Expression of recombinant proteins: Eukaryotic hosts

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Our mission is to discover and characterize, alone or in partnership, novel anti-cancer drugs

Strong expertise in the discovery, preclinical profiling and clinical characterization of small molecule inhibitors of kinase and non-kinase targets

Product pipeline focused on cell cycle and signal transduction regulators which are abnormally expressed in cancer cells

Capabilities range from target validation to phase II (proof-of-principle) clinical studies
The drug discovery process & the protein production impact

Protein Production required

HTS: High Throughput Screening
SBDD: Structure-Based Drug Design
LBDD: Ligand-Based Drug Design
SAR: Structure-Activity Relationship
MoA: Mechanism of Action
ADME: Administration, Disposition, Metabolism, Excretion
Eukaryotic hosts: presentation outline

• Eukaryotic hosts: general features
• Yeasts
• Insect cells and the protein production at NMS
• Mammalian cells
• Conclusions
Several eukariotyc hosts can be used

Pichia pastoris
Several eukariotyc hosts can be used

Insect cells

Spodoptera frugiperda

Trichoplusia ni
Several eukaryotic hosts can be used.

Mammalian cells

CHO

HEK293
Several eukaryotic hosts can be used

plants
Several eukariotyc hosts can be used

AdCEV™ Vectors

Express your proteins in hen eggs!

Recombinant product development.

AdCEV™ vectors can provide an efficient and convenient way to make high fidelity viral antigens, diagnostic reagents, mAbs or research reagents rapidly and safely. With easy scale up, we can help you develop a reliable supply source for any quantity! Our vectors extend the range of high-value proteins that can be produced in eggs to include subunit vaccines, serum proteins, viral glycoproteins, interleukins, recombinant antibodies, and a host of other valuable biological products you may wish to produce.
<table>
<thead>
<tr>
<th>Host</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Many references and much experience available</td>
<td>No post-translational modification</td>
</tr>
<tr>
<td><em>e.g.</em> Escherichia coli</td>
<td>Wide choice of cloning vectors</td>
<td>Biological activity and immunogenicity may differ from natural protein</td>
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<td></td>
<td>Gene expression easily controlled</td>
<td></td>
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<tr>
<td></td>
<td>Easy to grow with high yields (product can form up to 50% of total cell protein)</td>
<td>High endotoxin content in gram negative bacteria</td>
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<td></td>
<td>Product can be designed for secretion into the growth media</td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>Secretes fusion proteins into the growth media</td>
<td>Does not express such high levels as <em>E. coli</em></td>
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<tr>
<td><em>e.g.</em> Staphylococcus aureus</td>
<td></td>
<td>Pathogenic</td>
</tr>
<tr>
<td>Mammalian cells</td>
<td>Same biological activity as native proteins</td>
<td>Cells can be difficult and expensive to grow</td>
</tr>
<tr>
<td></td>
<td>Mammalian expression vectors available</td>
<td>Cells grow slowly</td>
</tr>
<tr>
<td></td>
<td>Can be grown in large scale cultures</td>
<td>Manipulated cells can be genetically unstable</td>
</tr>
<tr>
<td>Yeasts</td>
<td>Lacks detectable endotoxins</td>
<td>Low productivity as compared to micro-organisms</td>
</tr>
<tr>
<td></td>
<td>Generally Regarded As Safe (GRAS)</td>
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<tr>
<td></td>
<td>Fermentation relatively inexpensive</td>
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<tr>
<td></td>
<td>Facilitates glycosylation and formation of disulphide bonds</td>
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<tr>
<td></td>
<td>Only 0.5% native proteins are secreted so isolation of secreted product is simplified</td>
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<tr>
<td></td>
<td>Well established large scale production and downstream processing</td>
<td></td>
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<tr>
<td>Cultured insect cells</td>
<td>Many processing mechanisms similar to eukaryotic cells</td>
<td>Lack of information on glycosylation mechanisms</td>
</tr>
<tr>
<td><em>Baculovirus vector</em></td>
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<td></td>
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<tr>
<td></td>
<td>Safe, since few arthropods are adequate hosts for baculovirus</td>
<td>Product not always fully functional</td>
</tr>
<tr>
<td></td>
<td>Baculovirus vector received FDA approval for a clinical trial</td>
<td>Few differences in functional and antigenic properties between product and native protein</td>
</tr>
<tr>
<td></td>
<td>Virus stops host protein amplification.</td>
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<tr>
<td></td>
<td>High level expression of product</td>
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</table>
Yeast as expression host

The first yeast used was *Saccharomyces cerevisiae* (well-known model organism, abundance of molecular biological tools, genetic markers, site-specific chromosomal integration, variety of inducible-GAL1- and constitutive -GAPDH- promoters)

The need for stronger promoters and better secretion of recombinant proteins led to the use of other yeast species.

In the last years *Pichia pastoris* has become the yeast of choice:

- Biology (and protocols) similar to baker’s yeast
- *Pichia* has a strong and tightly regulated inducible promoter
- The *Pichia pastoris* expression vectors integrate in the genome (higher stability)
- Extremely high cell density can be obtained
- Ability to obtain both secreted and intracellular recombinant proteins
- Purification of secreted recombinant proteins easier
- *Pichia* can glycosylate proteins and several “*ad hoc*” strains are now available
- Disulfide bonds are efficiently generated

Warning:
The induction medium requires methanol. In large scale culture, this might become a fire hazard
Yeasts: not only Pichia

Other species developed:

- **Hansenula polymorpha**, (methylotrophic yeasts)
- **Pichia methanolica** (methylotrophic yeasts),
- **Kluyveromyces lactis** (the first yeast after *Saccharomyces coelicolor* to be developed for recombinant protein production, used in the rennin production for cheese preparation)
- **Arxula adeninivorans**, 
- **Yarrowia lipolytica**.

Also some filamentous fungi are described as expression host, such as

- **Neurospora crassa**, **Aspergillus**, etc.
History of development of Pichia pastoris as expression system

- During the 1970’s Phillips Petroleum company developed media and methods for growing P. pastoris on methanol (cheap production from natural gas methane). They wanted to obtain high cell densities to generate yeast biomass or Single Cell Protein to be marketed as a high protein animal feed. During the same period, however, the price of methanol increased dramatically due to the oil crisis and PP never used this technology.

- In the 1980’s PP developed in collaboration with SIBIA, the protocols to use Pichia as expression system for heterologous proteins.

- In 1993 PP sold its patent position to RCT (Research Corp. Technologies) and to Invitrogen to sell media and tools.

- Today, to use Pichia (working in an industrial environment) requires the payment of a licensing fee.

- The use is free for Academia, provided that it cannot be transferred to any industrial company.

<table>
<thead>
<tr>
<th></th>
<th>Class A</th>
<th>Class B</th>
<th>Class C</th>
<th>Research Only (Valid for 5 years)</th>
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<tbody>
<tr>
<td>License Issue Fee</td>
<td>$75,000</td>
<td>$40,000</td>
<td>$20,000</td>
<td>$50,000</td>
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<tr>
<td>Annual Minimum Royalty</td>
<td>$30,000</td>
<td>$20,000</td>
<td>$10,000</td>
<td>NA</td>
</tr>
<tr>
<td>Royalty Rate (initial two products)</td>
<td>3%</td>
<td>4%</td>
<td>5%</td>
<td>NA</td>
</tr>
<tr>
<td>Royalty Rate</td>
<td>3%</td>
<td>3%</td>
<td>3%</td>
<td>NA</td>
</tr>
</tbody>
</table>
Pichia pastoris is a methyltropic yeast: in the absence of glucose it uses methanol as a carbon source. The promoter AOX1 controls the expression of alcohol oxidase, which catalyses the first step in methanol metabolism. AOX1 is a strong inducible promoter: up to 30% of total soluble protein upon methanol induction is alcohol oxidase.

Pichia inducible vectors use AOX1 promoter

To have a constitutive expression the GAPDH promoter is used
Pichia strains and genetic markers

• The ADE2 gene is used as a genetic marker. ADE2 codes for aminoribosyaminoimydazole carboxylase, necessary in the biosynthesis of purines. Loss of function mutations in ADE2 cause the accumulation of purine precursors, that accumulate in the vacuole and produce a red pigment. The colonies colour can be used as a selection screening method. Furthermore, the picha strains are ade2-/- (auxotrophe, need media supplemented with adednine); the expression vectors harbour the ADE2 gene and the transformants are able to grow on minimal /Ade drop out medium. The color intensity is inversely proportional to the amount of protein expressed: pink corresponds to very low amount, white, high amounts

• Another auxotrophic marker used is his4

• AOX1 and or AOX2 genes are frequerntly deleted in Pichia strains (lower amount of methanol necessary to induce heterologous protein expression, better recombinant protein production)

• KM71 strain: his4, arg4, aox1::ARG4: this strain relies on the much weaker AOX2 gene to utilise methanol and grows on methanol at slower rates (MutS phenotype) When both aox1 and aox2 genes are deleted, the strain has a mut- phenotype and is unable to grow on methanol
The $\alpha$-factor prepro signal peptide from *Saccharomyces coelicolor* is largely used as secretion signal peptide.

The advantage of using *Pichia pastoris* in producing recombinant secreted proteins is that *Pichia* secretes very low levels of endogenous proteins (Note: your protein will be glycosylated if contains glycosylation sites, such as N-X-S/T)

prb1 and pep4 protease knock-out strains can be adopted to reduce as much as possible the use of protease inhibitors (reduce consumption for large scale cultures)
Clone GOI into pPink-HC or pPink-LC

linearize DNA

Electroporate into Strain 1 ade2
Strain 2 ade2 pep4
Strain 3 ade2 pbb1
Strain 4 ade2 pep4 pbb1

Plate transformants

Incubate 3-7 days

Select 3-8 white colonies

Small-scale expression or

3-4 days

Select strain for large-scale expression
Characterization of yeast-derived antibody

Recombinant glycoproteins represent 70% of all approved therapeutic proteins and have been usually produced in CHO or HEK293 cells. Still, the end product differs in glycans composition from their human counterpart.

Human antibodies produced in glycoengineered lines of Pichia pastoris

Li H. et al, Nature Biotechnology 2006
Figure 3 Receptor binding assays of rituximab glycovariants. (a-f) Binding to human FcγRIIb (a,b), FcγRIIla 158V (c,d), FcγRIIla 158F (e,f).
Expression of recombinant protein in Pichia: pros and cons

• High expression levels
• High cell densities
• Controllable expression
• Variety of strains to modulate expression
• Post-translational modifications
• Possibility to secrete your protein

• Licensing required (industrial environment)
• Post-translational modifications might be different from the ones of higher eukaryotes
Insect cells (baculovirus) as expression host

- Baculovirus (Autographa californica) is a lytic dsDNA virus
- In vitro, baculoviruses are reproducing in insect cells lines from Spodoptera frugiperda (Sf9 or Sf21)
- Expression of recombinant proteins occur either in Sf9/Sf21 cells or in a cell line called H5
- The technique of creating recombinant baculoviruses has evolved a lot: from rare recombination events to site-specific transposition or improved homologous recombination (100% efficiency)
- Virus titer determination: from plaque assay to qPCR and ELISA
- It is possible to obtain both secreted recombinant proteins (adding a signal peptide and using a special medium) and intracellular recombinant proteins
- Disulfide bonds are efficiently generated. Usually presence post-translational modifications found in mammalian cells.
- Glycosylation different from the one of mammalian cells (new generation of insect cell line to produce correctly glycosylated proteins)
- 4 weeks from co-transfection to expression test
Protein production workflow at NMS. Proteins are required for different applications:

**Biochemical Assays**
- Enzymes for HTS and primary screening
- Enzymes for secondary assays
- Substrates

**AIM: evaluate potency and selectivity of Hits and Leads**

**Structural Studies**
- Crystallizable constructs for X-ray
- Proteins suitable for NMR studies
- Proteins for Biophysical Studies (ITC, Thermal stability etc.)

**AIM: evaluate binding mode of compound on target protein**
Two main requirements

Practical consequence: two different approaches

Assay

NEED = ACTIVE PROTEIN
- Active:
  - Post-translational activating modifications
  - Activating mutations
  - Activation by upstream regulators or chemical agents
- Native or fusion proteins (tagged)
- Purity > 85%

Structure

NEED = CRYSTALS
- Minimal folded domain
  - Untagged
  - Purity > 95%
  - Monodisperse
  - Homogeneous
  - ...Crystallizable!

Insect cells/E.coli

E.coli/ Insect cells
Workflow

- Obtaining cDNA
- Select boundaries
- Cloning
- Small-scale test expression
  - Try prokaryotic and eukaryotic hosts (i.e. E.coli and insect cells)
- Scale up
- Protein purification
- Protein characterization
### Baculovirus vs. E. coli

<table>
<thead>
<tr>
<th>Insect cells/Baculovirus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time consuming</td>
<td>Fast, easy</td>
</tr>
<tr>
<td>Expensive</td>
<td>Cheap</td>
</tr>
<tr>
<td>Postranslational modifications</td>
<td>No post-translational modifications</td>
</tr>
<tr>
<td>(similar to mammalian)</td>
<td></td>
</tr>
<tr>
<td>High solubility</td>
<td>Low solubility</td>
</tr>
<tr>
<td></td>
<td>Low success rate for eucaryotic proteins and MW &gt; 60 kDa</td>
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</table>

In many cases Insect cells are the system of choice, as it results to give active enzymes with correct fold and modifications.
Baculoviruses (family baculoviridae) are dsDNA viruses that infect many different insect species. Baculoviruses strains are highly species-specific and are not known to propagate into non-invertebrate host. The genome is replicated and transcribed in the nuclei of the cell host and then packaged into rod-shaped nucleocapsids. As the nucleocapsids is flexible, it can contain larger DNA molecule. There are two main baculoviruses used: *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the *Bombyx mori* nuclear polyhedrosis virus, but the first is commonly used.
Baculovirus vectors contain:

- An E.coli ori
- Amp resistance marker
- A promoter (polyhedrin, p10 or basic protein AcNPV gene)
- A cloning region
- A large tract of AcNPV genome to allow homologous recombination

in vitro
Isolation of recombinant viruses:

- Firstly done by selecting plaques occlusion body negative (obtained by deletional or insertional inactivation of the polyhedrin gene). Recombination efficiency low (1:1000 = 0.1%). Today obsolete method.

- Recombination efficiency improved (30%) by linearizing AcNPV DNA (behind the polyhedrin or p10 promoter).

- White/blue selection of plaques (in X-gal plates): the lacZ gene under the polyhedrin promoter was inserted in the AcNPV genome. The linearization and consequent recombination, produces white plaques. The blue plaques are those where the linearization and consequent recombination did not occur.

- The deletion of essential portions of the viral genome is complemented by recombination with a transfer vector during a co-transfection process: not recombinant virus cannot replicate. Recombination efficiency >99%.

- Baculogold Bright: a gfp gene is cotransfected: green cells are also those efficiently infected by the virus.
Baculovirus transfer vector carrying the gene of interest is generated by cloning Baculovirus transfer vector and baculogold DNA are co-transfected in insect cells and generate a complete baculovirus genome by homologous recombination. Competent viral particles are generated in the infected cell, and can be recovered from the medium. Normally 2-3 rounds of viral amplification are required for a high titer viral stock. Now the virus is ready to infect cells.
Sf9 cells were co-transfected with BD BaculoGold™ Bright and hMip-1α (A) or BD BaculoGold Bright alone (B). 5 days after transfection cells were analyzed using light and fluorescence microscopy.
Workflow

Obtaining cDNA
- Extracting from library, purchasing from commercial sources, asking somebody…..

Select boundaries
- Literature and/or alignment and/or LP

Cloning
- Gateway cloning

Small-scale test expression
- Try E.coli and Insect cells, test several conditions
- Lyse by sonication

Scale up
- Flasks or wave bioreactors
- Lyse by Gaulin homogeniser

Protein purification
- Affinity tags, on-column cleavage with PP, IEX, SEC, …

Protein characterization
- SDS-PAGE, native gels, analytical SEC, stability, LC-MS, MALDI-ToF-Tof, activity, etc
The Gateway strategy

[Diagram showing the Gateway strategy with gene entry clones and various cell types and fusion proteins.]
**E.coli**

- **Molecular Biology**: 2 weeks
- **Test Expr & Optim**: 1-2 weeks
- **Purification Set up**: 1 week
- **Scale up**: 1-2 weeks
- **Total**: 4-8 weeks

**Baculovirus**

- **Molecular Biology**: 2 weeks
- **Virus ampl**: 3 weeks
- **Test Expr & Optimization**: 1-2 weeks
- **Purification Set up**: 1 week
- **Scale up**: 2-4 weeks
- **Total**: 9-12 weeks
Small scale expression tests are used to determine which clone actually produces soluble protein and which are the optimal expression conditions.

**Bacteria**
- Temperature of induction (from 16°C to 37°C)
- time of induction (from 3hrs to 48Hrs)
- bacterial strain (BL21 Rosetta, BL21 pLysS, BL21codon+)

**24 different conditions**

**Insect cells**
- amount of virus (different molteplicity of infection –MOI)
- time of infection (48 hrs, 72 hrs)
- cell line (Sf9, Sf21, H5)
- Growth temperature (21C, 27C)

**36 different conditions**
Robotic stations

**Manual** 24 wells insect cell expression tests: done manually until July 2008 (time consuming; few plates/week; preferably one cell lines/plate; human errors)

EP- motion 5070 placed in the cell room under sterile hood

**Automatic** 24-wells insect cell expression test (Dispense cells in 24-wells plates, dispense viruses to the cells) (20 min/plate, possibility to plate different cell lines/plate; less errors; increase number of expression conditions)
Robotic stations

24 wells-expression tests centrifuged

Packard multiprobe II

24 tips sonicator

Caliper
Large scale production

Most frequently used systems to grow large volume of liquid cell culture:
- 3 l Erlenmeyer flasks
- Wave bioreactor

Sterile cellbag filled with media and cells
Gas impermeable. Single use

Provide optimal environment for cell growth and expression
Cell counter - monitor cell size to optimize protein production

Time 0 (before infection)

- Infection not efficient: less homogenous cell population
  - Ø Mean: 17.28 µ μ
  - Standard dev.: 2.52 µ μ

72 hrs

- Efficient Infection: homogenous cell population, larger cells
  - Ø Mean: 24.54 µ μ
  - Standard dev.: 5.15 µ μ
**Cell counter – another example (protein2)**

**Time 0 (before infection)**

- **0 hrs**
  - Mean: 17.25 µ
  - Standard dev.: 2.5 µ

**72 hrs**

- **Infection not efficient: less homogenous cell population**
  - Mean: 20.83 µ
  - Standard dev.: 6.55 µ
  - 1 mg/10⁹ cells protein1

- **Efficient Infection: homogenous cell population, larger cells**
  - Mean: 24.28 µ
  - Standard dev.: 5.5 µ
  - 10 mg/10⁹ cells protein1
Virus titer determination by qPCR

Primers specific to gp64, a gene that encodes a essential virus envelope protein.

Gp64 is involved in membrane fusion during viral entry and is incorporated into budded virus particle during virus egress

Hitchman et al, 2007
One example: protein1

**Expression conditions:** 3 billion Hi5, infection at 27°C for 48h

**Purification protocol:** GSH-affinity column + tag removal by Prescision Protease

GSH-sepharose purification

Yield: 100mg/billion

LC-MS: Correct MW 100% 0P
cloning and expression in *E.coli* of protein2

**Tags**
- His
- His GST
- Trx His
- His SUMO

**Strains**
- *BL21*(DE3)
- *BL21*(DE3) pLys
- Origami2, B (DE3)
- Rosetta-gami2, B (DE3)
- Rosetta (DE3) pLac

**Temperatures**
- 30°C
- 20°C

Example in *BL21*(DE3) at 20°C

Selected condition: TRX_His in *BL21*(DE3) at 20°C
protein2 in bacteria: low yield and chaperone contamination

**Ni Affinity**
- Most of the protein in inclusion bodies
- Low recovery of soluble protein
- Purity <60%

**Ion exchange**
- Final yield: 1 mg/L
- Purity >70%

**LC-MS characterization**
- Correct MW
- 100% 0P
- Chaperones contamination

DNAK

The expression was tested in *E. coli* in 37 different conditions:
- 5 tags
- 3 strains
- 2 medias
- 3 temperatures

Protein is aggregated in all the conditions evaluated
His_GST was chosen for Insect cell expression.
protein2 in insect cell: acceptable yield

GSH Affinity + prescission cleavage

Yield: 15 mg/10^9
50% pure
54% 1P

Yield: 1 mg/10^9
50% pure
67% 2P

Further steps of purification needed
Modulating protein expression and optimizing purification protocol gave a final yield of 15 mg/10^9 of **homogeneous, stable, monodisperse** protein.

Optimization steps:
- Infection at low temperature
- Addition of cofactors and detergents during purification
- Identification of optimal storage buffer

**Production of >1g of protein**
>90% pure by coomassie staining
**Gst-kinase1: expression set-up in insect cells**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Cell lines</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gst_kinase1</td>
<td>SF9</td>
<td>SF21</td>
</tr>
</tbody>
</table>

48h and 72h time points are marked for each cell line.

Hi5 seems to express very low level of kinase1.

**Expression of kinase1 is toxic for insect cells:** very low viability and very low expression of recombinant protein upon infection.
**kinase1: expression set-up and large scale production**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Strains</th>
<th>Temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>His$_{10}$ _kinase1</td>
<td>BL21(DE3)pLys</td>
<td>21°C</td>
</tr>
<tr>
<td>His-Gst _kinase1</td>
<td>BL21(DE3)star</td>
<td>30°C</td>
</tr>
<tr>
<td>His$_{6}$-SUMO _kinase1</td>
<td>BL21(DE3)cd+</td>
<td></td>
</tr>
<tr>
<td>His$_{6}$-TRX _kinase1</td>
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</tbody>
</table>

**HIS_GST** at N-terminus was chosen for expression in *Bacteria*

**Expression conditions:**
2L *BL21* (DE3)pLys, 21°C for 22h using AI medium

**Purification:** GSH-sepharose+ GSH elution

Yield: **2.5mg/L**

His-Gst-kinase1 pool showed presence of several bacterial contaminants
2d  HisGst-kinase1 production

Expression conditions: 4L BL21 (DE3)pLys, 21°C for 22h using Al medium

Purification: GSH-sepharose + GSH elution (3L) → 4.6 mg + Resource Q
           + SDX200
           + Cleavase o.n.(1L) → 0.7 mg + Resource Q

Second step of purification doesn’t improve purity vs bacterial contamination
3rd HisGst-kinase1 CD production

Expression conditions: 4L BL21 (DE3)pLys, 21°C for 22h using Al medium
Purification: Ni-GSH-sepharose + GSH elution or cleavage o.n

Double-affinity purification protocol increases purity
LC-MS analysis: correct MW, very highly phosphorylated
4th HisGst-kinase1 production

Expression conditions: 6L (flasks) + 9L (Wave) of BL21 (DE3)pLys, 21°C for 22h

Purification: Ni-sepharose + GSH-sepharose + PPcleavage o.n

Ni-sepharose  GSH-seph.

E: elution with 400mM Imidazole
FT: FT after GSH-sepharose
R1: GSH-resin before elution
E1: elution after cleavage ON
R2: GSH-resin after elution

Yield: 80 mg (5.3 mg/L)

*Red indicates samples from Wave fermentation
**substrate1 expression in Bacteria**

**substrate1 full length**

- Presence of contaminants
- Tested as kinase1 substrate
- Not suitable as kinase substrate: contaminant ATPases from E.coli consuming ATP in the kinase assay

**Substrate1 short construct**

- No elution: Protein is aggregated on the resin

3.3 mg/L
substrate1 purification from Insect cells

Expression conditions: 1 X10^9 Hi5 cells infected for 72h at 27°C

Purification: GSH-sepharose + Tag cleavage by Prescission protease O.N.

Pool was concentrated

LC/MS analysis:
MW confirmed (100% 0P)
Tested in kinase assay:
suitable for monitoring kinase activity
Mammalian cells as expression host

• Considered the least efficient host

• Recent advances have improved the expression levels (stably trasfected CHO cell lines reported to express up to few grams/L of recombinant Abs)

• HEK293 (human embryonic kidney) and CHO (chinese hamster ovary) are the most used cells lines

• Scale-up (and consequent large scale transfection) can be technically challenging

• CHO cells are more often used to produce large amounts of recombinant proteins. Most of the therapeutic Abs on the market are produced in CHO cells

• The standard method to obtain stable CHO expression is to transfec a CHO DHFR-deficient cell line with a vector harboring a DHFR cassette and your G.O.I. Methotrexate is used as a selection agent: it binds and inhibits DHFR and only those cells in which the DHFR cassette integrates in the genome, will survive. Infact treatment with increasing concentrations of Methotrexate cause DHFR locus amplification along with the linked G.O.I. The stably transfected pools of cells are subcloned and analysed for expression levels: only few clones will express good-high levels of recombinant proteins, as the majority of integration events will occur in heterochromatic-transcriptionally inactive-regions. This process takes ~3 months.
Recombinant protein expression in mammalian cells today

Several features can be considered:

- Constitutive or Inducible expression
- Transient gene expression or Stably transfected expressing cell lines (vector integration: random, site-specific)
- Gene delivery by transfection or by adenovirus, lentivirus, baculovirus
- From suspension cell culture (FreeStyle CHO-S)
Mammalian vector for constitutive expression

**Figure 2.** High Yields of Recombinant Protein. We used pcDNA™3.3-TOPO® vector to clone and express milligram levels of erythropoietin (EPO), Factor IX (FIX), and IgG in FreeStyle™ CHO-S cells. pcDNA™3.3-TOPO® constructs containing the relevant PCR-derived gene sequences were transfected into FreeStyle™ CHO-S cells in 30 ml volumes. Heavy and light chain genes were co-transfected for IgG expression. Four to six days posttransfection, EPO and FIX were quantified using immobilized goat anti-human IgG antibody. Each bar shows the average of two expression runs.

**Figure 3.** pcDNA™3.3 vector map. The latest version in the pcDNA vector family, pcDNA™3.3 offers you the flexibility to express a native protein or add your favorite purification tag to the gene of interest prior to TOPO® cloning.
1. Tet repressor (tetR) protein is expressed from pcDNA6/TR in cultured cells.

2. TetR homodimers bind to Tet operator 2 (TetO2) sequences in the inducible expression vector, repressing transcription of the gene of interest.

3. Upon addition, tetracycline (tet) binds to tetR homodimers.

4. Binding of tet to tetR homodimers causes a conformational change in tetR, release from the Tet operator sequences, and induction of transcription from the gene of interest.

Modulate expression 0.1-1 ug/ml 8-24 h
It is also possible to obtain stable cell lines constitutively expressing the T-rex and inducibly expressing your g.o.i.
Stable cell lines generation by Flp-in system

1. pFRT/lacZeo is stably transfected into the mammalian cells of interest to generate the Zeocin™-resistant Flp-In™ Host Cell Line(s).

2. The pcDNA5/FRT expression vector containing your gene of interest (GOI) is cotransfected with pOG44 into the Flp-In™ Host Cell Line.

3. The Flp recombinase expressed from pOG44 catalyzes a homologous recombination event between the FRT sites in the host cells and the pcDNA5/FRT expression vector.

4. Integration of the expression construct allows transcription of the gene of interest (GOI) and confers hygromycin resistance and Zeocin™ sensitivity to the cells.
• Baculoviruses modified by insertion of a mammalian expression cassette

• Baculoviruses do not replicate in mammalian cells: safety profile

• Gene expression begins within 4-6 hours of transduction and in many cell types is completed after an overnight period.

• Depending on transduction efficiency, cell type and cell division rate, the transgene remains detectable from 5 to 14 days.

• Efficient infection of mammalian immortalized and primary cells, stem cells and non-mammalian cells

How to choose the expression host to express/purify a recombinant protein?

1. Which protein do I have to express?
2. Which host(s) do I have at my disposal?
3. Has anyone else expressed it before? Check literature/PDB/etc
4. Cytoplasmic or secreted protein? Check literature/PDB/etc
5. Disulfide bonds? Check literature/PDB/etc
6. Membrane protein? Check literature/PDB/etc
7. Toxic protein? Check literature/PDB/etc
8. For which application?
9. ....
Biochemistry activities in 2008: some numbers……

Proteins for Assays:
>40 proteins delivered to assay development, 10 proteins for HTS

Proteins for Structural Chemistry:
>35 constructs delivered to Structural Chemistry group, corresponding to 15 different proteins, 11 crystals obtained

- >250 expression vectors, corresponding to >60 different genes
- >140 recombinant viruses generated
- >1500 expression conditions tested
- >200 large scale productions (from 1 up to 15-30 billion-scale) to produce >60 different proteins (in some cases different constructs per each protein)
- >1200.000.000.000 insect cells infected
- >250 L E. coli

Total >7.7 grams of proteins
http://www.pichia.com