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Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions

Gaurav Backliwal[1], Markus Hildinger[2], Sebastien Chenuet[1], Sarah Wulhfard[1], Maria De Jesus[2] and Florian M. Wurm[1,2,*]

[1]Ecole Polytechnique Fédérale de Lausanne, Laboratory of Cellular Biotechnology, Institute of Bioengineering, Faculty of Life Sciences, Lausanne, Switzerland and [2]ExcellGene SA, Monthey, Switzerland

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ABSTRACT

Transient transfection allows for fast production of recombinant proteins. However, the current bottlenecks in transient transfection are low titers and low specific productivity compared to stable cell lines. Here, we report an improved transient transfection protocol that yields titers exceeding 1 g/l in HEK293E cells. This was achieved by combining a new highly efficient polyethyleneimine (PEI)-based transfection protocol, optimized gene expression vectors, use of cell cycle regulators p18 and p21, acidic Fibroblast Growth Factor, exposure of cells to valproic acid and consequently the maintenance of cells at high cell densities (4 million cells/ml). This protocol was reproducibly scaled-up to a working volume of 2 l, thus delivering >1 g of purified protein just 2 weeks after transfection. This is the fastest approach to gram quantities of protein ever reported from cultivated mammalian cells and could initiate, upon further scale-up, a paradigm shift in industrial production of such proteins for any application in biotechnology.

INTRODUCTION

Recombinant proteins are of great commercial and scientific interest. Yet, most current production methods in mammalian cells involve the time- and labor-consuming step of creating stable cell lines (1). In some instances, it might not even be feasible to generate a stable cell line expressing a particular protein of interest. Here, production methods based on transient gene expression can offer a solution (2–6).

However, the major bottlenecks in transient transfection are low titers and low specific productivity compared to stable cell lines (7,8): whereas transient transfection yields titers in the range of 20–40 mg/l with a specific productivity of 1–4 pg/cell/day, stable cell lines reach 1–2 g/l with a specific productivity of 20–50 pg/cell/day (1).

Here, we report an optimized transient protein production method that yields titers exceeding 1 g/l in HEK293E cells. The HEK293E cell line used is a suspension adapted human embryonic kidney-293-based cell line stably expressing the Epstein–Barr virus nuclear antigen (EBNA1) (6,9). Titers were obtained by combining rational vector design with multi-pathway modulation based on previously performed systematic optimizations of each transfection parameter (10–12) in HEK293E cells. In short, cells were transfected at high cell densities (20 million cells/ml) with 25-kd linear polyethyleneimine (10,13,14) with a total of five HEK 293-optimized expression vectors encoding IgG heavy chain, IgG light chain, the cell cycle regulators p18 and p21 and the growth factor acidic Fibroblast Growth Factor (aFGF). Upon adjustment of cell density to 4 million cells/ml, cells were subsequently exposed to valproic acid for 10–14 days.

Titers obtained in small-scale experiments were reproduced in orbitally shaken bioreactors (15) with a working volume of 2 l obtaining a yield of 860 mg/l, thus delivering >1 g of purified protein just 2 weeks after transfection. This is the fastest approach to gram quantities of protein ever reported from cultivated mammalian cells and could initiate, upon further scale-up, a paradigm shift in industrial production of such proteins for any application in biotechnology.

*To whom correspondence should be addressed. Tel: +41 (0) 21 693 61 41; Fax: +41 (0) 21 693 61 40; Email: florian.wurm@epfl.ch

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

MATERIALS AND METHODS

Vector construction

pEAK8-LH39 and pEAK8-LH41 carrying the full-length cDNAs of the anti-Rhesus D light and heavy chain IgG genes, respectively, were described previously (16). pEAK8 was purchased from Edge Biosystems (Gaithersburg, MD). Cloning of pXLG^{HEK}-RhHC and pXLG^{HEK}-RhLC, carrying the full-length cDNAs of the anti-Rhesus D heavy and light chain IgG genes, respectively, as well as cloning of pXLG^{HEK}-p21h (encoding the human cell cycle regulatory protein p21), pXLG^{HEK}-p18h (encoding the human cell cycle regulatory protein p18) and pXLG^{HEK}-aFGF (encoding the human acidic Fibroblast Growth Factor) were achieved as previously described (17).

To summarize: vector pXLG^{HEK}-p21h was chemically synthesized (GENEART AG, Regensburg, Germany) based on sequence information provided (Supplementary Data). The human cDNAs coding for aFGF (acidic Fibroblast Growth Factor or Fibroblast Growth Factor 1) and p18h were purchased from RZPD GmbH (Berlin, Germany). pXLG^{HEK} vectors were then cloned by replacing p21h in pXLG^{HEK}-p21h with the transgene of interest, where the transgene of interest was cloned by PCR. All forward and reverse PCR primers were designed by using the first or last 15 bp of the corresponding cDNA sequences. The forward primers were extended with the sequence 5′-AAAGCGGCCGCC-3′, which harbors a NotI restriction site; the reverse primers were extended with the sequence 5′-TAAGCTTAA-3′, which harbors a HindIII site. PCR was performed using Pfu Polymerase according to supplier instructions. The fragments were then cloned after restriction digestion into the pXLG^{HEK} vector backbone from pXLG^{HEK}-p21h, which was opened via digestion with NotI and HindIII, which removed the p21h cDNA cassette. The correctness of the cDNA sequences was then verified by DNA sequencing.

pXLG^{HEK} vectors comprise the human CMV promoter, an artificial intron followed by an optimized 5′-untranslated region with Kozak consensus sequence, two stop codons, the woodchuck post-regulatory element (WPRE) as 3′-untranslated region (18) as well as the bovine growth hormone polyadenylation signal. The gene expression cassette is flanked by two inverted terminal repeats. The full sequence of pXLG^{HEK} including a vector map can be found in the Supplementary Data.

Plasmid DNAs were purified on a Nucleobond AX anion exchange column (Macherey-Nagel, Dueren, Germany) according to the manufacturer's protocol.

Transfection

Expansion of cell culture. Suspension-adapted HEK293E cells (6) were adapted to suspension growth under serum-free conditions in Ex-Cell 293 CDM (Cat. No. 14571-1000M; Lot No. 4L1122; SAFC Biosciences, Lenexa, Kansas, USA, 'Ex-Cell' medium) supplemented with 4 mM glutamine. The cells were routinely grown in square-shaped glass bottles (Schott Glass, Mainz, Germany) in Ex-Cell medium (15,19). Medium was kept at levels equal to or less than 1/3 of the bottle volume. The bottles were fixed to a horizontal Model ES-W orbital shaker with a rotational diameter of 2.5 cm (Kühner AG, Birsfelden, Switzerland) using double-sided adhesive transfer tape (3M Corp, Minneapolis, MN) and agitated at 110 r.p.m. (19). Cells were cultured at a density of 10^6 cells/ml up to 3×10^6 cells/ml. Cell passaging was done by pelleting the cells and re-suspending them at the appropriate density in fresh medium.

Transfection with 'XLG protocol'. Cells were centrifuged prior to transfection and resuspended at a cell density of 20 million cells/ml in FreeStyle 293 Expression Medium ('FreeStyle' medium, Invitrogen AG, Basel, Switzerland). Then DNA was added to a final concentration of 50 μg/ml directly to the cells (with a heavy chain to light chain ratio of 1:1 w/w), followed by addition of the appropriate amount of a 1 mg/ml solution of 25-kd polyethyleneimine (PEI; Polysciences, Eppelheim, Germany; pH7) to a final concentration of 100 μg/ml. Three hours after transfection, cells were diluted with Ex-Cell medium to a cell density of 4×10^6 cells/ml, and valproic acid (VPA, Sigma-Aldrich Chemie GmbH, Industriestrasse 25, CH-9471 Buchs SG, Catalog Number P4543) was added to a final concentration of 3.8 mmol/l. The cells were then propagated for 10–14 days under agitation. The ratio of plasmids used under our optimal production conditions was as follows: 37.5% pXLG^{HEK}-RhHC, 37.5% pXLG^{HEK}-LC, 10% pXLG^{HEK}-p18h, 10% pXLG^{HEK}-p21h, 5% pXLG^{HEK}-aFGF.

All small-scale transfections were made in triplicates with 10 ml final volume after dilution in the tube spin system [TPP AG, Trasadingen, Switzerland; (20)] under orbital shaking in a Kuhner ISF4-X orbital shaker at 180 r.p.m. in a 5% CO_2 atmosphere—unless specified otherwise. For the large-scale experiment, cells were cultivated in a 5-l Schott glass round bottle in a Kuhner ES-W orbital shaker at 110 r.p.m. in a 5% CO_2 atmosphere.

Transfection with 'standard protocol'. Prior to transfection, cells were pelleted, washed once with Phosphate Buffered Saline (PBS), and resuspended at a density of 2×10^6 cells/ml in RPMI 1640 with 25 mM Hepes (pH 7.1) and 4 mM L-glutamine (Cat. No. BE12-115Q, Lot No. 2MB0221; BioWhittaker Europe/Cambrex, Verviers, Belgium) and 1% Pluronic F68 ('RPMI F68'). The transfection cocktail was prepared as follows (quantities are per milliliter of resuspended cells): 7.5 μl of a 1 μg/μl solution of 25-kd polyethyleneimine (PEI; Polysciences, Eppelheim, Germany; pH7) was added to 42.5 μl of 150 mM NaCl (pH7). Separately, 2.5 μg of DNA was dissolved in 150 mM NaCl (pH7) to a final volume of 50 μl. Then, the DNA was added to the PEI solution and resuspended by pipetting up and down. The complex was incubated for about 8 min at room temperature, and then added to the cells. Four hours after transfection, an equal volume of Ex-Cell medium was added (i.e. 1 ml of Ex-Cell medium for each milliliter of RPMI medium.).

Protein analysis

The IgG concentration in the culture medium was determined by sandwich ELISA as previously described (9).

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

In order to detect the Fc-tagged fusion proteins, goat anti-human IgG (Fc Fragment specific; Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA; Code Number 109-006-098; Lot No. 72349) was used for coating the ELISA-plates. As a detection antibody, we used AP-conjugated goat anti-human gamma chain IgG (Biosource, Dielsdorf, Switzerland). NPP was used as a substrate for the alkaline phosphatase. Absorption was measured at 405 nm against 490 nm using a microplate reader (SPECTRAmax^{TM}340; Molecular Devices, Palo Alto, CA, USA). Antibody was also purified using Streamline Protein A beads according to manufacturer's instructions (GE Healthcare, Uppsala, Sweden) and quantified spectrophotometrically.

Cell mass and viability

Cell mass and viability were determined as previously described. In short, the voluPAC system (Sartorius AG, Göttingen, Germany) was used to determine packed cell volume (PCV) which was correlated to cell density as previously described (21). In order to transform Packaged Cell Volume (PCV) into million cells/ml, we used a factor of 0.55 for HEK293E; for example, a PCV of 1.1 (i.e. 1.1% packaged cell volume) corresponds to 2 million HEK293E cells/ml.

Analysis of variance (ANOVA)

Design of experiments strategy was used in order to determine statistically significant independent and synergistic effects of the components (factors) of the XLG transfection protocol. The factors considered were cell density, p21, p18, aFGF and VPA. Full factorial experimental design ($2^5 = 32$ experiments) was selected over other experimental designs as this allowed fitting of the data to a linear model with all possible interaction terms for each of the factors. The coefficients of this model provide us with the magnitude and the statistical significance of the effects due to each of the factors, alone and in combination. A two-level design was chosen where each factor could have a high or low value ($+1$ or -1), where $+1$ indicates presence and -1 indicates absence of that factor, except in the case of cell density where $+1$ indicates a PCV of 2.2 and -1 indicates a cell density of 1.1. The set of 32 experiments was performed thrice on three separate days in order to remove any biases due to condition of cells or other human factors on the day of transfection. Samples were collected 12 days after transfection and ELISA was performed as described above. The results were normalized with respect to one of the 32 experiments (same experiment in all three cases) as an internal control in order to remove any biases due to ELISA variations. Upon collection of data from all three experimental sets, the coefficients of the parametric model were calculated using a least squares fit. Analysis of Variance (ANOVA) was performed on these coefficients in order to identify statistically significant effects by choosing only those coefficients whose *P*-values were $<5\%$.

RESULTS

Rational vector design tailored to HEK293E cells is essential for high-yield recombinant protein production

In the first set of experiments, we used a rational vector design approach to specifically tailor vectors for high-yield recombinant protein production in HEK293E cells. For that purpose, we compared different high-yield enhancer/promoters (human eIF1α, mouse CMV, human CMV) in the context of HEK293E cells. In order to make a valid comparison, we cloned each enhancer/promoter into the same vector backbone of an IgG heavy chain and IgG light chain expression vector, respectively. We then transfected HEK293E cells with the corresponding constructs using a previously published (standard) protocol. As depicted in Figure 1, the human CMV enhancer/promoter performs the best in HEK293E cells. We then further optimized the vector backbone by introducing an intron

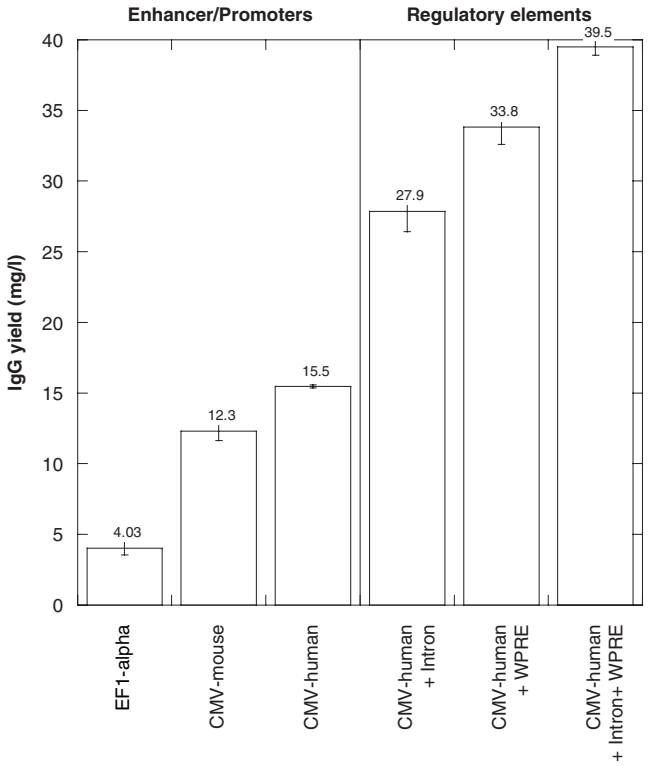

Figure 1. Rational vector design tailored to HEK293E cells is essential for high-yield recombinant protein production. Comparison of different high-yield enhancer/promoters (human eIF1α, mouse CMV, human CMV) and regulatory elements (intron, WPRE). All elements were cloned into the same vector backbone of an IgG heavy chain and IgG light chain expression vector, respectively. We then transfected HEK293E cells with the corresponding constructs using the standard protocol and measured IgG yields via ELISA at day 5.

and woodchuck post-transcriptional regulatory element (WPRE) (17), resulting in the pXLG^{HEK} vector backbone, which further increased recombinant titers by a factor of ~10 compared to the use of the eIF1α promoter alone. We also tested different polyadenylation signals [from SV40, bovine growth hormone as well as an artificial polyadenylation signal (17)], but did not detect a statistically significant difference (data not shown).

Multi-pathway modulation of HEK293E cells in combination with step-wise optimization of a standard transfection protocol increases recombinant antibody titers by a factor of ~27 from 40 mg/l to 1.1 g/l

In the second set of experiments, we performed a multi-pathway modulation of HEK293E cells in combination with step-wise optimization of a standard transfection protocol to increase recombinant antibody titers by a factor of 27 from 40 mg/l to 1.1 g/l. For that purpose, we compared five critical parameters for high-yield transient gene expression in HEK293E cells: cell density (from 1 million cells/ml to 8 million cells/ml), expression of human cell cycle regulatory proteins (p15, p16, p18, p19, p21, p27, p57), expression of human growth factors [IGF-1; transferrin; TGF-β; FRS2; FRS3; acidic Fibroblast Growth Factor (aFGF); FGF7, 9, 12, 13, 14, 18, 19, 20, 21, 23] and the addition of histone deacetylase inhibitors (valproic acid, sodium butyrate, MS-275, trichostatin) and inhibitors of DNA methyltransferases [azacytidine (azaC), RG-108, procainamide, hydralazine].

Based on our initial screen of the individual components (10–12) (data not shown), we have identified the following parameters as crucial: cell density (4 million cells/ml), expression of p18h and p21h (in terms of cell cycle regulatory proteins), expression of aFGF (in terms of growth factors) and addition of VPA (in terms of histone deacetylase and DNA methyltransferase inhibitors). We then directly compared this optimized protocol (henceforth referred to as the XLG protocol) with a previous standard protocol (see 'Materials and Methods' section). In short, for the XLG protocol, we transfected HEK293E cells at a density of 20 million cells/ml in FreeStyle medium by directly adding plasmid DNA to a final concentration of 50 μg/ml and PEI to a final concentration of 100 μg/ml. Three hours after transfection, cells were diluted to 4 million cells/ml with Ex-Cell medium, and VPA was added to a final concentration of 3.8 mmol/l. The following plasmids were used (w/w ratio): 37.5% pXLG^{HEK}-RhHC, 37.5% pXLG^{HEK}-RhLC, 10% pXLG^{HEK}-p18h, 10% pXLG^{HEK}-p21h, 5% pXLG^{HEK}-aFGF.

For the standard protocol, cells were transfected at a density of 2 million cells/ml in RPMI medium with 2.5 μg/ml DNA and 7.5 μg/ml PEI (final concentrations). Four hours after transfection, the cells were diluted to 1 million cells/ml with Ex-Cell medium. Only two plasmids were used for the transfection (w/w): 50% pXLG^{HEK}-RhHC and 50% pXLG^{HEK}-RhLC.

As one can see (Figure 2A), the XLG protocol increases recombinant protein titers by a factor of 27 from 40 mg/l to 1.1 g/l (which corresponds to an average specific productivity, calculated over the entire duration of the culture, of 3.9 pg cell^{-1} day^{-1} and 22.1 pg cell^{-1}day^{-1}, respectively). This is most likely driven by improving cell viability through the addition of p18h, p21h and higher specific productivity through the addition of aFGF. VPA as a chemical additive exerts wide ranging effects on various aspects of protein expression, some direct, (transcription, translation, secretion), and some indirect (effects on cell cycle and cell division)—similar to those shown for other chemical additives such as sodium butyrate (22). In order to fully elucidate the role of VPA, further analysis would be required using high-throughput methods such as DNA microarrays. In summary, our experiments show that VPA significantly impacts both cell viability and specific productivity (see also Figure 3B; data not shown). Our results also indicate that cell division decreases recombinant yields—due to loss of plasmids during cell division, i.e. not all plasmids relocate into the nucleus; once the cells are growth arrested, the number of plasmids per nucleus stays rather constant (data not shown). To our knowledge, this is the first study to describe titers based on transient gene expression in the range of 1 g/l, a productivity level, which has previously been demonstrated only for stable cell lines.

In order to quantify and statistically validate the impact and interaction of the productivity levers identified, we performed an Analysis of Variance (ANOVA) based on the XLG protocol varying each of these five factors using factorial design (see 'Materials and Methods' section). The data set obtained from 3×32 experiments (32 experiments on three different days) was fitted to a linear model (e.g. $a_0 + a_1x_1 + a_2x_2 + a_{12}x_1x_2$) with all possible interaction terms using a least squares fit, thus giving us 32 coefficients indicative of the effects of each of the factors, individually or in combination with other factors. An ANOVA was performed for these 32 coefficients allowing us to calculate the *P*-values, i.e. the probability that the value of the coefficient was obtained due to random errors (background noise) in the experimentation, and these values were plotted versus the magnitude of the coefficient (Figure 2B). All the factors with a *P*-value <5% are considered statistically significant. In summary, VPA had the strongest positive impact on transient gene expression (an independent effect of 4.51 with significant interactions, Figure 2B), followed by co-expression with aFGF (an independent effect of 0.81 with significant interactions, Figure 2B). In order to compare the effects of the various co-expressed genes, we re-calculated the coefficients assuming VPA was always used and cell density was always 4 million cells/ml, as these were the conditions which gave us the highest yield (Figure 2C). Out of the genes used, presence of aFGF and p21h had the strongest positive impact. p18h only showed a significant impact in combination with aFGF whereas p21h in the same setting had a slightly negative impact (Figure 2C), and therefore the two effects cancelled each other at a given cell density.

Our protocol is scalable to the 2-l scale and applicable to other transgenes and vector backbones

In the third set of experiments, we demonstrated scalability of the XLG protocol to the 2-l scale and applicability to other transgenes and vector backbones. In a first step,

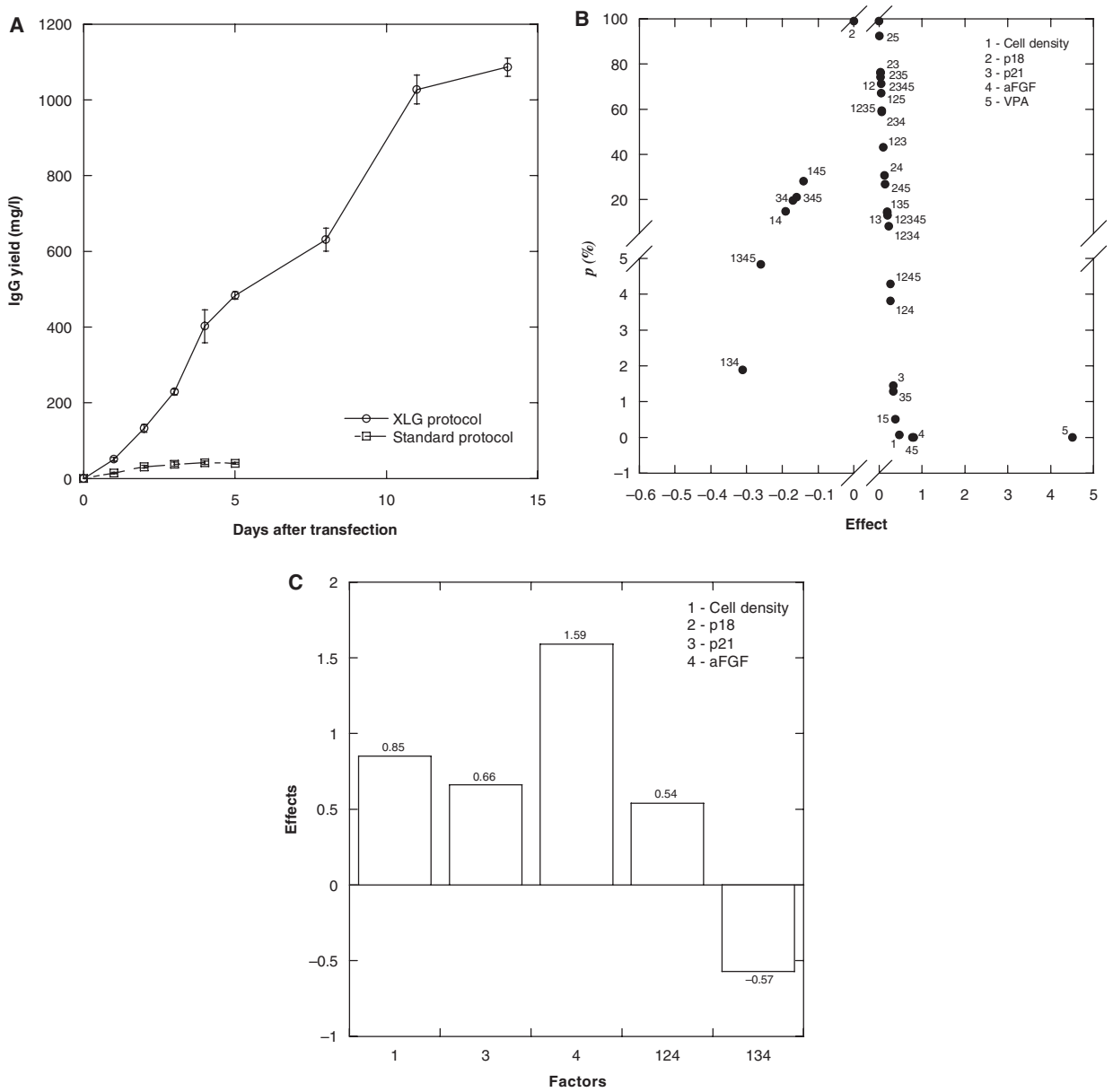

Figure 2. Multi-pathway modulation of HEK293E cells in combination with step-wise optimization of a standard transfection protocol increases recombinant antibody titers by a factor of 27 from 40 to 1.1 g/l. (**A**) Comparison of the standard protocol to the XLG protocol. HEK293E cells were transfected as described in 'Materials and Methods' section. IgG titers were measured every day until cell viability dropped below 50%. (**B** and **C**) Results of ANOVA based on a design of experiments strategy in order to determine statistically significant independent and synergistic effects of the components (factors) of the XLG transfection protocol. The factors considered were cell density, p21, p18, aFGF and VPA. Full factorial experimental design ($2^5 = 32$ experiments) was selected. Transfections were performed in triplicates as described in 'Materials and Methods' section. (**B**) Summary data of all 32 experiments. (**C**) Assumes VPA is always used, i.e. the VPA effect is included in the constant.

we reproduced our small-scale experiment at the 2-l scale (Figure 3A). For that purpose, we transfected 8 billion HEK293E cells in 400 ml FreeStyle medium according to the XLG Protocol (as described in 'Materials and Methods' section) with 20 mg DNA and 40 mg PEI at a concentration of 1 mg/ml each. Three hours after transfection, we diluted the cells by adding 1.6 l Ex-Cell medium. Titers and cell viability were determined daily over a 10-day period. As depicted in Figure 3A, titers reached ~860 mg/l at day 10 after transfection with cell viability at around 50%. This is roughly in the range of our small-scale experiments. After Protein A purification, we were able to recover 1.2 g of total recombinant antibody, which corresponds to a recovery rate of ~70%, confirming our ELISA results. In addition, the final product dominated in both the crude supernatant and the Protein A purified product as verified by SDS-PAGE; there were no major signs of antibody degradation (data not shown).

We then assessed if our protocol is also applicable to non-IgG proteins and tested it in combination with Fc-fusion proteins to the soluble receptors TNFR (Tumor Necrosis Factor receptor), FGFR1, FGFR2, FGFR3 and FGFR4 (Fibroblast growth factor receptor 1, 2, 3, 4 respectively) in the context of the pXLG^{HEK}

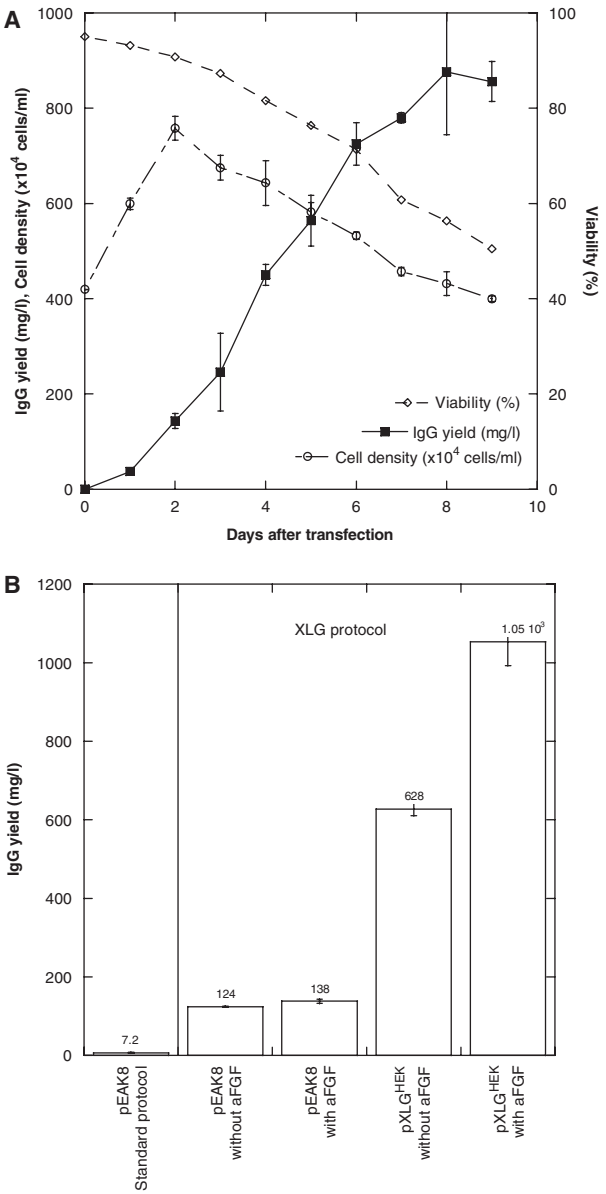

Figure 3. The XLG protocol is scalable to the 2-l scale and specifically adapted to the pXLG^{HEK} vector backbone. (**A**) Results of large-scale transient transfection experiment with XLG protocol (2-l scale). Eight billion HEK293E cells were transfected in 400 ml FreeStyle medium according to the XLG Protocol (as described in 'Materials and Methods' section). Titers and cell viability were determined daily over a 10-day period. (**B**) Comparison of pEAK8 and pXLG^{HEK} vector backbone when used in combination with vectors coding for p18h, p21h and with and without a FGF.

expression vectors. For that purpose, we transfected 40 million cells in 2 ml FreeStyle medium according to the XLG protocol (as described in 'Materials and Methods' section) and diluted afterwards by adding 8 ml of Ex-Cell medium. Titers were determined 14 days after transfection. Titers of up to 941 mg/l were obtained with significant variance between the different proteins (TNFR-Fc 941 ± 92 mg/l; FGFR1 341 ± 19 mg/l, FGFR2 766 ± 8 mg/l, FGFR3 244 ± 38 mg/l, FGFR4 266 ± 4 mg/l).These differences could most likely be due to suboptimal fusion of the Fc-part to the soluble receptors,

Table 1. Transient gene expression of five different vectors pairs, each encoding a different recombinant antibody

Vector(s)	Titer (mg/l)		Fold increase
	Standard protocol	XLG protocol	
pMyk K4/M4	34.8 ± 2.7	636.3 ± 76.4	18.3
pMyk R1/R2	2.4 ± 0.3	42.7 ± 6.9	17.7
pMyk V1/V2	0.3 ± 0.1	17.1 ± 5.8	64.2
pMyk 301/302	0.1 ± 0.02	21.0 ± 1.1	167.9
pEAK LH39/40	7.2 ± 0.6	138.2 ± 9.0	19.2

Titers including standard deviation are shown based on transfection with the standard protocol and the XLG protocol as well as the increase achieved by applying the XLG protocol.

suboptimal codon usage in some of these constructs or lower secretory potential of some of these proteins. It is also possible that the magnitude of effects of the factors delineated above and analyzed by ANOVA, might be different for different proteins depending on the nature of the limiting step for the specific proteins or vector constructs. Still, the XLG protocol in all instances yielded titers high enough to produce sufficient material for pre-clinical experiments.

Last, we evaluated how the XLG protocol performs in the context of vector backbones that performed poorly with the standard transfection protocol (Table 1). For that purpose, we chose five vectors expressing various antibodies in the context of pMyk and pEAK8 vector backbones with titers ranging from 0.1 to 35 mg/l with the standard transfection protocol (see 'Materials and Methods' section). With the XLG protocol, titers could be increased by a factor of 18 to 168—with a more significant increase at lower 'standard' titers. For example, the titer for pMyk 301/302 was increased from 0.1 mg/l to 21 mg/l, which—although still low—would at least enable the production of sufficient material to conduct preclinical experiments.

We then tested if changing the vector backbone to pXLG^{HEK} would further increase titers. For that purpose, we cloned the cDNA for the heavy and light chains from pEAK8-LH39 and pEAK8-LH41 into pXLG^{HEK}, leading to vectors pXLG^{HEK}-RhHC and pXLG^{HEK}-RhLC. As can be seen in Figure 3B, the addition of the aFGF expression plasmid to the transfection cocktail did not affect pEAK8-driven gene expression, but increased expression from the pXLG^{HEK} vector backbone by a factor of ~1.7. Altogether, we increased expression from ~7 mg/l for pEAK-LH39/41 with a standard transfection protocol to 1053 mg/l with the XLG protocol for pXLG^{HEK}-RhHC/RhLC, which is a factor of ~150.

DISCUSSION

Reported titers from transient transfections of mammalian cells, small scale or large scale, are at minimum 1 log lower than those reported from stably transfected, optimized cell lines used in manufacturing of recombinant proteins in bioreactors. Here, we report a method that brings transient expression titers into the range of those

of stable cell lines. In addition the timeline to establish such a process is short and can be reproduced in a matter of days, not months (as is the standard for stable production).

Our method will be useful in many areas of basic and applied biotechnology research, where large amounts of different proteins will have to be produced in a short period of time such as proteomics applications or screening of antibody leads. Due to the speed of establishment of high level production, once the appropriate conditions have been assembled, our protocol will enable certain biodefense applications, where an antibody or vaccine will have to be produced in large quantities within weeks. One could even imagine scenarios in which proteins are regularly produced under good manufacturing practices (GMP) conditions by means of transient transfection—eliminating the time-consuming steps of establishing and banking stable cell lines and performing process development for each individual clone.

Furthermore, instead of purchasing expensive growth factors, growth factors can be expressed *in situ*. Transient gene expression also allows for the introduction of factors into mammalian cells that are incompatible with long-term cell survival or biomass expansion (such as p18 or p21) without the need for a tight, inducible gene expression system: as biomass expansion takes place prior to transfection, there are no limitations of this kind. Indeed, one can independently optimize each step of the manufacturing cycle, from biomass expansion via transfection to protein production.

In summary, our scalable expression system yields titers of up to 1 g/l with recovery of more than 1 g of Protein A purified antibody at the 2-l scale, performs similarly for other recombinant proteins and significantly increases titers also in other vector contexts to the extent that preclinical experiments become feasible. We are optimistic that the XLG protocol will further accelerate the development of biotechnology drugs and lead to a paradigm shift in manufacture of recombinant proteins for commercial use.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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