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Large-scale transient transfection of mammalian cells: A newly emerging attractive option for recombinant protein production

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Abstract

Mammalian expression systems have an undisputed long-standing and very successful history for the generation of recombinant proteins, mainly as biopharmaceuticals. However, for use as 'tool proteins' in, e.g. assay development and screening, for structure elucidation and as antigens these expression systems were generally regarded as being cumbersome, tedious and expensive. This bias has largely been overcome with the very recent development of large-scale transient transfection (LST) approaches. Especially the HEK.EBNA expression system described here has contributed significantly to this success. The simplicity and speed of this approach compares well with expression trials using the widely applied Baculovirus/insect cell system. In addition, proteins generated in mammalian cells are usually correctly folded, fully processed and functionally active.

Introduction

The introduction of foreign genetic material into cultured cells on a transient basis is a standard procedure and routinely applied on small-scale to analyze the effect of genes/proteins upon over-expression, pathway analyses and to produce small amounts of recombinant protein or conditioned medium. In contrast to the development of clonal, well-characterized host cell lines for production of, e.g. antibodies as biopharmaceuticals this approach offers many advantages: it is rapid, simple and, with a plethora of different transfection reagents and protocols available, can be applied to a multitude of cell lines.

While in theory many commonly used cell lines can also be subjected to transient transfection on large-scale for recombinant protein production, published examples are scarce as yet. In a few cases, COS cells have been used, albeit with limited success due to difficulties of adapting COS cells to serum-free suspension cultivation as well as the cytopathic effect exerted by high numbers of episomally replicating plasmids [1, 2]. Cho et al. [3, 4] described the use of HKB11 cells, a hybrid cell line derived from a fusion of 293S cells and a Burkitt lymphoma cell line, 2B8, in large-scale transient transfection experiments. However, the most widely applied cell lines in conjunction with large-scale transient transfection approaches are EBNA-1 transformed HEK293 cells (HEK.EBNA) cells and CHO cells. For both cell systems $CaPO_4$-mediated and polyethylenimine (PEI)-mediated transfection on large-scale have been described [5–11]. Here we present our integrated approach to generate recombinant proteins using PEI as transfer reagent in suspension-adapted HEK.EBNA cells.

Materials and methods

Recombinant plasmids and expression hosts

The most frequently used host cell line in LST experiments is a modified HEK 293 cell line, featuring a stably integrated copy of the EBNA-1

gene of Epstein-Barr virus (HEK.EBNA or 293-EBNA cells). The EBNA-1 gene product is capable of driving episomal replication of expression plasmids carrying the oriP origin of replication derived from EBV [12]. The cell line is commercially available from the ATCC (Cat. No. CRL-10852, 293 c18; also until recently available from Invitrogen). Suitable expression plasmids can also be obtained from Invitrogen (pREP4 and pCEP4). We have developed an in-house expression vector, which harbours an intron splice element upstream of the gene-of-interest to enhance translation, and carries a Zeocin resistance marker for selection of stable transfectants, if desired.

For large-scale transient expression, the recombinant expression vector is propagated in the *E.coli* strain DH5α in 1–20 l high-density cultures; isolation of plasmid DNA is performed by alkaline lysis and anion-exchange chromatography using NucleoBond™ cartridges (Macherey-Nagel, Oensingen, Switzerland).

Small-scale protein expression

In order to screen recombinant plasmids for functionality and expression levels a small-scale transfection trial in plate format (6-well–96-well plates) is useful as initial screen. HEK.EBNA cells cultivated adherently in Dulbecco's Modified Eagle medium supplemented with 10% foetal calf serum are seeded one day prior to transfection into the wells, which are pre-coated with Poly-D-lysine (Sigma, 0.1 mg/ml) to facilitate attachment of the cells. Transfection is performed using the transfection reagent Lipofectamine 2000™ (Invitrogen), essentially as described by the vendor. Three days post-transfection the cells (for intracellular or membrane-bound proteins) or supernatants (for secreted proteins) are harvested and subjected to protein analysis.

Large-scale transient transfection

Several essential pre-requisites exist for transfection on large- scale: (a) the availability of a transfection procedure that is highly efficient yet cost-effective, (b) a cultivation protocol for serum-free suspension culture of HEK.EBNA cells that is compatible with the transfection method applied and (c) the availability of multi-milligrams of purified plasmid DNA. The latter has briefly been addressed above.

The adaptation of HEK.EBNA cells to serum-free suspension culture by sequential 'weaning' from Dulbecco's Modified Eagle medium plus 10% foetal calf serum under agitated cultivation in shake flasks, roller bottles or bioreactors is performed following protocols described in relevant text books and manuals. However, not all commercially available serum-free media for HEK 293 cells are compatible with suitable transfection reagents for LST, such as $CaPO_4$ and PEI. Therefore, we chose to develop our own proprietary medium (M11V3) for transfection. Alternatively, the commercially available FreeStyle™ medium marketed by Invitrogen Corp. (Carlsbad, CA) gives satisfactory results.

Our transfection reagent of choice for LST approaches is the cationic polymer PEI, which was first described by Boussif et al. [13]. A stock solution of 1 mg/ml of 25 kD linear PEI (Polysciences, Warrington, PA) is prepared in water, the pH is adjusted to 7.0, the solution is sterile-filtered, aliquotted and stored at −80 °C until use. Careful optimization studies revealed that a ratio of 1 mg of plasmid DNA and 2 mg of PEI per litre of culture at a density of 1×10^6 cells/ml yielded optimal transfection results (see Results section). The plasmid DNA is complexed with PEI molecules in cultivation medium during an incubation period of 15 min at room temperature prior to addition to the cells. Following an incubation period of 4 h at 37 °C, the cell culture is then diluted 1:2 with cultivation medium to a density of 5×10^5 cells/ml as starting cell density for the production phase.

Transfection in bioreactors

The transfection protocol can be applied to cultures grown in shake flasks, roller bottles or bioreactors. Our reactor of choice is the Wave™ bioreactor (Wave Biotech, Tagelswangen, CH) at 10-l scale. Pre-cultures of HEK.EBNA cells adapted to suspension cultivation are grown in roller bottles until the desired cell density for transfection is reached. An inoculum of 3.6 l of cells at a density of 1.4×10^6 cells/ml is then transferred into the 10-l Wave bag and the DNA–PEI complexes in 1.4 l volume are added. The Wave settings, i.e. the rocking rate is reduced to 10 rpm at an angle of 7°.

Following 4-h incubation, the culture is adjusted to the final volume of 10 l by addition of 5 l of cultivation medium and the rocking rate is set back to 18 rpm at 7° angle. Subsequently, during the actual production run, cell density, cell viability as well as pH, glucose consumption and lactate production are monitored daily using an automated cell counter (Cedex, Innovatis AG, Bielefeld, FRG) and the BioProfile 200 Bioanalyser (Nova Biomedical, Waltham, MA); protein titres are determined by protein-specific analytics. The time point of harvest of the culture is dependent on the nature of the protein expressed: secreted proteins are harvested usually after 6–7 days of fermentation, while intracellularly expressed proteins may be harvested at an earlier time point to ensure highest integrity and prevent proteolytic damage of the protein brought about by release of proteases from dying cells.

Harvesting the cells or the culture supernatant is conveniently done by cross-flow filtration (Fresenius Medical Care AG, Bad Homburg, FRG), giving rise to a 500–1000 ml concentrate or cleared lysate which is subjected to further downstream processing following conventional protein purification protocols.

Results

Prior to attempting LST for a given gene a small-scale expression evaluation is routinely performed. We developed miniaturized transfection protocols for different well formats by carefully optimizing the number of cells plated and the ratio of transfection reagent vs. plasmid DNA and were able to readily detect secreted proteins in the supernatant of 96-well cultures. Also transfection of two plasmids into the same cell population to generate heterodimeric recombinant proteins is possible using this system. For this early expression evaluation, neither transfection under serum-free conditions nor cheap transfection reagents are necessary, as the consumption of reagent is low. Lipofectamine 2000™ has proven to be a very efficient transfection reagent in conjunction with HEK.EBNA cells. Transfection efficiencies of ≥90% after 3 days of incubation are routinely obtained, as evidenced by reporter gene expression such as eGFP. Pre-coating of the wells with poly-D-lysine greatly enhances the success of this experiment by firmly attaching the HEK.EBNA cells to the surface and thus minimizing cell losses during the washing steps.

Following the preparation of plasmid DNA in sufficient quantity and quality, transfection on the multi-litre scale is done using PEI as transfer reagent. Polyethylenimine (PEI) is commercially available as branched and linear molecules of different molecular weights. The linear 25 kD PEI reagent that has been shown to be most effective in transient transfection approaches [5, 10] is our reagent of choice.

The ratio of plasmid DNA to PEI reagent is probably one of the most critical parameters to be assessed. As shown in Figure 1, a two-fold excess of PEI reagent over DNA is essential; the absolute quantities of plasmid and PEI appear to be less critical. A further reduction to 1 mg plasmid DNA and 2 mg of PEI reagent per litre culture yields similar results, while enhancing the excess of PEI beyond two-fold does not increase productivity (data not shown). The supplementation of the culture with FCS prior to transfection to facilitate the uptake of PEI complexes into the cells, as stated in some transfection protocols [5] is not necessary. During the incubation time of 4 h, the rocking rate of the Wave platform is reduced to 10 rpm to allow attachment and uptake of the PEI particles; settling of the cells at the bottom of the bag should, however, be avoided to prevent a heat-shock of the cells exerted by the thermoplate. A prolongation of the incubation time to 24 h prior to addition of feed medium does not lead to increased yields (data not shown).

Finally, the cell density at transfection was chosen such that the density of cells at the beginning of the production phase at 10-l scale reaches $4–5 \times 10^5$ cells/ml, in order to prevent a lag phase in cell growth and production.

These optimization efforts resulted in the protocol described above with transfection efficiencies of approx. 80% in a highly reproducible process. We have expressed more than 30 proteins following this approach and obtained titres ranging from 0.5 mg/l to ≥12 mg/l depending on the individual nature of the gene/protein. A typical transfection/production run for a secreted protein is depicted in Figure 2.

The integrated expression approach from small-scale to large-scale is shown in Figure 3. Pre-selection of a suitable expression construct as well as assessment of expression levels allows all

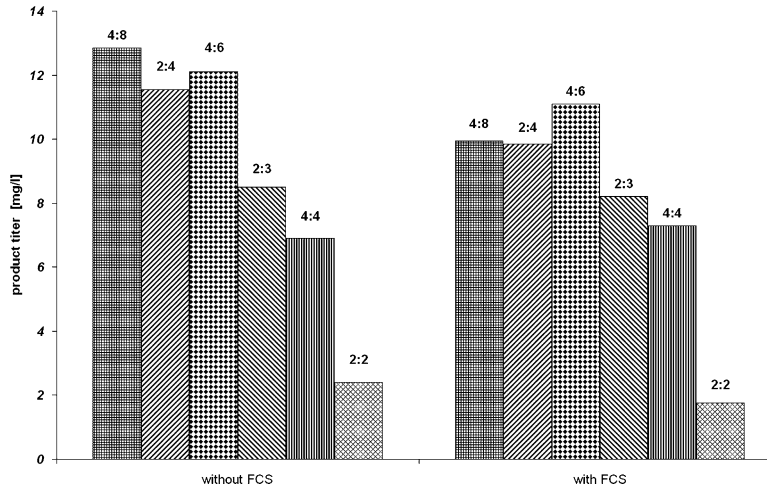

Figure 1. Determination of plasmid DNA and PEI concentrations used in transient transfection. The indicated ratios correspond to µg quantities of DNA:PEI per ml culture. Results with and without addition of 1% FCS to the medium prior to transfection are shown. The product titre was determined 6 days post-transfection.

subsequent steps, e.g. scale of plasmid preparation and transfection into mammalian cells to be adapted to the required scale.

Discussion

Large-scale transient transfection approaches using mammalian cells as hosts have not yet found broad application in industrialized recombinant protein production, but their enormous potential clearly sets the stage for future trends especially in the field of tool protein production [14]. Mammalian host cells bear the unbeatable advantage of being able to process and modify proteins of human and higher eukaryotic origin to full functionality without the necessity for codon optimization and protein refolding [15]. Even for structural analyses, where secondary modifications of a protein are often thought to negatively influence successful crystallization, protein preparations derived from insect cell and mammalian sources have been

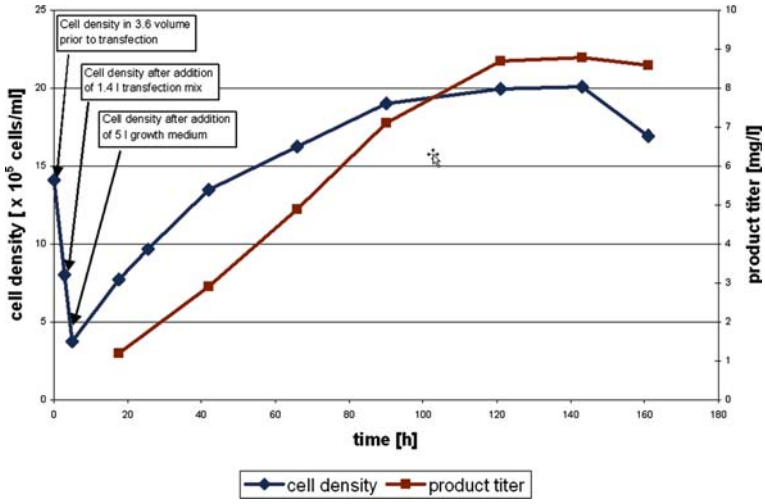

Figure 2. The graph shows a typical fermentation run of HEK.EBNA cells transfected and cultivated in the Wave™ bioreactor in M11V3 (Novartis proprietary medium) and fed with 5 l of ExCell VPRO (JRH Biosciences, Lenexa, KS) medium for the production phase. Final volume at harvest: 10 l.

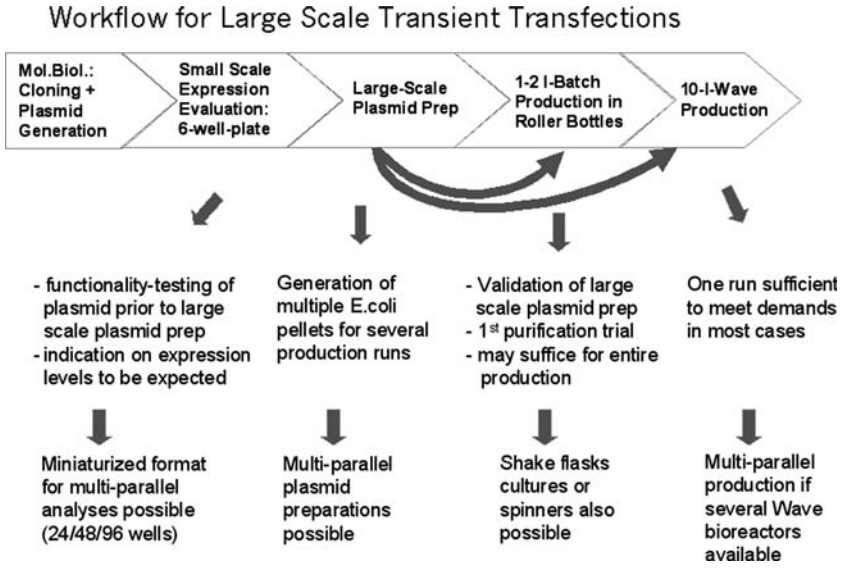

Figure 3. Workflow for the large-scale transient transfection process. For details see text.

shown to give rise to high quality crystals and subsequent structures, provided that sufficient protein can be produced.

The most successful cell line currently employed in transient expression trials is HEK.EBNA. Several genetic features of this human cell line contribute to its success, e.g. the presence of the Adenovirus-derived E1a protein in the cells driving and enhancing transcription from a CMV promoter preceding the gene of choice, and the EBNA-1 protein allowing episomal replication of oriP-containing expression plasmids. Additionally, these cells are easily transfectable, easy to cultivate and not very demanding with respect to nutrients and growth factors in the culture medium.

Commercially available transfection reagents such as Lipofectamine 2000^{TM}, despite their usefulness for routine small-scale experiments, cannot be considered as cost-effective on large- scale, but two methods described for LST meet the above mentioned criteria: $CaPO_4$-mediated transfection and the application of the cationic polymer PEI as complexing agent. $CaPO_4$-mediated and PEI-mediated gene transfer have both been performed successfully on large-scale (1–100-l scale) using HEK.EBNA cells [5, 16].

In both instances, the choice of medium for cultivation of HEK.EBNA cells in suspension is of prime importance for success. Not only should the cultivation medium support good cell growth as single cells or small aggregates, but transfection using PEI-complexed plasmid DNA should also give rise to transfection efficiencies of ≥80% which is not always the case when using serum-free suspension media advertised for HEK 293 cell cultivation. Unfortunately, most of the commercially available culture media with proprietary composition preclude successful transfection with PEI for unknown reasons. This provoked us to develop our own serum-free suspension culture medium M11V3. It can be expected, though that with increasing success in PEI-mediated transfection the relevant industries will develop PEI-compatible cell culture media.

$CaPO_4$-mediated gene transfer on the other hand, carries the drawback that suspension culture media mostly feature a low calcium content to prevent cell aggregation. This leads to an immediate precipitation of the $CaPO_4$ complexes when added to the cells. Thus, a change of medium to, e.g. an intermediate transfection medium (such as DMEM/F12) with normal calcium content is mandatory, rendering the process not highly feasible and prone to contaminations.

In comparison to the widely applied Baculovirus/insect cell expression (BEVS) system, large-scale transient transfection into mammalian cells offers several advantages. While for successful expression using BEVS two parameters, i.e. the generation of viral particles and recombinant protein production require optimization, the LST approach is directly geared towards optimal protein expression. Additionally, the storage of large plasmid preparations

vs. the appropriate storage of virus stocks (and the potential deterioration of viral particles over time) is advantageous. Both systems allow the rapid assessment of different expression constructs featuring, e.g. different domain lengths and the impact of positioning of (cleavable) protein tags at the N- or C-terminus of the gene.

Finally, by transient expression not only secreted, but also intracellularly expressed proteins can conveniently be produced; stable recombinant mammalian cell lines usually do not overexpress recombinant proteins intracellularly, unless regulated by inducible expression [15].

Thus, the development of transient production processes abrogates many, although not all of the prejudices towards mammalian expression systems for recombinant protein production.

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