Introduction to Protein Purification

Input for Protocol Development

Guidelines/Flowchart for Protein Production

Know your Protein

Detection and quality: PAGE-SDS, others

Day 1 (29/10) **Introduction** to Protein Purification Facility. Flowchart and input for Purification Protocol Development - Guidelines for Protein Purification –Know your Protein

Day 2 (05/11) **Basic principles on protein expression and bacterial systems**

Day 3 (12/11) **Sample Preparation before Chromatography.** Cell disruption considerations. Centrifugation. Filtration - Chromatography: General Considerations. Other basic tools in protein production: Ultrafiltration and other protein concentration methods. Dialysis. Protein quantification: different methods. Protein analysis (PAGE-SDS, native gels, IEF, others)

Day 4 (19/11) **Ion Exchange**: Main stages in chromatography. Basis for selectivity. Operational considerations. Determination of start conditions. Parameters for optimization. Examples.

Day 5 (26/11) **Gel Filtration Chromatography** and desalting: Main stages in chromatography. Basis for selectivity. Operational considerations. Determination of start conditions. Parameters for optimization. Examples. SEC-MALS

Day 6 (03/12) **Hydrophobic Exchange** Chromatography: Basis for selectivity. Determination of start conditions. Operational considerations. Parameters for optimization. Examples.

Other Purification Techniques: Multimode Resins (HIC + IEX & GF + IEX), Hydroxyapatite, Salt tolerant, Reverse Phase and more

Day 7 (10/12) **Affinity chromatography**: Main stages. Advantages and Disadvantages. Type of Affinities. Designing and preparing an affinity gel. Antibody purification for Therapy. Future Trend in Pharma.

Day 8 (17/12) **Genetically engineered Tags, recombinant proteins**. Cleavage Sites. Parameters for optimization. Recombinant Proteins and aggregation problems. Examples

Day 9 (24/12) **Mammalian Cell expression**

Day 10 (31/12) Selection and combination of purification techniques. Examples. Storage.

Day 11 (07/01) **Aggregation problems**. Mechanism of aggregation. Methods for screening solubility. Considerations when selecting buffer components. Additives that increase yield and prevent aggregation and/or stabilize proteins

Day 12 (14/01) **Refolding**: Major steps. Refolding methods. Screening. Strategies and examples.

Day 13 (21/01) **Protein characterization (QC)**. Major requirements for purification of proteins for structural studies: crystallography, NMR, others. Minimal QC criteria in Academic and Pharma

Day 14 (28/01) **Membrane protein** purification. Main features, use and removal of detergents. General approach for purification of **big protein complex**, virus, nano-vesicles

PURIFICATION STRATEGY I

- The Protein Purification Facility
- Pipeline for Purification
- **General approach**: Input for Protocol Development
- Guidelines for Protein Purification. Commonly confronted decisions. Properties of Target Protein: Know your protein
- Detection and quality: PAGE-SDS, others

The Protein Expression and Purification Facilities

http://wolfson.huji.ac.il/purification

Educational and Research Center for Protein Expression & Purification

Users:

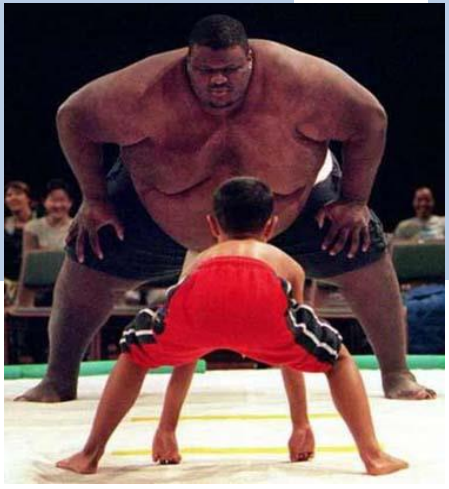
Hebrew University
Academic Institutes
Biotech/Pharma industry

Mode of Operation

1. Expression and purification services
2. Individual training and supervision of projects
3. Annual workshop and course
4. Repository of specialized reagents, vectors and cells

Large and small scale projects

- Large-scale high purity
- Structure analysis
- Animal studies
- Therapy prove of concept
- Vaccines
- Ab production



- Small scale
- Enzymatic assays
- Cellular systems
- Protein characterization
- Protein-Protein Interaction

Protein Production and Purification Partnership in Europe

Network of > 150 protein facilities
Mainly but not exclusively in Europe

- Started in 2010
- Share expertise
- Exchange materials & protocols
- New tools & technologies
- Establish standards
- Benchmarking
- Enable collaboration
- Access to external facilities
- Training of staff/users
- 15 Meetings and few courses

http://wolfson.huji.ac.il/purification/

האוניברסיטה העברית בירושלים
The Hebrew University of Jerusalem

Faculty of Science

The Wolfson Centre for Applied Structural Biology

Home | NMR | X-Ray | Protein Purification | Protein Expression | Academic Committee | Wolfson Foundation | Centre Personnel | Search

The Protein Purification Facility

HOME Table of Content Selected Protocols	EXPRESSION SYSTEMS	EXTRACTION AND CLARIFICATION	PURIFICATION	CHARACTERIZATION	OTHERS

Dr. Mario Lebendiker

Entries Since September 2006 80382
mariol@cc.huji.ac.il

Tel: 972-2-6586920

The Protein Purification Facility is a resource of information and assistance available to researches and students as well as biotech and pharmaceutical companies that are interested in protein purification. Our Unit assists researches to overcome the major bottleneck in structure determination by X-Ray crystallography or NMR, that is the preparation of suitable crystalline samples. Mainly we provide consultation and active support for researchers and students who are interested in using the equipment, methods and materials of the Facility.

The unit offers a complete and fully automated liquid chromatography system designed for method development and research applications, that simplifies the transition from laboratory to full-scale production. We have columns and resins for purification according to size, charge, hydrophobicity and substrate affinity. Gel electrophoresis and IEF (isoelectric focusing) apparatus; cell disruption, blotting and ultrafiltration systems.

The facility assists researchers, students and people from the biotechnology industry in resolving their protein purification problems. We are actively involved in many collaborations for structural and biochemical studies; isolation and identification of new proteins.

Due to the unique equipment and wide experience in the field, the protein purification facility is the only place in Israel that provides comprehensive services in this area, and also functions as a learning station.

Protein Information Submission Form (pdf)

Table of Contents

PURIFICATION PROTOCOLS

poly His-Tagged Fusion Proteins

NOVAGEN: pET purification manual (pdf)
NOVAGEN: Ni-NTA Hi-Bind Resins Protocols (pdf)
NOVAGEN: His-Tag GST-Tag purification and Detection tools (pdf)
QIAGEN: Ni-NTA purification manual (pdf)
QIAGEN: NiNTA cell lysis under nature conditions (pdf)
MERCK: Metal Chelate Affinity Chromatography (pdf) (pdf-II)
IBA Tools for protein expression & purification. 6xHis-tag & Ni-NTA technology: The optimal partner for Strep-tag in double tag proteins (pdf)
IBA: Expression and purification of proteins using Strep-tag and/or 6xHistidine-tag. A comprehensive manual. (pdf)
IBA Mammalian expression and purification system using Strep-tag and/or 6xHistidine-tag (pdf)
CLONTECH: Talon Resin Protocol (pdf)
CLONTECH: Talon Products (pdf)
CLONTECH: BD Talon™ Metal Affinity Resins User Manual (pdf)
CLONTECH: Purification in the presence of Beta Mercaptoethanol (pdf)
CLONTECH: Protein Purification Products (pdf)
CLONTECH: BD HAT Protein Expression and Purification System User Manual (pdf)
AMERSHAM-BIOSCIENCES Hi Trap Chelating (pdf)
AMERSHAM-BIOSCIENCES Hi Trap Chelating Application Note (pdf)
AMERSHAM-BIOSCIENCES Chelating Sepharose Fast Flow Instructions (pdf)
AMERSHAM-BIOSCIENCES His GraviTrap prepacked, single-use column for purification of histidine-tagged proteins by immobilized metal affinity chromatography (IMAC) without any need for a purification system. Negligible nickel leakage and is compatible with denaturing and reducing agents as well as a wide range of additives. Short purification times. High protein binding capacity, ≈ 40 mg/column. Purifies unclarified samples (pdf)
AMERSHAM-BIOSCIENCES His SpinTrap™ is a prepacked, single-use spin column for purifying histidine-tagged proteins by immobilized metal ion affinity chromatography (IMAC). The resin has high protein binding capacity, low nickel ion (Ni2+) leakage, and excellent compatibility with denaturing agents plus a wide range of additives. His SpinTrap is used with a standard microcentrifuge and one purification run takes approx. 10 min. His SpinTrap columns allow direct purification of unclarified cell lysates. (pdf-I) (pdf-II)
AMERSHAM-BIOSCIENCES HisTrap™ FF crude. This pre-prepacked column is intended for preparative purification of histidine-tagged recombinant proteins from unclarified lysate without precentrifugation and filtration of the sample. Leakage of Ni2+ is very low. The medium is compatible with all commonly used aqueous buffers, reducing agents, denaturants such as 6 M Gua-HCl

EXTRACTION and CLARIFICATION

Preparation of Cell Lysates / Different Disruption Methods Mitochondria, Nuclear & Membrane Isolation	Choice of Buffers	Inhibitors Apoptosis Inhibitors Cell Division/Cell Cycle/Cell Adhesion Inhibitors Lipid Signal Inhibitors Neurobiology/Neurodegeneration Inhibitors Nitric Oxid/Oxidative Stress Inhibitors Phosphorilation/Dephosphorilation Protease Inhibitors Others	DNA removal Endotoxin removal	Detergents and detergent removal	Concentration / Ultrafiltration / Dialysis / Desalting columns

Preparation of Cell Lysates / Different Disruption Methods

NOVAGEN - A comprehensive selection of sample preparation tools for three main areas of protein research: expression proteomics, functional

The Protein Purification Facility
The Wolfson Centre for Applied Structural Biology
The Hebrew University of Jerusalem
Dr. Mario Lebendiker
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EXPRESSION SYSTEMS

Bacterial Exression Systems		
GST	Drosophila Expression System	In-Vitro Translation
poly His	Mammalian	
Maltose	Insect	
Calmodulin	Leishmania	
Intein	Yeast	
Biotinylated		
Two Step: poly His and Strep-Tag		
Solubility enhancement tags (SETs)		
SUMO		
Cold Expression		
Single Protein Production		

Bacterial Expression Systems

CHARACTERIZATION PROTOCOLS

Protein Quantitation

Absorbance 280nm
BCA
Biuret
Bradford
CBQCA
Comparision of Methods
Coomasie
ESL
Biotinylated proteins
Glycoprotein Carbohydr.
Interfering Substances
Lowry
Nano Orange
Non-Interfering
OPA (fluorescent)
Preparation Reagent
Protein Precipitation

Gel Electrophoresis

PAGE-SDS (Laemmli)
Basic-Native
Acidic-Native
Tricine

Proteomics Sample Preparation

Protein Precipitation

Protein extraction from Polyacrylamide Gel

Gel Stain

Coomasie Blue
Fluorescence
Glycoprotein
Lypopolysaccharide
Oligohistidine
Phosphoprotein
Silver Stain
Zinc Stain

Proteases

Analytical Ultracentrifugation

Protein Quantitation

Comparision of Different Protein Determination Methods

Absorbance 280nm According toProtein Protocols in CD Rom
Bradford
Lowry According toProtein Protocols in CD Rom

Selected Protocols of The Protein Purification Unit and Others

Preparation of Cell Lysates / Different Disruption Methods

Bacterial Protein Extraction (mini-scale) Sonication
Bacterial Protein Extraction (mini-scale) Using B-Per
PROTEIN EXPRESSION FACILITY of THE HEBREW UNIVERSITY *Sonication of bacterial samples*

Choice of Buffers

AMERSHAM BIOSCIENCES *Recommended buffers for Anion Exchange Chromatography*
AMERSHAM BIOSCIENCES *Recommended buffers for Cation Exchange Chromatography*
AMERSHAM BIOSCIENCES *Recommended Volatile buffers systems for Ion Exchange Chromatography*
PROTEIN EXPRESSION AND PURIFICATION FACILITY OF THE EUROPEAN MOLECULAR BIOLOGY LABORATORY *Choice of lysis buffer and additives*
PROTEIN EXPRESSION AND PURIFICATION FACILITY OF THE EUROPEAN MOLECULAR BIOLOGY LABORATORY *Solubility Studies*
Preparation of soluble/insoluble protein from cells

DNA removal

Removal of Nucleic Acids - Different Protocols

Purification Strategy - Others

Test Tube

Purification of Recombinant Proteins

Small scale GST-fusion protein purification under nature conditions
Small scale His-Tag fusion protein purification under nature conditions
Small scale His-Tag fusion protein purification under denaturative conditions
Small scale MBP-fusion protein purification

Protein purification is diverse

Basic purification is the same……strategy change

Several types of target protein

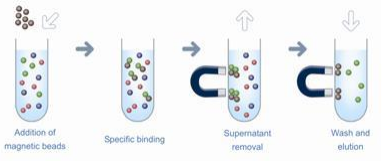
- ✓ Recombinant
- ✓ From natural source
- ✓ Affinity tagged
- ✓ Non-tagged
- ✓ Membrane protein
- ✓ Soluble protein
- ✓ Protein complex

Different objectives

- ✓ Functional studies
- ✓ Structural studies
- ✓ Antigen to prepare Ab
- ✓ Therapy prove of concept
- ✓ Human Therapy

Several scales

Various equipment



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

For each application you need:

✓different quantities

✓different protein purity

✓start material is different

✓Different timeline

✓different strategy

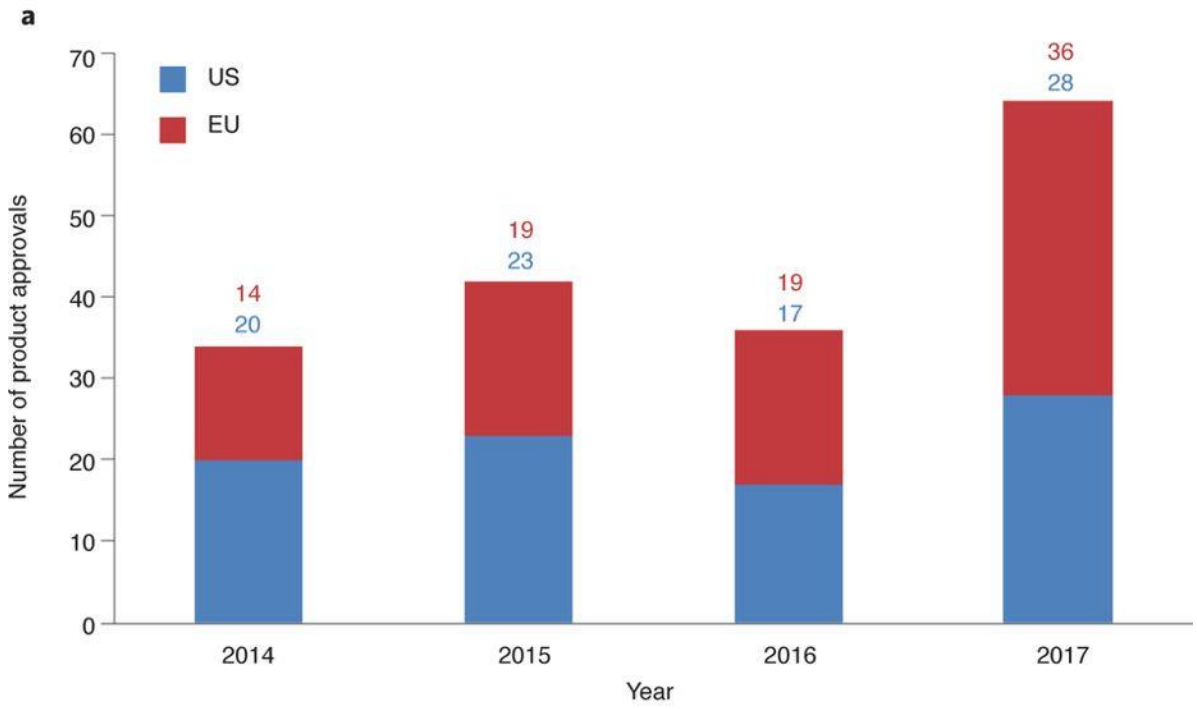
- Post-translational modification tests
- N-terminal sequencing
- Electromobility shift assay (band shift)
- DNA footprinting
- Protein cross-linking studies
- Vaccine development/production
- Probes for protein arrays/chips
- Expression library screening
- Other

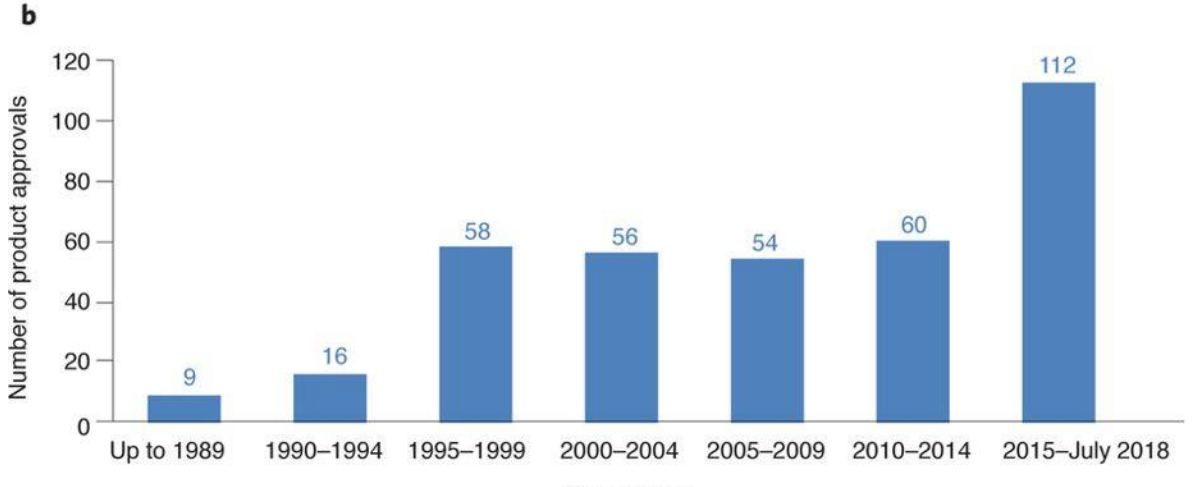
Each purification project must be adapted to your start material and your final needs

Check your final protein: Don’t waste clear thinking on dirty or not healthy proteins!!!!

Biopharmaceutical approvals products in 2017

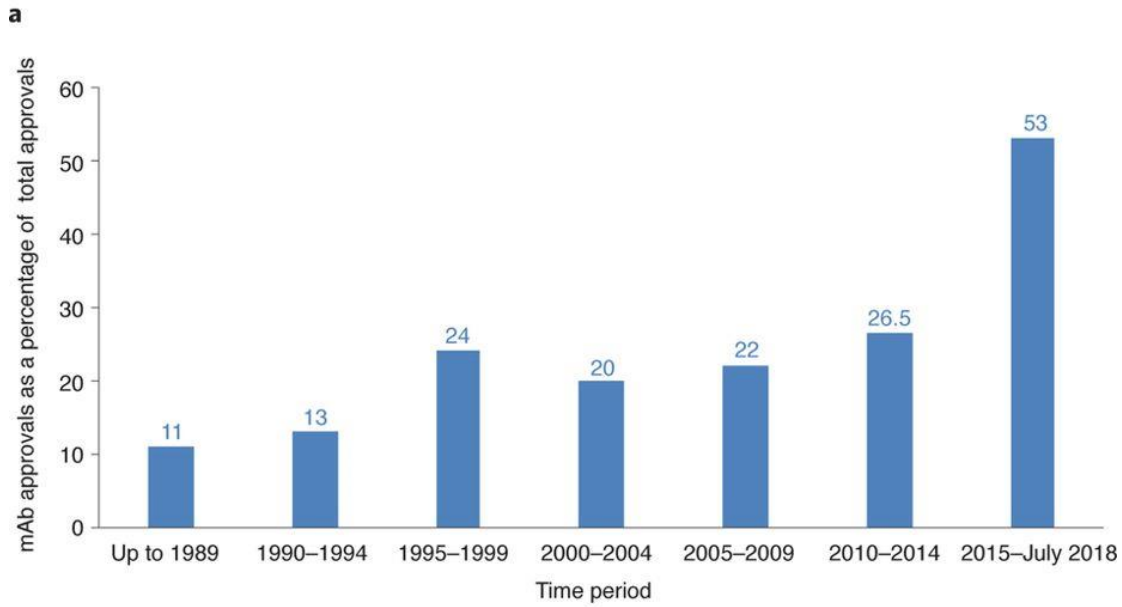
Biopharmaceutical benchmarks 2018 Gary Walsh *Nature Biotechnology* ***volume36****, pages1136–1145 (2018)*

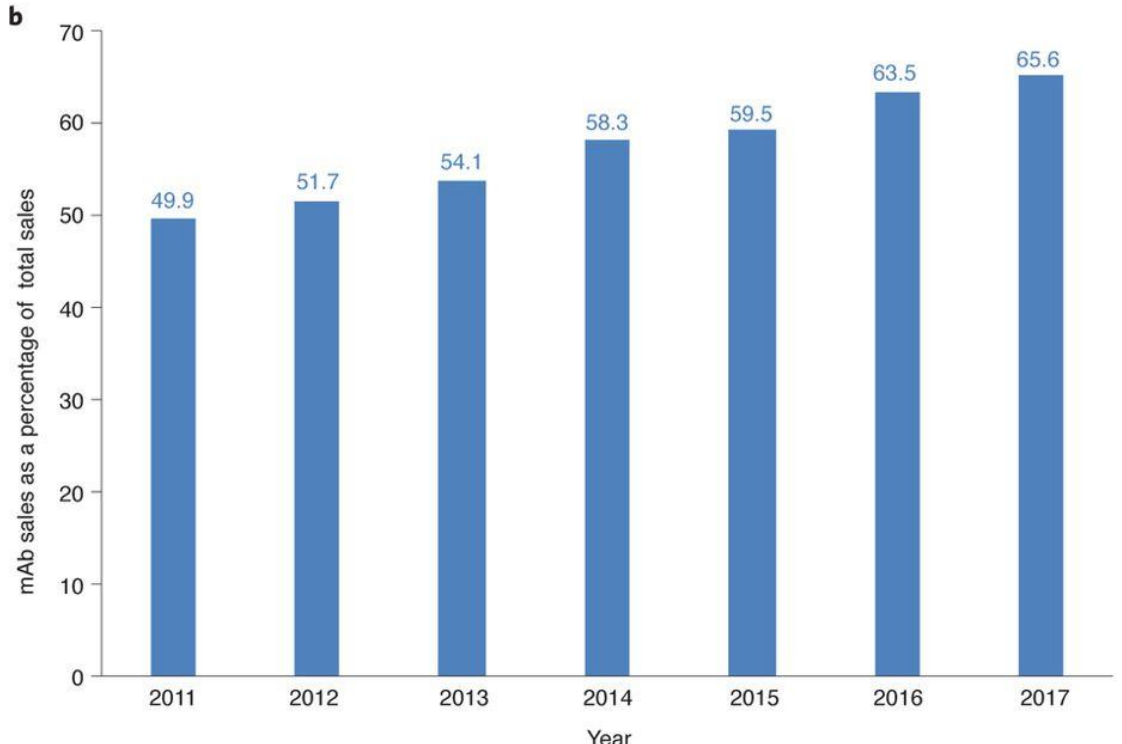




Overview of mAb approvals

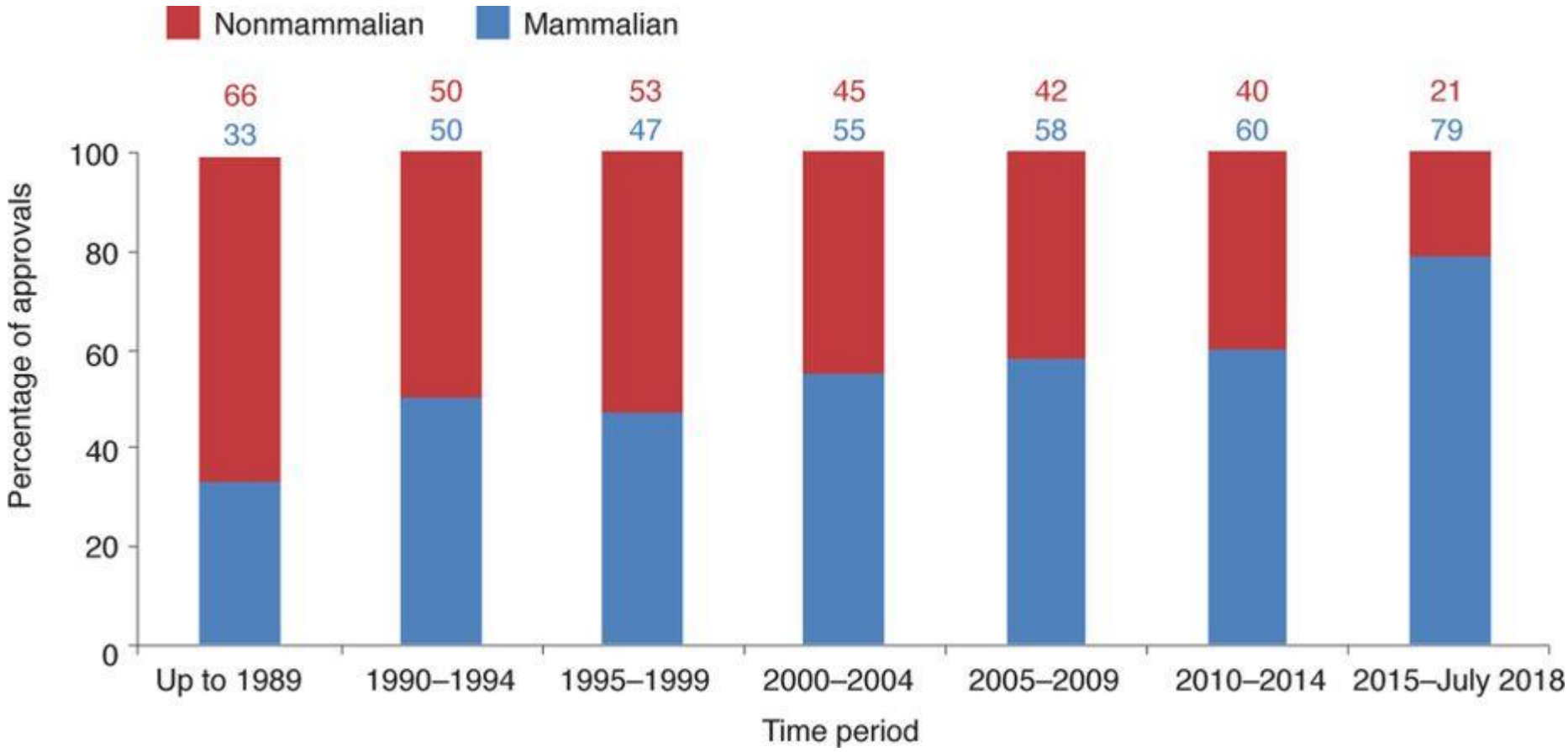
Biopharmaceutical benchmarks 2018
Gary Walsh Nature Biotechnology
***volume36**, pages1136–1145 (2018)*

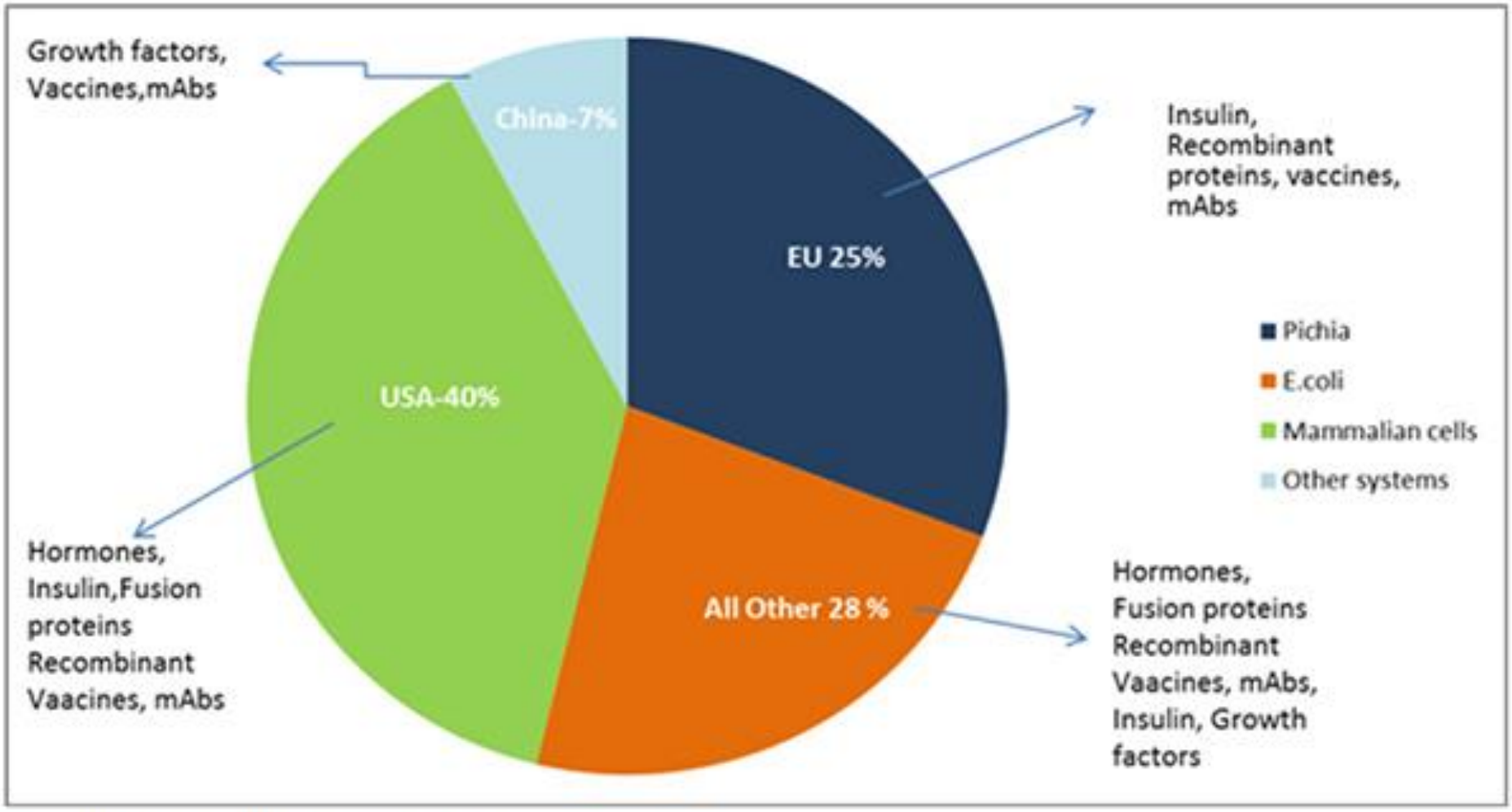




Manufacture of biopharmaceuticals: Relative use of mammalian- versus nonmammalian-based production cell lines

Biopharmaceutical benchmarks 2018 *Gary Walsh* *Nature Biotechnology* ***volume36****, pages1136–1145 (2018)*





Global Therapeutic Protein Market

Proteins in Pharma: sales

#1. Humira® (adalimumab)
AbbVie **% Change:** 8.2%
2018 Sales: $19.936 billion
2017 Sales: $18.427 billion

#2. Eliquis® (apixaban)
Bristol-Myers Squibb and Pfizer
2018 Sales: $9.872 billion ($6.438 billion BMS + $3.434 billion Pfizer) [4]
2017 Sales: $7.395 billion ($4.872 billion BMS + $2.523 billion Pfizer)

#3. Revlimid (lenalidomide)
Celgene **% Change:** 18.3%
2018 Sales: $9.685 billion
2017 Sales: $8.187 billion

#4. Opdivo® (nivolumed)
Bristol-Myers Squibb and Ono Pharmaceutical **% Change:** 31.4%
2018 Sales: $7.570 **2017 Sales:** $5.763 billion

#5. Keytruda® (pembrolizumab)
Merck & Co.
2018 Sales: $7.171 billion
2017 Sales: $3.809 billion

#6. Enbrel® (etanercept)
Amgen and Pfizer **% Change:** –9.6%
2018 Sales: $7.126 billion ($5.014 billion Amgen + $2.112 billion Pfizer) [3]
2017 Sales: $7.885 billion ($5.433 billion Amgen + $2.452 billion Pfizer) [3]

#7. Herceptin® (trastuzumab)
Roche (Genentech) **% Change:** –0.5%
2018 Sales: $6.981 billion (CHF 6.982 billion)
2017 Sales: $7.013 billion (CHF 7.014 billion)

#8. Avastin® (bevacizumab)
Roche (Genentech) **% Change:** 2.4%
2018 Sales: $6.847 billion (CHF 6.849 billion)
2017 Sales: $6.686 billion (CHF 6.688 billion)

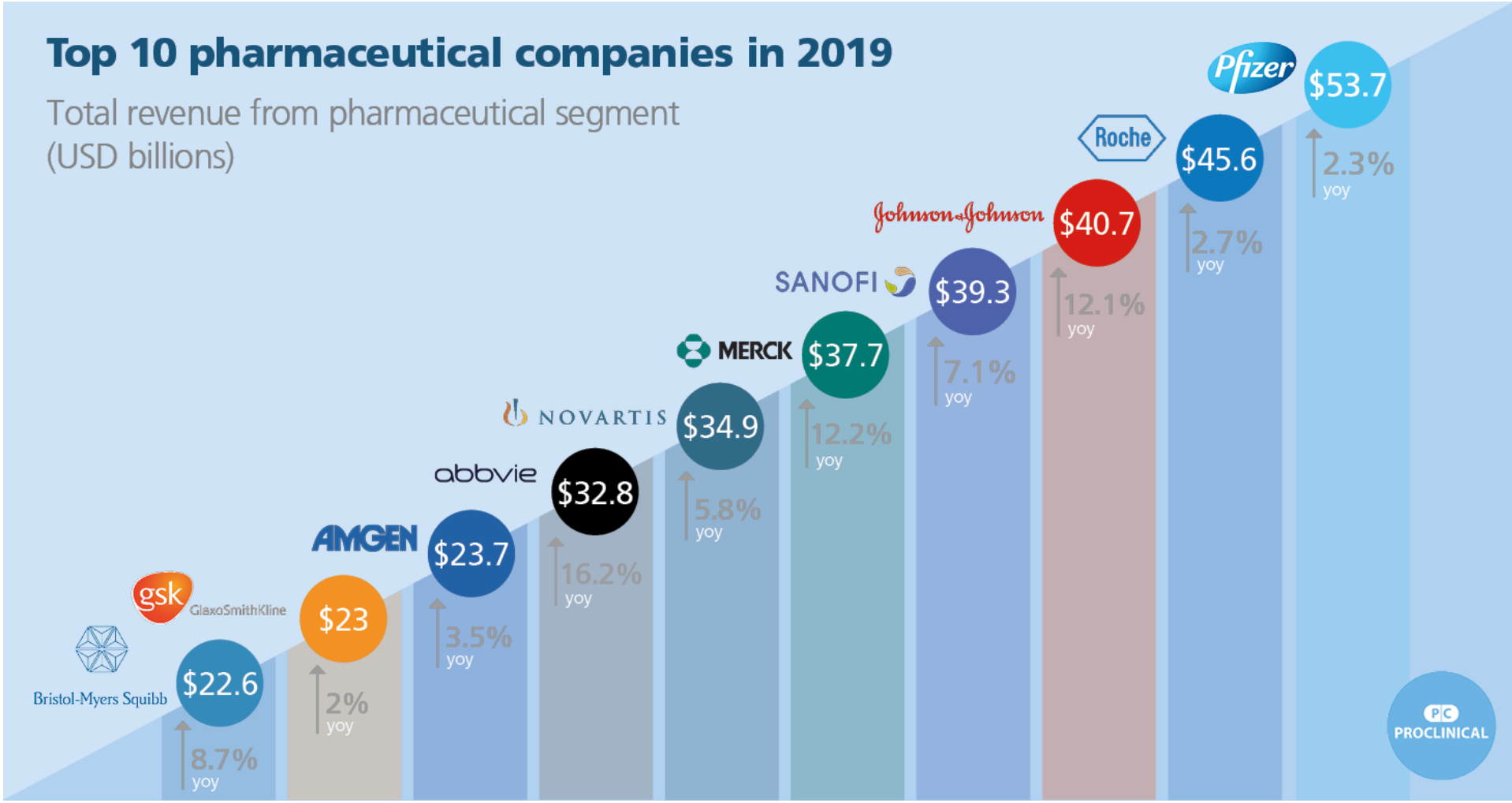
#9. Rituxan® (also sold as MabThera; rituximab)
Roche (Genentech) and Biogen [2]
2018 Sales: $6.750 billion [CHF 6.752 billion] [2]
2017 Sales: $7.298 billion [CHF 7.300 billion] [2]
% Change: –7.5%

GEN Genetic Engineering & Biotechnology News
Mary Ann Liebert, Inc. publishers

Top-selling biopharmaceutical products in 2017

Biopharmaceutical benchmarks 2018 Gary Walsh *Nature Biotechnology* ***volume36****, pages1136–1145 (2018)*

Rank	Product	Sales, 2017 ($ billions)[a]	Cumulative sales, 2014–2017 ($ billions)	Year first approved	Company	Patent expiry[b]	Biosimilar version(s) approved
1	Humira (adalimumab; anti-TNF)	18.94	62.6	2002	AbbVie, Eisai	2016 (US) 2018 (EU)	Halimatoz/Hefiya/Hyrimoz,Amgevita/Amjevita/Solymbic, Cyltezo, Imraldi
2	Enbrel (etanercept; anti-TNF)	8.34	35.4	1998	Amgen, Pfizer, Takeda Pharmaceuticals	2015 (EU) 2028 (US)	Erelzi, Benepali
3	Rituxan/MabThera(rituximab; anti-CD20)	7.78	29.1	1997	Roche, Biogen Idec	2013 (EU) 2016 (US)	Blitzima/Truxima, Ritemvia, Rituzena, Rixathon/Riximyo
4	Remicade (infliximab; anti-TNF)	7.77	35.6	1998	Johnson & Johnson, Merck, Mitsubishi Tanabe Pharma	2015 (EU) 2018 (US)	Zessly, Ixifi, Renflexis/Flixabi, Inflectra/Remsima
5	Herceptin (trastuzumab; anti-HER2)	7.39	27.1	1998	Roche	2014 (EU) 2019 (US)	Herzuma, Kanjinti, Trazimera, Ogivri, Ontruzant
6	Avastin (bevacizumab; anti-VEGF)	7.04	27.0	2004	Roche	2017 (US) 2019 (EU)	Mvasi
7	Lantus (insulin glargine)	6.72	27.4	2000	Sanofi	2014 (EU & US)	Semglee, Lusduna, Abasaglar/Basaglar
8	Eylea (aflibercept; anti-VEGF)	5.93	18.0	2011	Regeneron, Bayer	2020 (EU) 2021 (US)	
9	Opdivo (nivolumab; anti-PD-1 receptor)	5.79	11.4	2014	Bristol-Myers Squibb, Ono Pharmaceutical	2027 (US) 2026 (EU)	


Top 10 pharmaceutical companies in 2019
Total revenue from pharmaceutical segment
(USD billions)
Bristol-Myers Squibb
$22.6
8.7% yoy
GlaxoSmithKline
$23
2% yoy
AMGEN
$23.7
3.5% yoy
abbvie
$32.8
16.2% yoy
NOVARTIS
$34.9
5.8% yoy
MERCK
$37.7
12.2% yoy
SANOFI
$39.3
7.1% yoy
Johnson & Johnson
$40.7
12.1% yoy
Roche
$45.6
2.7% yoy
Pfizer
$53.7
2.3% yoy
PROCLINICAL

Manufacture of biopharmaceuticals: Future directions

Biopharmaceutical benchmarks 2018 *Gary Walsh* *Nature Biotechnology* ***volume36****, pages1136–1145 (2018)*

Some 40% of the 6,000 or more products currently in clinical development globally are biopharmaceuticals

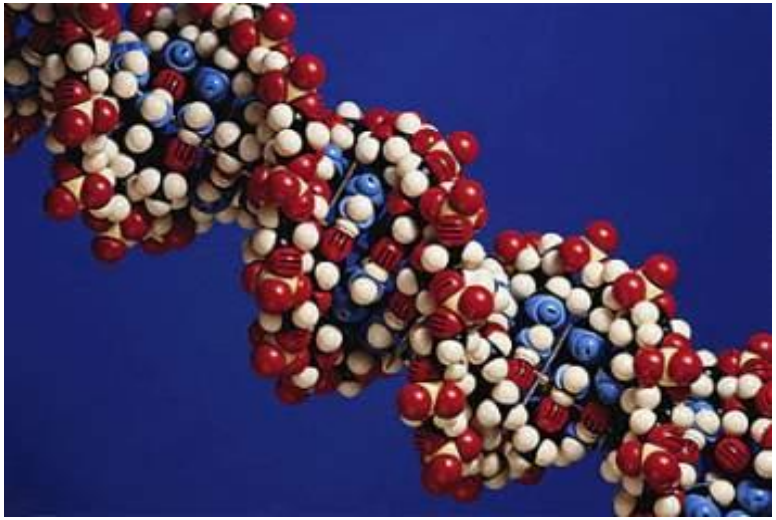
Biopharmaceutical approvals over the next few years will continue to be predominantly protein-based (rather than nucleic acid- or cell-based), that they will be produced largely using conventional mammalian cell expression systems

Biosimilars will continue to feature with increasing prominence in the global biopharmaceutical landscape, but their greatest impact will continue to be in regions outside the more developed markets, such as the US and EU. It seems likely that the rapid growth of biosimilar products will continue over the years to come.

Advances in adeno-associated virus (AAV) and lentiviral gene therapy modalities (particularly in *ex vivo* cellular therapy contexts)—together with increasing interest in CRISPR endonuclease-based gene editing

Some Situations Demand Higher Purity

Laboratory Scale



Raising antibodies or
Biochemical studies: > 90-95%

Structural (X-Ray, NMR)
or Functional Studies > 95%

Characterization: > 99%

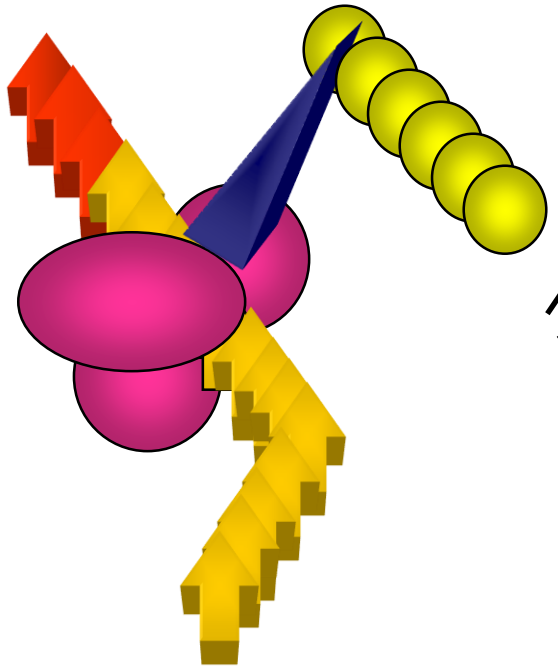
Production Scale

- Authority regulated
- e.g. FDA
- Impurities to check:
- DNA
- Endotoxins
- Host cell proteins
- Modified forms
- Dimers
- Misfolded forms
- Virus

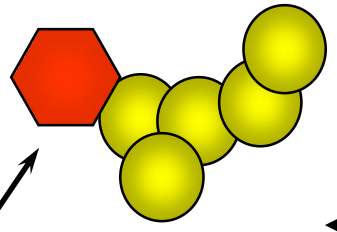
Need economical and robust proceses

Validation is important (FDA, others)

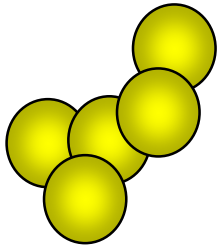
Protein Purification Strategy



EXPRESSION



FUSION PROTEIN



NON-FUSION PROTEIN

Simple Purification

ONE STEP Affinity

70-95% Purity
(not always)

For higher purity

Multi-Step Purification

Capture
Intermediate Purification
Polishing

PURIFICATION STRATEGY I

- The Protein Purification Facility
- Pipeline for Purification
- **General approach**: Input for Protocol Development
- Guidelines for Protein Purification. Commonly confronted decisions. Properties of Target Protein: Know your protein
- Detection and quality: PAGE-SDS, others

Protein Production Flowchart

EXPRESSION LEVEL

Target Selection (Literature)

Target Optimization
Main domain, partial/random truncations, rational design, solubility tags, #tags, others

Gene Cloning

Selection of Expression Vector

Selection of Expression Host

Expression Analysis

Solubility Analysis
Soluble/insoluble
Binding to capture resin
Oligomeric conformation

Scaling-up Fermentation

PURIFICATION LEVEL

Buffer choice

Low Scale Purification

Cell disruption and clarification method

(Test tube, batch purification)

First protein characterization

Medium Scale

Capture
Affinity columns

Intermediate Purification
IEX / HIC or MMC

Final polishing : SEC

Purification Optimization

Characterization
Purity / Homogeneity / Identity / Integrity

Concentration & Storage

Scale-up Purification

Structural Studies:
Crystallization – NMR- etc

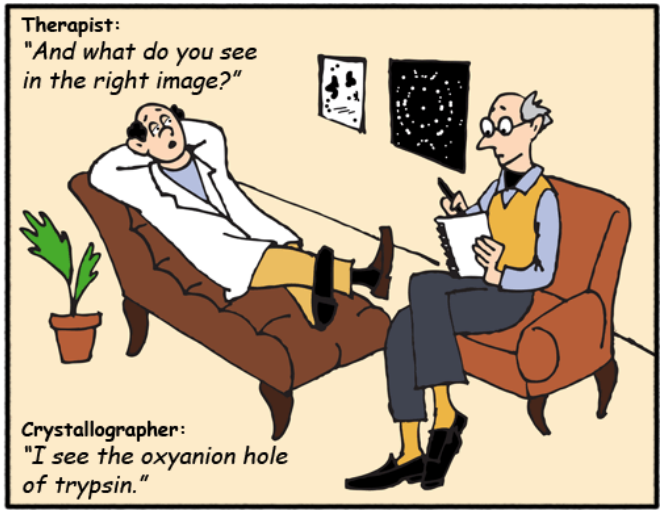
Pharmaceutical Studies

Biochemical Studies

Batch reproducibility

Protein Purification - Aims

- Satisfactory
  - ✓ expression levels
  - ✓ protein activity
  - ✓ purity
  - ✓ homogeneity
  - ✓ stability
- Economical use of reagents/equipment/time

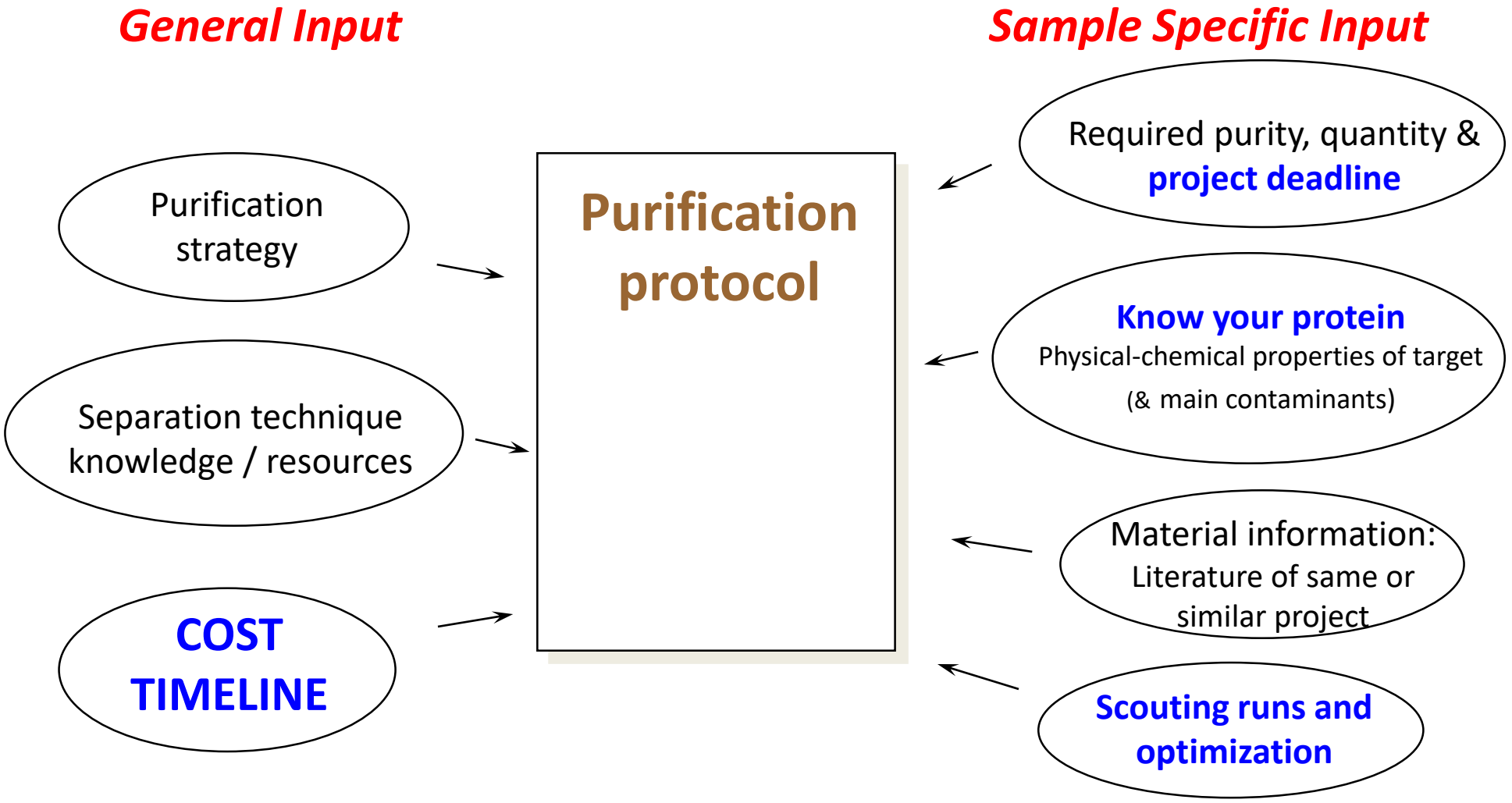


- Goal to Success:
- Selection or optimization of the best source or best expression conditions
- A good understanding of the protein needs
- Selection & optimization of the most appropriate technique for each step
- A rapid and reliable assay for the target protein: biological assay, enzymatic, SDS-PAGE, Western, etc

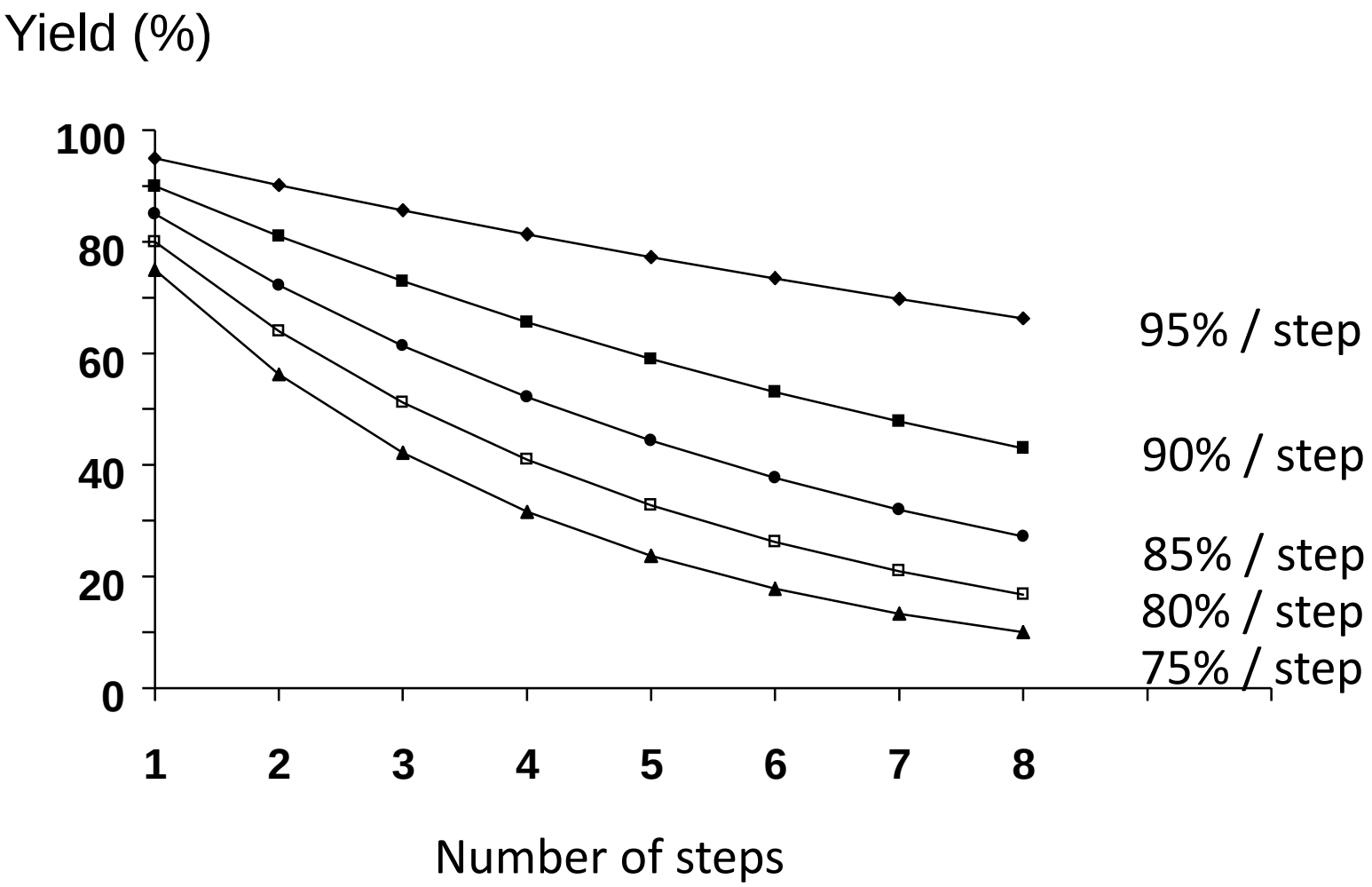
Commonly confronted decisions

- 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)? Intra or extracelular?
- 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 do I want my sample? Can I concentrate it? How much? Buffer?
- How to keep activity, solubility and homogenicity of my sample?

Input for Purification Protocol Development



Yields from Multistep Protein Purifications



Define Properties of Target Protein (I)

- Temperature Stability Need to work rapidly at low temperature
- pH Stability Selection of buffers for each step
- Organic Solvents Stability Selection of Conditions for RPC
- Salt Stability Selection of Conditions for all steps
- Co-factors for Stability or Activity Selection of Additives, pH, Salts, Buffers
- Protease Sensitivity Fast removal of proteases. Protease Inhibitors
- Sensitivity to Metal Ions Need of EDTA or EGTA in Buffers

Define Properties of Target Protein (II)

- Redox Sensitivity Need of reducing agents to protect reduce Cys: DTT, DTE or on the contrary, need to protect disulfide bridges
- Molecular Weight/Oligomeric State Selection of Gel Filtration Media / UF
- Charge Properties Selection of Ion Exchange Conditions
- Biospecific affinity Selection of ligand for Affinity Medium
- Post Translational Modifications Selection of Group Specific Affinity: Lectins
- Hydrophobicity Solubility prediction - Selection of medium for HIC - Need of detergents

Initial Bioinformatics Investigation

Using Bioinformatic Tools to Strategically Design Expression/Purification Projects

Dr. Nurit Kleinberger-Doron

http://wolfson.huji.ac.il/expression/software.html

Bioinformatics Tools-I

Physical and chemical parameters

http://www.expasy.org/tools/protparam.html

Computation of various physical and chemical parameters for a given protein: molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY)

http://www.scripps.edu/~cdputnam/protcalc.html

Generates molecular weight information (including scanning mass spectrometry results), estimated charges (including pI estimation), uv absorption coefficients, crystallographic solvent content percentage and Vm, and counts atoms and residues based on the protein sequence

Proteolytic Cleavage

http://www.expasy.org/tools/peptidecutter/

Predicts potential cleavage sites cleaved by proteases or chemicals in a given protein sequence

http://www.cf.ac.uk/biosi/staff/ehrmann/tools/proteases.index.html

Protease database of E.Coli

Bioinformatics Tools-II

Post-translational modification prediction

http://www.expasy.org/tools/#ptm

Prediction of Ser, Thr and Tyr phosphorylation sites in eukaryotic proteins

Prediction of N-acetyltransferase A (NatA) substrates (in yeast and mammalian proteins

Prediction of O-GalNAc (mucin type) glycosylation sites in mammalian protein

Prediction of N-glycosylation sites in human proteins

Prediction of N-terminal myristoylation by neural networks

Recombinant Protein Solubility Prediction **:** The statistical model predicts protein solubility **assuming the protein is being overexpressed in *Escherichia coli*.**

http://www.biotech.ou.edu/

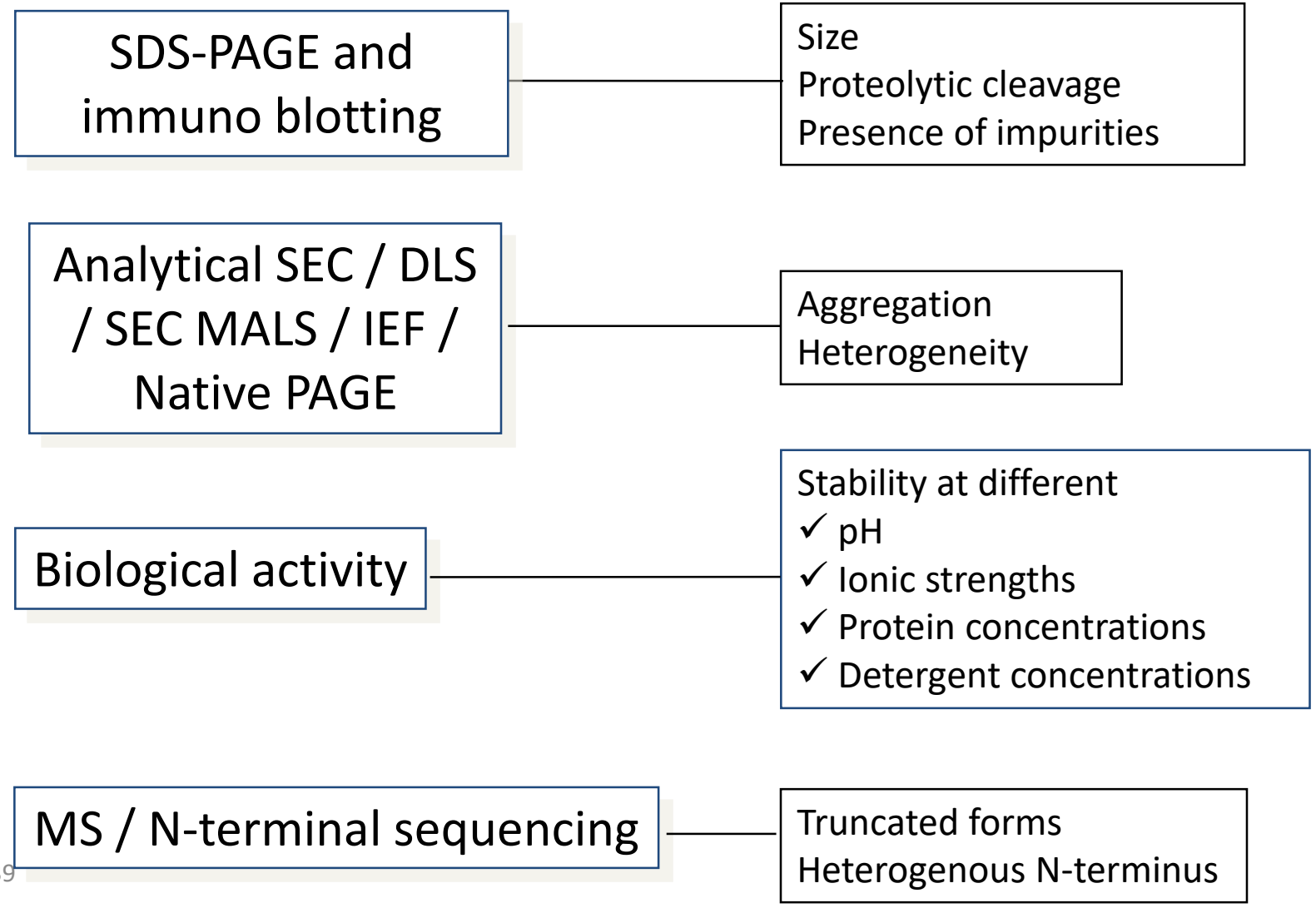
S-S bonds: Predicts cysteins that are likely to be partners in cysteine bridges

http://clavius.bc.edu/~clotelab/DiANNA/

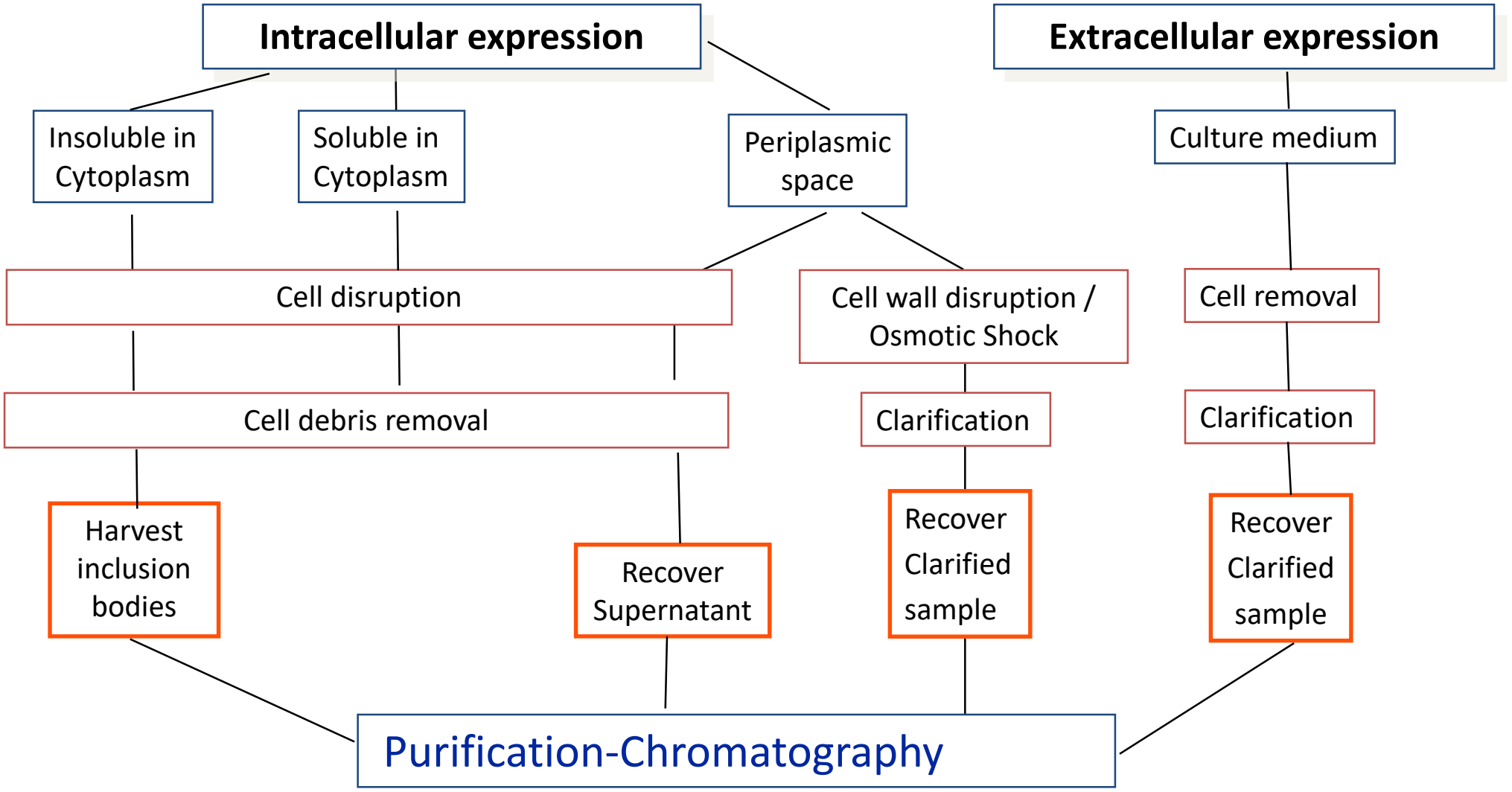
http://gpcr.biocomp.unibo.it/cgi/predictors/cyspred/pred_cyspredcgi.cgi

FoldIndex© tries to answer to the question: *Will this protein fold?*
http://bip.weizmann.ac.il/fldbin/findex

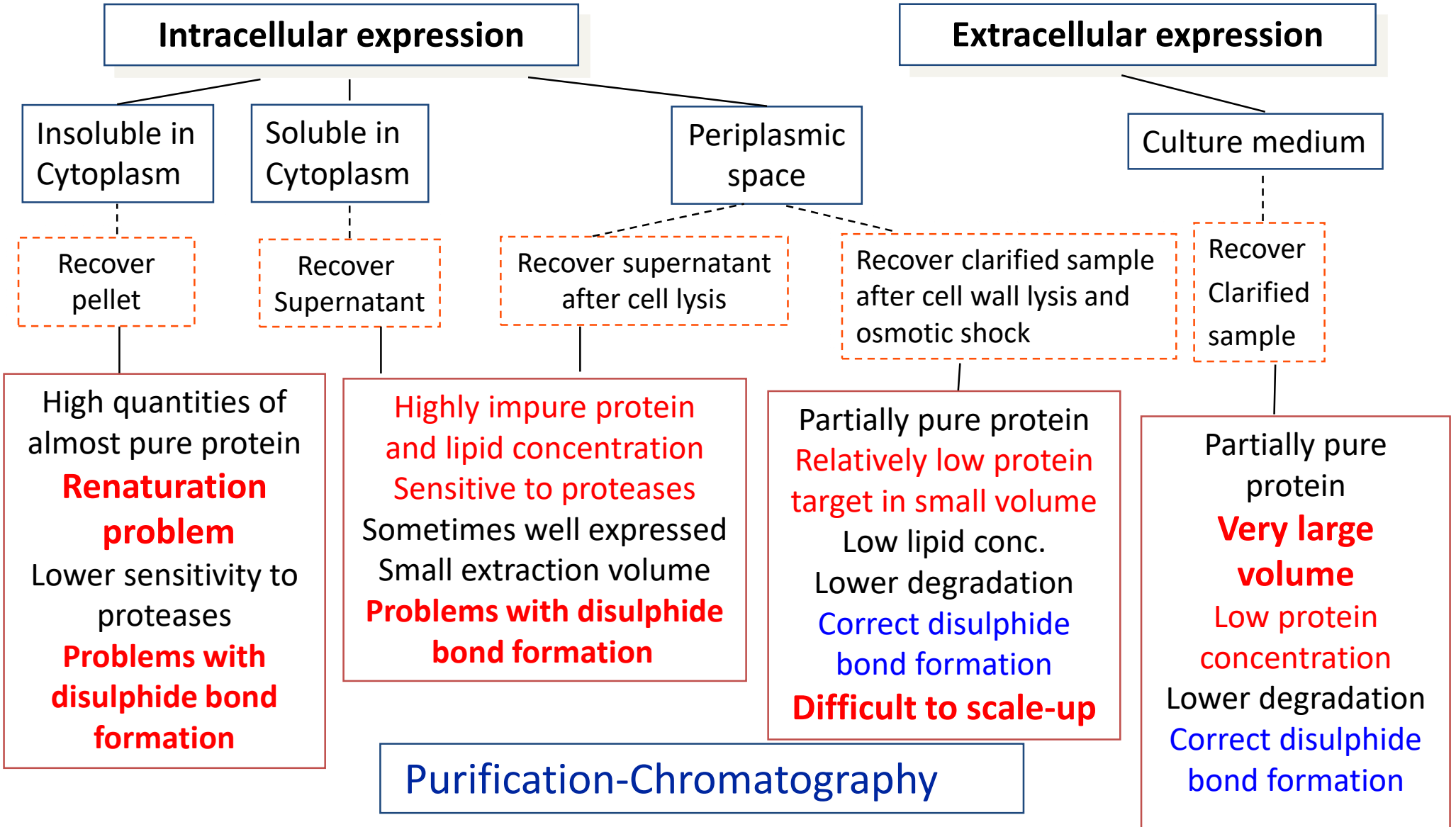
Is the Recombinant Protein Correctly Expressed



Advantages or Disadvantages of Intra or Extracellular Expression - I



Advantages or Disadvantages of Intra or Extracellular Expression - II



Extraction and Clarification

- Definition: Primary isolation of target protein from source material. Removal of debris or other contaminants which are not compatible with chromatography.
- Goal: Preparation of clarified sample for further purification.
- The chosen technique must be robust and suitable for all scales of purification.
- Choice of additives and buffers must be carefully considered before scaling up
- *Use additives only if essential for stabilization of product or improved extraction; select those that are easily removed.*

Common Substances Used in Sample Preparation

- Buffer: Tris HCl 20-50mM pH 7.5-8.0 or other buffers (HEPES, Phosphate, etc)
- Salt/conductivity: NaCl/KCl 0.3-0.5M (to maintain ionic strength). For soluble proteins NaCl can lower to 50mM. For some insoluble proteins it can be increase till 1M
- Stabilizers: Glycerol 5-10% to stabilize prone to aggregate proteins (can be increase till 20%). Increase viscosity and back flow of columns. Other stabilizers: detergents, amino acids, chaotropic or kosmotropic agents
- DNaseA 25-50µg/ml (or Benzonase): degrade DNA. Reduce viscosity. Eukaryotic cells could need more Dnase
- Lysozyme 0.2mg/ml for wall lysis of bacterial cells
- Detergents (NP40, Triton X100, Tween 20, OG, DDM etc) for solubilization of some insoluble proteins or extraction of membrane proteins. Use only if it does not affect protein stability!!!!
- Reducing agents: 1-15mM BME, up to 2mM DTT or DTE, 1-5mM TCEP. Use **only** for Cys containing proteins without disulfide bridges (maintain Cys in reduce form). Not all the IMAC columns can be use with all the reducing agents
- EDTA 1-10mM Reduce oxidation damage. Chelate metal ions. Metalloprotease inhibitor. Do not use with IMAC.
- Sucrose or Glucose 25mM Stabilize lysosomal membranes in eukaryotic cells. Reduce protease release.
- Protease or Phosphatase inhibitors if necessary

Minimize use of additives: they must be removed in extra purification steps or may interfere with activity assays

Protease Inhibitors

Protease Inhibitor	Specificity of inhibition	Working concentration
Antipain-dihydrochloride	Papain, Trypsin, Cathepsin A and B	1-100µM
Aprotinin	Trypsin, Plasmin, Chymotrypsin, Kallikrein	2µg/ml
Benzamidine HCl	Serine Proteases	0.5-4µM
Bestatin	Aminopeptidases	
Chymostatin	Chymotrypsin and Cysteine Proteases	10-100µM
E-64	Cysteine Proteases	10µM
EDTA (or EGTA)	Metalloproteases (Calcium)	2-10mM
Leupeptin	Serine and Cysteine Proteases such as Plasmin, Trypsin, Papain, Cathepsin B	10-100µM
PMSF and AEBSF	Serine Proteases	0.1-1mM
Pepstatin	Aspartic Proteases	1µg/ml
Phosphoramidon	Metalloproteinases, specifically, Thermolysin	1-10µM

- Serine proteases are widely distributed in most types of cells.
- Bacterial extracts typically contain serine and metalloproteases.
- Extracts from animal tissues contain mainly serine-, cysteine-, and metalloproteases. (some also contain aspartic proteases).
- Plant extracts contain large amounts of serine and cysteine proteases

Protease inhibitors are not enough: Work quickly and at low temperature!!

Remove proteases early in the first purification step!!: load on capture column immediately after lysis and clarification.

Protease Inhibitor Cocktail Set III (Cat. No. 539134)

MERCK – EMD

Recommended for mammalian cells/tissue

1 ml sufficient for 20 g cells (~1 L). Dilution 1:100 to1:300

EDTA-free (good for His-Tag® protein purification)

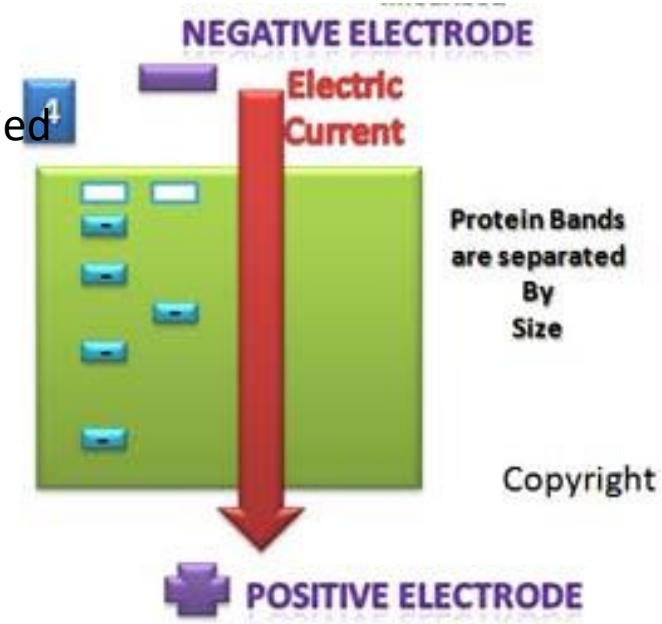
Product	Cat. No.	Recommended Application
Protease Inhibitor Cocktail Set I	539131	General use
NEW Protease Inhibitor Cocktail Set I, Animal-Free	535142	General use and for applications that require the use of animal-free reagents
Protease Inhibitor Cocktail Set II	539132	Bacterial cell extracts (except those intended for metal chelation chromatography)
Protease Inhibitor Cocktail Set III, EDTA-Free	539134	Mammalian cells and tissue extracts purified using metal chelation chromatography; samples to be analyzed by 2-D gel electrophoresis
NEW Protease Inhibitor Cocktail Set III, Animal-Free	535140	Mammalian cells and tissue extracts and for applications that require the use of animal-free reagents
Protease Inhibitor Cocktail Set IV	539136	Fungal and yeast cell extracts
Protease Inhibitor Cocktail Set V, EDTA Free	539137	Mammalian cells and tissue extracts purified using metal chelation chromatography; samples to be analyzed by 2-D gel electrophoresis
NEW Protease Inhibitor Cocktail Set V, Animal-Free	535141	Mammalian cells and tissue extracts and for applications that require the use of animal-free reagents
Protease Inhibitor Cocktail Set VI	539133	Plant cell extracts
Protease Inhibitor Cocktail Set VII	539138	Proteins containing His•Tag® sequences
Protease Inhibitor Cocktail Set VIII	539129	Broad range cysteine protease inhibition
Serine Protease Inhibitor Cocktail Set I	565000	Broad range serine protease inhibition

PURIFICATION STRATEGY I

- The Protein Purification Facility
- Pipeline for Purification
- **General approach**: Input for Protocol Development
- Guidelines for Protein Purification. Commonly confronted decisions. Properties of Target Protein: Know your protein
- Detection and quality: PAGE-SDS, others

SDS-PAGE*electrophoresis*

- Electrophoresis is defined as the movement of ions and charged macromolecules through a medium when an electric current is applied
- The matrix are primarily made of agarose or polyacrylamide
- Polyacrylamide gel electrophoresis (PAGE) is a powerful tool for separating and identifying mixtures of proteins and peptides.
- In SDS-PAGE samples are completely denature and molecules are separated only by their MW



- Proteins are detected by Coomassie staining (Silver stain, or others) or by Western Blot using specific antibodies. Coomasie sensitivity ~100ng. Silver is more sensitive and can detect as little as 5-10 ng. Silver is not quantitative as Coomasie (less linear); moreover, can stain nucleic acids
- SDS-PAGE do not give information regarding the oligomeric state of the protein. Different oligomeric conformations will run as a monomer. Oligomeric conformations can be separated by SEC because of the native conditions
- *Western blot **detect** presence of specific protein. No information regarding purity.*

Electrophoresis

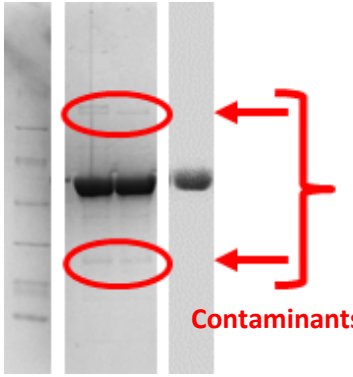
SDS-PAGE - native- IEF- BN-PAGE - 2D

- Macromolecules are separated through the matrix based on size
- The SDS of the sample buffer gives same charge to all proteins,

while structure is disrupt because of the SDS, the heat and the reduction agent. Some proteins needs Urea (up to 6M

- % Acrylamide is selected according to MW of target protein
- Gradient gels: Thin bands. Higher MW spectrum. But, lower resolution around target.
- Native gels (acidic, basic, neutral), others. Difficult to predict results. Non-denaturative. No MW markers. Separation is according to charge and length. Use: aggregation, isomers, complex
- Blue Native gels (BN-PAGE) : partially non-denaturative. for Analysis of Multiprotein Complexes
- Isoelectric Focusing (IEF): separation according to charge
- Two Dimensional Electrophoresis: First dimension IEF, and second dimension SDS-PAGE
- Capillary Electrophoresis (CE): Similar information to SDS-PAGE

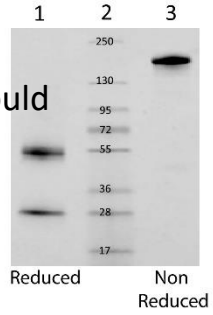
SDS-PAGE: Protein sample



- Evaluating protein purity

Ideally no other band other than the expected one for your protein of interest should be detected, as illustrated example figure. The staining should be chosen according to the amount loaded on the gel in order to be able to detect contaminants of 1% or less of the total protein load. Detection limit of Coomassie blue staining is approximately 100ng per band, for reverse zinc staining approximately 10ng per band, while fluorescent or silver stains have a detection limit of approximately 1ng of protein per band. If you load 10µL of a solution at 1mg/ml, you will load in total 10µg of protein meaning. In order to detect contaminants you will need sensitivity lower than 100ng per band and you should therefore use reverse zinc, fluorescent or silver stain to be able to assess contamination.

Very dense bands can hide other bands: repeat sample loading less protein sample



- Protein concentration before SDS-PAGE: Denaturative methods. *Do not use if you need active protein*

http://wolfson.huji.ac.il/purification/Protocols/ProteinPrecipitation.html TCA, DOC/TCA, acetone, ethanol or other precipitation easy and cheap methods to concentrate ten or more times the sample. Same methods could be use to eliminate interferents (high salt, guanidine HCl, high sugar, others)

Common staining protocols can be found in:
Quality assessment and optimization of purified protein samples: why and how? Bertrand Raynal, Pascal Lenormand, Bruno Baron, Sylviane Hoos and Patrick England Microbial Cell Factories ***2014 ; 13:180***.

More detail about other techniques can be found in:
Applications of capillary electrophoresis in characterizing recombinant protein therapeutics. *Zhao SS, Chen DDY Electrophoresis* ***2014; 35:96–108.***
Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products. Fekete S, Veuthey JL, Beck A, Guillarme D. J Pharm Biomed Anal. ***2016; 130:3-18.***

Avoiding Proteolysis

Discriminate between proteolysis during expression or during purification

During expression

- Alternative expression systems / host

Lon and OmpT protease deficiency stains from NEB and Novagen

- Targeting extracelular or periplasmatic
- Reduce protein misfolding
- Use of fusion proteins

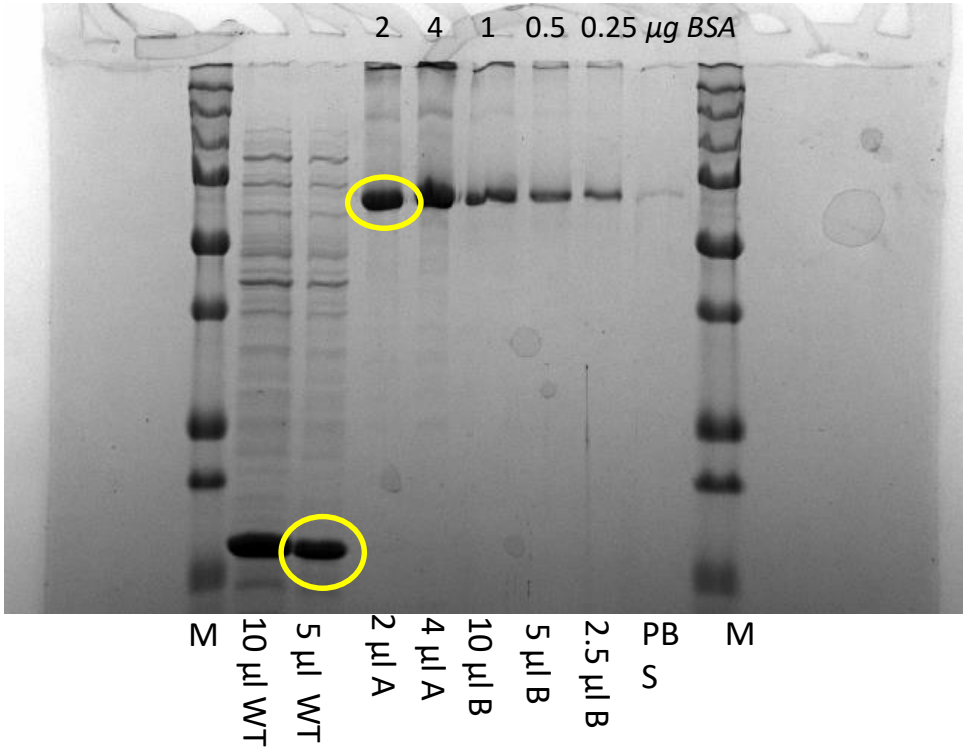
During purification

Combination of PI + low temp + fast purification is the way to avoid or reduce proteolysis

- Remove proteases early in the first purification step!!
- Strength specific PI if necessary
- EDTA (1-10mM): metallo-protease inhibitor; reduce oxidation damage and chelates metal ions

Internal Nicks: covalent nicks that not necessarily affect protein conformation
Combine SDS-PAGE and SEC information

How to calculate target protein quantity In crude extract



2 µl BSA 1 mg/ml = 2 µg

5 µl WT= 2 µg
10.5 ml=4200 µg =4.2 mg

C=0.4 mg/ml

A= BSA 1 mg/ml
B= BSA 0.1 mg/ml

Paz Drori
Lerner lab

Literature

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- GE-HEALTHCARE Protein purification Applications
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- GE-HEALTHCARE Protein and Peptide Purification - Technique selection guide
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/AMERSHAM_ProteinPurificGuide.pdf

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- GE-HEALTHCARE ÄKTAdesign Purification Method handbook. ÄKTAexplorer™ or ÄKTApurifier™ chromatography
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- TOSOH: General Principles of Chromatography
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/T0SOH_PrinciplesChromatPoster.pdf

- Mario Lebendiker Website http://wolfson.huji.ac.il/purification/index.html

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http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/GE_AKTAdesignPurification%20%281%29.pdf

- GE-HEALTHCARE Packing of Gel Filtration column (movie) http://wolfson.huji.ac.il/purification/Purification_Protocols.html

- GE-HEALTHCARE Packing of IEX, Affinity or HIC column (movie) http://wolfson.huji.ac.il/purification/Purification_Protocols.html

- GE-HEALTHCARE Column evaluation (movie) http://wolfson.huji.ac.il/purification/Purification_Protocols.html

- MERCK_MILLIPORE Protein Concentration and Diafiltration by Tangential Flow Filtration
http://wolfson.huji.ac.il/purification/PDF/dialysis/MILLIPORE_TFF.pdf

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- Preparative Purification of Recombinant Proteins: Current Status and Future Trends. Review Article - **M. Saraswat** - *(2013) http://dx.doi.org/10.1155/2013/312709*
- Protein production and purification – **Core Facilities Europe** – *(2008) - NATURE METHODS | VOL.5 NO.2 | FEBRUARY 2008 | 135*