Development and Optimization of CHOgro® Transient Expression Technologies for High Titer Antibody Production in Suspension CHO cells

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Abstract

During early stage drug development, quickly obtaining relevant candidate proteins through transient transfection can accelerate drug discovery. High titers are often obtained from Human Embryonic Kidney (HEK) 293 derived cell types; however, the use of different host cells between early stage transient and later stable protein production is a concern and can lead to the advancement of false-positive candidates. Chinese hamster ovary (CHO) cells are a desirable target cell type due to growth characteristics and a history of regulatory approval; however, their use has been hampered by low transient gene expression levels. To address this short-coming, we have created a robust and simple CHO transient protein expression system enabled by critical media attributes such as high density cell growth, quick adaptation, and minimization of cell clumping post-transfection. The CHOgro® Expression System was developed through systematic optimization of transfection protocol parameters including: cell density, transfection reagent, media formulation and culture temperature leading to a commercially accessible high titer CHO transient transfection platform. Through this optimization, antibody titers increased 2-10 fold over existing technologies with higher amounts of antibody secreted per cell. Six different representative antibody constructs were also successfully expressed using the CHOgro® Expression System. Notably, even CHO cells maintained in other commercially available media formulations (e.g. FreeStyle™ CHO Expression Medium) can be seamlessly adapted with a full media exchange to the CHOgro® Expression Medium 24 hours prior to transfection and yield multi-fold increases in transient expression levels. With the CHOgro® Expression System, high protein titers can now be achieved in suspension CHO cells through high density transient transfection.

Introduction

For the development of a suspension CHO transient transfection platform, many parameters require consideration including: cell type, transfection reagent formulation, growth media compatibility and the interplay of a multicomponent complex system.

There have been many reports of engineered CHO derived cells improving product yield (refs 1-4); however, these cells are not commercially available or require prohibitive licensing agreements. We sought to find a platform that was widely accessible; therefore we optimized the conditions using a popular suspension CHO cell type, FreeStyle™ CHO-S. In our experience these cells gave higher titers than their CHO-K1 counterparts.

Chemical transfection methodology is dependent on electrostatic interactions between the negatively charged nucleic acid (e.g. plasmid DNA) and positively charged polymer and/or lipid. This allows for ample coating and condensation of the nucleic acid with a positive charge to promote binding of the transfection complex to negatively charged cell surface receptors (e.g. heparin sulfate proteoglycans) and based on the size restrictions of other endocytic pathways, internalization is likely mediated through macropinocytosis. Typically a transfection complex grows larger over time; therefore it is important to add the transfection complexes at an optimized time point so they are compatible with rapid internalization into the cell. This may be one factor that contributes to the transfectability of a particular cell type.

Media formulation is essential for cell growth and viability. Serum-free complete medium formulations contain a myriad of components to mimic the attributes of undefined serum for *in vitro* culture conditions. Many commercial serum-free medium formulations contain compounds that interfere to varying levels with chemical transfection methods. This is likely due to negatively charged compounds disrupting complex formation between the transfection reagent and nucleic acid or destabilizing the complex as it is internalized into the cell. Since the recipes of commercial formulations for serum free media are frequently maintained as trade secrets, we sought to develop a chemical transfection system where the culture medium and transfection formulation act synergistically to promote high efficiency transfection and protein yield in batch culture.

Cell density at the time of transfection is a key parameter for increasing protein titers. Considerations when increasing the cell density include: transfection reagent and media compatibility, sufficient transfection complexes per cell, as well as ample nutrients in the media formulation to support the increased cell population. If a high efficiency transfection reagent is utilized and a large percentage of the population is transfected, then more aggregate cellular resources are available to make the protein of interest. Without an episomal replication system (e.g. EBNA and oriP) plasmids expressed during transient transfection will be lost during cell

division; therefore greater transfection efficiency is desirable. In addition, the ability of EBNA and OriP to maintain plasmids in cells of hamster origin (e.g. CHO) has been questioned, although it may have other unrelated benefits (ref 1). At higher cell densities, medium nutrients will be depleted faster therefore a rich medium is required. The CHOgro® Expression Medium supports prolonged high density cultures in fed batch culture (Figure 1.). During normal cell maintenance of CHO-S cells grown in CHOgro® Expression Medium, the doubling time is 18 hours compared to 24 hours with CHO-S cells grown in FreeStyle™ CHO Expression Medium grown using the same culture conditions.

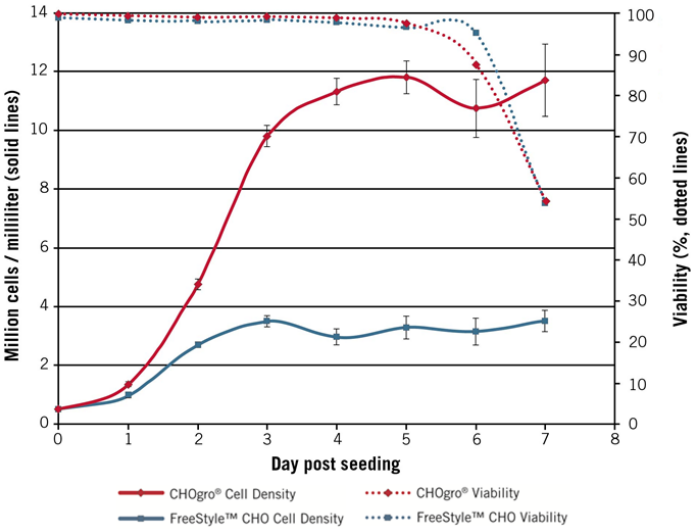


Figure 1. Suspension CHO Cells Grow to High Density in the CHOgro® Expression Medium. Triplicate flasks of FreeStyle™ CHO-S cells were seeded in CHOgro® Expression Medium (red line) or FreeStyle™ CHO Expression Medium (blue line) at a cell density of 0.5 x 10^6 cells/ml, 40 ml per 125 ml shake flask (Thomson). Cell counts (solid line) and viability (propidium iodide staining, dotted line) were measured daily using a Guava easyCyte™ 5HT flow cytometer (EMD Millipore). Error bars represent the standard deviation of three readings of biological triplicates.

The CHOgro® Expression Medium elicits a positive effect on cellular health and more rapid cell division which translates to higher protein expression levels during transient transfection. Three fold or greater differences in protein yield are observed when CHO-S cells are transfected in CHOgro® ™ Expression Medium independent of the transfection reagent used (Figure 2A).

The selection of a high performance transfection reagent, such as *Trans*IT-PRO® Transfection Reagent, further increases the transient transfection titer. *Trans*IT-PRO® was identified through empirical testing of proprietary lipid and polymer libraries for high performance with suspension CHO and 293 cell types. During the development of the CHOgro® Expression System we screened hundreds of polymer and lipid combinations and *Trans*IT-PRO® Transfection Reagent yielded the highest performance. This validated our previous findings where *Trans*IT-PRO® was the top reagent for transient transfection of suspension CHO and 293 cell types.

In contrast to many candidate compounds, *Trans*IT-PRO® was able to maintain high transfection efficiencies at elevated cell densities. In addition, significantly less cell clumping is observed post-transfection with the CHOgro® Expression System which leads to accurate cell counts and increased culture viability through the availability of nutrients (Figure 2B).

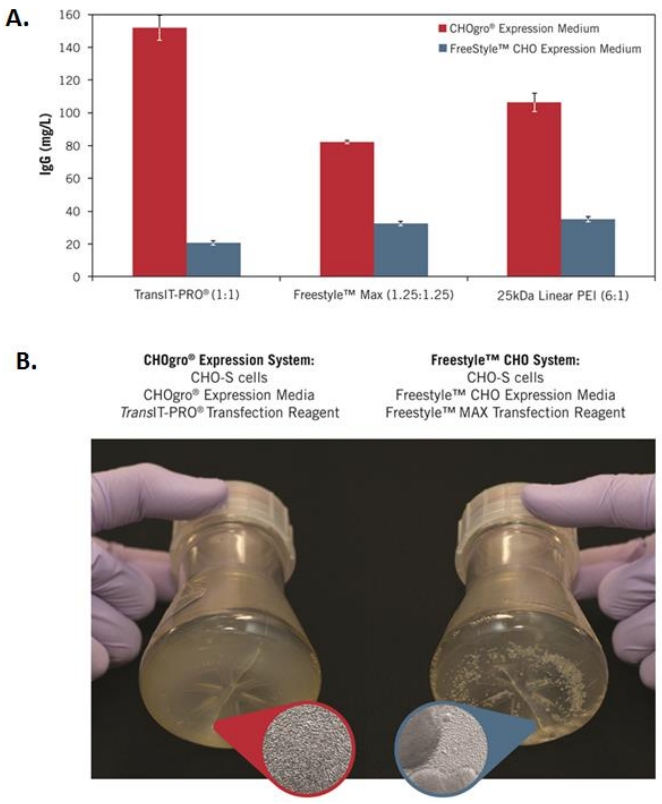


Figure 2. CHOgro® Expression Medium Yields Multi-fold Increases in Antibody Titer. (A) Human IgG1 was produced by transient transfection using *Trans*IT-PRO® (1:1), FreeStyle™ MAX (1.25:1.25) or 25kDa linear PEI (6:1) transfection reagents according to the manufacturers' or published protocol (reagent:DNA ratio). Transfections were performed using 1 µg plasmid DNA per milliliter of culture and cell densities of 2 x 10^6 cells/ml or 1 x 10^6 cells/ml for the CHOgro® Expression Medium (red bars) or FreeStyle™ Expression Medium (blue bars), respectively. At the time of transfection. FreeStyle™ CHO-S cells were cultured in CHOgro® Expression Medium or FreeStyle™ CHO Expression Medium and plated into non-treated 6-well plates (2ml/well) for transfection. Antibody levels were also analyzed from clarified day 6 supernatants using a human IgG ELISA (Zeptometrix). Error bars represent the standard deviation of triplicate technical replicates. (B) The same experimental conditions described in (A) were used to photograph transfected cells in flasks, inset show microscopic images of cells.

Reagent-to-DNA Ratio

The transfection reagent-to-DNA ratio is a critical parameter for high efficiency. For ease of use, we have formulated the *Trans*IT-PRO® Transfection Reagent to work at a 1:1 ratio (volume: weight), thereby reducing the amount of reagent required per milliliter of culture. Similarly, we have found a total plasmid concentration of 1 µg/ml to work best with the *Trans*IT-PRO® Transfection Reagent. It should be noted that

this is substantially lower than flow electroporation methods which can be upwards of 10 µg per milliliter of culture (ref 5).

High Density Transfection

CHOgro® Expression Medium is capable of simultaneously facilitating high density cell growth and transient transfection. Cell density titrations were performed at 1, 2 and 4 million cells/ml at the time of transfection with CHO-S cells grown in CHOgro® Expression Medium and FreeStyle™ CHO Expression medium. The transfections were performed with *Trans*IT-PRO® or FreeStyle™ Max Transfection Reagents and a plasmid encoding a human IgG1 antibody. Greater than two-fold enhancements in titers were observed by increasing the initial cell density from one to two million cells/ml. When the CHOgro® Expression Medium is combined with the *Trans*IT-PRO® Transfection Reagent; a greater than four-fold enhancement in titer is observed compared to the FreeStyle™ CHO Expression System (Figure 3).

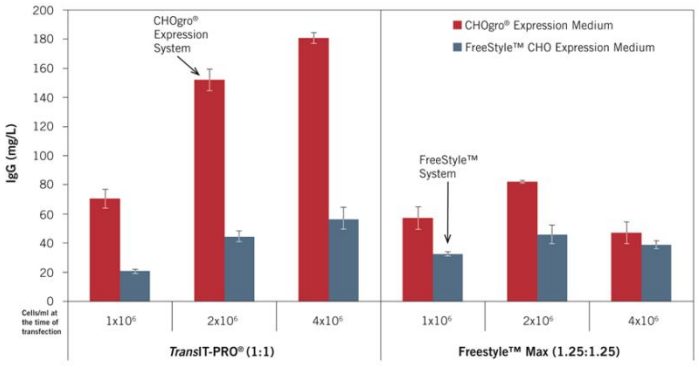


Figure 3. Higher Cell Densities Leads to Higher Titers Using the CHOgro® Expression System. Human IgG1 was produced by transient transfection using *Trans*IT-PRO® (1:1) or FreeStyle™ MAX (1.25:1.25) transfection reagents according to the manufacturers' protocol (reagent:DNA ratio, volume:weight). Transfections were performed using 1 µg plasmid DNA per milliliter of culture and cell densities of 1, 2 or 4 x 10^6 cells/ml at the time of transfection. FreeStyle™ CHO-S cells were cultured in CHOgro® Expression Medium (red bars) or FreeStyle™ CHO Expression Medium (blue bars) and plated into non-treated 6-well plates (2ml/well) for transfection. Day 6 supernatants were clarified and analyzed using a human IgG ELISA (Zeptometrix). Error bars represent the standard deviation of triplicate technical replicates. The arrows the standard protocol for the CHOgro® Expression System and FreeStyle™ CHO Expression System are designated by the arrows.

High Levels of Antibody Secretion

Antibodies are temporarily retained at the cell surface during secretion and can be captured by simple cell staining with a phycoerythrin conjugated "secondary" antibody and then measured using flow cytometry (Figure 4A). In brief, cells are gently pelleted and washed twice with CHOgro® Expression Medium, and incubated with a staining solution comprised of CHOgro® Expression Medium, polyvinylpyrrolidone (PVP, 1%, blocking agent) and a 1:100 dilution of the Anti-Human IgG R-phycoerythrin antibody for 30 minutes in the dark. Cells are pelleted and washed twice with CHOgro® Expression Medium and analyzed by flow cytometry. The level of antibody secreted, measured by mean fluorescence intensity, correlates well with antibody titers measured by ELISA at day 6 post-transfection. Higher levels of antibody secretion and titers measured by ELISA were observed with the CHOgro® Expression System at 6 days post-transfection (Figure 4B).

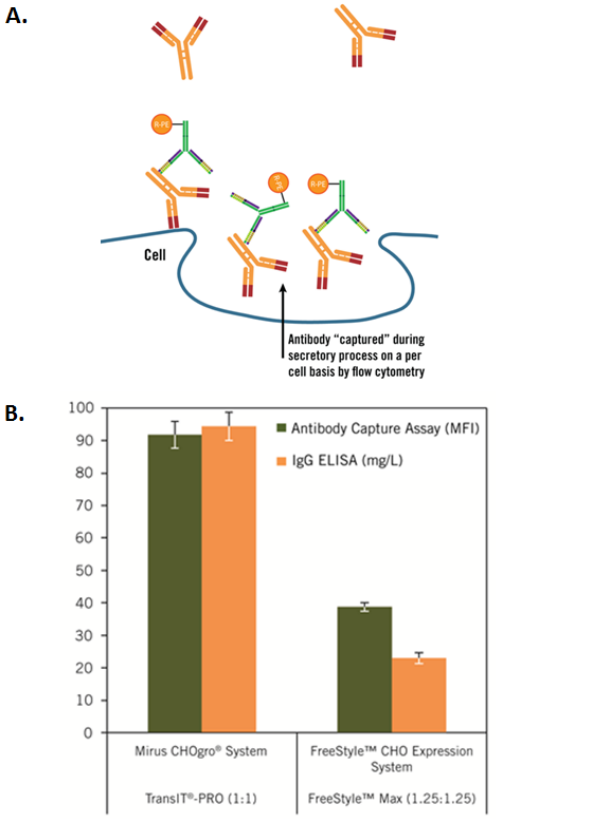


Figure 4. More Antibody is Secreted Per-Cell With the CHOgro® Expression System. Human IgG1 was produced by transient transfection using *Trans*IT-PRO® (1:1) or FreeStyle™ MAX (1.25:1.25) transfection reagents according to the manufacturers' protocol (reagent:DNA ratio). Transfections were performed using 1 µg plasmid DNA per milliliter of culture and cell densities of 2 x 10^6 cells/ml or 1 x 10^6 cells/ml for the CHOgro® or FreeStyle™ System, respectively, at the time of transfection. FreeStyle™ CHO-S cells were cultured in CHOgro® Expression Medium or FreeStyle™ CHO Expression Medium and plated into non-treated 6-well plates (2ml/well) for transfection. (A) Cartoon schematic antibody capture assay. Briefly, an aliquot of cells was washed, and incubated with an anti-IgG-PE antibody and blocking agent, washed and assayed for fluorescence. (B) Fluorescence was measured using a Guava easyCyte™ 5HT flow cytometer. Antibody levels were also analyzed from clarified day 6 supernatants using a human IgG ELISA (Zeptometrix). Error bars represent the standard deviation of triplicate technical replicates.

Medium Exchange for Quick Adaptation

Cell adaptation to a new formulation of serum-free complete medium can be an arduous and lengthy process. For the CHOgro® Expression Medium we sought to make this transition as fast and seamless as possible, allowing researchers' greater flexibility in upstream processes. In our experience stock vials of CHO-S cells can be brought out of cryopreservation and diluted directly into the CHOgro® Expression Medium with high viability and doubling times. Alternatively, CHO-S cells maintained in FreeStyle™ CHO Expression Medium or FreeStyle™ F17 Expression Medium

can be centrifuged and resuspended in 100% fresh CHOgro® Expression Medium without adverse effects (Figure 5A). Indeed, within 24 hours of the media exchange to the CHOgro® Expression Medium, antibody titers increase up to 10-fold over the cells grown in the FreeStyle™ CHO Medium and are similar to cells maintained continuously in CHOgro® Expression Medium (Figure 5B). This illustrates the robustness of the CHOgro® Expression Medium for CHO-S cell culture and transient transfection.

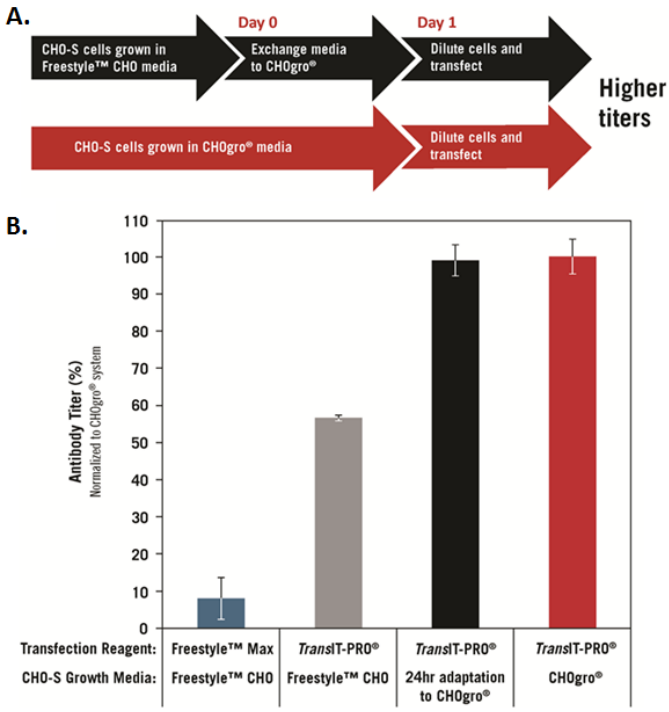


Figure 5. Media Exchange Leads to Higher Protein Production. FreeStyle™ CHO-S cells were cultured in FreeStyle™ CHO Expression Medium or CHOgro® Expression Medium and 24 hours prior to transfection a subset of the cells grown in FreeStyle™ CHO Expression Medium were centrifuged and exchanged with 100% fresh CHOgro® Expression Medium. The cells were allowed to grow and adapt for 24 hours prior to transfection with FreeStyle™ MAX (1.25:1.25) or *Trans*IT-PRO® (1:1) transfection reagents according to the manufacturers protocol (reagent:DNA ratio) and a hIgG1 encoding construct. Transfections were performed using 1 µg plasmid DNA per milliliter of culture and cell densities of 1 x 10^6 cells/ml for cells transfected with FreeStyle™ Max and 2 x 10^6 cells/ml for cells transfected with *Trans*IT-PRO®. All cells were plated into non-treated 6-well plates (2ml/well) for transfection. (A) Workflow schematic of media exchange of CHO-S cells from FreeStyle™ CHO Expression Medium to CHOgro® Expression Medium (black arrow) or the normal CHOgro® Expression System (red arrow) (B) Day 6 supernatants were clarified and analyzed using a human IgG ELISA (Zeptometrix). Data is normalized to the complete CHOgro® Expression System (red bar). Error bars represent the standard deviation of triplicate technical replicates.

Ease of use and Robustness

High antibody titers can be achieved within 7 days of transfection using a simple workflow (Figure 6A). No additional supplements are added post-transfection reducing the risk of contaminating your culture. A separate incubator at 32°C is not required; however, up to a 2-fold increase in titer is observed at longer time points when cells are shifted to mild hypothermic conditions (32°C) at 24 hours post-transfection (Figure 6B). It is well established that lower temperatures cause changes in cell metabolism and gene expression (refs 6-7). With the CHOgro® Expression System we observe increased viable cell counts over extended incubation times (> 7 days), as well as, specific production rates. In addition, less cell death at the time of protein harvest leads to higher quality protein through decreased release of cell protease. The broad applicability of the CHOgro® Expression System is demonstrated with the transient expression of five different clinically relevant IgG antibodies (Figure 6C). Actual titers vary depending solely on the protein that is encoded. All 10 vectors (individual heavy and light chains) were created in the same vector backbone using codon optimization parameters for both CHO and 293 cells types (DNA 2.0, Menlo Park USA).

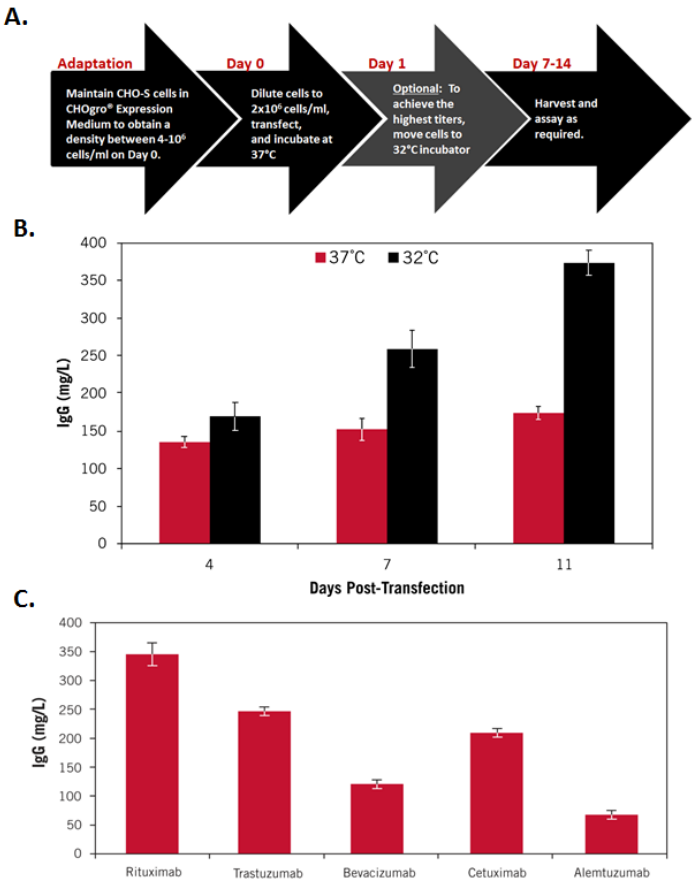


Figure 6. CHOgro® Expression System is easy to use and robust. (A) Workflow schematic of transient transfection process (B) Human IgG1 was produced by transient transfection with the *Trans*IT-PRO® Transfection Reagent and 1 µg plasmid DNA per milliliter of culture at a 1:1 reagent:DNA ratio. Cells were transfected at a density of 2 x 10^6 cells/ml in 20 ml of CHOgro® Expression Medium in 125 ml shake flasks (Thomson). Antibody levels were also analyzed from clarified day 4, 7 and 11 supernatants using an in-house human Fc ELISA with a full-length human IgG standard. All flasks were incubated at 37°C for 24 hours; at that point designated parallel flasks were switched to 32°C for the remainder of the experiment. Error bars represent the standard deviation of triplicate technical replicates. (C) Five different antibodies were produced by transient transfection using *Trans*IT-PRO® as specified in (B); all flasks were shifted to 32°C at 24 hours post-transfection. Day 11 supernatants were clarified and analyzed using a human IgG ELISA (Zeptometrix). Error bars represent the standard error of the mean of triplicate technical replicates.

Cost Considerations

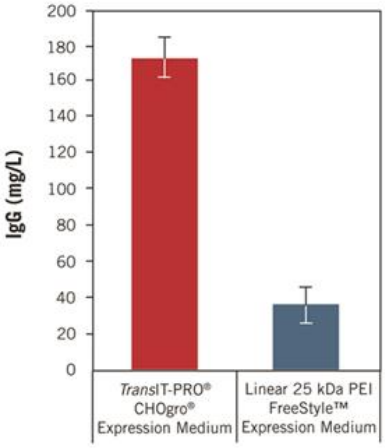
Basic polymeric transfection methods, such as 25 kDa linear PEI, are commonly employed in biotherapeutics largely due to their seemingly low cost; however, actual costs are much higher than the CHOgro® Expression System when considering lower protein yield, labor commitment and material costs. In a head-to-head comparison, the *Trans*IT-PRO® Transfection Reagent in combination with the CHOgro® Expression Medium gave 4-fold higher yields than cells transfected with 25 kDa linear PEI in the FreeStyle™ CHO Expression Medium (Figure 7A). In a hypothetical scenario where a researcher requires 150 mgs of protein, only 1L of total culture would be required; whereas for 25 kDa linear PEI, 4 liters would be required. Increased material cost (4x) and slightly increased labor cost (1.5x) leads to a 40% higher cost when using 25 kDa linear PEI (Figure 7B). Additional savings for the *Trans*IT-PRO® Transfection Reagent can be obtained if it is purchased in bulk quantities. Increases in overall productivity are also observed with the CHOgro® Expression System since lower culture volumes allow incubators to accommodate additional culture vessels. The CHOgro® Expression System is compatible with many suspension CHO-derived cell types allowing researchers to use their current cell line or a cell type, such as FreeStyle™ CHO-S, that is commercially available for R&D use. In addition, with the *Trans*IT-PRO® Transfection Reagent and the CHOgro® Expression Medium, no licensing is required for further manufacturing use, giving researchers' peace of mind and contributing to a lower bottom line.

Conclusions

In summary, we have created a pre-optimized system where the transfection reagent and growth medium work in concert to allow high density suspension CHO cells growth, high efficiency transfection, and increased antibody secretion per cell. Fast adaptation to the CHOgro® Expression Medium saves time and agony; many suspension CHO cells double within 24 hours of a full media exchange. The CHOgro® Expression System is a turn-key platform that allows all researchers, regardless of their experience level, to achieve high titers via transient transfection in suspension CHO cells.

For more information about the CHOgro® Expression System visit: www.mirusbio.com/CHOgro

A.



B.

Materials needed for 1L Transfection, 150mg desired yield		
	CHOgro® System	25 kDa Linear PEI w/ FreeStyle™ materials 4x, labor 1.5x
1L media	$100.00	$365.00
100 mL Complex Formation Media	$25.00	$100.00
1 mg DNA	$100.00	$400.00
Transfection Reagent		
Trans IT-PRO® (1:1)	$350.00	
25 kDa linear PEI (6:1)		$1.80
Disposable 1L Culture Flask	$102.00	$442.00
Time in hours * 150 per hour	$750.00	$1,125.00
TOTAL	$1,427.00	$2,433.80

Figure 7. The CHOgro® Expression System Outperforms 25 kDa linear PEI. (A) Human IgG was produced by transient transfection using *Trans*IT-PRO® (1:1) or 25 kDa linear PEI (6:1) in either the CHOgro® ™ or FreeStyle™ Expression System. CHO-S cells were grown in designated medium and split to 30 ml per 125ml shake flask (Thomson). Clarified supernatants were analyzed using a human IgG ELISA (ZeptoMetrix). Error bars represent the standard error of the mean of triplicate technical replicates. (B) In the hypothetical scenario where a researcher needed to produce 150 mg of an IgG protein, 4X more culture volume would be required if 25kDa linear PEI was used with the FreeStyle™ CHO Expression Medium compared to the CHOgro® ™ Expression System. The cost comparison of 4 times the materials and 1.5x the labor costs leads to a 40% reduction in costs if the CHOgro® ™ Expression System is utilized.

References

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