Affinity Tags for Protein Purification

I. Overview of Protein Expression and General Strategies for Protein Purification

II. Affinity tags for Protein Purification
   A. Characteristics of tags
   B. Common affinity tags
      • GST-tag
      • FLAG-tag
      • His-tag
   C. Cleavage of affinity tags

III. SDS gel analysis of purified proteins
Our progress so far for H396P Abl(229-511) expression and isolation:

Prior to Session 2, BL21(DE3) expression cells co-transformed with
1) an H396P Abl-encoding _kan_-resistant vector and
2) a YopH Tyr phosphatase-encoding _strep_-resistant vector
were spread onto a LB-agar plate with antibiotics for colony selection.

(Session 5)
Lyse cells and Isolate the H396P Abl protein

**Diagram:**
- BL21(DE3) cell
- kan/strep plate
- Pick a single colony
- grow up overnight
- spin down and freeze cell pellet
- starter culture
- 500-mL culture
- Induce protein over-expression
Successful Abl kinase domain expression in bacteria requires co-expression with a phosphatase!

High yield bacterial expression of active c-Abl and c-Src tyrosine kinases
Markus A. Seeliger 2 1, Matthew Young 2 1, M. Nidanie Henderson 2 1, Patricia Pellicena 2 1, David S. King 1, Arnold M. Falick 1, John Kuriyan 3 2 1 *

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Prior to 2005, expression of active Abl kinase domain was carried out

- in insect cells. Insect cells yield milligram quantities of protein, but are time consuming and expensive to maintain.

- in bacteria with very low yields (micrograms). While biochemical studies can be carried out with tiny amounts of protein, milligram quantities are required for biophysical and structural studies, such as crystallography and NMR.
Successful Abl kinase domain expression in bacteria requires co-expression with a phosphatase!

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Seeliger and co-workers hypothesized that the low yields of soluble Abl in E. coli were due to the toxicity of Tyr kinase activity in bacteria.

- Phosphatase co-expression prevents high levels of toxic kinase activity.
- YopH is a non-selective Tyr phosphatase, meaning it dephosphorylates most phosphotyrosine (pY) substrates, regardless of the specific sequence.
- Yields of purified Abl K domain protein using the co-expression method in BL21-DE3 cells range from 5 to 15 mg/L.
In Session 5 you will **lyse** (split open) your BL21-DE3 cells and isolate the H396P Abl protein.

Crude cell lysate has many components. Your mixture will include:

- Overexpressed **His**-tagged H396Abl-kinase domain *\(^{\ast}\)*
- Overexpressed Yop phosphatase X
- E. Coli proteins, DNA, and metabolites X

*\(^{\ast}\)* what we want
X what we don’t want
Strategies for protein purification

Solubility
1. Salting in
2. Salting out

Ionic Charge
1. Ion exchange chromatography
2. Electrophoresis
3. Isoelectric focusing

Polarity
1. Adsorption chromatography
2. Hydrophobic interaction chromatography

Size
1. Dialysis
2. Gel electrophoresis
3. Size exclusion chromatography

Binding Specificity
1. Affinity Chromatography

Protein characteristics
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Affinity Chromatography

Chromatography involves a mobile phase (ie. cell lysate with overexpressed protein) and a stationary phase (the column with ligand-bound beads)

Three steps in column chromatography:

1) **Binding** the desired protein to the affinity column
2) **Washing** away unwanted proteins, DNA etc.
3) **Eluting** the desired protein
Affinity Chromatography

Examples of protein/ligand pairs

- Avidin/ or streptavidin --- biotin
- Glutathione-S-transferase --- glutathione
- Dihydrofolate reductase --- methotrexate

However,

- Not all proteins have known binding partners
- Known binding interactions might not be sufficiently strong to withstand washing steps.
- Raising antibodies for all proteins?—no way.

Is there a general purification strategy that lends itself to a diverse set of proteins?
Affinity Tags

In affinity tag purification, the protein of interest (POI) is expressed with a polypeptide affinity “tag” on the N- or C-terminus of the protein.

Purification is based on the highly specific interaction between the polypeptide affinity tag and a binding counterpart that is immobilized on solid support.

Tags are introduced at the DNA level, either supplied by the expression vector or using primers.
Summary of sequence, size, matrices, and elution conditions of commonly-used affinity tags

Figure removed due to copyright restrictions. See tables 1 and 2 from Terpe, K. “Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems.” Appl Microbio Biotech 60 (2003): 523-533.

An ideal affinity tag:

• requires a simple purification process that is free of harsh reagents.

• does not affect (or only minimally affects) the tertiary structure and biological activity of the POI.

• can be easily and specifically removed from the POI following purification to produce native protein.

• can be used with diverse types of proteins.

Some tags confer additional benefits, such as increasing protein solubility and increasing the yield of recombinant proteins.
Small tags versus large tags:

Small peptide tags

- Examples: poly-Arg, FLAG-, poly-His, Strep
- Advantages:
  - Minimal interference with the fused protein
  - Depending on the location and amino acid composition of the tag, removal of the tag may not be required
  - Not as immunogenic as large tags—recombinant proteins can be used directly as an antigen in antibody production

Large peptide tags

- Examples: GST, MBP
- Can increase solubility and expression of the POI
- In most cases, large tags need to be removed after purification.
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IV. SDS gel analysis of purified proteins
Glutathione S-transferase-tag (GST-tag)

- A 26-kDa (220 aa) protein that binds to glutathione (a tripeptide).
- GST-fusion proteins can be purified from crude lysate by affinity chromatography on immobilized glutathione.

- Bound fusion proteins can be eluted with 10 mM reduced glutathione under non-denaturing conditions.
- Advantages: The tag can help to protect against intracellular protease cleavage and stabilize the recombinant protein.
- Due to its large size, the GST-tag should be cleaved from the fusion protein after purification.
FLAG-tag

• A short hydrophilic octapeptide, DYKDDDDK
• The FLAG-tag binds to a monoclonal antibody purification matrix (typically anti-FLAG antibody bound to sepharose resin.)

![](image.png)

• The tagged protein is commonly dissociated by adding competing FLAG peptide, or by transiently reducing the pH (to \(~3.5\)).

• Advantages: The FLAG tag/antibody interaction is highly specific resulting in highly pure proteins. The tag can be appended to the N or C terminus.

• Diadvantage: cost!
Polyhistidine tag (His-tag): Binding

- A short polyhistidine peptide (from 4-10 residues; normally 6)
- His is the amino acid that binds most strongly to metal ions
  - (Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$) — use column with immobilized metal ions.
  - Common immobilizer is NTA (nitriloacetic acid)
His-tag: Washing and Eluting

Washing
The washing buffer typically contains 10 to 30 mM imidazole. The imidazole competes with His residues for metal chelation.

Elution. There are three options:
• Reduce pH to 4.5-5.3
  Protein can be damaged by reduction in pH
• Use metal chelators (e.g. EDTA)
  Recycling of Ni-NTA resin is much harder when the Ni is chelated
• 100-250 mM imidazole ***
  Mildest conditions. Imidazole can be subsequently removed by dialysis.
Considerations in using His-tags

- Ni-NTA resin is damaged by high concentrations of strong reducing agents.
- In certain cell systems (e.g. insect), acidic media is required, which can prevent His from binding to Ni-NTA.
- Certain proteins have native polyHis patches.

Advantages to using His-tags.

- Ni-NTA resin is FAR less expensive than FLAG-resin or other anti-body based resins.
- His tags can be added to the N- or C-terminus of a protein and often do not need to be removed after purification.
The H396P Abl(229-511) protein has an N-terminal hexahistidine (His$_6$) tag

- The tag was introduced by the pET-28a vector (see map).
- The Yop phosphatase is NOT His-tagged.

You will use affinity tag purification to isolate the H396P Abl kinase domain.

Studies have shown that an N-terminal His tag does not significantly affect Abl kinase domain activity, so we will not remove the tag.
Dialysis of the purified protein

Long-term storage of certain proteins in high-concentration imidazole buffers can lead to degradation.

Consider the sizes (molecular weights) of the components in your elutions:

32 kDa

68 Da

Therefore we’ll use a 10 kDa MWCO (molecular weight cut off) dialysis device.
Site specific proteases can be used for tag cleavage. Commonly used proteases with cleavage sites indicated:

- **Enterokinase**: DDDDK \( \rightarrow \) X
- **TEV protease**: ENLYFQ \( \rightarrow \) S(or C).
- **Thrombin**: X4-X3-P-R \( \rightarrow \) X1'-X2', where X4 and X3 are hydrophobic aa’s and X1', X2' are non-acidic aa’s
- **Factor Xa**: I(or E)-DGR \( \rightarrow \) X
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III. SDS gel analysis of purified proteins
SDS-PAGE

- **SDS**: Sodium dodecyl sulfate: a detergent that binds strongly and confers negative charges to proteins

  \[
  \text{SDS} = H_3C-(CH_2)_{10}CH_2\cdot OSO_3\cdot Na^+ 
  \]

- **PA**: Polyacrylamide

- **GE**: Gel Electrophoresis
  Separation (by molecular mass) using a current applied to gel matrix
Stacking gel vs. Resolving gel

Difference in pore size as a result from different % acrylamide added

Stacking gel has larger pore size
• Proteins are not yet separated by mass. Will be concentrated and thinned.

Resolving gel allows separation according to size
• Difference in % acrylamide can be used to separate different ranges of proteins
• 8%-20% gel
• Higher % acrylamide ⇒ smaller pores ⇒ suitable for smaller proteins
Electrophoresis

- Negatively-charged proteins move towards the positive end
- Smaller proteins run faster
- Estimation of size based on the molecular weight markers

- After electrophoresis, the gel can be stained (e.g. by Coomassie stain) or further processed (e.g. Western blot)
Session 4  prepare purification and gel analysis buffers  
Session 5  Lyse your cells and isolate the Abl protein. Dialyze.  
Session 6, 7, and 8  
  Run a gel to visualize your protein fractions.  
  Concentrate your purified and dialyzed protein.  
  Quantify protein concentration.