Transient Gene Expression: Recombinant Protein Production with Suspension-Adapted HEK293-EBNA Cells

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Abstract: Transient gene expression (TGE) in mammalian cells at the reactor scale is becoming increasingly important for the rapid production of recombinant proteins. We improved a process for transient calcium phosphate-based transfection of HEK293-EBNA cells in a 1–3 L bioreactor volume. Cells were adapted to suspension culture using a commercially available medium (Bio-Whittaker, Walkersville, MD). Process parameters were optimized using a plasmid reporter vector encoding the enhanced green fluorescent protein (EGFP/CLONTECH, Palo Alto, CA, USA). Using GFP as a marker-protein, we observed by microscopic examination transfection efficiencies between 70–100%. Three different recombinant proteins were synthesized within a timeframe of 7 days from time of transfection to harvest. The first, a human recombinant IgG1-type antibody, was secreted into the supernatant of the cell culture and achieved a final concentration of >20 mg/L. An E. coli-derived DNA-binding protein remained intracellular, as expected, but accumulated to such a concentration that the lysate of cells, taken up into the entire culture volume, gave a concentration of 18 mg/L. The third protein, a transmembrane receptor, was expressed at 3–6 × 10^6 molecules/cell.


Keywords: transient transfection; HEK293-EBNA; calcium phosphate; suspension

INTRODUCTION

Various biological expression systems are currently in use for the large-scale production of recombinant proteins. They involve microbial as well as eukaryotic host cells. Mammalian cells are becoming increasingly important as novel transfection and clonal selection methods allow the generation of cell lines expressing recombinant protein at higher concentrations. Widely used stable expression systems based on chromosomal integration of foreign DNA is costly and time-consuming. As a more rapid alternative, we present here an efficient transient expression system, which was successfully applied for the synthesis of a number of different recombinant proteins.

Transient expression systems have thus far been exploited only for the synthesis of smaller quantities of recombinant proteins using different cell lines, e.g., COS cells (Blasey et al., 1996), HEK293 (Jordan et al., 1998), and BHK cells (Wurm and Bernard, 1999). To make transient expression approaches more efficient for industrial purposes, improvements have to focus on scale-up to larger volumes, simplification or reduction of handling steps, and reduction in the amount of DNA for transfection for economic reasons.

Here we present our effort to address these issues in studies on a transient gene expression (TGE) technology for suspension cultures based on calcium-phosphate transfection. It is hoped that this methodology would eventually become feasible at a 100-L scale or larger, yielding commercial quantities of recombinant proteins. Recombinant HEK293 cells, constitutively expressing the Epstein-Barr virus nuclear antigen (EBNA), had to be adapted to growth in suspension. These cells were then used for transfection with an improved CaPi coprecipitation method and subsequently maintained in a simple batch culture for up to 1 week. Using 100 ml spinner flasks and stirred bioreactors at the 1–3 L scale we achieved transfection efficiencies of up to 80% using EGFP (CLONTECH, Palo Alto, CA, USA) as a reporter protein. To demonstrate the broad applicability of our method for large-scale production, we chose recombinant proteins with different target sites for localization. With the medically important serotonin receptor 5-HT3 (Gandara et al., 1998), a transmembrane receptor, we reached receptor densities of 3–6 × 10^6 molecules per cell 2 days posttransfection. This expression level is comparable to

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those reported earlier for the same protein when using the very efficient Semliki Forest virus expression system (Blasey et al., 1998). The second example concerns the production of a bacterial protein: the DNA-binding domain of the Tetracycline repressor, which is produced intracellularly upon transfection. For reasons unknown, it had failed to be produced in significant quantities in E. coli, yet we achieved up to 18 mg/L within 2 days. Finally, as an example of a secreted recombinant protein, we produced an IgG1 immunoglobulin molecule with titers of up to 40 mg/L. This antibody is directed against RhD-antigen (Miescher et al., 1998) and is a candidate for a prophylactic drug.

MATERIALS AND METHODS

Vectors

Full-length cDNA of the heavy (5.3 kb) and light chain (4.1 kb) of anti-human RhD-IgG1 (provided by M. Zahn, UNILausanne, Switzerland) were cloned into the eukaryotic expression vector pEAK8 (Edge BioSystems, Gaithersburg, MD, USA), hereafter designated pEAK8-LH39 (encoding for the light chain) and pEAK8-LH41 (encoding for the heavy chain).

TetR DNA-binding domain (800 bp) was previously amplified by PCR from pTA plasmid (CLONTECH) for expression in bacteria and cloned into the pEAK8 vector (hereafter pEAK8-TetR).

Full-length 5-HT3 cDNA (1.5 kb; provided by K. Lundström, F. Hoffmann-La Roche, Basel, Switzerland) was cloned into the eukaryotic expression vector CMVβ (CLONTECH, Palo Alto, CA, USA) by replacing the β-galactosidase reporter gene.

DNA Preparation

All plasmids were produced in E. coli strain DH5α grown in LB medium + Ampicillin (50 μg/ml). Plasmid DNA was isolated and purified using plasmid preparation kits (Macherey-Nagel, Oensingen, Switzerland). Quality and quantity of plasmid DNA were verified by gel electrophoresis and UV-spectrometry.

Transfection in Spinner Flasks

Transient transfection of suspension adapted HEK293-EBNA cells was carried out in DMEM/F12 medium supplemented with 29 mM sodium bicarbonate, 10 mM HEPES, 2.5 mg/L human transferrin, 2.5 mg/L insulin, 0.1 mM diethanolamine, 0.1 mM L-proline, and 1% FCS (hereafter DMEM-based medium). Prior to transfection, cells were expanded in 0.5-3 L spinner flasks in 293G medium supplemented with 1% FCS. Cells were maintained in this medium at 37°C for 2 h. 125 μg of supercoiled plasmid DNA was precipitated in a 5-ml transfection mix consisting of 2.5 ml 250 mM CaCl2 and 2.5 ml 1.4 mM phosphate in 50 mM HEPES + 280 mM NaCl that had been combined and mixed rapidly. After an incubation period at RT of exactly 1 min the precipitation mix was added rapidly to the cell suspension. Four hours later, 45 ml fresh medium were added in order to facilitate dissolution of the precipitate.

Transfection efficiencies (number of cells showing expression in percent) were determined by using pEAK8-EGFP vector as a reporter. The assessment of transfection efficiency was executed about 24 h after transfection using laser scanning confocal microscopy.

For production of anti-human RhD-IgG1, cells were cotransfected with the two plasmids pEAK8-LH39 and pEAK8-LH41 at a ratio of 3:7. At days 3 and 6 posttransfection, samples were taken and IgG1 titers analyzed by ELISA. HEK293-EBNA cells transfected with pEAK8-TetR were harvested at day 2. The expression of the TetR protein was evaluated by SDS-PAGE and Western blotting.

Transfection in Bioreactors

Suspension-adapted HEK293-EBNA cells were expanded in 0.5–3 L spinner flasks in 293G medium supplemented with 1% FCS. For transfection, cells were centrifuged (Heraeus) in 1 L bottles for 5 min at 400g and the 3 L bioreactor (Applicon) was inoculated with 3 × 10^5 cells/ml freshly resuspended in 800 ml of DMEM-based medium containing 1% FCS. Cells were maintained in this medium for 2 h (37°C, pH 7.4; 200 rpm stirrer speed). Five minutes prior to transfection, 2 ml 2.5 M CaCl2 was injected through a septum into the bioreactor to elevate the calcium concentration in the medium to 7.25 mM. Transfection was carried out with 1 mg of total DNA precipitated in a 40 ml transfection cocktail (20 ml 250 mM CaCl2 containing 1 mg plasmid DNA were mixed with 20 ml 1.4 mM phosphate in 50 mM HEPES + 280 mM NaCl, pH 7.05). One minute after rapid mixing of the transfection cocktail the solution was taken up into a 60 ml syringe and injected through a septum into the bioreactor. Four hours later, 200 ml fresh DMEM-based medium were added and the pH was slowly reduced from 7.4 to 7.1 (dissolution of the DNA precipitate). Temperature, dO2, and pH of the medium of the bioreactor were controlled and measured on-line. Cell density, glucose, and lactate as well as the concentrations of recombinant products were analyzed off-line by taking samples once per day. After cotransfection of HEK293-EBNA cells with pEAK8-LH39 and pEAK8-LH41 (at a ratio of 3:7), daily samples were collected and kept at −20°C. IgG1 titers of all the supernatants were subsequently analyzed by ELISA. Two days after transfection the expression of 5-HT3 receptor was evaluated by radioligand-binding assay (according to Hovius et al., 1998).
Anti-human RhD-IgG1 ELISA

Goat anti-human kappa light chain IgG (Biosource) was used for coating the ELISA-plates and with AP-conjugated goat anti-human gamma chain IgG (Biosource) the synthesized anti-human RhD-IgG1 was detected. NPP was used as a substrate for the alkaline phosphatase. Absorption was measured at 405 nm against 490 nm using a microplate reader (SPECTRAmax™340; Molecular Devices, Palo Alto, CA, USA).

Laser Scanning Confocal Microscopy

The fluorescent signals were obtained using a Zeiss LSM 510 laser scanning confocal microscope. Suspension cell samples were transferred to the perfusion chamber (Attofluor, Zeiss). EGFP signal recording was achieved with an appropriate filter set (488 fluorescein). Scanning speed and laser strength were adjusted to avoid photobleaching, cell damage, or morphological changes.

RESULTS

Adaptation of HEK293-EBNA Cells to Suspension Culture

Adherent HEK293-EBNA cells (Invitrogen, La Jolla, CA, USA) were cultivated in DMEM/F12 (1:1) medium, supplemented with 2.4 g/L sodium bicarbonate and 2% fetal calf serum (FCS). In the first step of adaptation of these cells to growth in suspension we used a medium specially developed for serum-free suspension culture of HEK293 cells (293G formula from BioWhittaker). At the start of the culture, cells were subcultivated (1:2) in 293G medium supplemented with 2% FCS, in adherent mode, using a trypsin treatment twice a week. Trypsinized cells were transferred into spinner flasks at a density of 5 × 10^5 cells/ml. Cells were kept in suspension at a stirrer speed of 80 rpm. Using 2% FCS in the medium, formation of large aggregates (>200 cells per aggregate) was observed. Reducing FCS concentration to 1% drastically reduced the size of these cell aggregates to about five cells per aggregate. Subsequently, cells were subcultivated twice a week with seeding densities of 2–3 × 10^5 cells/ml. After an adaptation phase of 6 weeks, cells achieved maximal cell densities of 1.5–2 × 10^6 cells/ml and were growing as single cells or small cell aggregates (<5 cells) with an overall viability of over 95%.

For transfection, we used a protocol for CaPi coprecipitation, originally developed by Jordan et al. (1996) for adherent HEK293 cells in DMEM/F12 medium supplemented with 2% FCS. Initially, we tried to transfect cells in BioWhittaker 293G medium but did not observe significant transfection efficiency. However, after carrying out a medium exchange to a DMEM/F12-based medium, the suspension-adapted cells appeared highly transfectable (Table I). Since exposure to the high calcium concentration in DMEM/F12 medium during the first 4 h posttransfection resulted in the formation of large cell aggregates (>20 cells), the assessment of transfection efficiency was done by using laser scanning confocal microscopy with cells that had been transfected with pEAK8-EGFP. Confocal slices of the aggregates revealed high transfection efficiencies (up to 80%) at any position (Fig. 1).

Transient Transfection in Spinner Flasks

Transfection of anti-human RhD-IgG1 or TetR encoding DNA vectors were initially done in 200 ml spinner flasks. For each transfection of the recombinant protein of interest, control transfections were carried out using pEAK8-EGFP in parallel spinner flasks inoculated with a fraction of the same cell population. The percentage of cells expressing microscopically visible green fluorescent protein were used as an indication of transfection efficiency per transfection (Table I). For a final volume of 100 ml we used 125 μg of plasmid DNA. In order to produce recombinant anti-human RhD-IgG1, cells were cotransfected as described in Materials and Methods. At days 3 and 6 posttransfection cell-free supernatants were collected and IgG1 concentrations were analyzed by ELISA. Up to 40 mg/L of antibody were observed within 6 days (Table I).

For the production of TetR protein, cells were harvested 2 days posttransfection. Histidin-tagged TetR protein was purified from the cell-lysate by affinity chromatography. Samples were analyzed by SDS-PAGE (Fig. 2A) and West-
ern blot (Fig. 2B) before, during, and after purification. The concentration of the purified TetR protein was evaluated in a spectrophotometer at 280 nm; 18 mg/L of this protein, expressed intracellularly, were produced within 2 days.

### Transient Transfection in Bioreactors

Transient transfections in bioreactors were performed at scales from 1–3 L, using pEAK8-EGFP as a reporter plasmid for development efforts concerning the various transfection and production steps. For a final harvest volume of 1 L cell-supernatant 1 mg of plasmid DNA was used and transfection efficiencies of 60–80% (percentage of green cells) were observed (Table I).

### IgG1 Production

For the production of the recombinant anti-human RhD antibody, HEK cells were cotransfected with 300 µg pEAK8-LH1 and 700 µg pEAK8-LH2 at a cell density of 3 × 10^5 cells/ml. Figure 3A shows on-line measurements of oxygen levels, pH-values, and temperature control of a typical transfection experiment performed in a 3 L Applicon-Bioreactor.

We observed that HEK293 cells were sensitive to shear forces and air bubbles, especially during the transfection process. For that reason the cell cultivation was initiated with a high oxygen level of 100% (relative to air) and the aeration was stopped directly after transfection onset. The aeration was only continued when the oxygen level reached a limit value of 35% oxygen relative to air, which generally occurred around 40 h posttransfection. For the following cultivation time the oxygen level was kept between 35 and 40%. The pH set-point was 7.4 at the time of inoculation and was maintained during the transfection period (4 h). After the addition of fresh medium at the 4 hour time point posttransfection, the pH set-point was lowered to 7.2 ± 0.1, therefore pH 7.3 was rapidly reached by the addition of CO₂. In a second step the pH was adjusted to 7.1 to dissolve residual DNA precipitate and was kept at this level during further cultivation. Figure 3B shows off-line measurements of cell densities and antibody titers from the same reactor run. The initial cell density was reduced from 3 × 10^5 to 2.5 × 10^5/ml due to the dilution effect caused by the addition of fresh medium at the 4-h time point. Subsequently, transfected HEK293-EBNA cells show a growth behavior that is typical for a batch culture; however, with a reduced maximal cell den-
We have routinely seen cells to perform about one population doubling, reaching $6.5 \times 10^5$ cells/ml. Significant antibody titers were observed from about 50 h post-transfection. The kinetics of product formation appear to closely follow the observed kinetics of cell growth. While at about 120 h a drop in cell mass begins to appear (the subsequent cell count reveals a viable cell density of only $4.5 \times 10^5$ c/ml), the product accumulation continues, reaching 16 mg/L at the time of harvest (Fig. 3B). Transiently expressed IgG1 was purified and subsequently tested in a number of biological and biochemical assays (antigen-binding, antibody dependent cell-mediated cytotoxicity (ADCC)) and showed full functionality indistinguishable from IgG1 materials derived from stably transfected CHO cells (data not shown).

5-HT3 Serotonin Receptor Production

Transfection experiments with adherent HEK293-EBNA cells revealed highest transmembrane 5-HT3 serotonin receptor expression 2 days posttransfection (Table I). Therefore, for large-scale production in suspension we transfected HEK293-EBNA cells in a 1 L bioreactor with 1 mg of plasmid DNA and harvested the cells at the expected production peak. The purification of the 5-HT3 receptor by immobilized metal-ion chromatography was performed in one step exploiting a hexahistidine tag (according to Hovius et al., 1998). The receptor yield was determined by radioligand binding using a 5-HT3 specific antagonist $[^3H]GR65630$. We reached levels of $3–6 \times 10^6$ receptor molecules per cell. The protein preparation showed high purity by SDS-PAGE as one band at 65 kD (Fig. 4), corresponding to the expected size of a glycosylated receptor.

DISCUSSION

Transient gene expression is an emerging technique, with the potential to speed up recombinant protein production in mammalian cells. In comparison to stable expression the transient system is less time-consuming and more cost-effective and offers increased versatility for approaches such as combinatorial gene expression using several protein-encoding DNAs in a single transfection. We used an improved CaPi DNA coprecipitation technique, which has been shown to be applicable for a wide range of cells, including CHO, HEK, BHK, HeLa, and primary neuronal cells.

Initially, this method was optimized for adherently growing producer cell lines like CHO and HEK293 cells (Jordan et al., 1996). Later studies indicated that this technique was also applicable for suspension-adapted HEK cells (Jordan et al., 1998). However, the obtained transfection efficiency and productivity was not yet competitive to stable expression systems.

In the present study we have described for the first time the adaptation of HEK293 cells expressing EBNA1 (Invitrogen) to growth in suspension and their subsequent transfection, resulting in commercially relevant quantities. We chose HEK293-EBNA cells because EBNA1 expression can lead to the replication of oriP-based plasmids (Yates et al., 1985). We obtained transfection efficiencies equivalent to those previously reported for adherently growing cells. For a bioreactor-based approach we used a suspension growth medium provided by BioWhittaker. However, in this medium transfection with CaPi coprecipitation was not possible, presumably because of unknown Ca$^+$ and PO$_4^{2-}$ concentrations in the medium that are incompatible with the maintenance of an effective CaPi precipitate for transfection. In spite of considerable effort, we were not able to identify conditions that allowed transfection with CaPi at reasonable efficiencies. We therefore decided to perform a medium exchange to a DMEM/F12-based medium prior to transfection, thus providing optimal calcium and phosphate concentrations. As a further improvement, the time between cell seeding and transfection could be reduced from 16–20 h for the adherent system to 2 h in the suspension mode. In addition, the volume of the transfection mix and DNA amount could be reduced by 50%. While still not entirely overcome as a potential bottleneck, the amount of plasmid DNA needed for transfections with our new protocol (1 mg
DNA per 1 L of cell suspension) appears now reasonable and practical, at least up to the 10-L scale.

To show the general applicability of our method for rapid expression we produced a variety of recombinant proteins in different cell compartments. Both soluble and membranous proteins were shown to be expressed in high amounts within a few days. The inherent advantage of calcium-phosphate as a nonviral transfection system allowing expression of multiple polypeptides (separate plasmid vectors) is significant.

As an example for an intracellular protein, we expressed the DNA-binding domain of the bacterial tetracycline repressor (TetR). This protein failed to be expressed in *E. coli* at reasonable levels, presumably because of rapid degradation. In HEK293-EBNA cells we observed an increasing expression of the TetR protein, which leveled out after 2 days and remained stable as shown by SDS-PAGE. Using our newly developed method in a 100 ml spinner flask, enough recombinant protein could be produced for starting phage display or other analytical experiments (e.g., EMSA, band shift assay).

The second recombinant protein we chose was the medically important serotonin (5-HT<sub>3</sub>) receptor, which is composed of five identical units forming an ion channel (Maricq et al., 1991). We determined the quantity of receptor molecules posttransfection to reach up to 6 million per cell, a density comparable to numbers previously reported for viral expression systems like the Semliki Forest virus system (Lundström et al., 1997).

As an example of a secreted recombinant protein, a clinically relevant antibody molecule, anti-human Rhesus D IgG1, was transiently expressed in our suspension-adapted HEK293-EBNA cells. Here we reached antibody production levels comparable to stable CHO cell lines (5–15 mg/L) (De Jesus et al., 1999), which usually need many months of research and development work before becoming useful in a bioreactor-based production facility.

Overall, we showed the feasibility of transfection with the CaPi-DNA-coprecipitation method to be reliable, versatile, and reasonably productive in a bioreactor-based suspension system with HEK293-EBNA cells. Scale-up to the 10- and 100-L scale of this technology now appears within reach. Also, further improvements in technology and a better understanding of gene transfer in mammalian cells will provide opportunities for even higher expression levels. This makes transient gene expression based on the low-cost calcium-phosphate delivery system for DNA an economical and extremely fast technology for the generation of milligram to gram quantities of any protein of interest. Whether this technology could eventually become a pharmaceutical production approach has yet to be explored.

![Figure 3](image-url) **Figure 3.** Transient expression of the secreted anti-human Rhesus D immunoglobulin in HEK293-EBNA cells after transfection with 1 mg of plasmid DNA. Cells were transfected with the CaPi coprecipitation method in a 3 L bioreactor (1 L working volume) and cultivated for 6 days in DMEM/F12-based medium (+1% FCS). A: On-line data of one representative bioreactor run (temperature, pH, DO<sub>2</sub>). B: Corresponding off-line data of viable cell counts and product concentrations (anti-human RhD IgG<sub>1</sub>) are shown as a function of time.

![Figure 4](image-url) **Figure 4.** SDS-PAGE analysis of the expressed and purified 5-HT<sub>3</sub> receptor. Proteins were separated on a 12% SDS-PAGE gel. The purified protein is visible as one single band at 65 kDa (lane 2) and at 49 kDa the nonglycosylated protein appeared. Lane 1: molecular marker; lane 2: purified 5-HT<sub>3</sub> protein.
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References