Purification of Vaccine Antigens & Carriers Expressed in *Pseudomonas fluorescens*

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**Background: The Company**

*Protein production company located in San Diego, California*

- Complete process development capabilities from molecular biology to downstream processing
- Non-GMP production at site; cGMP production at partner sites
- Four key business units
  - Lead Proteins
  - Reagent Proteins
  - Biosimilars/Biobetters
  - Biodefense/Government Programs
The Pfēnex Toolbox: The Next Generation for Bacterial Strain Engineering

Thousands of unique combinations are harmoniously combined to enable strain engineering for optimal protein production

- 2 plasmids
- 4+ promoters
- 3 ribosome binding sites
- 25+ secretion leaders
- 45+ chaperone/disulfide bond isomerase overexpression plasmids
- 130+ protease clean deletion mutants, multiple deletions

Elements combined to generate >100 rapid cloning, off the shelf, expression vectors
Proteins produced with Pfēnex expression technology

- Messenger Proteins (e.g., cytokines, interferons)
- Antibody Derivatives
- Enzymes
- Vaccine Components
  - *P. aeruginosa* exotoxin A (rEPA)
  - *C. difficile* toxin B (TcdB)
  - Cholera toxin B subunit (CTB)
  - *E. coli* heat-labile toxin (LT)
  - CRM197

<table>
<thead>
<tr>
<th>Protein</th>
<th>Used as</th>
</tr>
</thead>
<tbody>
<tr>
<td>rEPA</td>
<td>adjuvant/carrier</td>
</tr>
<tr>
<td>TcdB</td>
<td>antigen</td>
</tr>
<tr>
<td>CTB</td>
<td>adjuvant/carrier</td>
</tr>
<tr>
<td>LT</td>
<td>adjuvant/carrier</td>
</tr>
<tr>
<td>CRM197</td>
<td>carrier</td>
</tr>
</tbody>
</table>
Purification of Vaccine Antigens/Carriers: Rapid Purification Process Development

Objective: Produce high purity/quality product to support preclinical development

- Strains/processes allow for easy transition to clinic as the projects advance to clinic
- Process optimization is minimal, while maintaining focus on quality
1) *P. aeruginosa* exotoxin A (rEPA)

- **Exotoxin A:** ADP-ribosyltransferase from *Pseudomonas aeruginosa*
  - Inhibits elongation factor in protein synthesis; similar to diphtheria toxin
- **rEPA** = non-toxic mutant of exotoxin A (deletion of Glu-553)
  - Immunoreactive, with $10^6$ lower enzymatic activity
- **Molecular weight** = 66.6 kDa; isoelectric point = 5.3

SDS-CGE of fermentation samples

**Objective:** Purify rEPA to > 95% purity
rEPA: Anion exchange

- Resin: Q Sepharose HP (GE)
- Flow: 60 cm/h = 2.5 min residence time
- Buffer A: 20 mM Tris, pH 8
- Buffer B: 20 mM Tris + 1 M NaCl

Chromatography:
(A) Load filtered lysate on column;
(B) Wash with buffer A;
(C) NaCl gradient from 0 – 20% B;
(D) Steps at 20% B, 100% B;
(E) Strip with sodium hydroxide

<table>
<thead>
<tr>
<th></th>
<th>Concentration (mg/mL)</th>
<th>Pool purity (avg., CGE)</th>
<th>Endotoxin (EU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution pool</td>
<td>5.7</td>
<td>97.7%</td>
<td>197</td>
</tr>
<tr>
<td>D6-E10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

→ High titer enables rapid purification process development
2) *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

SDS-CGE of strain screening samples

**Objective:** Purify active TcdB to >95% purity, while avoiding degradation
C. difficile Toxin B: Anion exchange

resin: Q Sepharose FF (GE)
flow: 50 cm/h = 3 min residence time
buffer A: 20 mM Bis-Tris + 0.25 M NaCl
buffer B: 20 mM Bis-Tris + 1 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

<table>
<thead>
<tr>
<th>Elution pool C12-D2</th>
<th>Concentration (mg/mL, CGE)</th>
<th>Pool Purity (CGE)</th>
<th>Endotoxin (EU/mg, CGE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.2</td>
<td>~97%</td>
<td>~600</td>
</tr>
</tbody>
</table>

→ Low pl leveraged for improved resolution from HCPs
→ High MW allowed final buffer exchange to increase purity to ~99%
→ Active by cell rounding assay (down to 0.2 ng/mL)
3) Cholera toxin B subunit (CTB)

- Ganglioside-binding subunit from *Vibrio cholerae*
  - Non-toxic; cytotoxicity from A subunit
- B subunit forms pentamer (linked to binding)
- Molecular weight = 11.5 kDa (monomer), 57 kDa (pentamer)
- Isoelectric point = 8.0

SDS-CGE (reduced) of strain screening samples

**Objective:** Purify active CTB to >95% purity
Cholera toxin B: Cation exchange

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**Resin:** SP Sepharose HP (GE)

**Flow:** 100 cm/h = 8 min residence time

**Buffer A:** 30 mM Na phosphate, pH 7

**Buffer B:** 30 mM NaPi + 1 M NaCl

**Chromatography:**

(A) load filtered lysate on column;
(B) wash with buffer A;
(C) NaCl gradient from 0 – 50% B;
(D) step at 100% B;
(E) strip with sodium hydroxide

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<table>
<thead>
<tr>
<th>Elution pool</th>
<th>Concentration (mg/mL, CGE)</th>
<th>Purity (CGE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4-C6</td>
<td>1.6</td>
<td>~95%</td>
</tr>
</tbody>
</table>

→High pI leveraged for improved resolution from HCPs
CTB: Analytical on lyophilized material

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

- **Endotoxin**
  - Endotoxin-removal step added to reduce endotoxin in final material to < 100 EU/mg

- **Purity**
  - SDS-CGE analysis >95% purity

- **Quality**
  - LC-MS

Deconvoluted mass spectra
4) *E. coli* heat-labile toxin (LT)

- Toxin from enterotoxigenic *Escherichia coli*
  - Similar in structure and function to cholera toxin
- Holotoxin formed by A subunit and B pentamer
- Molecular weight = 11.7 kDa (B subunit);
  27.8 kDa (A subunit)
- Isoelectric point = 8.0 (B subunit);
  6.5 (A subunit)

SDS-CGE of strain screening samples (run alongside standard)

Objectives: Purify LT & LTB separately to >95% purity
**E. coli LT: Cation exchange**

- **Resin:** SP Sepharose FF (GE)
- **Flow:** 37 cm/h = 4 min residence time
- **Buffer A:** 30 mM Na phosphate, pH 7
- **Buffer B:** 30 mM NaPi + 1 M NaCl

**Chromatography:**
1. Load filtered lysate on column;
2. Wash with buffer A;
3. NaCl gradient from 0 – 50% B;
4. Step at 100% B;
5. Strip with sodium hydroxide

➔ **High pl leveraged for improved resolution from HCPs**
   - Similar to cholera toxin B purification
   - Pool purity > 90% by SDS-CGE

➔ **Separation between LT and LTB still needed**
E. coli LT/LTB separation, option 1

- **Original CEX (009)**

  ![Graph showing A280 and cond fractions](image)

  - Shallow NaCl gradient separates two forms
    - RP-HPLC analysis of peak containing A and B indicates formation of AB₅ holotoxin

  - Separation between LT and LTB still needed

- **CEX employing shallower gradient (010)**

  ![Graph showing A280 and cond fractions](image)

  - Mol ratio of B:A is ~5.8

  - RP-HPLC of 010 sample (on Zorbax 300SB-CN column)

  - Analyzed by RP-HPLC

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**E. coli LT/LTB separation, option 2: CEX → HIC**

- **Resin**: Phenyl Sepharose HP (GE)
- **Flow**: 37 cm/h = 4 min residence time
- **Sample**: 566-009 B9-D4 + 1.0 M ammonium sulfate (AS)
- **Buffer A**: 30 mM NaPi + 1 M AS, pH 7
- **Buffer B**: 30 mM NaPi, pH 7
- **Chromatography**: wash w/ A11;
  - (A) load sample on column;
  - (B) wash with buffer A;
  - (C) reverse AS gradient (0-100% B);
  - (D) step at 100% B;
  - (E) strip with sodium hydroxide

- LT proteins present in two peaks
  - → 1st peak: B pentamer
  - → 2nd peak: AB₅ holotoxin

- HIC cleanly separates the two forms
- Acts as concentration step
LT/LTB: Analytical on lyophilized material

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

- **Purity**
  - SDS-CGE analysis >95% purity

- **Quality**
  - LC-MS

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**Diagram Description**

- LT (A+B)
  - A and B subunits
  - Range: $2.412 \times 10^1$

- LTB
  - B subunit
  - Range: $2.90 \times 10^1$
5) 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

→ 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

SE-HPLC

RP-HPLC
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

• Employing ion exchange as primary column increases purity and reduces endotoxin, while allowing additional columns to be added without need for buffer exchange

• Leveraging available information (such as theoretical pI) to tailor purification methods to each protein reduces time to reach final process

• 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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