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Comparative Investigations of Codon Engineered Anti-EGFR Antibody to Enhance Protein Expression in Mammalian Host

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Abstract

Monoclonal antibodies are extensively used in biomedical research, in diagnosis of diseases, and in the treatment of such diseases as infections and cancers. Molecular cloning and expression in mammalian host is the most efficient method for the expression of therapeutic monoclonal antibodies. Production of biotherapeutics like monoclonal antibodies in the mammalian host requires optimization of complex molecular parameters to accomplish desired expression such as codon optimization. Codon optimization is a competent gene engineering approach for effective protein expression. Gene designing enables exploitation of degeneracy of genetic code to boost target recombinant protein expression. The present work describes comparative analysis of codon optimization from two different sources. Trastuzumab genes were codon optimized by using GeneArt (Thermo fisher scientific) codon optimization services as well as in-house codon optimization algorithm. Trastuzumab genes were cloned and expressed in pBG-SVII(pgk) mammalian expression vector. In-house codon optimized Trastuzumab demonstrated proficient results in pBG-SVII(pgk) mammalian expression vector when compared to GeneArt codon optimization services (Thermo fisher scientific) codon optimization. Current study reveals that in-house codon optimization algorithm is advantageous to achieve higher level of monoclonal antibody expression in mammalian host.

Keywords
Codon optimization, Expression vector, monoclonal antibody, Trastuzumab.

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1. INTRODUCTION:

Monoclonal antibodies have made transformation from scientific tool to powerful human therapeutics.

The rapid development of high yielding and robust manufacturing process for monoclonal antibodies is an area of significant research. Several antibodies were developed in last two decades. Numerous recombinant antibodies were used in the treatment of inflammatory diseases and types of solid tumors [1].The use of parallel cloning strategies employing different constructs are used to increase the rate of heterologous protein expression [2][3][4]. There are several hosts accessible which are used for heterologous protein expression studies [5]. However for expression of monoclonal antibodies, mammalian cell culture technology is preferred as a production platform owing to the quality of post translational modifications, specially glycosylation patterns [6]. Chinese Hamster Ovary (CHO) cells have become predominant host of choice for the production of most marketed recombinant monoclonal antibodies [7]. Even though, mammalian cell culture is an efficient technology for the expression of monoclonal antibodies, [8] few limitations are still present for the expression of heterologous protein including slow growth rate, low productivity and high production costs [9], [10] [11]. To overcome these restrictions of monoclonal antibodies expression, it is strongly important to enhance production yield by optimizing the molecular factors like codon optimization, choice of signal sequence and elements of expression vector hence must be considered in protein expression studies [12],[13],[14], [15]. Codon optimization is a gene designing approach to enhance heterologous gene expression and functions without altering the amino acid sequence of the protein for improvement in recombinant gene expressions [16].

Generally, the protein comprises 20 different standard amino acids [17]. However total 64 codons code for these 20 amino acids including stop codons. A three nucleotide codon in the nucleic acid codes single amino acid [18]. All amino acids are encoded by more than one synonymous codons except Methionine and Tryptophan [19]. Synonymous codons are different codons those encode same amino acids. This aspect of genetic code is referred as degeneracy of code [18] [20]. Numerous studies have demonstrated use of synonymous codons as nonrandom process [21-23]. Several strategies ideally used for codon optimization like synonymous codon usage [24], gene copy number [25], mRNA secondary structure [26], removal of instability motifs, elimination of direct repeats, abundance of tRNA, [24] removal of splicing sites should be taken into accountability [27]. Optimization of these factors is responsible to enhance transcription, translation and protein folding efficiency leading to a remarkable heterologous protein expression [15].

The phenomenon of appearance of synonymous codons at non-random frequencies is called as codon usage bias (CUB). CUB is always measured by the relative synonymous codon usage (RCUB) [28, 29]. Codon usage bias (CUB) varies from species to species, within a genome, or even within a host. Each host organism has its own preferred choice of nucleotide usage to encode any particular amino acid referred as codon usage [30]. Another measure of a codon optimization is Codon adaptation index (CAI). Codon adaptation index defines a relative adaptiveness to genes. Mammals and prokaryotes vary in their codon preferences for translation of mRNA into proteins. It was observed that coding sequence of the DNA affects mRNA level, mRNA degradation and the host cell growth rate. Synonymous codon substitution at the 5' of the mRNA can impact mRNA structure and stability. Higher percentage of GC in genes affects the protein translation via formation of secondary mRNA structure and impact protein expression of the heterologous gene. This secondary structure may reduce or stop the protein translation by reducing thermodynamic stability of mRNA. Computational analysis of the protein structure database identified that high frequency codons are associated with structural elements such as α helices where clusters of low frequency codons are more likely to be associated with β strands, random coils and structural domain boundaries [31], therefore alpha helices are encoded by the rapidly translating and beta sheets are preferentially coded by the slow translating codons [28]. Minimization of GC content in the gene of interest up to 40 to 60 % can eliminate the secondary structure formation and increase the yield of protein [32]. This may be achieved by using AT rich synonymous codons. Synonymous codons for the same amino acids may exhibit the dissimilar translational efficiency [19]. Different host organism has different codon preference and amount of tRNA are available for recognizing different codons These features may independently reduce the speed of ribosomal in open reading frame. Studies also discovered that translation proceeds via trans locational pause cycle. When the length of pause is variable, the rate of translocation is fast. Such pauses are recommended to appropriate administer protein folding in vivo [33] [28]. Low level of protein expression is also usually accompanying with the use of increased number of rare codons. The major reason behind the concept is abundance of tRNA pool. In most organisms, highly expressed genes contain a greater percentage of codons comparable to

abundant tRNA. Translational elongation is major determinant of composition of the proteome affecting the amount and quality of each protein. Abundance of tRNA to decode the synonymous codons varies in amount and requirement of some codons for wobble interactions between the third base of the codon and first base of the tRNA anticodon. Studies suggested that an optimal codons subset is decoded by abundant tRNAs, efficiently translated and used nearly exclusively in many high expressed genes in yeast and E. coli. The translation elongation rate at different codons depending upon the supply of tRNA and that slower translation rate and reduce translation efficiency [34]. Rare codons usually translate less rapidly. Consistent with this observation the theory of mRNA translation predicts that rare codons are preferred within first 50 amino acids because they slow down the rate of elongation and prevent ribosomal crowding and thereby provide additional layer to thermodynamically less stable mRNA [15]. Recently various web-based, basic and advanced softwares are available at hand. GeneArt, Genewiz, Eurofins, Genescript DNA 2.0 are top existing suppliers for synthetic gene designing and codon optimization [35].

Some of the possible features of codon sequences are enlisted that potentially influence protein expression level are listed in Table No. 1

Table 1:Possible features of codon which influence protein expression level.

Sequence feature	Possible effect	Reference
G+C content		[16, 27]
High G+C content (>70%)	mRNA secondary structure formation, slow down or inhibition of translation	
Very low G+C content (<30%)	Can slow down transcription elongation	
mRNA secondary structure		[34, 36-38]
Global	can promote m RNA stability	
Near RBS	Reduction in translation initiation,	
Repetitive sequence	inhibition of translation through formation of mRNA stem loops	
Rare codons		[24, 39, 40]
Global	can result in translational pausing, tRNA depletion resulting in low protein yield, mRNA degradation	
At protein domain boundaries	Promotes accurate Co-translational folding	
Alternative start codons	Shorter protein (if in frame), mixture of proteins, incorrect protein if out of frame	

In 1998 Trastuzumab was approved by the US Food and Drug Administration (FDA) and by the European medicines Agency (EMA) in 2000, in with Her 2+ MBC to Genentech\ Roche. Trastuzumab is a humanized IgG monoclonal antibody used in the treatment of metastatic breast cancer (MBC) [41]. Breast cancer is most common type of cancer in women. Passive targeted therapies using monoclonal antibodies against breast cancer specific tumors markers have made significant progress in recent years. [42] Trastuzumab specifically binds to external domain of Her2 receptor and blocks the downregulation pathways of the cells [43, 44][45].

In the current study of codon optimization, Trastuzumab heavy and light chain genes were codon optimized differently by two sets of optimal codons and cloned and expression in pBG-SVII(pgk) mammalian expression vector using CHO cells. One set was codon optimized from commercial gene optimization service provider (GeneArt – Thermo fisher scientific). The another set of Trastuzumab was codon optimized by in house developed codon optimization algorithm.

2. MATERIALS AND METHODS:

2.1 Generation of trastuzumab heavy and light chain codon optimized genes:

Full length Trastuzumab amino acid sequence was obtained from Drug bank database. (Accession number: DB00072). Appropriate secretory signal sequence was selected for heavy and light chain genes. One set of codons optimized Trastuzumab heavy and light chain genes was synthesized by GeneArt (Thermo scientific.) For in-house codon optimization, amino acid sequence translations were performed by using Kazuka database according to codon preference of Chinese Hamster ovary (CHO). The preferred codons were analyzed according to the in-house codon optimization algorithm. Final designed constructs from in-house codon optimized Trastuzumab (Trastuzumab heavy chain -pBG015, Trastuzumab light chain-pBG014) sequences and

GeneArt codon optimized Trastuzumab (Trastuzumab heavy chain -pHBHC, Trastuzumab light chain-pBG003) were synthesized. Gene synthesis were performed by GeneArt (Thermo scientific) for both the sequences. Four genes of codon optimized Trastuzumab were synthesized which is schematically represented in figure no. 1 to 4

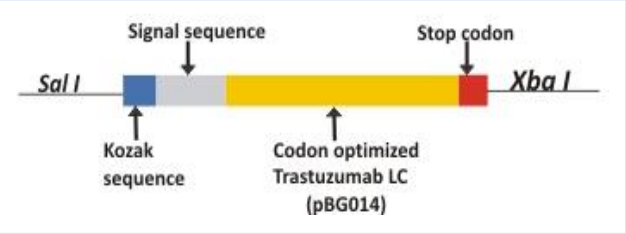

Figure 1: Schematic representation of in-house codon optimized Trastuzumab Light chain gene.

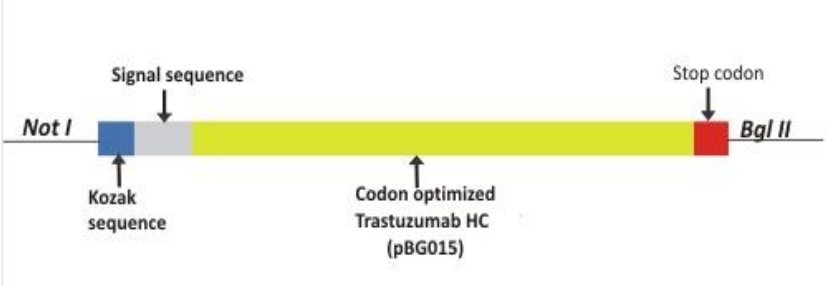

Figure 2: Schematic representation of in-house codon optimized Trastuzumab heavy chain gene.

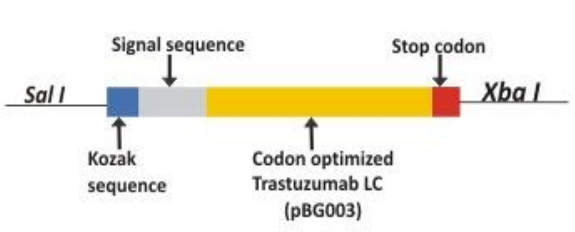

Figure 3: Schematic representation of GeneArt codon optimized Trastuzumab Light chain gene.

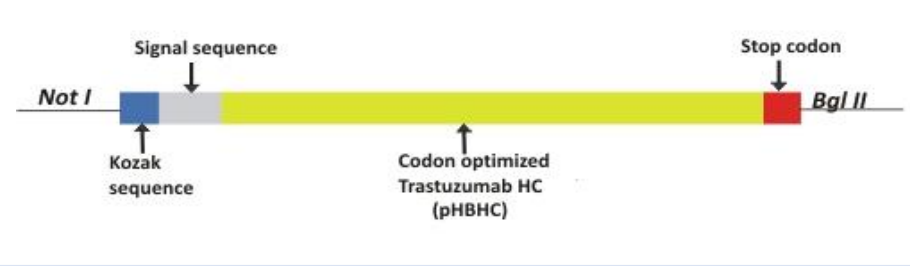

Figure 4: Schematic representation of GeneArt codon optimized Trastuzumab heavy chain gene.

2.2 Cloning codon optimized trastuzumab heavy and light chain genes in pBG-SVII(pgk) expression vector:

The codon optimized heavy and light genes (GeneArt optimized and In-house codon optimized) were cloned into pBG-SVII(pgk) mammalian expression vector. The GeneArt optimized and in-house optimized Trastuzumab Light chain genes were cloned between *Sal I* (Thermo scientific) and *Xba I* (Thermo scientific) restriction sites and expressed *via*

CMV promoter. Similarly, the GeneArt optimized and In-house optimized Trastuzumab heavy chain genes were cloned between *Not I* (Thermo scientific) and *Bgl II* (Thermo scientific) restriction sites and expressed through pEF1α promoter. Summary of the both codon optimized Trastuzumab heavy and light chain genes is enlisted in table 2.

Table 2: Summary of gene optimization parameters of In-house and GeneArt codon optimized Trastuzumab.

Sr. No.	Parameters	Light chain		Heavy chain	
		pBG003 (GeneArt optimized)	pBG014 (In-house optimized)	pHBHC (GeneArt optimized)	pBG015 (In-house optimized)
1	Codon adaptation Index %	0.98	0.93	0.98	0.92
2	GC content %	64.24	60.68	63.38	59.93
3	CIS acting elements	0	0	0	0
4	Repeat sequence	0	0	0	0

2.3 Stable expression scale up and fed batch studies of geneart and in-house codon optimized trastuzumab:

To generate stable cell pool of GeneArt codon optimized Trastuzumab and In-house codon optimized Trastuzumab, purified plasmid DNA were transfected using freedom CHO-S kit (Thermo scientific). Plasmid DNA of each codon optimized combination were transfected in CHO-S cells according to freedom CHO-S Kit manual. Establishment and generation of stable pools for selection of high producers were performed by three-phase amplification process. Increasing concentrations of Puromycin (Gibco) and Methotrexate hydrate (Sigma) were used to obtain the stable pool (10 µg/mL Puromycin + 100 nM Methotrexate; 20 µg/mL Puromycin + 200 nM Methotrexate and 30 µg/mL Puromycin + 500 nM Methotrexate). Single cell cloning was performed using the final amplified pool of 30 µg/mL Puromycin + 500 nM Methotrexate selection pressure from each codon optimized DNA pool as the combination of selection pressure illustrated highest protein concentration. 120 single cell clones were screened primary by ELISA (primary screening on Day 4) from each combination and 15 high producing clones were selected for further studies. The selected clones from each combination were scaled up 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 % cell viability were determined until the culture viability drops to 65%. The protein expression was evaluated by Enzyme linked immuno sorbent assays (ELISA).

3. RESULTS:

3.1 Trastuzumab gene optimization and cloning in pBG-SVII(pgk):

GeneArt codon optimized and In-house codon optimized Trastuzumab genes were cloned in pBG-SVII(pgk) mammalian expression vector using standard molecular biology cloning methods. Obtained positive clones of In-house and GeneArt codon optimized bicistronic clones were characterized by restriction digestion method. A single clone of each codon optimized Trastuzumab was selected for the transfection and further protein expression studies. The vector map of final Trastuzumab clone construct is shown in Figure 5.

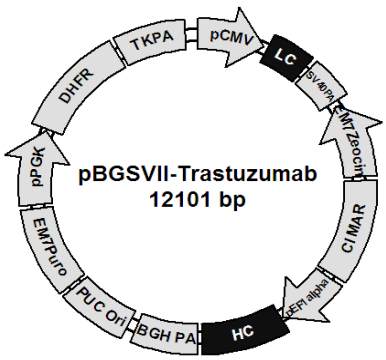

Figure 5: Schematic representation of pBG-SVII(pgk)-trastuzumab clone

Detailed description of expression cassette of MCS I and MCS II of pBG-SVII(pgk) is shown in figure 6 and 7.

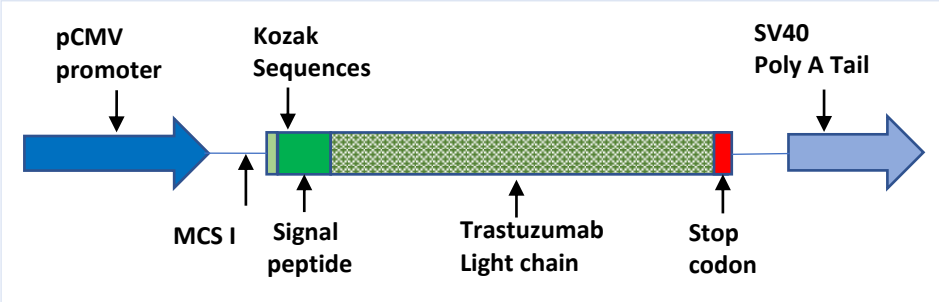

Figure 6: Expression cassette of MCS I of pBG-SVII(pgk) expression vector.

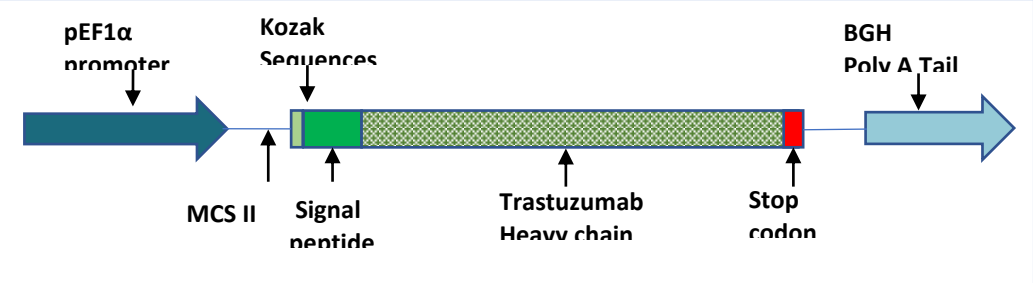

Figure 7: Expression cassette of MCS II of pBG-SVII(pgk) expression vector.

3.2 Stable expression scales up and fed batch studies of in-house and geneart codon optimized trastuzumab: Protein expression of In-house codon optimized Trastuzumab and GeneArt codon optimized Trastuzumab were performed by ELISA. Figure 8 and figure 9 shows single cell cloning fed batch results of the protein express ion of codon optimized Trastuzumab.

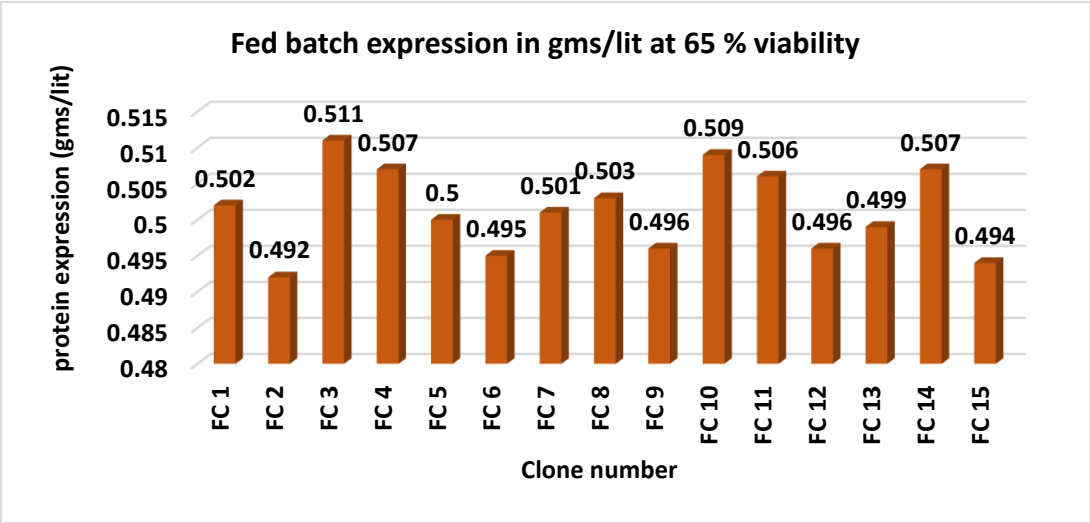

Figure 8: Graphical representation of final yield for Geneart codon optimized Trastuzumab obtained in the fed batch culture.

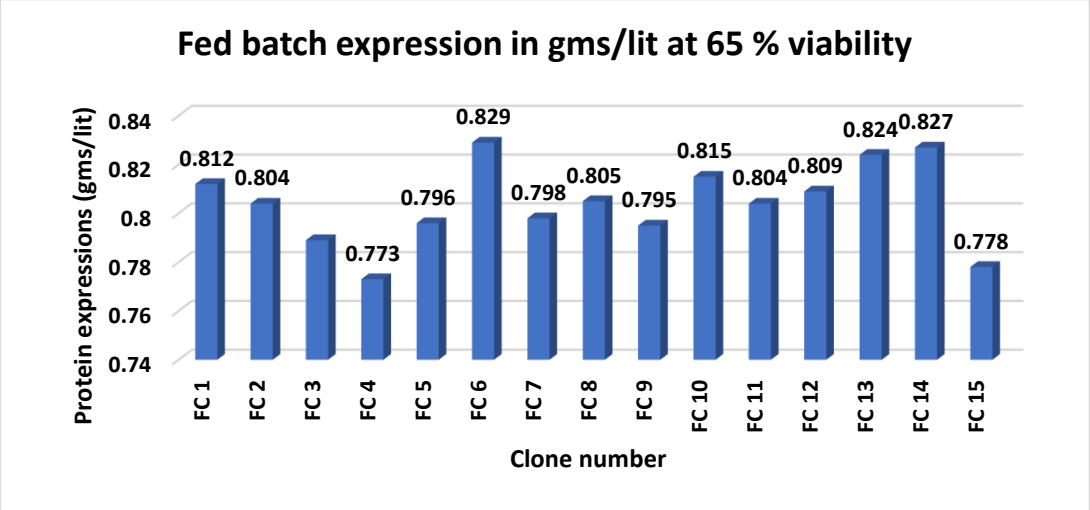

Figure 9: Graphical representation of final yield for In-house codon optimized Trastuzumab obtained in the fed batch culture.

4. DISCUSSION:

A common strategy for optimization of codons is usage of biased codons. Species specific difference in the codon biased results in tRNA depletion and effectively compensated by the expression host [46]. Manipulation of codon biased to enhance the rate of protein expression is based on codon adaptation index (CAI). The best result using CAI optimization can be obtained when subset of less frequently codons are replaced [47]. Current study indicates that codon adaptation index of in-house codon optimized and GeneArt codon optimized trastuzumab lies between 0.92 to 0.94 whereas the standard value shows 0.8 to 1.0 range. Recent studies indicate that rare codon analysis as well as mRNA secondary structure are effective factors of codon optimization to achieve high protein expression [48][49]. In the present study, the GC content of both combinations of codon optimized genes are maintained in the range of 60 to 64 %. During in-house codon optimized Trastuzumab genes development, the high frequency synonymous codons were selected from the Kazuka database and distributed in genes. Repeated sequences, restriction cloning sites, start codons, high GC containing codons were precisely eliminated from gene sequence so that mRNA secondary structures and rate of translation will not affect gene expression. Rare codons were eliminated, and high frequency codons were selected in order to reduce poor translational efficiency.

In this study of strategy I, GeneArt sequence optimizer [35] have been used for the GeneArt codon optimized sequence while many online tools have been used for the in-house codon optimization analysis. According to Figure 8, codon optimization based on the GeneArt sequence optimizer algorithm, in CHO-S cells enhanced Trastuzumab expression and highest Trastuzumab protein expression of single cell clone obtained at the 65% viability of the fed batch culture was 0.511gms/lit. Remaining clones reported the protein expression in the range of 0.490 to 0.510 gms/lit Similarly in strategy II (Figure 9), In-house codon optimization of Trastuzumab exhibited the enhancement of Trastuzumab protein production in the range of 0.770 to 0.830 gms/lit. The highest protein production in the strategy II was 0.829 gms/lit. A total of 15 clones were selected for fed batch studies from each combination of codons optimization. There have been reports of similar increase in protein production following gene optimization reported ~1.8- fold increase in the amount of antibody after gene optimization using geneart sequence optimizer compared to nonoptimized construct [13]. Increase in the preferred codon usage for host cells improves translational efficiency of heterologous protein which is performed by placing high frequency codons in gene of interest instead of any other synonymous codons. Also decrease in the GC content and removal of repeated sequences plays a very important role in decreasing the m RNA secondary structure and improving mRNA stability.

5. CONCLUSION:

The aim of the present study was to enhance Trastuzumab monoclonal antibody expression by In-house codon optimization. From the obtained results, it was concluded that strategy II of in-house codon optimization by designed algorithm exhibited

more proficient results than the GeneArt codon optimization (strategy I) in pBG-SVII(pgk) mammalian expression vector.

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