Extracellular Secretion and Non-canonical Amino Acid (ncAA) Incorporation: New Capabilities Supporting Conjugate Vaccine Discovery and Development

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Pfenex Expression Technology® by primrose bio

Engineered *P. fluorescens* host strains enable extracellular protein localization and high-fidelity ncAA incorporation for next-generation conjugate vaccine development.

ABSTRACT

Efficient production of vaccine carrier proteins and antigens is paramount to ensuring access to affordable conjugate vaccines. Streamlined conjugation technologies improve product quality and consistency and allow lower antigen input needs. Development of extracellular (EC) localization and non-canonical amino acid (ncAA) incorporation within Primrose Bio's *Pseudomonas fluorescens*-based expression platform accelerates the discovery and development of affordable vaccine candidates.

We have developed engineered host strains for EC localization that move properly folded proteins from the periplasmic space to the culture medium where they can be readily separated from microbial cell contaminants without a cell lysis step, reducing downstream purification steps.

Contrary to what has been observed with other recombinant protein expression platforms, *P. fluorescens* has been shown to be a very robust protein expression engine, capable of incorporating ncAAs with high fidelity and high titers. In some examples, we have demonstrated titers of up to 15 g/L target protein with precise, 100% ncAA incorporation.

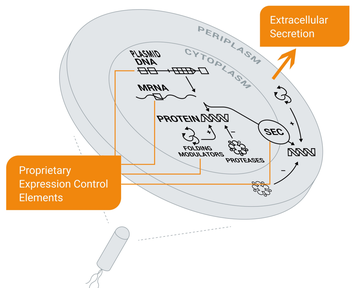
OBJECTIVE

Demonstrate EC localization of multiple example proteins, including mutant toxin proteins for use as carrier proteins. Further demonstrate efficient ncAA incorporation and production of several vaccine-relevant target proteins using the *P. fluorescens*-based Pfenex Expression Technology® platform.

PFENEX EXPRESSION TECHNOLOGY®

Purpose-built platform for high-titer protein expression

- Accelerated development: rapid, automated combinatorial screening to identify manufacturing strains for soluble, properly folded protein
- High cell density fermentation with short cultivation time; periplasmic titers up to 30 g/L
- Regulatory friendly: animal-origin free, no antibiotics
- Six approved, marketed biologics (FDA, EMA, WHO)



Toolbox components based on *P. fluorescens* biology

Off the-shelf expression plasmids
- No antibiotic resistance genes
- Various promoter and ribosome binding site strengths
- Periplasmic targeting secretion leaders
- Fusion partners

Frozen banks of engineered host stains
- Folding helper overexpression
- Protease knock-outs

Engineered hosts for advanced, complex protein expression
- ncAA incorporation
- Streamlined recovery

Proven track record in high quality vaccine antigen and carrier protein production

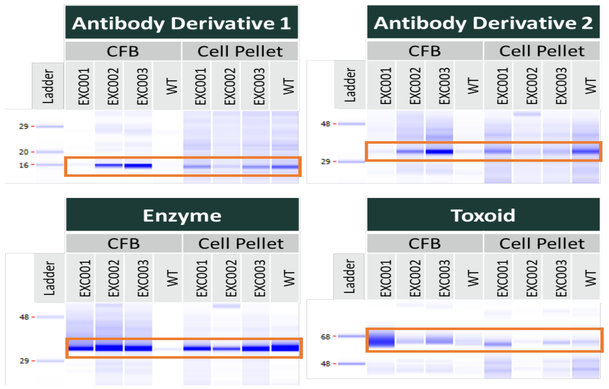
Protein Type	Alternative Host and Challenges	Pfenex Results
VLPs: Cowpea Chlorotic Mottle Virus	Plants	Multiple g/L assembled particles
Microbial outer membrane protein	*E. coli* : no expression	Soluble active expression 20 g/L
HIV Nef protein	Yeast: low yield, degradation, glycosylation	Multiple g/L, high quality
HIV Gag protein	*E. coli* : low yield	10X yield improvement
Circumsporozoite protein (CSP) from *Plasmodium falciparum*	*E. coli* : inclusion bodies, no soluble expression, truncation	3-5 g/L soluble expression, no refolding
Chimeric flu antigen-adjuvant	*E. coli* : low insoluble yield, inefficient refold	Multiple g/L, soluble, antigenic fusions
C. difficile toxin B (TcdB)	Native organism, *E. coli* : low yield	> 2 g/L, soluble, active
B. anthracis recombinant PA (rPA)	Native organism, lower yield, containment	Mid double-digits g/L soluble

RESULTS

Extracellular Localization

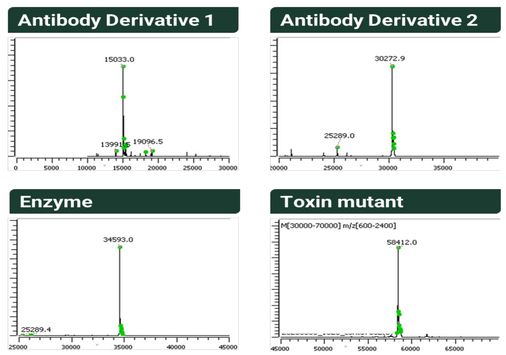
Engineered host strains move properly folded proteins from the periplasmic space to the culture medium, enabling recovery without cell lysis. This reduces downstream purification steps, **saving time and costs**.

Multiple host cell targets identified influencing EC expression levels



Screening Methods:
- Evaluate four targets ranging from ~15kDa to 60kDa
- Three EC hosts (EXC001, 002, 003) and wild type (WT) Pfenex host strain evaluated
- SDS-CGE analysis performed on cell-free broth (CFB) and soluble lysate from cell pellet (above)

High quality protein in culture supernatant



Intact Mass Analysis of Secreted Samples: Supernatant samples analyzed by LC-MS. All observed masses matched the expected theoretical sequence within 1 Dalton.

Extracellular Localization Key Features

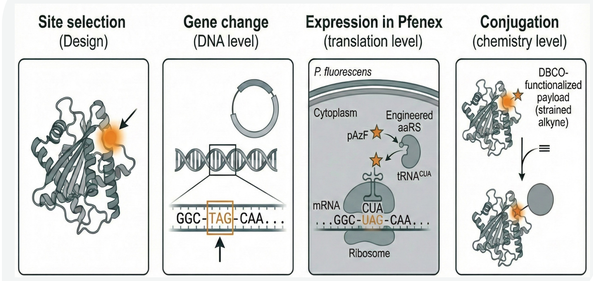
Periplasm First -> High Quality Target Protein
- Folding in the periplasm for proper disulfide formation; reduced exposure to intracellular proteases

Simplified Target Recovery
- Eliminates lysis step; reduced HCP, HC-DNA, and endotoxin

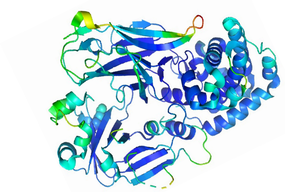
Real-Time Expression Monitoring
- In-process sampling without cell disruption

NEXT GENERATION CARRIER PROTEINS

Controlled, site specific conjugation through ncAA incorporation

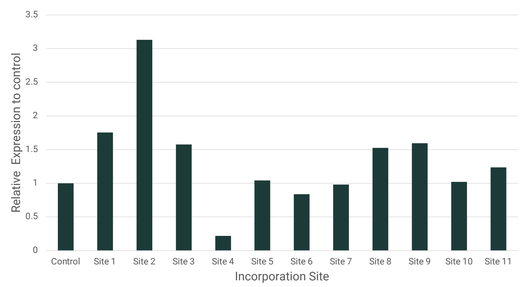


Site Selection:
Hydrogen-Deuterium Exchange (HDX) Analysis of PeliCRM197®



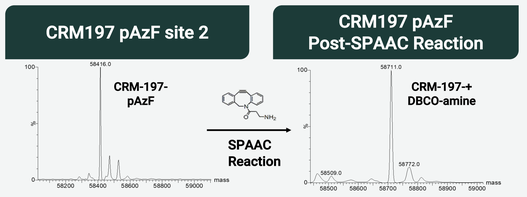
- Four regions of CRM197, marketed by Primrose as PeliCRM197, were identified by HDX-MS analysis and molecular modeling to exhibit characteristics favorable for site-specific conjugation, facilitating next-generation conjugate vaccine development
- None of the regions identified are found on the binding interface of the monomeric subunit which drives dimer formation

Expression of CRM197:
With para-azidophenylalanine (pAzF) incorporation



Site-Specific ncAA Incorporation in CRM197: CRM197 was engineered for para-azidophenylalanine (pAzF) incorporation at 11 unique sites and expressed in P. fluorescens. CRM197 expression in the presence of pAzF was assessed at 2L scale. 10 of 11 constructs demonstrated soluble titers at or greater than native CRM197 protein.

pAzF incorporation and reactivity confirmed



CRM-197 pAzF (Da)		CRM-197 + DBCO-amine (Da)	
Theoretical Mw	Observed Mw	Theoretical Mw	Observed Mw
58412.0	58415.0	58713.0	58711.0

CRM197-pAzF Intact Mass Analysis:
- Intact mass analysis confirmed incorporation of pAzF into PeliCRM197 (left panel)
- SPAAC reaction was performed, confirming reactivity of the incorporated pAzF (right panel)

Next-Gen CRM Key Features

Orthogonal techniques for site selection
- Accessible sites for conjugation identified through wet lab and dry lab analysis

Maintain manufacturability
- Expression titers similar to or great than control protein (no ncAA incorporation) achieved with 10/11 sites selected

Ensure simplified conjugation strategy
- pAzF accessible for click chemistry

Next steps: finalize site(s) and immunogenicity testing

CONCLUSION

The Pfenex Expression Technology® platform demonstrates two key capabilities for conjugate vaccine development:

1. Extracellular secretion strains enable efficient recovery of properly folded proteins from the culture medium, simplifying purification and improving product quality.

2. Pfenex is a robust protein engine capable of incorporating ncAAs with high fidelity and high titers (up to 15 g/L with 100% ncAA incorporation), enabling site-specific bioconjugation for next-generation carrier proteins.

These capabilities accelerate the discovery and development of affordable conjugate vaccine candidates.

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