Uncovering Methods for the Prevention of Protein Aggregation and Improvement of Product Quality in a Transient Expression System

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Mammalian expression systems are used routinely for the production of recombinant proteins as therapeutic molecules as well as research tools. Transient expression has become increasingly popular in recent years due to its rapid timeline and improvements in expression level. While improvements to transient expression systems have focused mainly on the level of protein expression, the aspect of protein quality has received little attention. The removal of undesirable products, such as aggregation, depends primarily on purification, requiring additional cumbersome steps, which can lead to a lower product yield and longer timelines. In this study, we show that reducing the level of transcription by transfecting at a lower gene dose improves the quality of secreted molecules prone to aggregation. For gene dosing to have this effect, it is critical for the carrier DNA to be an empty vector containing the same elements as the gene containing plasmid. This approach can be used in combination with a temperature shift to hypothermic conditions during production to enhance the effect. The observed improvements not only minimized aggregation levels, but also generated products with overall superior quality, including more homogeneous signal peptide cleavage and N-linked glycosylation profiles. These techniques have produced a similar improvement in product quality with a variety of other molecules, suggesting that this may be a general approach to enhance product quality from transient expression systems. © 2014 The Authors. Published by Wiley Periodicals, Inc. on behalf of American Institute of Chemical Engineers

Keywords: transient expression, 293-EBNA1, mild hypothermia, gene dosage, aggregation

Introduction

In the past few years, the use of transient gene expression (TGE) systems in mammalian cells for the generation of recombinant antibodies has gained widespread acceptance and popularity.¹⁻³ In comparison with producing stable cell lines, TGE is particularly useful in early research when a number of potential therapeutic candidates are needed for assessment or when a molecule is needed in a short time frame. The broad application of TGE was made possible due to advances in commercially available cell culture media that supported the growth and maintenance of mammalian cells under serum-free suspension conditions. Concurrently, the development of economic and efficient gene transfer processes has made large-scale transfection of mammalian cells for the generation of recombinant proteins practical and cost-effective.²⁻⁴ Such processes enable the very rapid generation of up to gram quantities of recombinant antibodies in a very short time frame, typically 1 to 3 weeks, which is in stark contrast to the establishment of stable cells for the same application that often takes several months.⁶ The Human Embryonic Kidney 293 (HEK293) cell line expressing Epstein–Barr virus nuclear antigen 1 (EBNA1) is well established for recombinant expression of secreted proteins.⁷ Binding of EBNA1 to an expression vector containing OriP allows for episomal replication of the vector during cell division. High expression in 293-EBNA1 cells is furthermore obtained by using optimized OriP-based expression vectors containing the strong CMV promoter.⁷ Protein production up to 1 g/L per liter of cell culture has been reported.⁶ However, not all molecules are well behaved. While the diversity of molecules limits generalizations of good and bad features, some red flags that warn of potential aggregation include extracellular domain truncations, engineered protein fusions, or proteins with a large or odd number of cysteine residues. Transient expression can exacerbate the problems, leading to poor quality and low purification yields. While properly folded molecules can be isolated from these aggregated species during purification, it does require additional method development, potentially impacting the benefit of fast timelines associated with TGE.

This study was conducted primarily as a case study for the expression of Activin Receptor IIB (ECD) with human IgG1 fused to the C-terminus (ActRIIB). ActRIIB is a high affinity activin type 2 receptor which mediates the signaling
of a subset of TGF-β family ligands including myostatin, activin, and GDF11. ActRIIB acts a decoy receptor to potently inhibit these pathways. When expressed transiently in 293-EBNA1 cells, ActRIIB showed high levels of aggregation, which greatly impacted purification recovery and protein quality. These observations prompted our study to identify transient expression conditions that improve the quality of molecules prone to aggregation. We show that lowering the rate of protein expression, through the combination of reduced gene copy number and hypothermic conditions, has a marked impact on aggregation as well as other quality attributes such as glycosylation and signal peptide processing. The expression conditions that were identified enable the generation of high quality protein from TGE. We also show this approach to be applicable to other aggregation prone molecules.

Materials and Methods

Molecules

Activin Receptor IIB (ECD) (1-134) was fused to wild-type human IgG1 Fc at the C-terminus. The correctly folded molecule forms a dimer through dimerization of the Fc region. ActRIIB begins with the native signal peptide. ActRIIB has three N-linked glycosylation sites per peptide with two of them located in the Activin Receptor portion and one in the Fc portion. The EGFR(ECD)-His molecule is a monomer of the extracellular domain of a human epidermal growth factor receptor and with a native signal peptide and a 6-His extension at the C-terminus for purification. The Fc-HwTxIV is a dimer of a wild type human IgG1 Fc fused to a spider Huwentoxin IV peptide with a human VK1 signal peptide. Dimerization takes place between the Fc domains.

Molecular biology

The cDNAs of target proteins were cloned into the pTT5 vector using conventional cut and ligate molecular biology techniques. Expression vector pTT5 is licensed from the National Research Council of Canada (NRCC). pTT5 and pBluescript SK+ (pBSSK) vectors (Agilent Technologies, Santa Clara, CA) lacking a coding DNA insert, and sheared salmon sperm (ssDNA) (Thermo Scientific, Waltman, MA) were used as carrier DNA.

Hybrid vectors, in which pBSSK elements were swapped into pTT5 vectors, were generated by traditional PCR followed by restriction digestion and ligation cloning. Those pTT5/pBSSK hybrid vectors include versions of pTT5 in which the origin of replication, the promoter, and the combination of origin and promoter were replaced with counterparts from pBSSK (Figure 1). To replace the CMV promoter cassette in pTT5, the Lac promoter b-Gal was amplified.
from pBSSK by PCR with SpeI and EcoRI restriction sites added to the 5' and 3' ends. It was then cloned into pTT5 using cut and ligate methods. To replace OriP in pTT5, the Ori F1 was amplified by PCR from pBSSK with BglII added to the 5' end and the PvuI site was amplified from pTT5. The two PCR fragments were assembled by overlapping PCR before being inserted into pTT5 using cut and ligate methods at BglII and PvuI sites.

**Tissue culture and analysis**

The human embryonic kidney (HEK) 293 cell line, stably expressing Epstein Barr virus Nuclear Antigen-1 (293-EBNA1), and the pTT5 expression vector were obtained from the National Research Council (Montreal, Canada) and used according to the detailed published guideline. Before transfection, cells were passaged by dilution into fresh Freestyle F17 media (Thermo Scientific) with seeding densities of 2.0e5 to 3.0e5 cells/mL. Cells were grown to a transfection density of 1.1e6 cells/mL in disposable plastic shake flasks (Corning, Enfield, CT) at 110 RPM at 37°C. Transfections were performed as previously described (Durocher et al., 2002). Twenty-four hours after transfection, 25 μL of a 20% mixture of Tryptone N1 (Organotechnie, France) in growth media was added per mL of culture and temperature shifts to 32°C were implemented 24 h after transfection as noted. Samples were collected 4 and 7 days after transfection.

**Protein purification and characterization**

Small scale purification for titer analysis and size exclusion chromatography (SEC) was performed using a Protein A HPLC column (Grace Vydac; 4.6 × 50 mm) connected to a Hewlett Packard 1050 liquid chromatograph with detection set at a wavelength of 280 nm. After conditioned medium was loaded on the column, the column was washed with PBS and a solution of 0.1 M sodium citrate and 4 M urea at pH 6.0. These are used to achieve higher binding specificity from ion strength and hydrophobicity respectively. Protein was eluted from the column with IgG elution buffer (Thermo Scientific, Catalog: 21004). The eluate was collected in 70 mM tris, pH 8.0. SEC was performed using the Superose 12 column (GE/Pharmacia Biotech Inc., 1.0 × 30 cm) equilibrated in 2× PBS. Chromatography was carried out using an Agilent 1100 liquid chromatograph.

Western blot analysis was done using Tris-Glycine gels (Thermo Scientific) with a 4 to 20% concentration gradient. Samples were mixed with loading buffer and they were heated at 85°C for 5 min. Blots were probed using horseradish peroxidase (HRP) conjugated rabbit anti-human IgG Fc (Thermo Scientific), and treated with Supersignal West Pico substrate (Thermo Scientific). Images of the Western blots were generated using the Gnome XP imager (Syngene, Frederick, MD) with an exposure time of 0.3 s. For Western blasts of cell lysates, samples were reduced using the NuPAGE reducing agent by Thermo Scientific. Blots were also probed with an anti-GAPDH antibody (Thermo Scientific) as a loading control. Western blot band intensity was measured using Visionworks software (UVP, Upland, CA).

For mRNA quantification of ActRIIB expression, a branched DNA (bDNA) binding assay was performed using the nucleic acid hybridization method by QuantiGene (GeneSpeca, Fremont, CA). Seven days post transfection, 2.5e5 cells were added to PBS and lysis buffer (Panomics, Santa Clara, CA). Samples of lysate were transferred at a dilution of 1:100 to 96-well plates of Luminex beads containing probes for ActRIIB and the housekeeping gene human Cyclophilin B (huCyB). The mRNA signal readings of ActRIIB were normalized to those of huCyB. To generate a strong detection signal, biotin labeled bDNA was added.

**Figure 2.** Secreted protein and aggregate levels of ActRIIB observed by western blot of nonreduced CM samples.

(A) Transiently expressed samples of ActRIIB show a contrast in aggregation in response to a titration of gene doses. The left lane is a purified dimeric ActRIIB from a stable CHO cell line. The lowest band of CM samples consists of the dimer form of the molecule and is at the expected molecular weight, running at the same size as the purified control. All other bands are high molecular weight aggregate species of ActRIIB. (B) Transiently expressed ActRIIB shows a reduction in aggregation upon temperature shift. ActRIIB was transfected at a 100% gene dose. The temperature shift to 32°C was implemented 24 h after transfection as noted. Samples were collected 4 and 7 days after transfection.

![Figure 2](image-url)
higher molecular weight multimers (Figure 2). This aggregation is thought to involve a disulfide shuffle, in which the cys29 does not correctly bind to the cys59 residue as it does in the native structure, but rather covalently binds to another molecule of ActRIIB (data not shown). With the natural pairing of the Fc portion as a dimer, the second binding site on the surface of some of the molecules results in multimer assemblies of the molecule at various lengths.

We hypothesized that the level of ActRIIB expression was exceeding the secretory capacity of the cell, resulting in the misfolding of ActRIIB and incorrect disulfide pairing. The pTT5 expression vector utilizes a strong modified CMV promoter, which results in very high levels of gene expression in 293-EBNA1 cells. In highly optimized TGE practices with 293-EBNA1 cells, we and others can achieve upwards of 90% transfection efficiency. Although a transcriptional limitation in HEK cells has been observed, for difficult to express molecules, the post-translation bottlenecks are considered the limiting factors in transient expression. To test this, we explored alternate conditions that might slow the expression rate, to match the secretory capacity of the cell and allow for improved product quality.

Transfection with gene dosing reduces aggregation of ActRIIB

Past experiments with gene dosing have revealed that levels of DNA in an optimized transient transfection protocol most directly affect DNA uptake and endosomal release. This has been demonstrated in previous literature with the use of various combinations of DNA and reagent achieving comparable transfection efficiency and expression levels. In one example, coding DNA was decreased and supplemented with carrier DNA. In the other example, the transfection complex was made with lower amounts of DNA and reagent, followed by the addition of more reagent before transfection. These results not only showed that less plasmid DNA can be used for transfection, but also that DNA and reagent amounts can be modified without having a negative effect on expression. In more recent studies to examine the rate limiting steps in transient expression, it was reinforced that intracellular DNA amounts were often not the limiting factors in the process. In this case, a titration of gene dosage had a very minor impact on protein expression until a certain point, upon which the titer dropped significantly with each change in gene dose. The point at which the relationship between the gene dose and the titer changed was hypothesized to be the gene dose at which the rate of transcription replaced translational/post-translational processing as the rate-limiting step. Indeed, it was recently shown that lowering the amount of coding plasmid for transfection and replacing it with an empty vector resulted in a lower aggregate level of a nonsecreted cytoplasmic protein. Together, this suggests that performing a transfection at a gene dose that limits gene expression to match the secretory capacity.

Results and Discussion

Upon transient expression of ActRIIB-Fc into 293-EBNA1 cells, a large percentage of the secreted protein consisted of

Table 1. Quantification of Aggregate by SEC

| SEC Trace | Experimental Conditions | Total Protein (mg/L) | Aggregate (%) | Nonaggregated Protein (mg/L) |
|-----------|-------------------------|----------------------|---------------|-----------------------------|
| 1         | 100% ActRIIB Plasmid    | 46.7                 | 36            | 29.8                        |
| 2         | 100% ActRIIB Plasmid    | 17.5                 | 20            | 14                          |
| 3         | 20% ActRIIB Plasmid     | 36.9                 | 14            | 31.7                        |
| 4         | 20% ActRIIB Plasmid     | 12.4                 | 4             | 11.9                        |
of the host cell has the potential to improve product quality of secreted molecules.

In our experiments, the gene dose was lowered by reducing the amount of coding plasmid and replacing it with an equal amount of carrier DNA to maintain the optimized ratio of cells, DNA, and reagent for transfection. Unless otherwise noted, the carrier DNA used in these studies was an empty pTT5 vector. In our initial gene dose titration of ActRIIB, aggregate species were prominent at gene doses of 100% and 50%, and declined rapidly among the lower gene doses. The presence of nonaggregate species was prominent at all gene doses and remained at comparable levels at gene doses of 100%, 50%, 30%, and 20%. At a gene dose of 10%, both nonaggregate and aggregate species decreased significantly compared with the higher gene dose levels (Figure 2A).

A temperature shift to 32°C decreases aggregation at the cost of lower protein titers

The use of mild hypothermic expression conditions has been widely reported to have a positive effect on recombinant protein production. This advantage is namely higher productivity and lower cell growth in CHO expression systems. Of particular interest to us, mild hypothermic production has been observed to reduce the degree of molecular aggregation. ActRIIB expressed in the 293-EBNA1 expression system, followed by a temperature shift from 37°C to 32°C at 24 h after transfection resulted in a decrease in the percentage of the aggregated species at harvest, from 36% of total protein produced under standard conditions down to 20% of the total protein (Figure 2B). The benefit of a hypothermia-induced productivity was not observed in this system, as expression was two to threefold lower.

Gene dosing and hypothermic production have a complementary effect on reducing aggregate

We further assessed the impact of gene dose and hypothermic conditions on aggregate levels of the ActRIIB molecule through protein A purification followed by SEC (Figure 3, Table 1). When low gene dose (20%) or low production temperature (32°C) were used separately, each resulted in a reduction of aggregate species, from 36% to 14% or 20%, respectively (Figure 3, Table 1). In both the 20% gene dose or 32°C culture condition, total protein expression levels were lower than the standard conditions, but despite the lower overall expression, the 20% gene dose generated a similar amount of nonaggregated protein as compared to standard production conditions, 31.7 mg/L versus 29.8 mg/L, respectively. The hypothermic production conditions on the other hand resulted in a lower amount of nonaggregated recombinant protein at 14 mg/L. The 20% gene dose therefore appeared to be more beneficial than the hypothermic production as it resulted in lower amounts of aggregate and more than double the purified yield of the desired product. The combined use of a 20% gene dose and a 32°C production temperature showed a synergistic impact on reducing the overall level of aggregate as it decreased the amount of aggregated species down to 4% of the total protein. However, the impact of hypothermic conditions on overall yield was still observed when used in combination with gene dosing with 11.9 mg/L of total protein after a protein A capture (Table 1).

Gene dosing and hypothermic production result in a more fully processed and homogeneous protein

The ActRIIB material generated both from standard conditions and the 20% gene dose with a 32°C production condition were further analyzed for quality attributes (glycosylation and signal peptide processing). The ActRIIB molecule has three potential N-linked glycosylation sites per peptide with sequences of NQSG, NSSG, and NSTY in series. In the MALDI-MS profiles of samples generated under standard conditions using TGE, there are two significant peaks of 94.66 kDa and 85.40 kDa, which correspond to the fully glycosylated and nonglycosylated forms of the NQSG site on the molecule, respectively (Figure 4). In the MALDI-MS profile of ActRIIB generated with a 20% gene

| Experimental Conditions | Observed Sequences | Ratio (%) |
|-------------------------|--------------------|-----------|
| 100% ActRIIB plasmid, 37°C | Primary start point (EAETRE) | 72 |
|                         | Secondary start point (ETRE) | 14 |
|                         | Noncleaved signal peptide | 14 |
| 20% ActRIIB plasmid, 32°C | Primary start point (EAETRE) | 75 |
|                         | Secondary start point (ETRE) | 25 |
|                         | Noncleaved signal peptide | 0 |
dose and at a 32°C production temperature, there is a single 94.89 kDa peak, demonstrating a marked increase in the ratio of occupied N-linked glycosylation sites. Hence, this expression condition generated protein with a more homogeneous profile of glycoforms than our standard approach.

Improved signal peptide processing was also observed in the low gene dose and hypothermal production conditions (Table 2). According to signal peptide predictions made using the Hidden Markov Model, ActRIIB is expected to have a heterogeneous cleavage of its signal peptide with the majority of the mature protein starting at residues EAETRE. The remainder of the mature protein should start two residues later with ETRE. However, TGE derived protein under standard conditions yielded a 14% noncleaved signal peptide in addition to the correctly processed forms with 72% starting at residues EAETRE and 14% at ETRE. ActRIIB produced with a 20% gene dose and a 32°C production temperature lacked this nonprocessed species, with 75% of the protein starting at residues EAETRE and 25% started at residues ETRE.

Gene dosing and hypothermic production lowered aggregation with additional molecules

As with ActRIIB, when low gene dose or low production temperature was applied to other aggregation prone molecules, each approach resulted in a reduction of aggregated species. With EGFR(ECD)-His, the combined use of a 20% gene dose and a 32°C production temperature had the greatest impact on aggregate levels (Figure 5A). When approaches to reduce aggregation were tested on Fc-HwTxIV, a peptide toxin fused to Fc, the temperature shift during production resulted in a substantial decrease in total expression. Using lower gene doses, on the other hand, decreased the amount of aggregation with little if any effect on the expression of the properly folded molecule (Figure 5B). We find that the use of gene dosing and hypothermic production is a generally applicable technique that reduces the level of aggregation in many of the aggregation prone molecules that we have tested. It is considered to be particularly applicable in cases where improper assembly of the molecule results in free cysteine residues or hydrophobic patches on the surface of the protein.

Gene dosing with different carrier DNA forms varies in expression and aggregation profiles

Previous utility of gene dosing has primarily been implemented with the use of sheared genomic DNA, such as salmon sperm (ssDNA) as carrier DNA. Gene dosing with ssDNA has been recognized to decrease the necessary amount of plasmid DNA (pDNA) for transient expression. This not only reduces the cost of transient expression, but can streamline the process by enabling expression of high throughput panels from small amounts of pDNA. Our initial experiments with gene dosing were supplemented with an empty pTT5 vector as carrier DNA. To determine if the benefits observed with pTT5 as carrier DNA could be applied in a more cost-effective manner, the technique was tested with ssDNA. The pBSSK vector was also included as a control due to it being in supercoiled plasmid form like pTT5 but lacking eukaryotic regulatory elements (the CMV promoter and OriP). Comparing the secreted protein of gene dose titrations supplemented with these forms of carrier DNA, there was a clear contrast in which aggregate decreased significantly in pTT5 supplemented cultures, but was a prominent portion of the total protein in all ssDNA supplemented cultures. Cultures supplemented with pBSSK closely resembled those supplemented with ssDNA, indicating that the advantages of using pTT5 were due to the vector elements in the carrier DNA and not the supercoiled structure of the vector (Figure 6A).
A hypothesis as to how the aggregate could be lower in transfections supplemented with pTT5 carrier DNA is that transcriptional elements in pTT5 carrier DNA are competing for transcription factors or other regulatory elements that would otherwise interact with the ActRIIB carrying plasmid. The carrier DNA would essentially be acting as a transcription factor decoy. This phenomenon has been widely described in literature and can very efficiently reduce transcription from a competing promoter plasmid.\textsuperscript{27,28} To test this, mRNA was measured from a titration of gene doses that were supplemented with pTT5 or pBSSK vectors. Results showed significantly less ActRIIB mRNA in cultures supplemented with pTT5 than in cultures supplemented with pBSSK at gene doses of 10\%, 5\%, and 2\% (Figure 6B). This difference correlates with expression and aggregate levels, supporting the hypothesis that vector elements in pTT5 carrier DNA are acting as a decoy and decreasing transcription of the gene of interest. Intriguingly, even when two transfection conditions using different carrier DNA forms resulted in similar mRNA levels, the product quality was distinctly

Figure 6. Comparison of ActRIIB with gene dosage supplemented with different forms of carrier DNA.

Samples were harvested on day 7. (A) Western blots of nonreduced CM from a titration of gene doses comparing pTT5, pBSSK, and ssDNA as carrier DNA. Samples were loaded at a volume of 5 \( \mu \)L each. Purified dimeric ActRIIB is loaded in the first lane as a control. (B) ActRIIB mRNA levels from gene dose titrations supplemented with pTT5 or pBSSK. Samples were measured by bDNA binding analysis and normalized to endogenous cyclophilin B bDNA measurement \( ([\text{ActRIIB}]/[\text{CycB}])\). (C) Reduced samples of lysate from pTT5 and pBSSK supplemented gene dose titrations were compared by western blot. Samples in the western blot were normalized to 1.0e5 cells per well and probed with anti-huFc and anti-GAPDH.
of interest, and in turn amplifies the effect of reduced gene and protein. This likely affects the transcription of the gene interact with gene containing plasmids to generate mRNA competing for transcription factors that would otherwise port our hypothesis that the pTT5 carrier DNA could be protein aggregate levels, though the CMV promoter has a CMV appear to be required for the full effect on mRNA and differences in expression and aggregation. Both the OriP and presence of the CMV promoter cassette and OriP that cause c closely to cultures in which transfections were supplemented dOri, ActRIIB mRNA levels and aggregate levels correlated dOri), or both CMV and OriP (pTT5 dPro, dOri) (Figure 1). This indicates that it is the nuclear retention of the carrier DNA, thereby prolonging the competition for transcription factors. Further investigation is underway to better define these mechanisms.

This was measured, using densitometry analysis of CM and lysate western blots. At each of the gene doses that were tested, the amount of nonsecreted ActRIIB was significantly lower in cultures supplemented with pTT5 than those supplemented with pBSSK (Figure 6C, Table 3). In this comparison, the ratio of secreted to nonsecreted protein increased at lower gene doses in pTT5 supplemented transfections, but not in pBSSK supplemented transfections (Table 3; Figure 6C). This measurement of secretion efficiency correlates more closely to aggregation levels than it does to mRNA levels. This data suggest that the transfections supplemented with pTT5 carrier DNA result in protein that is more efficiently processed and secreted than those supplemented with pBSSK carrier DNA. Lowering the transcription rate to match the folding and secretory capacity of the endoplasmic reticulum is a likely mechanism, but further investigation of transcription, translation, and secretion rates is underway to address this question more fully.

Vector hybrids reveal that elements in carrier DNA affect transcription and protein expression

To further investigate the role of vector elements in lowering transcription, a series of carrier DNA vectors were engineered in which elements of pTT5 were removed and replaced with stuffer DNA from pBSSK. These removed elements and resulting vectors include the CMV promoter cassette (pTT5 dPro), the OriP origin of replication (pTT5 dOri), or both CMV and OriP (pTT5 dPro, dOri) (Figure 1). In gene dose transfections supplemented with pTT5 dPro, dOri, ActRIIB mRNA levels and aggregate levels correlated closely to cultures in which transfections were supplemented with pBSSK (Figures 7A,B). This indicates that it is the presence of the CMV promoter cassette and OriP that cause differences in expression and aggregation. Both the OriP and CMV appear to be required for the full effect on mRNA and protein aggregate levels, though the CMV promoter has a greater impact than OriP (Figures 7A,B). These results support our hypothesis that the pTT5 carrier DNA could be competing for transcription factors that would otherwise interact with gene containing plasmids to generate mRNA and protein. This likely affects the transcription of the gene of interest, and in turn amplifies the effect of reduced gene dose as compared with pBSSK, or even ssDNA. The observed relevance of the origin of replication may involve the nuclear retention of the carrier DNA, thereby prolonging the competition for transcription factors. Further investigation is underway to better define these mechanisms.

Conclusions

We have shown that cell culture conditions can be strategically modified to groom protein quality. Combining transfection at a low gene dose with hypothermic production conditions resulted in dramatic improvements in the characteristics of difficult to express proteins. The improvements in product quality extend beyond aggregation to include aspects such as more homogeneous signal peptide cleavage and N-linked glycosylation. The data presented here suggest that this approach has a broad application among difficult to express proteins.

As a means to improve product quality, gene dosing with a matching empty vector enables the user to optimize a number of attributes with a single approach. In that aspect, it provides significant advantages over isolating the desired product during purification. In addition to the ActRIIB, the above described approach was also tested on other aggregate prone recombinant proteins in our repertoire. From a series of experiments, we found that this approach has broad applications to effectively reduce aggregation with optimal gene doses, using pTT5 as the carrier DNA. While there are a number of means to reducing the rate of transcription, such as the use of weaker promoters, the use of carrier DNA to supplement reduced amounts of coding DNA has some distinct advantages. The most prominent of those advantages is the ability to titrate down the amount of coding DNA used in order to reach the rate of transcription most suitable for a given molecule. The data also suggest that using pTT5 as a carrier DNA may have effects beyond lowering transcription that result in enhanced product quality. With more difficult to express molecules, such as ActRIIB, the use of gene dosing can be coupled with mild hypothermic expression conditions to achieve an enhanced improvement in quality. We consider this approach to be an important advancement in the practice of transient expression in which issues of product quality can be addressed effectively at the expression phase of production rather than being limited to methods of protein purification and/or protein engineering.

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Table 3. Densitometry Analysis of ActRIIB at 6 Gene Doses Supplemented with pTT5 and pBSSK

| Experimental Conditions | Secreted Protein (%) | Nonsecreted (%) | Secreted/Nonsecreted |
|-------------------------|---------------------|----------------|---------------------|
| pDNA (%)    | Carrier DNA (%)    | pTT5     | pBSSK     | pTT5     | pBSSK     | pTT5     | pBSSK     |
| 100         | 0                   | 100      | 100       | 100      | 100       | 1.0      | 1.0       |
| 50          | 50                  | 90       | 84        | 31       | 57        | 2.9      | 1.5       |
| 20          | 80                  | 83       | 92        | 11       | 59        | 7.3      | 1.6       |
| 10          | 90                  | 22       | 90        | 5        | 66        | 4.8      | 1.4       |
| 5           | 95                  | 18       | 74        | 2        | 54        | 9.1      | 1.4       |
| 2           | 98                  | 9        | 41        | 1        | 40        | 9.5      | 1.0       |
Literature Cited

1. Pham PL, Kamen A, Durocher Y. Large-scale transfection of mammalian cells for the fast production of recombinant protein. Mol Biotechnol. 2006;34:225–237.

2. Schlaeger E, Christensen K. Transient gene expression in mammalian cells grown in serum-free suspension culture. Cytotechnology. 1999;30:71–83.

3. Wurm FM, Bernard A. Large-scale transient expression in mammalian cells for recombinant protein production. Curr Opin Biotechnol. 1999;10:156–159.

4. Durocher Y, Perret S, Kamen A. High-level and high throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. 2002;30:59.

5. Pham PL, Perret S, Cass B, Carpentier E, St-Laurent G, Bisson L, Kamen A, Durocher Y. Transient gene expression in HEK293 cells: peptone addition post-transfection improves recombinant protein synthesis. Biotechnol Bioeng. 2005;90:332–344.

6. Backliwal G, Hildinger M, Chenuet S, Wulfhard S, De Jesus M, Wurm FM. Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. Nucleic Acids Res. 2008;36 (15): e96.

7. Massie B, Moser DD, Koutroumanis M, Vitte-Mony I, Lamoureux L, Couture F, Paquet L, Guilbault C, Dionne J, Chahla D, Jolicoeur P, Langelier Y. New adenovirus vectors for protein production and gene transfer. Cytotechnology 1998;28:53–64.

8. Lee S-J, McPherron AC. Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci USA. 2001;98:9306–9311.

9. Souza TA, Chen X, Guo Y, Sava P, Zhang J, Hill JJ, Yaworsky PJ, Qiu Y. Proteomic identification and functional validation of activins and bone morphogenetic protein 11 as candidate novel muscle mass regulators. Mol Endocrinol. 2008;22:2689–2702.

10. Zhou X, Wang JL, Lu J, Song Y, Kwak KS, Jiao Q, Rosenfeld R, Chen Q, Boone T, Simonet S W, Lacey DL, Goldberg AL, Han HQ. Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. Cell. 2010;142:531–543.

11. Zhang A, Pastor L, Nguyen Q, Luo Y, Yang W, Flagella M, Chavli R, Bui S, Nguyen CT, Zheng Z, Wueh I, McMaster G, Witney F. Small interfering RNA and gene expression analysis using a multiplex branched DNA assay without RNA purification. J Biomol Screen. 2005;10:549–556.

12. Flagella M, Bui S, Zheng Z, Nguyen T, Zhang A, Pastor L, Ma Y, Yang W. Crawford KL, McMaster GK, Witney F, Luo Y. A multiplex branched DNA assay for parallel quantitative gene expression profiling. Anal Biochem. 2006;352:50–60.
13. Hacker DL, Kiseljak D, Rajendra Y, Thurnheer S, Baldi L, Wurm FM. Polyethyleneimine-based transient gene expression processes for suspension-adapted HEK-293E and CHO-DG44 cells. Prot Expr Purif. 2013;92:67–76.
14. Carpentier E, Paris S, Kamen A, Durocher Y. Limiting factors governing protein expression following polyethyleneimine-mediated gene transfer in HEK293-EBNA1 cells. J Biotechnol. 2007;128:268–280.
15. Mason M, Sweeney B, Cain K, Stephens P, Sharfstein ST. Identifying bottlenecks in transient and stable production of recombinant monoclonal-antibody sequence variants in Chinese hamster ovary cells. Biotech Prog. 2012;28:846–855.
16. Hasegawa H, Wendling J, He F, Trilisky E, Stevenson R, Franey H, Kinderman F, Li G, Piedmonte DM, Osslund T, Shen M, Ketchem RR. In vivo crystallization of human IgG in the endoplasmic reticulum of engineered Chinese hamster ovary (CHO) cells. J Biol Chem. 2011;286:19917–19931.
17. Stoops J, Byrd S, Hasegawa H. Russell body inducing threshold depends on variable domain sequences of individual human IgG clones and the cellular protein homeostasis. Biochim Biophys Acta 2012;1823:1643–1657.
18. Kichler A, Leborgne C, Danos O. Dilution of reporter gene with stuffer DNA does not alter the transfection efficiency of polyethyleneimines. J Gene Med. 2005;7:1459–1467.
19. Halff EJ, Versteeg M, Brondijk THC, Huizinga EG. When less becomes more: optimization of protein expression in HEK293-EBNA1 cells using plasmid titration – a case study for NLRs. Prot Expr Purif. 2014;99:27–34.
20. Galbraith DJ, Tait AS, Racher AJ, Birch JR, James DC. Control of culture environment for improved polyethyleneimine-mediated transient production of recombinant monoclonal antibodies by CHO cells. Biotechnol Prog. 2000;70:25–31.
21. Kaufmann H, Mazur X, Fussenegger M, Bailey JE. Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. Biotechnol Bioeng. 1999;63:573–582.
22. Underhill MF, Marchant RJ, Carden MJ, James DC, Smales CM. On the effect of transient expression of mutated elf2alpha and elf4E eukaryotic translation initiation factors on reporter gene expression in mammalian cells upon cold-shock. Mol Biotechnol. 2006;34:141–149.
23. Wulhfard S, Tissot S, Bouchet S, Cevey J, De Jesus M, Hacker DJ, Wurm FM. Mild hypothermia improves transient gene expression yields several fold in Chinese hamster ovary cells. Biotechnol Prog. 2008;24:458–465.
24. Andersen DC, Bridges T, Gawlitzek M, Hoy C. Multiple cell culture factors can affect the glycosylation of Asn-184 in CHO-produced tissue-type plasminogen activator. Biotechnol Bioeng. 2000;70:25–31.
25. Rodrigo J, Spearman M, Huzel N, Butler M. Enhanced production of monomeric interferon-B by CHO cells through the control of culture conditions. Biotechnol Prog. 2005;21:22–30.
26. Rajendra Y, Kiseljak D, Manoli S, Baldi L, Hacker DL, Wurm FM. Role of nonspecific DNA in reducing coding DNA requirement for transient gene expression with CHO and HEK-293E cells. Biotechnol Bioeng. 2012;109:2271–2278.
27. Tomita S, Tomita N, Yamada T, Zhang L, Kaneda Y, Morishita R, Ogihara T, Dzau VJ, Horuchi M. Transcription factor decoy to study the molecular mechanism of negative regulation of renin gene expression in the liver in vivo. Circ Res. 1999;84:1059–1066.
28. Brown AJ, Mainwaring DO, Sweeney B, James DC. Block decoys: transcription-factor decoys designed for in vitro gene regulation studies. Anal Biochem. 2013;443:205–210.

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