REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
31-03-2023	Final Report	27-Jun-2019 - 26-Jun-2022

4. TITLE AND SUBTITLE
Final Report: Expression of Recombinant Products with Butyrylcholinesterase (BChE) Activity in Pichia pastoris

5a. CONTRACT NUMBER
W911NF-19-2-0211

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHORS

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Massachusetts Institute of Technology (MIT
77 Massachusetts Avenue
NE18-901
Cambridge, MA 02139 -4307

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
75327-BB-DRP.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
2 Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			J. Christopher Love
UU	UU	UU	UU		19b. TELEPHONE NUMBER 617-324-2300

RPPR

as of 23-May-2023

Agency Code:

Proposal Number: **Agreement Number:**

Organization:
Address: , ,
Country:
DUNS Number: EIN:
Report Date: Date Received:
for Period Beginning and Ending
Title:
Begin Performance Period: **End Performance Period:**
Report Term: -
Submitted By: Email:
Phone:

Distribution Statement: -

STEM Degrees: **STEM Participants:**

Major Goals:

Accomplishments:

Training Opportunities:

Results Dissemination:

Plans Next Period:

Honors and Awards:

Protocol Activity Status:

Technology Transfer:

I certify that the information in the report is complete and accurate:
Signature:
Signature Date:

Contract #W911NF1920211
Institution: Massachusetts Institute of Technology

Title: Expression of Recombinant Products with Butyrylcholinesterase (BChE) Activity in *Pichia pastoris*

Final Report for 27-June-2019 thru 26-June-2022

Date of Report: 31-March-2023

Total Dollar Value: $1,799,348

DARPA Program Manager: Dr. Anne Cheever, DARPA/BTO
Cooperative Agreement Manager (CAM): Dr. Stephanie McElhinny, ARO

Submitted by:
J. Christopher Love, Ph.D. – Principal Investigator
77 Massachusetts Avenue (76-253)
Cambridge, MA 02139
Telephone: 617-324-2300
Fax: 617-258-5042
Email: clove@mit.edu

Distribution List and Addresses

One report to:
Dr. Anne Cheever
Program Manager
Defense Advanced Research Projects Agency
Email: anne.cheever@darpa.mil

One report to:
Dr. Stephanie McElhinny
Cooperative Agreement Manager (CAM)
Email: stephanie.a.mcelhinny.civ@army.mil

1. Executive Summary:

Background/Project Significance: Butyrylcholinesterase (BChE) is a serine hydrolase used as a prophylactic countermeasure against organophosphate nerve agents, like V-series gases and sarin. BChE binds and neutralizes nerve agents in the bloodstream before the toxins can inhibit native cholinesterase function in the nervous system. BChE is currently the only therapeutic agent effective in protecting humans against the entire spectrum of organophosphate nerve agents. Procurement of measurable quantities of this reagent for human treatment remains challenging mainly due to its complex biophysical characteristics: native circulating BChE is a ~270 kD tetramer of heavily glycosylated subunits bearing N-linked glycans at each of nine different sites.

Current recommendations for use of BChE as a prophylactic measure against nerve agents require doses of ~400 mg per person (provided in 8-16 mL intravenous bolus at 25 mg/mL) with neutralizing activity of 621 U/mg and a circulating half-life of 10 days. While BChE's functional activity is independent of both BChE glycosylation and tetramerization, longevity is largely determined by glycosylation, specifically sialylation, and may be improved with increased tetramerization. To best achieve this target product profile (TPP), BChE typically is isolated from human plasma Cohn fraction IV. Given the overall protein complexity of human plasma and starting purity of BChE within it, the isolation process requires expensive affinity resins like procainamide or huprine and requires an excess of 400 kg starting material to yield only ~8g of pharmaceutical-grade BChE product. Given these low product yields and the associated costs, prophylactic doses of BChE rarely are provided beyond those given to at-risk active military personnel. Development of a transgenic system for the expression of pharmaceutical-grade BChE (or suitable analogs) could enable efficient manufacturing of large quantities of product for prophylactic administration to larger populations of at-risk individuals (e.g., civilian populations in at-risk regions like Syria).

The overall goal of this project was to demonstrate a proof-of-concept for the end-to-end manufacturing of a protein biologic with potential for prophylaxis against nerve agents. Specifically, we aimed to develop a methodology that enables the efficient distributed and scalable production of a protein with the same neutralizing activity (621 U/mg) and circulating half-life (10 days) as serum-derived human butyrylcholinesterase (BChE), the current standard of care for at-risk patients. BChE is currently the only therapeutic agent effective in protecting patients against a broad spectrum of chemical weapons, but inefficiencies in procurement and purification of serum-derived material severely limits the treatable patient cohort. Given the global rise in emergent situations involving chemical weapons, we need adept and agile means to manufacture products with BChE activity.

2. Milestone Summary:

Milestone or Deliverable Description *taken from your SOW*	% Complete	Issues and Status
Task 1: *P. pastoris* w/ minimal N-linked glycans	100%	
Task 2: *P. pastoris* w/ upregulated chaperones	100%	
Task 3: Reagents for rBChE detection (mAbs)	100%	
Task 4: Analytical methods for rBChE activity/quality	100%	
Task 5: rBChE expression in glyco-eng. *P. pastoris*	85%	rBChE products remain sequestered within organelles (likely the ER) which inhibits successful folding & correct glycosylation; method development for Cryo-EM would be required for improved analysis of localization & cellular dynamics
Task 6: Molecular engineering of rBChE for higher titers	100%	
Task 7: Improved rBChE expression in glyco-eng. *P. pastoris*	85%	Based on RNA sequencing and activity assays, we hypothesize that protein folding and/or translation remain the most crucial bottleneck to production of an active rBChE. Additional strain engineering required.
Task 8: *P. pastoris* with fully humanized *N*-glycans	100%	
Task 9: Expression of rBChE-nanobody fusion protein	50%	Further analysis of lysis & assays to determine effects of HSA-fusion on improved passage from ER required
Task 10: Expression of rBChE-Fc domain fusion protein	50%	Further lysis & additional screening of Fc-monomer to look for improved transport from ER required
Task 11: Draft DSP to support non-clinical testing of rBChE products	50%	Minimal purification process developed; protein required to test/iterate.
Task 12: Expression of rBChE with bi-antennary glycans in *P. pastoris*	80%	First round integration of human chaperones did not improve glycan profiles, more candidates identified
Task 13: Multimerization of fully-glycosylated rBChE		No-Go: Activities not pursued
Task 14: Molecular engineering of rBChE products		No-Go: Activities not pursued
Task 15: POC expression of manufacturable rBChE products in perfusion		No-Go: Activities not pursued
Task 16: Selective control of *N*-glycosylation in *P. pastoris*		No-Go: Activities not pursued
Task 17: Selective control of *O*-glycosylation in *P. pastoris*		No-Go: Activities not pursued
Task 18: rBChE product expression with selective control		No-Go: Activities not pursued
Task 19: Optimization of rBChE product perfusion process		No-Go: Activities not pursued
Task 20: Development of rBChE product purification process		No-Go: Activities not pursued
Task 21: End-to-end manufact. process for rBChE product		No-Go: Activities not pursued

Table 1: *Milestone Summary shows %-complete and tasks that were not pursued. A full discussion of each task is given in Section 3.*

As a result of the global COVID-19 pandemic that emerged in the beginning of 2020, we were directed by MIT to ramp down all non-essential activities, including the experimental work on this program on 20-March-2020. In addition, the Institute imposed immediate measures to control costs. In doing so, MIT paused hiring for sponsored research to conserve resources for existing personnel. While on-campus research ramped up to 25% capacity on 15-June-2020, the immediate impact was the completion of Phase 1 activities (Tasks 5, 6 and 7) by 27-June-2020. Subsequently, we could not begin work on Phase 2 Tasks.

On 27-July-2020, MIT increased to 50% capacity (with restricted hours) and lifted restrictions on weekly hours on 14-October-2020 but did not return to pre-pandemic operation until 7-June-2021. With hiring restrictions and extensive lead time on lab supplies due to supply chain disruptions, Tasks 5 and 7 (Base Period) and Tasks 9-15 (Option 1) could not be completed by 27-June-2021. We leveraged technical status update meetings to re-evaluate program goals, mitigate risks and reprioritize efforts. As a result, Option Period 2 Tasks were not pursued and funding in the amount of $818,000 was deobligated on 21-July-2022. Subsequently Task 5 (Base) was discontinued, Tasks 9 and 10 (Option Period 1) were paused and resources were reallocated to Tasks 7 and 12 for the remainder of the program.

3. Comprehensive Detailed description of accomplishments, results and conclusions

Phase 1 (Months 1-12): Proof-of-concept expression of bioactive recombinant butyrylcholinesterase (rBChE) in *Pichia pastoris* (Tasks 1-7)

Task 1. *Engineering of a P. pastoris strain with minimal N-linked glycosylation.*

Completion criteria. Detection of uniform Man5GlcNAc2 *N*-glycosylation on K3 via LC-MS

Status. Complete. We achieved uniform $GlcNAcMan_5GlcNAc_2$ and glycosylation of a reporter peptide (K3) using CRISPR-Cas9 modifications to the *K. phaffii* genome. Glycosylation of K3 confirmed by liquid chromatograph mass spectrometer (LC-MS) (Figure 1).

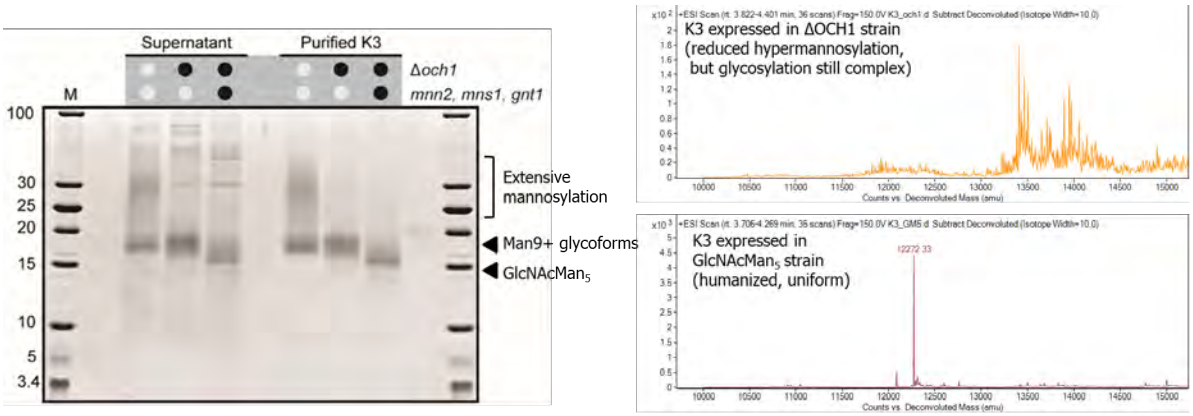
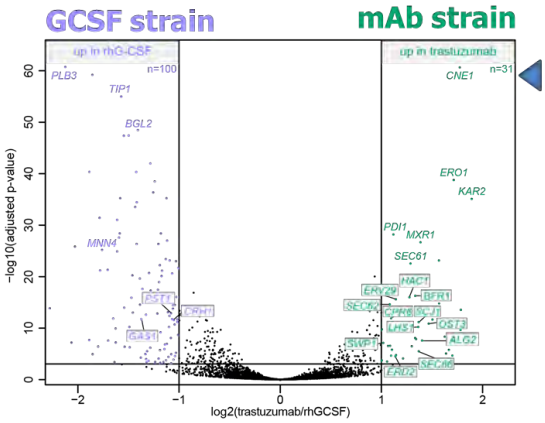


Figure 1. *K3 peptide integrated into humanized glycoengineered yeast strain; small scale cultivations performed to confirm glycan differences.*

Task 2. *Engineering of an improved glyco-engineered yeast strain with upregulated glycan-specific chaperones*

Completion criteria. Improved titers of uniformly Man5GlcNAc2 glycosylated K3 by LC-MS and SDS-PAGE.

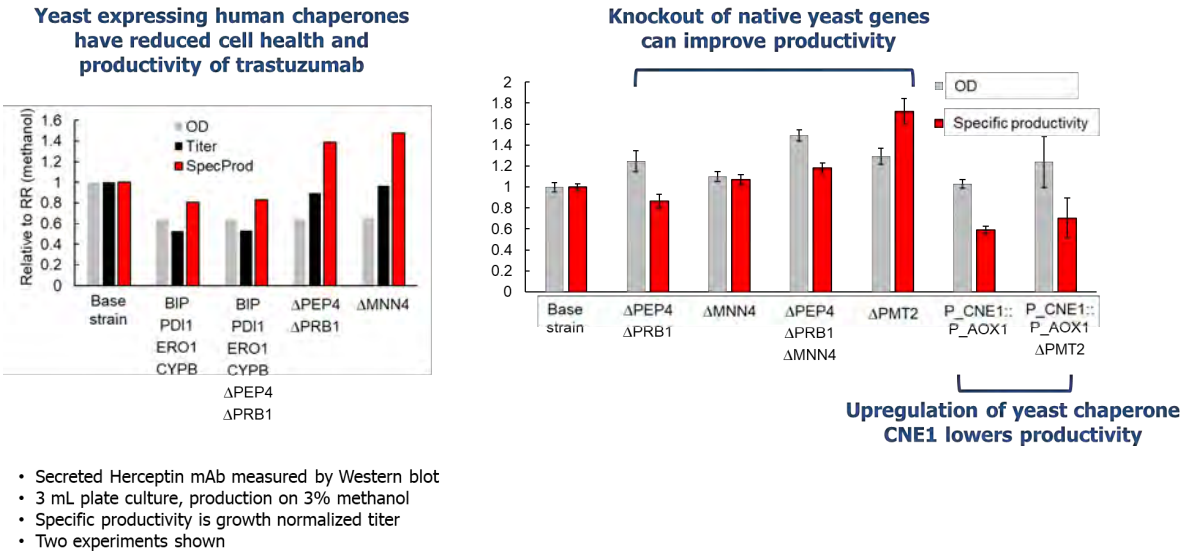
Status. Complete. Human chaperones appeared to have had a negative effect on cell health. To test our hypothesis that yeast chaperones may improve productivity with less burden, we used RNASeq to identify potential genes of interest. We observed "common" yeast genes as engineering targets and selected the top upregulated gene, Calnexin (CNE1), to test this hypothesis (Figure 2).



Top upregulated genes:
- **CNE1 (Calnexin)**
- ERO1
- KAR2 (BiP)
- PDI1
- SEC61
- MXR1
- STE6
- HAC1

Figure 2. *Calnexin (CNE1) is shown as the top upregulated gene.*

As shown in Figure 3, we observed the upregulation of Calnexin (CNE1) lowered productivity.



- Secreted Herceptin mAb measured by Western blot
- 3 mL plate culture, production on 3% methanol
- Specific productivity is growth normalized titer
- Two experiments shown

Figure 3. *Expression of human chaperones and overexpression of native chaperones reduced titers of specific productivities of expressed BChE.*

Learnings from these studies on expression of exogenous genes were applied to exogenous glycosidases to improve the homogeneity of Man5GlcNAc2 glycosylation. Improved glyco-engineered strains and chaperones could be applied as needed in future efforts.

Task 3. *Development of reagents for detection of rBChE expression in P. pastoris cultivation supernatants*

Completion criteria. Production of milligram quantities of at least 2 mAbs with rBChE affinity ≥ 1 µM and verification of reagent suitability for Western blot and ELISA using BChE standard purified from human serum.

Status. Complete. We confirmed performance of commercial antibodies for BChE detection and quantification using serum-derived material; (4) suitable reagents with varied epitope specificity identified (Figure 4). Commercially available BChE-specific ELISA antibody pair validated and linear range of BChE detection determined using serum-derived material. Protocols completed for Western blot, enzyme-linked immunosorbent assay (ELISA) and automated quantitative Western (Protein Simple, Jess™ system).

Primary Antibody	Supplier	Host	M/P	Procedure	Dilution	Epitope	Sensitivity
BChE monoclonal TrueMAB™	Thermo Fisher	mouse	monoclonal	WB	1:1000	Full-length	ng
BChE antibody (PA527385)	Thermo Fisher	rabbit	polyclonal	WB, Jess	1:1000	Pos. 59-297	pg - ng
BChE antibody (PA514971)	Thermo Fisher	rabbit	polyclonal	WB, Jess	1:1000	Pos. 385-415	ng - ug
BChE Antibody (D-5)	Santa Cruz Biotechnology	mouse	monoclonal	WB, Jess, ELISA	1:1000	Pos. 85-119	pg - ng

Figure 4. *Four suitable reagents with varied epitope specificity identified.*

Automated quantitative Western assay shows improved BChE detection using vendor optimized secondaries (Protein Simple); automated quantitative Westerns can detect both monomer and dimer forms of BChE (Figure 5)

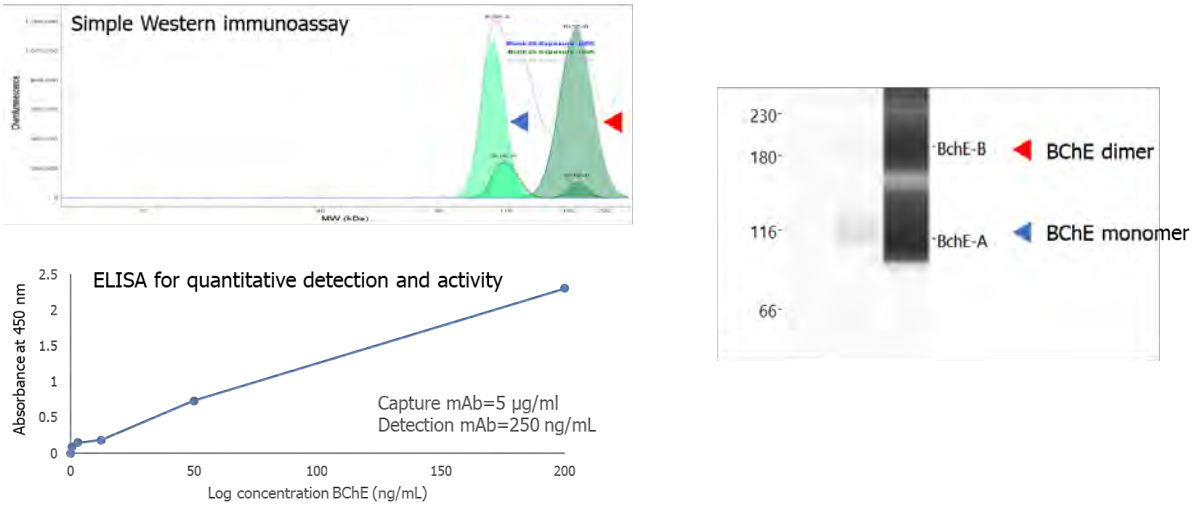


Figure 5. *BChE detected using commercially available antibodies.*

Task 4. ***Development of analytical methods for assessment of rBChE activity and quality***

Completion criteria. A draft target product profile (TPP) for rBChE activity and purity relative to product-related variants and protein contaminants, including a ratio of tetramer to monomer.

Status. Complete. We developed a draft Target Product Profile (TPP) for a BChE product focused on clinically relevant product attributes, not product-related variants of serum-derived material (Figure 6)**.**

Target	Minimum essential	Ideal
Indication	Post-exposure treatment of exposed patients	Prophylactic treatment of populations in high-risk areas
Administration route	Infusion	Injection
Population	Warfighters	Warfighters and civilians
Purity	>90%	>95%
Product-related impurities	<15% at expiration (Deamid., Oxidation)	<10% at expiration
Clinical efficacy	Bioactivity equivalent to serum-derived BChE	Bioactivity and pK/pD equivalent to serum-derived BChE
Safety	Aggregates at same level as serum-derived BChE	Aggregates < 1%
Formulation	Liquid with typical excipients	Liquid with minimal excipients
Stability	24 h at 25°C, 3 mo. at 2-8°C	24 h at 25°C, 12 mo. at 2-8°C

Figure 6. *Target Product Profile for a BChE product*

Drafted butyrylthiocholine colorimetric assay protocol (Ellman's assay) for testing activity of BChE products. Confirmed activity of 534 $\pm$ 30 U/mg for serum derived BChE from the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) (Certificate of analysis listed 500 U/mg). Established target concentration/amount of protein product needed for an effective assay (≤0.001 mg BChE per sample) (Figure 7).

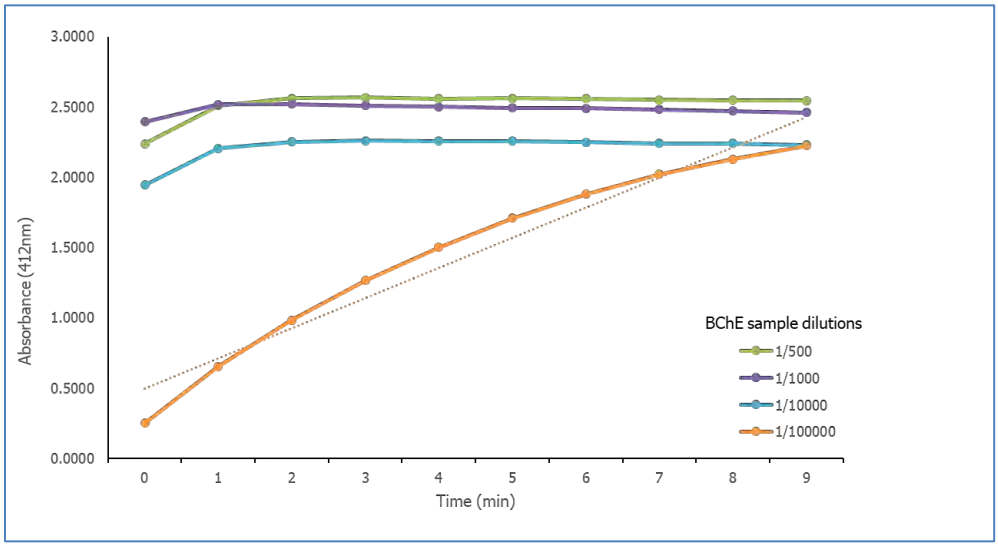


Figure 7. *Optimization of BChE dilutions*

Size-exclusion chromatography (SEC) analysis of serum-derived BChE indicates >95% tetramer (Figure 8).

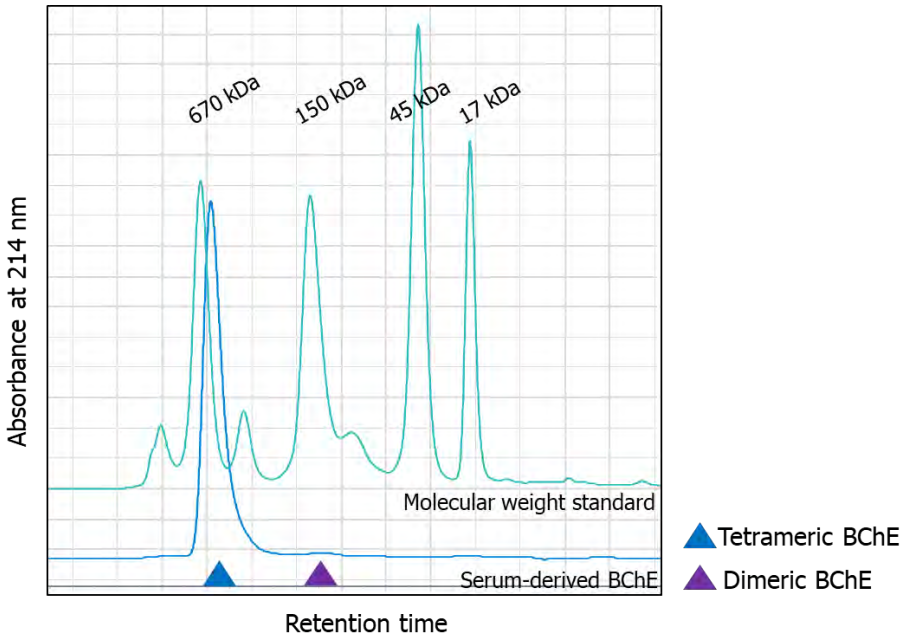


Figure 8. *Size-exclusion chromatography (SEC) analysis of serum-derived BChE*

Task 5. ***Expression of rBChE in an improved glyco-engineered strain of P. pastoris***

Completion criteria. Detection of rBChE in cultivation supernatants by intact LC-MS, SDS-PAGE, or Western blot (using mAbs generated in Task 3). Confirmation of rBChE activity in unpurified supernatants relative to BChE standard at similar concentrations using Ellman's assay.

Status. Deprioritized (85% complete). Experiments confirmed that rBChE products remained sequestered within organelles of the yeast (likely the endoplasmic reticulum (ER)), potentially inhibiting successful folding and correct glycosylation. Our previous findings suggested that integration of rBChE mutPRAD into a strain with three secretome-related genes (TOS1, CRH1, EXG1) knocked out resulted in some heavily glycosylated rBChE product being released from the ER-Golgi, based on the automated Western blot of the lysed fractions (Figure 9).

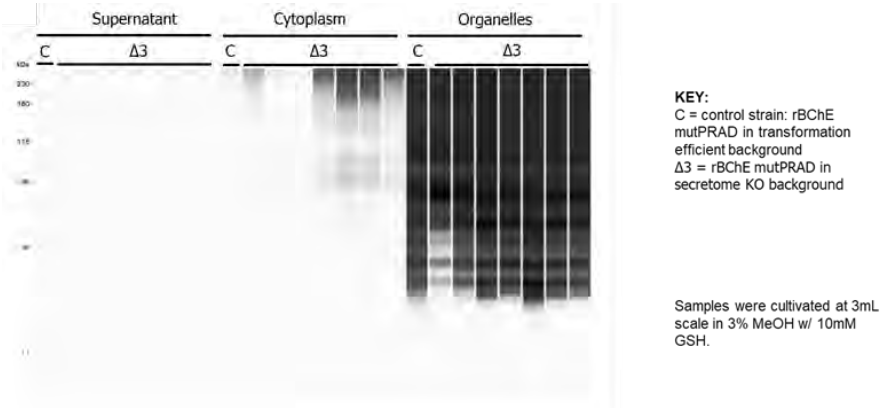


Figure 9. *Automated Western blot of the lysed fractions*

Alternate transformation protocols to improve integration into engineered strains were evaluated (i.e., rBChE mutPRAD monomer into another secretome knockout (KO) strain (TOS1, CRH1, EXG1, GQ5326, LSC2, TFS1) but no improvement to secretion was detected. Localization of the product was determined through two rounds of chemical lysis, approximating the lysis of the cell wall and the internal organelle membranes respectively. The bulk of the product was detected in the second lysis fraction, confirming that reduced traffic in the ER from secretory knockouts did not alleviate sequestration.

We used fluorescent microscopy to test our hypothesis that the bulk of the product remained sequestered within the ER: the signal from labelled BChE remained highly clustered around the nucleus (Figure 10).

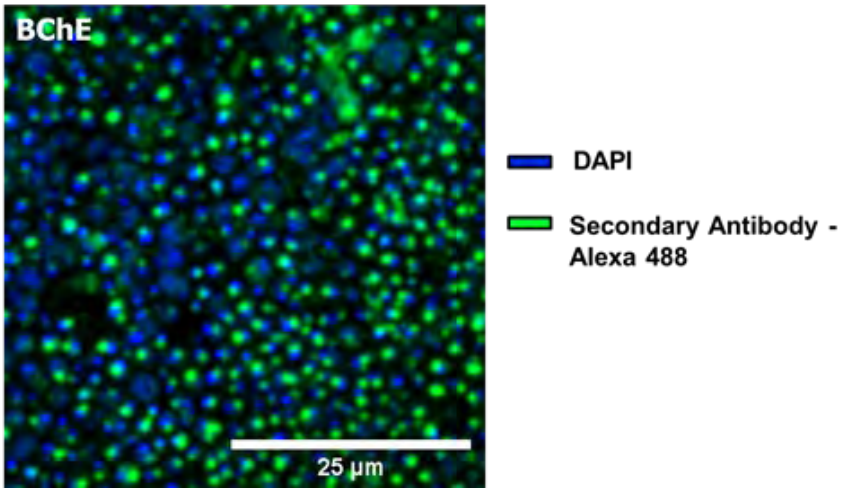


Figure 10. *Fluorescent microscopy indicates nascent BChE was clustered around the nucleus.*

Due to the small size of *P. pastoris* cells (4-6µm), localizing the sequestered protein at the organelle in question required electron microscopy (EM). We developed EM-based techniques to visualize the labelled proteins. We used a rBChE mutPRAD strain (MIT-Strain-243) in a 200 mL shake flask cultivation and subsampled the cultivation at the production stage for expression controls.

Initial data regarding the degradation of cell structure following digest with zymolase indicated that cellular structure was largely maintained (Figure 11).

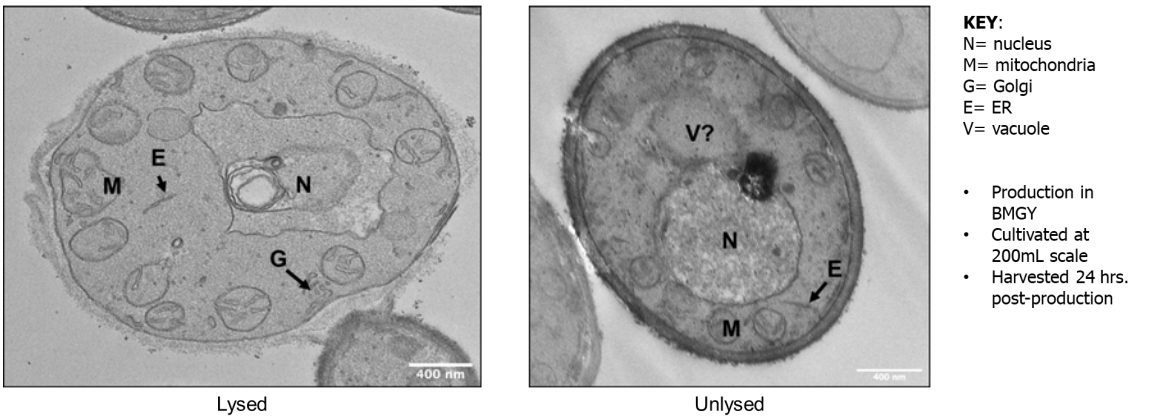
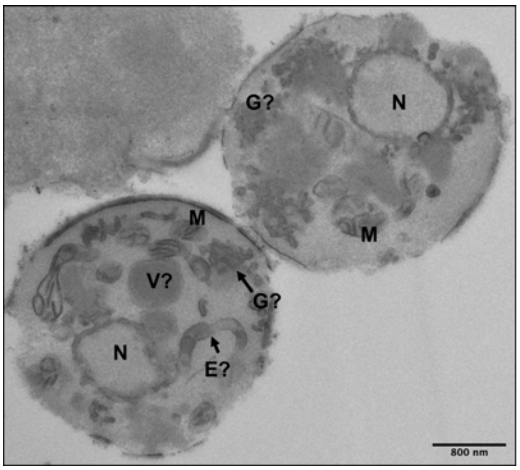


Figure 11. *Permeabilization with zymolase does not fully degrade cellular structure.*

Sample shown with methanol-induced expression of BChE—cells that have made BChE and were subsequently lysed (without immunostaining) (Figure 12).



KEY:
N= nucleus
M= mitochondria
G= Golgi
E= ER
V= vacuole or vesicle

- BChE expression induced with 3% MeOH + 10mM GSH
- Cultivated at shake flask scale
- Harvested at 24 hrs. post-production

Figure 12. *Sample shown with methanol-induced expression of BChE.*

Initial RNA analysis affirmed resource sequestration when producing full monomeric BChE, regardless of secretory signal used (Figure 13). We hypothesized that the full monomer of BChE is difficult for *P. pastoris* to effectively translate and fold with the yeast's native glycosylation, resulting in sequestration of cellular resources and reduced ribosome availability.

Gene ontology-biological process	preOST1/proMFalpha-BChE vs K3: NES	MFalpha-BChE vs K3: NES
cellular response to DNA damage stimulus	1.512766428	
chromatin organization	1.384620737	
cytoplasmic translation	**2.358801772**	
DNA recombination	1.6792042	
DNA repair	1.623966221	
DNA replication	1.810442498	
mitochondrial translation	**2.664409876**	**1.984194217**
mitochondrion organization	**2.08072281**	**1.550598225**
mRNA processing	1.836986319	
nuclear transport	**1.718378284**	
nucleobase-containing compound transport	1.997903044	
peptidyl-amino acid modification	1.613524564	
protein acylation	1.79995572	
protein folding	**1.793758413**	**2.204796928**
protein targeting		1.512365955
regulation of translation	**2.23597129**	
ribosomal large subunit biogenesis	**2.070081464**	**1.790788233**
ribosomal small subunit biogenesis	**2.456655838**	**2.058004644**
ribosomal subunit export from nucleus	**1.770393188**	
ribosome assembly	**1.989478047**	
RNA catabolic process	1.633016398	
RNA modification	2.501822872	2.001030533
RNA splicing	1.950627415	
rRNA processing	2.413593215	2.297021781
translational initiation	**2.135666363**	**1.820518144**
transmembrane transport		**1.7165261**
transposition	1.707233579	
tRNA processing	2.200321662	1.586704391
vitamin metabolic process		2.035577126

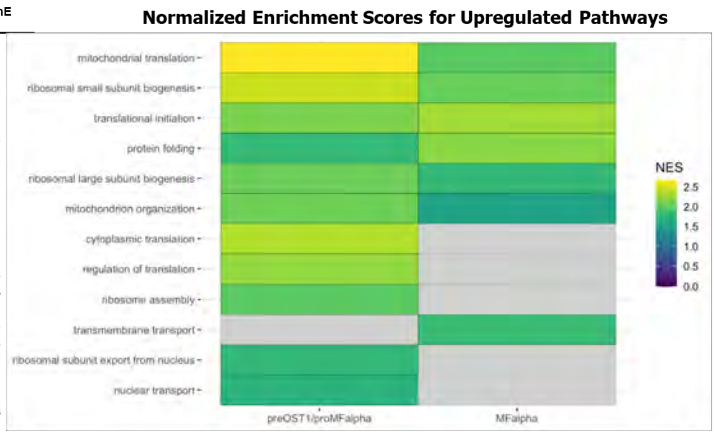


Figure 13. *RNA data shows resource sequestration when producing full monomeric BChE.*

To alleviate stress on translation and ribosomal availability, we transitioned to using the mutPRAD as our primary variant but continued using preOST1/proMFalpha signal peptide to increase product quantity. Despite that, the EM results suggest that further analysis of organelles and BChE localization would be needed to address improving translocation and cellular stress involved in rBChE production.

Task 6. ***Molecular and strain engineering for improved rBChE titers.***

Completion criteria. In-silico development of at least 5 alternative sequences for further expression and activity testing. Identification of cellular pathways modulated during rBChE production in P. pastoris.

Status. Complete. We tested numerous protein variants of rBChE to reduce sequestration of product within organelles, reduce product variation generally, and improve the activity and secretion of the recombinant BChE. Our results showed modified base strains and improvements to cultivation conditions could modestly improve quality and quantity of recombinant BChE. Molecular engineering to reduce protein size or mitigate problematic features of biochemistry were not sufficient to improve activity or secretion, suggesting that rBChE production requires more modifications of the host organism to produce a correctly folded and secretable rBChE of any size.

Molecular Engineering

In total, 14 new rBChE designs/strains were created for a more "secretable" rBChE focused on the substrate binding pocket (SBP). addressing combinations of 1) mitigation of N-linked glycosylation sites; 2) inclusion of additional portions of N-terminus or C-terminus to protect hydrophobic core of BChE; and 3) mitigation of an unpaired cysteine in the SBP.

Key takeaways:

- We tested full monomeric and monomer-PRAD mutant (A)
- We tested removal of some portions of the N- and C-terminus (B).
- We determined that the SBP can be further truncated while still maintaining activity (C & D) and tested these variants.

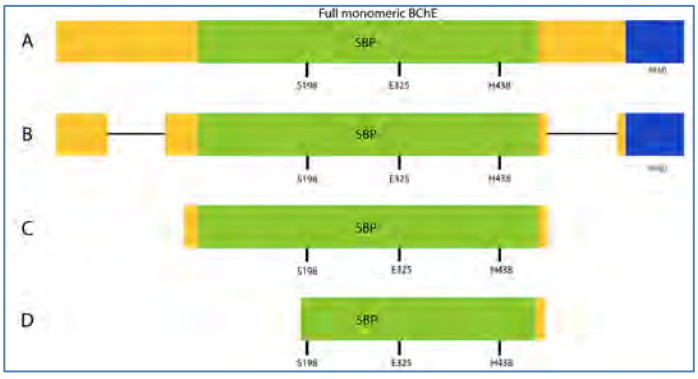


Figure 14. *Schematic representing the domain architecture of BChE, and series of truncation variants targeting domains and features responsible for enzymatic activity.*

Development of assay for BChE activity

We developed an assay to detect activity (Task 4) which was performed using spent supernatant using serum derived BChE. However, spent supernatant displayed no activity on its own (Figure 15).

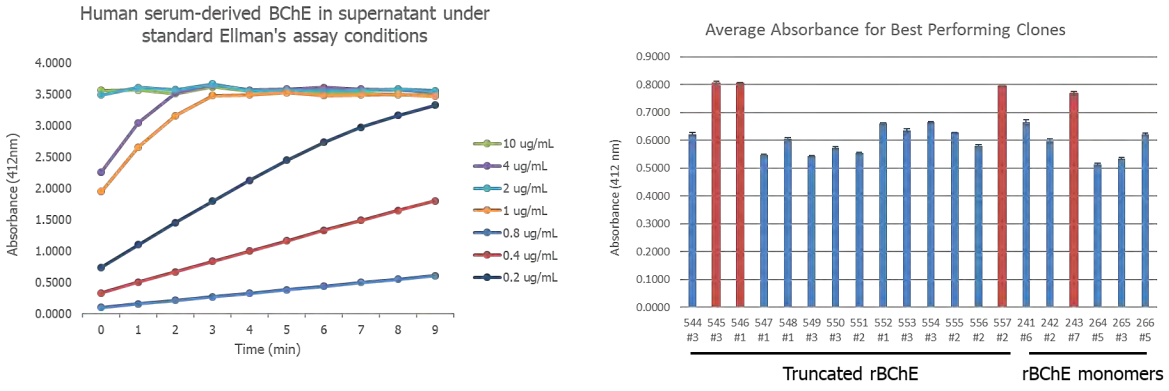
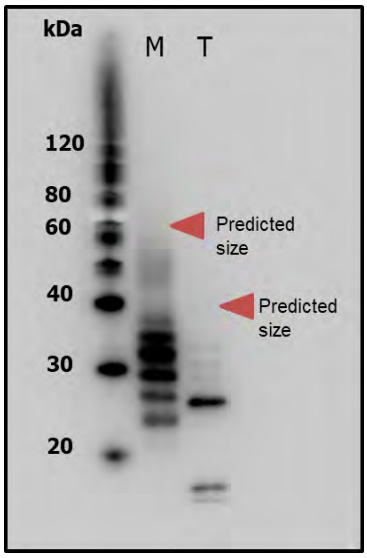


Figure 15. ***(****Left) Ellman's assay of serum-derived material suspended in spent supernatant to determine efficacy of detecting BChE activity in unfiltered supernatant. (Right) Average absorbance readings of strains producing rBChE monomers and truncation variants over ten minute Ellman's assay. These strains did not produce sufficient active, secretable product to measure activity via Ellman's.*

With the goal to purify sufficient material for Ellman's and ELISA from cell lysate material, we cultivated 12mL of the most promising monomeric (rBChE-PRAD) and subunit variants (substrate binding pocket, SBP) based on 1) activity of supernatant material in preliminary Ellman's; and 2) features predicted to be crucial for correct activity (inclusion of N241)

Subunit rBChE detected using same polyclonal primary antibody used on monomeric variants in automated Westerns. As shown in Figure 16, gels suggest that both intended products are being internally cleaved, with no full-length products detected.



KEY:
M - BChE monomer S-243 (-PRAD); predicted size ~61.2 kDa
T - BChE truncation S-546; predicted size ~38.2 kDa

12% Tris-Glycine gel using nickel-column purified material

Primary antibody: rabbit polyclonal targeting SBP (Thermo Fisher PA514971)

Figure 16. *Western blot of lysate material indicating extensive product variation and cleavage of both monomeric and truncated variants.*

Engineering the BChE monomer to remove the tetramerization domain or minimize contact residues were not shown to appreciably increase activity or improve secretion. Subunit screens showed that further truncation to the substrate binding pocket did not measurably improve secretion of rBChE. Learnings from this study showed that modifications to the rBChE by itself may not appreciably improve titer; secretion and purification likely to be improved by additional machinery (Fc- and nanobody-tagged fusions).

Assessment of Protein Disorder

Analysis of protein disorder in Protein DisOrder Prediction System (PrDOS) showed that butyrylcholinesterase is highly structured, which may complicate folding in *Pichia*. Literature suggests that glycosylation may not be crucial for folding or activity, except for one site: Asn241-required for correct structure of active gorge site* (Bernardi et al. 2017 *PLoS ONE* 12(11)) (Figure 17).

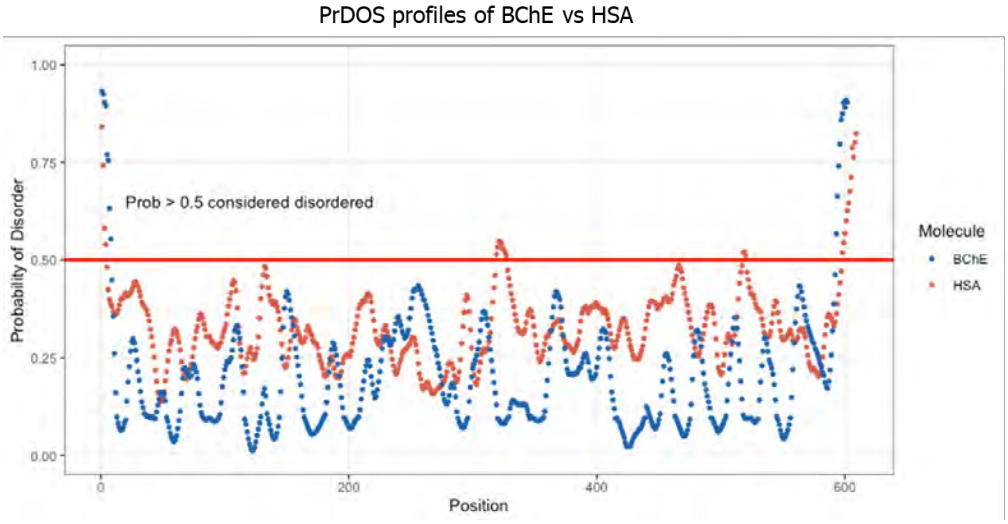


Figure 17. *Analysis of structural disorder of human BChE protein compared to HSA, a protein of comparable size secretable in Pichia pastoris. The BChE protein does not posess any disordered regions.*

Crystal structure analysis has shown that N-linked glycosylation sites appear to be located on the exterior of the folded molecule. New molecular designs started with substrate binding pocket (SBP) – removing 6 of 9 N-linked glycosylation sites. SBP-only designs focused on combinations of N-linked gly-site mitigations and removal of free cysteine (Figure 18)

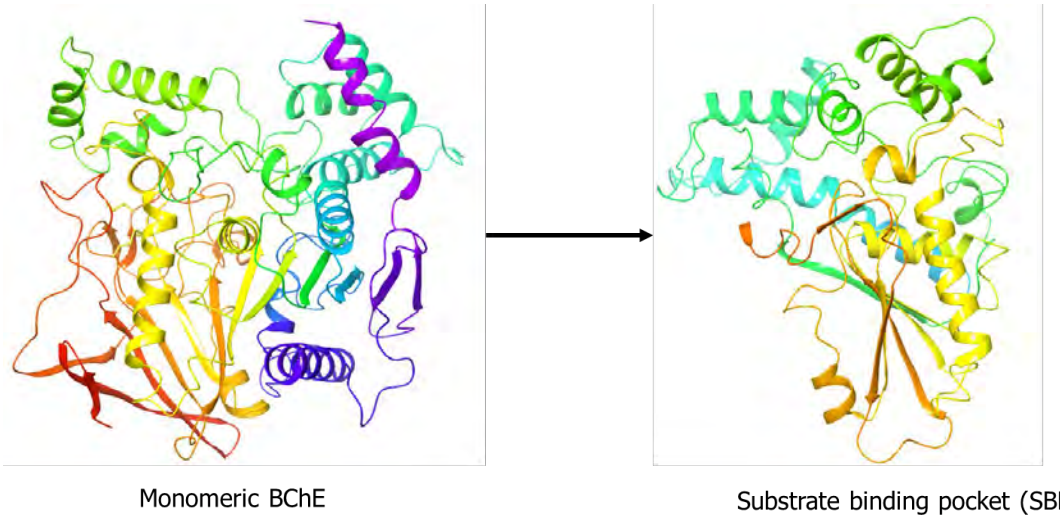


Figure 18. *(Left) 3D structure of fully-folded monomeric BChE rendered in Schrödinger. (Right) 3D structure of truncated BChE variant including only the substrate binding pocket. Truncated product contains the key structures of the binding pocket in a more condensed structure.*

Designs featuring SBP-only were adapted to shield the hydrophobic core of the BChE monomer (i.e., additional pieces of the N-terminus and C-terminus were included to mitigate exposure of hydrophobic patches that might lead to aggregation or incorrect folding). Additional sequence inclusions also required mitigation of N-linked glycosylation sites (Figure 19).

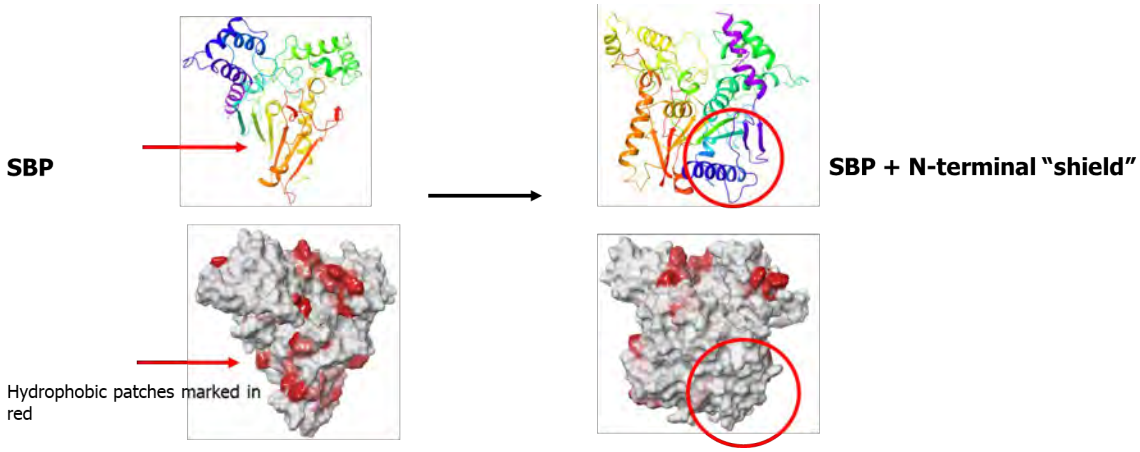


Figure 19. *3D structures of substrate binding pocket highlighting the exposure of hydrophobic residues which could be detrimental to product secretion (left) vs next-generation variant which retained a segment of the N-terminal sequence ahead of the substrate binding pocket to "shield" these hydrophobic regions and mitigate inappropriate aggregation (right).*

Strain Engineering

Engineered base strain enables detection of rBChE.

We performed expression testing of rBChE variants in engineered *P. pastoris* at plate scale (3 mL). rBChE was detected in cytoplasm and within organelles, but not secreted into cell culture fluid (likely due to mannosylation (Figure 20). Data shown used preOST1-proMF1 signal peptide.

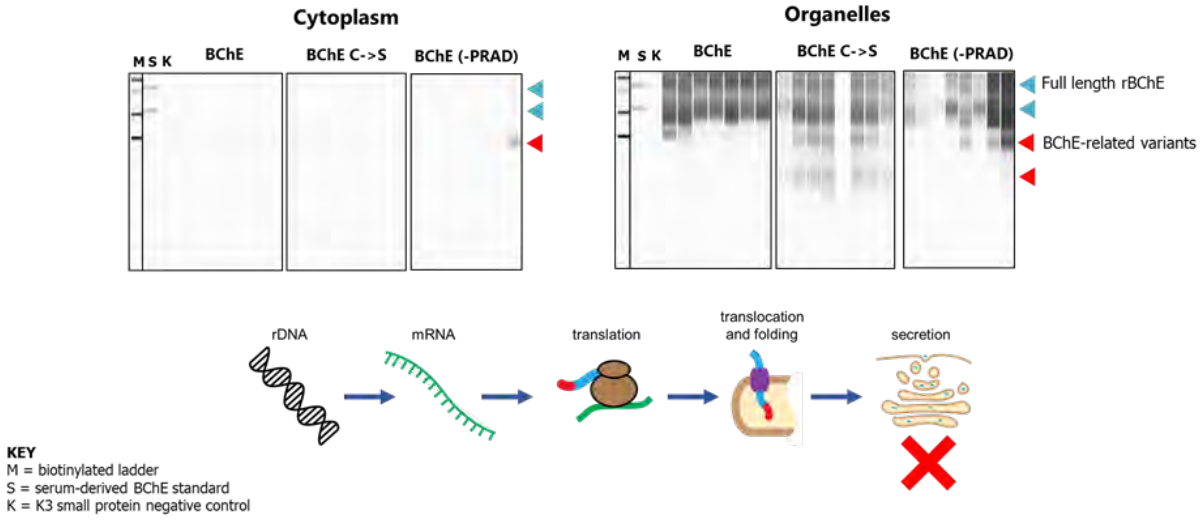


Figure 20. *rBChE detected but not secreted.*

Chemical chaperones may improve protein assembly in the ER:

Addition of chemical chaperones to cultivation media may reduce stress when expressing human chaperones and BChE variants. Glutathione (GSH) has been shown to improve production/folding and improve metabolism of methanol.

Shown in Figure 21, increased production of monomeric BChE modestly improved by adding 10mM glutathione (GSH) to production media (intracellular, organelles). Reduced glycosylation variation and improved production of monomeric BChE were observed when cultivated with GSH at plate scale (3 mL) (strain shown uses preOST1/proMFalpha BChE).

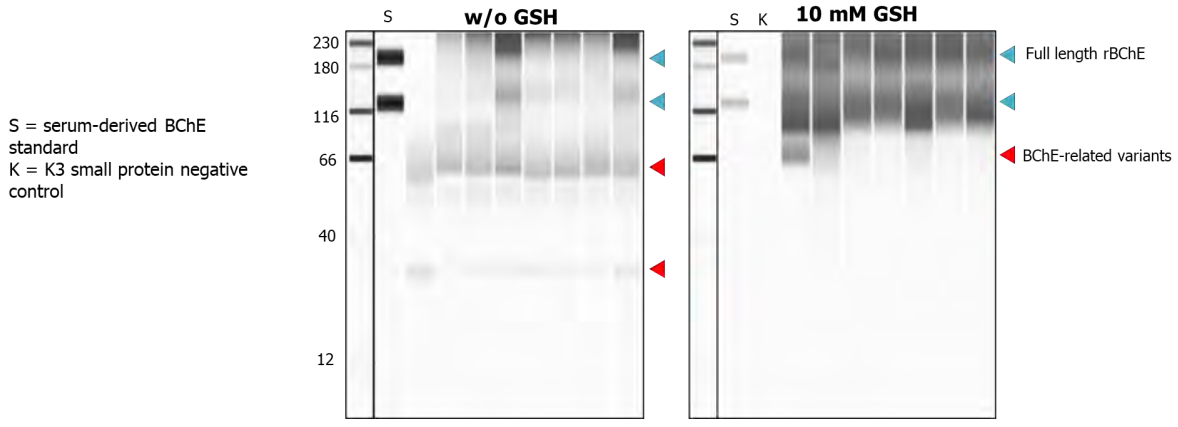


Figure 21. *Production of monomeric BChE is improved by adding 10mM glutathione (GSH) to production media.*

Task 7. ***Expression of an optimized rBChE sequence in P. pastoris for improved rBChE titers.***

Completion criteria. Detection of increased titers of rBChE in cultivation supernatants by LC-MS, SDS-PAGE, or Western blot (using mAbs generated in Task 3) relative to the strains from Task 5 (~1-100 mg/L). Confirmation of rBChE activity in unpurified supernatants relative to BChE standard at similar concentrations using Ellman's assay.

Status. (85% complete) Based on ribonucleic acid (RNA) sequencing and activity assays, we hypothesized that protein folding and/or translation remain the most crucial bottleneck to production of an active rBChE. We completed our scout screens and lysis of codon-optimized, codon-tuned, and native human coding BChE in transformation efficient *P. pastoris*. We determined via Western blot that both codon-tuned and native human BChE improve protein production and accessibility of the BChE monomer within the cell and show increased protein levels in the cytoplasm relative to codon-optimized BChE.

We detected possible hypermannosylation or aggregation of BChE monomer for all variants. In these initial draft strains, the native human sequence yielded the greatest improvement to BChE production. We also completed our lysis and initial scout screen of codon variant BChE in the secretome knock-out strain △6 (TOS1, CRH1, EXG1, GQ5326, LSC2, TFS1) and detected secreted fragments of BChE in the supernatant, primarily in the codon-tuned and native human coding sequence strains. Scouts of the cytoplasm lysis fraction showed that in the △6 (as above) background, protein levels between the coding variants seemed to be consistent in its quantity and accessibility, but the codon-tuned variant may reduce aggregation or mannosylation of the BChE production (Figure 22).

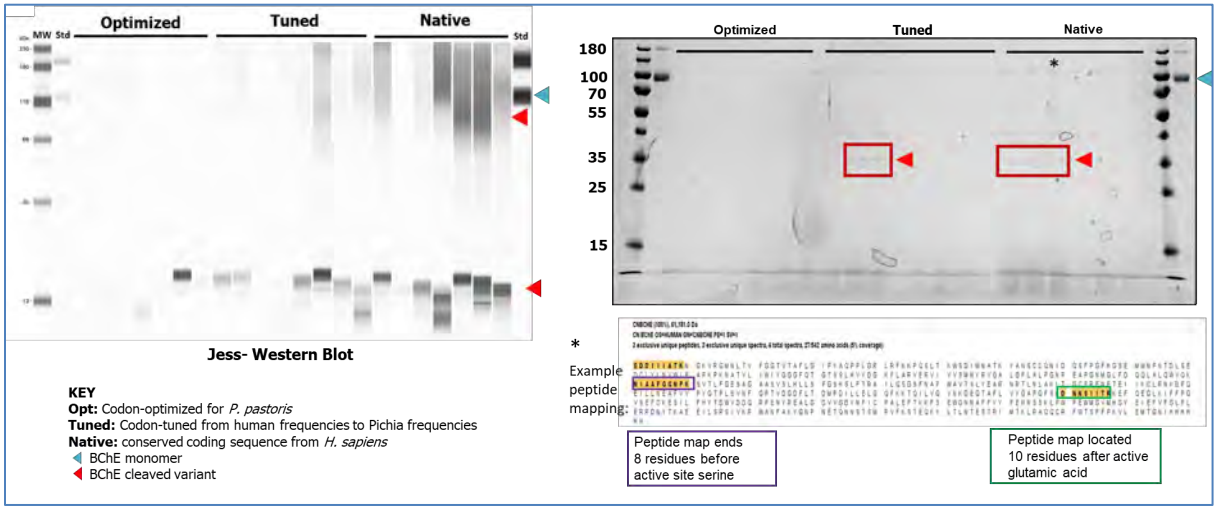


Figure 22. *Initial codon tuning on BChE yields secreted variant.*

Fundamental biological questions crucial to improving production of difficult recombinant molecules could be answered with expanded next generation sequencing (NGS). We hypothesize that transcriptomic comparison for a range of molecules, including integration of ribosomal footprinting as a standard part of analytics pipeline, yields insights to host pathways as a function of protein size and complexity. Next steps would include screening variants for activity using Ellman's assay and sampling for RNAseq and ribosomal footprinting.

Phase 1 Milestone: *P. pastoris*-expressed monomeric rBChE with target neutralizing activity
Deliverable: Proof-of-concept expression of bioactive rBChE in *P. pastoris.*

Phase 2/Option Period 1 (Months 12-24)

Task 8. Extension of humanized glycosylation to include uniform terminal sialylation of N-linked glycans.

Completion criteria. Detection of uniform ~~Sia2Gal2~~GlcNAc2Man3GlcNAc2 glycosylation on K3 via LC-MS.

Status. Complete. We completed glycan profiling for GlcNAcMan5 and GlcNAc2Man3GlcNAc2 ("G0") strains with further reduced MNS1 promoter activity. Uniform G0 glycan profile achieved. MNS1 mutant and less active promoters enable G0 engineering. MNS1 mutant can generate very homogeneous "G0" glycan. Similar GlcNAc2Man3GlcNAc2 "G0" glycan profile can also be achieved by using wildtype sequences with less active promoters (Figure 23).

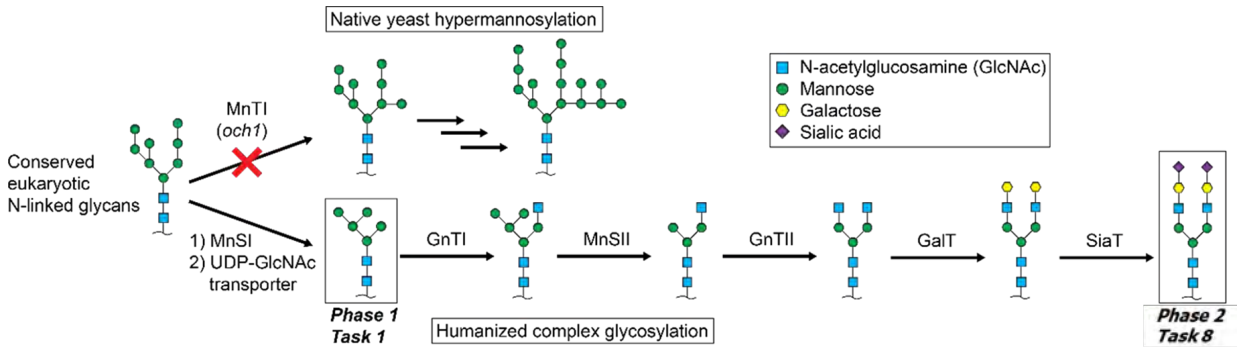


Figure 23. *Humanization of N-linked glycosylation in P. pastoris*

Success of GNT1 integration was affected by promoter choice. There was no obvious trend relating promoter strength and transformation success rate. As such, we proceeded with pENO1 and pMNN4 constructs for K3 glycosylation characterization and further glycoengineering (Figure 24).

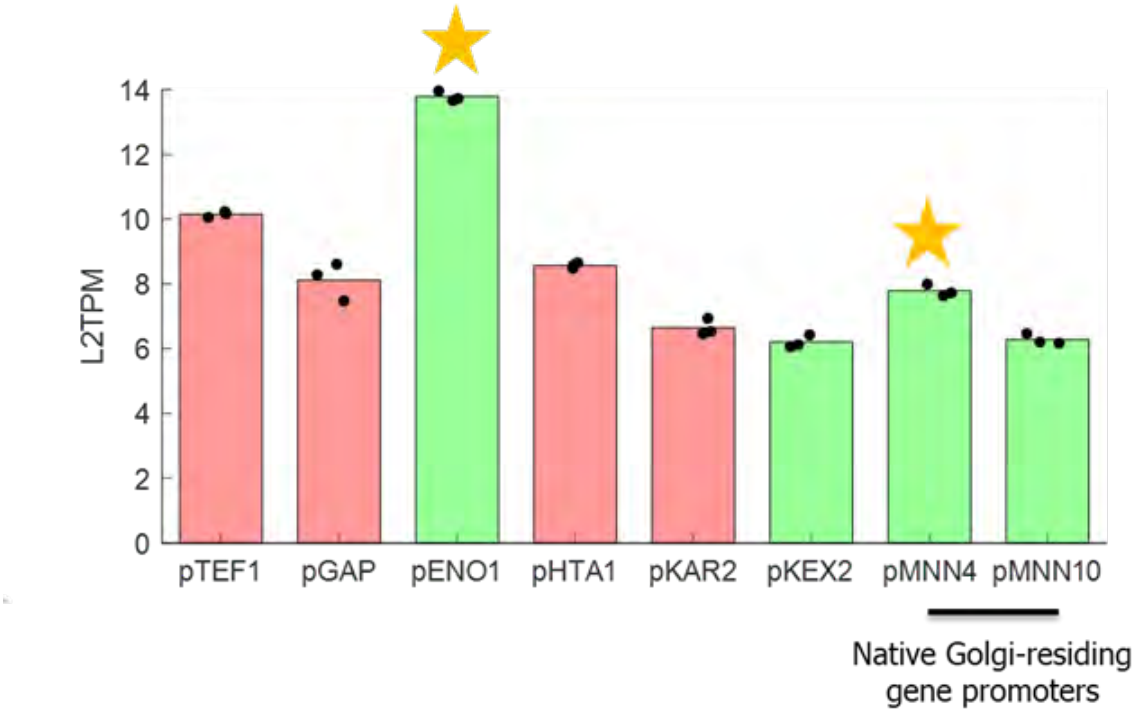


Figure 24. *Success (shown in green) of the transformation of GNT1 constructs under different promoters. Successful transformants of two constructs (with pENO1 and pMNN4) were carried forward to the next step of glycoengineering.*

Success of MNS2 integration shows similar promoter dependency (Figure 25).

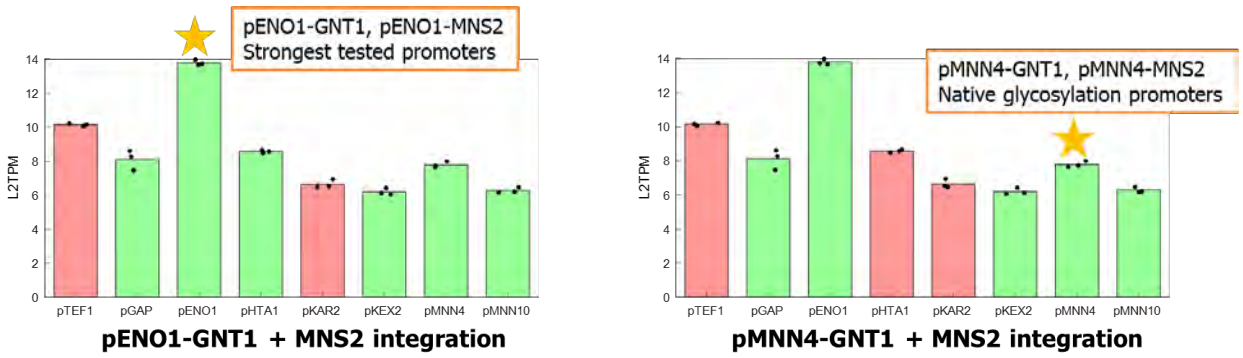
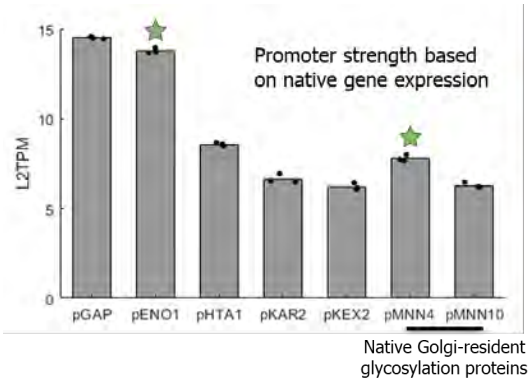


Figure 25. *Success (shown in green) of the transformation of MNS2 constructs under different promoters in either pENO1- or pMNN4-GNT1 parent strain. Successful transformants of two constructs (starred) were carried forward to the next step of glycoengineering.*

Glycoengineering with strong constitutive gene expression is not viable. After integrating the first two genes under strong pGAP/pTEF1 promoter, pGAP-GNT1 integration attempts yielded no positive transformants. We then generated two sets of glycoengineered strains (pENO1: stronger constitutive expressions, slightly weaker than pGAP; pMNN4: mimicking native glycosylation pathway gene expression) (Figure 26).



	GNT1	MNS2	GNT2	Expected glycan
pENO1	●			GlcNAcMan5
	●	●		GlcNAcMan3
	●	●	●	G0
pMNN4	●			GlcNAcMan5
	●	●		GlcNAcMan3
	●	●	●	G0

Base strain: pTEF1-*MNN2*, pGAP-*MNS1*

Figure 26. *(Left) Panel of promoters included in glycoengineering edits. (Right) List of strains generated with different promoters and their expected glycan structure.*

Further reducing the promoter activity for MNS1 can still generate homogeneous GlcNAcMan5 glycan profile (Figure 27). Similarly, wildtype MNS1 expression driven by these less active constitutive promoters enables further glycoengineering to G0 glycosylation (Figure 28).

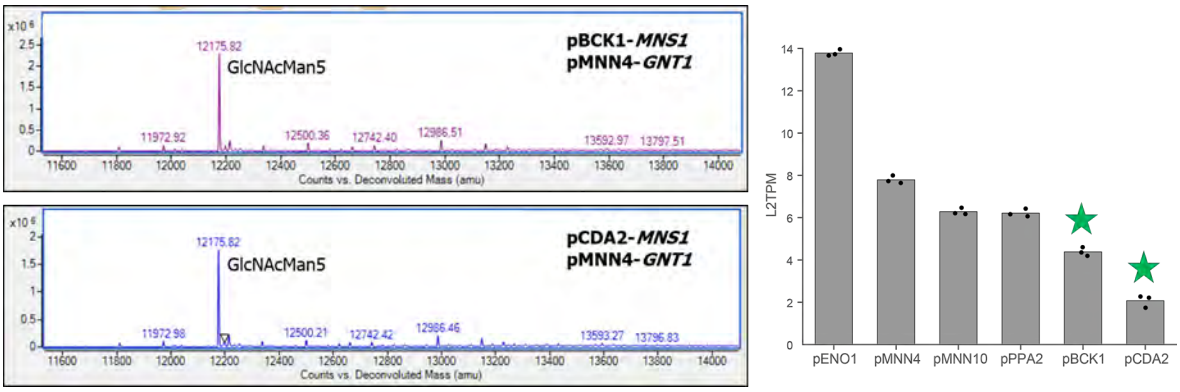


Figure 27. *(Left) MNS1 with less active promoters (pBCK1 and pCDA2) can generate homogeneous GlcNAcMan5 glycan profile on K3 peptide. (Right) List of less active promoters included to test the viability of wildtype MNS1 expression.*

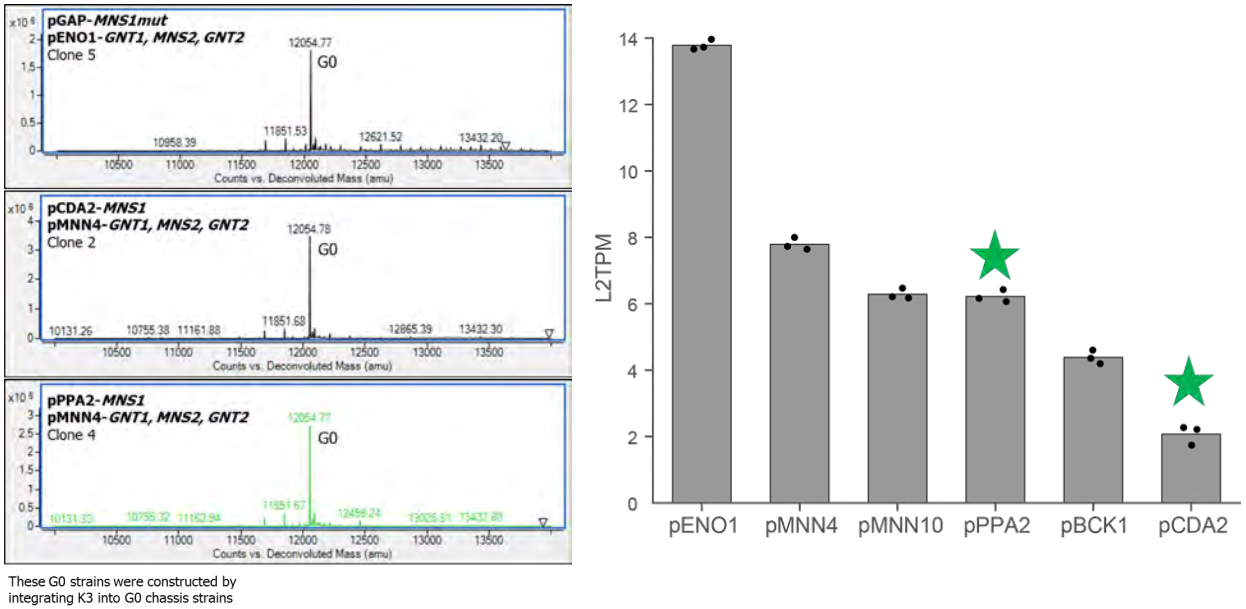
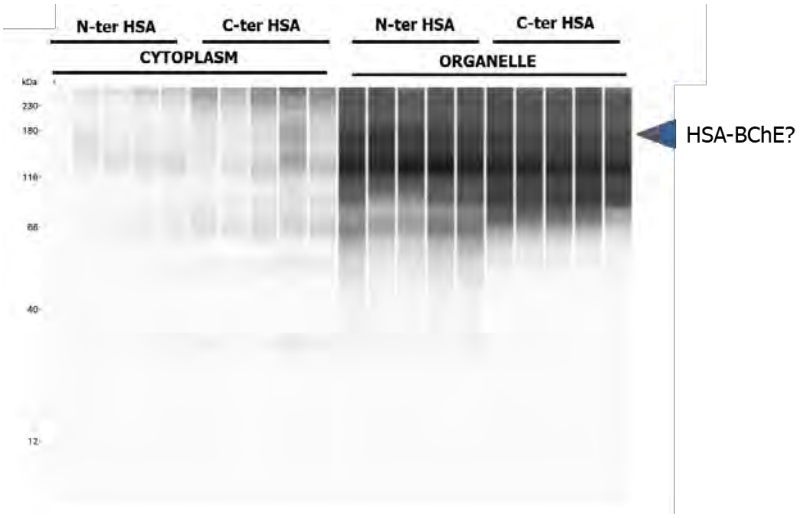


Figure 28. *MNS1 mutant and less active promoters both enable G0 engineering.*

Task 9 Expression of an rBChE-nanobody fusion protein in *P. pastoris*

Completion criteria. Detection of rBChE-nanobody fusions in cultivation supernatants by intact LC-MS, SDS-PAGE, or Western blot (using mAbs generated in Task 3). Confirmation of rBChE activity of unpurified materials relative to BChE standard using Ellman's assay. Confirmation of rBChE-nanobody fusion binding to HSA and/or guinea pig serum albumin by ELISA.

Status. Paused (50% complete). We successfully transformed human serum albumin (HSA)-mutPRAD constructs and N-terminal HSA-mutPRAD as a Consensus Coding Sequence (CCDS) variant. We cultivated scout screens of HSA-mutPRAD draft strains and screened for successful secretion, which did not improve secretion. Preliminary data from lysis and further Western blot analysis suggests that fusion with HSA may have improved transport from the ER; Western blot assays with HSA materials confirmed this is not the case. Currently, it is unknown if HSA-fusion improves folding and activity (Figure 29).



Key Takeaways:

- HSA-fusion may have 'dragged' some BChE from the ER
- C-terminally fused HSA may be more effective for expulsion from the ER

Figure 29. *Automated Western blot of HSA-fused rBChE in lysis fractions. C-terminally fused HSA showed modest improvement in product sequestration over N-terminal fusion.*

Task 10. Expression of an rBChE-Fc domain fusion in *P. pastoris*

Completion criteria. Detection of rBChE-Fc fusions in cultivation supernatants by intact LC-MS, SDS-PAGE, or Western blot (using mAbs generated in Task 3). Confirmation of rBChE activity of unpurified materials relative to BChE standard at similar concentrations using Ellman's assay. Confirmation of rBChE-Fc fusion binding to human and/or guinea pig Fc receptors by ELISA.

Status. Paused (50% complete). Upon further evaluation of current bottlenecks, new iterations of Fc-rBChE fusion proteins and their integration into *P. pastoris* strains were paused. We began to develop and test strategies to improve sequestration of rBChE products in the ER. The efficacy of Fc-fusion on improving transport from the ER, improving half-life, and improving secretion would need to be reassessed following these efforts. Construction of further Fc-fusion molecules was also paused.

The first round of Fc-truncation constructs was cloned. We successfully created a draft strain of Fc-CAT3 using the CCDS sequence. Transformations continued further Fc-tagged truncation constructs, testing N- and C-terminal Fc domains with optimized and CCDS variant SBPs and CAT3 sequences. Monomeric constructs were completed but the Fc-monomers did not result in secretion. Considering potential improvements to HSA-fusion, we would recommend using chemical lysis to assess a potential impact of Fc-fusion on transport from the ER.

Task 11. Development of a draft purification process to support non-clinical testing of rBChE products.

Completion criteria. Confirmation of suitable rBChE purity with respect to process related variants to support non-clinical testing (≤1000 ppm HCP/dose; ≤1000 ppb host cell DNA/dose) using ELISA (HCP detection) and Picogreen assays (host cell DNA)

Status. (50% complete) A method for minimal purification of *K. phaffii* secreted proteins was developed. The method was validated for several proteins: severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) receptor-binding domain (RBD) (pI 8.57), human growth hormone (hGH) (pI 5.27), nonreplicating rotavirus vaccine (NRRV) subunit P[8] (pI 5.92), 2KD1 nanobody (pI 6.71), human serum albumin (HSA) (pI 5.67), Interferon (IFN) (pI 5.97), Granulocyte colony-stimulating factor (GCSF) (pI 5.65). The method uses a positively-charged Q-membrane filter that binds DNA and low pI HCPs. In our tests, the method can yield >80% pure protein (<1000PPM) and reduce DNA to <0.1ng/ml. The pI of rBChE is 6.59, which suggests that the method should be applicable and could support the purification of supernatant protein for non-clinical assessment.

Task 12. Expression of fully glycosylated rBChE in *P. pastoris*

Completion criteria. Detection of fully-sialylated rBChE in cultivation supernatants by intact LC-MS, SDS-PAGE, or Western blot (using mAbs generated in Task 3). Confirmation of rBChE activity in unpurified supernatants relative to BChE standard at similar concentrations using Ellman's assay.

Status. (80% complete) We completed cultivations for different G0 strains expressing select recombinant proteins using alternative outgrowth and production media conditions. We also integrated two human chaperones, calnexin and calreticulin, under the control of yeast native chaperone promoter. This genetic change did not improve the glycan profiles of SARS-CoV-2 RBD or of trastuzumab, possibly due to the lack of co-chaperones. We selected more chaperone candidates for further engineering and testing.

We also knocked out native phosphomannosyltransferases mnn4 and mnn6 in strains expressing K3, mouse Granulocyte Macrophage Colony Stimulating Factor (mGM-CSF), and a SARS-CoV-2 RBD variant. We observed a decreased amount of phosphomannosylation but no significant decrease in the degree of hypermannosylation. We also performed preliminary analysis of the RNA sequencing transcriptomic dataset and observed that strong endoplasmic reticulum associated degradation (ERAD) response is mediated by weaker mns1 constructs and further glycoengineering beyond mns1 integration (Figure 30).

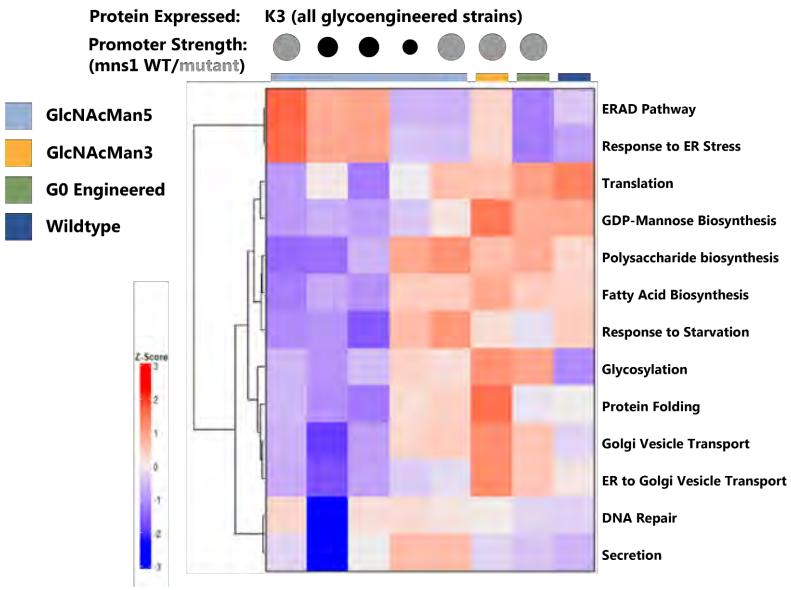


Figure 30. *Gene set enrichment analysis of glycoengineered strains.*

Task 13. Tetramerization of fully glycosylated rBChE from *P. pastoris*

Completion criteria. Detection of rBChE tetramers in cultivation supernatants, either pre- or post-treatment by intact LC-MS, SDS-PAGE, or Western blot (using mAbs generated in Task 3). Confirmation of rBChE activity of unpurified materials relative to BChE standard at similar concentrations using Ellman's assay

Status. No-Go: Required completion of Task 12

Task 14. Molecular engineering of rBChE for improved product quality

Completion criteria. Detection of rBChE products in cultivation supernatants with reduced product-related variation by intact LC-MS, SDS-PAGE, or Western blot (using mAbs generated in Task 3). Confirmation of rBChE activity of unpurified materials relative to BChE standard at similar concentrations using Ellman's assay.

Status. No-Go: Required completion of Task 7.

Task 15. Proof-of-concept expression of manufacturable rBChE product(s) in perfusion culture

Completion criteria. Selection of 2 rBChE product candidates for further process development based on achievement of product activity and baseline titers ≥ 0.1 g/L.

Status. No-Go: Required completion of Task 11.

Phase 2 Milestone: *P. pastoris-expressed rBChE with a relevant in vivo circulating half-life…*
Deliverable: Development of an rBChE product having a clinically relevant circulation and manufacturable using *P. pastoris.*

Phase 3/Option Period 2 (Months 25-36):

Task 16. ***Development of a strategy for selective control of N-linked glycosylation***

Completion criteria. Increased cell densities following the biomass accumulation phase of cultivation relative to cell densities achieved in Tasks 1 and 8. Improved titers of uniformly glycosylated K3 as detected by LC-MS and SDS-PAGE.

Status. "No-Go" - Phase 2/Option Period 1 criterium was not met.

Task 17. ***Development of a strategy for selective control of O-linked glycosylation***

Completion criteria. Improved titers of aglycosylated rG-CSF relative to a base strain without glycosylation control as assayed by LC-MS and SDS-PAGE.

Status. "No-Go" - Phase 2/Option Period 1 criterium was not met.

Task 18. ***Expression of an rBChE product in an improved glyco-engineered strain of P. pastoris with complete glycosylation control***

Completion criteria. Increased cell densities following the biomass accumulation phase of cultivation relative to cell densities achieved in Tasks 15. Detection of increased titers and improved quality of rBChE product in cultivation supernatants by LC-MS, SDS-PAGE, or Western blot (using mAbs generated in Task 3) relative to the strains tested in Task 15 (> 0.1 g/L).

Status. "No-Go" - Phase 2/Option Period 1 criterium was not met.

Task 19. ***Optimization of a perfusion-based process for rBChE production***

Completion criteria. Perfusion expression processes for 2 rBChE products and detection of rBChE product in cultivation perfusates at titers >0.1 g/L with suitable product activity and quality based on the draft TPP established in Task 4.

Status. "No-Go" - Phase 2/Option Period 1 criterium was not met.

Task 20. ***Development of a straight-through downstream purification process for rBChE product from P. pastoris cultivation perfusates***

Completion criteria. Purification processes for (2) rBChE products enabling purified materials with suitable product activity and quality based on the draft TPP established in Task 4.

Status. "No-Go" - Phase 2/Option Period 1 criterium was not met.

Task 21. ***Demonstration of end-to-end production of an rBChE product using InSCyT.***

Completion criteria. Purified and formulated rBChE product with suitable product activity and quality based on the draft TPP established in Task 4; rBChE product activity and half-life comparable to BChE purified from human serum when tested in a guinea pig model (partnered work with AMRI ChemDef labs).

Status. "No-Go" - Phase 2/Option Period 1 criterium was not met.

Phase 3 Milestone: Development of integrated rBChE mfg. process on InSCyT
Deliverable: End-to-end mfg. process for a high-quality, bioactive, long half-life rBChE product

4. <u>Productivity:</u> Updated Work Breakdown Structure shown in Table 2.

	Task Name	Start	End	Completion
1	Expression of Recombinant Products with BChE Activity in Pichia pastoris	6/27/2019	6/26/2022	0%
2	**POC expression of bioactive rBChE in P. pastoris (Base)**	**6/27/2019**	**6/26/2020**	**93%**
3	Task 1-P. pastoris with minimal N-linked glycosylation	6/27/2019	8/19/2019	100%
4	Task 2-P. pastoris with upregulated chaperones	8/27/2019	11/26/2019	100%
5	Task 3-Reagents for rBChE detection	6/27/2019	12/16/2019	100%
6	Task 4-Analytical methods for rBChE activity/quality	6/27/2019	11/15/2019	100%
7	Task 5-rBChE expression in glyco-engineered P. pastoris	4/24/2020	1/10/2022	85%
8	Task 6-Molecular engineering of rBChE for higher titers	12/27/2019	12/15/2020	100%
9	Task 7-Improved rBChE expression in glyco-eng P. pastoris	12/15/2020	12/15/2020	85%
10	**Development of rBChE product with circulating half-life (Option 1)**	**6/27/2020**	**6/26/2021**	**73%**
11	Task 8-P. pastoris with fully humanized N-glycans	3/9/2020	7/26/2021	100%
12	Task 9-Expression of rBChE-nanobody fusion protein	12/16/2020	10/31/2021	50%
13	Task 10-Expression of rBChE-Fc domain fusion protein	12/1/2020	12/31/2021	50%
14	Task 11-Draft DSP to support non-clinical testing of rBChE products	1/27/2021	3/26/2021	50%
15	Task 12-Expression of fully-glycosylated rBChE in P. pastoris	2/1/2021	6/26/2022	80%
16	~~Task 13-Tetramerization of fully glycosylated rBChE~~	~~1/27/2021~~	~~3/26/2021~~	~~0%~~
17	~~Task 14-Molecular engineering of rBChE products~~	~~2/27/2021~~	~~6/26/2021~~	~~0%~~
18	~~Task 15-POC expression of manufacturable rBChE products in perfusion~~	~~2/27/2021~~	~~2/27/2021~~	~~0%~~
19	**Development of an integrated mfg process for production of an rBChE product using InSCyT (Option 2)**	**6/27/2021**	**6/26/2022**	**0%**
20	~~Task 16-Selective control of N-linked glycosylation~~	~~6/27/2021~~	~~9/27/2021~~	~~0%~~
21	~~Task 17-Selective control of O-linked glycosylation~~	~~8/27/2021~~	~~11/26/2021~~	~~0%~~
22	~~Task 18-rBChE product expression with selective control~~	~~8/27/2021~~	~~12/27/2021~~	~~0%~~
23	~~Task 19-Optimization of rBChE product perfusion process~~	~~6/27/2021~~	~~2/26/2022~~	~~0%~~
24	~~Task 20-Development of rBChE product purification process~~	~~6/27/2021~~	~~6/26/2022~~	~~0%~~
25	~~Task 21-End-to-end manufacturing process for rBChE product~~	~~3/27/2021~~	~~6/26/2022~~	~~0%~~

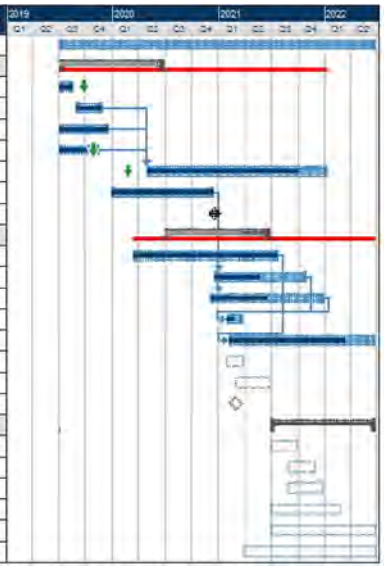


Table 2. *Updated Work Breakdown Structure shows task duration, completion and tasks not pursued.*

5. Technology Transitions: N/A

6. Innovations/Patents: See Appendix for Form DD882

7. Publications, Press Releases, Abstracts, Talks, Awards Relevant to this Effort: N/A

8. Meetings and Events:

24-10-2019: Biostasis Program Review, Joint Base San Antonio, TX

Prof. J. Christopher Love provided an overview of the program, members of the team presented the project's progress to-date and Prof. Love shared the team's plans for the next six months. Those in attendance included fellow performers, military personnel from the Army/Army Medical, Air Force, Office of the Secretary of Defense, Joint Base San Antonio stakeholders, transition partners and members of the U.S. Food & Drug Administration.

28-04-2020: Biostasis Spring 2020 PI Meeting, Virtual

Prof. J. Christopher Love provided an overview of the program, members of the team presented the project's progress since the previous review and Prof. Love shared the team's plans for the next six months.

30-09-2020: Biostasis Fall 2020 PI Meeting, Virtual

Prof. J. Christopher Love provided an overview of the program, members of the team presented the project's progress since the previous review and Prof. Love shared the team's plans for the next six months.

30-03-2021: Biostasis Spring PI Meeting, Virtual

Prof. J. Christopher Love provided an overview of the program, members of the team presented the project's progress since the previous review and Prof. Love shared the team's plans for the next six months.

9. IACUC and/or IRB documentation status

IACUC/IRB Protocol No.	ACURO Approval* IACUC only	NIWC Submission and Approval	Amendments	ACURO Approval	NIWC submission and approval	Protocol closeout documentation submitted
75327-LS DRP.02	26-Sept-2019	N/A	N/A	N/A	N/A	16-Nov-2022

10. Financial Summary

Financial Summary Reporting Period: 27-June-2019 – 26-June-2022		
Expenditures	**Proposed Costs**	**Cumulative Costs**
Cost Element		
Personnel & Benefits	$885,736.36	$613,707.74
Supplies	$629,396.33	$451,622.48
Equipment	$111,196.00	$111,196.00
Travel/Program Admin Costs	$271,351.07	$72,725.97
Subaward	$243,691.57	$0
Indirect Total	*$852,873.27*	*$542,764.33*
Total Direct	*$2,141,371.26*	*$1,249,252.19*
Total	**$2,994,244.53**	**$1,792,016.52**

11. Equipment purchases*

Agilent 4200 TapeStation: We requested $6,336.45 to support the purchase of this system to improve our ability to identify key bottlenecks in our workflow related to cloning and strain construction. With an increased emphasis on the use of Next Generation Sequencing techniques and RNA sequencing, we were able to expedite our library process and improve quality control in library preparation (serial number: DEDAA03279).

Fisher Scientific Innova 42R Incubators (Model M1335-0014): We requested $31,129.55 for the purchase of two incubators to improve our workflow by increasing our capacity to cultivate more strains in parallel (serial numbers: Si42iN204582; Si42iN004584)

ProteinSimple (Model 004-650): We requested $73,730 to purchase the "Jess-Chemiluminescent & Fluorescent Western Blotting" system to support the expansion of the team's analytical capabilities. This model is capable of automating protein separation and immunodetection of traditional Western blotting and was necessary to facilitate rapid detection and quantification of our protein products (serial number: JS-3370).

*See Appendix for each invoice.

APPENDIX

Agilent Technologies: 4200 TapeStation system
Invoice 118359677

Fisher Scientific
Invoice 2275055
Invoice 5382505
Invoice 5797416

Protein Simple
Invoice 0121323-IN

Report of Inventions and Subcontracts
DD Form 882

INVOICE 118359677

REMIT TO:
Agilent Technologies, Inc.
4187 Collections Center Drive
Chicago, IL 60693

ORDER DATE	INVOICE DATE	DUE DATE	PAYMENT TERMS	AGILENT TECH. ORDER NUMBER
12/12/2019	12/26/2019	01/25/2020	NT30	309042279

Purchase Order Number
402018

US: 77-0518772
Customer Number: 70055304
Contact Phone: 617-253-8348
Contact Name: Cheryl Williams

INVOICE TO:
Massachusetts Institute of
Technology
PO Box 9169
CAMBRIDGE MA 02139-9169

Special Instructions:
* PO 402018

INQUIRIES TO:
AGILENT TECHNOLOGIES
CHEMICAL ANALYSIS GROUP - M/S 37
2850 CENTERVILLE RD
WILMINGTON DE 19808
Phone: (800) 227-9770

SUMMARY:

Items Total	: $	51,098.00
Total Discount	: $	9,187.42-
Invoice Sub Total	: $	41,910.58
VAT/Tax 0.00 %	: $	0.00
Invoice Total	: $	41,910.58

ITEM	PRODUCT	DESCRIPTION	UNIT-AMT	QTY	UM	EXTD-AMT	EXTD-DISC	EXTD-NET	TAX
1000	G2991AA	4200 TapeStation system	51,098.00	1	EA	51,098.00	9,187.42-	41,910.58	
		SERIAL # DEDAA03279 , BOX # 26831 4200 Tapestation							
		SERIAL # 5CG9388J3P , BOX # 26831 TapeStation Laptop							
		SERIAL # DEW0689136 , BOX # 26831 TapeStation SW and User Information							
	UP_FRONT_SERVICE_H	Up Front Support Services for Hardware	1	EA					
		Purchase Agreement number: G8S94							
		Item Total	**51,098.00**			**51,098.00**	**9,187.42-**	**41,910.58**	**0.00**

SHIP TO:
Massachusetts Institute of Technolo
Customer Ref 402018
32 Vassar St
CAMBRIDGE MA 02139-4309

Shipped via - Federal Express Freight LTL
Shipping Date - 12/26/2019
Bill of lading - 4211851836

INVOICE 118359677

ORDER DATE	INVOICE DATE	DUE DATE	PAYMENT TERMS	AGILENT TECH. ORDER NUMBER
12/12/2019	12/26/2019	01/25/2020	NT30	309042279

US: 77-0518772

Customer Number: 70055304

Contact Phone: 617-253-8348

Contact Name: Cheryl Williams

Orders for the sale of Agilent Products and Services referenced in this Invoice are subject to the then current version of Agilent's Terms of Sale, and any Supplemental Terms or Occasional Reseller Terms of Sale or other applicable terms referenced in the Quotation and Acknowledgement. The sale of Microplates Products shall be subject to Microplates Terms of Sale and any Supplemental Terms or other applicable terms referenced herein and in the Quotation and Acknowledgement. The sale of Microplates Tooling Products shall be subject to Microplates Tooling Terms of Sale and any Supplemental Terms or other applicable terms referenced herein and in the Quotation and Acknowledgement. All of the above "Terms" as applicable. If you have a separate agreement in effect with Agilent covering the sale of Products and Services referenced in this Invoice, the terms of that agreement will take precedence for those Products and Services.

11/07/2019000011

fisher scientific
part of Thermo Fisher Scientific

REMIT TO:
ACCT# 508355-004
P.O. BOX 3648
BOSTON MA
02241-3648

INQUIRE AT: (800) 766-7000
3970 JOHNS CREEK COURT
SUWANEE GA
30024

D-U-N-S-00-432-1519
FEIN 23-2942737
ORIGINAL INVOICE

PLEASE REFER TO THIS INVOICE NUMBER ON YOUR REMITTANCE

2275055

CUSTOMER PURCHASE ORDER NUMBER - RELEASE NUMBER	INV. DATE
381363	10/31/2019

ORDER NO.	ACCOUNT NO.	CSO	F.O.B.	ORDER ENTRY DATE	PAGE	DUPLICATE
H93012680	508355-004	ATL	DESTINATION	10/28/2019	1	

SOLD TO:

ACCOUNTS PAYABLE
MASSACHUSETTS INSTITUTE
OF TECHNOLOGY
PO BOX 9169
CAMBRIDGE MA 02139-9169

SHIP TO:
CARMEN ELENBERGER(76-231)
MASSACHUSETTS INSTITUTE
OF TECHNOLOGY
SHIPPING AND RECEIVING
32 VASSAR ST
CAMBRIDGE MA 02139-4309

INVOICE TYPE: NOR FON CON
THIS IS A PARTIAL SHIPMENT [X]

DUE: 11/30/2019
TERMS: NET 30 DAYS
PAYABLE IN U.S. CURRENCY.

Visit: www.fishersci.com

DESCRIPTION	CATALOG NUMBER	QUANTITY SHIPPED	UNIT PRICE	AMOUNT
CALLER-CHERYL WILLIAMS PHONE-617-253-5595				
SHIPMENT NBR: 001 FROM: VND ON: 10/31/2019				
LINE # 01 ORDERED PART # M13350014				
INNOVA 42R W/1 ORBITW/120V/60 VN00114060 M1335-0014 EPPENDORF 3P NORTH AMERICA ORIG CAT NBR M13350014	50 117 9943	2 EA	11,540.27	23,080.54
LINE # 02 ORDERED PART # M13350800				
STACKING KIT F/42R VN00001355 M13350800 EPPENDORF NORTH AMERICA BIOTOO ORIG CAT NBR M13350800	NC1195565	1 EA	305.01	305.01
LINE # 03 ORDERED PART # M12509902				
18INX18IN PLTFORM UNIVERSAL ORIG CAT NBR M12509902	14 278 42	2 EA		NO CHARGE
TOTAL INVOICE AMOUNT				23,385.55

FOR YOUR PROTECTION, OUR COMPANY DOES NOT ACCEPT CREDIT CARD NUMBERS VIA FAX OR EMAIL

TELL US ABOUT YOUR RECENT CUSTOMER SERVICE EXPERIENCE BY COMPLETING A SHORT SURVEY. THIS SHOULD TAKE NO LONGER THAN THREE MINUTES. ENTER THE LINK INTO YOUR BROWSER AND ENTER THE PASSCODE SHOWN.
http://survey.medallia.com/fishersci PASSCODE: USA-PGH-CS2

E-INVOICE @HTTPS://WWW.E-SCICOM.COM/THERMOFISHER/REGISTER.ASPX

See reverse side for complete terms and conditions or visit http://www.fishersci.com/salesterms

PAST DUE BALANCES ARE SUBJECT TO A **FINANCE CHARGE.** THIS SHIPMENT WAS DELIVERED IN PERFECT CONDITION AND SIGNED FOR BY THE TRANSPORTATION COMPANY. CONSIGNORS RESPONSIBILITY CEASES UPON DELIVERY OF GOODS TO CARRIER. DO NOT ACCEPT SHIPMENT SHOWING EVIDENCE OF DAMAGE OR SHORTAGE UNTIL AGENT OF CARRIER ENDORSES NOTATION TO THIS EFFECT ON FACE OF TRANSPORTATION RECEIPT. WITHOUT THIS DOCUMENTARY EVIDENCE CLAIM CANNOT BE FILED. SELLER CERTIFIES THAT ALL GOODS (OR SERVICES) COVERED BY THIS INVOICE WERE PRODUCED IN COMPLIANCE WITH ALL APPLICABLE REQUIREMENTS OF SECTIONS 6, 7, AND 12 OF THE FAIR LABOR STANDARDS ACTS OF 1938, AS AMENDED, AND OF THE REGULATIONS AND ORDERS OF THE UNITED STATES DEPARTMENT OF LABOR ISSUED UNDER SECTION 14 THEREOF.

NO CREDIT WILL BE ALLOWED FOR MERCHANDISE RETURNED WITHOUT PRIOR AUTHORIZATION.

THE PRICES SHOWN ON THIS INVOICE ARE NET OF DISCOUNTS PROVIDED AT THE TIME OF PURCHASE. SOME PRODUCTS MAY BE SUBJECT TO ADDITIONAL DISCOUNTS AGREED UPON BETWEEN THE PARTIES.

95 178 0 0 3011601 0gOoDz9000H2ZAS 000187581

12/17/2019000029

fisher scientific
part of Thermo Fisher Scientific

REMIT TO:
ACCT# 508355-004
P.O. BOX 3648
BOSTON MA
02241-3648

INQUIRE AT: (800) 766-7000
3970 JOHNS CREEK COURT
SUWANEE GA
30024

D-U-N-S-00-432-1519
FEIN 23-2942737
ORIGINAL INVOICE

PLEASE REFER TO THIS INVOICE NUMBER ON YOUR REMITTANCE

5382505

CUSTOMER PURCHASE ORDER NUMBER - RELEASE NUMBER	INV. DATE
381363	12/09/2019

ORDER NO.	ACCOUNT NO.	CSO	F.O.B.	ORDER ENTRY DATE	PAGE	DUPLICATE
H93012680	508355-004	ATL	DESTINATION	10/28/2019	1	

SOLD TO:

193
ACCOUNTS PAYABLE
MASSACHUSETTS INSTITUTE
OF TECHNOLOGY
PO BOX 9169
CAMBRIDGE MA 02139-9169

SHIP TO:
CARMEN ELENBERGER(76-231)
MASSACHUSETTS INSTITUTE
OF TECHNOLOGY
SHIPPING AND RECEIVING
32 VASSAR ST
CAMBRIDGE MA 02139-4309

INVOICE TYPE:
NOR FON CON
THIS IS A PARTIAL SHIPMENT [X]

DUE: 01/08/2020
TERMS: NET 30 DAYS
PAYABLE IN U.S. CURRENCY.

Visit: www.fishersci.com

DESCRIPTION	CATALOG NUMBER	QUANTITY SHIPPED	UNIT PRICE	AMOUNT
CALLER-CHERYL WILLIAMS PHONE-617-253-5595 SHIPMENT NBR: 002 FROM: VND ON: 12/05/2019 LINE # 04 ORDERED PART # 008208000 4 SHAKER PERF PLAN	NC1624941	2 EA	985.00	1,970.00
VN00114060 0082080004 EPPENDORF 3P NORTH AMERICA ORIG CAT NBR 0082080004				
TOTAL INVOICE AMOUNT				1,970.00

FOR YOUR PROTECTION, OUR COMPANY DOES NOT ACCEPT CREDIT CARD NUMBERS VIA FAX OR EMAIL

TELL US ABOUT YOUR RECENT CUSTOMER SERVICE EXPERIENCE BY COMPLETING A SHORT SURVEY. THIS SHOULD TAKE NO LONGER THAN THREE MINUTES. ENTER THE LINK INTO YOUR BROWSER AND ENTER THE PASSCODE SHOWN.
http://survey.medallia.com/fishersci PASSCODE: USA-PGH-CS2

E-INVOICE @HTTPS://WWW.E-SCICOM.COM/THERMOFISHER/REGISTER.ASPX

See reverse side for complete terms and conditions or visit http://www.fishersci.com/salesterms

PAST DUE BALANCES ARE SUBJECT TO A **FINANCE CHARGE.** THIS SHIPMENT WAS DELIVERED IN PERFECT CONDITION AND SIGNED FOR BY THE TRANSPORTATION COMPANY. CONSIGNORS RESPONSIBILITY CEASES UPON DELIVERY OF GOODS TO CARRIER. DO NOT ACCEPT SHIPMENT SHOWING EVIDENCE OF DAMAGE OR SHORTAGE UNTIL AGENT OF CARRIER ENDORSES NOTATION TO THIS EFFECT ON FACE OF TRANSPORTATION RECEIPT. WITHOUT THIS DOCUMENTARY EVIDENCE CLAIM CANNOT BE FILED. SELLER CERTIFIES THAT ALL GOODS (OR SERVICES) COVERED BY THIS INVOICE WERE PRODUCED IN COMPLIANCE WITH ALL APPLICABLE REQUIREMENTS OF SECTIONS 6, 7, AND 12 OF THE FAIR LABOR STANDARDS ACTS OF 1938, AS AMENDED, AND OF THE REGULATIONS AND ORDERS OF THE UNITED STATES DEPARTMENT OF LABOR ISSUED UNDER SECTION 14 THEREOF.

NO CREDIT WILL BE ALLOWED FOR MERCHANDISE RETURNED WITHOUT PRIOR AUTHORIZATION.

THE PRICES SHOWN ON THIS INVOICE ARE NET OF DISCOUNTS PROVIDED AT THE TIME OF PURCHASE. SOME PRODUCTS MAY BE SUBJECT TO ADDITIONAL DISCOUNTS AGREED UPON BETWEEN THE PARTIES.

193 1045 0 0 3035463 0gcxkNW000EsogR 000287511

12/26/2019000030

fisher scientific
part of Thermo Fisher Scientific

REMIT TO:
ACCT# 508355-004
P.O. BOX 3648
BOSTON MA
02241-3648

INQUIRE AT: (800) 766-7000
3970 JOHNS CREEK COURT
SUWANEE GA
30024

D-U-N-S-00-432-1519
FEIN 23-2942737
ORIGINAL INVOICE

PLEASE REFER TO THIS INVOICE NUMBER ON YOUR REMITTANCE

5797416

CUSTOMER PURCHASE ORDER NUMBER - RELEASE NUMBER	INV. DATE
381363	12/16/2019

ORDER NO.	ACCOUNT NO.	CSO	F.O.B.	ORDER ENTRY DATE	PAGE	DUPLICATE
H93012680	508355-004	ATL	DESTINATION	10/28/2019	1	

SOLD TO:

ACCOUNTS PAYABLE
MASSACHUSETTS INSTITUTE
OF TECHNOLOGY
PO BOX 9169
CAMBRIDGE MA 02139-9169

SHIP TO:
CARMEN ELENBERGER (76-231)
MASSACHUSETTS INSTITUTE
OF TECHNOLOGY
SHIPPING AND RECEIVING
32 VASSAR ST
CAMBRIDGE MA 02139-4309

INVOICE TYPE: NOR FON CON
THIS IS A PARTIAL SHIPMENT ☐

DUE: 01/15/2020
TERMS: NET 30 DAYS
PAYABLE IN U.S. CURRENCY.

Visit: www.fishersci.com

DESCRIPTION	CATALOG NUMBER	QUANTITY SHIPPED	UNIT PRICE	AMOUNT
CALLER-CHERYL WILLIAMS PHONE-617-253-5595				
SHIPMENT NBR: 003 FROM: VND ON: 12/05/2019				
LINE # 05 ORDERED PART # 901513564 WARRANTY SHAKER GRP 3 VN00001355 901513564 EPPENDORF NORTH AMERICA BIOTOO ORIG CAT NBR 901513564	NC1517922	2 EA	2,887.00	5,774.00
TOTAL INVOICE AMOUNT				5,774.00

FOR YOUR PROTECTION, OUR COMPANY DOES NOT ACCEPT CREDIT CARD NUMBERS VIA FAX OR EMAIL

TELL US ABOUT YOUR RECENT CUSTOMER SERVICE EXPERIENCE BY COMPLETING A SHORT SURVEY. THIS SHOULD TAKE NO LONGER THAN THREE MINUTES. ENTER THE LINK INTO YOUR BROWSER AND ENTER THE PASSCODE SHOWN.
http://survey.medallia.com/fishersci PASSCODE: USA-PGH-CS2

E-INVOICE @HTTPS://WWW.E-SCICOM.COM/THERMOFISHER/REGISTER.ASPX

See reverse side for complete terms and conditions or visit http://www.fishersci.com/salesterms

PAST DUE BALANCES ARE SUBJECT TO A **FINANCE CHARGE.** THIS SHIPMENT WAS DELIVERED IN PERFECT CONDITION AND SIGNED FOR BY THE TRANSPORTATION COMPANY. CONSIGNORS RESPONSIBILITY CEASES UPON DELIVERY OF GOODS TO CARRIER. DO NOT ACCEPT SHIPMENT SHOWING EVIDENCE OF DAMAGE OR SHORTAGE UNTIL AGENT OF CARRIER ENDORSES NOTATION TO THIS EFFECT ON FACE OF TRANSPORTATION RECEIPT. WITHOUT THIS DOCUMENTARY EVIDENCE CLAIM CANNOT BE FILED. SELLER CERTIFIES THAT ALL GOODS (OR SERVICES) COVERED BY THIS INVOICE WERE PRODUCED IN COMPLIANCE WITH ALL APPLICABLE REQUIREMENTS OF SECTIONS 6, 7, AND 12 OF THE FAIR LABOR STANDARDS ACTS OF 1938, AS AMENDED, AND OF THE REGULATIONS AND ORDERS OF THE UNITED STATES DEPARTMENT OF LABOR ISSUED UNDER SECTION 14 THEREOF.

NO CREDIT WILL BE ALLOWED FOR MERCHANDISE RETURNED WITHOUT PRIOR AUTHORIZATION.

THE PRICES SHOWN ON THIS INVOICE ARE NET OF DISCOUNTS PROVIDED AT THE TIME OF PURCHASE. SOME PRODUCTS MAY BE SUBJECT TO ADDITIONAL DISCOUNTS AGREED UPON BETWEEN THE PARTIES.

89 163 0 0 3040188 0gfV4LN000GXpZd 000187516

10/21/2019000017

Invoice

Page: 1

ProteinSimple
3001 Orchard Parkway
San Jose, California 95134
A/R Phone#: 408-510-5544
A/R Fax#: 408-510-5599
TAX ID: 94-3396256
DUNS #: 140594966

Invoice Number: 0121323-IN
Invoice Date: 10/17/2019

Order Number: 0096860
Order Date 10/11/2019
Salesperson: RCAN
Customer Number 10-0024664
RMA Number:

Sold To:	Ship To:
MASSACHUSETTS INST. OF TECH. ATTN: ACCOUNTS PAYABLE P.O. BOX 9169 CAMBRIDGE, MA 02139 UNITED STATES	MIT ATN:CARMEN ELENBERGER(76-231), PO# 374254 32 VASSAR STREET CAMBRIDGE, MA 02139 UNITED STATES **Confirm To:** CARMEN ELENBERGER

Customer P.O.	Ship VIA	INCOTERM	FOB	Terms
374254	TRUMP CARD	FOBD	FREIGHT PREPAID	NET 30

Item Number	Unit	Ordered	Shipped	Back Ordered	Price	Amount
004-650	EACH	1.000	1.000	0.000	68,907.000	68,907.00
SYSTEM, JESS						
Serial Number: JS-3370			1.000			
PS-N007	EACH	1.000	0.000	1.000	4,308.000	0.00
Jess Training Support						
055-302	EACH	1.000	1.000	0.000	0.000	0.00
Wes Pwr Cord Set, 110v US/JP/TW/PH/SA						

Please Remit ACH/Wire Payments To:
ProteinSimple
Swift Code: HATRUS44
ABA: 071000288
Bank Account: 3728672

Please Remit Checks To:
ProteinSimple Checking BIN #39
P.O. Box 1150
Minneapolis, MN 55480-1150

Net Invoice:		68,907.00
Less Discount:		0.00
Freight & Handling:		515.00
Sales Tax:		0.00
Invoice Total:	US$	69,422.00

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

Form Approved
OMB No. 9000-0095
Expires Oct 31, 2004

PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THE ABOVE ORGANIZATION. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.

1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT *(X one)*
Massachusetts Institute of Technology	W911NF1920211			a. INTERIM ✗ b. FINAL

b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	4. REPORTING PERIOD *(YYYYMMDD)*
77 Massachusetts Avenue, Room NE18 Cambridge, MA 02139 (Account 6941398)	20220626		20220626	a. FROM 20190627 b. TO 20220626

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
	NONE							

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*	(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME OF SUBCONTRACTOR(S) a.	ADDRESS *(Include ZIP Code)* b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES *(YYYYMMDD)* f.	
			(1) CLAUSE NUMBER	(2) DATE *(YYYYMM)*		(1) AWARD	(2) ESTIMATED COMPLETION
NONE							

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))* | | SMALL BUSINESS or | ✗ | NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Colleen Mazzeo Leslie	Sr. Director, Research Administration & Compliance	Colleen M. Leslie	9/6/2022

DD FORM 882, DEC 2001 PREVIOUS EDITION IS OBSOLETE. Reset