Video Article

High Yield Expression of Recombinant Human Proteins with the Transient Transfection of HEK293 Cells in Suspension

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Abstract

The art of producing recombinant proteins with complex post-translational modifications represents a major challenge for studies of structure and function. The rapid establishment and high recovery from transiently-transfected mammalian cell lines addresses this barrier and is an effective means of expressing proteins that are naturally channeled through the ER and Golgi-mediated secretory pathway. Here is one protocol for protein expression using the human HEK293F and HEK293S cell lines transfected with a mammalian expression vector designed for high protein yields. The applicability of this system is demonstrated using three representative glycoproteins that expressed with yields between 95-120 mg of purified protein recovered per liter of culture. These proteins are the human FcγRIIIa and the rat α2-6 sialyltransferase, ST6GalI, both expressed with an N-terminal GFP fusion, as well as the unmodified human immunoglobulin G1 Fc. This robust system utilizes a serum-free medium that is adaptable for expression of isotopically enriched proteins and carbohydrates for structural studies using mass spectrometry and nuclear magnetic resonance spectroscopy. Furthermore, the composition of the N-glycan can be tuned by adding a small molecule to prevent certain glycan modifications in a manner that does not reduce yield.

Video Link

The video component of this article can be found at https://www.jove.com/video/53568/

Introduction

Producing high yields of appropriately folded and post-translationally modified human proteins for detailed analysis of structure and function remains a significant challenge. A large number of expression systems are available that produce recombinant proteins with native-like function and behavior. Bacterial expression systems, predominantly *Escherichia coli* strains, represent the most accessible and commonly used tools in the research arena, due to the simplicity of these expression systems, though yeast, plant, insect and mammalian systems are also described[1-4]. However, the majority of these systems are incapable of appropriate post-translational modification of the target proteins. A fundamental interest of the Barb and Moremen laboratories is producing eukaryotic proteins with appropriate glycosylation. Many human proteins require appropriate glycosylation for proper function (see[5]).

The eukaryotic glycosylation machinery is extensive and capable of making a diverse range of modifications, including both asparagine(N)- and serine/threonine(O)-linked complex glycans[6]. It is estimated that >50% of human proteins are N-glycosylated[7]. Glycans are essential components of many proteins including therapeutic monoclonal antibodies, erythropoietin, and blood clotting factors like factor IX, to name a few. Though multiple methods exist to prepare appropriately N-glycosylated proteins and range from purely synthetic[8-10], to chemoenzymatic[11-14] or recovery from engineered recombinant systems[15-20], not surprisingly, human expression systems have thus far proven to be the most robust methods for generating human proteins.

Many therapeutic human glycoproteins are produced in recombinant systems using mammalian cells. Systems of note are the Chinese Hamster Ovary (CHO), mouse myeloma (NS0), Baby Hamster Kidney (BHK), Human Embryonic Kidney (HEK-293) and human retinal cell lines that are employed in adhesion or suspension culture for protein production[4,21,22]. However, mammalian protein expression systems have required the generation of stable cell lines, expensive growth media and substrate assisted transfection procedures[23].

Mammalian cell transfection is achieved with the aid of numerous agents including calcium phosphates[24,25], cationic polymers (DEAE-dextran, polybrene, polylysine, polyethylimine (PEI)) or positively charged cationic liposomes[26-29]. PEI is a polycationic, charged, linear or branched polymer (25 kDa)[26] that forms a stable complex with DNA and is endocytosed. Upon acidification of the endosome, PEI is thought to swell, leading to the rupture of endosomes and release of the DNA into the cytoplasm[26,30].

Until recently, transient transfection in suspension culture was carried by prior DNA/PEI complex formation followed by addition to the cell culture[29]. However, Würm and coworkers reported a highly efficient protocol optimized for recombinant protein production in HEK293 cells that formed a DNA/PEI complex *in situ*[31,32]. This avoided preparation, sterilization of the complex, and buffer exchange into a culture medium. Further

optimization by including expression-enhancing plasmids led to significant yield increases[33]. Herein is a method that builds upon these advances and is broadly applicable. Expression conditions may also be altered to impact the N-glycan composition.

The HEK293S cell line, with a gene deletion that halts N-glycan processing at an intermediate stage, leads to the expression of proteins with uniform N-glycans consisting of 2 N-acetylglucosamine residues plus five mannose residues ($Man_5GlcNAc_2$)[34,35]. These cells lack the N-acetylglucosaminyl transferase I (GntI) gene which is required for downstream N-glycan processing[36,37]. The use of glycosyltransferase inhibitors including kifunensine, sialic acid analogs and the fucose analog and 2-deoxy-2-fluoro-fucose has similar effects and limits N-glycan processing[38-41].

The protocol reported here uses the pGEn2 vector as shown in **Figure 1**[42,43], PEI assisted transient transfection into mammalian cells lines (HEK293F or HEK293S cells), and the recovery of high yields of appropriately glycosylated protein. This system is robust and can accommodate various factors including isotope labeling and glycan engineering for the production of large titers of recombinant proteins.

Protocol

This protocol is sufficient for expression using either HEK293F or HEK293S cells.

1. Cell Establishment

1. Culture Inoculation
   Note: All culture manipulation procedures must be carried in a BSL-2 facility and each item brought into the biosafety cabinet must be sterilized by spraying with a 70% ethanol in water solution.
   1. Operate the incubator shaker at 135 rpm, 80% humidity and at 8.0% CO_2 and 37 °C. Turn "on" the UV lamp of the biosafety cabinet at least 1 h prior to working. Prewarm the sealed Medium A and Medium B bottles in the water-bath at 37 °C for 1 hr.
   2. Sterilize 125 ml Erlenmeyer growth flasks with a vented cap, pipettes, pipettors, and prewarmed Media bottles by spraying with the 70% ethanol in water solution. Place these items in the biosafety cabinet. Note: Sterilize only the outer plastic covering the 125 ml Erlenmeyer culture flask, and then remove only when it is inside the biosafety cabinet.
   3. For a 30 ml culture, withdraw 26 ml of Medium A and 3 ml of Medium B (10% of the final culture) and transfer into the 125 ml Erlenmeyer culture flask and gently mix by shaking (hereafter termed as fresh culture medium).
   4. Transfer one vial of cells containing HEK293F cells, frozen at -80 °C, onto ice and move to the culture room. Note: HEK293F cells are supplied at a density of 1 x 10^7 cells/ml.
   5. Warm the vial gently in water-bath at 37 °C to partially thaw the cells (it takes approximately one min and cells should not be completely thawed). Sterilize the outside of the vial with the 70% ethanol in water solution and move it into the biosafety cabinet.
   6. Using 1 ml pipette, withdraw the cell suspension (approximately 0.7 ml) and transfer into the 125 ml Erlenmeyer flask containing 29 ml of fresh culture medium. Close the cover of the culture flask and move it into the incubated shaker.

2. Cell Maintenance: Check Cell Density, Viability and Cell Passages
   1. Check the cells density after 24 hr of thawing of the culture by following the protocol below. Note: Cells are grown for 24 hr after thawing to ensure essential cells growth and viability of >80%.
   2. Sterilize the culture flask with the 70% ethanol in water solution and move it into the biosafety cabinet.
   3. Using a 1 ml pipette and pipettor, slowly withdraw 100 µl of suspension culture and transfer into a sterile 0.5 ml eppendorf tube. Close and transfer the culture flask back into the shaker as soon as possible.
   4. Mix 7.5 µl of a Trypan Blue Solution with 7.5 µl of cells. Mix vigorously with 7.5 µl of the cell/Trypan blue mixture and transfer it to the counting slide.
   5. Turn on the automatic cell counter and place the counting slide into the loading chamber. The auto reader is activated and cells are counted automatically in the units of number of cells per ml and percent viability
   6. Determine the volume of the donor culture required for the transfer to a fresh culture medium at the cells density of 0.3 x 10^6 live cells/ml.
      Note: See a sample calculation in Supplementary Materials.
   7. Repeat steps 1.1.2 and 1.1.3 of the "1.1 Culture Inoculation" protocol above to prepare materials required for fresh culture medium.
   8. Transfer Medium A (23 ml) and Medium B (3 ml) to a fresh 125 ml culture flask. Remove the stock bottles of Medium A and Medium B from the biosafety cabinet.
      Note: Stocks of media bottles should not be kept in the biosafety cabinet at the same time as the HEK 293F cells. This limits the possibility of contaminating the stock medium bottles.
   9. Remove the HEK293 cell culture from the incubator and spray with the 70% ethanol in water solution. Using a new pipette, withdraw the aliquot of cells (4 ml, this volume was determined according to step 1.2.6) from the culture and transfer it to the flask containing fresh culture medium.
   10. Transfer both the cultures from the biosafety cabinet to the incubator shaker set according to 1.1.1. Grow cells to an approximate density of 2-3 x 10^6 live cells/ml.
       Note: The approximate doubling time of cells is 32 hr. It will take 3-4 days to reach this density. Incubate the cells for 3-4 passages to have stable growth pattern of cells prior to the first transfection.

2. Prepare Materials for Transfection

1. Preparing HEK293 Cells (Day 1)
   1. Subculture cells to a density of 1 x 10^6 cells/ml in fresh Medium B 24 hr prior to transfection according to Step 1.2.

Note: the volume of the transfection culture will be dependent upon the number of cells. Larger transfection volumes (>20 ml) will require a greater volume of culture at this stage.

2. Preparing Stocks of Materials
    1. Prepare stock solution of linear Polyethylenimine (PEI) at a concentration of 1 mg/ml in a buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 150 mM NaCl (pH 7.5). Dissolve PEI completely; this may take 30-60 min at RT. Sterilize through 0.22 µM syringe filter and store at -20 °C for long-term use.
    2. Prepare sterile plasmid DNA (according to steps 2.2.2.1-2.2.2.4). The plasmid construction procedure is described elsewhere[42]. A brief procedure for DNA preparation is explained here:
        1. Grow a culture of chemically-competent *Escherichia coli* cells transformed with the pGEn2 vector in 1 L of LB medium (Tryptone- 10 g/L, yeast extract- 5 g/L and sodium chloride- 10 g/L) supplemented with 100 µg/ml ampicillin. *E. coli* is grown at 37 °C in a non-humidified, shaking incubator.
        2. Purify the pGEn2 vector encoding target plasmid DNA according to the manufacturer's DNA purification protocol.
        3. To ensure complete sterility of the plasmid DNA, perform the final isopropanol precipitation and resuspension steps of the DNA extraction procedures inside the biosafety cabinet.
        4. Add the volume of isopropanol (specified in the plasmid preparation kit) to the eluted DNA and centrifuge at 10,000 x g for 10 min. Sterilize the outside of the DNA container with 70 % ethanol in water solution and transfer it inside the biosafety cabinet. Discard the isopropanol supernatant using a pipette and air-dry the DNA pellet. Once dry, resuspend the pellet in sterile 10 mM Tris buffer, pH 8.0.
    3. Prepare stock solution of 220 mM valproic (VPA) acid in water and sterilize by passage through a sterile 0.22 µm filter. Store the solution at -20 °C until further use.

3. Establishing a Transient Transfection

1. Transfection (Day 0)
    1. Check the cell density by following steps 1.2.2 through 1.2.5 of "1.2. Cell Maintenance" above. Determine the amount of cells for the transfection (2.5-3.0 x 10^6 live cells/ml with the viability >95%). See a sample calculation in Supplementary Materials.
    2. Transfer the volume of suspension culture cells calculated in the previous step (here 67 ml) to a 250 ml centrifuge tube using a sterile serological pipette. Collect cells by centrifugation for 5 min at 100 x g.
    3. In the meantime, prepare a fresh transfection culture medium following step 1.1.3 according to "1.1. Culture Inoculation" above. See the Supplementary Materials for a sample calculation. Also, transfer 5 ml of fresh culture medium to a sterile 15 ml tube and set aside.
    4. Transfer tubes containing the pelleted cells into the biosafety cabinet after sterilizing the outside of the tubes with the 70% ethanol in water solution and use a sterile pipette to decant the supernatant consisting of spent culture medium.
    5. Vigorously resuspend the pelleted cells by pipetting up and down using 10 ml of fresh culture medium and transfer to the flask with fresh transfection culture medium (prepared in step 3.1.3 above). Screw the cap on the vented cell culture flask and move the flask to the incubator (as set in 1.1.1) for 15 min-1 hr with shaking.
    6. During this time, dilute the plasmid DNA and PEI stocks using fresh culture medium (using the 5 ml volume withdrawn from step 3.1.3 above) to a final concentration of 0.5 µg/µl. See sample calculations in Supplementary Materials to determine the amount of DNA and PEI. Transfer the culture flask with the dispersed cells into the biosafety cabinet.
    7. Add plasmid DNA (300 µl of 0.5 µg/µl) to the culture using micro-pipette and mix by gentle manual shaking. Add PEI (900 µl of 0.5 µg/µl) to the culture, mix by gently shaking. Add 3.8 ml of fresh culture medium, from step 3.1.3, above and transfer flask to the incubator shaker for 24 hr.
    8. Clean the biosafety cabinet according to Step 1.1.

2. Dilution (Day 1) (For a 50 ml transfection volume)
    1. Incubate the transfected culture for 24 hr.
    2. Withdraw 1 ml of prewarmed 220 mM Valproic Acid (VPA; prepared according to step 2.2.3.) using a sterile 1 ml serological pipette and transfer it to a sterile 50 ml tube.
    3. Withdraw 5.0 ml of prewarmed Medium B and 44 ml of prewarmed Medium A and add this to the VPA solution using a sterile serological pipette to prepare 50 ml of fresh culture medium with a final concentration of 4.4 mM of VPA.
    4. Move the transfected culture into the biosafety cabinet after sterilizing the exterior of the flask with a 70% ethanol in water solution, add the prepared dilution medium and transfer the flask back to the incubated shaker.

3. Expression and Harvest (Days 2-6)
    1. Incubate the cells for an additional 4-5 days (5-6 days total since transfection).
    2. Withdraw aliquots of culture medium following step 1.2.2. to 1.2.5. described in "1.2. Cell Maintenance," above using a sterile 1 ml serological pipette, to monitor cells viability and save aliquots to analyze protein expression at each day by SDS-PAGE (see Section 5, below).
    3. Harvest cells after 5-6 days by centrifuging the cells plus medium at 1,000 g for 5 min. Additionally, harvest the supernatant if the cell viability falls below 50% (see steps 1.2.2-1.2.5).
    4. Pour off the supernatant that will contain secreted protein. Add 5 ml of a 10% bleach to the HEK cell pellet and discard as a biohazard.

4. Protein Purification

1. Prepare a column with 5 ml of Protein-A sepharose. Equilibrate the column with 5 column volumes of buffer A (20 mM of 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.4, 100 mM NaCl).

2. Centrifuge the collected supernatant with expressed protein at high speed (14,000 g for 10 min) to remove any remaining cell debris. Collect by decanting into a fresh tube. Discard the pellet as a biohazard.
3. Collect a 10 µl aliquot of the supernatant and additionally collect a small sample at each protein purification step to analyze the sample by SDS-PAGE (described in Section 5). Load the clarified supernatant and collect the flow through.
4. Wash the column with 3-column volumes of buffer A and elute the protein with 5-column volumes of 100 mM glycine, pH 3.0. Collect the eluate in tubes containing one-half volume of 1 M TRIS buffer, pH of 8.0 to rapidly neutralize the pH.
5. Wash the column with 10 column volumes of buffer A and store for future use.
6. Buffer exchange the eluted protein with at least a 10-fold excess of buffer A using 10 kDa molecular weight cut-off centrifugal filters. Note: Do not concentrate the eluted sample to a very low volume (<2 ml) in the eluted buffer. Use the centrifugal filters to exchange the buffer with buffer A.
7. Store purified protein at 4 °C until next use.
   Note: Protein expressed in the supernatant can be purified by affinity columns selected on the basis of protein's properties or the use of affinity tags. For a typical purification procedure, Protein A column can be used for IgG or Fc fragments or nickel column can be used for proteins expressed with a poly-histidine tag. Here, is a description of the purification steps for IgG1-Fc using a Protein A column.

5. Analysis of Protein by SDS-PAGE

1. Dilute the aliquots (7.5 µl) of each sample with an equal volume of 2x loading buffer (0.125 M Tris, pH 8.0, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.01% bromophenol blue).
2. Boil the mixture at 90 °C for 5 min using a heat block or water bath. Centrifuge the samples at 10,000 g for 1 min. Load the prepared gel with 5 µl of marker protein and 14 µl of samples for analyses using a 20 µl pipette with a disposable tip.
3. Run one gel at 25 milliamps for 60 min. Once the electrophoresis step is complete, transfer the gel to a container with 1 L of water and microwave for 5 min.
4. Stain the gel with staining solution (40% ethanol, 10% acetic acid and 0.1% coomassie brilliant blue) for 2 hr and destain in water.

6. Glycans Analysis by Mass Spectrometry

1. Analyze glycans as described previously[44]. Briefly, 75 µg of IgG1-Fc was trypsinized, then treated by PNGaseF for the release of glycans[45]. Released glycans were permethylated and analyzed by using matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS).

7. Protocol Adjustments for Special Scenarios

1. Labeling Proteins with ^{15}N-labeled Amino Acids (for a 50 ml transfection volume)
   1. Dispense 5 mg of each amino acid into a single sterile glass vial. Resuspend with 4 ml autoclaved water (note Tyr does not completely solubilize, unlike Lys and Phe). Sterilize the solution by autoclaving at 121 °C for 15 min. Allow the solution to cool approximately to 50-60 °C.
   2. For a 50 ml transfection volume, follow step 3.1.3 according to "3.1 Establishing a transient transfection". See the Supplementary Materials section for an example with appropriate volumes.
   3. For transfection, follow steps of "3. Establishing a transient transfection" using fresh culture medium substituted with labeled amino acid residues as prepared above.
   4. After 24 hr of transfection, on the day of culture dilution, withdraw 5.0 ml of prewarmed Medium B, 40 ml of prewarmed Medium A and a freshly autoclaved 4 ml aliquot of the amino acid mixture (prepared as in step 7.1.1. above) and add 1 ml prewarmed stock solution of VPA solution to prepare 50 ml of fresh dilution culture medium with a final concentration of 4.4 mM of VPA. Add this medium to the transfection culture using a sterile serological pipette.
   5. Transfer the transfected culture into the biosafety cabinet after sterilizing the exterior of the flask with a 70% ethanol in water solution, add the prepared dilution medium and transfer the flask back to the incubated shaker.
      Note: Medium A is a chemically defined, serum-free medium. Thus, it is possible to prepare a different formulation. Contact the supplier to obtain a custom Medium A preparation that lacks certain amino acids. It is possible to have all amino acids left out, however, we prefer to use medium that lacks only few select residues because a complete dropout medium would require adjustment of osmolarity after supplementation. We chose a Lys-Tyr-Phe dropout medium that can be supplemented and does not require osmolarity adjustment. Furthermore, the Medium A supplier does not freely share the concentrations of medium components; we used 100 mg/L each for Lys, Tyr and Phe with success. Volumes of the transfection and expression medium must be adjusted to account for the added amino acids. Here are the adjustments to the protocol above for isotope labeling

2. Supplement the Transfection with a Small Molecule
   1. Prepare 100 mM stock solution of 2-deoxy-2-fluoro-l-fucose using autoclaved water and sterilize this solution using a 0.22 µM filter.
   2. For a 50 ml culture, add 125 µl (at 250 µM) of the fucose analog solution to the transfection and dilution media. Note: We neglected to perform any further volume adjustment because adding the substituent at this concentration accounts for only 0.25% of the total culture volume .It is possible to modify the expression using small molecule inhibitors of glycan processing. Here we describe the conditions for including 2-deoxy-2-fluoro-l-fucose, an inhibitor of glycan fucosylation. We found >90% reduction in fucosylation by adding the fucose analog at a concentration of 250 µM in the transfection and dilution media.

Representative Results

High-level protein expression and purity

This optimized expression system generated a high yield of glycosylated proteins. A typical pattern is shown in the expression of IgG1-Fc (**Figure 1**). In this case, Day 0 is the transfection day followed by Day 1 (dilution) and subsequent culture days up to Day 5. Protein expression is analyzed using the soluble expression fraction in the crude medium. A very small amount of protein expression was observed in Day 1 as the culture aliquot was withdrawn 3 hr after culture dilution. This is easier to visualize with GFP-tagged proteins that display a distinct green color in the culture medium. Such an increase was observed for in the expression of the GFP-FcγRIIIa and GFP-ST6GalI from Day 2 to Day 5. No significant increase in the expression was observed between Day 4 and Day 5. At Day 5, the cells were <50% viable and hence culture was harvested to limit proteolysis. Affinity purification using a Protein A column for IgG1 Fc or nickel column for GFP-FcγRIIIa resulted in proteins with high purity (>99%; **Figure 2**), yield (**Table 1**) and complete glycosylation (data not shown).

^{15}N or ^{13}C Isotope labeling of proteins and glycans

This system efficiently expressed isotopically-enriched IgG1 Fc according the described protocol. Although a small reduction in protein yield was observed (25-50%), well-dispersed signals were seen in a 2d NMR spectrum that correlates the resonance frequency of ^{1}H atoms and the directly bonded amide ^{15}N atoms (**Figure 3**). This level of expression permits efficient protein production on the scale required for structure-based studies (typically 2-20 mg of protein) and illustrates the successful incorporation of the labeled isotopes into the protein. The high degree of similarity between this spectrum and published spectra indicate a high degree of protein labeling occurred with minimal scrambling of the ^{15}N labels[43].

Producing proteins with different N-glycans

Different glycoforms were produced with the HEK293F and HEK293S cells. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analyses of the enzymatically-released N-glycans showed the expected glycoforms (**Figure 4**). Proteins expressed from HEK293S cells harbored N-glycans that were of the $Man_5GlcNAc_2$ form and contained no fucose (**Figure 4**). Glycans from HEK293F-expressed material were complex-type, biantennary forms with core fucose and mostly having terminal N-acetylglucosamine (GlcNAc) or small proportion of mono- or di-galactosylated (Gal) forms. Though it is not know what the native N-glycans of ST6GalI and FcγRIIIa are, the glycan profile for IgG1 Fc is highly similar to IgG1 Fc purified from human serum[46]. The major differences include the degree of modification; IgG1 Fc from human serum shows a higher degree of galactosylation than observed for the HEK293F expression. Addition of 2-deoxy-2-fluoro-l-fucose, an inhibitor of glycan fucosylation, to an expression conducted using the HEK293F cell line showed a dramatic >90% reduction in fucose incorporation (**Figure 4**).

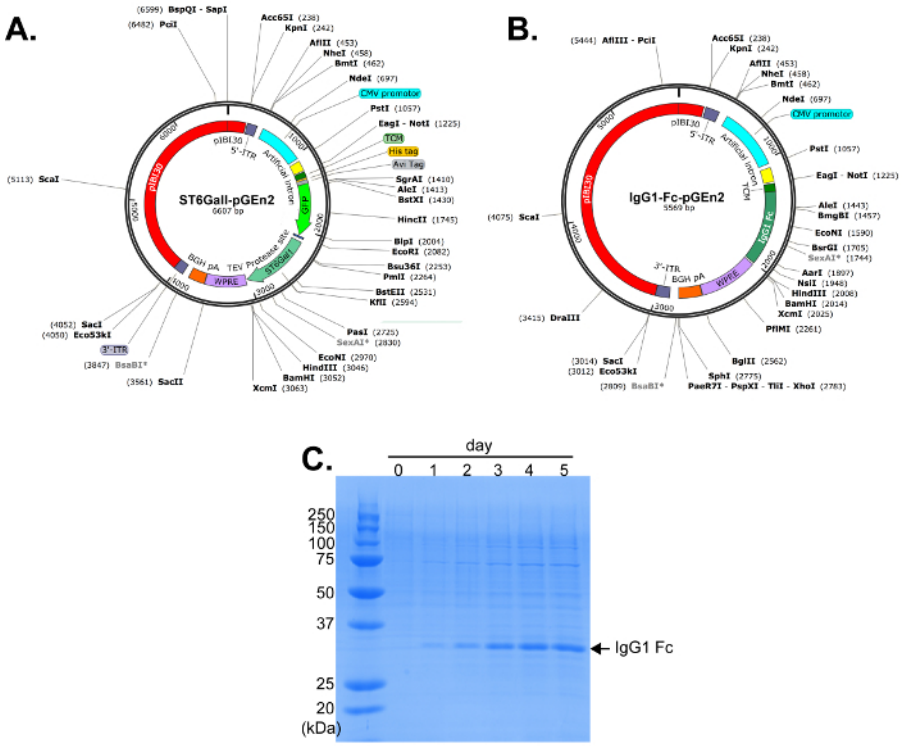

Figure 1: High yields of recombinant proteins are recovered by expressing from the pGEn2 vector. (**A**-**B**) Two expression vectors used in this study were generated from a pIBI30 plasmid that contains an amp^{R} cassette and an *E. coli* replication origin. (**C**) Expression level of the secreted IgG1 Fc protein after transient transfection of HEK293F cells. SDS-PAGE analysis shows the accumulation of expressed proteins from Day 0 to Day 5 and is indicated by the arrow. Please click here to view a larger version of this figure.

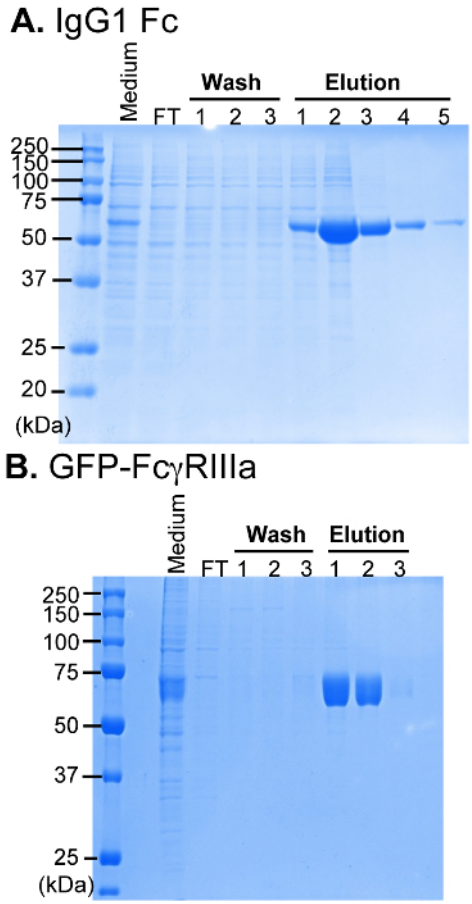

Figure 2: Recovery of expressed protein from the culture medium. (**A**) IgG1-Fc, (**B**) GFP-FcγRIIIa. "Medium" refers to the culture medium after centrifugation; FT = flow through fraction from the purification column. SDS-PAGE samples are prepared in non-reducing conditions without β-mercaptoethanol. Please click here to view a larger version of this figure.

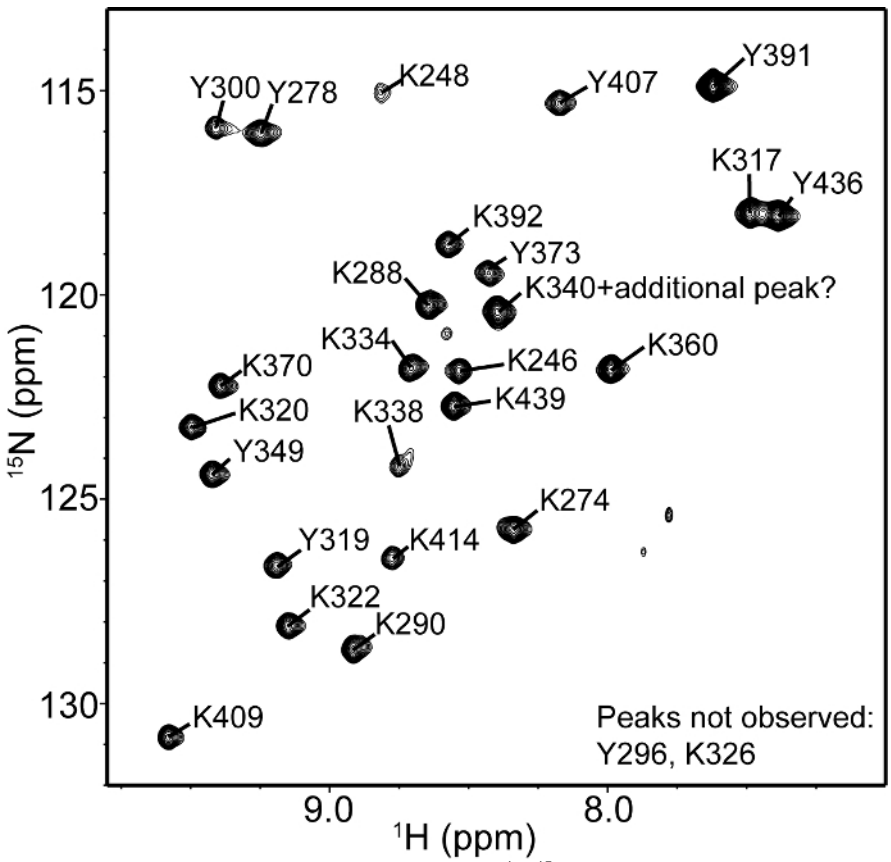

Figure 3: Amino acid selective labeling of IgG1 Fc. ^{1}H-^{15}N heteronuclear single quantum coherence spectrum of [^{15}N-Tyr; ^{15}N-Lys]-labeled IgG1 Fc expressed using HEK293F cells in custom Medium A supplemented with (^{15}N) labeled l-Tyr and l-Lys. Crosspeaks were assigned based on previous reports[43,47]. Please click here to view a larger version of this figure.

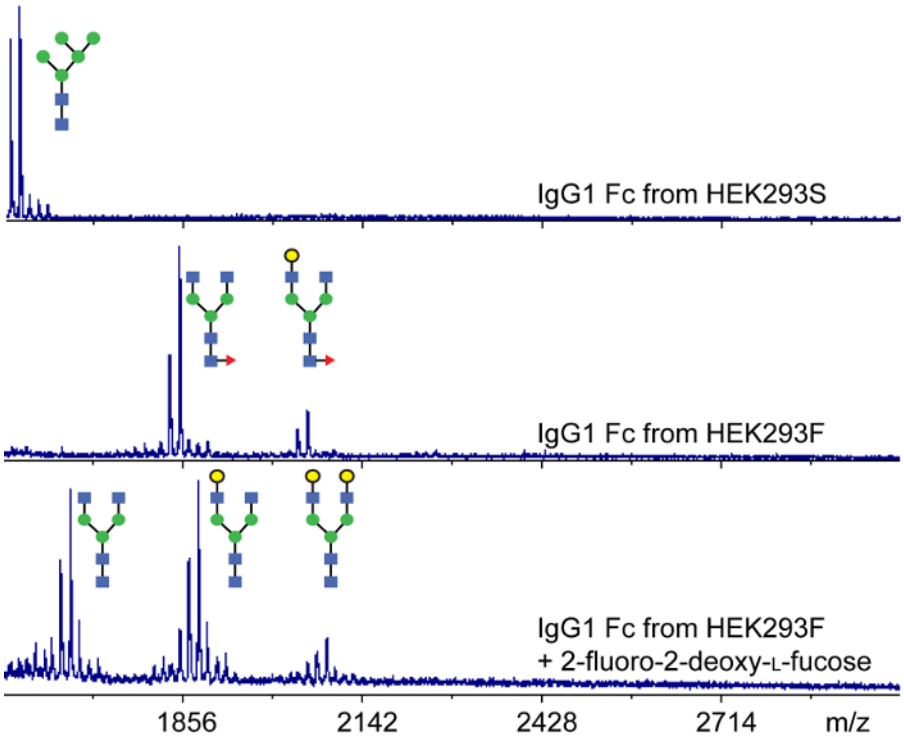

Figure 4: Protein expression in the presence of small molecule modulators of glycan composition. N-glycan profiles of IgG1 Fc expressed in HEK293 cells using different culture conditions. The top spectrum was prepared using glycans isolated from IgG1 Fc expressed in HEK293S cells. The center spectrum reveals the IgG1 Fc glycoforms from material expressed in HEK293F cells and the bottom spectrum was prepared similarly expect cells were grown in the presence of an inhibitor of GDP-fucose biosynthesis, 2-deoxy-2-fluoro-l-fucose. N-glycans are presented as cartoon diagrams following the CFG convention[5]: *N*-acetylglucosamine (GlcNAc), blue squares; Mannose (Man), green circles; Fucose (Fuc) red triangles; Galactose (Gal), yellow circles. Please click here to view a larger version of this figure.

Protein	Yield (mg/L)
IgG1 Fc	120
GFP-FcgRIIIa	100
GFP-ST6Gall	95

Table 1: Yield for different proteins expressed in HEK293F cells.

Discussion

This protocol illustrates protein expression via the transient transfection of HEK293F or S cells. The optimal transfection conditions established in the Barb and Moremen labs employ a critical combination of cell density and reagent concentrations to achieve high efficiency transfection. Critical considerations when implementing this protocol include: maintaining a stable culture prior to transfection (with consistent culture doubling times); transfection of actively growing cells (achieved by diluting cells to 1 × 10^6 cells/ml 24 hr prior to transfection) with cell viability greater than 95%; cell density at transfection should be between 2.5-3.0 x 10^6 live cells/ml with a viability >95% (transfection density) in a culture containing 90% Medium A and 10% Medium B; and, adding DNA prior to PEI addition at 3 µg/ml and 9 µg/ml, respectively[31]; at 24 hr post-transfection the culture is diluted 1:1 in medium containing 4.4 mM valproic acid to result in a concentration of 2.2 mM valprioc acid in the diluted culture; the production phase growth then continues in a humidified shake flask at 37 °C and 8% CO_2 for an additional 4-5 days. The vector described here is optimized and an important feature of the soluble protein expression system[32,42].

If a target protein fails to express, in addition to the factors listed above, multiple other variables may contribute to low yields. Ensure the protein coding DNA sequence contains codons optimized for human cells. This can be assessed with multiple online resources. Sterility throughout the procedure is paramount. An actively growing and pure culture of HEK293F cells should appear slightly grainy to the naked eye, and the medium should be mostly clear (particularly at cell densities <1.0 ×10^6 cells/ml).

Cultures contaminated with bacteria or fungus should be immediately removed to prevent spread. Is it important to maintain a healthy stock culture. This is achieved by inoculating these cultures at a cells density of 0.3 × 10^6 cells/ml. Lower inoculation densities can slow the growth rate by limiting the supply of various growth factors required for cell growth and division[48]. As cultures have been passaged for more than 25 or 30 times, a decrease in expression was observed. For this reason, cultures passaged more than 30 times are discarded.

It is possible the protein is being degraded in the expression medium, or the expression timeframe is too long, leading to protein degradation. For these reasons it is recommend to monitor the culture viability and protein expression at each day to determine an optimal expression timeframe.

It is important to purify proteins as quickly as possible after harvesting the medium. For some proteins, it is helpful to include protease inhibitors during purification. In total, these adjustments have improved the recovery of proteins in the Barb and Moremen labs[42,43].

The transient transfection of HEK293 cells has shown great utility for producing soluble proteins and protein domains that are naturally targeted to the secretory pathway. It is likely that production of cytoplasmic or integral membrane proteins will require further optimization. A primary advantage of this system is the native substrates and machinery for protein production are present and available in the HEK293 cells[49]. This largely accounts to its advantages over other recombinant protein production methods such as bacteria with no or limited post-translational modifications, or yeast and baculovirus systems with correctly folded but different N-glycoforms incorporated[1,50,51].

Furthermore, the protocol described here shows the added capability to include low molecular weight compounds such as labeled amino acids, labeled glucose or small molecules designed to modify the N-glycan composition. The HEK293 cells also prove adept at expression of non-mammalian proteins, including plant enzymes, and supports co-expression of multiple polypeptides for the recovery of protein complexes (data not shown).

The structural and functional characterization of glycoproteins is often hampered by sample production challenges. The protein expression system described here surmounts this drawback by accomplishing post-translational modifications using the natural complement of sophisticated intracellular glycan processing enzymes[6]. This robust and simple protocol is proven for the transient transfection of suspension human HEK293 cells. This method demonstrated significant glycoprotein yield (>95 mg/L) with three N-glycosylated proteins and can accommodate supplements such as labeled amino acid residues or the small molecule chemical inhibitor 2-deoxy-2-fluoro-l-fucose. The expressed glycoproteins can be further remodeled post-purification to prepare a wide range of defined glycoforms[52].

Disclosures

All authors declare no competing financial interests in this manuscript.

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