Temasek POLYTECHNIC

Major Project Written Report

CHE1617- 135

Development of a biobetter for Gaucher Disease

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Submission Date	:	14 Oct 2016

Diploma in Chemical Engineering

AY 2016/2017

Temasek Polytechnic

School of Applied Science

Diploma in Chemical Engineering

AY 2016/2017

Subject: Major Project (Level 3)

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Name	Admission Number	Signature	Date
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Acknowledgement

This internship opportunity from Bioprocessing Technology Institute, A*STAR was a great one. It has allowed me to research on the development of a biobetter for gaucher disease. At the same time, this was also an eye opening experience for me to understand how a research institute operates and carries out their various experiments. I am honoured and thankful to be able to work with researchers and meet inspiring people in this company.

First and foremost, I would like to thank Dr Song Zhi Wei for allowing me to do my internship in his lab.

Secondly, I would like to express my deepest gratitude to my supervisor, Dr Tan Yun Lei. Despite her busy schedules and my limited knowledge in this field, she took time to listen and patiently guided me onto the correct path. This project would not be possible without her.

Furthermore, I would also like to extend my thanks to Dr Lin Pao Chun, who advised me in the absence of my supervisor.

Lastly, I want to thank all my other colleagues, Mr. Chan Kah Fai, Mr. Ryan Haryadi, Mr. Wahyu Shahreel, Mr Rizal Ismail, Dr Natasha Ann Pereira, Ms. Helen Pu for creating an enjoyable working experience and willingly helped me out with their abilities.

This opportunity will be a stepping stone for my future career. I will continue to use the knowledge that I have learnt here and improve it. I hope to work everyone again in future.

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Abstract

Gaucher disease is a lysosomal storage disorder and enzyme replacement therapy (ERT) is an established treatment for Gaucher disease patients. The recombinant enzyme is intravenously administered and delivered to the lysosomes of macrophage cells through cell-surface mannose receptors. Imiglucerase is the only protein drug expressed in wild-type Chinese Hamster Ovary (CHO) cells, which requires exo-glycosidase treatment to expose core mannose glycan residues that can be recognised by the macrophages. Our CHO-gmt4D cell line allows the production of mannose-terminated N-glycosylated proteins directly from CHO cell culture. The aim of this study was to develop CHO-gmt4D cells that express high yields of glucocerebrosidase with mannose-terminated N-glycans.

We have tested two recombinant plasmids that expressed the glucocerebrosidase enzyme with either a full-length signal peptide or a truncated signal peptide in the CHO-gmt4D cell line. We have also tested different plasmid conformation during transfection. This CHO-gmt4D cell line also has a defective dihydrofolate reductase (DHFR) gene, allowing for gene selection and amplification. Methotrexate (MTX) was used to amplify glucocerebrosidase production in pools, as quantified by an enzyme activity assay and by Western blot analysis.

Results have shown that higher concentration of recombinant enzyme was produced in the cell line harbouring the plasmid encoding for the full-length signal peptide compared to the one encoding for shorter signal peptide. In addition, amplification also had showed promising results, all the adherent cell pools had increase of protein expression levels.

It can be concluded that using the DHFR system can greatly increase glucocerebrosidase expression levels through amplification, and that linearizing the plasmid, modifying the native signal peptide does not affect the protein expression level greatly in CHO-gmt4D cell. Future studies should be conducted on overcoming the chromatin positional effects as well as to test on different non-native signal peptides combination to improve protein expression levels.

1. Introduction

1.1 Background Information

Gaucher Disease is the most common lysosomal storage disorder (LSD) in the world that happens to 1 in 57000 people. [1, 2] There are different approaches in treating this disease and enzyme replacement therapy (ERT) is one of them. [1] Patients suffering from Gaucher Disease inherit a mutated form of the enzyme glucocerebrosidase. Therefore, they require frequent infusions of the recombinant wild type enzyme to maintain normal lysosomal function, restoring patients' health. Recombinant glucocerebrosidase is taken up through mannose receptors on affected macrophage cells. As such, mannose-terminated glycans on recombinant glucocerebrosidase is necessary for efficient uptake into macrophages. [2] In the current market, imiglucerase or Cerezyme® is the only protein drug that is expressed in wild type Chinese Hamster Ovary (CHO) cells, which requires exo-glucosidase treatment to expose core mannose. The other biosimilars are expressed in carrot cells (taliglucerase alfa) and human cells (Velaglucerase alfa). [4] The problem now is that they can cost up to US$642,900 a year to treat one patient. [5]

1.2 Objective & Scope

There have been no studies conducted on glucocerebrosidase that is expressed in Chinese Hamster Ovary (CHO) cells that have a modified glycosylation process. Hence, the aim of this report was to use the DHFR system, modification of the native signal peptide and plasmid conformation, to obtain cells that have very high levels of glucocerebrosidase expression to fulfil the market needs in CHO cells that have a modified glycosylation process. This allows the production of predominantly mannose-terminated glycans in a single step. Thereby producing a biobetter that has greater efficacy and functionality over the existing drugs, while reducing cost to patients.

2. Literature Review

2.1 Gaucher's Disease

Lysosomal Storage Diseases(LSDs) are caused by "specific mutations in genes encoding lysosomal enzymes", which in turn prevent the degradation of their specific substrates, which include a wide variety of glycolipids, oligosaccharides and glycoproteins. There are more than 50 distinct lysosomal storage diseases, with most being inheritable autosomal recessive diseases. [2]

Gaucher Disease being one of the LSDs that is caused by the mutation of the glucosylceramidase beta(GBA) gene, is characterised by the accumulation of the glycolipid substrate glucosylceramide in lysosomes of macrophage cells. [3,6] There are three types of Gaucher Disease, with type I being the most common in patients. Some of the symptoms include enlarged liver and/or spleen, low blood platelet count, anaemia and low white blood cell count. Patients with type II and III Gaucher Disease also suffer extensive neurological symptoms, and often do not survive past early adulthood. At present, only type I Gaucher Disease can be treated by Enzyme Replacement Therapy. [6].

2.2 Enzyme Replacement Therapy

Enzyme Replacement Therapy (ERT) is the replacement of a mutated lysosomal enzyme by a recombinant wild type enzyme that will be taken into macrophages intravenously. Glucocerebrosidase is taken in through mannose receptors on macrophage cells. As such, the glycans on recombinant glucocerebrosidase have to be mannose-terminated for efficient uptake. [2]

The first drug approved by the United States Food and Drug Administration (FDA) for Gaucher Disease was Cerezyme® in 1994 [7]. Cerezyme® is expressed in wild type CHO cells, producing a heterogeneous population of glycans on the recombinant enzyme. A second step is required to modify the glycans on glucocerebrosidase to predominantly mannose-terminated forms. [1] In 2009, there was a virus contamination in the Cerezyme® manufacturing plant and it greatly affected the overall drug supply for Gaucher's Disease. [8] This incident catalysed the FDA approval of biosimilars velaglucerase alfa in 2010 [9] and taliglucerase alfa in 2012. [10] Since Gaucher's Disease is a rare disease, the treatment can cost up to US$642,900 annually. [5]

2.3 Chinese Hamster Ovary Cells

Chinese Hamster Ovary (CHO) cells are excellent for the expression of proteins and they are most commonly used for the mass production of recombinant protein products due to their capacity for single cell suspension culture. They can be genetically modified as well, for example, the knockout of genes can be performed with relative ease. [10] Furthermore, up to 70% of recombinant protein therapeutics are produced using CHO cells. One of the compelling reason is they have similar properties and post-translational modifications compared to that of native human proteins, which greatly decreases the likelihood of rejection when administered to patients. Thus, CHO cell lines are important in bioprocessing research and the manufacturing of therapeutic biopharmaceuticals by increasing protein productivity and reducing safety concerns. [11]

"Glycosylation, the process of attaching sugar moieties to proteins, is a post-translational modification that provides great proteomic diversity. Glycosylation is important for many biological processes which includes cell attachment to the extracellular matrix and protein-ligand interactions in the cell endogenously." [12] Glycosylation of recombinant protein drugs may also enhance drug efficacy and longevity. [11] In particular, modification of glycans on recombinant glucocerebrosidase is vital for its uptake into macrophages through the mannose receptors. CHO-gmt4, developed by the Song lab, is a mutant that has lost the gene N-acetylglucosaminyltransferase I (GnT1). This cell line was selected using Ricinus communis agglutinin-1 (RCA1) treatment. This mutant produces protein with mannose 5-terminated N-glycans, allowing us to bypass the second enzymatic deglycosylation step required in the production of Cerezyme. CHO-gmt4D is a mutant that has lost GnT1 and the dihydrofolate reductase (DHFR) gene. DHFR was knocked out by the Song lab using a zinc finger nuclease, allowing us to select for and amplify the expression of glucocerebrosidase. [13] By using the DHFR system, DHFR-deficient cells are cultured in high level methotrexate (MTX) as MTX inhibits the DHFR activity in cells. The cells have to amplify the copy number of DHFR locus to survive, and during the process the

glucocerebrosidase gene is also co-amplified increasing the chances of having highly producing strain. [11]

2.4 Signal Peptide

Signal peptides are chains of 15 to 30 amino acid residues that are responsible for translocation of the protein into the endoplasmic reticulum through the interaction of signal recognition particle (SRP), SRP receptor and the translocon. [14,15,16] The endogenous signal peptide plays a role in regulating the secretion level of the protein in the cell, thus, modifying the signal peptide of the protein can also improve the level of expression of the protein. [16,17] However, some signal peptides only work on specific protein while others are able to promote protein secretion on a variety of proteins. [14] In addition, using native signal peptide is a logical choice apparently but truncation of the peptide can also be considered. [14]

2.5 Linearizing plasmid

Most of the time, circular plasmids are used in transfection of DNA into mammalian cells. However, some studies have found that linearizing the plasmid before transfection can increase both the efficiency of stable clone and transgene expression. [18,19] Linearizing of the plasmid using a random restriction cut can cause the gene of resistance gene or the gene of interest to be destroyed. Whereas, using a particular restriction enzyme to cut at non-coding area can retain all important gene elements in the plasmid. [18]

2.6 Chromatin Positional Effect

Even with all the ways to increase protein expression levels and obtaining stable clones mentioned above, during transfection the plasmid DNA integrates in a random manner into the cell's genome. The site of integration can impact the transcriptional activity of the gene and this is known as chromatin positional effects. Thus expression levels can vary between individual clones. "Screening of large number of clones will be needed to find efficient and stable clones." [20]

3. Materials and Methods

3.1 Cell Culture

The CHO-gmt4D glycosylation mutant cells used in this project was provided by Dr Tan Yun Lei. Adherent CHO-gmt4D cells were cultured in Dulbecco's modified eagle media (DMEM) + Glutamax™ (Life Technologies) with 10% fetal bovine serum (FBS) and Hypoxanthine-Thymidine (HT) supplement (Life Technologies). Adherent cells were grown at 37°C in a humidified incubator with 5% CO_2. Suspension CHO-gmt4D cells were cultured in a 1:1 mixture of HyClone™ (GE, USA) and CD-CHO (Life Technologies) media supplemented with HT, 6 mM L-glutamine and 0.05% pluronic acid. Cells were grown at 37°C and 115 rpm in a humidified incubator with 8% CO_2.

3.2 Polymerase chain reaction of wild type glucocerebrosidase gene

The truncated glucocerebrosidase gene was provided by Dr Tan Yun Lei. The gene amplicon was obtained using polymerase chain reaction (PCR) with Pfx DNA polymerase (Invitrogen, USA).

Forward primer with Kozak sequence (Highlighted):

5′ GCCGCTAGCGCCACCATGGCTGGCAG 3′

Reverse primer:

5′ CGAGCGGCCGCTCACTGGCGACGCCACAG 3′

Followed by the purification of the gene amplicon using GeneJET PCR Purification Kit (Thermo Scientific, USA)

3.3 Polymerase Incomplete Primer Extension (PIPE) Cloning

3.3.1 Obtaining recombinant plasmid with truncated-signal peptide Glucocerebrosidase gene

Primers were designed according to the selected sequence in the pCID vector (provided by Dr Tan Yun Lei) and truncated-signal peptide glucocerebrosidase gene.

Forward Primer for pCID vector:

5′ CCTACCTGTGGCGTCGCCAGTGATGGCGGCCGCTCGAGTCTAGAC 3′

Reverse Primer for pCID vector:

5′GAGGCTGCCAGCCATGGTGGCCTGTGCTGGATATCTGCAGAATTCCACC 3′

Forward Primer for truncated signal peptide glucocerebrosidase gene:

5′ GGTGGAATTCTGCAGATATCCAGCACAGGCCACCATGGCTGGCAGCC 3′

Reverse Primer for truncated signal peptide glucocerebrosidase gene:

5′ GTCTAGACTCGAGCGGCCGCCATCACTGGCGACGCCACAGGTAGG 3′

DNA templates went through PCR using Pfu Turbo DNA polymerase (Agilent,USA). Primers were used with its respective DNA templates in the PCR reaction, with the following components and cycling information:

Components	**Volume**
DNA Template	Able to give 100 ng of DNA
Primer- Forward (10μM)	1.5 μL
Primer- Reverse (10μM)	1.5 μL
dNTPs (10μM)	1 μL
10x Buffer	1.5 μL
Pfu Turbo	1 μL
Water to	50 μL

Cycle information

No. of cycles for all reactions: 34

For vector pCID:

Denaturation:	95°C	for 30 seconds
Annealing:	55°C	for 1 min
Extension:	72°C	for 8 min

For truncated signal peptide glucocerebrosidase gene:

Denaturation:	95°C	for 30 seconds
Annealing:	53.5°C	for 1 min
Extension:	72°C	for 8 min

Subsequently, the PCR products were analysed by running 1% agarose gel electrophoresis at 110V for 30 min. PCR products were digested with Dpn1 (Agilent Technologies, USA) at 37°C for 2 h. 5 μL of each digested pCID vector and truncated-glucocerebrosidase gene sequence was mixed and incubated at 28°C for 2 h to get the recombinant plasmid. The recombinant plasmid was transformed using One Shot TOP10™ bacterial cells (Invitrogen, USA). The cells were then cultured overnight at 37°C on agar plates that consist of ampicillin (100 μg/mL). Positive colonies were inoculated into 1.5 mL of LB Broth with ampicillin (100 μg/mL) and incubated overnight at 37°C. The recombinant plasmid was extracted using QIAprep® Spin Miniprep Kit (Qiagen,USA).

3.3.2 Obtaining recombinant plasmid with full-signal peptide

Glucocerebrosidase gene

Primer was designed according to the sequence in the recombinant plasmid with truncated-signal peptide glucocerebrosidase gene.

Forward Primer for full-signal peptide glucocerebrosidase gene:

5′GATGCTTACCCTACTCAAAGGCTTGGGACATTCCTCTCTGGAAGGACTTGAAAAC TCCATGGTGGCCTGTGCTGGATATCTGCAG 3′

Reverse Primer for full-signal peptide glucocerebrosidase gene:

5′ATGGAGTTTTCAAGTCCTTCCAGAGAGGAATGTCCCAAGCCTTTGAGTAGGGTAA GCATC ATGGCTGGCAGCCTCACAGGATTGC 3′

Primers mentioned above were used in the same PCR reaction as the others. The cycling information was adapted from the truncated glucocerebrosidase gene, except that the extension time had to be changed to 9 min.

The recombinant plasmid was digested with Dpn1 (Agilent Technologies, USA) at 37 °C for 2 h. The recombinant plasmid was transformed using One Shot TOP10™ bacterial cells (Invitrogen, USA). The cells were then cultured overnight at 37°C on agar plates that consist of ampicillin (100 μg/mL). Positive colonies were inoculated into 1.5 mL of LB Broth with ampicillin (100 μg/mL) and incubated overnight at 37°C. The recombinant plasmid was extracted using QIAprep® Spin Miniprep Kit (Qiagen,USA).

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3.4 Linearizing Plasmid

Recombinant plasmids were linearized by digesting with ASCI restriction enzyme. 30 μg of DNA was added into a 1.5 mL microtube. 15 μL of ASCI restriction enzyme and 11.25 μL of CutSmart buffer were added (New England Biolabs,USA)into the same 1.5 mL microtube. 11.25 μL of water was topped up into the microtube. The mixture was incubated at 37°C for 2 h. The recombinant plasmids were analysed by running 1% agarose gel electrophoresis at 110v for 30 min. The agarose gel was viewed under ultraviolet light and the band size between 5000bp and 4000bp by referring to 1kb DNA ladder (1st BASE, Singapore) was excised out and put into a 1.5 mL microtube. 11.3 μL of 3M sodium acetate (sterile) was added into the microtube. 310 μL of ice cold 100% ethanol (sterile) was added into the microtube. The microtube was incubated at -20°C for 30 min and subsequently spun at 14800 rpm, 4°C for 10 min. Supernatant was removed by not disturbing the pellet. The pellet was washed with 500 μL of 70% ethanol. The tube was again spun at 14800 rpm at 4°C for 5 min. Supernatant was removed by not disturbing the pellet and left to air dry. The pellet was resuspended by adding double-distilled water.

3.5 DNA sequencing

10μL of 10-40(ng/μL) purified plasmids and 5 μL of 10-20μM primers were sent to 1st Base for DNA sequencing. Results received from 1st Base were analysed using the BioEdit software [21] and aligned using MultAlin [22].

CMV-F Primer can be found on 1st Base website:

5′CGCAAATGGGCGGTAGGCGTG 3′

Internal Primer:

5′GTTACAGTTCTGGGCAGTGAC 3′

End of gene Primer:

5′CTCACCTGGCTACTCCATTCAC 3′

End of IRES Primer:

5′CGATGATAAGCTTGCCACAACC 3′

End of XhoI Primer:

5′ CGAAAAGTGCCACCTGACGTC 3′

3.6 Transfection

CHO-gmt4D cells were counted using Vi-CELL™XR (Beckman Coulter, USA). The volume for 10 million CHO cells was aliquoted and transferred to a 50 mL tube. The tube was centrifuged at 1050 rpm for 5min. The supernatant was aspirated. The cell pellet was washed with 30 mL of Dulbecco's phosphate-buffered saline (Life Technologies), centrifuged at 1050 rpm for 5 min and the supernatant was aspirated (For adherent cells only). The cell pellet was resuspended in 4D Nucleofector™ Solution (Lonza, USA) at room temperature. 5μg of recombinant plasmid encoding glucocerebrosidase and DHFR was added to the solution. Subsequently, the solution was transferred into the Nucleocuvette™ Vessels(Lonza,USA). The vessel was placed into the retainer of 4D-Nucleofector™ Core unit (Lonza, USA) run with the selected CHO-K1 protocol on the machine. The cuvette was left at room temperature for 10 min. Adherent cells were transferred into T75 flask in 10 mL of normal culture medium and cultured at the same condition (stated in section 3.1). Suspension cells were seeded into a 6 –well plate with 2ml of normal culture medium. Suspension cells were cultured in a humidified incubator at 37°C and 5% CO_2 for 2-3 days.

3.7 Selection and Amplification

Transfected CHO-gmt4D cells were cultured in no HT supplement culture medium until it recovered in 20 mL of the medium in T150 flask for adherent cells and 12 mL of the medium in T25 flask for suspension cells. After the cells recovered from no HT supplement culture medium, multiple rounds of amplification were done using methotrexate (MTX). Concentration of MTX in no HT supplement culture medium was increased after each recovery phase of the cells in this order 50, 200, 500 μM. The cells were cultured in 20 mL and 12 mL of culture medium with MTX in the same condition for both adherent and suspension cells respectively. Cells were banked down in 1mL of 10% DMSO in no HT culture medium and kept in -80°C after each round of amplification.

3.8 Qualitative analysis of glucocerebrosidase using western blot

Supernatant of cell culture was mixed with Pierce™ Lane marking reducing sample buffer 5x (Thermo Scientific, USA) and incubated at 95°C for 5 min. After incubation, the supernatant was loaded into 4-12% Bis-Tris gel (Life Technologies). Gel Electrophoresis was performed at 30V for 10 min and subsequently 150V for 1 h in a gel electrophoresis tank filled with 1x NuPAGE® MES SDS running buffer. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using iBlot™ 2 (Invitrogen, USA) at 20V for 7 min. Blocking of the membrane was done using 5% non-fat milk in phosphate-buffered saline with 0.1% tween(PBST) for 1 h. The membrane was incubated with the primary rabbit polyclonal anti-glucocerebrosidase antibody (Sigma-Aldrich, G4171) at a dilution of 1:1000 in PBST with 1% milk for 2 h at room temperature. The membrane was washed 3 times, each time 5 min with PBST with 1% milk. Subsequently, the membrane was incubated with secondary antibody anti-rabbit IgG-HRP (Jackson, 111-036-003) in PBST with 1% milk for 2 h. Once again, the membrane was washed 3 times in PBST with 1% milk, 5 min each. The membrane was incubated with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo scientific,USA) for 2-3 min. Chemiluminescence produced was detected using ChemiDoc™ Touch Gel and Western Blot Imaging System (BIO-RAD, USA).

3.9 Quantitation of glucocerebrosidase

10 mg of 4-Methylumbelliferyl β-D-glucopyranoside (MUG) (Sigma, USA) was weighed and dissolved in 100 μL of dimethyl sulfoxide (DMSO). Dissolved MUG was mixed with 2.5 mL of citric buffer and 2.5 mL of deionised water, this was referred to as 2x assay buffer. 50 μL of each sample was added into 3-4 wells in 96-well. 1-2 wells were added with 100μL of high pH glycine buffer and 50 μL of 2x assay buffer, these samples were known at t=0 samples. The remaining 2 wells were added with 50 μL of 2x assay buffer, these samples were known as t=60 samples. The 96-well plate was incubated for 1 h at 37°C. 100 μL of 0.5 M high pH glycine buffer was quenched into the remaining wells. The fluorescence of the liberated 4-methylumbelliferone was measured (excitation and emission 445 nm) using Infinite® M200 PRO (Tecan, Switzerland). Readings taken at t=0 were subtracted from t=60 samples. The subtracted readings were compared with a standard curve using standards diluted between 1-100 μM of 4-methylumbelliferone sodium salt (Sigma, USA).

4. Results

4.1 Gel electrophoresis of pCID vector and glucocerebrosidase gene after PCR

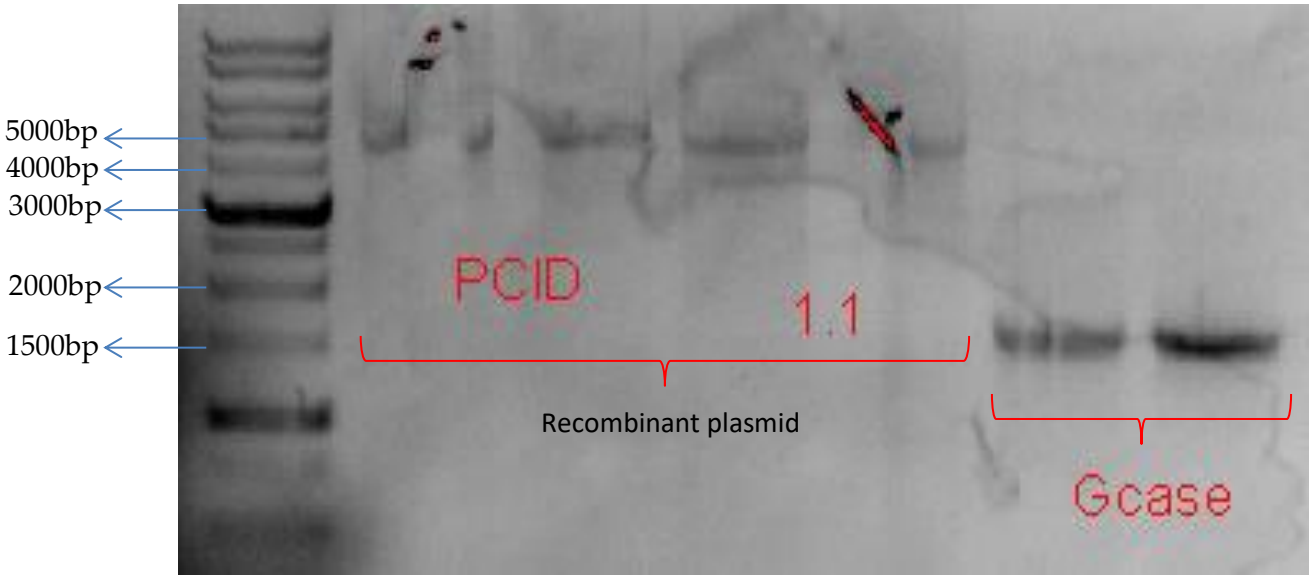

Fig 4.1 Shows the bands of recombinant plasmids and glucocerebrosidase gene (Gcase) with a 1kb DNA ladder

In fig 4.1, both pCID and 1.1 were the same vectors that went through PCR using the PIPE cloning method. The bands indicated the correct DNA band size for the recombinant plasmid of approximately 4500 bp and the Gcase gene of approximately 1500 bp.

4.2 DNA sequencing of recombinant plasmid

4.2.1 Truncated signal peptide

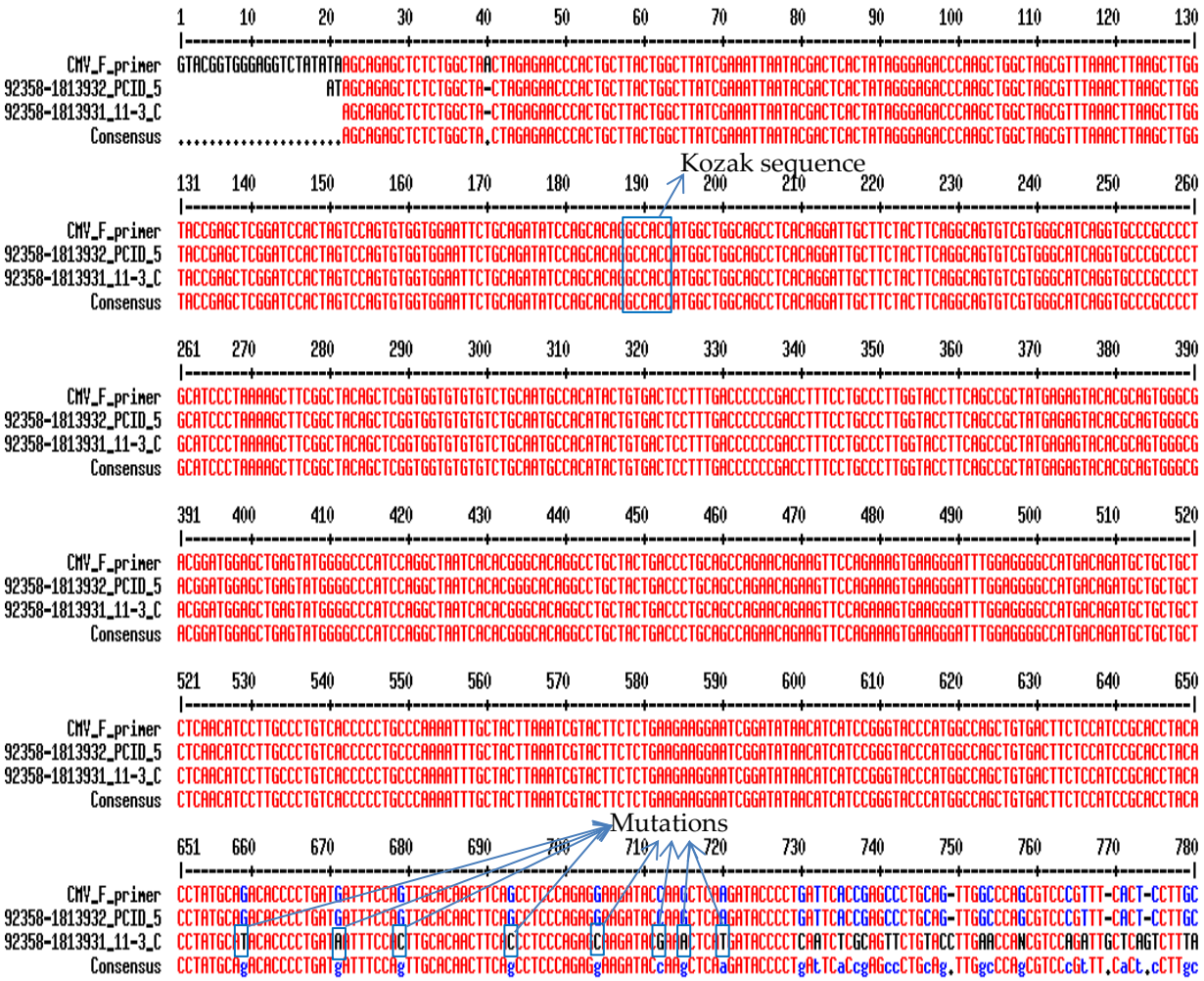

Fig. 4.2.1 Comparison of sequence of 2 identical clones PCID5 and 11-3 with the original sequence using CMV-F primer

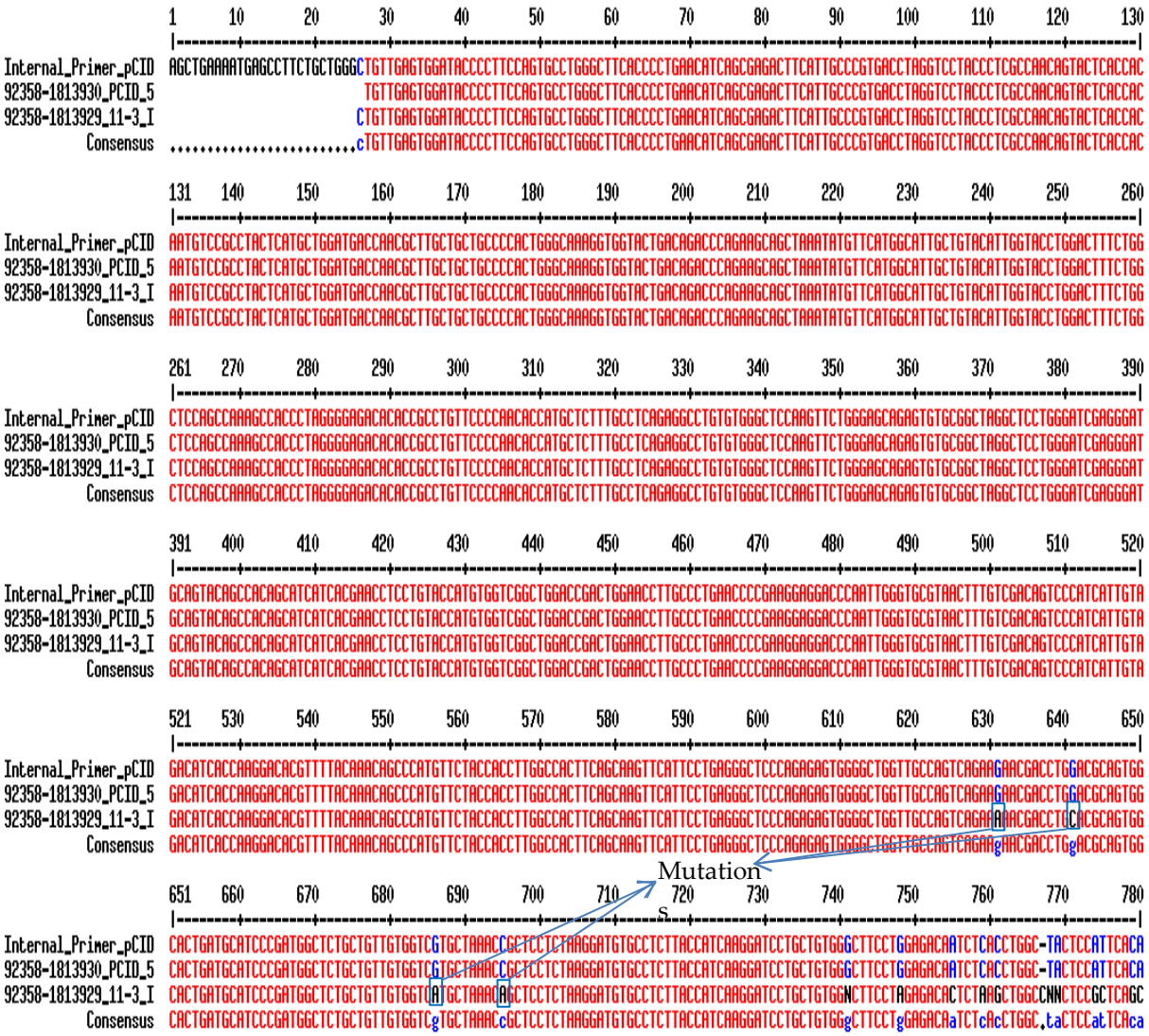

Fig 4.2.2 Comparison of sequence of 2 identical clones PCID5 and 11-3 with the original sequence using internal primer

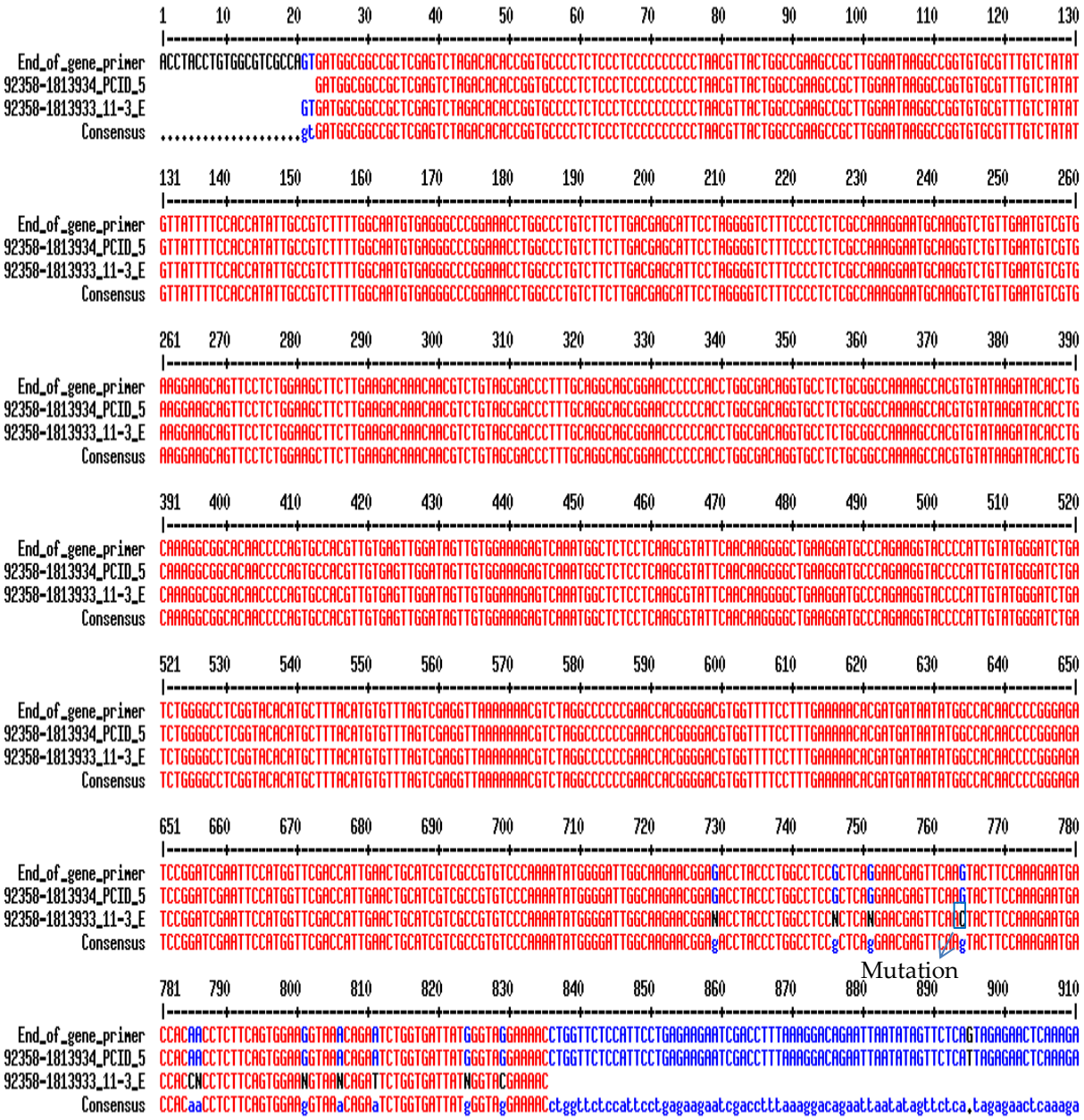

Fig 4.2.3 Comparison of sequence of 2 identical clones PCID5 and 11-3 with the original sequence using end of gene primer

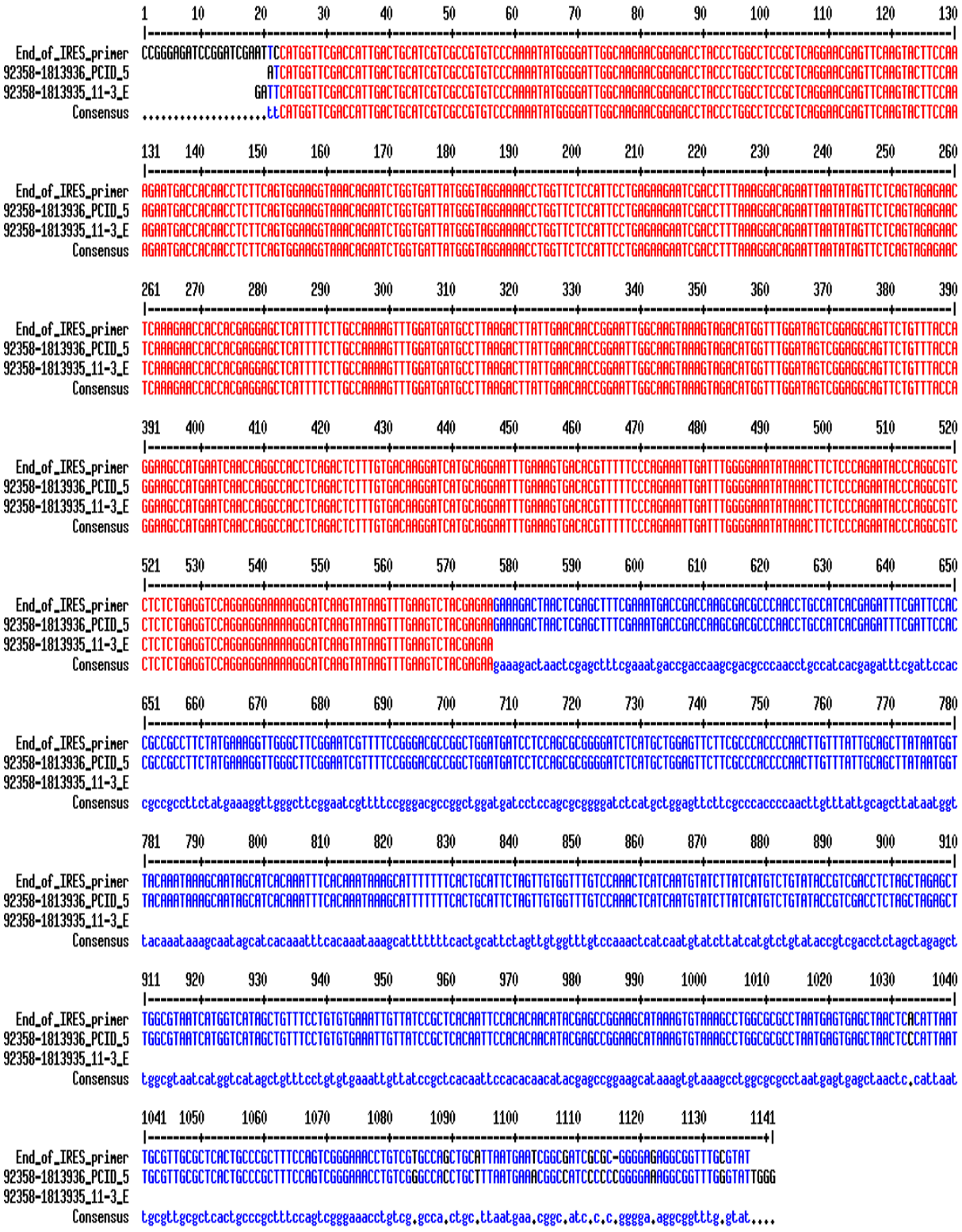

Fig 4.2.4 Comparison of sequence of 2 identical clones PCID5 and 11-3 with the original sequence using end of IRES primer

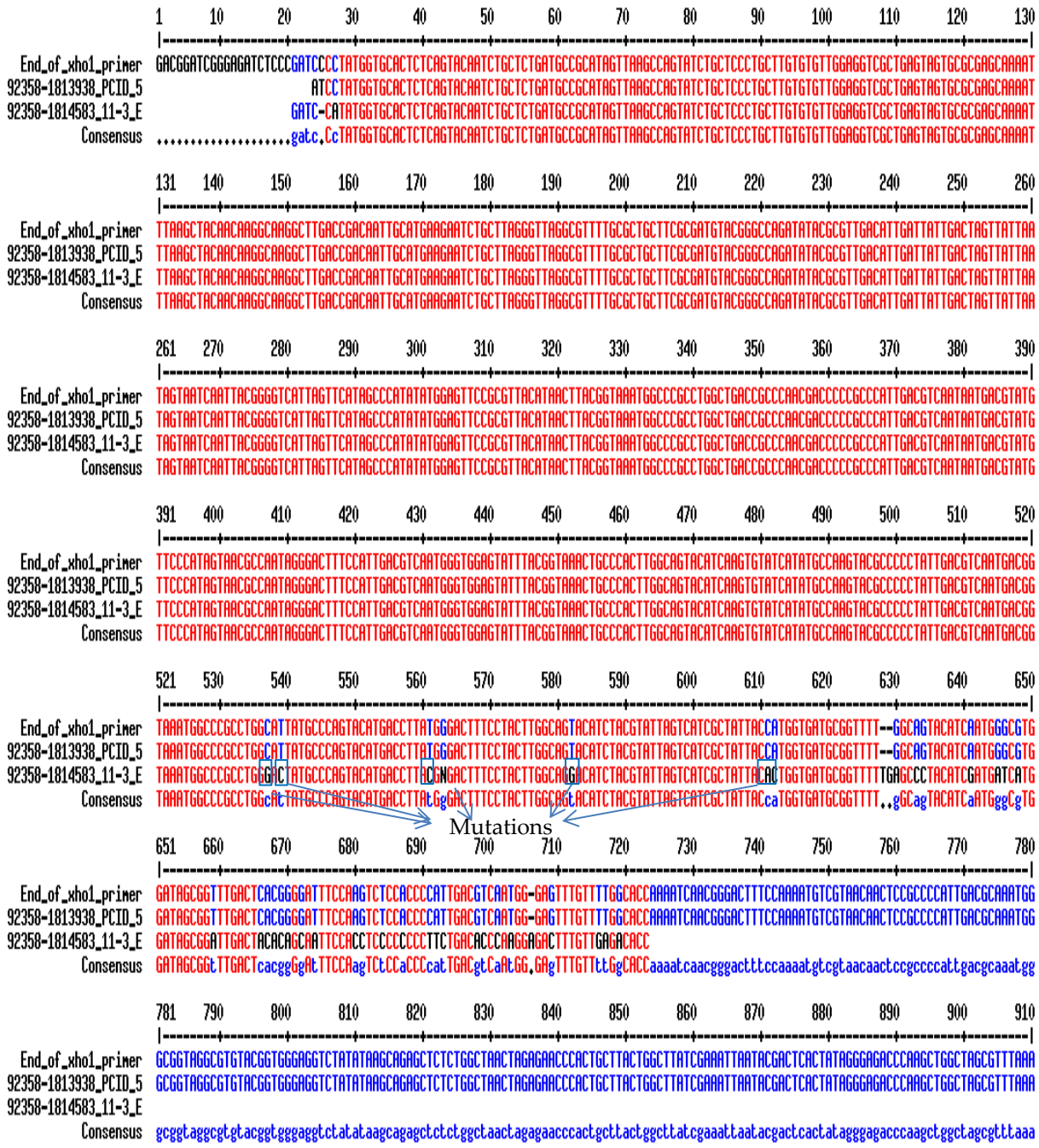

Fig 4.2.5 Comparison of sequence of 2 identical clones PCID5 and 11-3 with the original sequence using end of XhoI primer

The DNA sequence shown in figures 4.2 determined that the clone PCID5 had its sequenced aligned to the original sequence compared to the clone 11-3 that had mutations in it. This also shows PIPE was successful for clone PCID5.

4.2.2 Full signal peptide

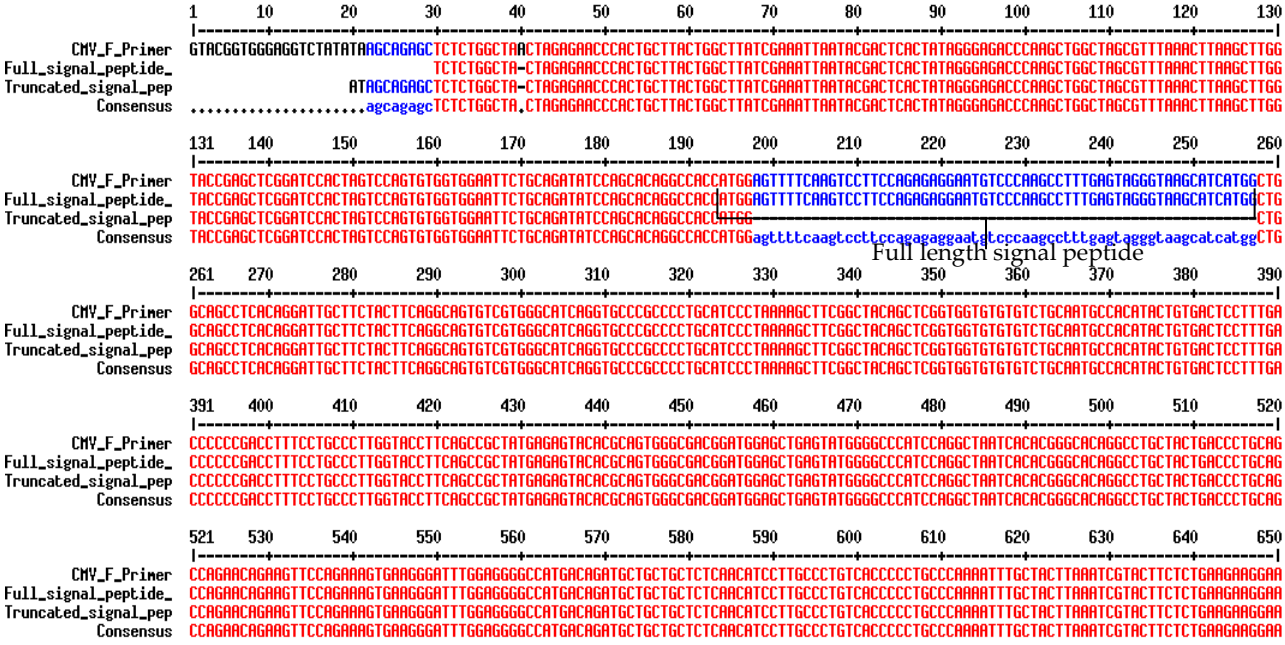

Fig 4.2.2 Comparison of sequence of 2 different clones full signal peptide and truncated signal peptide with original sequence using CMV-F primer

In fig 4.3, the DNA sequence showed that the full signal peptide had been fully incorporated into the plasmid using PIPE.

4.3 Linearization of plasmid

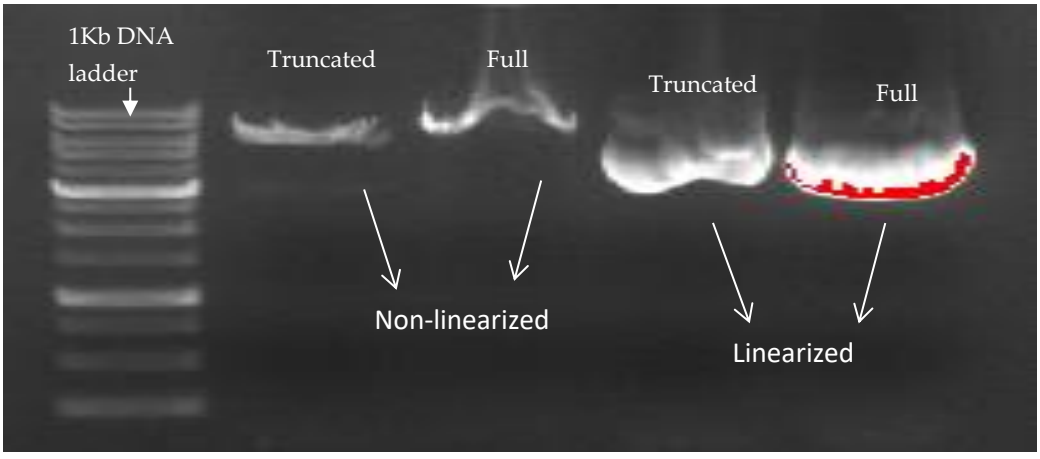

Fig 4.3 Bands of non-linearized and linearized plasmid

In fig 4.4, the bands showed that the plasmid for both truncated and full signal peptides had been linearized using ASCI restriction enzyme.

4.4 Gene amplification with methotrexate (MTX)

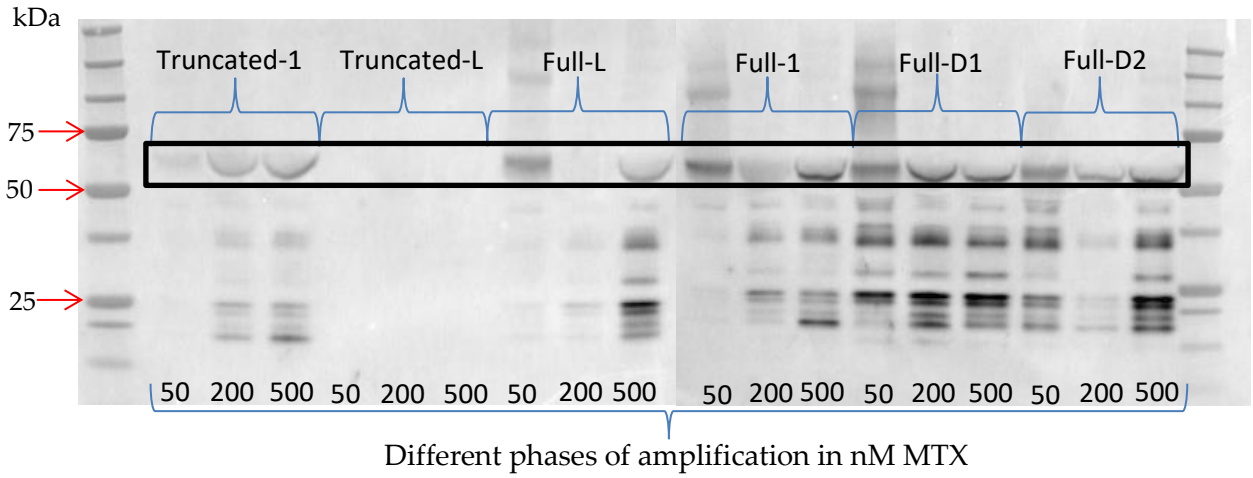

Fig 4.4.1 Glucocerebrosidase produced in CHO-gmt4D adherent cells

In fig 4.4.1, the blot quantified glucocerebrosidase in their various amplification stages were indicated in the black box. 5 out of 6 pools produced glucocerebrosidase except for the Truncated-L pool that did not produce protein as seen by the lack of bands in all amplification stages. Truncated-L and Full-L are pools that had their plasmid linearized before transfection. Other bands may possibly indicate the degradation of the glucocerebrosidase protein.

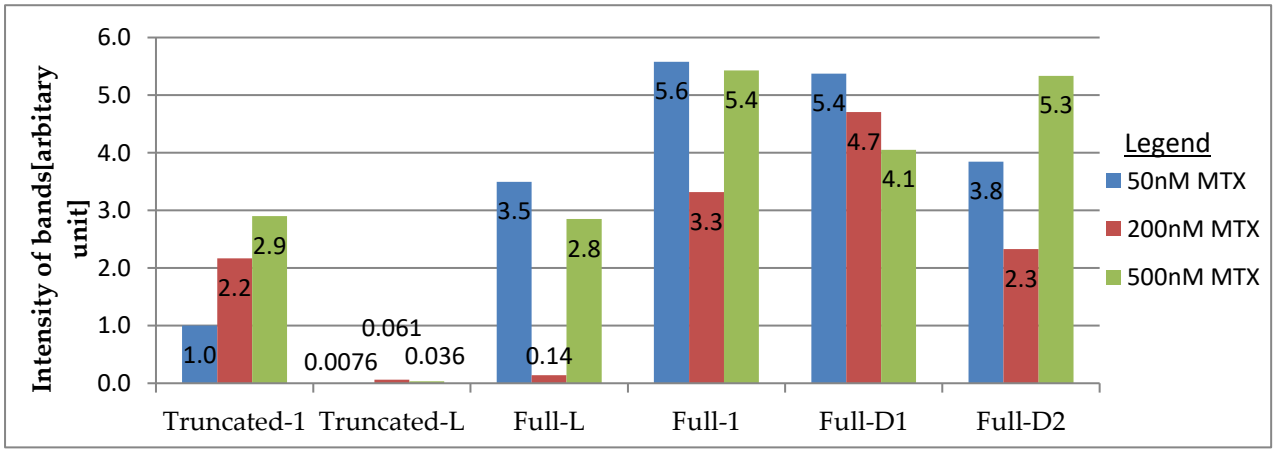

Fig 4.4.2 Graph of intensity of glucocerebrosidase bands

In fig 4.4.2, the graph showed the intensity of bands based on the blot (refer to fig 4.4.1), with Full-1 showing the highest reading at 50 nM and 500 nM MTX amplification when compared to other pools. At 200 nM MTX amplification, Full- D1 had the highest reading.

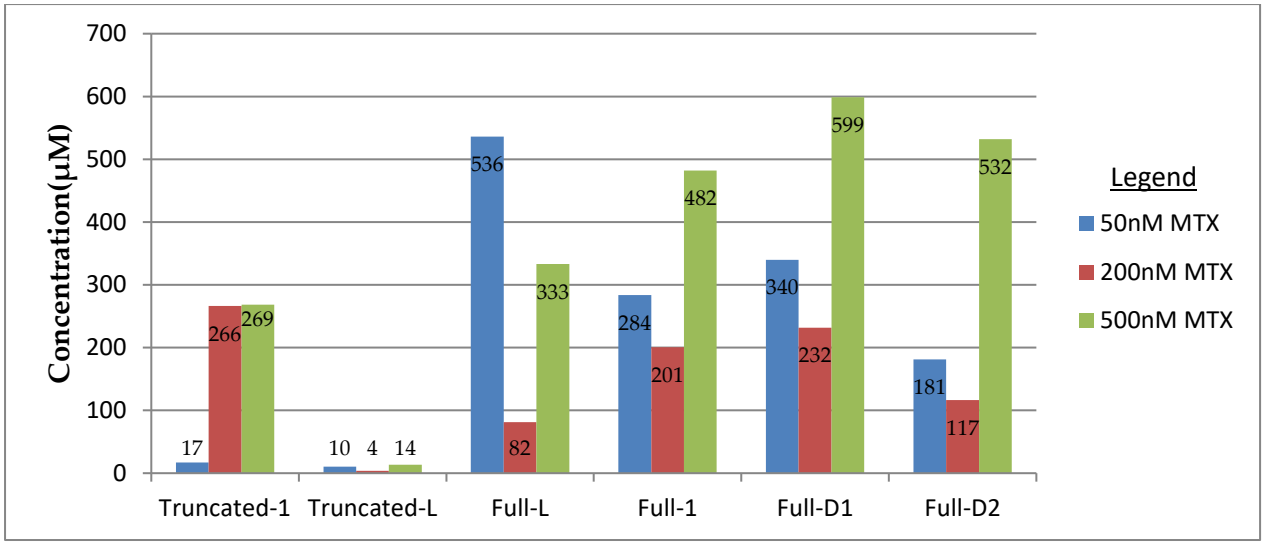

Fig 4.4.3 Graph of activity of glucocerebrosidase enzyme of adherent cells

In fig 4.4.3, the graph showed that Full-D1 had the highest glucocerebrosidase enzyme activity among the rest of the pools at 500 nM MTX amplification. Full-L had the highest glucocerebrosidase enzyme activity when compared to the rest at 50 nM MTX. For 200 nM MTX amplification, it seemed that most of the pools had similar enzyme activity except for Full-L.

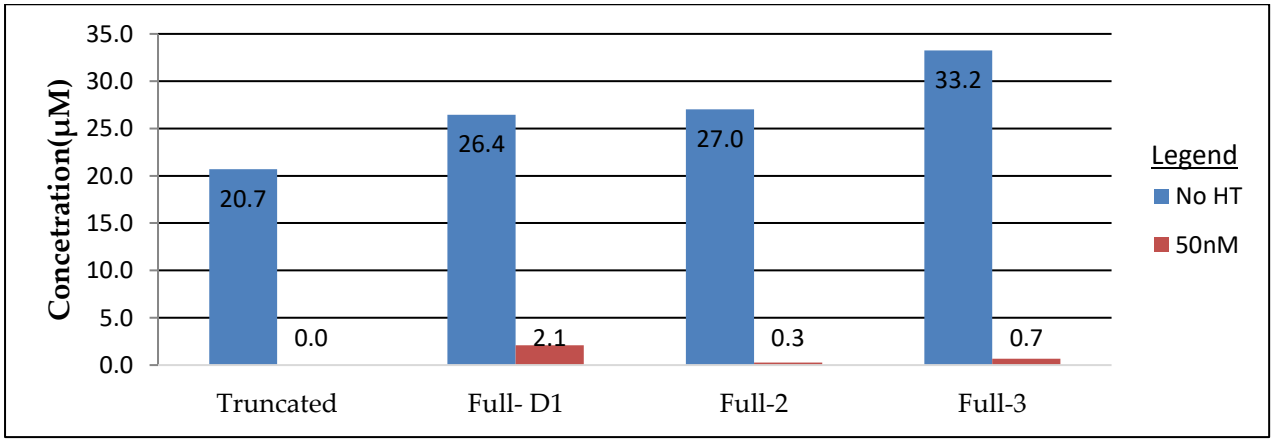

Fig 4.4.4 Graph of activity of glucocerebrosidase enzyme of suspension cells

In fig 4.4.4, the graph showed that all the pools expressed glucocerebrosidase in no HT media only.

5. Discussion

Overall, the study showed that amplification through increasing levels of MTX led to increase glucocerebrosidase expression level in CHO-gmt4D adherent cells. This could have been due to the increased gene copy number of DHFR in the selection pressure of MTX in which also increased glucocerebrosidase gene copy. This confirms what is stated in the literature, that using MTX to inhibit DHFR cause the cells to increase protein expression levels by increasing copy number of DHFR gene (Jayapal, Wlaschin, Hu, & Yap, 2007).

In this study, we have also shown that shortening of the glucocerebrosidase native signal peptide did not increase the expression level of the protein. This could be the incompatibility of the signal peptide on the protein. This confirms what is stated in the literature, that certain signal peptide only worked on specific protein (Dalton & Barton, 2014).

In our study, linearizing the plasmid did not show any significant increase of protein expression when compared to the non-linearized plasmid clones after going through amplification. This might be due to the chromatin positional effect. This confirms what is stated in the literature, that the site of the plasmid DNA that integrates into genome affects protein expression level (Zboray et al., 2015).

It was unexpected that all suspension pools did not express glucocerebrosidase in 50 nM MTX phase even when there was glucocerebrosidase expression during the no HT phase. This might due to the loss of the gene in the CHO cells genome or an error that occurred during the collection of the protein, low viability of the cells likely caused the protein to be extensively degraded and as such no observed activity.

This phenomenon can also be seen on Truncated-L of adherent cells.

It was also unexpected that all adherent cells that have the integrated full-signal plasmid have lower expression during the 200 nM MTX amplification. There could have been an error during the collection of the protein, the cells were not healthy which also means there were more lysed cells than healthy cells. Lysed cells release proteases that may affect protein quality (Goldman, James, Ison, & Bull, 1997).

With the use of CHO-gmt4D cells and the DHFR selection system, we have successfully attained pools of cells that highly express glucocerebrosidase. This demonstrates the utility of the widely used DHFR system and its effectiveness in producing highly expressing stable clones. However, it is a time consuming and tedious process. In the industrial setting 6 months or more might be needed for the process of selection and amplification for this system (Jayapal, Wlaschin, Hu, & Yap, 2007).

In the future, single clones can be isolated from the highest expressing pool. This is to find a clone with the highest expression. Since current results of the protein expression is just a representation of the average of all clones expressing glucocerebrosidase.

Stability test can also be conducted on the isolated single clones by removing MTX from the cell culture. So to prepare the clone for large scale industrial setting, as high expression of the protein needs to be maintained over many generations under no selection pressure.

Protein expression varies in all pools, and sometimes it does not increase with higher concentration of MTX. Hence, the results obtained from this study will contribute to future studies on gene amplification and glucocerebrosidase related studies.

6. Conclusion

In conclusion, the study has demonstrated that through the use of DHFR amplification and selection system we can obtain highly expressing glucocerebrosidase cell populations. The results also show that linearizing the plasmid and modifying the native signal peptide did not affect the protein expression level greatly in CHO-gmt4D cell.

However, there are more ways to optimize protein production. So, future studies should be conducted on how to overcome the chromatin positional effects as well as to test on different non-native signal peptides combination to improve protein expression levels.

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Appendix 1

Proposal

Major Project Proposal

Development of a biobetter for Gaucher's Disease

Name of Student	:	Lim Junwei Darien
Care Group	:	D6
Industrial Supervisor	:	Dr Tan Yun Lei
TP Liaison Officer	:	Dr Wuang Shy Chyi
Submission Date	:	6/5/16

Diploma in Chemical Engineering

AY 2016/2017

Table of Contents

1 Introduction

Gaucher's Disease is a type of Lysosomal Storage Disease (LSD) that affects lysosomal function. It is an inherited metabolic disease resulting in multi-systemic symptom, affecting 1 in 57 000 people in the general population. In this project, we will be aiding in Enzyme Replacement Therapy (ERT) for Gaucher's Disease (1). Patients suffering from Gaucher's Disease inherit a mutated form of the enzyme glucocerebrosidase. Therefore, they require frequent infusions of the recombinant wild type enzyme to maintain normal lysosomal function, restoring patients' health. Recombinant glucocerebrosidase is taken up through mannose receptors on affected macrophage cells. As such, mannose-terminated glycans on recombinant glucocerebrosidase is necessary for efficient uptake into macrophages. (1) We propose to express glucocerebrosidase in Chinese Hamster Ovary (CHO) cells that have a modified glycosylation process allowing the production of predominantly mannose-terminated glycans in a single step. In the current market, imiglucerase or Cerezyme® is the only protein drug that is expressed in wild type CHO cells, which requires a second step to modify the heterogeneous glycan population of glucocerebrosidase to predominantly mannose-terminated forms. (2) The other biosimilars are expressed in carrot cells (taliglucerase alfa) and human cells (velaglucerase alfa) (1). The current problem is that these protein drugs are extremely expensive, costing approximately US$200,000 per year. (3) This project aims to produce a biobetter that has greater efficacy and functionality over the existing drugs, while reducing cost to patients.

2 Literature review

2.1 Lysosomal Storage Diseases

LSDs are caused by specific mutations in genes encoding lysosomal enzymes, which in turn prevent the degradation of their specific substrates, which include a wide variety of glycolipids, oligosaccharides and glycoproteins. There are more than 50 distinct lysosomal storage diseases, with most being inheritable autosomal recessive diseases. (1)

2.2 Gaucher's Disease

Gaucher's Disease is characterised by the accumulation of the glycolipid substrate glucosylceramide in lysosomes of macrophage cells. There are three types of Gaucher's Disease, with type I being the most common in patients. Some of the symptoms include enlarged liver and/or spleen, low blood platelet count, anaemia and low white blood cell count. Patients with type II and III Gaucher's Disease also suffer extensive neurological symptoms, and often do not survive past early adulthood. At present, only type I Gaucher's Disease can be treated by Enzyme Replacement Therapy. (4)

2.3 Enzyme Replacement Therapy

ERT is the replacement of a mutated lysosomal enzyme by a recombinant wild type enzyme that will be taken into macrophages intravenously. Glucocerebrosidase is taken in through mannose receptors on macrophage cells. As such, the glycans on recombinant glucocerebrosidase have to be mannose-terminated for efficient uptake. (1)

The first drug approved by the United States Food and Drug Adminstration (FDA) for Gaucher's Disease was Cerezyme® in 1994(5). Cerezyme® is expressed in wild type CHO cells, producing a heterogeneous population of glycans on the recombinant enzyme. A second step is required to modify the glycans on glucocerebrosidase to predominantly mannose-terminated forms. (2) In 2009, there was a virus contamination in the Cerezyme® manufacturing plant and it greatly affected the overall drug supply for Gaucher's Disease. This incident catalysed the FDA approval of biosimilars velaglucerase alfa in 2010(6) and

taliglucerase alfa in 2012. (7) Since Gaucher's Disease is a rare disease, the treatment can cost up to US$200 000 annually (3).

2.4 Chinese Hamster Ovary Cells

CHO cells are excellent for the expression of proteins and they are most commonly used for the mass production of recombinant protein products due to their capacity for single cell suspension culture. They can be genetically modified as well, for example, the knockout of genes can be performed with relative ease. (8) Furthermore, proteins produced in CHO cells have similar properties and post-translational modifications compared to that of native human proteins, which greatly decreases the likelihood of rejection when administered to patients. Thus, CHO cell lines are important in bioprocessing research and the manufacturing of therapeutic biopharmaceuticals by increasing protein productivity and reducing safety concerns. (9)

Glycosylation, the process of attaching sugar moieties to proteins, is a post-translational modification that provides great proteomic diversity. Glycosylation is important for many biological processes which includes cell attachment to the extracellular matrix and protein-ligand interactions in the cell endogenously. (10) Glycosylation of recombinant protein drugs may also enhance drug efficacy and longevity. (9) In particular, modification of glycans on recombinant glucocerebrosidase is vital for its uptake into macrophages through the mannose receptors. CHO-gmt4, developed by the Song lab, is a mutant that has lost the gene *N*-acetylglucosaminyltransferase I (GnT1). This cell line was selected using Ricinus communis agglutinin-1 (RCA1) treatment. This mutant produces protein with mannose-terminated N-glycans, allowing us to bypass the second enzymatic deglycosylation step required in the production of Cerezyme. CHO-gmt4D is a mutant that has lost GnT1 and the dihydrofolate reductase (DHFR) gene. DHFR was knocked out by the Song lab using a zinc finger nuclease, allowing us to select for and amplify the expression of glucocerebrosidase. In this project, we will utilise the CHO-gmt4D cell line for the production of recombinant glucocerebrosidase. (11)

3 Aim of the project

The aim of the project is to produce a glucocerebrosidase biobetter, that has greater efficacy and functionality over the existing protein drugs in the market, while lowering the cost to patients. Molecular cloning will be performed to construct a plasmid encoding for glucocerebrosidase and DHFR. Next, a series of transfection, selection and amplification of these genes in CHO-gmt4D cells will be conducted to get the most desirable result. The objective is to obtain cells that have very high levels of glucocerebrosidase expression to fulfil the market needs.

4 Materials and Methods

4.1 Cell culture

The CHO-gmt4D glycosylation mutant cells used in this project was provided by Dr Tan Yun Lei. It produces glycoproteins with predominantly mannose-terminated N-glycans.Recombinant glucocerebrosidase produced in this line should have mannose-terminated N-glycans that will enhance its uptake into macrophages through the mannose receptors. Adherent CHO-gmt4D cells were cultured in Dulbecco's modified eagle media (DMEM) + Glutamax™ (Life Technologies) with 10% fetal bovine serum (FBS) and *Hypoxanthine-Thymidine (*HT) supplement (Life Technologies). Adherent cells were grown at 37°C in a humidified incubator with 5% CO_2. Suspension CHO-gmt4D cells were cultured in a 1:1 mixture of HyClone™ (GE,USA) and CD-CHO (Life Technologies) media supplemented with HT, 6 mM L-glutamine and 0.05% pluronic acid. Cells were grown at 37°C and 115rpm in a humidified incubator with 8% CO_2.

4.2 Molecular Cloning using restriction enzymes

The isolation of a DNA sequence (usually a gene) to be inserted into a vector suitable for transfection into mammalian cells. The pCID vector and the glucocerebrosidase gene were provided by Dr Tan Yun Lei. The gene amplicon was obtained using Polymerase Chain Reaction (PCR) through the use of Pfx DNA polymerase from Invitrogen, USA. Both pCID vector and glucocerebrosidase gene sequence were digested using Nhe1 and Not1 restriction enzymes from New England Biolabs, USA. The ligation of the pCID vector and the gene was done using T4 Ligase from New England Biolabs, USA. The resulting plasmid was transformed using *One Shot TOP10*™ bacterial cells from Invitrogen, USA. These cells were cultured on agar plates that consist of ampicillin to select for colonies that had the plasmid encoding the ampicillin-resistant gene.

4.3 Polymerase Incomplete Primer Extension (PIPE) Cloning

Another method of molecular cloning without the use of restriction enzymes. This method takes advantage of the fact that PCR generates DNA molecules that are incompletely extended during the last few cycles. This results in overhangs for each PCR product, allowing intermolecular complementation when mixed together, bypassing the use of a ligase.Primers were designed according to the selected sequence in the pCID vector and glucocerebrosidase gene. Both DNA templates will go through PCR using Pfu Turbo DNA polymerase (Agilent,USA). Subsequently, both pCID vector and the gene were digested with Dpn1 from Agilent Technologies,USA to remove methylated DNA. Digested pCID vector and glucocerebrosidase gene sequence was mixed to get the recombinant plasmid. The recombinant plasmid was transformed using *One Shot TOP10*™ bacterial cells from Invitrogen,USA. These cells were cultured on agar plates that consist of ampicillin to select for colonies that had the plasmid encoding the ampicillin-resistant gene.

4.4 Polymerase Chain Reaction (PCR)

To make multiple copies of selected DNA in a PCR machine.The DNA was denatured over 90°C and the primers will anneal to the single stranded DNA under the condition of 40°C to 60°C. The temperature was increased to 72°C for the DNA polymerase to extend and replicate the DNA strand. This cycle repeats 30 to 35 times, exponentially generating the desired product.

4.5 Transfection

Is the introduction of foreign DNA into cells, preferably to be inserted into the genome.

CHO-gmt4D cell line that has dysfunctional GnT-I and DHFR genes wil be used

Electroporation will be used to insert the recombinant plasmid into the CHO-gmt4D cells. Cell culture plates will be prepared. Desired density of cells will be measured using a cell counter and prepared for centrifugation. The cell pellet will be resuspended in room temperature 4D Nucleofector™ Solution (Lonza, USA). Recombinant plasmid encoding glucocerebrosidase and DHFR will be added to the solution. Subsequently, the solution will be transferred into the Nucleocuvette™ Vessels. The vessel will be placed into the retainer of 4D-Nucleofector™ Core unit (Lonza, USA) and run with the selected protocol on the machine. The cells will be grown in a humidified incubator at 37°C in the culture medium until further analysis.

4.6 Selection and amplification

DHFR is used as our genetic selection marker since our cell line CHO-gmt4D lacks DHFR activity. Transfected CHO-gmt4D cells will be cultured in normal culture medium. After recovery, culture medium will be changed to one that does not contain HT supplement to facilitate the selection of cells that have incorporated the recombinant plasmid. Multiple rounds of amplification using increasing concentration of methotrexate will allow us to select for cells that produce the highest levels of recombinant protein.

4.7 Western Blot

This is used for protein identification and analysis through the interaction of antibody and antigen.

The mixture of proteins in conditioned medium is separated on a Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel. Next, the proteins will be transferred from the gel to a polyvinylidene difluoride (PVDF) membrane. Blocking of the membrane will be done using non-fat milk to prevent non-specific binding. After blocking, the membrane will be incubated with the primary rabbit polyclonal anti-glucocerebrosidase antibody (Sigma,USA). After washing, the membrane is incubated with an anti-rabbit secondary antibody conjugated to *horseradish peroxidase* (*HRP*). After washing, the membrane is then incubated with an HRP substrate. Chemiluminescence produced by the cleavage of the HRP substrate will be detected on an imager.

4.8 Enzyme Activity Assay

Is the measure of enzyme activity by using a modified glucocerebrosidase substrate that when hydrolysed by the enzyme, releases a fluorescent molecule. By quantifying the fluorescence given off, we can quantify the concentration of glucocerebrosidase present in conditioned media.4-Methylumbelliferyl β-D-glucopyranoside (MUG) from Sigma in an acetate buffer, pH 4 will be added to each well containing conditioned media in a 96-well plate. The plate will be incubated at 37°C for an hour and the reaction will be quenched by the addition of a high pH glycine buffer to each well. The fluorescence of the liberated 4-methylumbelliferone will be measured (excitation 365nm and emission 445nm) using a fluorescence plate reader.

5 Time Schedule

Task		Wk1	Wk2	Wk3	Wk4	Wk5	Wk6	Wk7	Wk8	Wk9	Wk10	Wk11	Wk12	Wk13	Wk14	Wk15	Wk16	Wk17	Wk18	Wk19	Wk20	Wk21	Wk22	Wk23	Wk24	Wk25	Wk26
Commencement of MP	Plan																										
	Actual																										
Overview of MP and confirm project title	Plan																										
	Actual																										
Literature Review of Gaucher's Disease	Plan																										
	Actual																										
Searching for information regarding molecular cloning	Plan																										
	Actual																										
Searching for information regarding western blot	Plan																										
	Actual																										
Searching for information regarding polymerare chain	Plan																										
	Actual																										
Searching for information regarding enzyme activity assay	Plan																										
	Actual																										
Proposal development	Plan																										
	Actual																										
Expression Vector through molecular cloning	Plan																										
	Actual																										
Transfection	Plan																										
	Actual																										
Selection	Plan																										
	Actual																										
Amplification	Plan																										
	Actual																										
Final Report	Plan																										
	Actual																										

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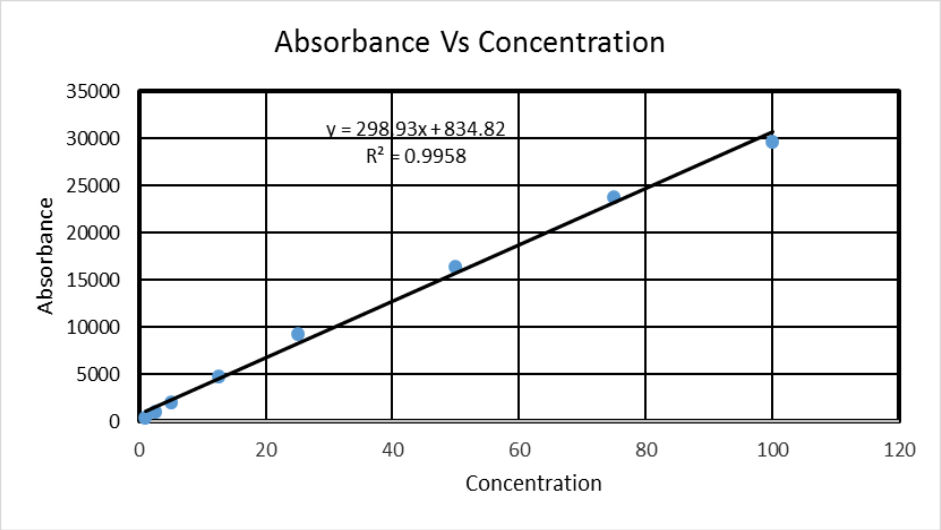
Appendix 2

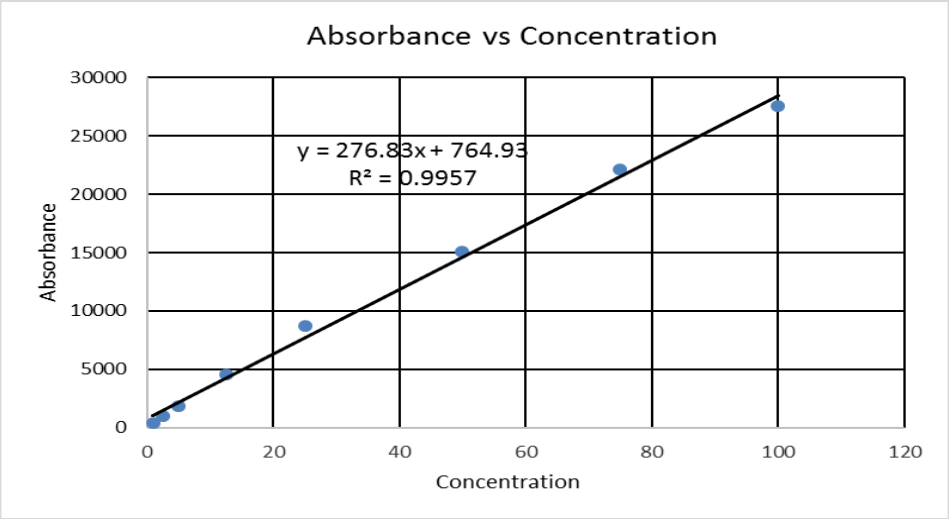
Enzyme Activity Assay after 50nM amplification of adherent cells

Bottom
Reading



Concentration(μM)	Absorbance
100	29708
75	23828
50	16481.5
25	9259
12.5	4860
5	2034.5
2.5	1060
1	457

Top
Reading

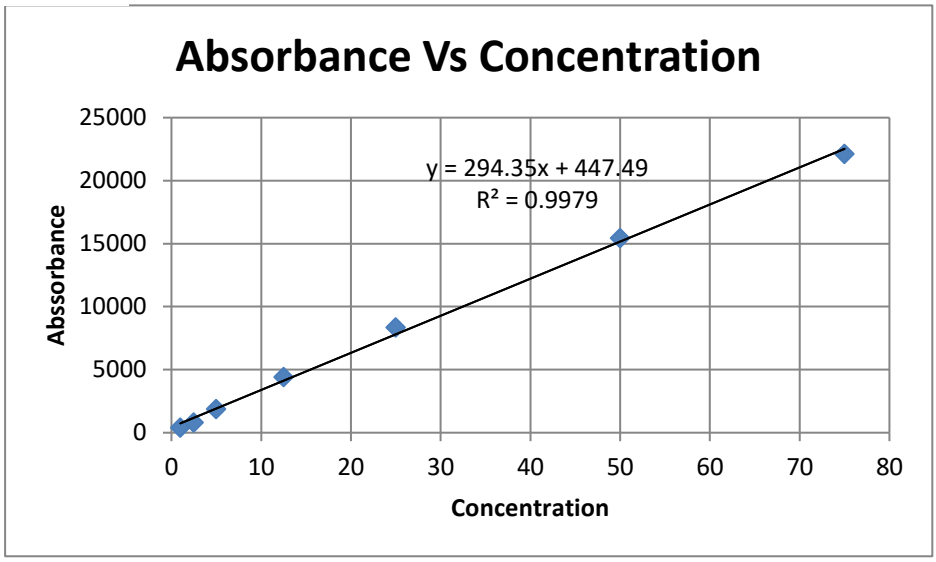


Concentration(μM)	Absorbance
100	27534.5
75	22110.5
50	15043
25	8690.5
12.5	4545.5
5	1847
2.5	961.5
1	407.5

		Truncated-1(50nmMTX)	Truncated-L(50nmMTX)	Full-L(50nmMTX)	Full-1(50nmMTX)
Absorbance (TOP)	STOP	262	237	137	107
	After 1 hr	5552	3730	14358.5	7843.5
	Actual(after 1hr-Stop)	5290	3493	14221.5	7736.5
Concentration	μM	16.346	9.855	486.095	251.836

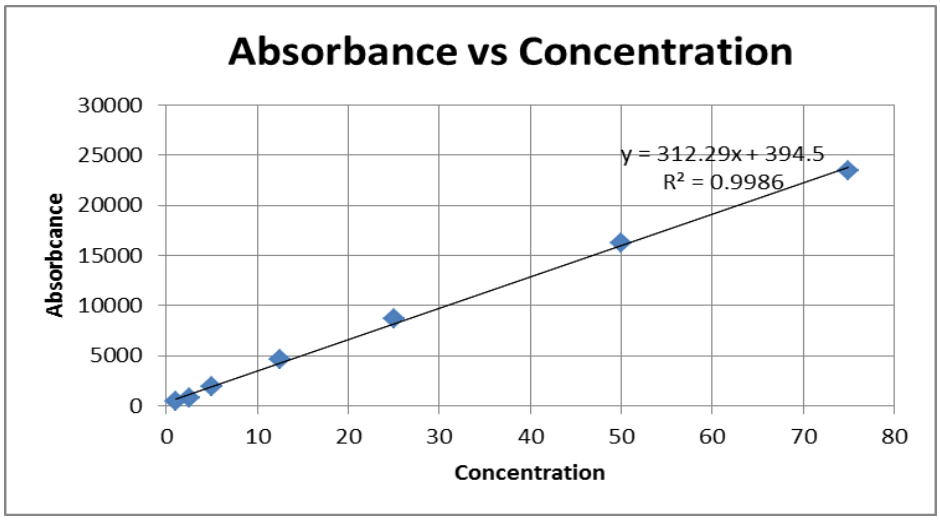
		Truncated-1(50nmMTX)	Truncated-L(50nmMTX)	Full-L(50nmMTX)	Full-1(50nmMTX)
Absorbance (Bottom)	STOP	303	275	170	141
	After 1 hr	5746	3863	15779.5	8755
	Actual(after 1hr-Stop)	5443	3588	15609.5	8614
Concentration	μM	16.899	10.198	536.234	283.534

Bottom
Reading



Concentration	Absorbance
75	22127
50	15441
25	8352
12.5	4427
5	1883
2.5	827.5
1	409.5

Top
Reading



Concentration	Absorbance
75	23489.5
50	16216
25	8669
12.5	4591.5
5	1942
2.5	842
1	413

		Full-D1(50nm MTX)20x	Full-D2(50nmMTX)20x
Absorbance (Top)	STOP	103	93
	After 1 hr	5562	3204
	Actual(after 1hr-Stop)	5459	3111
Concentration	µM	16.217	8.699
	20x	324.346	173.973

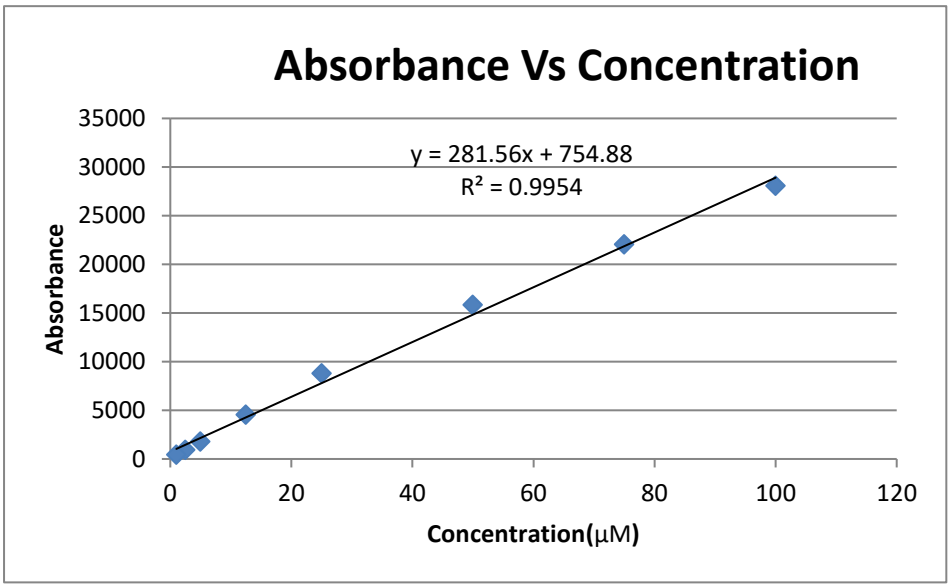
		Full-D1(50nm MTX)20x	Full-D2(50nmMTX)20x
Absorbance (Bottom)	STOP	109.5	104
	After 1 hr	5556	3220
	Actual(after 1hr-Stop)	5446.5	3116
Concentration	µM	16.983	9.066
	20x	339.664	181.315

Appendix 3

Enzyme Activity Assay after 200nM amplification of adherent cells

Bottom

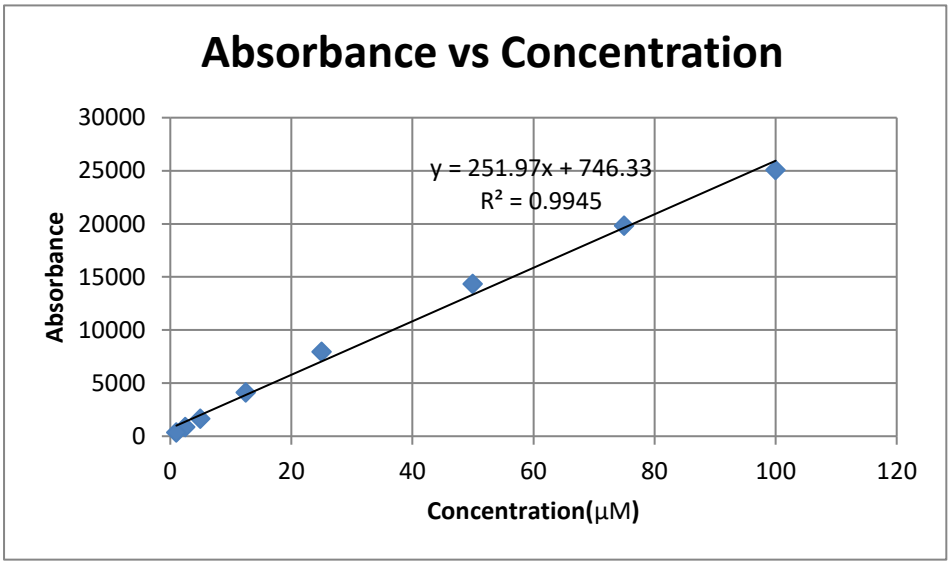
Reading



Concentration	Absorbance
100	28058
75	22030
50	15823.5
25	8778
12.5	4528.5
5	1781.5
2.5	919.5
1	423

Top

Reading



Concentration	Absorbance
100	25084
75	19823.5
50	14351.5
25	7955.5
12.5	4137
5	1649
2.5	878.5
1	375

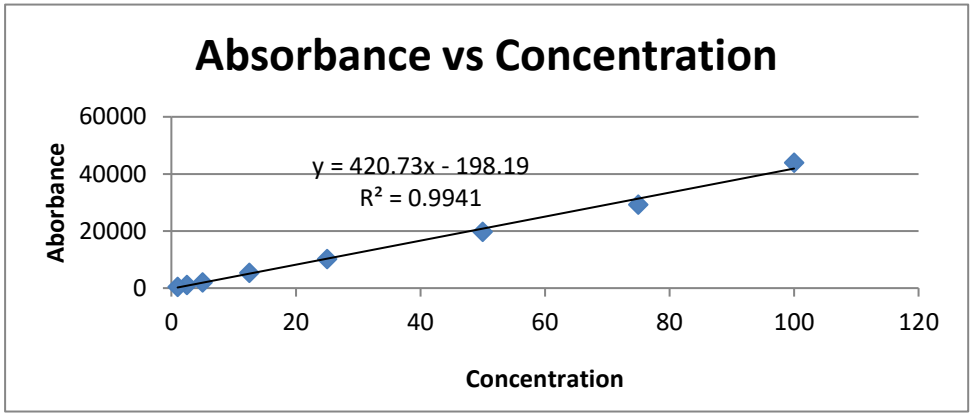
		Truncated-1	Truncated-L	Full-L	Full-1	Full-D1	Full-D2
Absorbance (Top)	STOP	87.00	142.50	270.00	82.50	76.00	75.50
	After 1 hr	3965.00	1744.50	21192.00	3157.00	3487.00	2115.50
	Actual(after 1hr-Stop)	3878.00	1602.00	20922.00	3074.50	3411.00	2040.00
Concentration	µM	12.43	3.40	80.07	9.24	10.58	5.13
	20x	248.57	-	-	184.80	211.51	102.68

		Truncated-1	Truncated-L	Full-L	Full-1	Full-D1	Full-D2
Absorbance (Bottom)	STOP	113.50	185.50	332.50	107.00	102.50	101.50
	After 1 hr	4615.50	2006.00	24021.00	3687.00	4117.00	2496.50
	Actual(after 1hr-Stop)	4502.00	1820.50	23688.50	3580.00	4014.50	2395.00
Concentration	µM	13.31	3.78	81.45	10.03	11.58	5.83
	20x	266.17	-	-	200.68	231.54	116.50

Appendix 4

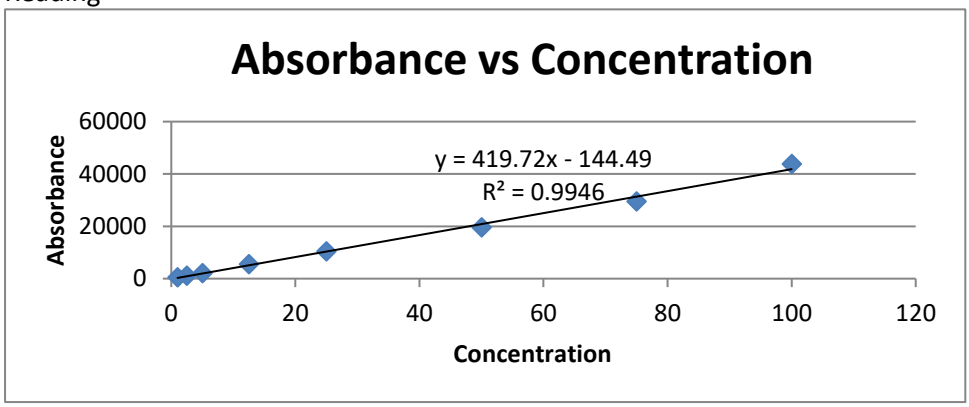
Enzyme Activity Assay after 500nM amplification of adherent cells with different collection dates

Bottom Reading



Concentration	absorbance
100	43944.5
75	29270.5
50	19732.5
25	10234.5
12.5	5448
5	2103
2.5	1172

Top Reading



Concentration	absorbance
100	43779.5
75	29452.5
50	19584
25	10419.5
12.5	5448
5	2144
2.5	1180.5

		Full-1(12/8-->17/8)	Full-1(25/8-->29/8)	Full-L(15/8-->18/8)	Full-L(29/8-->1/9)	Full-D1(22/8-->25/8)	Full-D2(15/8-->18/8)	Full-D2(25/8-->29/8)	Truncated-1(4/8-->8/8)	Truncated-1(28/8-->1/9)
Absorbance (Top)	STOP	198.00	143.00	143.00	148.00	200.00	201.00	181.00	163.00	152.00
	After 1 hr	10223.00	675.00	559.00	764.00	12612.50	11357.50	8609.50	5901.50	3800.50
	Actual(after 1hr-Stop)	10025.00	532.00	416.00	616.00	12412.50	11156.50	8428.50	5738.50	3648.50
Concentration	µM	23.54	0.92	0.65	1.12	29.23	26.24	19.74	13.33	8.35
	20x	470.81	18.47	12.94	22.47	584.58	524.73	394.74	266.56	166.97

		Full-1(22/8-->25/8)	Full1ATG-1(29-->1/9)	Full-L(25/8-->29/8)		Full-D1(30/8-->1/9)	Full-D2(19/8-->22/8)	Full-D2(29/8-->1/9)	Truncated-1(22/8-->25/8)	Truncated-L(29/8-->1/9)
Absorbance (Top)	STOP	200.00	212.00	184.00		171.00	273.00	224.00	158.00	140.00
	After 1 hr	8040.50	8467.00	7232.00		3518.00	8107.00	11263.50	4180.00	563.50
	Actual(after 1hr-Stop)	7840.50	8255.00	7048.00		3347.00	7834.00	11039.50	4022.00	423.50
Concentration	µM	18.34	19.32	16.45		7.63	18.32	25.96	9.24	0.66
	20x	366.72	386.47	328.96		152.60	366.41	519.16	184.77	13.30

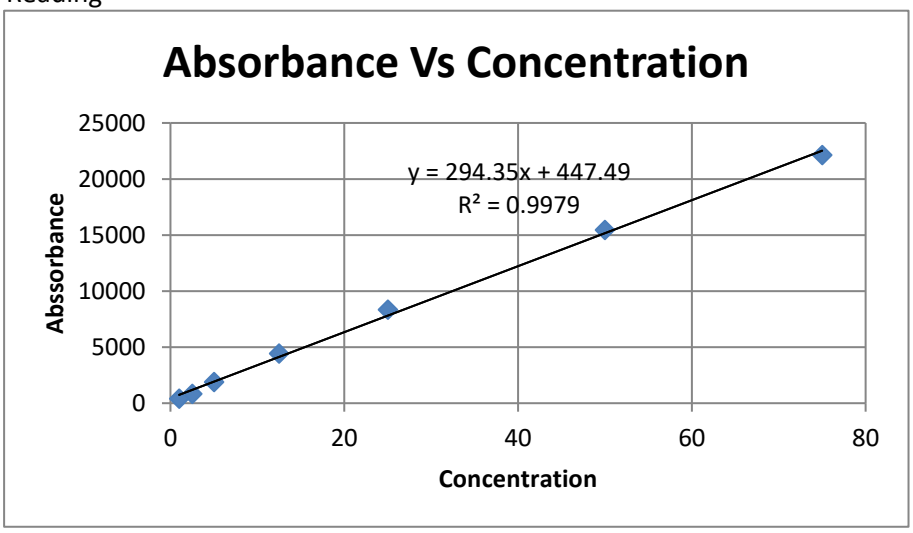
		Full-1(12/8-->17/8)	Full-1(25/8-->29/8)	Full-L(15/8-->18/8)	Full-L(29/8-->1/9)	Full-D1(22/8-->25/8)	Full-D2(15/8-->18/8)	Full-D2(25/8-->29/8)	Truncated-1(4/8-->8/8)	Truncated-1(28/8-->1/9)
Absorbance (Bottom)	STOP	223.00	169.00	168.00	247.00	229.00	227.00	207.00	194.00	176.00
	After 1 hr	10563.00	706.00	590.50	793.50	13027.50	11619.00	8834.00	6042.50	3900.50
	Actual(after 1hr-Stop)	10340.00	537.00	422.50	546.50	12798.50	11392.00	8627.00	5848.50	3724.50
Concentration	µM	24.11	0.81	0.53	0.83	29.95	26.61	20.03	13.43	8.38
	20x	482.11	16.11	10.66	16.56	598.97	532.11	400.68	268.60	167.63

		Full-1(22/8-->25/8)	Full-1(29/8-->1/9)	Full-L(25/8-->29/8)		Full-D1(30/8-->1/9)	Full-D2(19/8-->22/8)	Full-D2(29/8-->1/9)	Truncated-1(22/8-->25/8)	Truncated-L(29/8-->1/9)
Absorbance (Bottom)	STOP	225.00	234.00	214.00		182.00	306.00	245.00	176.00	157.00
	After 1 hr	8178.00	8818.50	7348.50		3580.50	8241.50	11491.00	4261.00	587.50
	Actual(after 1hr-Stop)	7953.00	8584.50	7134.50		3398.50	7935.50	11246.00	4085.00	430.50
Concentration	µM	18.60	20.11	16.65		7.75	18.56	26.45	9.39	0.68
	20x	372.08	402.17	333.08		155.06	371.25	529.00	187.77	13.63

Appendix 5

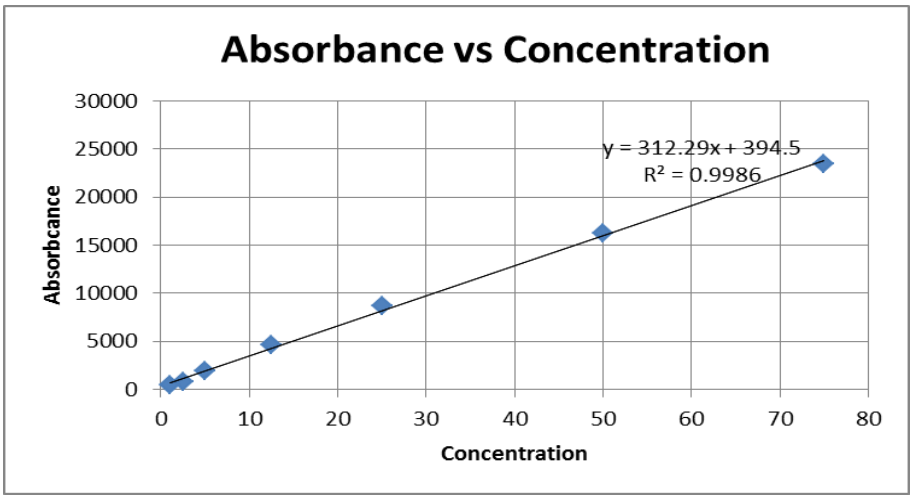
Enzyme Activity Assay after recovery from no HT media for suspension cells

Bottom Reading



Concentration	Absorbance
75	22127
50	15441
25	8352
12.5	4427
5	1883
2.5	827.5
1	409.5

Top Reading



Concentration	Absorbance
75	23489.5
50	16216
25	8669
12.5	4591.5
5	1942
2.5	842
1	413

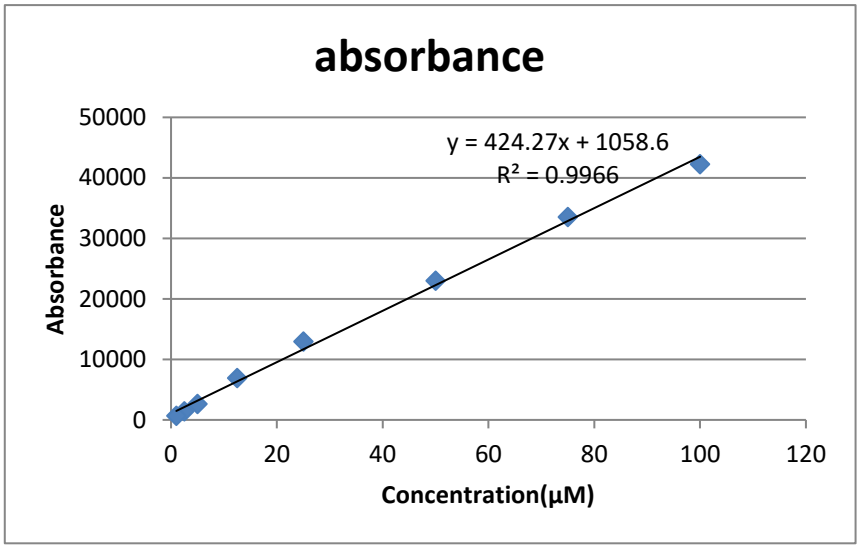
		Truncated[13/6](noHT)S	Full-D1[13/6](No HT)S	Full-2[27/6](No HT)S	Full-3[27/6](No HT)S
Absorbance (Top)	STOP	252	237	285.5	273.5
	After 1 hr	6852.5	8557	10532	11689
	Actual(after 1hr-Stop)	6600.5	8320	10246.5	11415.5
Concentration	µM	19.873	25.379	31.548	35.291
	20x	-	-	-	-

		Truncated[13/6](noHT)S	Full-D1[13/6](no HT)S	Full-2[27/6](No HT)S	Full-3[27/6](No HT)S
Absorbance (Bottom)	STOP	262.5	246.5	304.5	285.5
	After 1 hr	6801.5	8475	8705	10524
	Actual(after 1hr-Stop)	6539	8228.5	8400.5	10238.5
Concentration	µM	20.695	26.435	27.019	33.263
	20x	-	-	-	-

Appendix 6

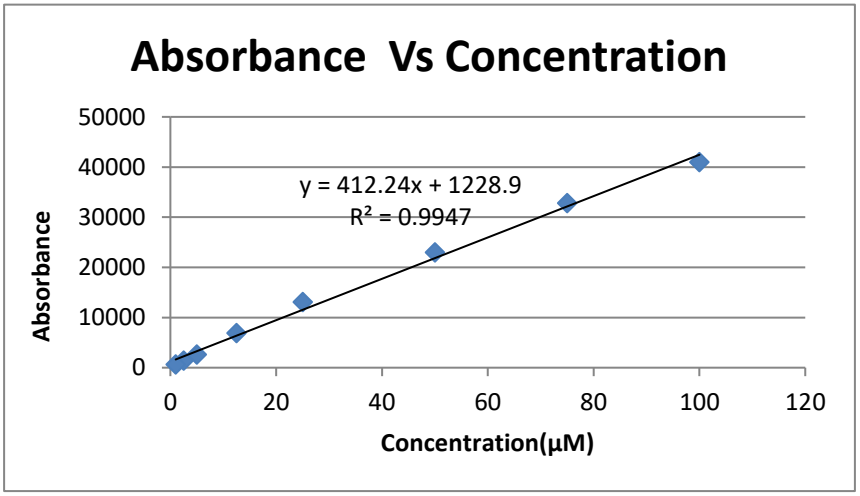
Enzyme Activity Assay after 50nM amplification for suspension cells

Bottom Reading



Concentration(μM)	absorbance
100	42277.5
75	33523
50	23030.5
25	12978
12.5	6920
5	2645
2.5	1410.5

Top Reading



Concentration(μM)	absorbance
100	40983.5
75	32806
50	22981.5
25	13131
12.5	6937
5	2644.5
2.5	1419.5

		Full-D1(27/6)	Full-2(27/6)	Full-3(27/6)	Truncated-(13/6)
Absorbance (Top)	STOP	355.00	330.00	351.00	336.00
	After 1 hr	1992.00	1448.50	1657.50	1021.00
	Actual(after 1hr-Stop)	1637.00	1118.50	1306.50	685.00
Concentration	μM	0.99	-0.27	0.19	-1.32

		Full-D1(27/6)	Full-2(27/6)	Full-3(27/6)	Truncated-(13/6)
Absorbance (Bottom)	STOP	382.00	362.00	382.00	367.00
	After 1 hr	2067.50	1528.00	1721.50	1062.00
	Actual(after 1hr-Stop)	1685.50	1166.00	1339.50	695.00
Concentration	μM	1.48	0.25	0.66	-0.86