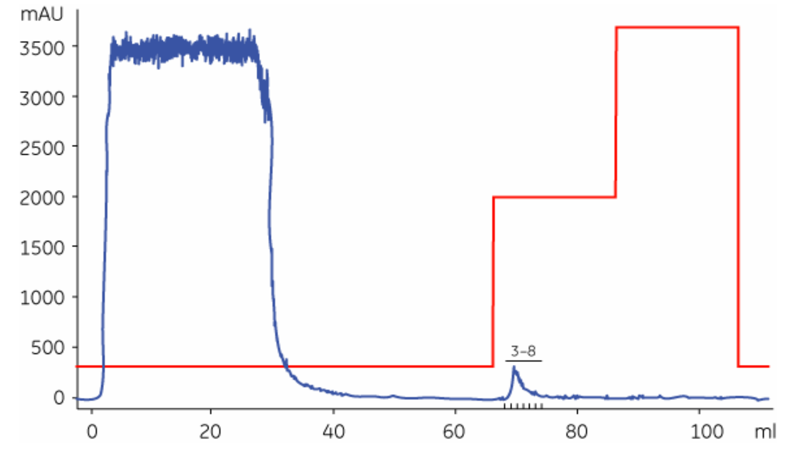
- Imidazole concentration

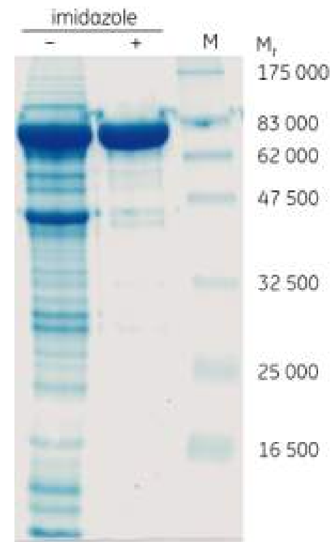
# Purity of target protein with/without imidazole

No imidazole




45 mM imidazole




Work done by:
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B. Felden de Neumann
GBC Biotech AG, Martinried, Germany

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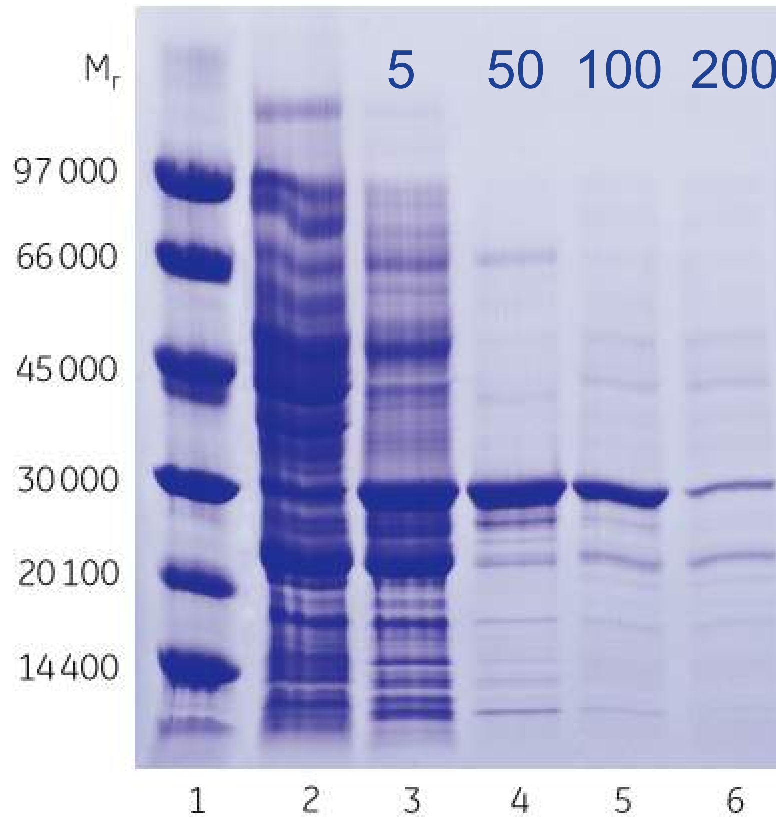
His SpinTrap™
Simple protein
mini-preps

# His SpinTrap™ – optimizing purification conditions




mM imidazole in sample and binding buffer

Lanes:

1. LMW markers
2. Start material (diluted 1:10)

3-6: Eluted pools

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Use
20–40 mM Imidazole
in sample
and binding buffer
FOR HIGHEST PURITY

# Optimize your histidine-tagged protein purity

- Imidazole concentration
- Different metal ions

# Selecting metal ion

- $Ni^{2+}$ most commonly used for purifying histidine-tagged proteins
- $Co^{2+}$ also used for histidine-tagged proteins
  - when a weaker binding of the target proteins is preferred
- $Cu^{2+}$ & $Zn^{2+}$ mainly for purification of untagged proteins
  - $Cu^{2+}$ gives strong binding to a range of proteins - some proteins will only bind to $Cu^{2+}$
  - $Zn^{2+}$ generally gives a weaker binding (could give more selective elution)
  - Both $Cu^{2+}$ & $Zn^{2+}$ can also be used for histidine-tagged proteins

# Purification of histidine-tagged proteins with YOUR choice of metal ion

HiTrap™ IMAC HP & HiTrap IMAC FF 1 ml (uncharged)

# IMAC

## Immobilized Metal ion Affinity Chromatography

**Transition metals : $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Cd^{2+}$ and H**

Matrix

$X^{2+}$

IMAC HP

$Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$

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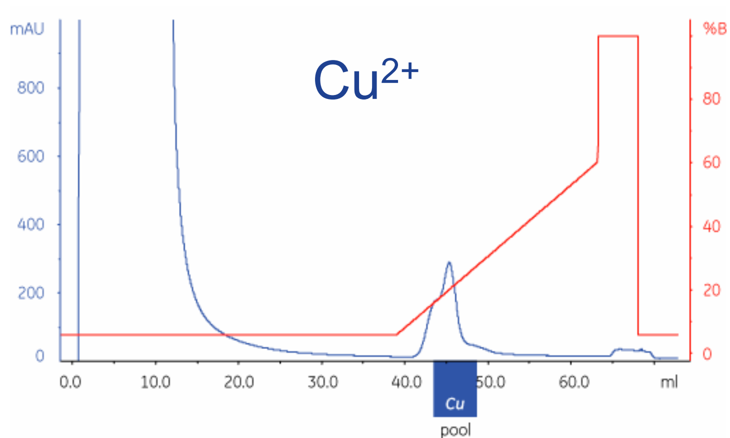
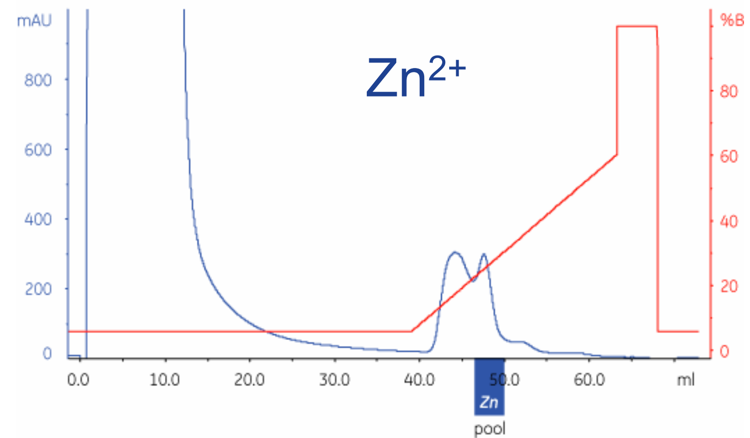
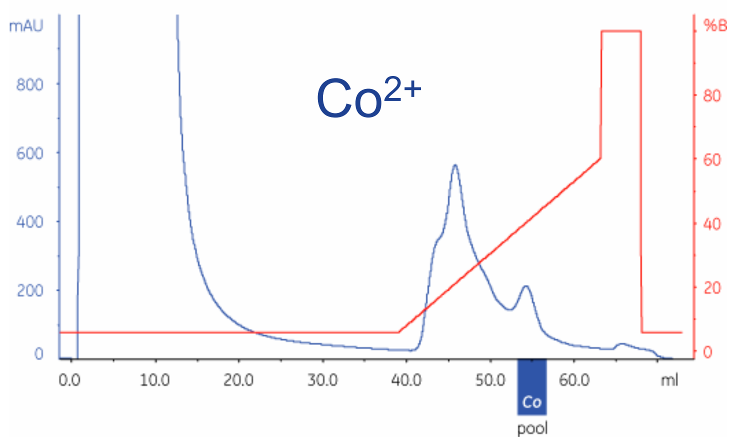
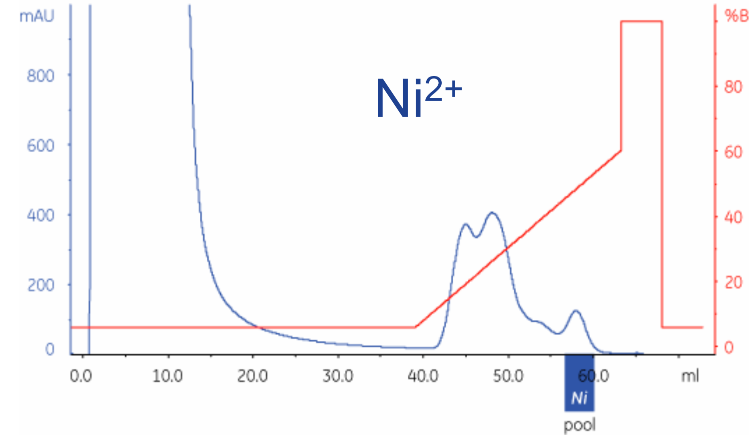
# Purification of a histidine-tagged protein using different metal ions

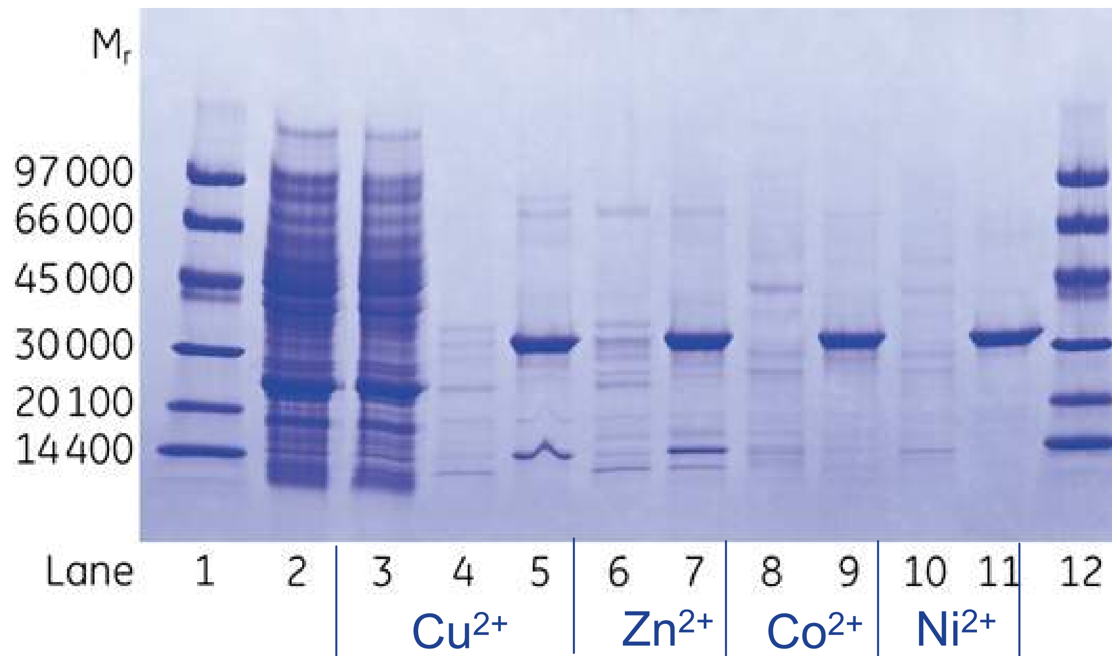


# SDS-PAGE analysis




1. LMW markers
2. Start material, diluted 1:10
3. Flowthrough, diluted 1:10, Cu
4. Wash, Cu
5. Eluted pool, Cu
6. Wash, Zn
7. Eluted pool, Zn
8. Wash, Co
9. Eluted pool, Co
10. Wash, Ni
11. Eluted pool, Ni
12. LMW markers

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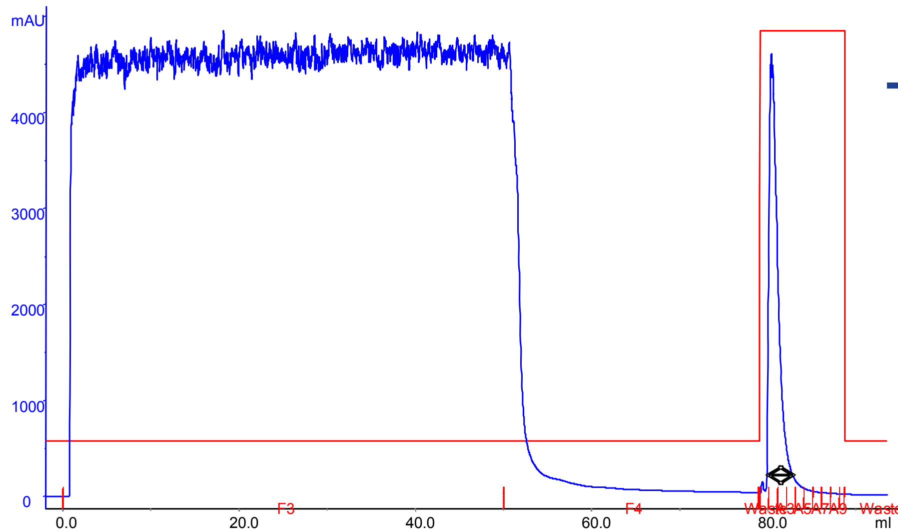
# Optimize your histidine-tagged protein purity

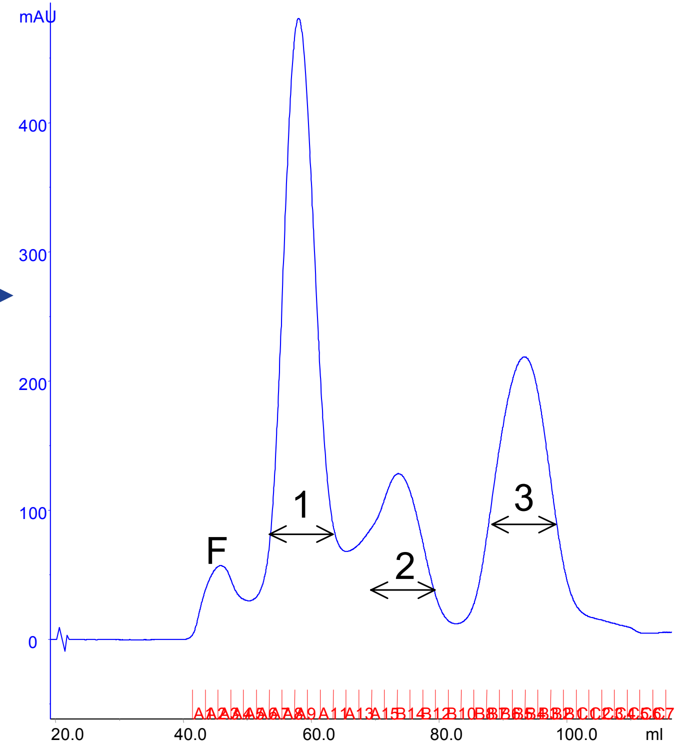
- Imidazole concentration
- Different metal ions
- Add more purification steps

# 2-step purification of a (histidine)$_{10}$-tagged protein

## 1. HisTrap™ FF




## 2. HiLoad™ 16/60 Superdex™ 200 pg

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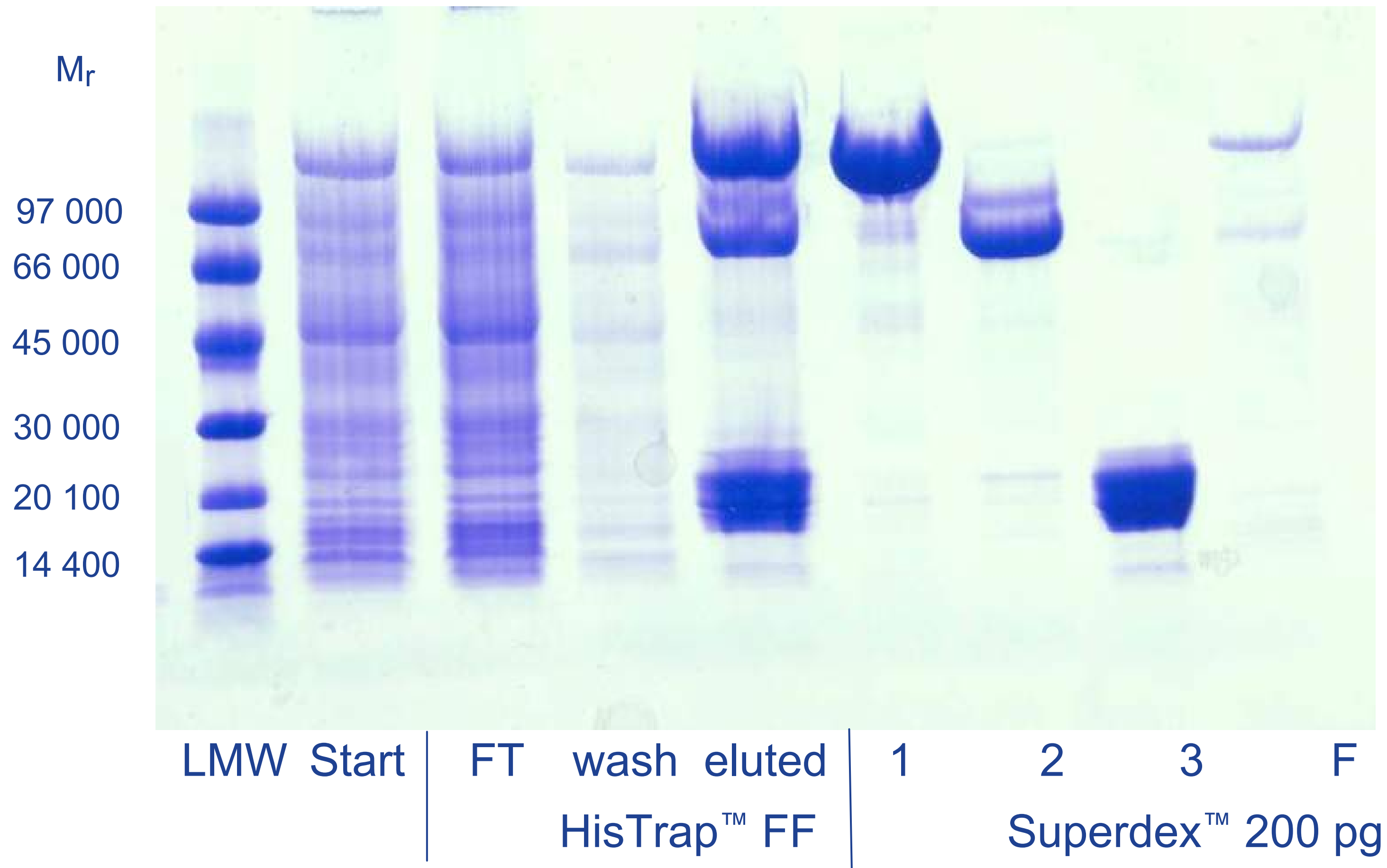
# SDS-PAGE analysis (reduced conditions)

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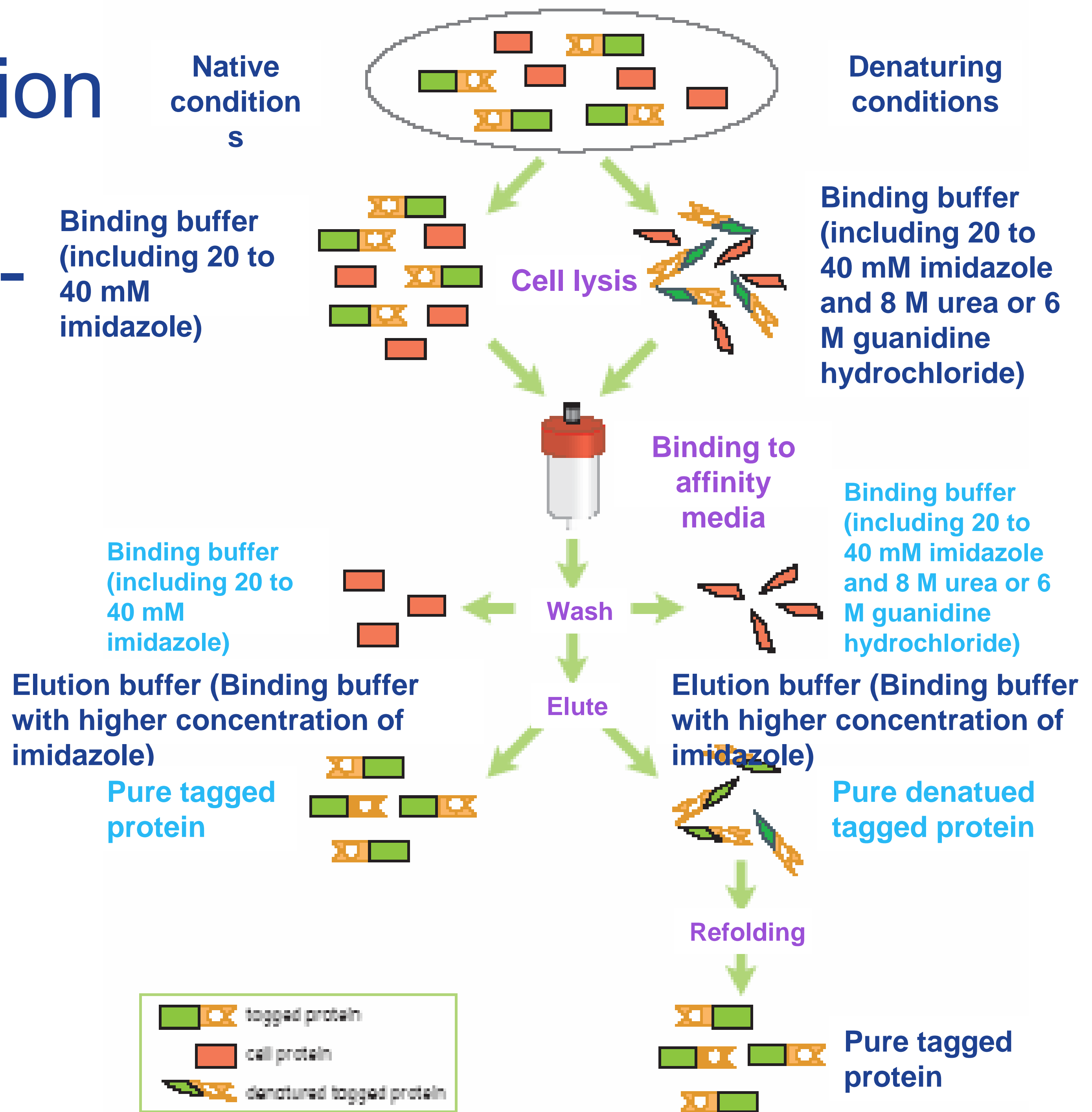
Prepacked columns-
Automated preparative purification
GRADIENT VALVE
HisTrap™ FF
crude 1 ml

# HisTrap™ FF crude
## 1-ml and 5-ml columns

- Prepacked with Ni Sepharose™ 6 Fast Flow
- Preparative purification of histidine-tagged proteins
- Load unclarified lysates
- High protein binding capacity > 40 mg/ml

# Purification of histidine-tagged protein

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# Ni Sepharose

Use
20–40 mM Imidazole
in sample
and binding buffer
FOR HIGHEST PURITY

*The best choice !!*

-**Greatest binding capacity**
**~40 mg/ml**
-**Tolerance of a wide range of additives**
**8 M urea or 6M GuaHCl**
-**Negligible nickel ion leakage**
-**Saving time & cost**

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# Ni Sepharose FF/ HP

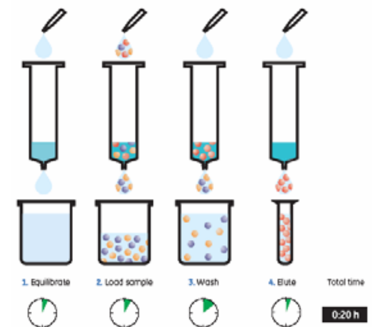
## Bulk media with empty column

PD-10 column

## Prepacked column

HisTrap

HisPrep FF




His GraviTrap

## Small scale/screening

His SpinTrap

His MultiTrap

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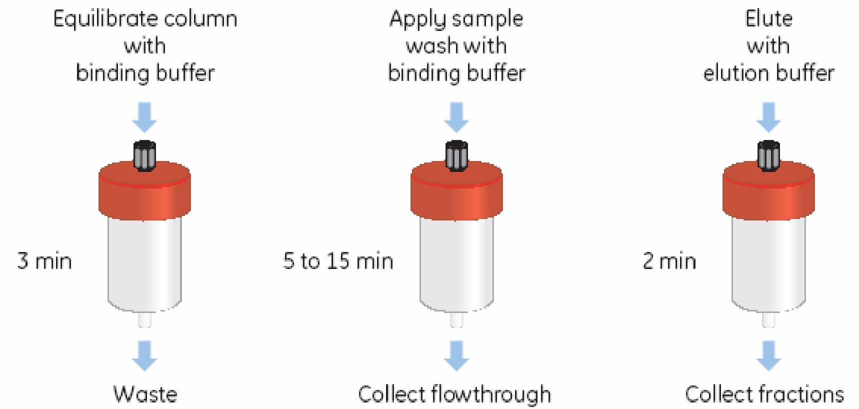
# HiTrap column

## Syringe, peristaltic pump, FPLC system



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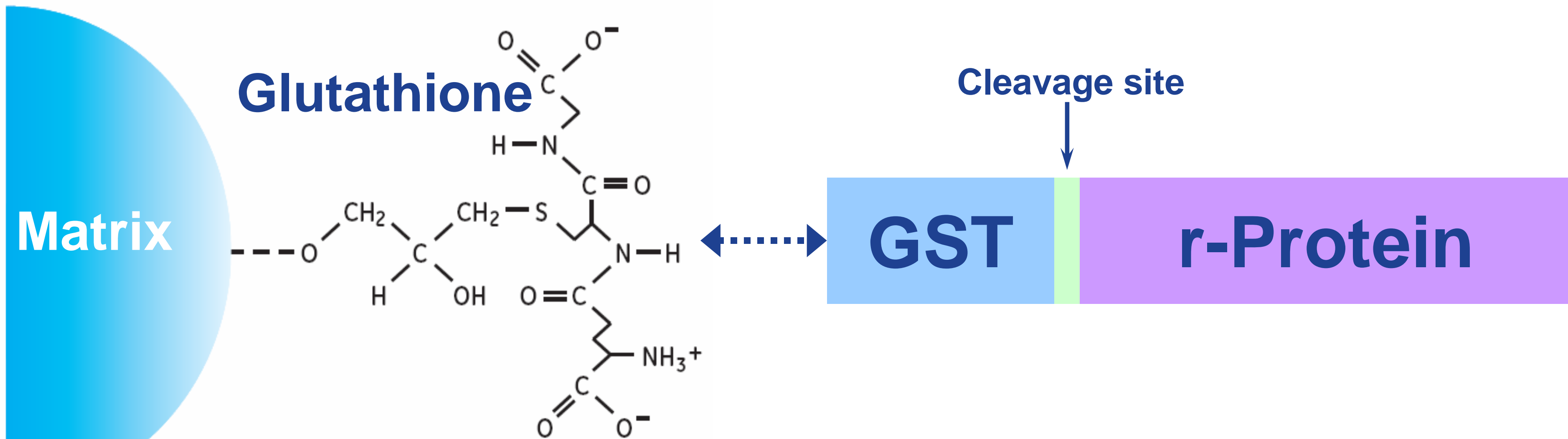
# GST-tagged Protein




- **Glutathione S Transferase (GST), 26 kD**
- GST is fused via its C terminus to the target protein
- Thrombin, Factor Xa and PreScission™ Protease cleavage site at C terminus of GST
- Elution : reduced glutathione (competition)
- GST activity is easily assayed.

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AFFINITY

# GST-tagged protein purification

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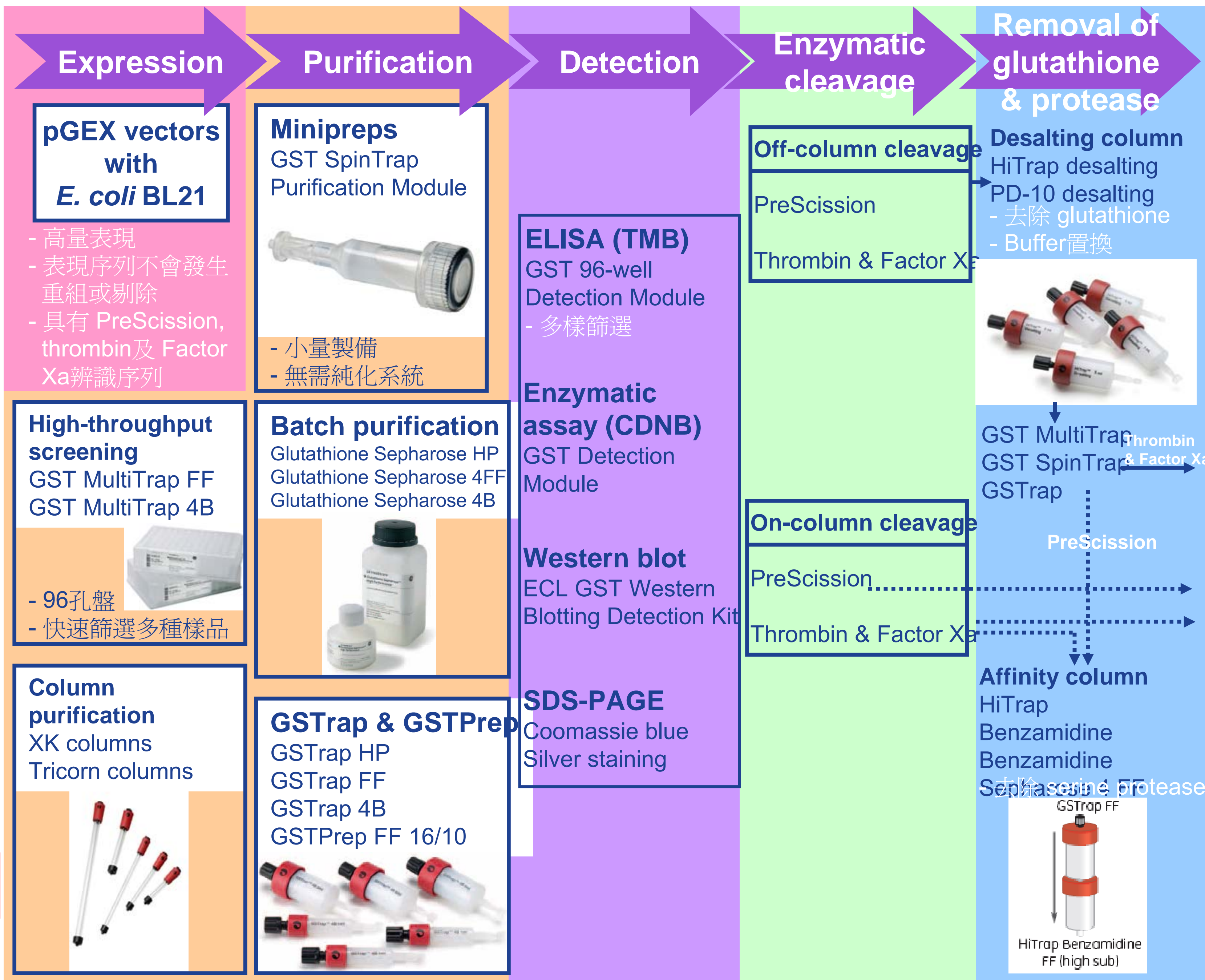
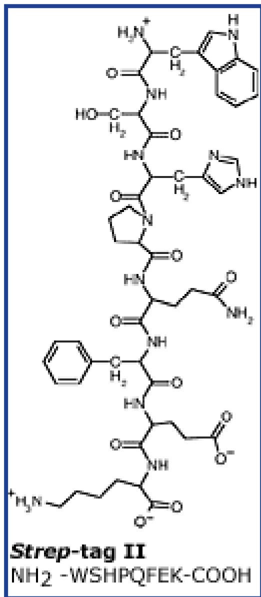
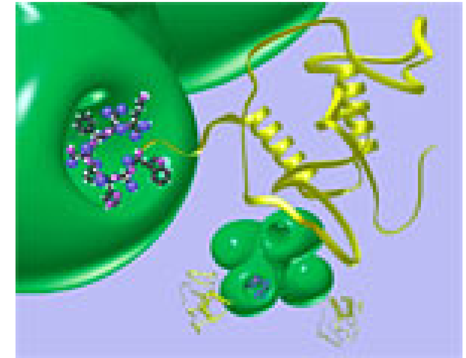


# StrepTrap™ HP

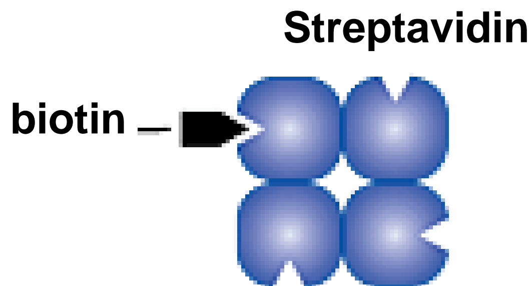
## Strep(II)-technology

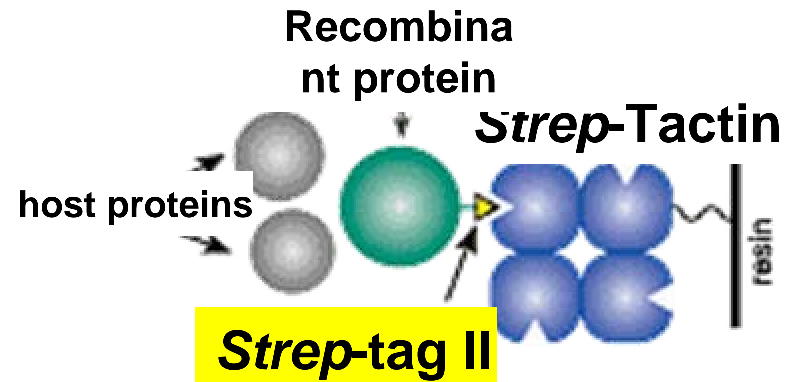



- -Strep(II)-tag, 8 aa, 1 kD
  (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys)
- -Often no cleavage of tag is necessary
- -Placement of tag both N- or C-terminal



***Strep*-tag** protein is binding to a ***Strep*-Tactin** tetramer.

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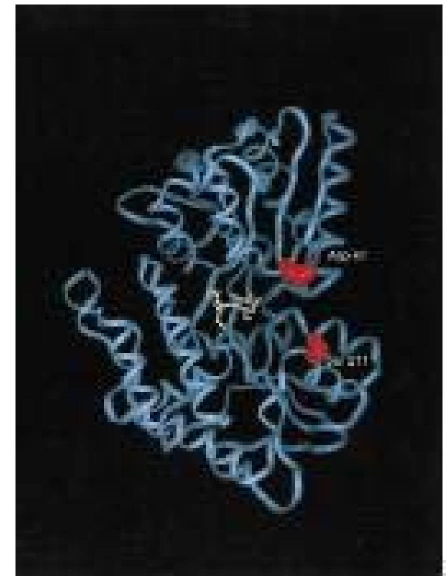
# MBPTrap™ HP

## MBP-technology

- MBP (maltose binding protein) - tag, 42.5 kD
- Cleavage of tag is often necessary- Enzymes: Factor Xa, Genease™, Enterokinase
- Most frequently placed in the N-terminal
- Expression in *E. coli*



Structure of MBP bound to maltose

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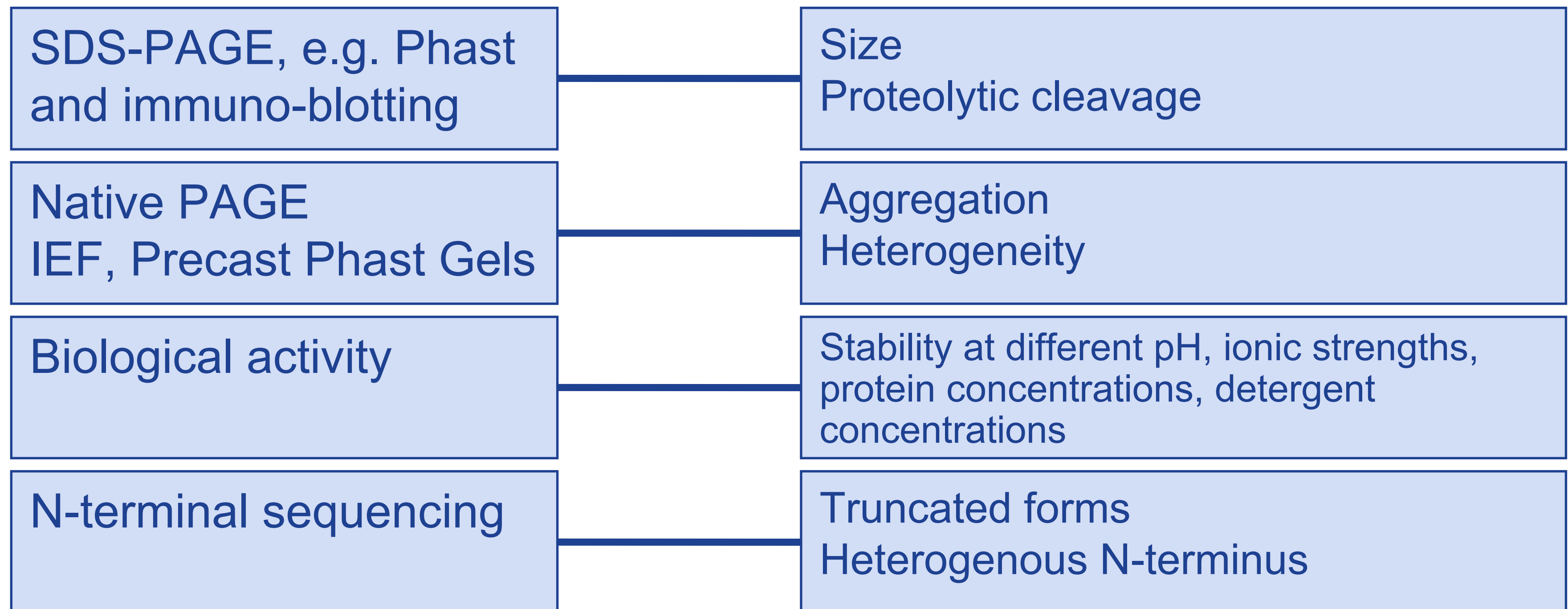
# Analytical tools

## Is the recombinant protein correctly expressed?

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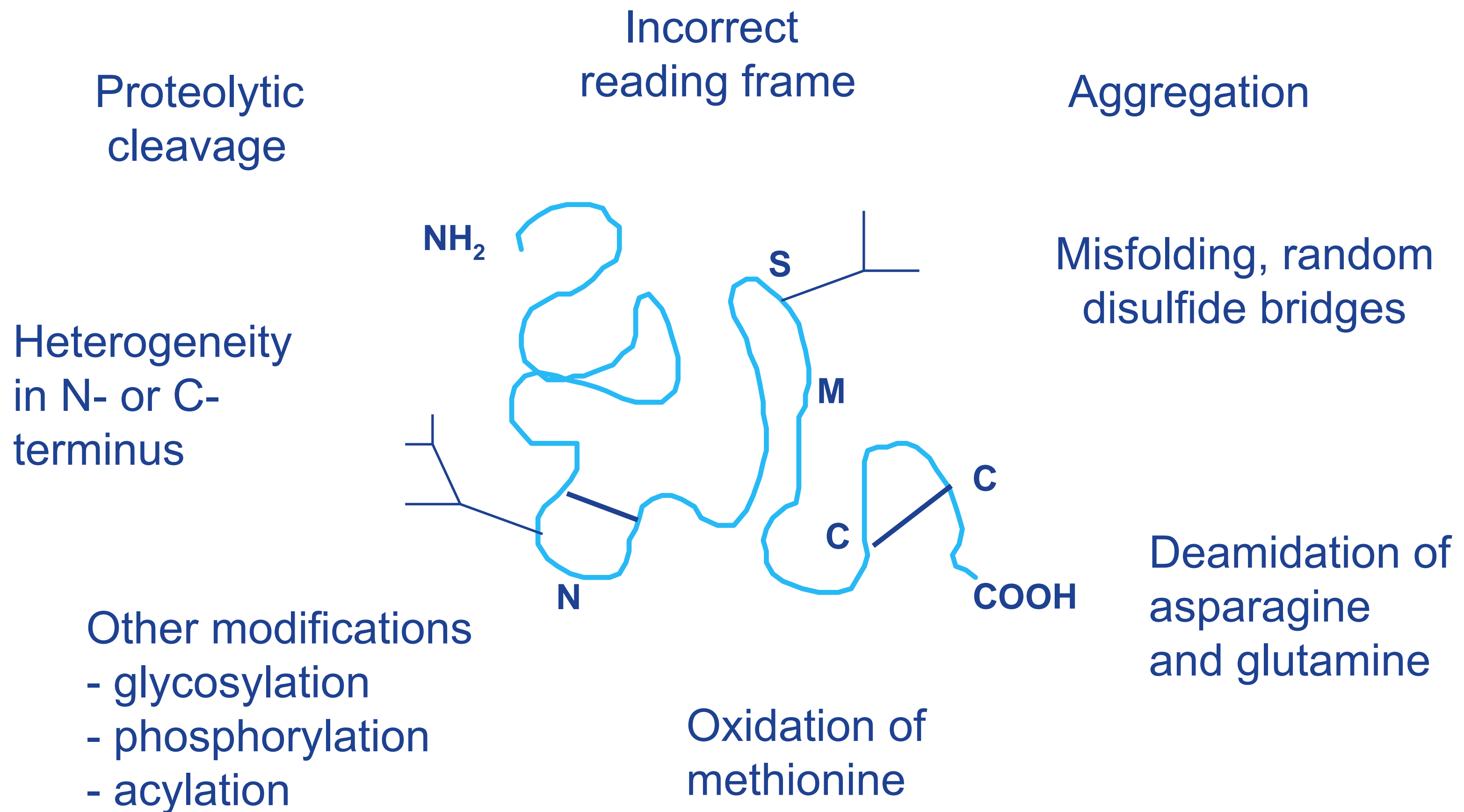
# Recombinant protein modifications



# Expression systems

| Processing | Bacteria | Yeast | Insect cells | Mammalian cells |
|---|---|---|---|---|
| Inclusion bodies | + | + | - | - |
| Secretion | +/- | + | + | + |
| Glycosylation | - | + | + | + |
| Proteolytic cleavage | +/- | +/- | - | - |

+ = Yes - = No

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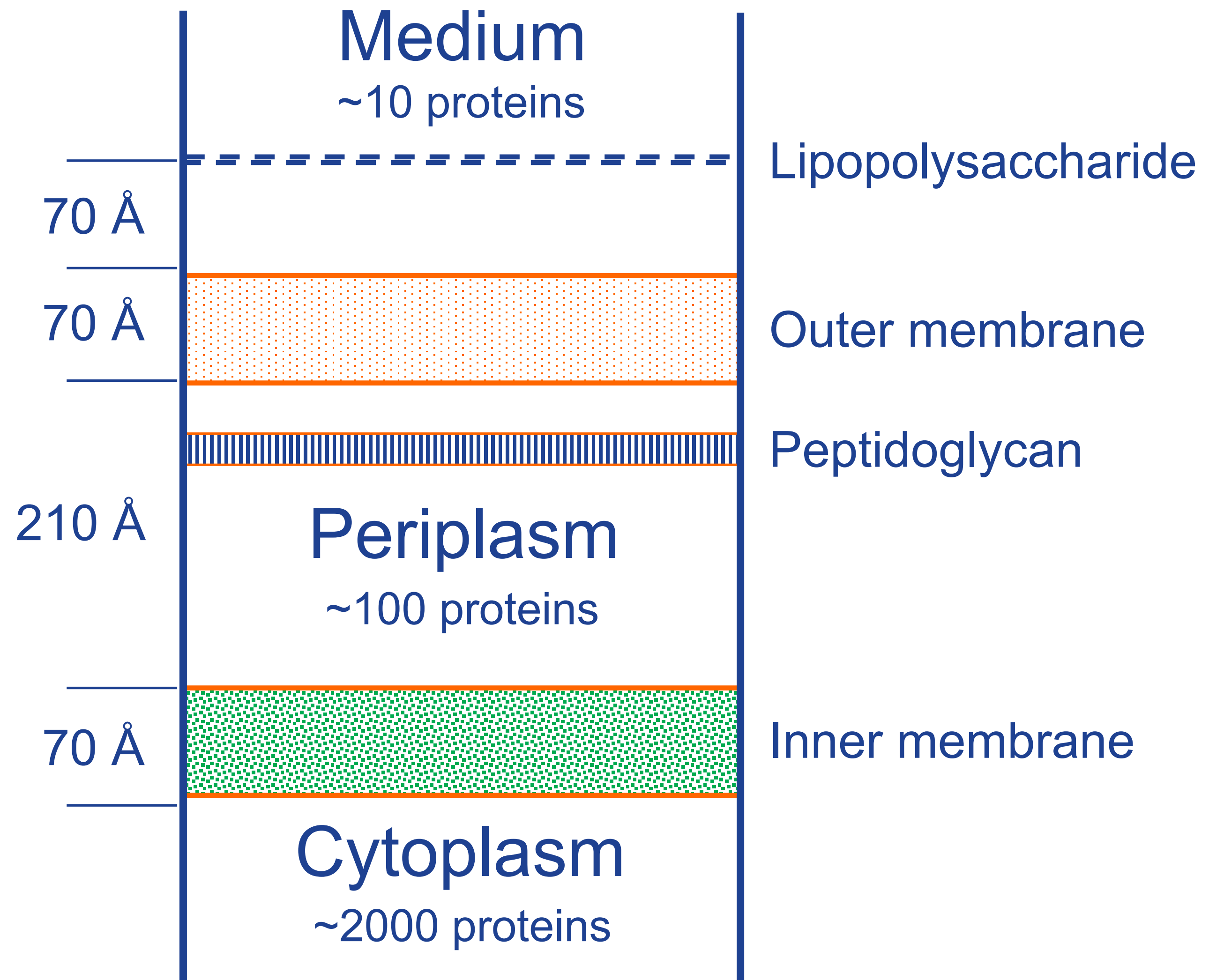
# Location of proteins in *E. coli*

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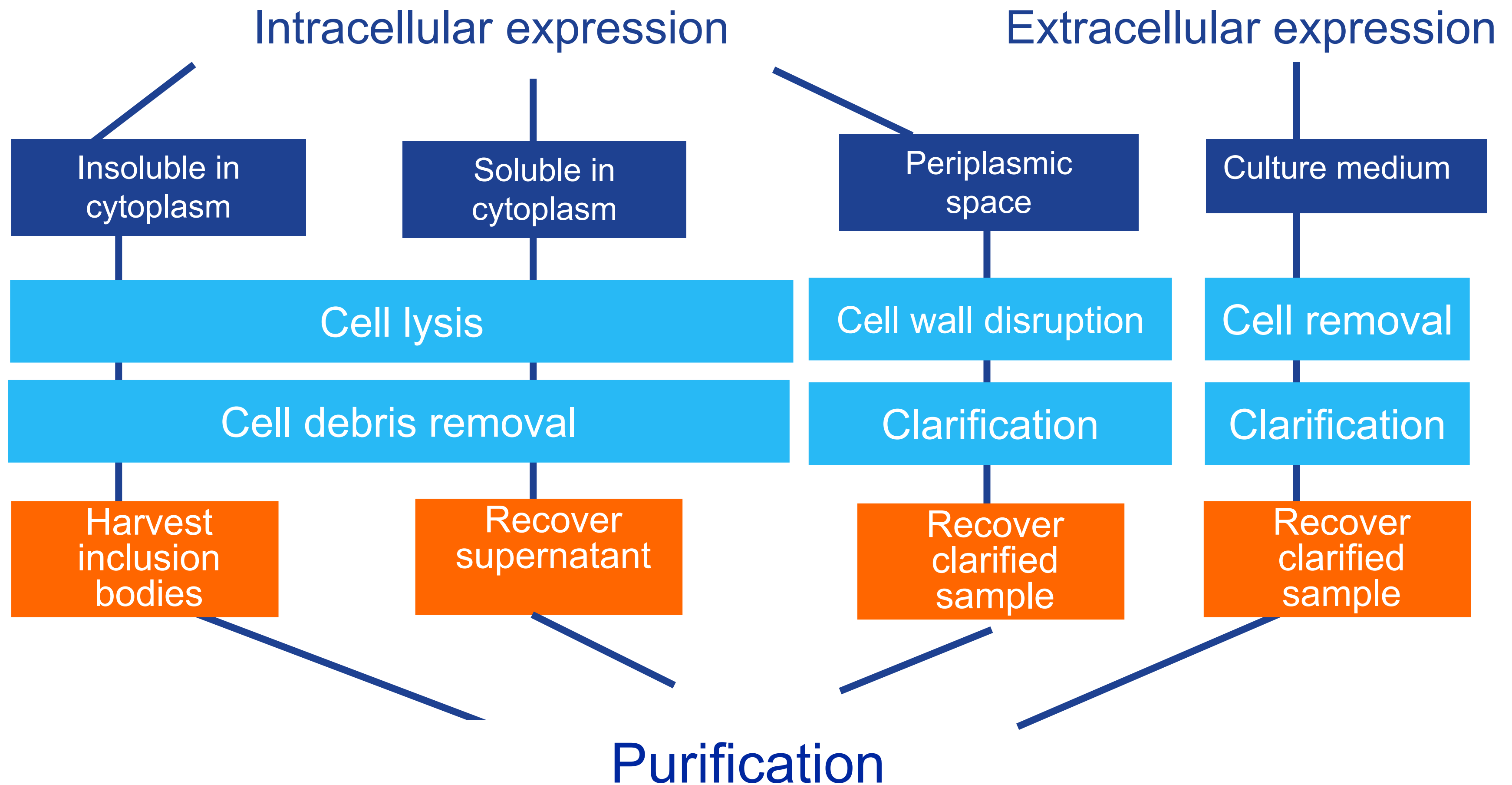
# Sample preparation

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# Refolding Proteins

## Refolding by chromatography

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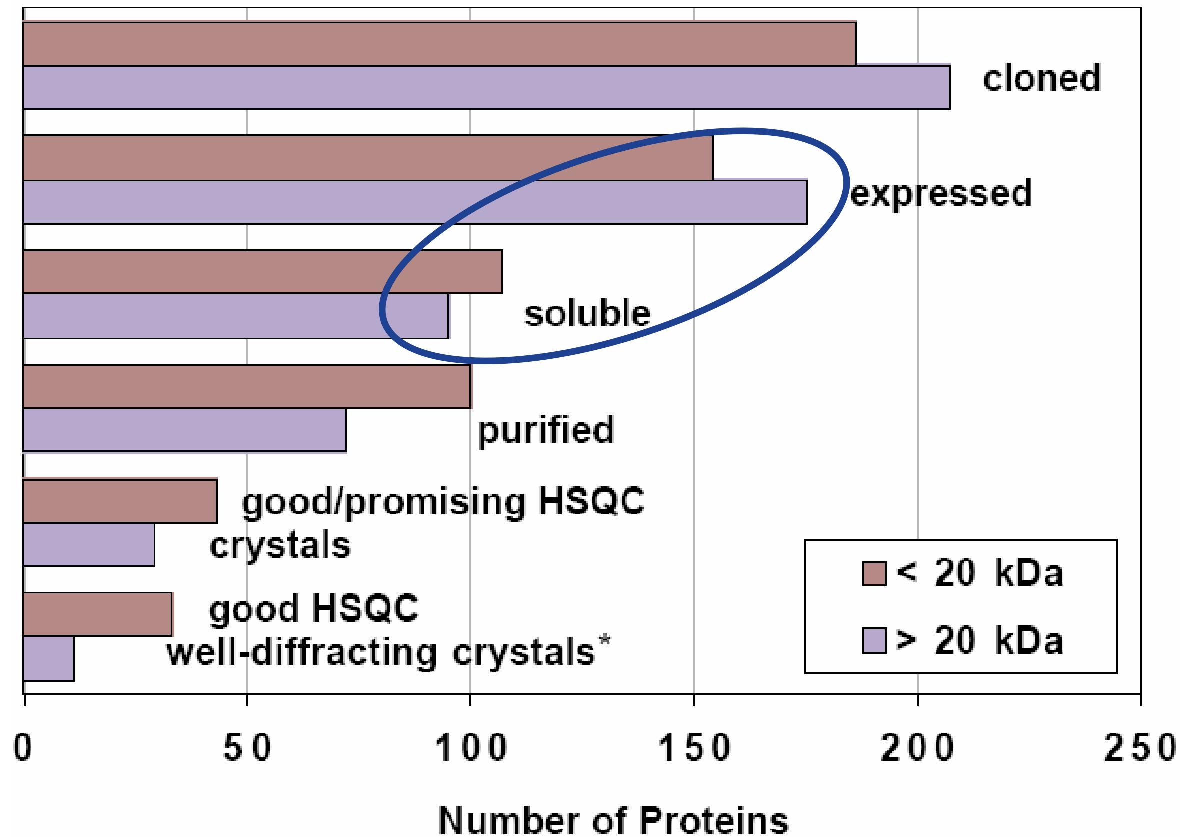
# Only a fraction of expressed proteins will be soluble....




**Non-membrane proteins from *Methanobacterium thermoautotrophicum* expressed in *E. coli.***

***30-50% of expressed proteins were insoluble.***

From: Christendat et al. Structural proteomics of an archaeon. Nature Struct. Biol. 7, 903-909 (2000). With permission of the publisher.

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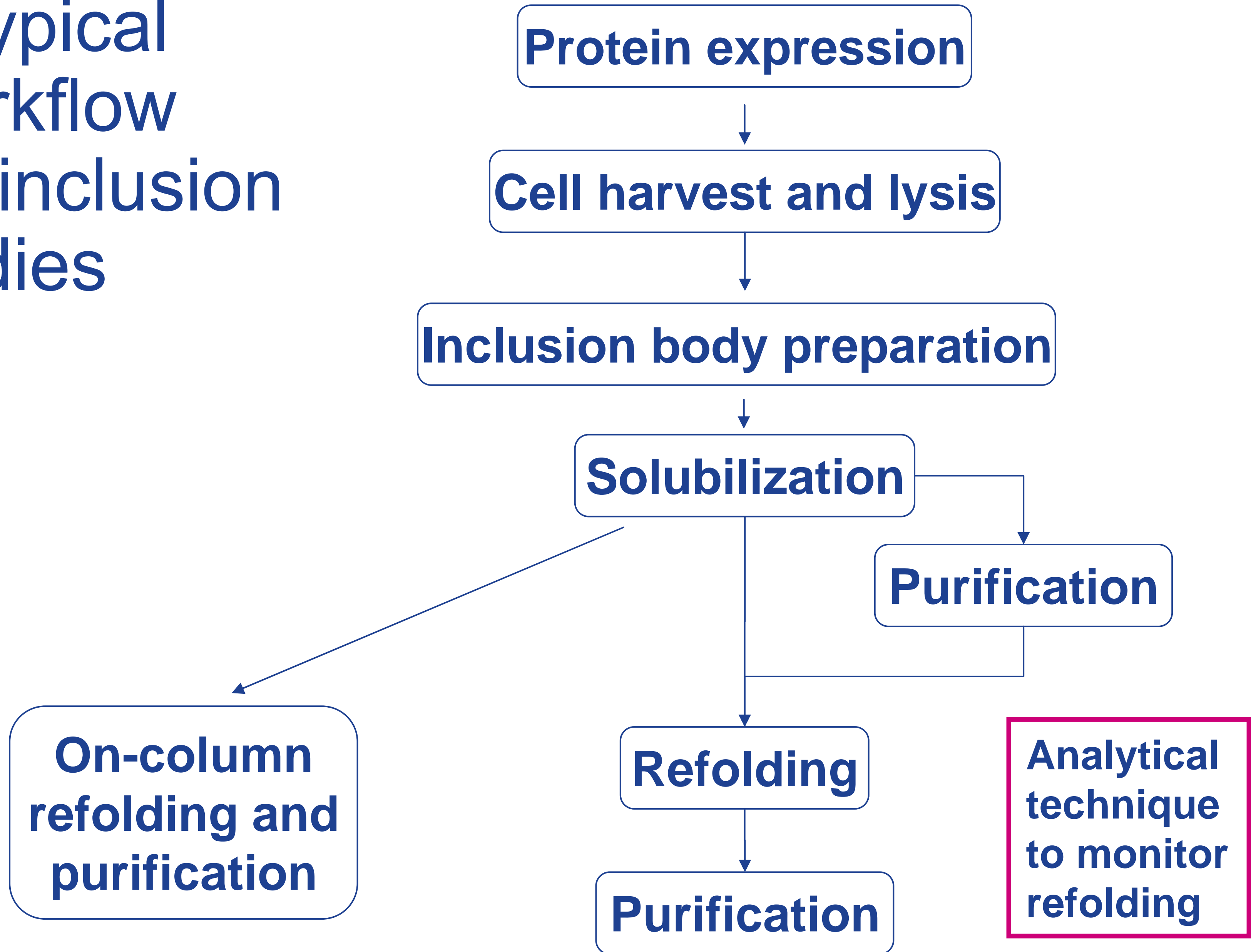
# A typical workflow for inclusion bodies

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# Techniques for refolding proteins from inclusion bodies

**Dilution**

+ Simple
- Slow
- Low sample concentration and large volumes

**Dialysis**

+ Simple
- Low sample concentration and large volumes

**Chromatography**

+ Fast, and can be combined with purification
+ Potential for automation
+ Higher initial concentration and higher yields

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# Refolding by Gel Filtration chromatography

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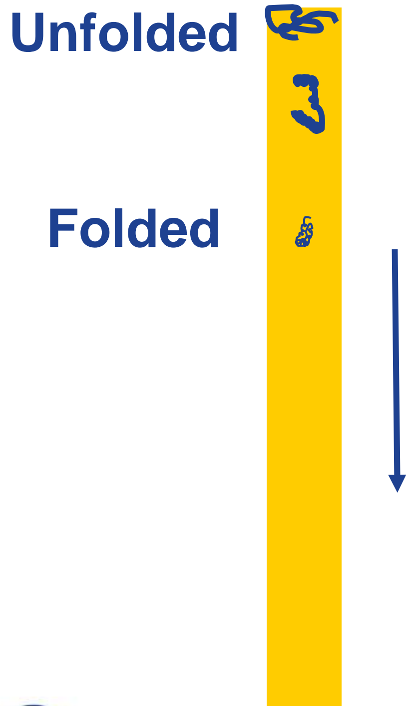
# Gel filtration can be used for…

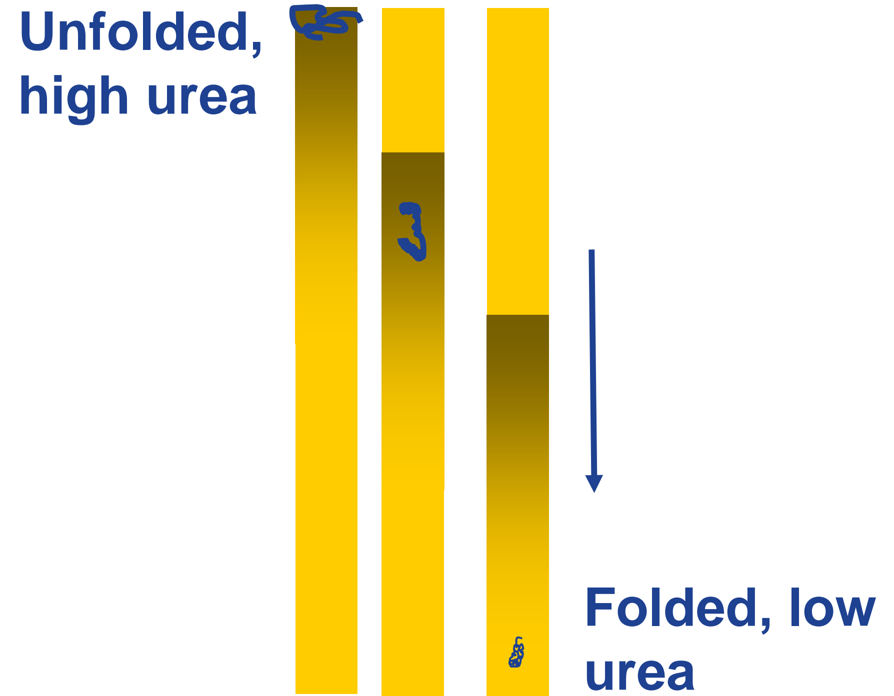
- Refolding
- Removing aggregates, thereby improving the refolding yield
- As an analytical technique to monitor folding

# Refolding by gel filtration

Gel filtration in refolding buffer (no denaturant).




Gel filtration on a column pre-equilibrated with a gradient of decreasing urea concentration.

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# Refolding by gel filtration

***Column:***
Superdex™ 75 10/300 GL
(Tricorn™)

***Sample:***
Lysozyme in 8 M urea, 0.2 M DTT

***Refolding buffer:***

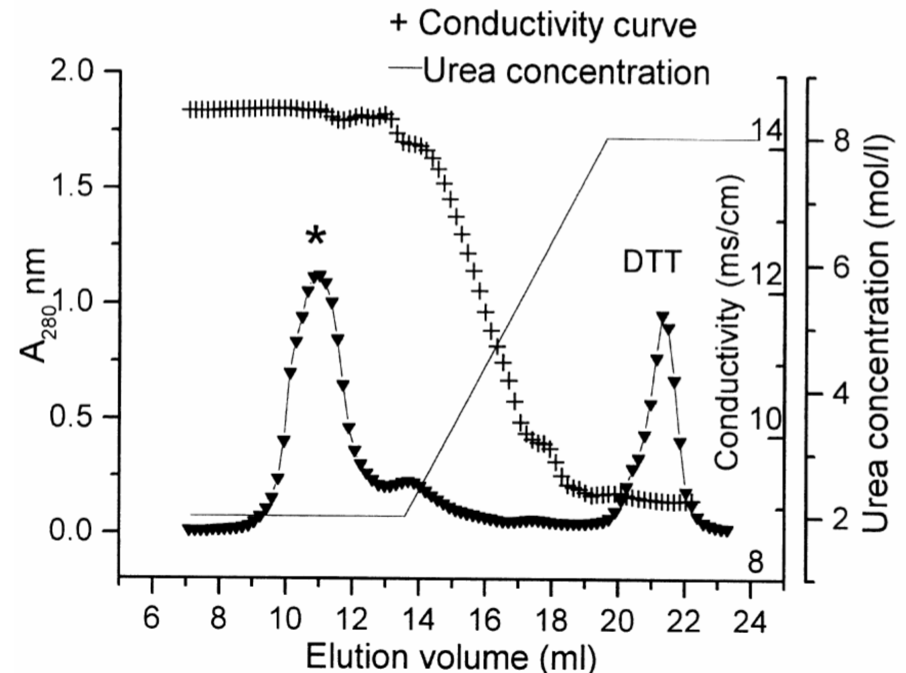
0.1 M Tris-HCl, pH 8.7,

1 mM EDTA, 150 mM NaCl,

3 mM GSH/0.3 mM GSSG

***Column equilibrated with***

6 ml gradient from 8 M to 2 M urea
in refolding buffer




Zhenyu et al. (2001) J. of Chromatogr. A, 918; 311–318.

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# Refolding by ion exchange chromatography

# Refolding by anion exchange chromatography

***Column:***
HiTrap™ Q FF

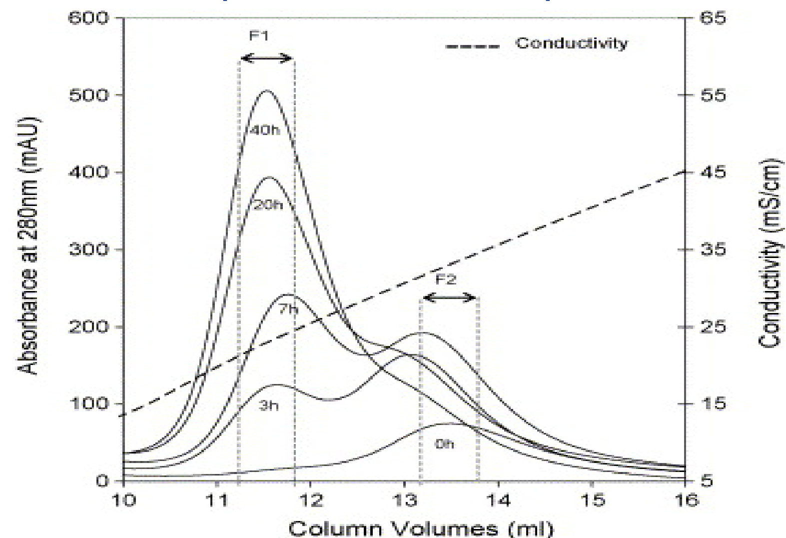
***Sample:***
1 ml BSA 0.5-20 mg/ml denatured-reduced

***Buffer A:***
50 mM Tris HCL, 3 mM EDTA,
8 M urea, pH 8.5

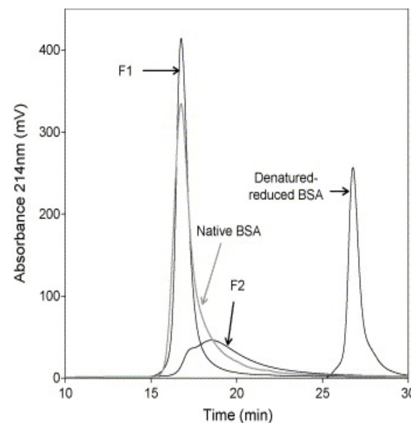
***Refolding Buffer:***
50 mM Tris HCl,
1 mM EDTA, 79 mM urea,
1.1 mM GSSG, 2.2 mM GSH, pH 8.5

***Elution Buffer:***
0-1 M NaCl in 50 mM Tris HCl, pH 8.5

Elution profile from Q Sepharose™ FF




Analysis on reverse phase column




Ref: Langenhof,M, et.al, (2005) Journal of Chromatography A, 1069 pp.195-201

# A three-buffer system…

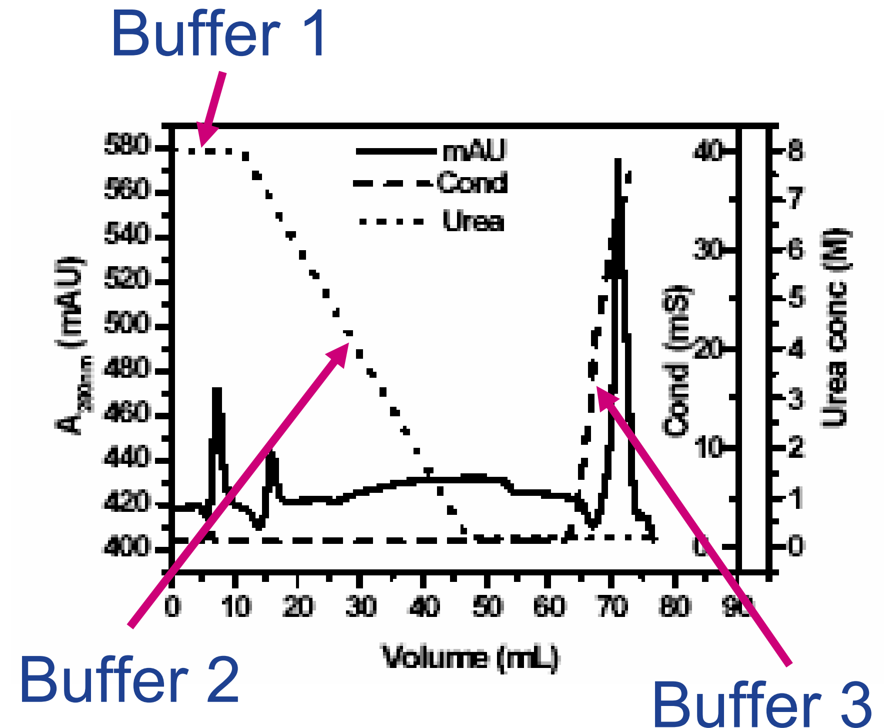
**Column:**
DEAE Sepharose™ FF (7 ml)

**Sample:**
Recombinant non-structural (NS3) protein from Hepatitis Virus C

**Buffer 1:**
50 mM Tris/HCl pH 9.0,
10 mM b-mercaptoethanol,
25 µM $ZnCl_2$, 8 M urea

**Buffer 2:**
50 mM Tris/HCl pH 9.0,
10% glycerol, 10 mM
b-mercaptoethanol, 25 µM $ZnCl_2$ ,
0.1% CHAPS

**Buffer 3:**
Buffer 2 + 0.5 M NaCl




Ref: Li, M et al. (2003) Biotech. Letters 25, pp. 1729-1734

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# Refolding by affinity chromatography

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# Refolding of extracellular superoxide dismutase from E. coli inclusion bodies

**Sample:**
his-SOD from E. coli IB

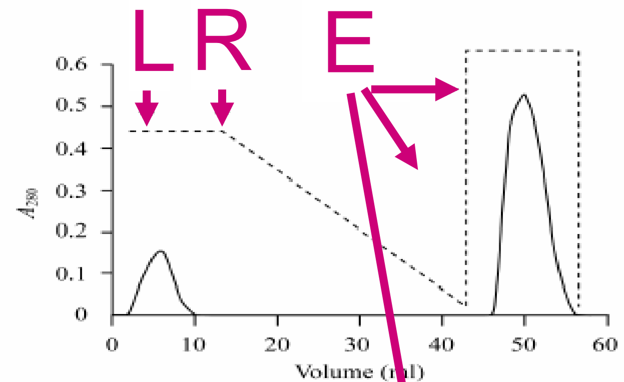
**Column:**
Chelating Sepharose™ FF (1.5 x 1 cm)

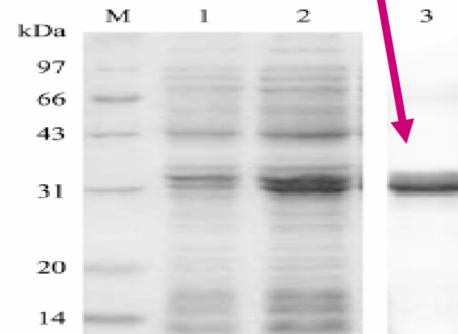
Flow during refolding: 0.6 ml/min

**L:**
Flow through in 50 mM Tris HCl, pH 8, 4 mM GSH,
1 mM GSSG, 8 M urea

**R:**
Urea gradient from 8 M to 1.5 M

**E:**
Elution with 100 mM histidine,
50 mM Tris HCl, pH 8




SDS-PAGE analysis of EC-SOD expressed in *E. coli*

Ref: Zhu X. et al. (2005), Acta Biochimica et Biophysica Sinica, 37 (4) 265-269

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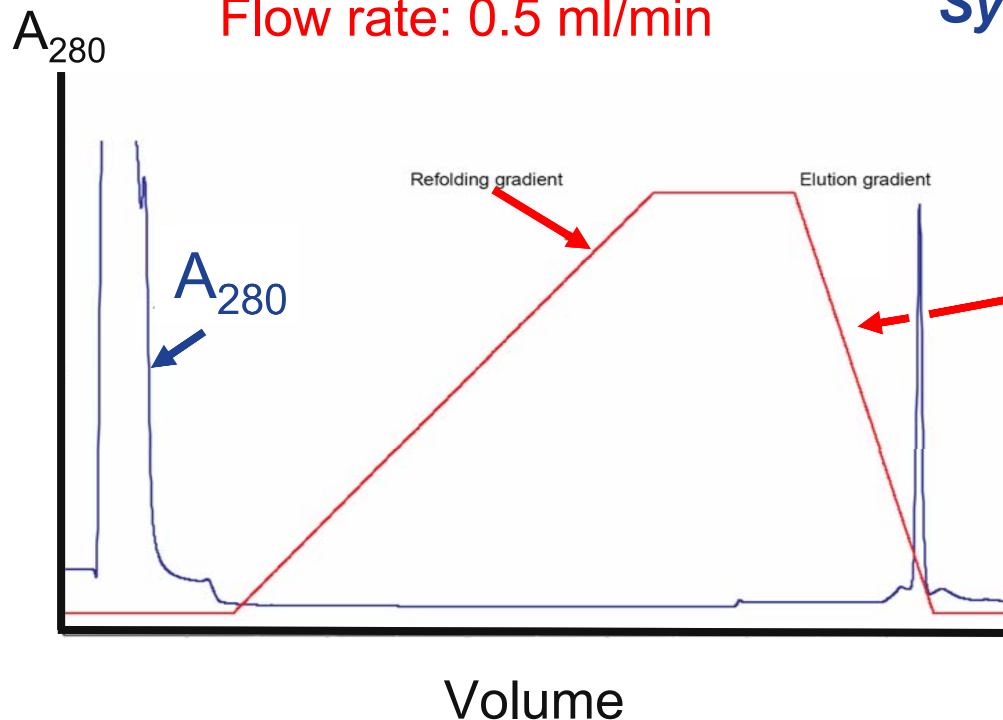
# On-column refolding and purification of a histidine-tagged protein

Refolding
Gradient: 6 M – 0 M urea
30 ml gradient volume
Flow rate: 0.5 ml/min

***IMAC Column:*** HisTrap™ (1 ml)

***System:*** ÄKTAprime™




Elution gradient:
20 mM – 500 mM imidazole
Flow rate: 1.0 ml/min

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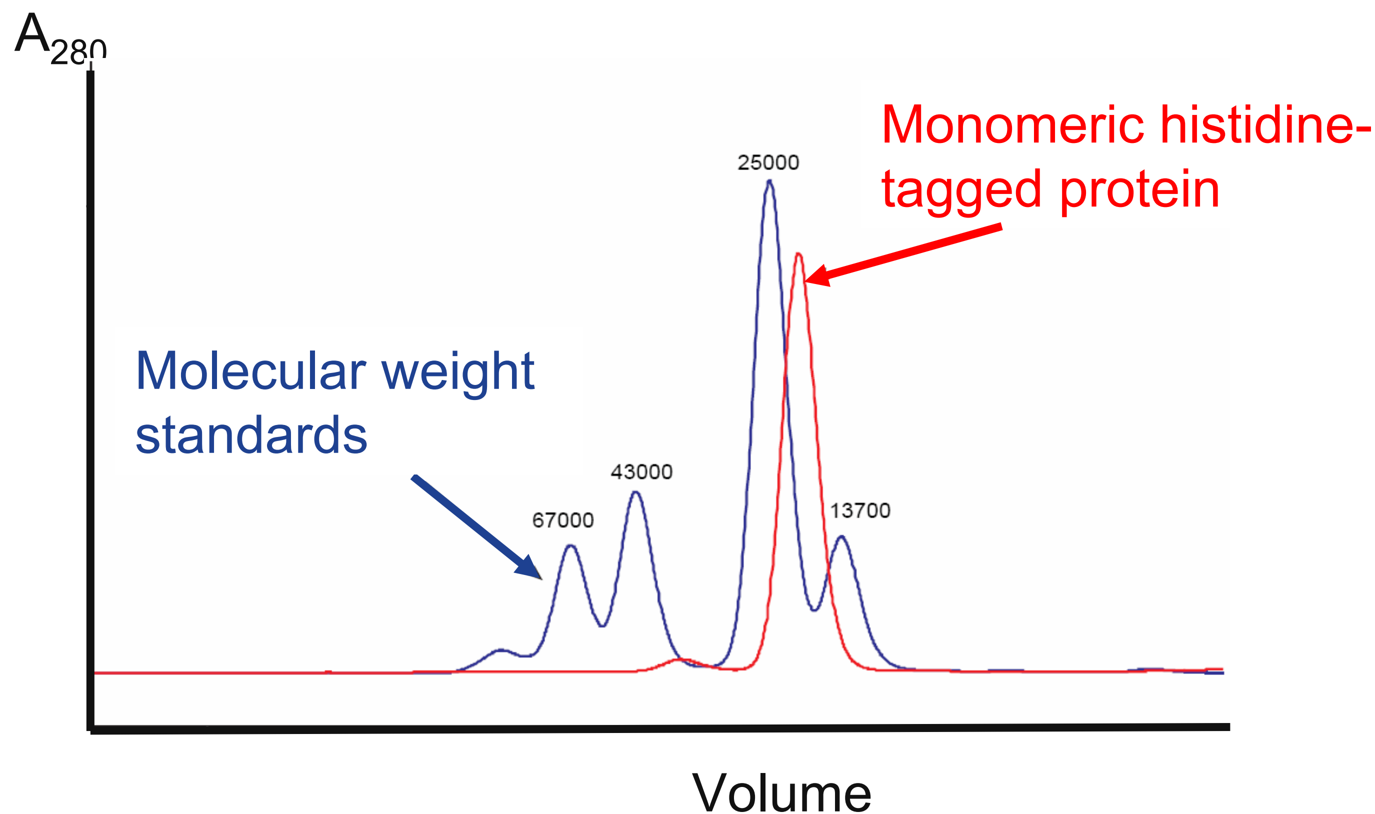
# Monitoring refolding

**Gel filtration column:** Superdex™ 75 10/300 GL

**System:** ÄKTAprime™

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# On-column refolding of a chemokine

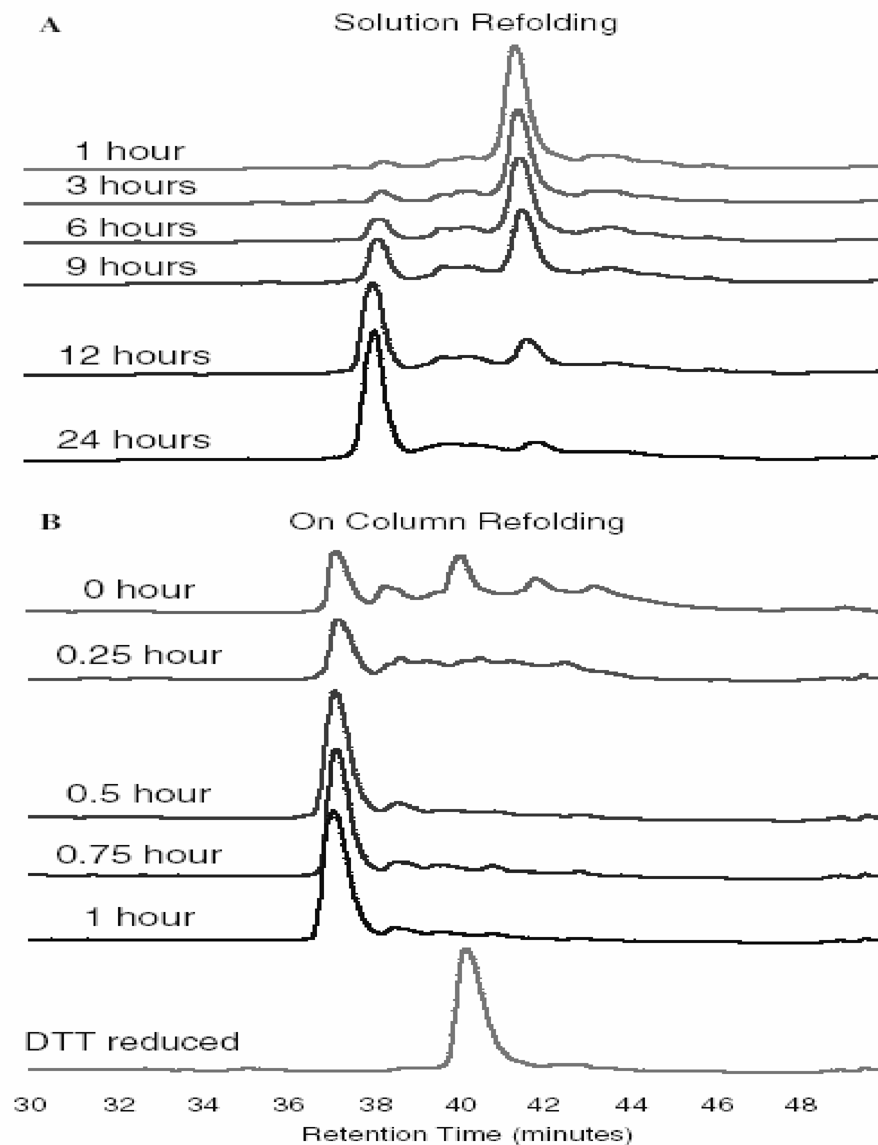
**Sample:**
Recombinant SDF-1α from E. coli inclusion bodies

**Column:**
Ni Sepharose™ 6 FF (2 ml in a gravity column)

**Figure:**
Refolding monitored by RP HPLC

41.5 min – protein reduced

38 min - completely oxidized.




Ref: Veldkamp, C. T. et al. (2007), Protein Expression and purification, 52, pp 202-209

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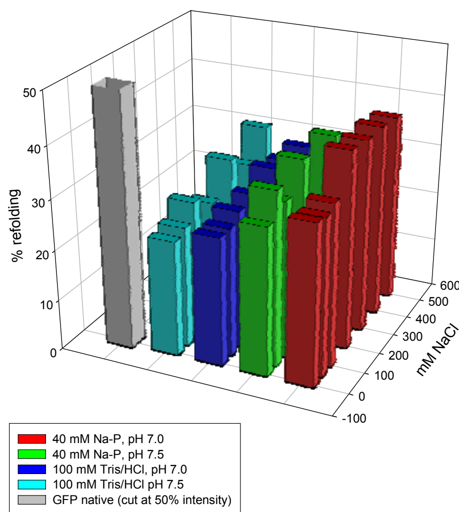
# Screening of refolding conditions can easily be done on MultiTrap

Fine screening of salt at optimal pH




Inclusion bodies denatured by 8 M guanidine HCl

**Target protein: GFP F64L S65T**

96-well His MultiTrap™ FF plates were used
Refolding criteria were fluorescence readings in 96-well reader and fluorescence spectrometer

**Screenings were done to select the best:**

- pH
- Salt
- pH/salt (Example shown on the left)
- Folding time
- Gradient types

Data kindly provided by J. Buchner, Munich Technical University, Germany.

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# Optimize your histidine-tagged protein purity

- Imidazole concentration
- Different metal ions
- Add more purification steps

# SUMMARY

There are no general methods for refolding every protein. Optimization and screening should be done on a case-by-case basis

Refolding by chromatography gives higher yields because it allows much higher starting concentrations and simultaneous removal of aggregates

Gel filtration, ion exchange, HIC and affinity chromatography can be used for refolding