Science Webinar Series

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Advances in Protein Expression:
High Throughput Tools for Improving Analysis

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Science Webinar Series

Advances in Protein Expression:
High Throughput Tools for Improving Analysis

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Sponsored by:
High-Throughput Protein Production at the Macromolecular Therapeutics Development Facility: Multi-Platform Expression Evaluation

Brandan Hillerich, Ph.D.
Managing Director of High-Throughput Protein Production

Macromolecular Therapeutics Development Facility
New York Structural Genomics Research Consortium (NYSGRC)
Albert Einstein College of Medicine
The **Protein Structure Initiative** (PSI) is an ongoing effort begun in 2000 to accelerate discovery in structural genomics and contribute to the understanding of biological function through atomic structure.

New York Structural Genomics Research Consortium (NYSGRC) is a PSI:Biology Large-Scale Center.

NYSGRC acts as the Protein Production and Structure Determination Facilities for The Immune Function Network (IFN) and two other Partnership Centers.

NYSGRC relies on the use of automation and high throughput small-scale expression screening to rapidly determine which domains, expression systems, cell lines, and purification conditions yield enough pure protein for structural studies. We are also using these methods to screen production conditions for full length proteins for functional characterization.
There are ~ 500 Ig superfamily genes in the human genome.

Members include:
- Co-stimulatory molecules (i.e. CD28:B7-1)
- Inhibitory molecules (i.e. PD-1:PD-L1, BTLN-2)
- Antigens & antigen receptors (i.e. PV-Rα, T-cell antigen CD7)
- Cell adhesion molecules & cytoskeletal regulators (i.e. JAM-1, RAGE)

- They all contain at least one Ig-like domain.
  - Good for Structural Studies

Many interact with other IgSF members and are involved in a wide range of human diseases.

New and critical interactions remain to be identified so screening methods are needed to:
- identify new interactions relevant to human physiology & disease
- elucidate mechanisms of known therapeutic targets
- provide new complexes to explore crystallographically
- develop novel reagents and potential therapeutics
Parallel Pipelines

PCR

LIGATION INDEPENDENT CLONING

pET based vector

12 Growth conditions
96-well-plate format

Refolding
96-well-plate format

Automated Small Scale Screening
384-well-format

Scale-up and Purification

BACTERIA

pLEX based vector

Viral generation
Transduction: 2 cell types
24-well-plate format

pEGFP-N1 based vector

Transient transfection:
HEK-293s cells
24-well-plate format

DAEDALUS based vector

Viral Generation:
HEK-293s cells
TPP format - 48 “well”

INSECT

MAMMALIAN

LENTIVIRAL
To expand the repertoire of proteins available from *E. Coli* hosts, a general method for HT refolding had to be developed.

IB’s containing human secreted proteins can often be successfully refolded in a single buffer system.

- Separate by SEC (20% success rate ... or 80% wasted effort)
IFN - Refolding

- **Current hurdle:** identifying which targets to scale up from a predictive small-scale screen.

- Developed a highly predictive small-scale screen
  - scout for proteins amenable to oxidative rapid dilution refolding.
  - 1 ml reaction volume
  - **highly predictive** for multi-liter production scale efforts.
Small-Scale Expression Evaluation
Parallel Pipelines

**PCR**

LIGATION INDEPENDENT CLONING

- **pET based vector**
  - 12 Growth conditions
  - 96-well-plate format
  - Refolding
  - 96-well-plate format

- **pLEX based vector**
  - Viral generation
  - Transduction: 2 cell types
  - 24-well-plate format

  Automated Small Scale Screening
  - 384-well-format

  Scale-up and Purification

**BACTERIA**

**INSECT**

**MAMMALIAN**

**LENTIVIRAL**

- **pEGFP-N1 based vector**
  - Transient transfection: HEK-293s cells
  - 24-well-plate format

- **DAEDALUS based vector**
  - Viral Generation: HEK-293s cells
  - TPP format - 48 “well”
Perkin Elmer Cell::Explorer
Tissue Culture Robotic Solution
Small Scale Expression – Ni IMAC
IFN Targets – Multiple cell lines

Hi5

Sf9
**Small Scale Screening**

**Disaggregation screening.** Twelve buffer conditions that span a variety of pHs (MES 6.0, Hepes 7.5, Tris 8.0) with different additive conditions (low salt, high salt, urea, L-arginine) are screened for their ability to disaggregate proteins.
Small Scale Screening

Monomer

Void
Recent Successes from BV

NYSGRC-005836
PDB Code 4HWN
Human Fc-receptor like A, Ig-C2 domain

NYSGRC-005912
PDB Code 4HWU
Mouse FGF receptor 2, Ig-C2 type 1 domain

NYSGRC-005691
In progress
Mouse Carcinoembryonic antigen related Cell adhesion molecule 15
Mammalian Expression

- Transient small – scale
  - *Inability to scale**
- Stable – ALL cells produce gene of interest
  - Linear expansion
- Select-and-Integrate
  - Time consuming
  - Variable/Unstable expression
- Lentivirus
  - High MOI, High Copy integration, High yield
    - Rapid (2 weeks)
      - Viral Generation
      - Packaging size

*Stably expressed Fc fusion proteins prior to magnetic selection of high expressing cells (SDS-PAGE + Coomassie stain following small scale nickel purification from supernatants)*
The DAEDALUS System

Ashok Bandaranayake**

From ~15 mg / Liter

Traditional

To ~90 mg/Liter

Daedalus

Unstable

Highly Stable

**Nucleic Acids Research, 2011, 1–11
Daedalus – HT Enhanced

• Problem
  – Adherent HEK 293
  – Serum
  – Purification of the virus by ultracentrifugation or ion exchange
    • High speed, overnight

• Solution
  – Suspension 293 Freestyle cells
    • Serum free
  – TPPs (50 ml tubespin bioreactors)
    • reducing space requirements
    • harvesting the virus, spinning down the cells in the TPPs and sterile filtering the supernatant
  – Direct PEI mediated transfection method
    • eliminates the need to form pre-complexes of DNA and PEI
Daedalus – HT Enhanced

Result:

- Rapid production of 30 ml of virus at titers of $1 \times 10^7$ TU/ml
- Facile generation of 48 viruses per week
- Production of 12 proteins per week with a yield of 10-20 mg
- From clone to protein in less than 2 weeks
Daedalus – HT Enhanced
Keys to Success

There are Ways to Overcome the Difficulties Associated with Production of Ig Superfamily Proteins

- Designing of Domains
- Trying Multiple Cell-lines and Expression Systems
- Screening Multiple Buffer Conditions

Start Small

- Better to Fail Quickly at Small-Scale than Slowly at Large-Scale
- Testing Constructs and Conditions at the mL Scale Saves Time and Money
- Automation Enhances Throughput and Reproducibility
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Production of Vaccine Antigens and Carriers Using Pfēnex Expression Technology™ and HTP Microchip CGE Analysis

Jason Payne
Science Webinar Series
April 17th, 2013
Background: The Company

• Protein production company, located in San Diego CA

• Platform based on *Pseudomonas fluorescens*

• Complete process development capabilities from molecular biology to downstream processing

• Non-GMP production at site; cGMP production at partner sites

• 35 FTE
• Expression of Lead Therapeutics/Vaccines: Macromolecules, Vaccine Antigens, Ab Fragments, Fusion Proteins, Scaffold Proteins, etc.
• Customer Segment: BioPharma

• Partnerships with BARDA and SAIC
• Projects include: *P. falciparum* based malaria vaccine and rPA based anthrax vaccine

• Over 5,000 catalog products, specializing in vaccine components and bacterial/viral antigens
• Custom protein expression services

• Product development partnerships in India/ROW
• GCSF, hGH, Interferon beta 1-b, and other programs

Pfenex Confidential – Do not share without permission
Strain Construction Pyramid

Thousands of combinations = thousands of samples =
Need for high-throughput sample prep and analysis
Robotically Enabled High Throughput Growth and Expression

1000+ strains evaluated in parallel

Replicate cultures will be grown for 24 h at 30 °C prior to induction
Temperature shift to 25 °C after induction and cultures incubated for 24 h
“Expression Space”: Plasmid and Host Strain Effects

Pfenex explores a large experimental design space, defined by interactions between expression strategy and host phenotype

- Some plasmid expression strategy combinations are more optimum than others
- Some host strain phenotypes are more optimum than others
Process Analytical: 1st and 2nd tier screens

challenge: to accurately analyze target in complex mixture of solids, protein, DNA, etc. in timely fashion

Quantity addressed

1st tier analysis

Number of samples: 1000s

HTP SDS-CGE

96-well format
90 min per plate

measure target mass yield

2nd tier analysis

Number of samples: 10s to 100s

HTP BLI

96-well format
30 min per plate

HTP Expression

Quality addressed

MS

HPLC

+ Western Blot and other methods

measure fragments, aggregates, etc.
Robotically Enabled Sample Preparation

Integrated robotic platform comprised of multiple workstations where sample plates are moved from station to station using a central robotic arm

- Plates (96-well) of strains harvested and stored in a chilled hotel
- Cultures are sonicated (24-pin) and then centrifuged to separate soluble and insoluble fractions
- Fractions are recovered and aliquoted using a liquid handling robot
- Samples prepped for HTP microchip SDS-CGE analysis

Parallel processed, robotically enabled strain engineering technology increases throughput and speed of development
SDS-CGE Every Day

- Strain screening
  - ~1000 per project/protein
- Fermentation samples
  - DOE samples in triplicate
- Purification fractions
  - Often need dilution
- Stability study samples

- ~100,000 samples in 2012
Fermentation Work Flow

- Micro-24 and parallel bioreactors are integral to Pfenex’s rapid fermentation development process

Minibioreactors (Micro-24)

- Scalable high cell density process in 2 L glass or 20 L stainless steel bioreactors

- Multiple strains evaluated in multiple fermentation conditions in DoE
- Micro-24 process can be predictably scaled

- Strain and process finalists scaled to higher cell densities in 2 L or 20 L bioreactors
- Production strain finalized and fermentation process further developed if needed
Downstream Processing

Robotic Batch Screen
PhyTip™ or filter plates containing resins
apply target, filter, wash, elute
with different parameters

Scouting and Optimization

HTP Analysis
Batch screen- robotically-enabled microtiter plate or PhyTip™ format; test resin for binding capacity/selectivity using varying conditions
Scouting- small columns to test screen leads; comparative test gradients; variable scouting; dynamic binding
Optimization- fine tune parameters using scaled-down larger column (pH, protein loading, flow velocity)

Scale-up
Pilot-scale Chromatography
Bench-scale Chromatography

Resin Screening

- 96-well format using filter plates, Sciclone liquid handler, and plate-centrifuge

- Can easily run Load, flow-through, wash, elution fractions using CGE
Proteins produced with Pfēnex expression technology

- Messenger Proteins (e.g., cytokines, interferons)
- Antibody Derivatives
- Enzymes
- Protein G

- Vaccine Components
  - *C. difficile* toxin B (TcdB) → antigen
  - *P. aeruginosa* exotoxin A (rEPA) → adjuvant/carrier
  - Cholera toxin B subunit (CTB) → adjuvant/carrier
  - *E. coli* heat-labile toxin (LT) → adjuvant/carrier
  - Tetanus toxin C-fragment (TTC) → adjuvant/carrier
  - CRM197 → carrier
  - Protective Antigen (rPA) → antigen
**C. difficile** Toxin B (TcdB)

- Cytotoxic glucosyltransferase from *Clostridium*
  - Induces cytoskeletal depolymerization
- Implicated in pseudomembranous colitis, AAD
- Molecular weight = 270 kDa; isoelectric point = 4.1
  - (HMW1 GX assay)
- SDS-CGE of strain-screening samples

Credit: Ng lab, University of Calgary
Tetanus toxin C-Fragment (TTC)

- Fragment of neurotoxin from *Clostridium tetani*
  - Binds to gangliosides on neuronal membranes
- Molecular weight = 51.6 kDa; pI = 6.4

**Objectives:** Purify active TTC to >95% purity
Tetanus toxin C: Anion exchange

resin: Q Sepharose FF (GE)
flow: 150 cm/h = 5 min residence time
buffer A: 20 mM Tris, pH 8.0
buffer B: 20 mM Tris + 0.2 M NaCl
chromatography:
  (A) load filtered lysate on column;
  (B) wash with buffer A;
  (C) NaCl gradient from 0-50% B;
  (D) steps at 50% B, 100% B;
  (E) strip with sodium hydroxide

• Purity increase needed; AEX elution fractions are pooled to go to 2nd column

• Initial HIC results showed loss of activity; subsequent salt-exposure experiments confirmed loss due to salt
  → alternative 2nd column to HIC needed to further purify protein
Tetanus toxin C: Anion exchange $\rightarrow$ Mixed-mode

- Resin: Capto adhere (GE)
- Column: 5 mL, HiTrap
- Flow: 60 cm/h = 2.5 min residence time
- Buffer A: 20 mM Tris, pH 8
- Buffer B: 40 mM acetate, pH 4
- Chromatography: wash w/ A11;
  (A) load CEX eluate on column;
  (B) wash with 10% A + 90% B;
  (C) 10-25% gradient over 10 CV
  (D) strip with sodium hydroxide

- Resin used exhibits properties seen in HIC resin without need for high salt
- Capto adhere peak fractions pooled
  (Endotoxin < 10 EU/mg)
TTC: Analytical of lyophilized material

- Hemagglutination activity
  - Ganglioside-binding activity confirmed, comparable to standard

- Purity
  - SDS-CGE analysis >95% purity

- Quality
  - LC-MS
CRM197

- Non-toxic mutant of diphtheria toxin
  - G52E point mutation
- Carrier protein in a number of approved vaccines, such as meningitis and pneumococcal bacteria infections
- Molecular weight = 58.4 kDa

- SDS-CGE of strain screening samples

- Process consists of a typical high cell density fermentation followed by three column chromatography steps
- \( \rightarrow \) cGMP manufacturing at partner site to produce high quality product
CRM197 Production

Streamlined & Scalable Path to Clinic & Commercial

- Product & process consistency from reagent grade (pre-clinical) to GMP grade (clinical)
- Capability to supply kg quantities
- Pfenex material currently being used by European based vaccine innovator to support Tox studies for new vaccine product under development

Regulatory Profile

- Animal-Origin-Free based process (compared with current technology)
- BMF access to support IND & BLA filings
- Vaccine product in Phase 1 based on Pfenex derived CRM197
## Protein Stability

<table>
<thead>
<tr>
<th>Ladder</th>
<th>WT</th>
<th>WT 2-8C 1 year</th>
<th>WT 25C 1 year</th>
<th>Mutant</th>
<th>Mutant 2-8C 1 year</th>
<th>Mutant 25C 1 year</th>
</tr>
</thead>
</table>

### CGE: E-gram

- [A1] WT
- [A2] WT 2-8C 1 year
- [A3] WT 25C 1 year

### RP-HPLC

Fluorescence vs. Aligned Time (sec)
Strain Genotyping using DNA chip

- Verify production strains
Summary

• Vaccine antigens and carrier proteins can be produced in Pfēnex Expression Technology strains at high titer and quality (full-length, very low degradation), which aids purification process development

• Using 96-well format for expression screening from growth to CGE allows us to screen 1000+ strains per project.

• CGE is also used daily for strain screening, fermentation, purification, and more.

• This approach has been used to purify a variety of vaccine antigens, with the intent of developing these processes further to produce GMP-grade vaccine components for vaccine developers globally

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