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Review

Polyethyleneimine-based transient gene expression processes for suspension-adapted HEK-293E and CHO-DG44 cells

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A B S T R A C T

Transient gene expression (TGE) from mammalian cells is an increasingly important tool for the rapid production of recombinant proteins for research applications in biochemistry, structural biology, and biomedicine. Here we review methods for the transfection of human embryo kidney (HEK-293) and Chinese hamster ovary (CHO) cells in suspension culture using the cationic polymer polyethylenimine (PEI) for gene delivery.

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Introduction

There is a growing interest in the rapid production of recombinant proteins in cultured animal cells for applications in medicine and fundamental research [1–6]. Currently, the two major approaches to rapid protein production are non-viral transient gene expression (TGE) using mammalian cells [7–11] and infection of insect cells with a baculovirus expression vector [12,13]. Cost-effective protein production is achievable by both methods when performed with suspension-adapted cells cultivated in simple and scalable systems.

One of the major differences between the two expression systems is the manner in which the gene of interest (GOI) is delivered to the production host. Construction of the recombinant baculovirus begins with the cloning of the GOI into a transfer vector that allows recombination-mediated transfer of the GOI into the baculovirus genome while it is maintained as an episomal DNA element (bacmid) in Escherichia coli [12,13]. After recovery of the recombinant bacmid DNA from E. coli, it is transfected into insect cells, resulting in a productive viral infection. The baculovirus stock is then used for the large-scale infection of insect cells to produce the recombinant protein of interest. Unfortunately, the baculovirus infection of insect cells is cytolytic, limiting the production phase to a relatively short period of ~2–5 days. In contrast, TGE requires the cloning of the GOI into a mammalian expression vector. After amplification of the plasmid in E. coli, it can be transfected into cells. A protein production phase of up to 2 weeks is possible depending on the protein and the culture conditions. In optimized cultures, volumetric yields up to 1 g/L have been reached [14]. A major drawback of this method, however, is the amount of plasmid DNA required, typically 1 mg or more, required per liter of transfection. By comparison, the generation of the recombinant baculovirus vector is time-consuming, but its continued propagation is relatively simple and inexpensive.

Both mammalian and insect cells support correct protein folding, multi-protein complex formation, and post-translational modifications. However, there is a major difference between the two animal cell hosts with regard to N-linked glycosylation. Insect cells mainly synthesize oligomannosidic and paucimannosidic glycans with low levels of galactose and sialic acid [13,15]. In contrast, mammalian cells synthesize complex glycans containing mannose, N-acetylglucosamine, galactose, and sialic acid [16,17]. For applications in structural biology, it is often beneficial to produce glycoproteins with homogeneous oligomannosidic glycans that can be efficiently removed by glycosidases [18–20]. For therapeutic proteins, on the other hand, it is preferable to have complex glycans to enhance both the half-life and functionality of the protein in vivo [21–23].

This article focuses on TGE methods with the two major mammalian production hosts, human embryo kidney 293 (HEK-293) and Chinese hamster ovary (CHO) cells, using polyethyleneimine (PEI) for DNA delivery. A non-exhaustive list of examples of proteins which have been produced in these cells is provided in Table 1. Since 2004, over 20 structures have been resolved using proteins produced transiently in HEK-293 cells. In addition, the method has been successfully applied to the production of virus vectors for the purpose of gene delivery (Table 1). Despite these successes, there may be perceptions that TGE is unaffordable for many academic labs and that the cultivation of mammalian cells in suspension is technically difficult. Fortunately, the production yields from transiently transfected mammalian cells have improved considerably in the last decade, and innovative cost-effective, non-instrumented cultivation systems for suspension-adapted mammalian cells have been developed [24–26]. These technical improvements have dramatically reduced protein production costs.

Our objective is to present a practical overview of the key components, methods, and limitations of PEI-based TGE production processes in HEK-293 and CHO cells based on our extensive experience in the Protein Expression Core Facility and the Laboratory of Cellular Biotechnology at the École Polytechnique Fédérale de Lausanne. The point is to provide access to the technology so that new users may better understand the critical steps in order to develop protocols to suit their own needs. We are not providing a comprehensive review of transient transfection methods with animal cells. Other recent reviews can be consulted for a broader perspective of TGE [2,10,11]. In addition, we and others have published step-by-step protocols on TGE using mammalian cells as hosts [27–33].

Cells

To achieve an economic transient production process, two properties of the host cell are essential. They must be able to grow to a high density (>5 × 10⁶ cells/mL) in single-cell suspension culture, and they must be efficiently transfected (DNA uptake in more than 50% of cells) with a low-cost DNA delivery vehicle. The ability to grow in the absence of serum is also highly desirable if the protein product is secreted. Although the TGE methods described here can be adapted to many other mammalian cell lines, most of them do not meet these criteria. Consequently, most efforts to develop TGE systems have focused on HEK-293 and CHO cells as hosts.

HEK-293 cells

HEK-293 cells (American Type Culture Collection, Molsheim, France) were generated from embryonic human kidney tissue by stable transfection with sheared human adenovirus DNA, resulting in cells overexpressing the adenovirus E1A and E1B genes [34]. The cells were initially used to propagate adenovirus mutants deficient in these genes. Due to their ease of cultivation and transfection, they eventually gained popularity as a TGE host. Subsequently, HEK-293T (American Type Culture Collection) and HEK-293E cells, resulting from stable transfection of the parental line with the simian virus 40 (SV40) large T antigen (LT) gene and the Epstein–Barr Virus nuclear antigen 1 (EBNA1) gene, respectively, were generated [35,36]. Both EBNA1 and SV40 LT function in viral DNA synthesis by binding to the cognate viral origin of DNA replication (ori) to recruit the cellular DNA replication machinery. These two cell lines were developed with the expectation that they would support the episomal replication and maintenance of a transfected plasmid DNA bearing the appropriate viral ori [37]. For the TGE system described here, HEK-293E cells were used. Interestingly, the highest protein yields in these cells were achieved under conditions of growth arrest following transfection with plasmids that did not bear the EBV ori [14]. However, there may be other TGE conditions in which episomal replication of the plasmid has a positive effect on recombinant protein yield [38–40].

More recently, HEK-293 subclones, adapted to suspension growth in commercial serum-free media, were made available. HEK-293F™ and Exp293F™ cells (Life Technologies Europe, Zug, Switzerland) were selected for high-density suspension growth in FreeStyle293™ and Exp293™ media (Life Technologies), respectively. A suspension-adapted cell line lacking
N-acetylglucosaminyltransferase-I activity (HEK-293S GnTI) was generated by chemical mutagenesis. These cells produce glycoproteins with immature N-linked glycans having a high-mannose (Man) content [18], and they have served as a host for both stable and transient recombinant protein production [20,41–45].

**CHO cells**

In the 1950s the original CHO cell lines were recovered as spontaneously immortalized cells from primary Chinese hamster ovarian cultures [46]. CHO-DG44 cells were derived from one of these

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**Table 1**

Examples of proteins and virus vectors produced by PEI-mediated transient transfection of HEK-293 and CHO cells.

| Protein                          | Host         | Affinity tag | Secreted | Refs. |
|----------------------------------|--------------|--------------|----------|-------|
| **A. Function studies**          |              |              |          |       |
| Alpha-factor receptor (Ste2p)    | 293E         | Fc or Strep/his | No       | [98]  |
| Transforming growth factor β receptor (TGF-βRII) (ED) and TGF-βRIII (MPD) | 293E | His | Yes | [99] |
| Vascular endothelial growth factor (VEGF) | 293E | None | Yes | [100] |
| Secreted clusterin (sCLU)        | 293E         | His          | Yes      | [101] |
| SLit homology protein 2 (Slit2)  | 293E         | His          | Yes      | [102,103] |
| Resistin (RSTN)                  | 293E         | His          | Yes      | [104] |
| Chimeric heavy chain antibody (chCAb) | 293E | None | Yes | [105] |
| Epidermal growth factor          | 293E         | Fc or His    | Yes      | [106] |
| Insulin-like growth factor binding protein 7 (IGFBP7) | 293E | His | Yes | [107] |
| Factor VIII                      | 293S-3F6     | None         | Yes      | [108] |
| C-terminal perlecan fragment (LG3) | 293E | His | Yes | [109] |
| Human IgG1 antibody              | 293E, CHO    | None         | Yes      | [14,79] |
| Nogo66                           | 293E         | None         | Yes      | [110] |
| Leucine-rich glioma inactivated 1 (LG1) | 293E | None | Yes | [110] |
| Respiratory syncytial virus fusion protein (RSV-F) | 293E | None | Yes | [96] |
| Bicyclic peptides                | 293E         | Fc           | Yes      | [111] |
| Dickkopf1 (Dkk1)                 | 293T         | His          | Yes      | [112] |
| CST complex (CCT1, STN1 and TEN1) | 293E | FLAG/His    | No       | [113] |
| Telomerase processivity factor (POT1-TPP1) | 293E | FLAG | No | [113] |
| HIV1 gp140 trimer                | 293S GnTI and 293T | None | Yes | [114] |
| Calsyntenin 1 (Cals1) (ED)       | 293E         | His          | Yes      | [80]  |
| HLA class II antigen             | 293E         | Fc           | Yes      | [80]  |
| Prion protein (PrP)              | 293E         | Fc           | Yes      | [80]  |
| HCMV UL18 (ED)                   | 293E         | Fc           | Yes      | [115] |
| Single chain antibody fragment (scFv) | 293E | His | Yes | [116] |
| CD1 (ED)                         | 293E         | His          | Yes      | [117] |
| CD1-antiHER2 scFv fusion          | 293E         | His          | Yes      | [117] |
| Erythrocyte membrane protein 1 (PMEP1 var2CSA ED) | 293E | His | Yes | [118] |
| Sonic hedgehog (Shh ED)          | 293T and 293S GnTI | None | Yes | [119] |
| Erythropoietin (EPo)             | 293E         | None         | Yes      | [120] |
| Coactivator-associated arginine methyl transferase 1 (CARM1) | 293T | Halo | Yes | [121] |
| Toll like receptor 2 (TLR-2) (EC) | 293E | His | Yes | [122] |
| Leukemia inhibitory factor (LIF) | 293E         | Fc           | Yes      | [89]  |
| Tumor necrosis factor receptor (TNFR ED) | 293E | Fc | Yes | [91]  |

| **B. Structure studies**         |              |              |          |       |
| Type IIB receptor protein tyrosine phosphatase (RPTPµ and RPTPπ) | 293T | His | Yes | [123–125] |
| Human IgG1 antibody              | 293T         | None         | Yes      | [126] |
| ORF-9B SARS coronavirus          | 293T         | His          | No       | [127] |
| Ephrin B2 (EphB2) (ED)           | 293T         | His          | Yes      | [128] |
| Nipah virus glycoprotein (NV-G) (ED) | 293T | His | Yes | [129] |
| Henda virus glycoprotein (Hev-G) (ED) | 293T | His | Yes | [129] |
| Hedgehog interacting protein (Hhip) (ED) | 293T | His | Yes | [130] |
| Ionotrophic glutamate receptor 2 (ED) | 293S GnTI | His | Yes | [42]  |
| Ephrin receptor A4 (EphA4) (ED)  | 293T         | His          | Yes      | [128] |
| Ephrin receptor A2 (EphA2) (ED)  | 293T         | None         | Yes      | [128] |
| Ephrin A5 ligand                 | 293S GnTI    | His          | Yes      | [131] |
| WIF domain of Wnt inhibitory factor 1 (WIF-1/WD) (ED) | 293S GnTI | His | Yes | [132] |
| Netrins G1 and G2 (ED)           | 293S GnTI    | His          | Yes      | [43]  |
| Netrin G ligands (NGL1 and NGL2) | 293S GnTI    | None         | Yes      | [43]  |
| LDL receptor related protein 6 (LRP6) (ED) | 293S GnTI | His | Yes | [112] |
| Chaperone mesoderm development (Mesd) | 293S GnTI | FLAG | Yes | [112] |
| IgG Fc                           | 293S GnTI    | None         | Yes      | [133] |
| Plexin-B1 (ED)                   | 293T         | His          | Yes      | [134] |
| Plexin-A2 (ED)                   | 293S GnTI    | His          | Yes      | [134] |
| Semaphorin 6A (ED)               | 293S GnTI    | His          | Yes      | [134] |
| Neurophin 1 (Nrp1)               | 293S GnTI    | None         | Yes      | [135] |
| Semaphorin 3A (ED)               | 293S GnTI    | His          | Yes      | [135] |
| Human telomerase                 | 293E         | FLAG         | No       | [136] |
| HIV1 gp140 trimer                | 293E         | None         | Yes      | [137] |
| Repulsive guidance molecule B (RGMB) (ED) | 293T | His | Yes | [138] |
| Neogenin (Neo1)                  | 293T         | His          | Yes      | [138] |

| **C. Virus vector**              |              |              |          |       |
| Lentivirus (LV) vector           | 293S-3F6 and 293 | None | Yes | [139] |
| Adeno-associated virus (AAV) vector | 293 and 293E | None | Yes | [140,141] |

Abbreviations: ED, ectodomain; MPD, membrane-proximal ligand-binding domain; Strep, streptavidin tag; His, 6×-histidine tag.
original lines by two rounds of gamma irradiation and then screened for the absence of dihydrofolate reductase (DHFR) activity [47]. These auxotrophic cells must be grown in the presence of hypoxanthine and thymidine (HT) whose synthesis requires the DHFR gene [47]. These cells were used as the host for the TGE method described here because they are efficiently transfected, grow to a high density in suspension culture, and are widely used in the biopharmaceutical industry to generate stable cell lines for the production of therapeutic proteins [1].

Two other lines, CHO-K1 and CHO-S, were separately derived from the original immortalized CHO cell lines with the latter being adapted to suspension culture [48-49]. Both have been transiently transfected with PEI [50]; Rajendra, unpublished data). Unfortunately, a commercially available cell line adapted for growth in suspension culture using FreeStyle™ CHO medium (Life Technologies) is also designated CHO-S, but it may not be the same as the CHO-S line developed in the 1960s. CHO-T cells were generated by stable transfection of CHO-K1 cells with the Polyomavirus (Py) LT gene for the purpose of supporting the replication of plasmids carrying the Py ori [51]. However, they have only been transfected in suspension culture with liposomes [52]. Lastly, CHO mutants deficient in lectin binding have been isolated [53,54]. These cell lines synthesize glycoproteins with immature N-linked glycans, but to our knowledge they have not been used for large-scale TGE.

Other host cells

Three human cell lines have recently emerged as potential TGE hosts, but the results are limited so far. CEVEC’s amniocyte production cell line (CAP-T™) (Köln, Germany) expressing the SV40 LT can be cultivated in suspension and efficiently transfected with PEI [55]. HKB-11 cells, a hybrid between HEK-293 and Burkitt’s lymphoma-derived 2B8 cells, and human Per.C6® cells (Crucell, Leiden, The Netherlands) have only been transiently transfected in suspension with liposomes [56-59].

Cell cultivation

Media

Suspension cultivation of HEK-293E and CHO-DG44 cells is usually performed in serum-free media that support densities of $5 \times 10^6$ cells/mL or more. We routinely cultivate HEK-293E cells in EX-CELL®293 (SAFC Biosciences, St. Louis, Missouri) and CHO-DG44 cells in ProCHO®5 (Lonza, Verviers, Belgium). However, many other serum-free media are available for high-density cell cultivation. As expected, the formulations of these high-performance media are not made available by the providers, but they often contain soy-derived peptides and one or more recombinant growth factors. Due to the lack of information on the formulation, choosing and trouble-shooting media are difficult since the various steps of the TGE process – cell growth, transfection, production, and purification – have different requirements, and unknown medium components may interfere with any one of them. Most, if not all, of the commercial media contain polymers such as Pluronic® F-68 (SAFC Biosciences) to reduce shear forces generated by the mixing of cells in suspension. To our knowledge, this polymer does not interfere with any step in the methods described here.

Cell cultivation system

One of the key factors required for the development of a cost-effective TGE method with mammalian cells is an efficient system that provides the physico-chemical environment for high-density cell cultivation. The major consumable cost in TGE processes is that of the medium. Therefore, maximizing medium utilization by growing cells to a high density is the best approach to making protein production more economical. This is not only important for the protein production phase but also for the provisioning of cells prior to transfection. Having a cultivation system that supports high-density cell growth is therefore necessary to minimize the overall costs of protein production.

Several options are available for suspension cell cultivation including orbitally shaken containers, stirred-tank bioreactors (STRs), spinner flasks, and WAVE bioreactors (GE Healthcare Europe, Glattbrugg, Switzerland) [1,60-62]. To select among these options, one must take into account the cost and engineering parameters of the equipment. Spinner flasks are inexpensive and available in nominal volumes of 100 mL-36 L. However, due to poor mixing and gas transfer, they do not adequately support high-density cell cultivation in the absence of active aeration. The WAVE bioreactor relies on disposable bags with nominal volumes of 500 mL-500 L [63-66]. The single-use bags are a major operating expense, and cultures in WAVE bags require active aeration for optimal cell growth. The other shortcoming of the WAVE bioreactor is that the simultaneous operation of multiple cultures requires multiple agitation/heating units, increasing the space and equipment needs. Finally, STRs are difficult to maintain, set-up and operate. They are also impractical for the performance of simultaneous cultures and for small-scale operations. Their cost may also be prohibitive for many potential users.

We use orbitally shaken bioreactors (OSRs) for cell cultivation and TGE due to the simplicity of operation from small to large volumetric scales and for their proven superior engineering characteristics for efficient mixing and gas transfer [26,67-70]. Orbitally shaken containers include the disposable TubeSpin® bioreactor 50 and 600 tubes (TPP, Trasadingen, Switzerland) with nominal volumes of 50 and 600 mL, respectively, cylindrical glass bottles with nominal volumes of 100 mL-5 L, square-shaped glass bottles with nominal volumes of 100 mL-1 L, and shake flasks with nominal volumes of 250 mL-5 L. We perform most of our operations in TubeSpin® bioreactors and in glass bottles [24,26,68]. Small-scale operations at volumes of 2-10 mL and medium-scale operations of 100-500 mL are well-suited to the TubeSpin® bioreactor 50 and TubeSpin® bioreactor 600, respectively (Fig. 1) [24,71]. These disposable containers are shaped like conical centrifuge tubes,
but they are equipped with a ventilated cap having a membrane filter as a sterility barrier. Cultures in these tubes are maintained in an incubator shaker with 5% CO₂ and 85% humidity in racks attached to the shaker platform (Fig. 1). It is important that the tubes fit tightly in the rack to prevent their rotation during agitation. The tubes double as centrifuge tubes to facilitate cell handling. Alternatively, it is possible to perform small-scale transfections in multi-well plates, but this cultivation system is limited by high evaporation rates [72,73].

When using cylindrical or square-shaped glass bottles, the working volume is 30–40% of the bottle's nominal volume [26]. The cultures are maintained on the shaker platform with double-coated removable foam tape (3M, Rüschlikon, Switzerland) (Fig. 2A) or with supports fastened to the platform in the case of 5-L bottles (Fig. 2B). It is best to maintain the cultures in an incubator with 5% CO₂. Suitable incubator shakers with CO₂ control are commercially available from several manufacturers. It is possible to substitute shake flasks for glass bottles, but they have a larger footprint than glass bottles of the same nominal volume.

In OSRs, gas exchange takes place at the liquid surface rather than by the sparging of gas into the liquid as in STRs. As a consequence gas transfer into and out of the culture is dependent on rapid mixing to create a high renewal rate at the liquid surface. At shaking frequencies suitable for cell culture, all the OSRs have a larger footprint than glass bottles of the same nominal volume.

An oxygen mass transfer coefficient (k_ao) of 7 h⁻¹ is needed to avoid oxygen limitations in high-density cultures of mammalian cells in OSRs [78]. The k_ao values for the OSRs described here are above this minimum level at shaking frequencies suitable for mammalian cell cultivation [69,78]. The efficient mixing and gas exchange in OSRs also means that the CO₂ produced by cells is effectively removed from the culture so that the dissolved CO₂ concentration is maintained by the CO₂ level in the incubator, keeping the pH in a suitable range (6.6–7.1) for cell cultivation [75,78]. Therefore, after appropriate optimization of the cultivation parameters, it is not necessary to continuously monitor and control the dissolved oxygen (DO) concentration and pH during bioprocesses performed in OSRs [75].
the medium [86], and time-dependent pre-complex formation between PEI and DNA is not required [29,87]. Cationic liposomes are arguably the most efficient DNA delivery vehicles [2], but their high cost is a limitation for their use at large scale [10,11].

Linear 25 kDa PEI (Polysciences, Eppelheim, Germany) is dissolved in water to generate a stock solution at a concentration of 1 mg/mL and pH 7.0. Dissolving PEI requires acidification of the solution to pH 3 by addition of 1 N HCl. When all the PEI is in solution, the pH is increased to 7.0 by addition of 1 N NaOH. The solution is filter sterilized and stored at −20 ºC. Linear 25 kDa PEI in this form usually has a number-average molecular weight (Mn) of 6–7 kg/mol with the range being 2–26 kg/mol [88]. Its polydispersity index (PDI) is about 1.9, an important property for efficient DNA delivery in HEK-293E cells [88]. In the synthesis of PEI from 2-ethylloxazoline, there is an acidification step to remove N-propionyl groups from the amine. However, this reaction may not be complete, resulting in batch-to-batch variation in the percentage of protonatable amine groups [88]. The presence of N-propionyl groups reduces both the charge density of PEI and the strength of its interaction with DNA. Nevertheless, we observed that the transfection efficiency of various batches of PEI with 4–15% N-propionyl groups did not vary [88].

Until recently, it was thought that pre-formation of PEI-DNA complexes (polyplexes) prior to their addition to the culture was critical for efficient transfection. However, it has been observed that the direct addition to the culture of DNA followed by PEI is possible when transfecting at a low or high cell density [29,87]. We have employed this approach with both HEK-293E and CHO-DG44 cells [30,89–91].

**Transfection processes**

**HEK-293E cells**

HEK-293E cells, grown in EX-CELL® 293 medium, are passaged to fresh medium on the day before transfection at a density of 1.5–2.5 × 10⁶ cells/mL. The next day, the appropriate volume of culture is centrifuged, and the cell pellet is resuspended at 20 × 10⁶ cells/mL in RPMI 1640 containing 0.1% Pluronic F-68 (transfection medium). As an example, for a 1-L (final volume) transfection, 1 × 10⁶ cells are resuspended in 50 mL of transfection medium in a 250-mL glass bottle (Fig. 3). The DNA is then added at 1.5 μg/million cells (1.5 mg total), and the culture is mixed by hand. Immediately, 3.0 mL of a 1 mg/mL PEI stock solution is added [87,90,91]. The culture is again swirled and then transferred to an incubator shaker with 5% CO₂ for 60–90 min at 37 ºC with agitation at 120 rpm. The transfection can also be performed in a TubeSpin® bioreactor 600 with agitation at 180 rpm. At the end of the transfection phase, the culture is transferred to a 5-L glass bottle containing 950 mL of pre-warmed production medium (see below) to give a density of 1 × 10⁶ cells/mL. The transfection can be performed at any volumetric scale with proportional adjustments of the cell number and the DNA and PEI amounts.

Valproic acid (VPA; SAFC Biosciences), a histone deacetylase inhibitor, can have a positive effect on the production of recombinant proteins in HEK-293E cells, but this is not universal [92,93]. The effect of VPA on yield must be determined for each protein, and the optimal amount of VPA must be optimized for each protein and each HEK-293 cell line. The stock solution is prepared by dissolving VPA in water at 0.5 M followed by filter sterilization and storage at −20 ºC in 50-mL aliquots. In general, the maximum level of protein accumulation is observed on day 2–3 post-transfection for transmembrane and intracellular proteins, and on day 3–7 post-transfection for secreted proteins. In the presence of VPA, cells double once post-transfection and then cease dividing [92]. Due to this effect, it is possible to dilute the transfected culture to a density of 2–4 × 10⁸ cells/mL instead of 1 × 10⁶ cells/mL. This allows for a greater accumulation of biomass in the culture, but it does not change the amounts of DNA and PEI added, since they are based on the total cell number rather than the final culture volume.

We use different media for production, depending on the protein. For all intracellular and transmembrane proteins we use EX-CELL® 293 or FreeStyle™ F17 (Life Technologies Europe). This is also the case for secreted proteins that are affinity-purified with protein A, protein G, or an anti-FLAG antibody. For secreted proteins that have a histidine-tag, the production medium is either FreeStyle293™ or Pro293™ (Lonza) since both EX-CELL® 293 and FreeStyle™ F17 contain a component(s) that interferes with nickel-affinity chromatography.

To estimate the transfection efficiency (defined as the percentage of transfected cells), we replace 2% of the plasmid carrying the GOI with one for the expression of the enhanced green fluorescent protein (eGFP) gene. At day 2 post-transfection, an aliquot of the culture is visually inspected by fluorescence and light microscopy to estimate the transfection efficiency [94]. Alternatively, an aliquot of the culture is centrifuged and resuspended in PBS, and the percentage of eGFP-positive cells is determined by flow cytometry. We expect 80–90% eGFP-positive cells by day 2 post-transfection.

**CHO-DG44 cells**

As with HEK-293E cells, a medium exchange must be performed the day before transfection for CHO-DG44 cells grown in ProCHO5™ medium. This culture is started at a density of 1.5–2.5 × 10⁶ cells/mL. The next day the appropriate volume of

![Fig. 3. Schematic diagrams of a one-liter TGE process in HEK-293E (top) and CHO cells (bottom). Production media for HEK-293E cells include EX-CELL® 293, Pro293™, FreeStyle™293, and FreeStyle™ F17. For cell scale-up, the approximate cell density one day after the inoculation is given. The clock indicates time-dependent steps. Abbreviations: F68, Pluronic®-F68.](image-url)
culture is centrifuged, and the cell pellet is resuspended at a density of $5 \times 10^{6}$ cells/mL in ProCHO5™ medium pre-warmed to 31°C. DNA and PEI are added sequentially at 0.6 µg/million cells and 3.0 µg/million cells, respectively. After each addition the culture is mixed by swirling. For a 1-L (final volume) transfection, $5 \times 10^{8}$ cells are resuspended in 1 L of medium in a 5-L cylindrical glass bottle (Fig. 3). Immediately, 3 mg of DNA and 15 mg of PEI (1 mg/mL stock solution) are added sequentially with brief mixing after each addition [89]. The culture is then transferred to an incubator shaker at 31°C with 5% CO₂ and agitated at 120 rpm with the bottle cap slightly open [79,89,95]. Under these conditions, the cells go through one doubling by day 1 post-transfection and then stop dividing [79,89]. The cells can be maintained at 31°C for up to 10 days before the cell viability falls below 50% [79,89,95]. We have not experienced problems with the affinity purification of secreted proteins in ProCHO5™ medium, and so this medium can be used for all steps of the TGE process. With CHO-DG44 cells the transfection efficiency, as determined by co-transfection with an eGFP expression vector as described for HEK-293E cells, is usually 50–60%. The maximum accumulation of transmembrane and intracellular proteins is usually observed at 2–4 days post-transfection, while for secreted proteins the maximum level can be observed at 4–10 days post-transfection.

**Limitations of TGE**

For TGE processes using CHO-DG44 and HEK-293E cells, they have observed yields up to 300 mg/L and 1 g/L, respectively, for a recombinant IgG antibody [14,89]. For membrane and intracellular proteins, the highest yields have been about 30 mg/L [96]. Note that these levels will not be achieved with every protein. For any given protein, the yield is usually higher from HEK-293E cells than from CHO-DG44 cells, but there are exceptions to this rule. Therefore, we routinely check the expression of each protein in both hosts.

Even though the TGE yields are satisfactory for most proteins and for most research applications, some aspects of the TGE processes described here are not ideal if a large amount of protein is needed. One of the main limitations of these methods is the need to passage the cells the day before transfection to achieve the optimal transfection efficiency and protein yield. This constitutes an important cost item, and it makes large-scale TGE (>10 L) very challenging due to the need to centrifuge large culture volumes.

We have investigated the molecular limitations of the TGE processes described here using a monoclonal antibody as the model protein. We did not observe a limitation to plasmid DNA uptake in either host. Instead, the steady-state level of transgene mRNA, as measured by quantitative PCR, did reach a plateau as the amount of plasmid DNA uptake increased [93]. A transcriptional limitation for transiently transfected HEK-293E has been independently reported [97]. For both CHO-DG44 and HEK-293E cells, most of the intracellular plasmid DNA remains intact, at least up to day 3 post-transfection [93]. However, the majority of this DNA is present as linear or nicked circular DNA rather than supercoiled DNA, as judged by southern blot analysis of total cellular DNA [93]. Unfortunately, it is not known if all three forms of plasmid DNA are competent for transcription in mammalian cells. From these results, it appears that further improvements in TGE yields will need to overcome the limitation in transgene transcription.

As stated before, there are problems with regard to some of the commercial media used for TGE. For example, EX-CELL® 293 inhibits its efficient transfection with PEI, and both EX-CELL® 293 and Free-Style™ F17 inhibit the affinity purification of secreted His-tagged proteins. We have also observed lot-to-lot variation in the quality of commercial media, leading to large variability in cell viability, maximum cell density, transfection efficiency, protein yield, and protein recovery by ion exchange chromatography. Therefore, it is advisable to test every lot of medium before purchase. Unfortunately, this may not be practical for users who only purchase a few liters at a time.

**Conclusion**

As summarized in Table 1 over 50 proteins have been overexpressed by PEI-mediated transfection of HEK-293 and CHO cells since 2004. This list includes over 20 proteins whose structure has been resolved. In addition, adeno-associated virus and lentivirus vectors have been transiently produced in HEK-293 cells by the methods described here. Undoubtedly, the number of proteins produced by TGE will continue to grow as more researchers see the benefits of this approach. It is for this reason that we have outlined a detailed guideline for developing an in-house cost-effective method for recombinant protein expression by TGE using suspension-adapted HEK-293E and CHO-DG44 cells grown in OSRs. Importantly, the methods described here have been optimized for our own cells and the corresponding medium formulation for their expansion. Variations from these “optimal” conditions are expected for CHO and HEK-293 cells maintained in other laboratories under different culture conditions.

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