Recent Advances in Transient Gene Expression Protocol

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The overall success of transient gene expression (TGE) in the production of milligram to gram quantities of recombinant proteins is largely based on the careful evaluation of process parameters on all scales up to 100 L working volume [1-4]. As briefly stated in the introduction and Figure 1.1, a typical TGE process starts with an established host cell bank such as human embryonic kidney 293 (HEK293) cells or Chinese hamster ovary (CHO) cells along with preparation of high quality transfection vectors, followed by cell growth, transfection and product expression. The entire TGE process, including cell growth and product expression phases, requires a total of one to two weeks duration depending on the production process scale. Cells are expanded through a seed train to reach the production volume, for instance 100 L for large scale production. In the late log phase cells are then infected or transfected with a viral or nonviral vector that carries coding sequence(s) of a gene of interest (GOI) for the expression of recombinant proteins, monoclonal antibodies (Mab), or protein complexes such as viral-like-particles. The soluble expressed proteins are usually secreted from cells and are present in the supernatant of the cell broth. The proteins are harvested through retention of them from the supernatant. After separation of the supernatant from the cells and cell debris, the product is recovered and purified through downstream processing (Figure 1.1). Product expression titre and production yield are significantly affected by several key factors such as host cell line, gene carrying plasmid or viral vector, expression vector construction, gene delivery method and reagent, cell culture, transfection, and the downstream production process. In this chapter, current protocols and critical steps in production processes including vectors, cell lines, gene transfer
methods, and typical large scale TGE protocols are described and discussed. Optimisation of culture medium and TGE protocols are updated in Chapter 3.

2.1 Vectors

A vector is a vehicle to carry and transfer genetic material or GOI into host cells. Besides GOI, a vector also contains other components in a vector ‘backbone’ region such as: promoters for gene transcriptions, terminators for ending transcription, components for gene translation, a replication unit for plasmid amplification in Escherichia coli (E. coli), and a component for plasmid replication in mammalian cells. Both viral and nonviral vectors have been used in TGE technology. Viral vectors, in general, deliver deoxyribonucleic acid (DNA) with higher efficiency compared with nonviral vectors. However, the preparation of viral vectors may require much effort and is labour-intensive. Biosafety concerns during production and residual viral DNA contamination to the final therapeutic protein are always negative impact factors when deciding which vector systems to use.

For expressing a complex protein composed of more than one peptide, such as a monoclonal antibody which includes heavy and light chains, a heterodimer protein such as a cytokine interleukin (IL)-12, and a ligand/receptor complex such as IL-15 and IL-15 receptor alpha complex, it is necessary to simultaneously introduce more than one transgene into a host. This can be accomplished by using two vectors (viral or nonviral) [5] with each coding for a single gene, such as heavy and light chains in the case of Mab. Otherwise, both coding sequences may be constructed in a single polycistronic vector. It is also a common approach to use one plasmid carrying two genes either under two independent promoters or under one promoter with an internal ribosome entry site inserted in between the two genes. A plasmid vector called a ‘supervector’, carrying light and heavy chains with both constant and variable regions, was constructed and used for Mab production [6]. The plasmid expresses both chains under two identical human Cytomegalovirus (hCMV)
Recent Advances in Transient Gene Expression Protocol

promoters and contains a dihydrofolate reductase (DHFR) deficient (dhfr-) marker for stable selection.

2.1.1 Viral Vector

Viruses that are used to deliver DNA into a host cell for recombinant protein expression include Adenovirus [9, 10], Lentivirus [11, 12], Baculovirus [13, 14], Vaccinia virus [15, 16] and Alphavirus [17, 18]. Viral vectors allow efficient delivery of DNA to a broad range of cells in the absence of chemical delivery agents. A master viral bank is required to maintain the consistency of virus seeds. Infected cells are normally kept for one production cycle, which usually lasts a few days. A comprehensive review article on virus-derived vectors for delivering genes into mammalian cells for gene expression and gene therapy was presented by van Craenenbroeck and co-workers [19]. Due to the infectious nature of all the viral vectors, they may pose a biosafety risk. For biosafety concerns, most operations from construction, expression, process scale-up, and downstream purification in a laboratory must be performed at biosafety level 2 or higher according to ‘National Institutes of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules’ [20].

2.1.1.1 Adenovirus

Adenovirus is a nonenveloped (i.e., has no outer lipid bilayer) virus, composed of a nucleocapsid and a double-stranded linear DNA genome. For Adenovirus as a gene carrier, the HEK293 cell is one of the hosts for Adenovirus-mediated protein production applications [21, 22], although the cell is primarily used as a host for Adenovirus production. The PER.C6 cell was also used as a host for both Adenovirus and Adenovirus-mediated protein production. Expression levels up to 63 and 21 mg/L of recombinant protein at a multiplicity of infection (MOI) of 500 and 50, respectively with high transduction efficiency (>80% at 30 viral particles (VP)/cell) have been achieved. Such a high efficiency resulted in high production yields up to 60 pg/
cell/day in a four day batch production protocol [9]. For a one litre culture at cell density $1 \times 10^6$/mL, $5 \times 10^{10}-5 \times 10^{11}$ viral infectious units would be needed [10]. Commercial expression kits, such as the ViraPower Adenoviral Expression System are available. The kits usually allow creation of a replication-incompetent adenovirus that can be used to deliver and transiently express a gene of interest in either dividing or non-dividing mammalian cells [7]. However, the use of an adenoviral vector-based transient protein production system raises the safety concern and hence it needs to be ensured that the vector virus does not contain replication competent \textit{Adenovirus} and replication deficient (E1 deleted [DE1]) \textit{Adenovirus}.

### 2.1.1.2 Lentiviruses

Lentiviral vectors (LV) are replicon defective retroviral vector systems based on the human immunodeficiency virus (HIV) [23]. LV are the most recently developed viral-derived vectors for gene therapy applications and have demonstrated much promise. The ability to transduce dividing and non-dividing cells and to sustain long-term transgene expression makes LV uniquely desirable as gene therapy vectors. LV have been used in clinical trials since 2002 [24]. With advances in vector design and large scale production, LV have become safer and more effective gene delivery systems. They can be used to express recombinant proteins in various cell types because they are able to efficiently and stably integrate their genome within the chromosome of the cells. One recent report of using LV to infect CHO cells resulted in $65 \text{ mg/L}$ of secreted recombinant alkaline phosphatase at $30 \, ^\circ\text{C}$ with a MOI of 200 [12]. Furthermore, three typical therapeutic molecules, fragment crystallisable Fc-fusion protein cluster of differentiation (CD)200/Fc, chimeric antibody chB43, and erythropoietin (EPO), were transduced with the expression system and resulted in yields of $235 \text{ mg/mL}$, $160 \text{ mg/mL}$, and $206 \text{ mg/mL}$, respectively, after 13 days post infection. The expression results were much higher than the average expression level from most reported results by TGE. To address the biosafety concerns of using HIV-based viral vectors, a ‘Biosafety Considerations for Research
Recent Advances in Transient Gene Expression Protocol

with Lentiviral Vectors’ was published by the Recombinant DNA Advisory Committee at NIH providing some general criteria to be considered when conducting risk assessments for research involving lentiviral vectors [8].

2.1.1.3 Baculovirus

Over the last 20 years, many recombinant proteins ranging from cytosolic enzymes to membrane-bound proteins have been successfully produced in Baculovirus-infected insect cells. Insect cells are able to glycosylate synthesised proteins in vivo but are unable to elongate the trimmed N-glycans to produce complex products containing terminal galactose and/or sialic acid residues [14]. Therefore, proteins produced from insect cells with different glycan structures may affect the biological function of the product. A technology called ‘BacMam’ was developed by incorporating a recombinant gene under the control of the hCMV promoter into the baculoviral genome [13, 14, 25]. Baculovirus-containing active mammalian transcriptional elements directly infect mammalian cells and have shown high expression levels of recombinant proteins in various cell lines including HEK293 and CHO cells. Unlike mammalian cell viruses, baculoviruses cannot replicate in mammalian cells, which makes baculoviruses an attractive vector for transient expression of recombinant protein in mammalian cells. A detailed protocol for gene expression in mammalian cells using BacMam was summarised by Fornwald and co-workers [26]. More examples of the use of BacMam technology to improve transient gene expression technology platforms will be described in the next chapter.

2.1.1.4 Vaccinia Virus

The Vaccinia virus is a large, complex, enveloped virus with a linear, double-stranded DNA genome. Vaccinia viral vectors have been used for high level protein synthesis in mammalian cells [27, 28]. To express transgenes, the gene is inserted downstream
of a bacteriophage T7 promoter within the *Vaccinia* genome and transcribed by the T7 ribonucleic acid (RNA) polymerase. Stringent control in the expression system that had previously been used successfully in a standard *Vaccinia* virus backbone was incorporated into the modified *Vaccinia* virus Ankara strain [27]. Using this system for expression of β-galactosidase resulted in production yields of about 15 mg protein from $10^8$ baby hamster kidney (BHK)21 cells over a 24 h period [27]. Several other proteins were also successfully expressed with this system [27].

Like other viral vectors used in research and development/manufacturing laboratories, certain safety concerns are raised because the virus can be pathogenic, and can cause serious problems in normal hosts. Therefore, production operations should be confined to biosafety level 2 facilities unless highly attenuated strains are used [29].

### 2.1.1.5 Alphavirus

Alphaviruses are positive-strand RNA viruses that can mediate efficient cytoplasmic gene expression in insect and vertebrate cells. Through recombinant DNA technology, the *Alphavirus* RNA replication machinery has been engineered for high level expression of heterologous RNA and proteins [18]. Semliki forest virus (SFV), an *Alphavirus*, has been used to express more than 100 recombinant proteins ranging from receptors, membrane proteins, enzymes, cytokines, chemokines, ion channel, and guanine nucleotide-binding proteins [18, 30].

To facilitate the production of large quantities of recombinant proteins in cells infected with SFV vectors, the adaptation to growth in suspension cultures has been established. A number of mammalian cell lines such as BHK21, CHOK1, HEK293, and rat C6 glioma cells were successfully cultured in a serum-free medium and infected with SFV. Production of Cyclooxygenase (COX)-2 at 1 L scale in
BHK cells cultured in spinner flasks generated high expression levels (16 mg COX-2/L) with high specific enzymatic activity [18]. Culture temperature may have a significant effect on expression levels: in one reported case, a 10- to 20-fold higher activity at 33 °C was achieved compared to the activity at 37 °C in BHK and CHO cells [18]. Using this technology platform, recombinant protein production has been carried out in bioreactors at an approximately 10 L scale [31]. To address safety concerns, a mutant SFV vector was developed which demonstrated significantly decreased cytotoxicity to host cells as well as a strictly temperature-sensitive phenotype. Engineering of novel temperature-sensitive mutant SFV vectors resulted in temperature-controlled transgene expression, which eliminates the risk of contaminating laboratory personnel [32].

### 2.1.2 Nonviral Vectors

Nonviral vectors, mainly plasmids, play a major role in transferring genes into host cells for protein expression. Plasmids as carriers have been involved in many published successful results [33, 34]. The most important advantages of using plasmids instead of viruses for protein production are biosafety for operators and product safety for patients. The safety requirements for large scale manufacturing of plasmid DNA (one gram of plasmid DNA is suitable for 1000 L transfection) are much lower than those required for working with a large quantity (>10^{14} infectious units) of potentially infectious virus. The entire production process of plasmid preparation, transfection, product harvest and purification can be managed in a regular production environment as for other recombinant proteins. The advantages of using plasmids for TGE also include ease of construction, amplification, and purification of plasmids. Delivery of the plasmid into cells is simple via inexpensive chemical agents. The disadvantages are lower transfection efficiency (20-80% with some chemical reagents) than viral vectors [35] as well as a size limitation that usually allows only two genes on a single plasmid.
Figure 2.1 Plasmid vector for transient gene expression.
Restriction enzyme sites in the plasmid are labelled outside the plasmid circle.

The plasmid map in Figure 2.1 represents a typical nonviral vector used for TGE. It contains a promoter CAG (a combination of the Cytomegalovirus (CMV) early enhancer element and chicken β-actin promoter) for expression of GOI, the plasmid origin of viral replication (oriP), neomycin-resistant gene (Neo) for selection in mammalian cells and ampicillin-resistant gene (Amp) for selection in E. coli. It is not necessary to construct a genetic selection marker in the vector for TGE; however, genetic selection can be used for expression by a stable transfection pool in future. The TGE plasmid can be used for selection of a stable cell line if a genetic marker is constructed.
2.2 Construction for Expression

Construction of protein expression for TGE can be a complex project involving the design including codon optimisation, promoter, lead signal sequence, and untranslated regions. It is worthwhile to think strategically during the designing stage, as this may result in a significant difference in product expression in terms of yield and quality. In this section, current practice of constructing an expression plasmid is described, while genetic optimisation of construction will be discussed in Chapter 3.

2.2.1 Promoter

The promoter is one of the biggest impact factors in protein expression using TGE. The hCMV promoter/enhancer is very powerful and highly active and is one of the strongest promoters for recombinant protein expression in mammalian cells, making hCMV very popular for production of recombinant antibodies, vaccine antigen, cytokines, fusion proteins, and others. Baldi and co-workers [36] reported that hCMV and elongation factor-1 a (EF-1a) promoters were used for transient expression of dozens of recombinant proteins by with either calcium phosphate or polyethylenimine (PEI) complex achieving maximum expression levels in the range of 2.5-28 mg/L. A particularly impressive result was achieved at 110 L batch culture with calcium phosphate to achieve 20 mg/L expression of a recombinant protein using the EF-1a promoter [36]. We evaluated a list of promoters when a r-protein is expressed in the laboratory as described in Chapter 3 and references [108, 124].

For expressing an antibody, there is a choice of either one single vector for both chains or dual vectors where the antibody heavy and light chain genes are expressed separately. A ‘supervector’ carrying light and heavy chains in one plasmid was constructed under identical CMV promoters for monoclonal antibody production [6]. Later reports revealed that when this promoter complex consisted of two identical CMV promoters, the antibody expression observed was
lower than expected based on the strength of the individual promoters [37]. Notably, a 12-fold increase in expression was found from the best of the truncated promoter complexes after stable site-specific integration when compared to the full length double CMV promoter complex [37]. Several mammalian CMV promoters (hCMV, mouse CMV, and mouse myeloproliferative sarcoma virus were compared in both HEK293E and CHOK1 cells. The full length hCMV, in the presence of intron A, provided the highest levels (approximately 10 mg/L) of protein expression in transient transfection in both cell lines.

### 2.2.2 Other Construction Components

Several leader sequences including human immunoglobulin (Ig) kappa, human IgG, CD33, CD5 used in TGE with HEK293 were summarised [36]. Several signal peptides including the native signal peptide, IgK, preprotrypsin, and tissue plasminogen activator were routinely evaluated to express a recombinant protein in HEK293 using TGE in our laboratory, and several-fold differences in product expression were observed (See Figure 3.1 in Chapter 3). Codon optimisation including balancing guanine-cytosine content and removal of potential splicing sites appeared to have a huge impact on expression of IL-15 in HEK293 cells [38].

### 2.2.3 Plasmid Preparation and Quality

Relatively large quantities of plasmid DNA are required by the TGE technology platform. Plasmid preparation requires *E. coli* fermentation followed by cell lysis and DNA purification. Many high plasmid yield (500-2600 mg/L) fed-batch fermentation processes [39-41] have been reported. However, many of these studies were based on proprietary fed-batch media and of heat inducible vector origin. Most plasmid production yields with *E. coli* DH5a strain in the biopharmaceutical industry are in the range of 100-250 mg/L [41]. One recent publication reported a simple large scale plasmid
DNA preparation for TGE usage [42]. Fed-batch growth of *E. coli* was carried out in a 5 L bioreactor by maintaining the glucose concentration below 1 g/L after the feeding had begun. Plasmid yields of 490 and 580 mg/L were achieved with *E. coli* TOP10 cells bearing two different plasmids respectively [42]. Furthermore, in the report, cell paste was lysed under alkaline conditions as a standard method [43] and one step of alcohol precipitation was used to purify the plasmids for TGE which resulted in high levels of endotoxin in the final product. Surprisingly, the plasmid DNA generated from the simple process showed excellent quality for TGE. Interestingly enough, both calcium phosphate and PEI-based transfection can tolerate a certain level of endotoxin that coexists with the plasmid. Furthermore, the HEK293 cell was able to accept plasmids with high endotoxin levels up to 10,000 IU/mg DNA through transfection [44]. However, the plasmid used in most protocols was purified through the EndoFree kit (Qiagen) with one step purification or multiple column chromatography and filtration steps.

![Figure 2.2 Plasmid production process, E.coli. MCB: master cell bank; WCB: working cell bank](image)

The production process starts from a plasmid which is transformed in to a host cell *E. coli* where it is amplified. The *E. coli* transformed with a GOI-containing plasmid is then prepared as MCB and WCB
for future production use. When large scale production begins, \textit{E. coli} cells in a WCB are inoculated into a flask containing culture medium. The seed in the flask is incubated in an environment-controlled shaker to grow the \textit{E. coli} as seed culture. When the seed culture reaches a desired density (usually overnight), it is inoculated into a fermenter where the plasmid is manufactured. Fermentation may take 10-30 h, depending upon the process used. The fermentation broth is harvested at the end of fermentation by centrifugation. The cell paste is collected and lysed. The plasmid DNA is released from the lysed cells and it is normally purified by at least one step of column purification followed by buffer exchange for final formulation. The product quality from this procedure is good enough for mammalian cell transfection. A plasmid vaccine product for therapeutic application can be purified by means of a similar procedure.

Most protocols in the last section of this chapter use plasmid concentrations of approximately 1-2 mg/L for transfections. For a large scale production of 100 g of Mab by TGE, a couple of runs of 100 L cell culture with the current highest expression level of 1 g/L will require 500-1000 mg of highly purified plasmid. This quantity of plasmid may be obtained from 10-20 L of \textit{E. coli} fermentation broth according to the current plasmid production yields in the biopharmaceutical industry. The process illustrated in Figure 2.2 can be easily scaled up to the 100 L fermentation scale for plasmid production. Overall, TGE is a viable option from plasmid to large scale transfection and expression to make 100 g of Mab for Investigational New Drug directed toxicology study and even early phase clinic trials.

2.3 Nonviral Gene Delivery

Transfection of DNA into mammalian cells involves forcing nucleic acids into cells using either physical methods, such as high voltage electric shock ‘electroporation’ [45, 46], or chemical methods with reagents such as calcium-phosphate, cationic lipids such as lipofectin [43], or cationic polymers, such as PEI [47-49]. A comprehensive