



RESEARCH ARTICLE

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Expression Enhancement of Trastuzumab in CHO Cells Using Codon Optimization and Promoter Selection for Mammalian Expression Vector

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ABSTRACT

Trastuzumab is recombinant humanized monoclonal IgG antibody used in the treatment of metastatic breast cancer. It blocks the overexpression of tyrosine kinase Her 2 family receptors and downregulation of pathways. Genetic optimizations like gene optimization and expression vector optimization are the key elements to enhance monoclonal antibody expression. Expression vector optimization includes major parameters like selection of right promoters, poly A tail selection of genome integrating elements. Codon optimization is gene designing approach without affecting the amino acid sequence of the protein. Current research work involves development of codon optimization algorithm for trastuzumab heavy and light chain gene and comparative analysis of expression in different vectors.

Keywords: Trastuzumab, mammalian expression, expression vector, codon optimization.

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INTRODUCTION

Several types of malignant tissues show overexpression of tyrosine kinase receptors. The receptors belong to ErbB family located on the cell surface and mainly function in the control of tumor growth. The Trastuzumab is recombinant IgG 1 antibody binds to specifically extracellular domain of the epidermal growth factor receptors (Her 2\new) receptors and blocks the pathway in tumor cells. [1] Trastuzumab was approved by the US food and drug administration

(FDA) in 1998 and also by the European medicines agency (EMA) in 2000 in women with Her 2 MBC to Genentech /Roche. [2]

The Increasing demand of stable and highly produced monoclonal antibody protein is major need of biopharmaceutical industries. Mammalian cells including Chinese hamster Ovary (CHO) cells, NSO and HEK have the ability of post translational modifications. The cells can produce large amount of monoclonal antibodies with consistent quality and

adaptation in large scale suspension bioreactors. [3-4] Among these cell lines, CHO cells are major choice as a host for the therapeutic protein production. The choice of the host can influence character, quality and cost of the final product of the therapeutics. [5]

Other factors like codon optimization and choice of expression vector also influence quality as well as quantity of protein produced. [6] Codon optimization is a gene designing approach to improve protein expression of recombinant gene by altering the amino acid sequence. The method is based on analysis of different genetic factors [7-8] like codon frequency, tRNA abundance, percentage of GC content, number of cis acting elements, mRNA secondary structure and rare codon analysis. [9-12] Different algorithms like CodonOpt, COOL, Visual gene developer, Optimum gene, Synthetic gene, Gene Optimizer, jCat, etc. are available to design a synthetic gene by codon optimization. [13] The codon adaptation index plays very important role in codon optimization and is widely used to predict expression level from gene and to approximate the success of heterologous gene expression. [14-15]

In addition to codon optimization, modification of expression vector also influences the amount of protein expression. [16] Strong promoters and strong poly a tail, Antibiotic selectable marker, Dihydrofolate reductase (DHFR) gene etc. are the key elements of mammalian expression vector. [17] Promoter is an element which drives the expression of the cloned gene of interest. Expression of mammalian genes requires a strong eukaryotic viral promoter like Cytomegaloviruses (CMV) and Simian virus 40 (SV40) promoter. [18] Selection of promoter is not a fully understood mechanism and recent studies suggests that insertion of chromatin modifying elements like insulator, Matrix attachment region (MAR), Scaffold matrix attachment region (SAR), Stabilizing anti repressor elements (STAR) in the expression vector increases the expression in many folds. [19-22] Matrix attachment region (MAR) is AT rich sequence of variable length with increased density of homologous sequence of topoisomerase II binding site and specific AT rich binding protein motifs (SATB1). [23] MAR is genetic element which insulates transgene from repressive effects linked to their site of integration within the host cell genome. Different Mar sequences like B globin MAR, Chicken lysozyme MAR (cl-Mar) are used in the expression vector to influence gene expression. [24-28]

Metabolic marker such as Dihydrofolate reductase (DHFR) is key element used in mammalian expression vectors. It involves in the nucleotide metabolism and catalyzes the conversion of the dihydrofolate to tetrahydrofolate. This DHFR allows the process of gene amplification with increasing concentration of the Methotrexate (MTX) and antibiotic inhibiting the DHFR enzyme. Transfected cells containing DHFR genes must increase their DHFR gene synthesis capacity by

amplifying the DHFR gene to survive. The amplification assembly is quite larger than DHFR gene, the gene of interest also amplifies with DHFR gene. [17, 29-30] In the current work, heavy and light chain genes of Trastuzumab were optimized by in-house developed codon optimization algorithm. They are cloned into expression vector pBG-SVII and its optimized version pBG-SVII(pgk). The CMV promoter driving DHFR gene expression is replaced with weaker phosphoglycerokinase (pgk) promoter in pBG-SVII(pgk) expression vector.

MATERIAL AND METHODS

Codon Optimization of Trastuzumab Genes

The trastuzumab heavy and light chain full length gene sequences are finalized from public databases Drug bank (Accession number DB00072). Suitable secretory signal sequences for heavy and light chain genes were carefully chosen. Trastuzumab light chain and heavy chain genes were optimized by using in-house developed codon optimization algorithm (Figure 1). The optimized full-length gene constructs of heavy and light chain genes were synthesized by GeneArt gene synthesis technology (Thermo). The light chain gene of Trastuzumab was labeled as (pBG014) and heavy chain was labelled as (pBG015) and schematically represented in figure 2.

Revival of Codon Optimized Trastuzumab Genes

Codon optimized Trastuzumab light chain (pBG003) and heavy chain (pHBHC) genes were reconstituted as per manufacturer's instructions. Each plasmid of codon optimized DNA was reconstituted in 50µl TE buffer. The plasmid containing codon optimized Trastuzumab genes (pBG014 and pBG015), pBG-SVII, and pBG-SVII(pgk) vectors were transformed into *E. coli* Top 10 competent cells. Transformants were selected on Luria agar plates containing Kanamycin and zeocin respectively as selection marker. Single transformant from each plate was selected and grown in Luria broth for 12-14 hours at 37°C. The plasmid DNA was extracted from each culture using Qiagen Kit using manufacturer's protocol, quantitated using UV spectrometer and analyzed by loading on 1% agarose gel.

Cloning Codon Optimized Trastuzumab genes into pBG-SVII and pBG-SVII(pgk) Expression Vector

The codon optimized light and heavy chain of Trastuzumab were cloned in pBG-SVII (Strategy 1) and pBG-SVII(pgk) (Strategy 2) mammalian expression vector. The in-house optimized Trastuzumab light chain genes were cloned between *Sal I* (Thermo) and *Xba I* (Thermo) restriction sites in both vector and are expressed via CMV promoter.

The in-house optimized Trastuzumab light chain gene, pBG-SVII and pBG-SVII(pgk) vectors were cleaved with *Sal I* (Thermo) and *Xba I* (Thermo) restriction enzymes in 2X tango buffer with 20 units of *Sal I* and 20 units of *Xba I*. The reaction was set in 40µl volume and incubated at 37°C for 45 mins for digestion. The

digested vectors pBG-SVII, pBG-SVII (pgk) and insert pBG014 were loaded on 1% agarose gel and resolved for separation of DNA fragment. The desired bands of vector and insert were excised from the gel under UV transilluminator and subsequently purified. Purification of the vector and inserts was done using QIAquick gel extraction kit (QIAGEN). The purified DNA were analyzed by agarose gel electrophoresis and quantitated from the gel by using standard molecular DNA ladder of known sizes. The concentrations of the purified DNA preparations were estimated by measuring the OD at 260 nm using spectrophotometer. The ligation reactions of pBG014 with both vectors were assembled in 10µl volume containing digested vector and insert with 1X T4 Ligase buffer and 5 Weiss units of T4 DNA ligase. The ligation reactions were set on ice and the ligation mixtures were incubated at 37°C for 1 hour. The ligation reactions were transformed into 100µl *E. coli* Top 10 competent cells and selected using Puromycin as antibiotic marker on Luria agar plates. The transformants obtained (Strategy1 and 2) were screened using colony PCR. The PCR samples were analyzed on 2% agarose gel for positive clones. The positive clones were isolated using QIAprep spin miniprep kit from QIAGEN. The isolated clones DNA (strategy 1 and 2) were analyzed on 1% agarose gel and quantitated using UV spectrophotometer. The clones DNA were confirmed for the presence of light chain gene by restriction digestion using *Sal I* and *Xba I* enzymes. Similarly, in-house optimized Trastuzumab heavy chain genes were cloned between *Not I* (Thermo) and *Bgl II* (Thermo) restriction sites and expressed through pEF1α promoter. Light chain positive clones

(From strategy 1 and 2) were used as vectors for heavy chain cloning to form bicistronic clones of strategy 1 and 2 trastuzumab.

Light chain positive clones from strategy 1 and 2, and heavy chain DNA were cleaved with restriction enzyme in 2X tango buffer with 30 units of *Not I* and 30 units of *Bgl II*. The reactions were set in 60µl volume and incubated at 37°C for 60 mins for digestion. The digested vectors and inserts were loaded on 1% agarose gel and resolved for separation of DNA fragment. The desired bands of vector and insert were excised from the gel under UV transilluminator and purified using QIAquick gel extraction kit (QIAGEN). The purified DNA were analyzed by agarose gel electrophoresis and quantitated from the gel by using standard molecular DNA ladder of known sizes. The concentrations of the purified DNA preparations were estimated by measuring the OD at 260 nm using spectrophotometer. The ligation reactions are assembled in 10µl volume containing digested vectors and inserts with 1X T4 Ligase buffer and 5 Weiss units of T4 DNA ligase. The ligation reactions were transformed into 100µl *E. coli* Top 10 competent cells and selected using Puromycin as antibiotic marker on Luria agar plates. The transformants obtained (strategy 1 and 2 bicistronic clones) were screened using colony PCR. The bicistronic clones DNA (strategy 1 and 2) were confirmed for the presence of heavy chain gene by restriction digestion using *Not I* and *Bgl II* enzymes. Details of the codon optimized Trastuzumab heavy and light chain genes are enlisted in table 1.

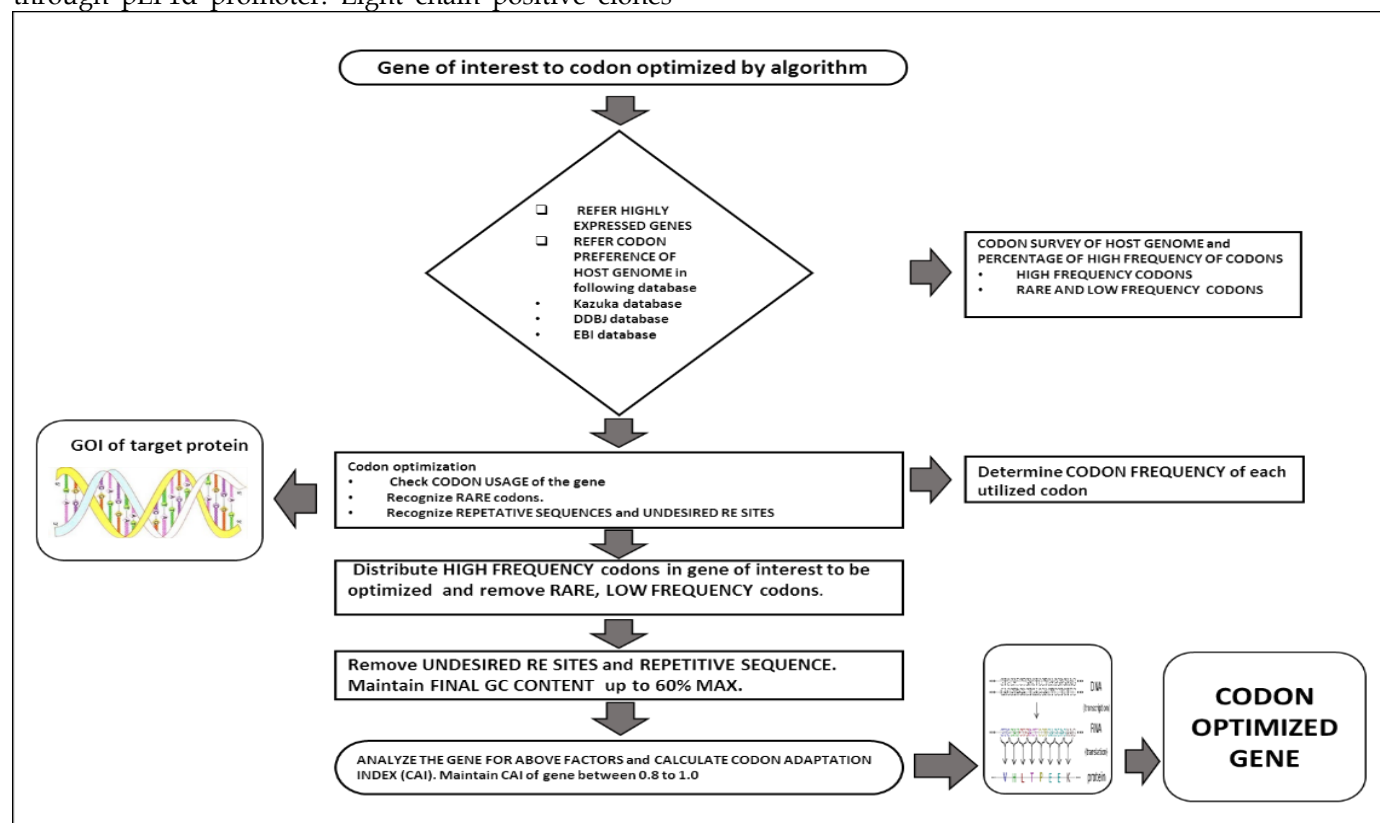


Fig. 1: In-house codon optimization algorithm

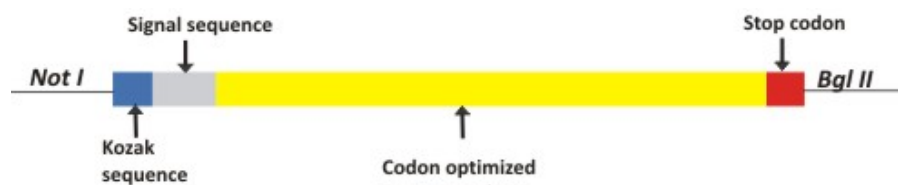


Fig. 2A: in-house codon optimized trastuzumab heavy chain gene cloned in pBG-SVII and pBG-SVII(pgk) expression vector

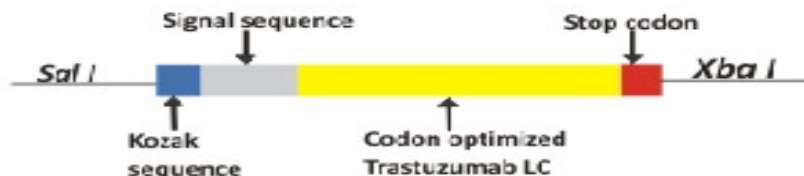


Fig. 2B: in-house codon optimized trastuzumab light chain gene cloned in pBG-SVII and pBG-SVII(pgk) expression vector

Table 1: Table showing summary of gene optimization parameters of in-house codon optimized Trastuzumab.

S. No	Parameters	Light chain gene(pBG014)	Heavy chain gene (pBG015)
1	Codon adaptation index (%)	0.93	0.92
2	GC content (%)	60.68	59.93
3	Cis acting elements	0	0
4	Repeat sequences	0	0

Table 2: Frequently used codon in the trastuzumab

Codes	Amino acid	Codon preferred in mammalian expression
M	Methionine	ATG
G	Glycine	GGC, GGA
V	Valine	GTC, GTG
H	Histidine	CAC
E	Glutamic Acid	GAG, GAA
C	Cysteine	TGT, TGC
P	Proline	CCT, CCA, CCC
A	Alanine	GCC
W	Tryptophan	TGG
L	Leucine	CTG
S	Serine	TCC, AGC
F	Phenylalanine	TTC
T	Threonine	ACC
R	Arginine	CGG, CGC
I	Isoleucine	ATC
D	Aspartic Acid	GAC
Y	Tyrosine	TAC
Q	Glutamine	CAG
N	Asparagine	AAT, AAC
K	Lysine	AAG
*	Stop Codon	TGA

Stable Expression, Scale up and Fed Batch Studies

The purified plasmid DNA of Trastuzumab from strategy 1 and strategy 2 were transfected in CHO-S cells according to freedom CHO-S Kit manual. The clones DNA were isolated using QIAGEN plasmid midi kit (QIAGEN 12143) and linearized with *Pvu I* and *Fsp I* restriction enzyme respectively. Stable pools from strategy 2 clones were established by selecting higher producers by three phase amplification process. Increasing concentrations of Puromycin (Gibco) and Methotrexate hydrate (Sigma) were used to obtain the stable pool from pBG-SVII(pgk) clones (10µg/mL Puromycin + 100 nM Methotrexate; 20µg/mL Puromycin + 200 nM Methotrexate and 30µg/mL Puromycin + 500 nM Methotrexate). Single cell cloning from strategy 2 clones was performed using the final

amplified pool of 30µg/mL Puromycin + 500 nM Methotrexate selection pressure as the combination of selection pressure showed highest protein concentration. Amongst the screened clones, 50 single cell clones were selected for fed batch expression primarily by ELISA. The selected clones from pBG-SVII(pgk) were scaled up at fed batch level. The study of selected clones was performed in 125 ml flasks with final volume of 20 mL Dynamis medium with glucose maintenance and feed additions. Cell density and percentage cell viability were determined until the culture viability dropped to 65%. The protein expression was evaluated by Enzyme linked immuno sorbent assays (ELISA).

RESULTS

Trastuzumab Codon Optimization and Cloning in pBG-SVII and pBG-SVII(pgk)

In-house codon optimized Trastuzumab genes were cloned in pBG-SVII and pBG-SVII(pgk) mammalian expression vector using standard molecular biology and cloning methods. The positive clones were characterized by restriction digestion. A single clone of Trastuzumab from strategy 1 and strategy 2 was selected for transfection and further protein expression studies. The vector map of final Trastuzumab construct from 2 is shown in figure 3. The finalized codons with higher frequency for trastuzumab genes are listed in following table 2.

Stable Expression, Scales up and Fed batch Studies of in-house Codon Optimized Trastuzumab

Plasmid midiprep of Protein expression of in-house codon optimized Trastuzumab (Strategy 1 and strategy 2) from transfection to third selection phase were performed by ELISA. The protein expression data and single cell cloning fed batch data of codon optimized Trastuzumab from strategy 1 and 2 by ELISA are shown in figure 4 and 5.

The protein expression at transfection level showed approximately same results for strategy 1 and 2. The cells transfected by clones from strategy 1, did not survived in first selection pressure of Puromycin and Methotrexate even after several media changes. Hence, expression protocol for strategy 2 clones only was continued to further study. The single cell clones of

strategy 2 achieved the highest titer 1.4 grams /lit by ELISA method. The protein expression of strategy 2 clones at single cell cloning stage was in the range of 0.6 to 1.4 grams/lit explained in figure 5.

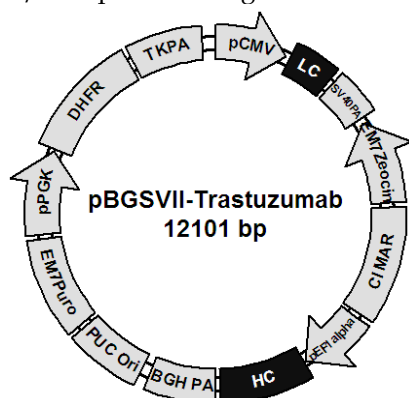


Fig. 3: The vector map of strategy 2 trastuzumab clone

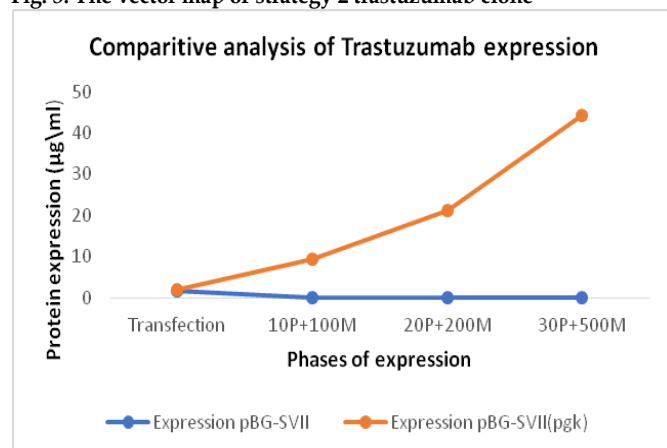


Fig. 4: Comparative analysis of protein expression of Trastuzumab from strategy 1 and strategy 2

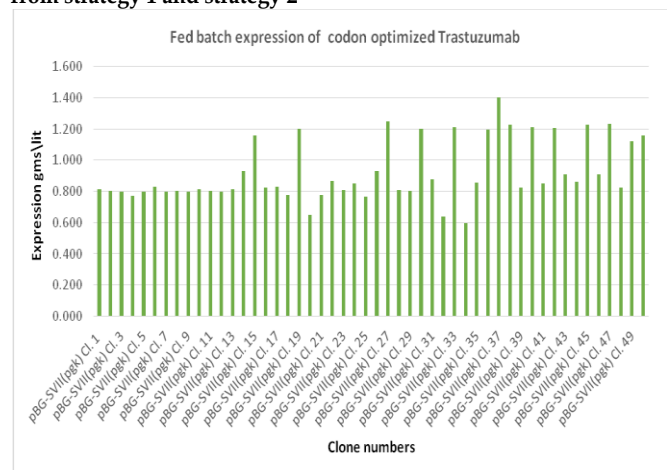


Fig. 5: Fed batch protein expression of strategy 2 trastuzumab clones

In-house developed algorithm of codon optimization was efficient as it increased trastuzumab expression up to 1.4 grams/lit. The promoter for metabolic marker in mammalian expression vector should be weaker promoter to efficiently amplify and increase gene copy number in the cells.

DISCUSSION

The in-house developed algorithm shows the CAI of both heavy and light chain gene of trastuzumab as 0.9.

Ideal standard for CAI is considered as 1. The GC content of the codon optimized genes should be around 65%. The GC content of in-house Trastuzumab genes is about 60%. Codon optimization of trastuzumab genes worked efficiently in mammalian expression systems. The clones from the strategy 1 are codon optimized but still they were unable to survive in first selection pressure. The CMV promoter is strong promoter used in DHFR expression cassette of pBG-SVII vector. The same codon optimized genes of Trastuzumab cloned in pBG-SVII (pgk) expression vector, expressed efficiently and had a titer of 1.4 grams/lit. The expression vector optimization by selecting appropriate promoter for DHFR in mammalian expression vector is one of the important aspects. The strong promoters should be used for multiple cloning sites for cloned gene expression. The weak promoter shall be used for metabolic markers. The strong promoters used for selection marker always imbalances the activity of DHFR gene, competes with the expression and effects the gene of interests. Hence, the use of weak promoter for selection marker of mammalian expression vector is always beneficial to enhance the protein expression.

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