

Identification of Non-standard Amino Acid Incorporation Sites for Half-life Extension of chemotherapeutic L-asparaginase

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Protein therapeutics have revolutionized modern medicine; however, their efficacy is limited by rapid clearance from the body. L-asparaginase, a chemotherapeutic enzyme used in the treatment of the most commonly diagnosed childhood cancer, exemplifies this challenge. Commercial strategies involve the attachment of polyethylene glycol (PEG) to extend protein half-life. However, complications such as hypersensitivity to PEG and reduction of function when modifying at the protein's terminal-end highlight the need for alternative strategies. Advancements in synthetic biology have enabled the incorporation of nonstandard amino acids (nsAAs) by genetically recoded *E. coli*. This allows for the engineering of modified proteins with intraprotein nsAA sites that facilitate the attachment of fatty acids via copper(I)-catalyzed azide-alkyne Huisgen cycloaddition (click-chemistry). These modifications aim to enhance the drug's pharmacokinetic profile, potentially reducing immunogenicity compared to PEGylation and enabling less frequent and more effective treatment. Preliminary data suggests the viability of at least three amino acid swap sites that conserve the bioactivity of L-asparaginase and allow for fluorophore attachment using copper-mediated click chemistry on the protein, setting proof of concept for fatty acid conjugation. Ultimately, this approach improves insight into L-asparaginase production conditions within C321 *E.coli*, investigates the effects of specific residues on enzyme function, and offers a promising approach for personalizing protein-based treatments.

Introduction

L-asparaginase, a protein chemotherapy, is a primary treatment for acute lymphoblastic leukemia (ALL), the most commonly diagnosed childhood cancer.^{1,2} This enzyme facilitates the breakdown of the vital amino acid L-asparagine into its components: L-aspartic acid and ammonium. By depleting L-asparagine needed for protein synthesis in cancer cells, the treatment leads to cell death, thereby controlling the infamously unrestrained proliferation characterizing cancer.³

Native L-asparaginase expressed from *Escherichia coli* demonstrates a weak pharmacokinetic profile, with the effective concentration diminishing approximately 24 hours after dosage. Commercially, a polyethylene glycol group (PEG) is added to create PEG-asparaginase, which extends the half-life to 6-14 days and helps combat L-asparaginase antibodies in patients with hypersensitivity issues.⁴ However, recent studies have identified the presence of anti-PEG in patients. These antibodies trigger the accelerated blood clearance of the drug and further immunogenic issues. Phenomena associated with anti-PEG in addition to issues with PEG accumulation in the liver make the pharmacokinetic profile difficult to predict, and these effects are concerning for a population that is already immunocompromised.^{5,6}

Recently, innovations in synthetic biology using nonstandard amino acids (nsAAs) to enhance therapeutics presents a promising alternative to PEGylation. These derivatives of the natural 20 amino acids possess the ability to precisely incorporate modifying groups through an azide-alkyne Huisgen cycloaddition (click-chemistry).⁷ By utilizing fatty acids already naturally abundant within the human body, it hopefully will induce less immunogenic results than PEG.

However, previous attempts using the phenylalanine derivative para-azidophenylalanine (pAzF) presented major issues such as azide degradation. Moreover, the treatment to reduce this reduction, IsAz, has been shown to be slightly toxic and highly explosive.⁸ Thus, we propose moving to alkyne-shaped nsAAs that can still undergo click-chemistry. This would involve finding an orthogonal translation system (OTS) promiscuous enough to incorporate the new nsAA. Another significant limitation of previous treatments is the sole incorporation of functional groups at a terminal end. Whether it be at the C or N terminus, bioactivity of the proteins is significantly reduced, calling for the necessity to test incorporation sites within the protein.⁹

In this study, we sought to conserve the activity of L-asparaginase when replacing native amino acids with nsAAs at select target sites. Specifically, we hijacked the native translation system of a recoded organism, C321 to read our orthogonal translation system (OTS) to site-specifically incorporate the non-standard amino acids p-propargyloxy-l-phenylalanine (pPaF), which will later serve as specific target sites to conjugate an azide-based palmitic acid.^{7,10,11} The conditions involved testing 11 sites of nsAA incorporation within the protein and 1 variant with 5 sites of conjugation mediated by an elastin-like protein (ELP). Limited effect of site switches on function would demonstrate that intra-protein conjugation is more favorable than the termini integration strategies like PEG-asparaginase and previous lipid functionalized treatments.¹² Additionally, we will modify the C321 strain to maximally produce bioactive protein through incorporating a shuttle vector, pelB.¹³ Ultimately, these comparative studies can be used to make protein therapeutic treatment less frequent, more effective, and more compatible with the human body.

Materials and Methods

Bacterial Strains

Wild type optimization tests were performed with the genomically recoded organism(GRO) (*E. coli* C321.A, CP006698.1, GI:54981157).^{7,10} Experimental tests involved a modified C321 strain with the orthogonal translation system (OTS) containing plasmid pAcFRS.1.t1(DG.OTS) with a p15A origin of replication incorporated and was selected by a chloramphenicol acetyltransferase marker.^{7,10} All proteins produced in the GRO (*E. coli* C321.A, CP006698.1, GI:54981157) grew at 34° C shaking at 220 rpm overnight.¹¹

Plasmid Constructions

The L-asparaginase gene, ansB, was expressed by a plasmid with a ptetR backbone which included a: pLtetO-1 promoter, tetracycline operator, colE1 origin, a ampicillin resistance marker, and was tagged with a 6-Histidine tag at the C-terminal (pTetR.ansb). This plasmid was then incorporated in the GRO (*E. coli* C321.A, CP006698.1, GI:54981157) with an OTS plasmid, pAzFRS.2.t1 selected by a chloramphenicol acetyltransferase marker and a ColE1 origin of replication.¹² Modified proteins were produced by this two-plasmid system.

PelB Integrated pTetR Plasmid

The first series of plasmids involved cloning the ansB sequence from a previous pLtetO plasmid without a tetracycline operator into the pTetR backbone with the necessary modifications such as a recovery pelB shuttle peptide.¹³ The original plasmids were expressed in the same C321 strain and were inoculated overnight at 34°C shaking overnight at 220rpm.

First, the template plasmids for ansB in C321 (*Appendix*, Fig. 1) were extracted through the QIAprep Spin Miniprep Kit by instruction with the following modifications: the spin condition for initial pelleting by centrifuging was done at 4000G for 10 minutes at 20° C and each spin for the kit was completed at 13000 rpm.

Then, each ansB fragment was then amplified by the primers oIG311, oIG312, oIG327, & oIG328 respectively (*Appendix*, Fig. 3). These primers were synthesized by IDT and resuspended to be 100uM then diluted to 10uM in nuclease free water. Additionally, the primers were designed to not amplify the first 66 nucleotides (the native *E. coli* signal sequence) to allow for the integration of a pelB signal peptide. Each 25µL reaction included 12.5µL 2x Kappa HiFi

HotStart Polymerase, 0.75 μ L forward primer, 0.75 μ L reverse primer, 1 μ L of the respective template fragment (*Appendix*, Fig. 1), and 10 μ L of water. The reactions were cycled in a thermocycler under the following conditions: 95 °C, 3 min → 98 °C, 20sec, 60 °C, 15sec, 72 °C, 15sec,) x 20 → 72 °C, 1 min. A 1% agarose gel stained with ethidium bromide was run on the products stained with 6X dye at 170V for 20 minutes to confirm amplification.

Finally, the plasmids with a template (*Appendix*, Fig.1) were constructed through a 4-part Golden Gate which included: the ansB fragment without the first 66 nucleotides, a pelB fragment, the pTetR backbone, and the terminator sequence. The fragment for pelB was synthesized by Twist Biosciences with the prefixes and suffixes with BsaI restriction site alongside a TEV cleavage tag (*Appendix*, Fig.2). This was resuspended to 50uM in nuclease free water. These strains were cloned through Golden Gate using NEB Golden Gate Mix, T4 Ligase Buffer, and nuclease-free water up to 10 μ L reaction volume. The Master Mix included 5.25 μ L of water, 1 μ L of T4 Ligase Buffer, and 1 μ L of NEB Golden Gate Master Mix (BsaI-v2) multiplied by the number of reactions run, totalling 7.25 μ L of master mix incorporated for each reaction. To the reaction 1.25 μ L of the ptetR backbone plasmid, 0.5 μ L of a terminator plasmid expressing the T1/T2 terminator sequence, .5 μ L of pelB fragment, and 0.5 μ L of the ansB fragment(WT) was added. This was then thermocycled at 37 °C, 1 min → 16 °C, 1 min) x 30 → 60 °C, 5 min.

The plasmid without the pelB, but with the first 66 nucleotides removed (d22N) was constructed similarly through Golden Gate Cloning at a10 μ L reaction volume. The Master Mix included 5.25 μ L of water, 1 μ L of T4 Ligase Buffer, and 1 μ L of NEB Golden Gate Master Mix (BsaI-v2), totaling 7.25uL. To the reaction 1.25 μ L of the ptetR backbone plasmid, 0.5 μ L of a terminator plasmid expressing the T1/T2 terminator sequence, and 0.5 μ L of the ansB fragment(WT) was added. This was then thermocycled at 37 °C, 1 min → 16 °C, 1 min) x 30 → 60 °C, 5 min.

Creation of New Variants

Additional target sites were selected for their lack of binding site affinity, tendency for being on the outside of the protein, or for being a derivative of potential non-standard amino acid through analysis on PDB and the NCBI Conserved Domain Database. This second set of variant

strains were created by site directed mutagenesis (SDM) on the wild type plasmid with pelB (pTetR.pelB-lead_ansB_d22N_C-His).

These variant plasmids were created through NEB Protocol for Q5 Site-Directed Mutagenesis with primers designed in Benchling (*Appendix*, Fig 4) to recode the target sites into a 5'TAG3'. These primers were created by IDT and resuspended then diluted to 10uM with nuclease free water. The thermocycler conditions were 98 °C, 30 sec → 98 °C, 10sec, 57 °C, 15sec, 72 °C, 100sec,) x 25 → 72 °C, 2 min. At this step, an agarose gel was run at 160V for 30 minutes to confirm amplification.

Transformations

Transformation into Cloning Strain

The plasmid products were then chemically transformed into NEB 10 beta cells using the NEB Chemical Transformation Protocol with the following modifications: 9μL of the NEB10 cells and 1μL of the PCR plasmid was used, 300μL of LB media was used to inoculate and incubate the cells, and the 200μL of the sample was plated on a Carbenicillin selection plate. This was then incubated at 37°C overnight.

The non fluorescent cells from these plates were inoculated into 5mL of LB media and incubated shaking at 220 rpm at 37°C overnight. The plasmids were then extracted through the QIAprep Spin Miniprep Kit by instruction with the following modifications: the spin condition for initial pelleting by centrifuging was done at 4000G for 10 minutes at 20° C and each spin for the kit was completed at 13000 rpm.

Then, the concentrations were confirmed by nanodrop. The plasmids were then sequence confirmed by Quintara BioSciences Nanopore Whole Plasmid Sequencing. Alignments were then analyzed using Benchling.

Wild Type Transformations in C321

Upon confirmation of the correct sequences, the plasmids were transformed into C321. For the 3 Wild Type Variants (with different leader sequences) that had no ansB TAG sites, the plasmids were integrated into a chemically competent C321 with no antibiotic resistance markers through the NEB Chemical Transformation protocol with the following modifications: 20μL of

the C321 cells and 2 μ L of the PCR plasmid was used, 300 μ L of LB media was used to inoculate and incubate the cells, and the 200 μ L of the sample was plated on a Carbenicillin selection plate.

Variant Transformations in C321 + DG.OTS

Upon confirmation of the correct mutations, the variant plasmids were transformed into the C321 strain with the DG.OTS already incorporated. The C321 + DG.OTS strain was grown overnight in LB media (1mL per transformation) and incubated shaking at 220 rpm at 34°C overnight. The cells were then spun down at 4000 rpm for 5 minutes and washed with SMQ water 4 times at 1mL resuspension each time. Each wash spin was done at 30 seconds at 12,000 rpm. At the last spin, the cells were resuspended in 50 μ L of SMQ water per transformation. In each electropuvette, 50 μ L of the resuspended cells and 1 μ L of the plasmid was added then electroporated under Laura Quinto's protocol. The cells were immediately recovered in 1mL of LB media and incubated for 2 hours shaking at 220 rpm at 30°C. Lastly, 200 μ L of each sample were plated on a Carbenicillin + Chloramphenicol selection plate. Test culture inoculations were derived from these plates or from glycerol stocks selected from these plates.

Protein Expression and Purification

Purification Optimization Experiment

Firstly, 5mL of LB media with Kanamycin at 30 μ g/ml was inoculated with C321[pLtetO.full_ansB_CHis] and incubated overnight shaking at 220 rpm at 34°C. Then, the culture was back diluted into a 50mL culture 1:20 with LB media with Kan to OD 0.1. Then, the culture grew for 3 hours until OD 0.6. Once OD 0.5-0.8 was met, the culture was induced with 100ng/ μ L aTc then incubated, shaking 220rpm at 34°C for 4 hours. The culture was then transferred into a 50mL Falcon tube and spun down.

The pellets were then resuspended in 2mL of 1X Bugbuster with protease inhibitor to lyse then spun at 13,000rpm for 5 min to collect the soluble portion.

150 μ L of the soluble portion was then purified with the Zymo Research HIS-spin Miniprep kit as per manufacturer instructions. The final elution was then collected.

500 μ L of the lysate (insoluble included) was purified using the Ni-NTA Protocol adapted from Vandersueren. 200 μ L of the Qiagen Ni-NTA Agarose Gel was added in an epi tube and

centrifuged at 13000rpm for 2 minutes. The supernatant was removed and the gel was resuspended in activation buffer (50mM NaH₂PO₄; 300mM NaCl; As much as NaOH (and HCl) as needed to pH 7.5). Then 500µL of the lysate was added and incubated for 10 minutes rotating. Then, it was centrifuged again for 2 minutes. The sample was then washed with 1mL Wash Buffer (activation buffer, 5mM imidazole) and incubated for 10 minutes. Then, the beads were resuspended in 200µL of Elution Buffer (activation buffer, 500mM imidazole) and sit for 5 minutes. Lastly, the sample was centrifuged at 13,000rpm for 2 minutes and the supernatant was collected.

Then, the samples from each purification protocol or just the lysate were buffer exchanged into solution of 25nM Tris (pH 8) and 300mM NaCl through a 30kDa MWCO Amicon filter as per instructions until a 1:1000 dilution.

Lastly, the resulting elutions were run through an SDS Page as per BioRad's Guide to Polyacrylamide Gel Electrophoresis and Detection.

PelB Leader Sequence Experiment

Firstly, 5mL each of LB media with appropriate antibiotic (*Appendix*, Fig. 5) were inoculated with the appropriate WT strain with different leader sequences (*Appendix*, Fig. 5) and incubated overnight shaking at 220 rpm at 34°C. Then, the cultures were back diluted into a 50mL culture 1:20 with LB media with antibiotic to OD 0.1. Once OD 0.5-0.8 was met, the culture was induced with 100ng/µL aTc then incubated, shaking 220rpm at 34°C for 4 hours. The culture was then transferred into a 50mL Falcon tube and spun down.

The pellets were then resuspended in 1mL of 1X Bugbuster with protease inhibitor to lyse then spun at 13,000rpm for 5 min to collect the soluble portion. 150µL of the soluble portion was then purified with the Zymo Research HIS-spin Miniprep kit as per manufacturer instructions. The final elution was then collected and buffer exchanged into solution of 25nM Tris (pH 8) and 300mM NaCl through a 30kDa MWCO Amicon filter as per instructions until a 1:1000 dilution.

Lastly, the resulting elutions were run through an SDS Page as per BioRad's Guide to Polyacrylamide Gel Electrophoresis and Detection and the Activity Assay.

Variant Analysis Experiments

Firstly, 5mL each of LB media with appropriate antibiotic (*Appendix*, Fig. 5) were inoculated with the appropriate variant (*Appendix*, Fig. 5) and incubated overnight shaking at 220 rpm at 34°C. In addition, the WT strain with pelB and the C321 + DG.OTS strain with no ansB plasmid were grown as a positive and negative control under the same conditions with their respective antibiotics.

Then, the control cultures were back diluted into a 50mL culture 1:20 with LB media with antibiotic to OD 0.1. The variant cultures were back diluted into a 50mL culture 1:20 with LB media with antibiotic to OD 0.1, 0.2% arabinose, 1mM pPaF, and 2mM HCl. Once OD 0.45-1.2 was met, the culture was induced with 100ng/ μ L aTc then incubated, shaking 220rpm at 34°C for 4 hours.¹⁴

The culture was then transferred into a 50mL Falcon tube and spun down. The pellets were then resuspended in 1mL of 1X Bugbuster with protease inhibitor to lyse then spun at 13,000rpm for 5 min to collect the soluble portion. After this point, every step was completed in the cold room. 150 μ L of the soluble portion was then purified with the Zymo Research HIS-spin Miniprep kit as per manufacturer instructions. The Negative Control strain was not purified.

The final elution was then collected and buffer exchanged into solution of 25nM Tris (pH 8) and 300mM NaCl through a 30kDa MWCO Amicon filter as per instructions until a 1:1000 dilution.

Lastly, the resulting elutions were run through an SDS Page as per BioRad's Guide to Polyacrylamide Gel Electrophoresis and Detection and the Activity Assay.

Asparaginase Functional Activity Assay

The bioactivity of the produced proteins were tested by measuring the OD570 colorimetric output of the BioVision Asparaginase Activity Assay Kit (ab107922) as per manufacturer instruction within a 96 well plate. 5 μ L of each sample was used as the 1X dilution and activity was thin calculated in nmol of aspartate converted per minute per mL of original volume. The standard curve as well as the variant well output readings were analyzed in excel. For each variant, 10 minute time frames were used to calculate activity with the time points being chosen 5 minutes after maximum turnover velocity and 5 minutes before.

Results and Discussion

Creation of Variant Strains Possible of Multi-site nsAA Incorporation

Top Candidate Sites Selected

Target Edits	
C-ter ELP (5TAG)	D40
K44	K101
Y47	K229
K51	E232
D128	Y272
K235	Y311

Figure 1. Preliminary Top 11 Candidate Sites of nsAA Incorporation

After database analysis, 11 sites were selected to trial.

Through an analysis on NCBI Conserved Domain Database and the Protein Data Bank (PDB ID: 6NXB) the 348 amino acids of L-asparaginase were screened to be replaced by nsAAs. Firstly, residues with known functions such as binding or mutagenesis were taken out of consideration. Then, sites with favorable qualities such as being a surface residue or having structural similarity to phenylalanine (due to pPaF being its derivative) were highlighted. This resulted in 11 candidate site swaps. Additionally, a C-terminal condition where 5 TAG sites will be incorporated by an elastin-like protein (ELP) was chosen as a comparison value between terminal-end and intra-protein integrations.^{7,12} These sites are the optimal preliminary sites to start testing site-specific swaps.

Creation of Candidate Vector

In order to express the ansB gene, which codes for L-asparaginase, a plasmid was created to incorporate the gene alongside replacing the first 22 amino acids with the pelB signal sequence. For better control of expression and selection, a tetracycline operator region was added to allow for inducibility, and an ampicillin resistance region was incorporated. Through Golden Gate Cloning mediated by the BsaI-v2 restriction enzyme and Site Directed Mutagenesis, the designed vector was created successfully and sequenced.

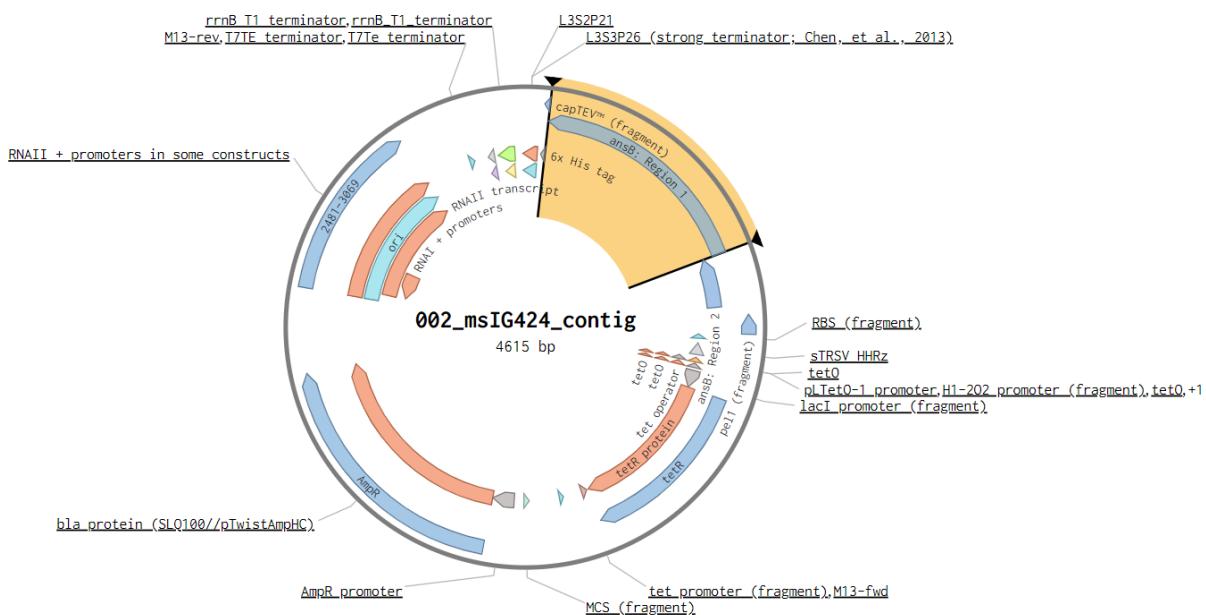


Figure 2. Whole Plasmid Sequencing of pTetR.pelB-lead_ansB_CHis Wild Type

Whole Plasmid Sequencing of the final plasmid displayed successful incorporation of the L-asparaginase coding region(highlighted) as well as the terminator, pelB, 6-HIS tag, antibiotic resistance, terminator sequence, and tetR region.

Successful creation of the WT plasmid allowed for a template for site directed mutagenesis of the target sites of interest though selectively designed primers as well as create a WT strain within C321 for testing with no TAG incorporation sites as a control.

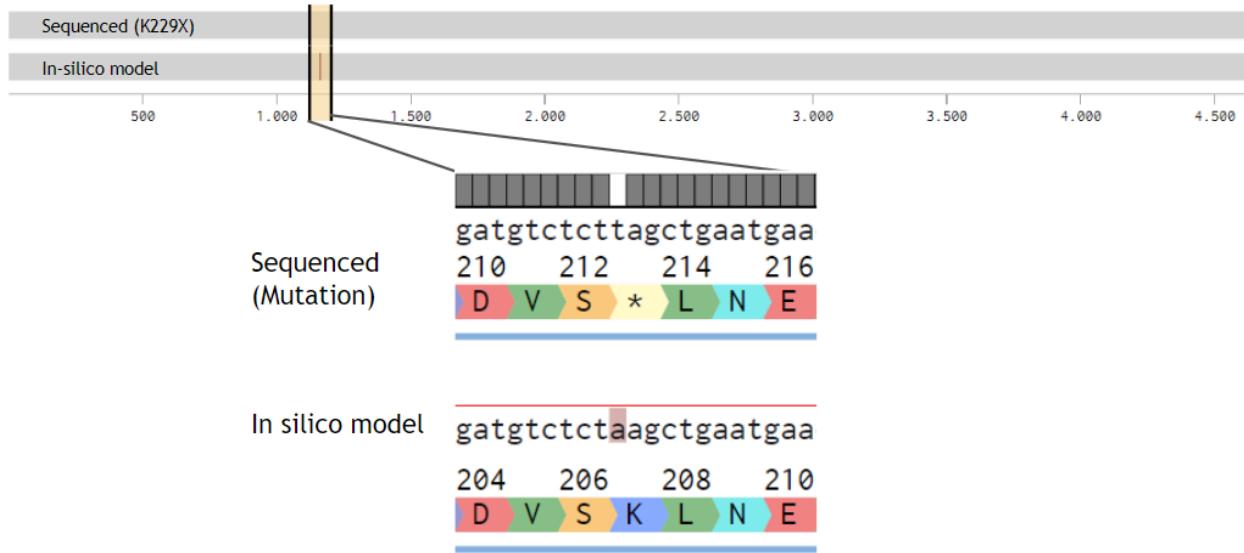


Figure 3. Sequencing Result of K229X Mutation

Whole Plasmid Sequencing of the edited vector displays successful integration of Lysine(K) residue at the location of interest into the TAG sequence with no off target mutations as demonstrated by a Benchling alignment to an in-silico WT plasmid.

However, not all variants had no off target mutations.

Variant Strain	Variant	Notes
pTetR.pelB-lead_ansB_d22N_C-His	pelB WT (no TAG)	1 mutation at in noncoding region-> C2370A
pTetR.pelB-lead_ansB_d22N_C-His (D40X)	D40X	1 mutation at in noncoding region-> C2370A, also has 5-HIS
pTetR.pelB-lead_ansB_d22N_C-His (K101X)	K101X	1 mutation at in noncoding region-> C2370A
pTetR.pelB-lead_ansB_d22N_C-His (K229X)	K229X	No mutations

pTetR.pelB-lead_ansB_d22N_C-His (E232X)	E232X	1 mutation at in noncoding region-> C2370A, has 8-HIS
pTetR.pelB-lead_ansB_d22N_C-His (Y272X)	Y272X	1 mutation at in noncoding region-> G1305T
pTetR.pelB-lead_ansB_d22N_C-His (Y311X)	Y311X	1 mutation at in noncoding region-> G1305T
pTetR.pelB-lead_ansB_d22N_C-His (Y348X)	Y348X	[NOT YET CLONED]
pTetR.pelB-lead_ansB_d22N_C-His (K44X)	K44X	1 mutation at in noncoding region-> G1305T, 5-HIS
pTetR.pelB-lead_ansB_d22N_C-His (Y47X)	Y47X	1 mutation at in noncoding region-> G1305T, 7-HIS
pTetR.pelB-lead_ansB_d22N_C-His (K51X)	K51X	[NOT YET CLONED]
pTetR.pelB-lead_ansB_d22N_C-His (D128X)	D128X	1 mutation at in noncoding region-> G1305T, 7-HIS
pTetR.pelB-lead_ansB_d22N_C-His (K235X)	K235X	[NOT YET CLONED]

Figure 4. Status and Mutations Found in Variant Strains

From Whole Plasmid Sequencing and Sanger Sequencing, it was found that most resulting plasmids had a one nucleotide mutation within a non-coding region in the backbone as well as some variants having more or less than 6-HIS terminals.

These mutations demonstrate that the reverse primer for amplification of template plasmids needs to be redesigned to not bind to a repeated unit such as the 6 Histidine code or the 5-TAG ELP as off-target binding occurred and created mutations in variant quantities of the repeated unit. While the non-coding region mutation should not be an issue, further testing such as a protein gel should be performed to determine if modified amounts of histidines affect purification binding ability.^{15,16} The unsuccessfully created variants should also be attempted to

be created again. However, sequencing of the variant plasmids that did work allowed for confirmation to move forward with C321 transformation alongside the Orthogonal Translation System(OTS) producing plasmid, DG.OTS to create a vessel for protein production.¹⁰

Optimization of Activity Assay and Protein Expression Conditions

HIS-SPIN Miniprep Purified Protein Better than Ni-NTA Gel

Once the WT protein was harvested, different purification protocols were used to isolate the protein of interest including the Zymo HIS-SPIN Miniprep and Ni-NTA Affinity Gel protocol. Both of which use nickel-affinity to isolate protein; however, a protein gel developed by Coomassie Blue stain demonstrated that while the protein of interest at ~36kDa was found in all three conditions, the HIS-SPIN protocol was more effective at isolating the protein of interest.

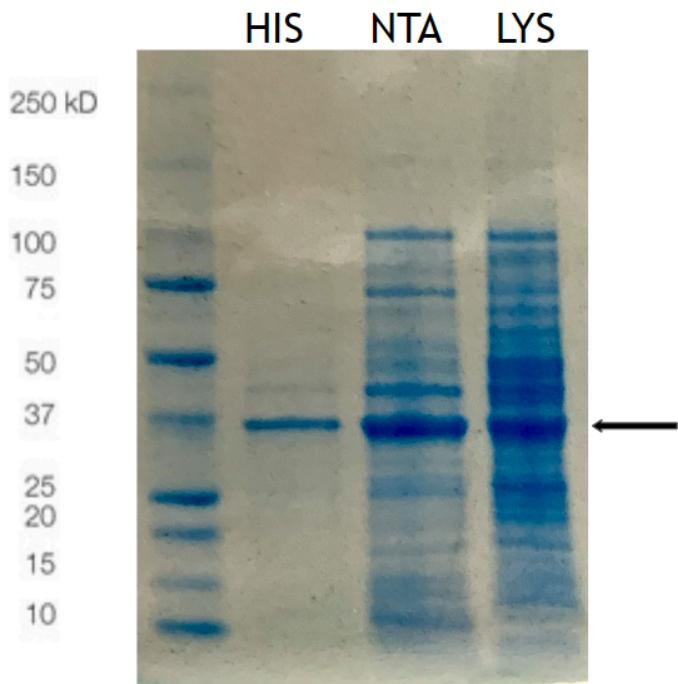


Figure 5. Protein Gel Analysis of Different Purification Conditions

Protein gel illustrates different purification levels of different purification strategies, from left to right, Zymo HIS-SPIN Miniprep, Ni-NTA Affinity Gel, and soluble fraction lysate with 5 μ L of sample loaded.

While the NI-NTA strategy extracted more of the protein band of interest, the clarity of the HIS-SPIN condition was much clearer, making future calculation of activity and purification of drug products easier to produce.

PelB Allows for Extraction of More Bioactive Asparaginase

Four conditions for WT activity were induced to produce protein. (variant with pelB, first 22 residues deleted (d22N), full native ansB, and an empty plasmid) After harvesting, the induced and purified proteins by a HIS-SPIN Miniprep, the resulting eluates were run on a protein gel at 5 μ L sample sizes.

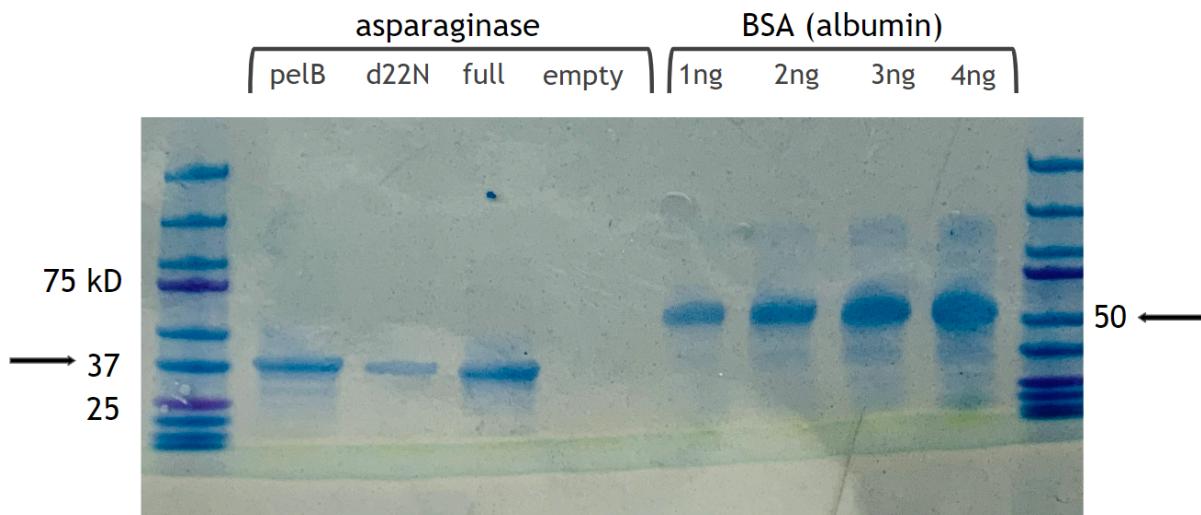


Figure 6. Protein Gel Demonstrating Induction and Purification of Asparaginase

Protein gel illustrates the presence of the purified L-asparaginase protein (~36kDa) in the induced conditions of ansB with pelB, the first 22 residues deleted (d22N), unedited full ansb, as well as no band on the uninduced C321 condition. Additionally, known values (1-4 ng) of Bovine Serum Albumin (BSA) are displayed as a positive control (50kDa) and as a comparison value for concentration.

The gel confirms the purification and induction of the protein of interest as well as giving a relative value of concentrations based upon comparison to the standard concentrations provided by BSA. Just visually speaking, the d22N conditions produce much smaller amounts of protein as compared to the two conditions with a full 348 residues. Between the pelB and full conditions,

the full seems to have a higher intensity band. Furthermore, all the samples look clean and consist mostly of ~35kDa proteins. Once confirmed to be present, the protein was then ready to be tested for bioactivity as what's necessary for analysis is the amount of active protein, not yield of extraction.

The Biovision Asparaginase Activity Assay measured the production of aspartate over time catalyzed by a commercial positive control and the produced proteins of interest by measuring the OD570 absorbance of the samples.

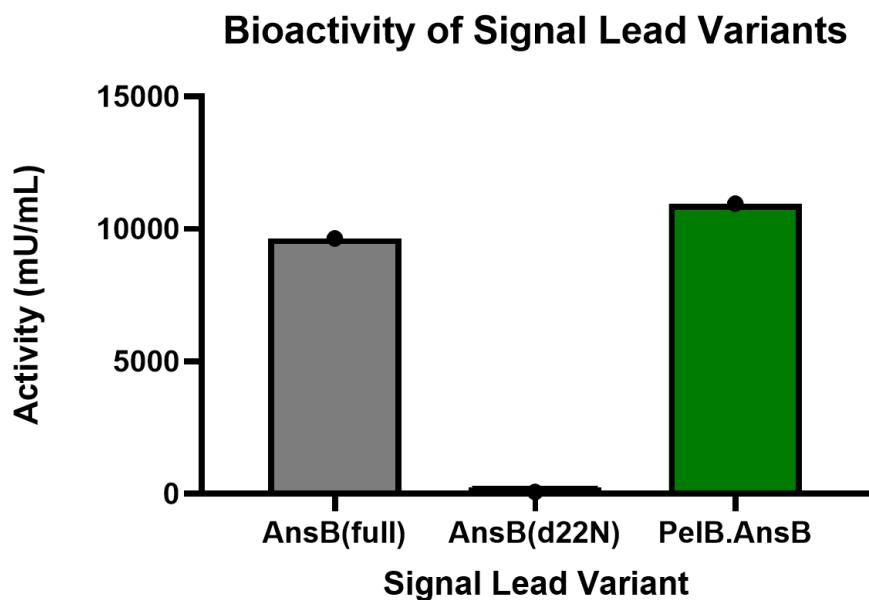


Figure 8. Activity Assay Demonstrates PelB Increasing Yield of Bioactive Asparaginase
Analysis of plate reader data demonstrates that the PelB condition had the highest bioactivity at 10,958 mU/µL, followed by ansB(full) at 9,641 mU/µL, then the 22 residue deletion conditions at 66 mU/µL where mU is defined as nmol Aspartate/min converted.

This initial data suggests that pelB does in fact allow for the extraction of more bioactive proteins in comparison to the d22N condition and the full native sequence condition. This suggests that the later modified proteins with TAG incorporations will also be produced in higher bioactive yields. While these results correlate with previous studies, this needs to be later confirmed in replicates for final consideration.¹³

Confirmation of Bioactivity with Variant Sites in L-Asparaginase

Variant Strains Produce Low Yield of Protein

After the expression and purification of the modified proteins, an SDS-Page Gel was run in order to gain preliminary insight on protein production.

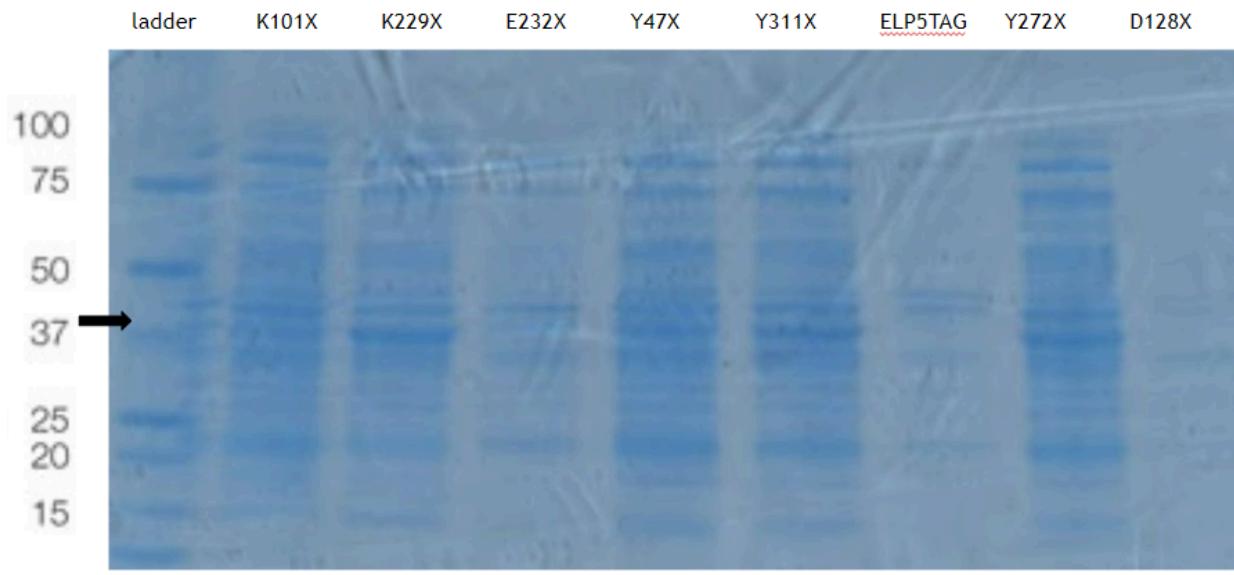


Figure 9. SDS Gel of Variants with pPaF Incorporated Demonstrate Low Protein Yield

The protein gel displays 5 μ L run of each of the tested variants. While a faint band can be seen in all of the lanes at the protein's expected weight, 36kDa, there are also many contaminant bands.

The presence of the expected band size (36kDa) demonstrates preliminary confirmation that the modified proteins were produced and that the pPaF was successfully incorporated as the lack of nsAA incorporation at these sites would signal for the degradation of the protein. However, the faintness of the bands as well as the presence of contaminant proteins suggest low yield of production. While stronger bands can be seen for the K229, Y47, Y311, and Y272 variants, the amount of protein is significantly decreased as opposed to the wild type's capacity for production (Figure 10). Additionally, more protein production could also impact the activity assay results as there is more protein to test the activity of.

Kit Positive Control Contains Extra Modifications than Native L-Asparaginase



Figure 10. SDS Gel of Proteins Suggest Modified Kit Positive Control

The protein gel displays 5 μ L run of each of the controls from left to right: the C321 with no pTetR.ansb plasmid as a negative control, WT(pelB) as a positive control, the kit positive control, and just assay buffer as a kit negative control. A strong band is demonstrated at the 36kDa for the WT and a strong band is demonstrated at the 65kDa range for the kit positive control.

While the gel demonstrates the expected condition of the controls with no distinct band for the negatives and a distinct band at the 36kDa for the positive, the band for the Biovision kit positive control appeared at a much higher band than expected at around 65kDa. This suggests that the Biovision positive control may have extraneous modifications to the protein, enlarging it. Thus, the pelB WT was selected as the control variant for downstream activity analysis as opposed to the kit control.

Bioactivity at 3 Sites is Conserved with pPaF Incorporation

After purification, the BioVision Asparaginase Activity Assay Kit was used to measure aspartate output over time was measured and compared to see which replaced sites conserved bioactivity.

BioActivity of Variants with pPaF Incorporation

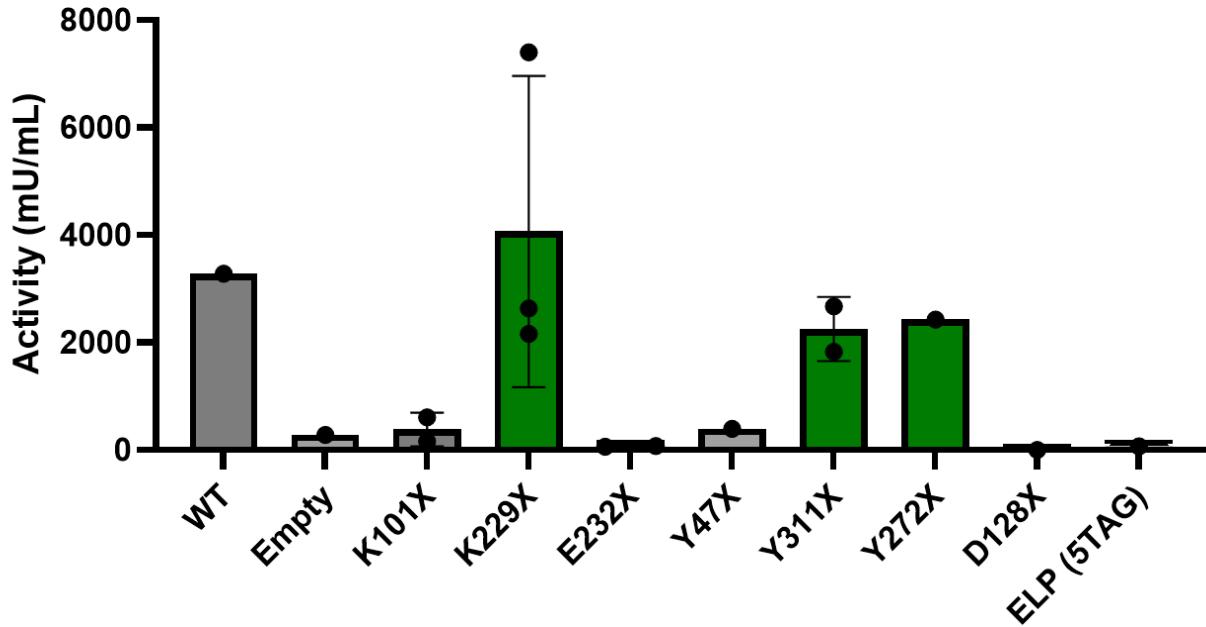


Figure 11. Activity Assay Demonstrates Bioactivity is Conserved at K229, Y311, and Y272
Analysis of plate reader data demonstrates the activities of the modified protein produced by each variant where mU/mL is defined as nmol of Aspartate converted per minute per original volume in mL. While most of the variants did not show much activity, K229, Y311, and Y272 showed comparable activity to the WT.

Preliminary results suggest that the sites K229, Y311, and Y272 are strong candidates for fatty acid conjugation as the conserved bioactivity demonstrates susceptibility to modification. These sites are additionally corroborated by previous studies conducted on lysate as opposed to purified samples, suggesting reliability of the results.¹² Furthermore, the conservation of bioactivity confirms the ability of DG.OTS to incorporate the linear alkyne pPaF successfully, suggesting further promiscuity than previously thought.

The next step includes using a Red Alexa Fluor F647-Azide and a CuAAC Biomolecule Reaction Buffer Kit (THPTA based) to click a fluorescence tag onto the sites with pPaF incorporation. This result will not only confirm successful creation of proteins containing the target nsAA of interest but also demonstrate proof of principle that an azide-based fatty acid can be conjugated and undergo copper-mediated click reactions with the pPaF alkyne structure as it is undergoing the same fusion mechanism.¹⁷

Additionally, these results also to a degree confirm the methodology used to select for the sites using protein database analysis due to finding multiple sites with conserved function. A comparison of the selection methodology can be further confirmed once all target variant strains are cloned. Even though the sites show more conserved function than the C-terminal TAG variant, at this point, the direct comparison between a terminal end modification and intraprotein swap can not be made due to the difference in TAG site quantities as more TAG sites probably require different growth conditions.

Future direction would involve a comparison with a 1 TAG ELP at the C-terminus and/or multiple sites of incorporation within the protein.¹⁰ Furthermore, PEG groups can be attached to these sites of incorporation and tested against the fatty acids in an immunogenicity test. Alternatively, the fatty acid conjugated proteins can be tested against each other in *in vivo* mouse trials to see to what extent fatty acids extend half-life. Ultimately, these sites can be utilized in a variety of ways but this research demonstrates the successful selection of intra protein sites susceptible of modification with minimal decrease in bioactivity. This research is the first step in many in the process to create the maximally efficient protein therapeutic for the most common childhood cancer.

Appendix

Figure 1: pLtetO Plasmid Templates

Plasmid	Concentration (ng/uL)
pLtetO.full.ansB_C-His	70.1
pLtetO.full.ansB_C-His (D40X)	56.3
pLtetO.full.ansB_C-His (K101X)	54.5
pLtetO.full.ansB_C-His (E232X)	58.7
pLtetO.full.ansB_ELP(10TAG)C-His	34.8

Figure 2: pelB Fragment

5'GCATCGTCTCATCGGTCTCATATGaaataacctattaccaacagcagcagctgggttattattgctcgctgcgcagccg
gccatggcgGATTACGATATCCAACGACCGAAAACCTGTATTTCAAGGGGCCGGTTCTT
GAGACCTGAGACGGCAT 3'

Component	Sequence (5' - 3')
T3a Prefix	GCATCGTCTCATCGGTCTCATATG
PelB Region	aaataacctattaccaacagcagcagctgggttattattgctcgctgcgcagccggcatggcg
TEV-linker	GATTACGATATCCAACGACCGAAAACCTGTATTTCAAGGGGCC
T3a Suffix	GGTTCTTGAGACCTGAGACGGCAT

Figure 3: Primers for ansB Amplification

Primer ID	F/R	Strain(s)	Sequence (5' -3')
oIG0311	FOR	pTetR.ansB(d22N)_C-His, pTetR.pelBlead_ansB(d22N) _ELP(10TAG)_C-His, pTetR.ansB(d22N)_C-His (D40X), (K101X), & (E232X)	GCATCGTCTCATCGGTCTCA TTCTttacccaatatcaccatttagc
oIG0312	REV	pTetR.ansB(d22N)_C-His, pTetR.ansB(d22N)_C-His (D40X), (K101X), & (E232X)	ATGCCGTCTCAGGTCTCAGG ATCCttaatgtatgtatgtatgatg
oIG0327	FOR	pTetR.ansB(d22N)_C-His	CATCGGTCTCATATGttacccaat atcaccattttagc
oIG0328	REV	pTetR.pelBlead_ansB(d22N) _ELP(10TAG)_C-His	GCATCGTCTCATCGGTCTC ATTCTttacccaatatcaccattttagc

Figure 4: Primers for Site Directed Mutagenesis

Primer ID	F/R	Strain(s)	Sequence (5' -3')
oIG0300	FOR	pTetR.pelBlead_ansB(d22N)_ C-His(Y47X)	aaatctaactagacagtggtaaagtggcgtag aa
oIG0301	REV	pTetR.pelBlead_ansB(d22N)_ C-His(Y47X)	ggttcggaggtcaccaccacc
oIG0302	FOR	pTetR.pelBlead_ansB(d22N)_ C-His(K44X)	cgcAACCTAGCTAACTACACAGTGGGT
oIG0303	REV	pTetR.pelBlead_ansB(d22N)_ C-His(K44X)	gagtcaccaccaccggcaatg
oIG0304	FOR	pTetR.pelBlead_ansB(d22N)_ C-His(K51X)	acagtgggttaggtggcgtagaaaatct

oIG0305	REV	pTetR.pelBlead_ansB(d22N)_C-His(K51X)	gtagtttagatttgttgcggagtccacca
oIG0306	FOR	pTetR.pelBlead_ansB(d22N)_C-His(D128X)	tgaaatgctagaaaccgggtgg
oIG0307	REV	pTetR.pelBlead_ansB(d22N)_C-His(D128X)	cgtcaggcgaggaaagtaagcagtt
oIG0308	FOR	pTetR.pelBlead_ansB(d22N)_C-His(D235X)	gaactgccgttaggtcggcattgt
oIG0309	REV	pTetR.pelBlead_ansB(d22N)_C-His(D235X)	attcagcttagagacatcgaatggcgt
oIG0317	FOR	pTetR.pelBlead_ansB(d22N)_C-His(K229X)	CGATGTCTCTtagCTGAATGAA C
oIG0318	REV	pTetR.pelBlead_ansB(d22N)_C-His(K229X)	AATGGCGTGTGCGCTG
oIG0321	FOR	pTetR.pelBlead_ansB(d22N)_C-His(Y272X)	CGGCAACCTGtagAAATCTGT GTTCGACACG
oIG0322	REV	pTetR.pelBlead_ansB(d22N)_C-His(Y272X)	TTACCCACACCAGCG
oIG0325	FOR	pTetR.pelBlead_ansB(d22N)_C-His(Y348X)	CTTCAATCAGCAtagTCATCAT CATCATCATTGATAAAAGC
oIG0326	REV	pTetR.pelBlead_ansB(d22N)_C-His(Y348X)	ATCTGCTGGATCTGCTG

Figure 5: Strains Used in Experiments

Experiment	Strain	Antibiotic
Purification Analysis, PelB Analysis	C321 [pLtetO.full_ansB_CHis]	Kan
PelB Analysis	C321 [ptetR_ansB(d22N)_C-His]	Cb
Variant activity assay, PelB Analysis	C321 [ptetR_pelB_ansB_C-His]	Cb
Variant activity assay, pelB Analysis	C321 [EMPTY]	NO ANTIBIOTIC
Variant activity assay	C321 [DG.OTS + pTetR.pelBlead.ansB(d22N)CHis (K101X)]	Cb+Cm
Variant activity assay	C321 [DG.OTS + pTetR.pelBlead.ansB(d22N)CHis (K229X)]	Cb+Cm
Variant activity assay	C321 [DG.OTS + pTetR.pelBlead.ansB(d22N)CHis (E232X)]	Cb+Cm
Variant activity assay	C321 [DG.OTS + pTetR.pelBlead.ansB(d22N)CHis (Y47X)]	Cb+Cm
Variant activity assay	C321 [DG.OTS + pTetR.pelBlead.ansB(d22N)CHis (Y311X)]	Cb+Cm
Variant activity assay	C321 [DG.OTS + pTetR.pelBlead.ansB(d22N)CHis (Y272X)]	Cb+Cm
Variant activity assay	C321 [DG.OTS + pTetR.pelBlead.ansB(d22N)CHis (D128X)]	Cb+Cm
Variant activity assay	C321 [DG.OTS + pTetR.pelBlead.ansB(d22N)CHis (5STAGELP)]	Cb+Cm
Transformation	C321 [DG.OTS]	Cm

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