Recombinant Protein Expression & Purification -- Challenges & Solutions

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2 Optimize Protein Expression

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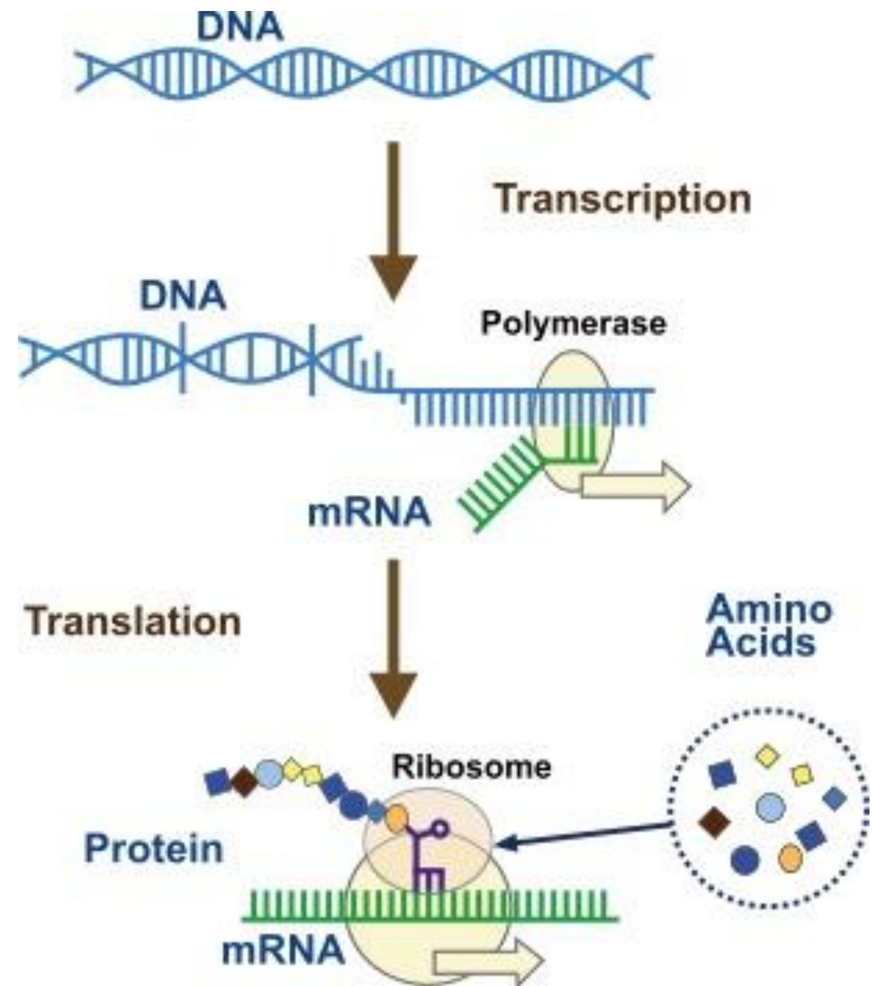
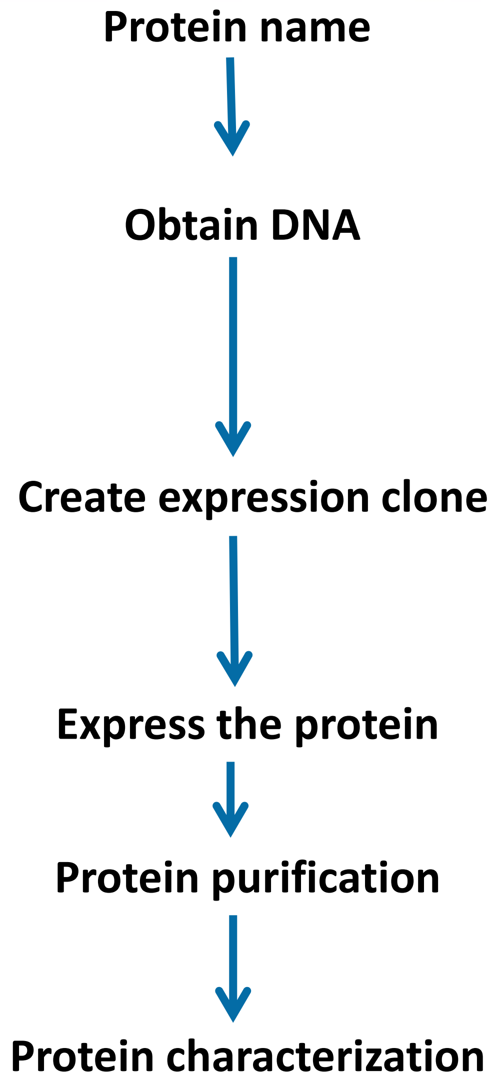
6 Advantages of GenScript Protein Services

Challenges in Protein Expression

- Soluble expression
- Correct conformation
  - Disulfide bond
  - Chaperonins
  - Refolding
- Production efficiency
- Purity
- Difficult-to-express proteins
  - Membrane protein
  - Toxic protein
  - Easy-to-degrade protein



Protein Expression Impacting Factors



- Sequence
- Expression system
  - Bacterial
  - Yeast
  - Insect
  - Mammalian
- Vector
  - Promoter
  - Tag
- Host strain
- Expression conditions
  - Medium component
  - Temperature
  - Inducer concentration & time
  - Inoculation volume

Protein Expression Common Questions

Protein name

- Full-length or fragment of the protein?
- PCR or gene synthesis?

Obtain DNA

- Expression system: bacterial, yeast, insect or mammalian?
- Expression vector ?
- Tag or no tag?
- Which tag?
- Tag placed at N or C-terminus?

Create expression clone

- Expression host?
- The best expression matrix?

Express the protein

Small scale test expression

- A necessary step?

Scale up protein expression

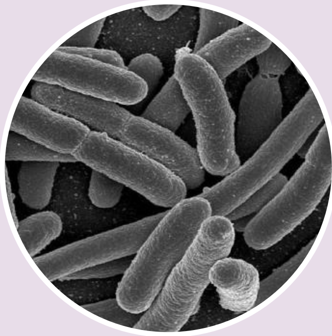
Protein purification

- Purification strategy?
- Refolding?
- Endotoxin removal?

Protein characterization

- QC methods?

Expression Systems



Bacteria

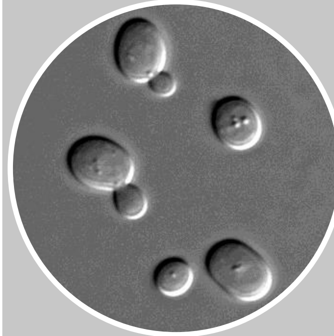
E. coli

1. "Work horse"
2. Well established
3. High expression
4. Simple genetics
5. Easy scale up
6. Speed
7. Costs
8. Equipment

Insect

Sf9, Sf21, S2, High-5

1. PTMs
2. Soluble proteins
3. High expressers



Yeast

S. cerevisieae

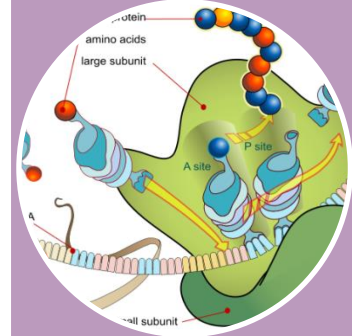
P. pastoris

1. PTMs
2. Soluble proteins
3. High expresser

Mammalian

CHO, HEK, COS

1. PTMs
2. Soluble proteins
3. Low expresser
4. Expensive



Cell Free

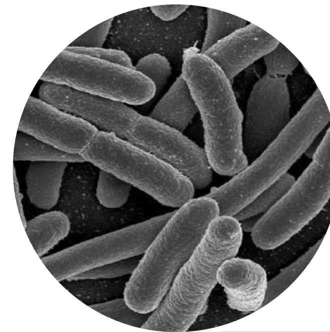
In vitro

1. Expensive
2. Not reproducible
3. Scalability issues

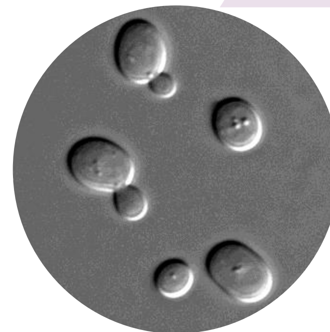
Which Expression System to Choose?

Factors to Consider:

- Protein property
  - MW
  - Disulfide bonds
  - Post-translational modifications
  - Homogeneity
- Intended applications
  - Structural biology
  - Functional assays
  - Therapeutic protein/vaccines
  - Antigens for Ab production
  - Protein-protein interactions
- Yield
- Cost



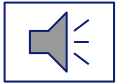
Which to Choose



Expression System Selection

Expression System	Pros	Cons	Intended Applications
Bacterial	• Relatively inexpensive • Simple genetics • Easy to manipulate • Easy scale up • Fast expression	• Lack of efficient post-translational modifications • Codon usage issues • Inclusion bodies • Low yield and activity for some eukaryotic proteins • Difficult to express higher MW proteins	• Structural biology • Functional assays • Antigen production • Protein-protein interaction (Bacterial & Yeast expression systems recommended) • Therapeutic protein (Yeast & Mammalian expression systems recommended)
Yeast	• Diverse post-translational modifications • Low cost of culture media • Industry-scale fermentation	• Improper glycosylation • Excessive glycosylation	
Insect	• Good secretion • Post translational modifications resemble mammalian system • Suitable for toxic gene products	• Long production time • Relative high media costs	
Mammalian	• Comprehensive post-translational modifications • Excellent method for the production of bioactive proteins	• Long production time • High media costs • Protein yields relatively lower	

GenScript Protein Standard Services



From sequence to purified protein - gene synthesis included!

Expression System	Deliverables	Timeline	Price
BacPower™	3mg purified protein guaranteed	6 -8 weeks	Staring from $2,200
InsectPower™	1mg purified protein guaranteed	8 -10 weeks	Staring from $3,950
MamPower™	3mg purified recombinant protein or 50mg purified antibody guaranteed	8 -12 weeks	Staring from $8,499
YeastHigh™	Customizable production up to 2000L	8 -10 weeks	Quote

PROTential™ - Expression Evaluation & Optimization

Eliminate the guesswork from your protein production work

- Evaluate whether your target protein expresses in your chosen system
- Identify the best expression system for your target protein
  - PROTential™ Standard packages
  - Before scale-up protein production, to avoid waste on your time & valuable resources

- Optimize your protein expression
  - PROTential™ Silver & Gold packages
  - When challenges arise – the most efficient & cost-effective way

One stop service at GenScript: gene synthesis → Subcloning →PROTential™ → Scale up protein production

GenScript's Solution for Expression Optimization

PROTential™ - Portfolios

Name	Service Type	Expression System(s)	Price	Timeline	Description
Standard	Protein Expression Evaluation	*E.coli* (SC1653-B)	Starting from $280	1-2 weeks	Test 1 condition for soluble expression in a customer chosen bacterial strain
		Insect (SC1653-I)	Starting from $400	3-4 weeks	Test 1 condition for soluble expression in Sf9, Sf21, S2 or Hi-5 cells
		Mammalian (SC1653-M)	Starting from $500	3-4 weeks	Test 1 condition for soluble expression in CHO or 293 cells
		All 3 Systems (SC1653-3S)	Starting from $950	3-4 weeks	Test soluble expression 1 condition/expression system (*E.coli*, Insect, Mammalian); total 3 systems
Silver	Protein Expression Optimization	*E.coli* (SC1667)	Login to inquire	2-3 weeks	• Test 8 different conditions • Optimize growth temperature, media components & inducer concentrations • Identify the best expression condition with your chosen vector and bacterial strain
Gold	Protein Expression Optimization	*E.coli* (SC1668)	Login to inquire	4-8 weeks	• Test 48 different conditions • Optimize growth temperatures, media components, inducer concentrations, promoters, host cell strains & fusion partners • Robust, industry-first, high-throughput expression and solubility optimization matrix

Case Study- Protein Expression Optimization

- **Challenges:**
  - Pilot purification (final yield 1mg/L)- ~ 28kDa protein;
  - The protein can be only purified from the soluble part
  - Large amount of protein with large scale fermentation (1000L) and purification is needed.
- **Strategies:**
  1. Expression improvement:
     a. Promoter optimization
     b. Strain optimization
     c. pH optimization
     d. Temperature and induction optimization
     e. Inoculated quantity optimization
  2. Recovery rate improvement during purification - Purification condition optimization

Case Study- Protein Expression Optimization

Strategy execution: HT expression testing

	1	2	3	4	5	6
A	X-1	X-2	X-3	X-4	X-5	X-6
B	X-7	X-8	X-9	X-10	X-11	X-12
C	X-13	X-14	X-15	X-16	X-17	X-18
D	X-19	X-20	X-21	X-22	X-23	X-24
E	X-25	X-26	X-27	X-28	X-29	X-30
F	X-31	X-32	X-33	X-34	X-35	X-36
G	X-37	X-38	X-39	X-40	X-41	X-42
H	X-43	X-44	X-45	X-46	X-47	X-48

Conditions X-1 to X-48

Case Study- Protein Expression Optimization

- 12X as much protein expression yield as original

1 mg/L starting protocol

2 mg/L T7 promoter/induction condition optimization

5 mg/L phoA promoter/induction condition optimization

6 mg/L growth condition optimization (pH)

12 mg/L seeding density optimization

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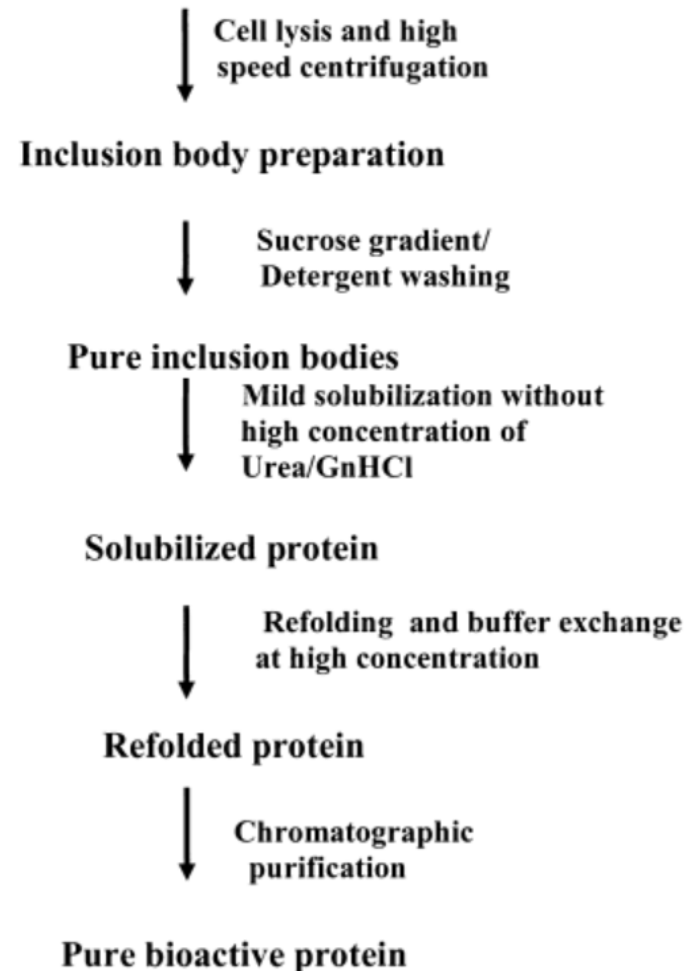
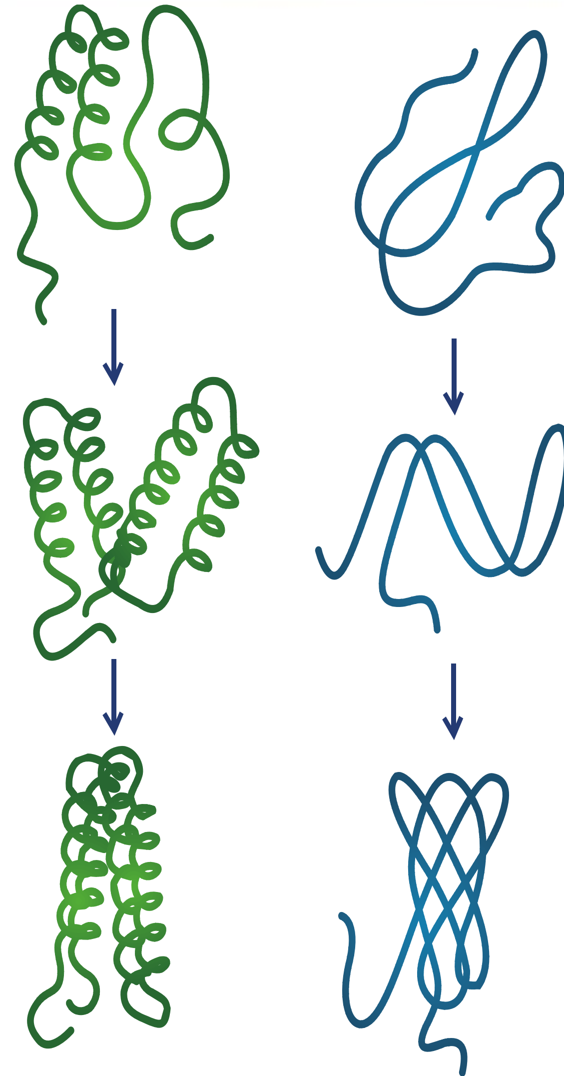
Protein Expression as Inclusion Bodies

- **What is an inclusion body?**
  - When E.coli is transformed to manufacture large amounts of recombinant protein, the protein sometimes forms dense aggregates of insoluble misfolded proteins, known as inclusion bodies.
- **Benefit**
  - allow high protein concentrations
  - protect sensitive proteins from proteolytic (enzymatic) degradation
  - protect the cell from any toxic proteins
- **Challenge**
  - to solubilise and refold this protein into its correct 'active' form



Protein Refolding Introduction

- All the information necessary for folding the peptide chain into its native structure is contained in the primary amino acid sequence of the peptide.
- The native form of a protein has the thermodynamically most stable structure.
- There are vastly too many different possible conformations for a protein to fold by a random search.
- A new view of protein folding suggested that there is no single route, but a large ensemble of structures follow a many dimensional funnel to its native structure.



GenScript's FoldArt™ Technology Overview

- Evaluation of target proteins' biochemical and biophysical properties
- Refolding optimizations
  - Selection of particular refolding strategy based on protein's sequence and the structural properties.
  - Buffer screening: Solutions for the inclusion body will be diluted to 20 different refolding buffers to determine which parameters affect the refolding results.
- Denaturant removal

  Techniques: dilution, dialysis, diafiltration, gel filtration, and chromatography (ion exchange, size exclusion, and affinity)
- Validation

  Refolding results will be validated by SDS-PAGE, HPLC and/or functional assay.

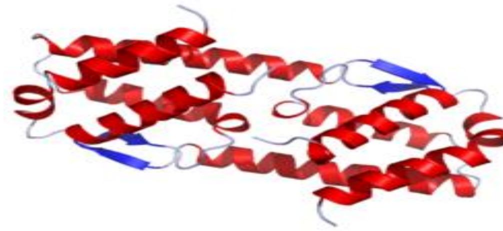
Refolding Buffer

- Refolding conditions must be optimized for each individual protein.
- Important variables are:
  - buffer type
  - pH
  - ionic strength
  - Additives, often in combination (glycerol, redox reagents, saccharides, amino acids, metal ion, detergents, chaperones)

Over 95% of the inclusion bodies can be solubilized and refolded by our proprietary FoldArt™ protein refolding technology

Case Study: Protein Refolding

- Human interleukin – 5: disulfide-bond linked homodimer as active form



Un-folded

Condition 1

Condition 2

Condition 3

Condition 4

Condition 5

Refolded

Ion exchange separation

Final product

Dimer

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Protein Purification

- Flexible purification methods
  - Affinity column (GST, Ni-NTA, protein A/G/L resins, etc.)
  - Ion exchange
  - Size exclusion
  - Hydrophobic interaction chromatography (HIC)

- Double Tag strategy for big protein isolation

Affinity Chromatography

- Affinity tags
  - His
  - GST
  - MBP
  - Flag
  - SUMO
- Matrices & elution conditions
- Methods
  - Column
  - Batch
- Tag removal by site-specific protease
  - TEV
  - Enterokinase
  - SUMO protease

Add mixture to column.
Discard flow through.

Add wash to column.
Discard flow through.

Add elution buffer to column.
Retain flow through.

Add mixture to batch to bind target molecule..

Remove mixture.

Add wash buffer.

Remove wash buffer.

Add elution buffer

Remove elution buffer and retain

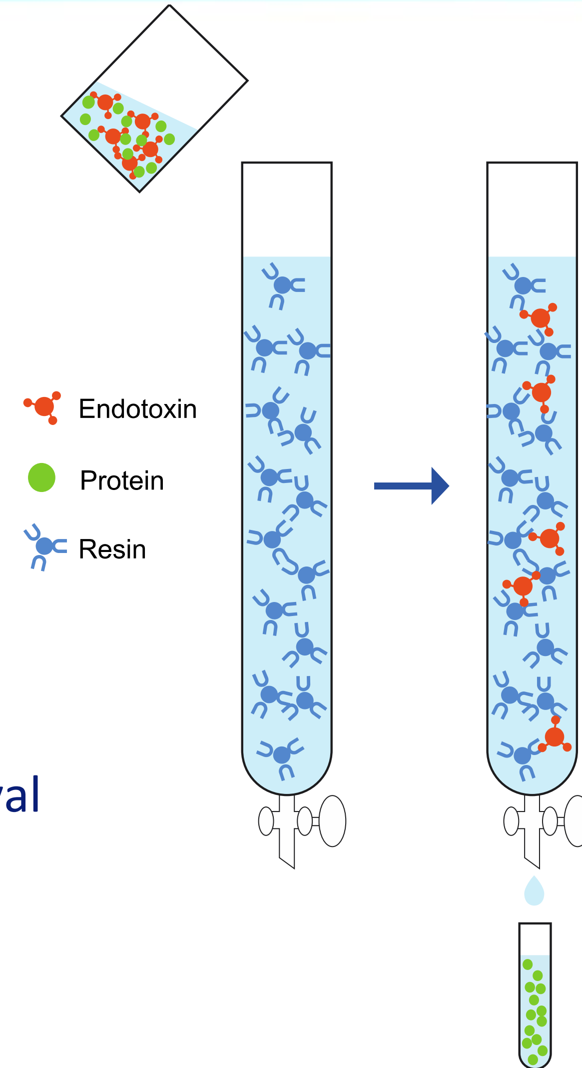
Endotoxin Removal

- **What are endotoxins?**

  - Endotoxins, also known as lipopolysaccharides (LPS), are large molecules found in the outer membrane of Gram-negative bacteria, which elicit strong immune responses in animals.

- **Detection**
  - Gel clot method
  - Chromogenic method

- **Removal methods:**
  - Polymyxin B (PMB) – affinity based ToxinEraser^{TM} (L00338) by GenScript allows highly efficient removal of endotoxin down to 0.1 EU/ml
  - Size exclusion chromatography (SEC)
  - Ion exchange chromatography (IEC)



Case Study: Endotoxin Removal

GenScript Endotoxin Removal Services: Endotoxin <= 1 EU/ug; <= 0.1 EU/ug; <= 0.01 EU/ug

	Protein 1	Protein 2	Protein 3
Volume of resins	3 ml	3 ml	3 ml
Volume of sample	15 ml	15 ml	15ml
Initial endotoxin	500,000 - 2,000,000 EU/ml	> 40,000,000 EU/ml	> 40,000,000 EU/ml
Final concentration	2.2 mg/ml	1 mg/ml	0.8 mg/ml
Final endotoxin	64 – 128 EU/ml	20 – 40 EU/ml	12.5 – 25 EU/ml
Final endotoxin	0.029 –0.058 EU/μg	0.02 –0.04 EU/μg	0.016 – 0.032 EU/μg

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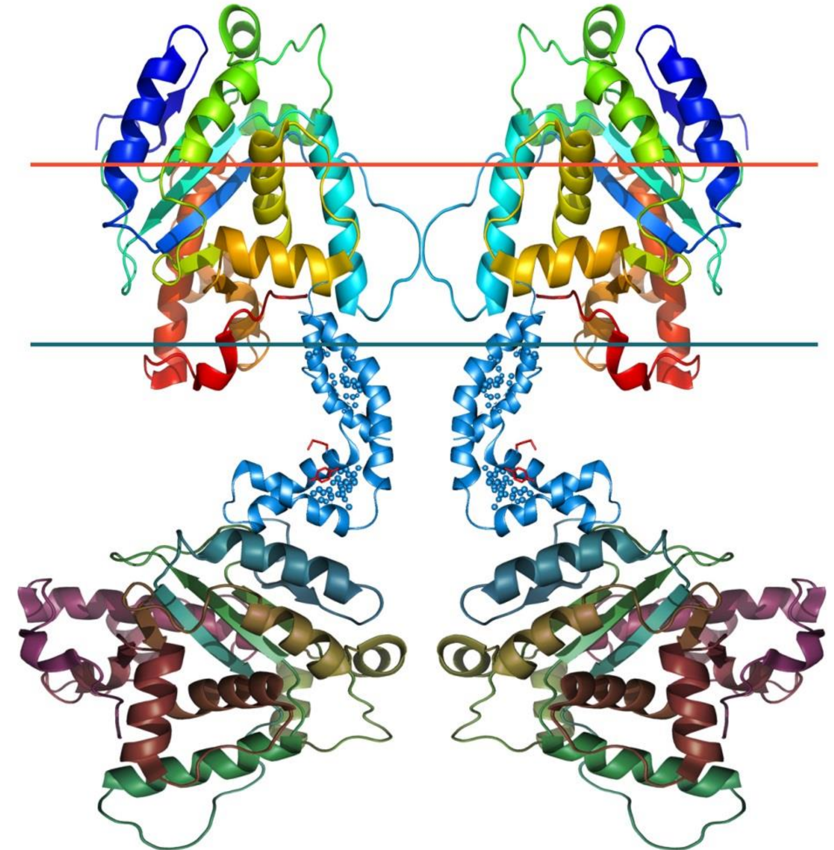
Difficult-to-Express Proteins

- Membrane protein
- Toxic protein
- Prone-to-degrade protein



Membrane Protein

Definition: Membrane proteins are proteins that interact with biological membranes.

- Targets of over 50% of all modern medicinal drugs.
- 20-30% of all genes in most genomes encode membrane proteins.

- Expression
  - Insect cells or mammalian cells;
  - Budded baculovirus or virus-like particle;
  - Cell-free
- Purification
  - Detergent screen;
  - Phosphate lipid ;
  - Nano-disc;

Membrane Protein Expression Work Flow

Gene synthesis and subcloning into transfer vector

↓

Generating the Recombinant Bacmid DNA

↓

Transfecting Insect cells

↓

Amplifying Baculovirus

↓

Expression optimization

↓

Large scale expression and harvest cell pastes

↓

Homogenizing cells with a glass-Teflon tissue homogenizer

↓

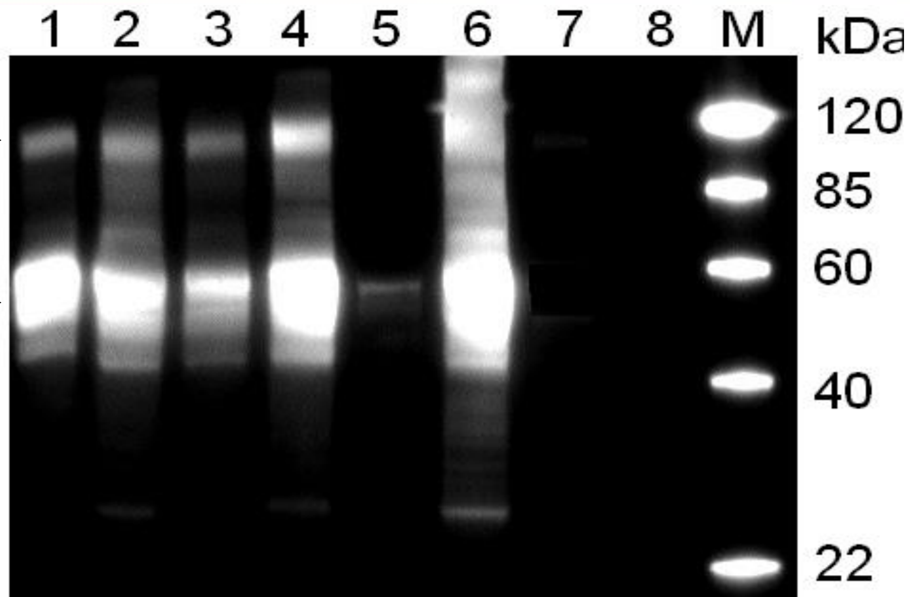
Centrifugation at low speed

↓

Centrifugation at ultra speed and re-suspend the resulting pellet

- Deliver recombinant Baculovirus, or cell pastes, or prepared membrane protein to the customer.
- Deliver functional budded virus containing membrane protein to the customer.
- Provide high throughput screening of membrane proteins and deliver expression evaluation report to the customer.

Case Study: Membrane Protein



❖25 L expression of GPCR,

Expression Level: 30-40 mg/L

❖Deliver the cell pastes in 6 weeks.

WB analysis of the membrane preparation

1. Whole cell lysate of GPCR before sonication
2. Whole cell lysate of GPCR after sonication
3. Supernatant after centrifugation of cell lysate for 10 minutes at 8k rpm
4. Pellet after centrifugation of cell lysate for 10 minutes at 8k rpm
5. Supernatant after ultracentrifugation of cell lysate for 45 min at 42k rpm
6. Pellet after ultracentrifugation of cell lysate for 45 min at 42k rpm
7. Negative control: supernatant after centrifugation of Sf9 cell lysate for 10 minutes at 8k rpm
8. Negative control: pellet after centrifugation of Sf9 cell lysate for 10 minutes at 8k rpm

Antibody: anti-His monoclonal-antibody (Genscript, Cat.No. A00186)

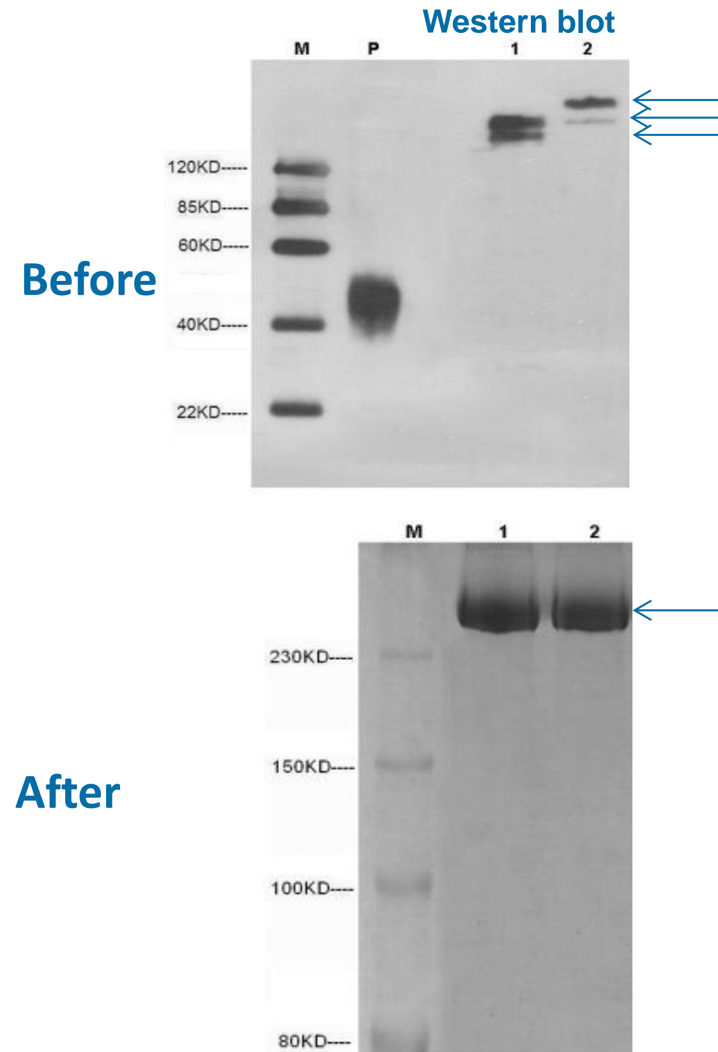
Toxic Protein Expression

- Definition: Toxic proteins defined here as proteins that cause cell death or severe cultivation and maintenance defects during the growth phase when their genes were introduced into E. coli strain.
  - Mostly due to leaking expression
  - ~80% protein growth and expression problems are caused by the toxicity of proteins

- Strategies in solving the problem
  - Promoter selection
  - Suppress basal expression from leaky inducible promoters
  - Tight control of plasmid copy numbers
  - Protein production as inactive (insoluble) forms

Case Study: Prone-to-Degrade Protein

- Inconsistency of measured concentration
- Trouble shooting:
  - Transfection methods
  - Cell lysis
  - Purification
- Challenge: DNase is only partially responsible for the protein degradation. This protein itself is prone-to-degrade.
- Solutions:
  - Remove DNase
  - Add protease inhibitor to every step
  - Optimize buffer components
  - Add protein stabilizers
  - Lyophilization immediately after protein purification
  - Storage temperature



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Advantages of GenScript Protein Services

◆ **Core in-house technologies for expression optimization & production efficiency.**

- OptimumGene™ – expression system specific codon optimization
- BacPower™ – increase bacterial soluble expression
- FoldArt™ – ensure appropriate protein refolding
- YeastHigh™ – high copy-number gene selection technique
- BacuVance™ – for protein secretion from baculovirus-infected insect cells
- MamPower™ – technology licensed from NRC for rapid recombinant protein production with high yield
- DoubleTag strategy – for big protein isolation

Advantages of GenScript Protein Services

◆ **One-stop service from sequence to purified proteins with large capacity.**

Guaranteed Protein Expression Package

- **3 mg** purified soluble protein from **$2,200**
- Subcloning
- Transformation
- Expression
- Refolding

Protein Expression and Purification Services

- Bacteria
- Yeast
- Baculovirus/insect cells
- Mammalian

Bioprocessing Services

- Mammalian protein expression services
- Stable cell line development & protein production

Large-scale Protein Production Services

- Bacterial fermentation up to 1,000 L
- Yeast fermentation up to 500 L
- Baculovirus/insect cell production up to grams
- Mammalian cell production up to grams

OptimumGene™ Gene Design Service

——Minimum 10-fold increase in protein express level

Advantages of GenScript Protein Services

- Flexible production scales
- Fast turn-around time (from sequence to purified protein in as little as 4 weeks)

Capacity:

Bacteria	Yeast	Baculovirus	Mammalian
1,000 L	500 L	100 L	500 L
Fermentor	Fermentor	Wave^{TM} Mixer	Wave^{TM} Mixer Sartorious bioreactor Hyclone SUB bioreactor

Variety of GenScript Protein Services

Protein Expression & Purification

Bacteria, Insect, Mammalian, Yeast, Customized protein services, Fermentation, Transient & Stable cell lines

Protein Expression Evaluation & Optimization

Small scale expression testing and optimization in bacterial, insect and mammalian expression systems

Large Scale Protein Production

Upstream & Downstream Process development, fermentation, GLP-compliant Bioprocess Services

Chemical Protein Synthesis

Alternative method to produce high purity functional proteins for hard-to-express proteins

High Throughput Protein Variants

Largest high-throughput capacity in the industry, proprietary platforms, 30 days for 1,000 protein variants

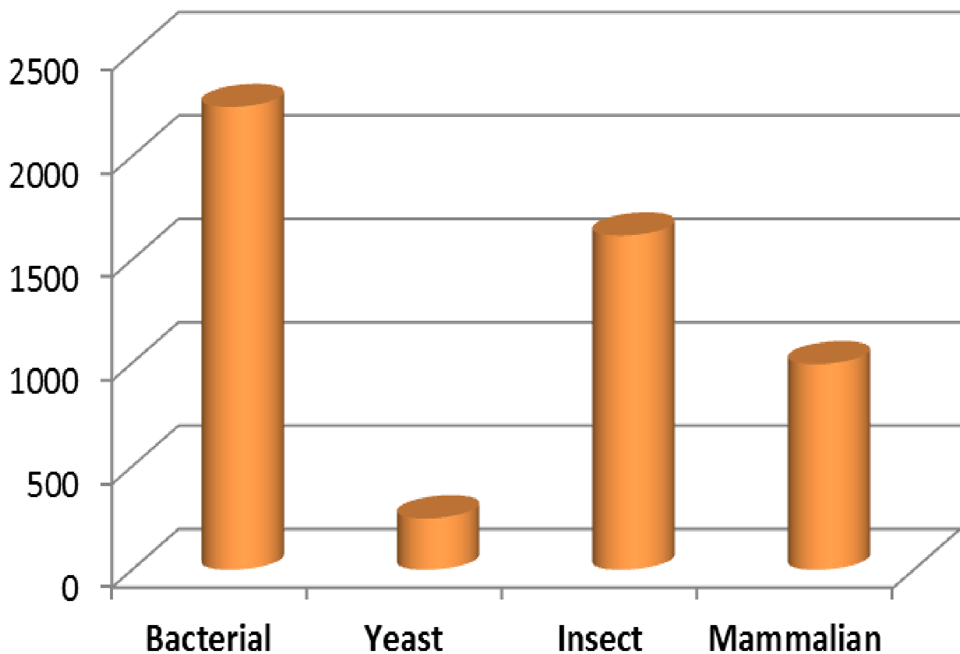
Structural Biology

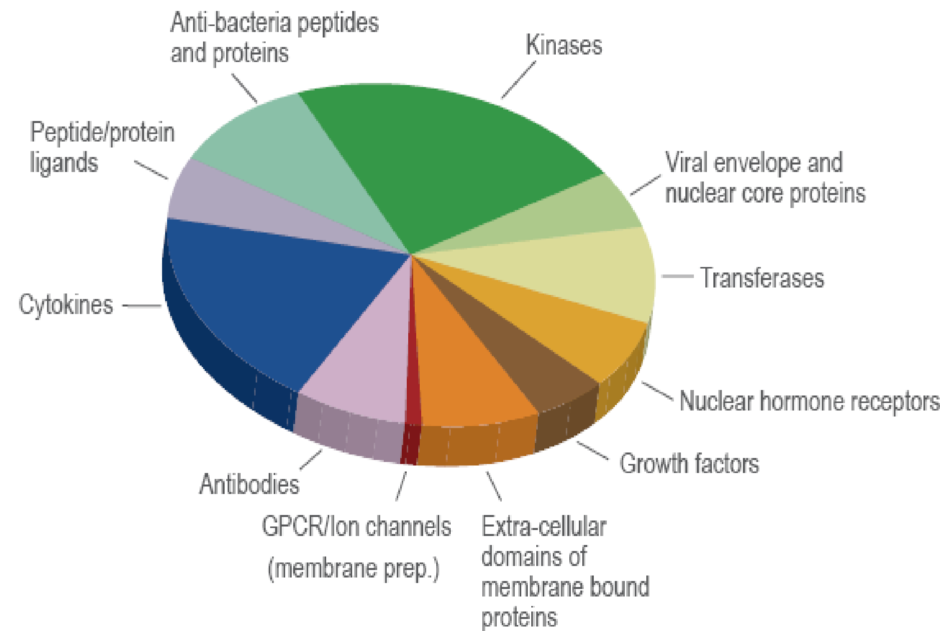
CrystalPro™ Gene-to-Structure Services, high purity protein preparation, crystal, co-crystal structure determination

Other Protein Services

Endotoxin removal, codon optimization, custom purification, protein characterization, refolding

GenScript's Experience in Protein Expression & Purification





GenScript has delivered over **5,000** proteins in four expression systems. Statistics showed **95%** success rate for all protein projects.

About GenScript

Gene

Cell Line

Peptide

GenScript

Products & Services

Make Research Easy

Discovery Biology

Protein

Antibody

Thank you for your participation

We wish you all success in your Research

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