Purification Strategy

Selection and combination of purification techniques

Guidelines for Protein Purification

Protein Characterization
PURIFICATION STRATEGY

- General approach: Protocol Development
- Guidelines for Protein Purification.
- Commonly confronted decisions.
- DoE: Design of Experiment
- Three Phase Strategy: Linking Chromatography Techniques
- Guidelines for Protein Purification
- Characterization Criteria - Quality control
- Storage
Applications of Protein Purification

- In vitro Activity assays
- Antibody development / production
- Protein:protein interaction assays
- Cell-based activity assays
- Ligand-binding assays
- Mass-spectrometric analysis
- Structural analysis
- In vivo activity assay

- Post-translational modification tests
- N-terminal sequencing
- Electromobility shift assay (band shift)
- DNA footprinting
- Protein cross-linking studies
- Vaccine development/production
- Probes for protein arrays/chips
- Expression library screening
- Other

For each application you need:

- different quantities
- different protein purity
- start material is different, etc
- different strategy

Each purification project must be adapted to your start material and your final needs

Don’t waste clear thinking on dirty or not healthy proteins!!!!
Protein Purification Strategy

- **FUSION PROTEIN**
  - **SIMPLE PURIFICATION**
  - ONE STEP Affinity
  - 70 - 95% Purity
  - For higher purity
  - **MULTI-STEP PURIFICATION**
    - Capture
    - Intermediate Purification
    - Polishing

- **NON-FUSION PROTEIN**

**EXPRESSION**
Protein Purification - Aims

- Satisfactory
  - expression levels
  - protein activity
  - purity
  - homogeneity
  - stability

- Economical use of reagents/equipment

- **Goal to Success:**
  - Selection or optimization of the best source or best expression conditions
  - A good understanding of the protein needs
  - Selection and optimization of the most appropriate technique for each step
Purification protocol development - step by step

1. Define the purification Objectives
2. Consider what’s known about the target protein
3. Select purification tools
4. Develop analytical assays
5. Develop the individual purification steps
Commonly confronted decisions

- Which is the best natural source?
- How much do we need?
- Active? Which assay?
- Purification grade?
- Which hosts: bacteria, yeast, insect cells or in human cells?
- Which expression vector should be used? Which strain(s) should be chosen?
- Should the protein be tagged? Which affinity tag is the best?
- Which is the best purification strategy?
- Which buffers should I use?
- Optimization of each purification step, where to stop?
- How much can I concentrate my sample?
- How to keep activity, solubility and homogeneity of my sample?
Protein Production Pipeline

- Target Selection
- Target Optimization
- Gene Cloning
  - Selection of Expression Vector
  - Selection of Expression Host
- Expression Analysis
- Solubility Analysis
- Scaling Up
- Fermentation
- Purification
  - Purification Optimization
- Characterization
- Concentration & Storage

Pharmaceutical Studies

Biochemical Studies

Structural Studies: Crystallization – NMR- etc
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

1. **Sequence:**

A. Gene ID from a known database (PubMed tools, etc.)

B. Domain determination of the specific gene ID, (i.e. aa 48-330 from Id xxx)

C. Specific elements (Signal peptide, Trans-membrane, cellular localization, specific protease sites, etc.)

D. Fusion tags (Full seq. + Ref.)

E. Protease sites for tag removal (Full seq. +ref. of)

F. Full translated region, DNA and AA seq.

2. **Cloning**

A. Vector name either commercial or from known database (such as link to AddGene). Should include: Full seq of the vector, annotated map with resistance, ori, cloning methodology, etc.

B. Cloning method (ligation, recombination, etc.)

C. DNA region into which the sequence described is inserted

D. Bacterial strain used for storage and propagation (commercial maker, genotype, cat. Number, or database)
3. **Expression conditions**
   A. Expression system (Host specification such as: bacterial genotype, cell line, cat. Number, and link to database)
   B. Growth & induction conditions: temperature, media & supplements [maker & cat numbers], if shaking : rpm, oxygenation (?), inducer, time, quantity
   C. Instrumentation used (Bioreactors, shakers, flasks, etc)

4. **Soluble / Insoluble**
   A. Cell lysis : buffers & lysis procedure
   B. Low scale purification (resin, buffers, yield )
   C. PAGE-SDS analysis: method (sup vs. pellet, and/or affinity binding: analyzed on Coomasie and/or Western)

5. **Scale-up and storage conditions**
   A. Initial growth volume + (OD, cell mass etc.)
   B. Lysis conditions: buffers, additives, lysis methodology, clarification procedure used
   C. Chromatography: Resin supplier, column volume, buffers use, loading, wash and elution conditions. Which criteria was used to select fractions
   D. Description of next chromatographic steps as before
   E. Final storage: protein concentration method use, storage buffer, storage conditions. Final yield
   F. Quality control: PAGE-SDS analysis, analytical SEC, protein quantification method, etc
5. Protein quality:

*Basic requirements for evaluating protein quality*

A. PAGE-SDS (coomassie and/or Western blot) provides multiple information regarding the quality of the protein such as the presence of degradation products as well as the absence of protein contamination

B. Analytical size exclusion chromatography (SEC) provides information regarding the correct oligomeric structure of the protein and the absence of soluble aggregates that can cause non-specific results in downstream experiments.

*Additional information for evaluating protein quality*

C. Protein quantification: procedure used

D. Functional activity: short description of the assay and results

E. Others: CD, OD spectrum, MS, analytical ion exchange (IEX), Reverse phase chromatography (RPC), DLS, SLS, SEC-MALS, etc
Purification protocol development

Develop the individual separation step

- Develop first step
- Develop second step
- Develop third step

- ✔ Screen different separation techniques
- ✔ Screen different resins
- ✔ Optimize conditions
**DoE: Design of Experiment**

A tool for easy method optimization

**Design of Experiments (DoE)**

- A statistical technique for planning and analyzing experiments
- Create a mathematical model of the process
- Used to understand the influence of experimental parameters and to find an optimum for the process
- Minimum number of experiments – maximum information

DoE measures the effect of each factor individually and in combination, allowing detection of interaction to give you more precise results quicker

Reach the same result in 16-32 experiments instead of 128 experiments using the traditional approach!
Integrated experimental design for quicker results

DoE could also be: "Design of Experience"

“I know that pH and conductivity are critical for my purification, how can I optimize the yield?”
Scouting runs for method optimization

Responses (Y’s):
- Capacity
- Yield
- Purity/Selectivity
- Molecular weight
- Activity
- HCP
- DNA
- Aggregates
- Protein A

Factors (X’s):
- Load pH
- Load conductivity
- Load concentration
- Mass load
- Wash volume
- Wash pH
- Wash conductivity
- Elution pH
- Gradient elution
- Step elution level
- Cut OD
- Elution Additive
- Media type
- Column size
- Bed Height
- Flow rate
- Residence time

External data:
- DBC (Frontal analysis)

Peak Data:
- Area
- Concentration
- Amount
- Resolution
- Asymmetry
- Plates per meter

Sample conditions
Wash conditions
Elution conditions
Entire process
Protein Purification Contaminants

- **Particulates**: Include cells and cell debris. Removed using centrifugation or filtration
- **Host Cell Contaminating Proteins**: Gross contaminants removed using chromatography techniques
- **Modified target protein**: Target protein modified through altered amino acid sequence, glycosylation, denaturation, etc. Removed using chromatography
- **Aggregates**: Natural aggregates or as a consequence of partial inclusion bodies solubilization. May be removed using gel filtration chromatography
- **Lipids, lipoproteins**: May be derived from host cells (membranes) or added to a fermentation (antifoams). Removed using chromatography
- **Small molecules**: Include salts, sugars and reagents added to a purification. Typically removed using gel filtration or diafiltration
- **Polyphenols**: Coloured compounds often derived from plant sources or in the fermentation ingredients. Removed by precipitation or chromatography
- **Nucleic acids**: Released during cell lysis. Remove using ion exchange, precipitation techniques such as protamine sulfate, or through hydrolysis with nucleases
- **Pyrogens**: Usually lipopolysaccharides derived from Gram negative bacterial cell walls. Removed using anion exchange chromatography and other methods
- **Virus**
Protein Modifications May Require Further Purification

- Misfolding, random disulfide bridges
- N or C terminal heterogeneity
- Desamidation of Asp and Glu
- Oxidation of methionine
- Aggregation
- Glycosylation
- Phosphorylation
- Acylation
- Proteolytic cleavage
Is the Recombinant Protein Correctly Expressed

- SDS-PAGE – Western blott
- Analytical GF / LS
- Native PAGE / IEF
- Biological activity
- MS or N-terminal sequencing

- Size
- Proteolytic cleavage
- Aggregation
- Heterogeneity
- Stability at different
  - pH
  - Ionic strengths
  - Protein concentrations
  - Detergent concentrations
- Truncated forms
- Heterogenous N-terminus
PURIFICATION STRATEGY

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Three Phase Strategy

- **Capture**
  - isolate product, concentrate, stabilize
  - remove bulk impurities

- **Intermediate Purification**

- **Polishing**
  - achieve final purity, remove trace impurities, structural variants, aggregates etc.
For Efficient Purification Strategies

- Resolution
- Polishing
- Intermediate Purification
- Capture
- Speed
- Capacity
- Recovery
Selection and combination of purification techniques

- Every technique offers a balance between resolution, capacity, speed and recovery
- So, resins should be selected to meet the objectives of the purification step
- GOAL: Fastest route to get a product of required purity
GOAL: Initial purification of the target molecule from clarified source material.
Rapid isolation, and concentration (volume reduction) of the target protein
BONUS: Concentration (smaller and faster columns). Stabilization (removal of proteases)
OPTIMIZATION: Speed and Capacity: Use Macroporous and Highly Substituted matrix
Most suitable techniques: IEX / HIC / (Industry)
or Affinity /IMAC/ IEX / HIC (Academics)
Maximize binding of the target proteins and minimize binding of contaminants during loading
Maximize protein purity during wash & elution
Higher speed that do not affect considerably the dynamic capacity of the column
Intermediate Purification

- **Goal:** Removal of major impurities
- Focus mainly on resolution
- Continuous gradient or multi-step elution
- Most suitable techniques: IEX / HIC or expensive affinity
- For good resolution use around 20% of column capacity with HIC or IEX
- Use a different technique (EIX, HIC, GF, Affinity),
- Or change the selectivity (same IEX at different pH, different ligands or salts concentra for HIC, etc.):
  - **Selectivity optimization**
  - Increase efficiency by using non-porous smaller beads
Final removal of trace contaminants, or separation of closely related substances, like structural variants of the target protein and aggregates.

End product of required high level purity and homogeneity (oligomeric conformation, post-translational modifications, phosphorylation, etc)

Suitable techniques: GF/IEX/HIC (RPC for suitable proteins)
# Properties of each chromatographic techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Capture</th>
<th>Intermed.</th>
<th>Polishing</th>
<th>Start Conditions</th>
<th>End Conditions</th>
<th>Considerations</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF</td>
<td>* * *</td>
<td>*</td>
<td>* * *</td>
<td>Small sample volume</td>
<td>Diluted sample (buffer change)</td>
<td>Limited Sample volume. Limited flow range</td>
</tr>
<tr>
<td>IEX</td>
<td>* * *</td>
<td>* * *</td>
<td>* * *</td>
<td>Low ionic strength</td>
<td>High ionic strength or pH change</td>
<td></td>
</tr>
<tr>
<td>HIC</td>
<td>* *</td>
<td>* * *</td>
<td>*</td>
<td>High ionic strength</td>
<td>Low ionic strength</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>* * *</td>
<td>* * *</td>
<td>* * *</td>
<td>Specific binding conditions</td>
<td>Specific elution conditions</td>
<td>Protein ligand is sensitive to harsh cleaning conditions</td>
</tr>
<tr>
<td>RPC</td>
<td>*</td>
<td>* * *</td>
<td>* * *</td>
<td>Harsh conditions</td>
<td>Use of organic solvents. Loss of biol.activ.</td>
<td></td>
</tr>
</tbody>
</table>
Linking Chromatography Techniques

1. IEX → HIC → GF
2. AC → GF
3. IEX (HIC) → AC → GF
4. \((\text{NH}_4)_2\text{SO}_4\) → HIC → IEX → GF
5. GF (desalting) → IEX → AC → GF
   → IEX or HIC → GF

Before or between chromatography:
- Concentrate sample by: ultrafiltration, AS precipitation, others
- Adjust conductivity and pH: dialysis, desalting, others

Ultrafiltration in large scale:
- Rapid concentration
- Get rid of low MW molecules
- Quick adjusting of conductivity and pH
Purification, crystallization and preliminary X-ray analysis of ferredoxin isolated from thermophilic cyanobacterium Mastigocladus laminosus.

Fish A, Lebendiker M, Nechushtai R & Livnah O

The SDS-PAGE gel analysis of fractions during purification:

1. Supernatant of *Mastigocladus laminosus*
2. After hydrophobic exchange column
3. After anion exchange column
4. After size exclusion column

Lane 5 - molecular weight markers

Crystals diffraction: 1.25 resolution using synchrotron radiation
Recombinant Fd, anion exchange runs.

rec Fd anion exchange on 1 cc Q FF

*Theoretical B concentration is Higher than observed by ~ 3%, due to the lag.*
Anion exchange Q FF  *rec Fd*, optimized run

Native Fd  Rec Fd

Gel fractions

POOL 13-18
**Native Fd, anion exchange runs.**

Open column

AKTA

Peak Fd 40-50% pract and 52-61 theor.

Peak bef. Fd. 35-40% pract. & 47-52% theor

Peak after Fd. 49-71% pract. & 61-78% theor.

Load + wash 20cv 0%B + 30cv 0-100%B

Native Fd. after AS 55% HIC and dial

Gel fractions
Anion exchange Q FF native Fd, optimized run

Native Fd

Rec Fd

Gel fractions

LMW sample

Waste

23 % B
30 % B
35 % B Grad 38 % to 48 % 10CV
38 % B
48 %B
100 % B
Size exclusion runs
On Superdex 75 HR

Native Fd

Rec Fd
The receptor for activated protein kinase C 1 (RACK1): an intracellular adaptor protein

**One step purification on Anion Exchange Column**

*Collaboration with E.Podoly, O.Livnah, H.Soreq*

Pellet of 1 liter culture of recRACK1
Cell Lysis with Microfluidizer
30 x 1.6cm (60ml) Q-Sepharose FF column
NaCl gradient elution in buffer
P38 INTERMEDIATE PURIFICATION
Anion Exchange
P38 FINAL POLISHING
Size Exclusion Chromatography

[Image of a gel electrophoresis and a chart with MW values and UV absorbance at 280 nm]
After Affinity
Highly resolute anion exchange
Resource 15Q 29 x 1cm ~23ml column


1st run main peak of Affinity column

2nd run: main peak of previous run

L16 loop
Optimization of ATOX1 purification

Michal Shoshan from Edit Tshuva lab

ATOX1: SUMO protease treatment after IMAC purification. Ammonium Sulphate precipitation. Load on 120ml Superdex 30 column (20mM MES pH 6.0 + 150mM NaCl)

ATOX1: SUMO protease treatment after IMAC purification. Concentration by UF instead of AS precipitation. Load on 200ml Superdex 30 column (20mM MES pH 6.0 + 150mM NaCl)

ATOX1: SUMO protease treatment after IMAC purification. Concentration by UF in the presence of 4M NaCl. Load on 200ml Superdex 30 column (20mM MES pH 6.0 + 250mM NaCl)
MBP fusion protein

Capture: combined IMAC and Dextrine-Sepharose Affinity Chromatography

Intermediate purification: Gel Filtration
**MBP fusion protein**

Protein Characterization: Analytical SEC and SEC-MALS (Multi-angle light scattering)

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**A**
Ve: 9.63ml

**B**
Ve: 14.70ml

**C**

- 130±60 kDa
- 68±10 kDa

**D**
Ve: 14.63ml

Ve: 14.77ml

Ve: 9.47ml
MBP fusion protein
Optimization of Size Exclusion Chromatography

BSA dimer

67
43

14 16 18 19 20 21 22 23 24 26 28
GTTP Pyrophosphokinase Capture: IMAC column

Chromatogram Questions

1. GTTPyrophosphoKinase Lysis Microfluidizer, spin and filter from 1L in ~50ml lysis buffer
2. column: NiSephFF 4ml
3. Aliq: &18 10mMHEPESpH7.4+0.3MNaCl+10%Glyc+10mMimid B1:500mMimid

POOL tubes 11-16 and add 50mM NaGlu & Arg - concentr tubes 9.10 & 17-21 to ~2.5 ml and add to pool
Chromatogram Questions

No 1: Sample: Nxl after Ni; concentr. & TEV ON (1:20 v:v)

No 2: Column: Sup12 2col.100 x 1cm (~200ml) - 3ml/fract

No 3: All: 10mMHEPESpH7.4 0.3MNaCl 5% Glyc

GTTP Pyrophosphokinase
Intermediate: SEC

POOL 21-31
Chromatogram Questions

No 1: GTTPyrophosphokinase after Ni, GF and dil 1:2 H2O pool 21-31 after GF dil. 1:1.8

No 2: Column: Fractogel EMD SO3 (M) 20x0.5cm=4ml

No 3: A11&A18: 20mM MES pH6.0 50mMNaCl B1: A + 1MNaCl

GTTP Pyrophosphokinase Polishing: CEIX

POOL 27-42
Strategy for Optimization of Complex Formation:
Co-purification of two proteins (separately expressed)
Complex stabilization: 0.01% Tween-20

Nadav Komornik / Oded Livnah lab

Strategy for Optimization of Complex Formation:
Co-purification of two proteins (separately expressed)
Complex stabilization: 0.01% Tween-20

Nadav Komornik / Oded Livnah lab
A Systematic Approach to Project Development Summary

- Invest time in strategic design of your expression/purification project: construct, host, vector, tag, bio-informatic, critical impurities, etc.
- Set the aims (purity and quantity)
- Establish a fast and reliable assay for the target protein
- Expression optimization or found best natural target source
- Low Scale Purification: Check different columns. Optimization conditions (buffers, etc)
- First characterization of the target protein: PAGE-SDS, Western blot, Activity, Analytical GF (oligomeric state)
- Medium Scale Purification: Select techniques and solutions compatible with sample stability
- Further steps: Use different separation principles or a different selectivity. Optimize. Use more resolutive small non-porous beads
- Use few steps and limit sample handling between purification steps: avoid lengthy procedures which reduce activity and recovery (dialysis, dilutions, long assays, etc)
- Suggestion: use size-exclusion chromatography as final purification step: further separation, final storage buffer, and separation of monomers from multimers
- Before scale up, optimize concentration and storage conditions for protein target (physico-chemical and biological properties)
- Final protein characterization: PAGE-SDS, Analytical GF (oligomeric state), MS
PURIFICATION STRATEGY

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A simple workflow for quality control

- Experiment can be performed in most biochemistry laboratories with the help of core facilities.

- Two part workflow:
  - Initial sample assessment
  - Sample optimization
Initial Sample Assessment: Purity and Integrity

- SDS-PAGE with sensitive staining
  - Coomassie Reverse zinc stain
  - Fluorescent or silver stain
  - >100ng >10ng >1ng
  - Detection of potential contaminants

- Capillary electrophoresis

- Mass spectrometry
  - Intact mass
  - N and C terminal sequencing
  - Post translational modifications
  - Contaminant identification

http://www.diarect.com/fileadmin/newsletter/archive/0210/
Initial Sample Assessment: Purity and Integrity

- Initial sample assessment
- Sample optimization
- Protein production and purification
- Purity and integrity assessment
- Passed
- Homogeneity assessment
- Failed
Initial sample assessment: Homogeneity

- Dynamic light scattering (DLS)
  - Can detect low amounts of aggregates
  - Low resolution: does not distinguish between monomer and dimer

- Size exclusion chromatography
  - Can detect oligomers
  - Can be coupled to a static light scattering to determine Molar Mass

Initial sample assessment: Homogeneity

Protein production and purification

- Purity and integrity assessment
  - Passed
  - Failed
- Homogeneity assessment
  - Passed
  - Failed
- Activity assessment
  - Failed
- Homogeneity and solubility optimization
  - Failed
Initial Sample Assessment : Activity

- Protein-specific functional assays
- When possible the method of choice should quantify the active concentration
  - When function involves binding, the “calibration-free concentration analysis” (CFCA) method, available in different SPR instruments allows to determine the active concentration
Initial Sample Assessment: Activity

- Initial sample assessment
- Sample optimization
- Protein production and purification
- Purity and integrity assessment
  - Passed
  - Homogeneity assessment
    - Passed
    - Activity assessment
      - Passed
      - Final optimum protein sample
The **minimum quality control** relies on

- SDS-PAGE (or similar)
- Size exclusion chromatography
- Mass spectrometry
- UV-Visible spectrometry

<table>
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<th>Step</th>
<th>First-line methods</th>
<th>Complementary methods</th>
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<td>Purity and Integrity</td>
<td>SDS-PAGE with sensitive staining and Mass Spectrometry (MS) or Capillary Electrophoresis (CE) in SDS</td>
<td>Isoelectric Focusing (IEF) UV spectroscopy (UV-Spec)</td>
</tr>
<tr>
<td>Homogeneity</td>
<td>Dynamic Light Scattering (DLS) and Size Exclusion Chromatography (SEC)</td>
<td>UV-Spec Fluorescence spectroscopy</td>
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<tr>
<td>Activity</td>
<td>Active concentration measurement: Functional assay, SPR, ... and Total concentration measurement: UV-Spec</td>
<td>Infrared spectroscopy Quantitative amino acid analysis (AAA)</td>
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<tr>
<td>Homogeneity and solubility</td>
<td>DLS</td>
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<tr>
<td>Time-stability and storage</td>
<td>DLS Functional assay</td>
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<td>Reproducibility (lot-to-lot consistency)</td>
<td>UV-Spec DLS MS SEC</td>
<td>Circular Dichroism (CD) Differential Scanning Calorimetry (DSC)</td>
</tr>
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</table>
Protein Characterization: Sample Homogeneity

- PAGE-SDS over a range of concentrations
  - Higher concentrations: information about few amount of contaminants
  - Lower Concentrations: reveal if there are more than one protein of similar MW
  - PAGE-SDS +/- BME or DTT +/- heat treatment
  - Western
- Isoelectric focusing (IEF)
- Non-denaturative native gel (basic, neutral, acid) +/- detergents
- Two-dimensional IEF/PAGE
- Protein Concentration
- Biological activity - Specific activity

- Analytical IEX & GF (FPLC) / RPC (HPLC) / capillary electrophoresis
- Mass spectroscopy

- Visible/UV/fluorescence spectroscopy/CD
  - Ratio 260/280nm for proteins complexed with nucleic acids or with nucleotide coenzymes
  - Some other ratio with proteins that contain porphyrin or other chromophoric cofactors
- Antibody reactivity
- Metal-ion content or cofactor content
- Pattern of protease digestion
Storage of biological samples
General recommendations for purified proteins

According to the website of The Protein Purification Service of The EMBL (European Molecular Biology Laboratory)

General advice
Cannot be applied to every biological sample. Consider properties of sample and its intended use before following any of these recommendations.

- For short term storage [up to 24 h], most proteins can be kept at 4°C.
- For storage times longer than 24 h at 4°C, it may be necessary to filter sterilize the protein preparation [through a 0.22 µm filter] or to add a bacteriostatic agent [e.g. 0.02% sodium azide] to avoid bacterial growth. **Note that not all proteins are stable at 4°C for longer periods.**
- For long term storage [more than a week]:
  It becomes necessary to freeze the protein preparation.
  Freeze it rapidly using liquid nitrogen to avoid denaturation.
  Freeze the solution in small aliquots to avoid repeated freezing and thawing which may reduce the biological activity or affect the structure.
  Several stabilizing agents can be added, such as glycerol [5-50% [w/v]], serum albumin [10 mg/ml], reducing agents [such as 1 mM DTT], and ligands [the nature and concentration depending on the nature and concentration of the target protein].
Storage of biological samples
General recommendations for purified proteins

According to the website of The Protein Purification Service of The EMBL (European Molecular Biology Laboratory)

Before anyone of these procedures, check stability of the protein with a little sample.
Aliquot before freezing

- **For several months storage at -20°C:** At this temperature it is recommended to add 50% glycerol to the solution to avoid freezing (or dialyze vs. buffer with 50% glycerol).

- **For longer periods storage [months to years],** freeze it at -70°C or even in liquid nitrogen. Although it is not really necessary to add glycerol at these temperatures, the addition of 5-50% glycerol could help to keep the protein stable.

- **Alternative methods are:**
  
  Storage of the protein at 4°C as an ammonium sulfate precipitate (4M).
  
  Storage of the protein at 4°C or lower in a lyophilized form (the protein could be dissolved in a volatile buffer [such as trimethylamine/HCl; pH range 6.8-8.8]. Note that not all proteins are stable during the freeze-drying process.

- **Proteins sensitive to temperature:** should not be stored at 4°C as they precipitate or lost ativity at this temperature. Keep at room temperature in the presence of a preserving agent.
Guidelines for Protein Purification

- Define objectives: Purity, activity and quantity of final product. Avoid over or under developing a method.
- Define properties of target protein and critical impurities: to simplify technique selection and optimization.
- Develop analytical assays: for fast detection of protein activity/recovery and to work efficiently.
- Remove damaging contaminants like proteases early: *work quickly, at 4ºC + Protease Inhibitors*.
- Use big macroporous and highly substituted matrix in the capture step: reduce the process volume significantly as an early-stage operation.
- Minimize number of steps. Combine steps logically. Use a different technique at each step (EIX, HIC, GF, Affinity), or change the selectivity (same IEX at different pH).
- Steps that exploit the greatest differences in the physical properties of the product and the impurities.
- Use non-porous smaller beads (very expensive) toward the end, leading to a lower cost of processing.
- Minimize sample handling at every stage: to avoid lengthy procedures which reduce activity and recovery (dialysis, dilutions, long assays, etc).
- Minimize use of additives: they must be removed in extra purification steps or may interfere with activity assays.

*KEEP IT SIMPLE!!!!!!*