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Review

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

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ABSTRACT

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.

Contents

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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)[1] 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 to 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, 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 homogenous 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 host [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 × 10^6 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 Expi293F™ cells (Life Technologies Europe, Zug, Switzerland) were selected for high-density suspension growth in FreeStyle293™ and Expi293™ media (Life Technologies), respectively. A suspension-adapted cell line lacking

[1] *Abbreviations used:* TGE, transient gene expression; CHO, Chinese hamster ovary; PEI, polymer polyethylenimine; GOI, gene of interest; HEK-293, human embryo kidney 293; EBNA1, Epstein–Barr virus nuclear antigen 1; DHFR, dihydrofolate reductase; HT, hypoxanthine and thymidine; Py, polyomavirus; OSRs, orbitally shaken bioreactors; k_La, oxygen mass transfer coefficient; DO, dissolved oxygen; hCMV, human cytomegalovirus; mIE, major immediate early; mCMV, mouse CMV; UTR, untranslated region; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; CaPi, calcium phosphate; PDI, polydispersity index.

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 ß receptor (TGF-ßRII) (ED) and TGF- ßRIII (MPD)	293E	His	Yes	[99]
Vacular 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	293SF-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 (LGI1)	293E	None	Yes	[110]
Respiratory syncytial virus fusion protein (RSV-F)	293E	None	Yes	[96]
Bicylic peptides	293E	Fc	Yes	[111]
Dickkopf1 (Dkk1)	293T	His	Yes	[112]
CST complex (CTC1, 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 (PfEMP1 var2CSA ED)	293E	His	Yes	[118]
Sonic hedgehog (Shh ED)	293T and 293S GnTi$^-$	His	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)	CHO	Fc	Yes	[89]
Tumor necrosis factor receptor (TNFR ED)	CHO	Fc	Yes	[91]
B. Structure studies				
Type IIB receptor protein tyrosine phosphatase (RPTPμ and RPTPσ)	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 (NiV-G) (ED)	293T	His	Yes	[129]
Hendra 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	His	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$^-$	His	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]
Neurophilin 1 (Nrp1)	293S GnTI$^-$	His	Yes	[135]
Semaphorin 3A (ED)	293S GnTI$^-$	His	Yes	[135]
Human telomerase	293E	FLAG	No	[136]
HIV1 gp140 trimer	293T	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	293SF-3F6 and 293	None		[139]
Adeno-associated virus (AAV) vector	293 and 293E	None		[140,141]

Abbreviations: ED, ectodomain; MPD, membrane-proximal ligand-binding domain; Strep, streptavidin tag; His, 6×-histdine tag.

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

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 5×10^6 cells/mL or more. We routinely cultivate HEK-293E cells in EX-CELL®293 (SAFC Biosciences, St. Louis, Missouri) and CHO-DG44 cells in ProCHO5™ (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 peptones 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,

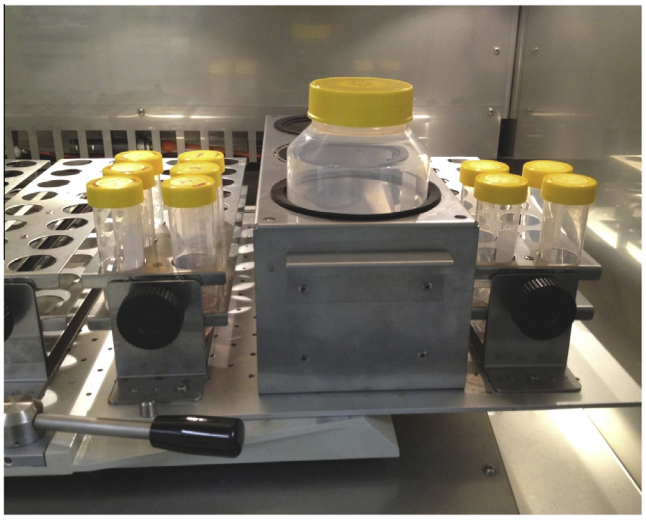


Fig. 1. Image of TubeSpin® bioreactor 50 and 600. The two disposable containers are shown in their appropriate racks fixed on the shaking platform of a incubator shaker.

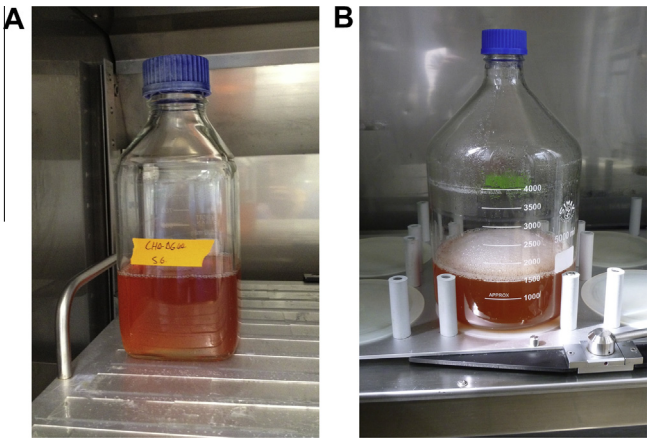


Fig. 2. Images of typical glass containers used for suspension cell cultivation in OSRs. (A) Square-shaped glass bottle of 1-L nominal volume attached to the shaker platform with double-sided tape. (B) Cylindrical glass bottle of 5-L nominal volume attached to the shaker platform with vertical supports.

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% CO_2 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% CO_2. Suitable incubator shakers with CO_2 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 mentioned above have mixing times (the time needed to mix to homogeneity) under 20 s [74]. Importantly, efficient mixing can be achieved with a low power input, resulting in less shear stress on cells, by an order of magnitude, than observed in STRs [25,70,75–77].

An oxygen mass transfer coefficient (k_La) of 7 h^{-1} is needed to avoid oxygen limitations in high-density cultures of mammalian cells in OSRs [78]. The k_La 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 CO_2 produced by cells is effectively removed from the culture so that the dissolved CO_2 concentration is maintained by the CO_2 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].

Routine maintenance of cells

Both HEK-293E and CHO-DG44 cells are subcultivated twice per week in EX-CELL®293 and ProCHO5™ media, respectively, at a cell density of 0.3×10^6 cells/mL. The cells are maintained as a 10-mL culture in a TubeSpin® bioreactor 50 or as a 100-mL culture in a 250-mL glass bottle with agitation at 180 or 120 rpm, respectively. All shaking speeds described here are based on a shaking diameter of 5 cm. For shakers with a smaller shaking diameter, the shaking speed must be higher.

We do not normally keep the cells in culture longer than 20 passages. For long-term storage, CHO-DG44 and HEK-293E cells are frozen in liquid nitrogen at a density of 15×10^6 cells/mL in aliquots of 1 mL or more, in the cultivation medium containing 10% DMSO (Sigma–Aldrich, Buchs, Switzerland).

Expression vector

Although many strong, constitutive promoters/enhancers are available for heterologous gene expression in mammalian cells, the human cytomegalovirus (hCMV) major immediate early (mIE) promoter/enhancer is usually the best choice for TGE in both CHO-DG44 and HEK-293E cells, and most commercially available expression vectors carry it. The mIE promoter/enhancer of mouse CMV (mCMV) and the human elongation factor 1-alpha (hEF-1α) promoter/enhances serve as adequate substitutes. We have found that the minimal expression vector can be constructed with the hCMV mIE promoter/enhancer, a polyadenlyation site, and either a splice site in the 5′ untranslated region (UTR) or the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in the 3′ UTR [14]. Currently, we use an expression vector ($pXLG^{HEK}$) that has the bovine growth hormone polyadenlyation site and a chimeric ß-globin-IgG splice site [14,79].

Modifications of the minimal expression vector are possible, depending on the needs of the user. For example, if the vector is frequently used for the production of secreted or membrane-associated protein, then it may be convenient to include the coding sequence for a signal peptide in the expression cassette. If this is necessary, the kappa light chain signal peptide (M E T D T L L L W V L L L W V P G S T G D) serves as a good choice. Otherwise, it is possible to include the native signal sequence of the protein of interest. An expression vector that includes the coding sequence of an affinity tag may also be desirable.

For transient antibody production, it may be advantageous to clone the genes into separate vectors in order to vary the ratio of the light and heavy chain genes to achieve the highest yield possible [80,81]. We have not observed higher antibody yields by expressing the light and heavy chain genes from a bicistronic vector with an internal ribosome binding site [82] or as a polyprotein with the heavy and light chains separated by the picornavirus 2A self-processing peptide [83].

Polyethyleneimine

Although several non-viral gene delivery methods are available for the transfection of mammalian cells, few of these are both scaleable and cost-effective. Among the scalable methods, the least expensive reagents are calcium phosphate (CaPi) and cationic polymers [11]. The former, however, requires the presence of serum in the transfection medium [84]. Since serum-free conditions are often preferred for the production phase, it is necessary to perform a medium exchange following gene delivery with CaPi [85]. Moreover, the formation of the CaPi-DNA complex is time-dependent, increasing the technical difficulty of its use at large scale. PEI-mediated gene delivery, on the other hand, does not require serum in

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].

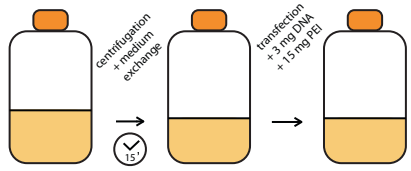
Linear 25 kDa PEI (Polysciences, Eppelheim, Germany) is dissolved in water to generate a stock solution at a concentration of 1 mg/mL and pH 7.0. Dissolving PEI requires acidification of the solution to pH 3 by addition of 1 N HCl. When all the PEI is in solution, the pH is increased to 7.0 by addition of 1 N NaOH. The solution is filter sterilized and stored at −20 °C. Linear 25 kDa PEI in this form usually has a number-average molecular weight (M_n) 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-ethyloxazoline, 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].

HEK-293E

Vessel volume [L]	1	0.25	0.25	5
Working volume [L]	0.4	0.05	0.05	1
Shaking speed [rpm]	110	120	120	120
Cell density [cells/mL]	4 x 10 ^{6}	20 x 10 ^{6}	20 x 10 ^{6}	1 x 10 ^{6}
Medium	EX-CELL ®293	RPMI + 0.1% F68	RPMI + 0.1% F68	Production
Temperature [°C]	37	37	37	37

CHO



Vessel volume [L]	5	5	5
Working volume [L]	1.5	1	1
Shaking speed [rpm]	120	120	120
Cell density [cells/mL]	4 x 10 ^{6}	5 x 10 ^{6}	5 x 10 ^{6}
Medium	ProCHO5 ™	ProCHO5 ™	ProCHO5 ™
Temperature [°C]	37	31	31

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, Pro293s®, 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.

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 × 10^6 cells/mL. The next day, the appropriate volume of culture is centrifuged, and the cell pellet is resuspended at 20 × 10^6 cells/mL in RPMI 1640 containing 0.1% Pluronic F-68 (transfection medium). As an example, for a 1-L (final volume) transfection, 1 × 10^9 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% CO_2 for 60–90 min at 37 °C 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 × 10^6 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 °C 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 × 10^6 cells/mL, instead of 1 × 10^6 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 × 10^6 cells/mL. The next day the appropriate volume of

culture is centrifuged, and the cell pellet is resuspended at a density of 5×10^6 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, 5×10^9 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% CO_2 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, we 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 efficient transfection with PEI, and both EX-CELL® 293 and FreeStyle™ 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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