Methods 65 (2014) 5–10

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

A simple, robust and highly efficient transient expression system for producing antibodies ☆

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ARTICLE INFO

Article history:
Available online 17 July 2013

Keywords:
Transient expression
HEK293
Antibody
SV40 large T antigen
Cell cycle
Experimental design

ABSTRACT

Transient expression systems in mammalian cells have become the method of choice for producing research quantities of antibodies. Both the speed and yield of the available transient systems and the natural posttranslational modifications favor these systems above expression in lower eukaryotes, prokaryotes or stable cell lines.

We describe an optimized mammalian transient expression system, capable of producing up to 400 mg/L of native secreted antibodies in less than a week. The system is composed of commercially available components and is based on expression in the fast growing suspension cell line, FreeStyle™ 293-F (HEK-293F).

The method depends on an optimal combination of a gene transfer method, an expression vector and cotransfection with expression enhancing plasmids, encoding the large T antigen of the SV40 virus and the cell cycle inhibitors p21 and p27.

Optimization of all components of the expression system, by experimental design techniques, yielded maximal expression levels (including antibody isotypes IgG1, 2, 3, 4 and Fab fragments of various species).

Expression volumes were scalable from 0.1 ml up to 1.2 L in a simple shaker flask system in animal component free, low protein medium, enabling consistent production of relatively high amounts of a large number of native antibodies.

1. Introduction

Antibodies require a complex cellular machinery to be properly produced and processed. Intra as well as inter chain disulfide bonds have to be formed and a number of posttranslational modifications have to be introduced, including glycosylation. Proper glycosylation is critical for proper functioning and minimizing potential immune reactivity of antibodies. Mammalian cells have the proper requisites enabling native folding and modification, and as such have been used to produce antibodies for research and clinic.

In the last decade, the use of transient mammalian expression systems for the production of native complex proteins has increased (see [1] for a recent review), boosted by the publication of efficient transfection protocols and the availability of suspension cell lines growing at high density. For transient expression, mainly the HEK-293 and CHO-K1 cell lines have been applied. Both cell lines can be adapted to suspension culture, and sub clones are available which grow in chemically defined medium at high cell densities. With its ease of transfection, high expression yield and native human glycosylation, the human embryonic kidney 293 cell line (HEK-293, [2]) is used most extensively.

Numerous expression vectors have been employed (see [1]), differing in promoters, episomal replication systems or other expression enhancing elements. In HEK-293 cells it appears that the long variant of the immediate early gene promoter of the human cytomegalo virus (CMV) results in especially strong transcriptional activity [3]. In addition, a large number of transfection methods have been described, ranging from lipofection with different formulations, electroporation, nucleofection, calcium phosphate transfection, and other methods [4]. Several methods for enhancing expression have been described. For instance, feeding strategies can prolong the production time and increase the

Abbreviations: HEK-293F, FreeStyle™ 293-F; HEK-293, human embryonic kidney 293 cell line; CMV, cytomegalo virus; SVLT, the large T antigen of the SV40 virus; DoE, design of experiments; Medium, FreeStyle™ 293 expression medium; Optimem, opti-MEM® I reduced-serum medium; 293Fectin, 293fectin™; HC, antibody heavy chain; LC, antibody light chain; p21, cyclin-dependent kinase inhibitor 1; p27, cyclin-dependent kinase inhibitor 1B.

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http://dx.doi.org/10.1016/j.ymeth.2013.07.018

number of cells, leading to higher production levels. Cotransfection of expression enhancing vectors, encoding proteins or RNA, can also increase expression levels, whereas coexpression of anti apoptotic proteins can boost expression levels by increasing viability and cell numbers [5,6]. Cell cycle inhibitors can augment production levels by stabilizing the cell line in a cell cycle stage most suitable for protein production [7–9]. Finally, chaperones can raise the rate and quality of protein folding [10,11] and a number of viral proteins have been described which can enhance production levels, for instance by inhibiting the unfolded protein response. Especially the large T antigen of the SV40 virus (SVLT) is a powerful enhancer of protein production [12–15].

Although much knowledge on transient mammalian expression system has been gained, it often remains obscure by which mechanism(s) specific enhancers work, and the performance of transient expression protocols can be specific for a cell line or even a sub clone of a cell line. For optimization of such complex processes with multiple interacting variables, the design of experiments (DoE) methodology [16], is especially suited. DoE can model and optimize complex processes using only a limited number of experiments.

We have developed a highly efficient and simple transient expression system by systematically optimizing all system components and introducing three expression enhancing proteins, SVLT, p21 and p27. Careful tweaking of the ratios of the antibody and enhancer expressing vectors using DoE techniques, led to an optimal, robust protocol. This system is capable of producing human IgG antibodies up to 400 mg/ml and is fully scalable from 0.1 up to 1200 ml. The simplicity of the system and the minimal amount of process steps make it very suitable for routinely producing high numbers and amounts of research antibodies.

2. Materials and methods

2.1. Cell culture

FreeStyle™ 293-F cells were obtained from Invitrogen (Carlsbad, CA). FreeStyle™ 293 Expression Medium (Medium) and Opti-MEM® I Reduced-Serum Medium (Optimem) were obtained from Invitrogen. Medium was pre warmed by incubating medium flasks in a 37 °C water bath. Proper pre warming of the medium is essential for good cell growth and different volumes of medium require different incubation times to reach 37 °C. Recommended minimal incubation times for pre warming medium are indicated in Table 1.

For cell culture volumes from 15–1200 ml, disposable polycarbonate erlenmeyer flasks from Corning (Tewksbury, MA) were used.

For low volume productions, we used the system described by Duetz [17] for bacterial culture and applied it to mammalian cell culture. This system is commercially available from Applikon Biotechnology (Schiedam, The Netherlands) as the Microflask system. It was used to culture either in low- or deep well, 96 well or 24 well plates.

Table 1
Minimal recommended time for medium to reach 37 °C.

Tube/bottle	Time in the 37.0 °C water bath (min)
15 ml tube with 1–15 ml medium	20
50 ml tube with 10–50 ml medium	25
500 ml flask with 50–250 ml medium	40
500 ml flask with 250–500 ml medium	60
1000 ml flask with 500–1000 ml medium	90

For agitation the Thermo Scientific (Waltham, MA) benchtop orbital shaker, model 416, was used (shaking amplitude of 25 mm).

293fectin™ (293Fectin), was obtained from Invitrogen.

2.2. Constructs

Expression plasmids encoding the human p21 and p27 (pORF-hp21 and hp27) were obtained from Invivogen (San Diego, CA, USA). A codon optimized version of the SV40 large T antigen coding region was constructed at GeneArt (Regensburg, Germany) and cloned into pcDNA3.3 (Invitrogen) and the resulting plasmid, named p33SVLT, can be obtained from the authors. Antibody heavy chain (HC) and light chain (LC) encoding regions were cloned in pcDNA3.3.

2.3. Preparation of vector DNA

Vector DNA was prepared using conventional plasmid DNA purification methods. We have used the systems of Qiagen (for example the HiSpeed plasmid maxi kit, cat. # 12662) and the automated system of Invitrogen (BenchPro 2100 plasmid purification system). Removal of endotoxins from the plasmid preparation is, in our hands, not necessary. Plasmid preparations were quantified by UV spectroscopy and stored in a suitable buffer at −20 °C.

2.4. Experimental design software

For design and analysis of DoE experiments, the Design-Expert software from Stat-Ease (Minneapolis, MN) was used.

2.5. Cell counting

Cell counting and viability were estimated using the Guava PCA system and the Viacount reagent from Millipore (Amsterdam, The Netherlands).

2.6. Quantitation of human IgG concentration

Concentrations of human IgG in supernatant were determined using a BNII Nephelometer (Dade Behring, Germany) and serum IgG as a reference.

2.7. Protocols

2.7.1. Basic transient expression protocol

Remove a cryovial of HEK-293F cells from the liquid nitrogen storage vessel and thaw quickly in a 37 °C water bath. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol. Transfer the entire contents of the cryovial into a 125 ml polycarbonate, disposable, sterile Erlenmeyer shaker flask containing 17 ml of pre warmed (37 °C) medium. Incubate the cells in a 37 °C shaker incubator with 85% humidity and 8% CO2 at 125 rpm. Once the culture has reached higher than 1×10^6 viable cells/ml (typically 3–5 days), transfer the cell suspension, aseptically, into a centrifuge tube and vortex for 10 s. Determine the viable and total cell counts by cell counting. Subculture the HEK-293F cells by seeding a shaker flask at 3×10^5 viable cells/ml in pre

Table 2
Transfection conditions for typical volumes.

Volume	3 ml	20 ml	200 ml
DNA/Optimem	3 μg/100 μl	20 μg/670 μl	200 μg/6.7 ml
293Fectin/Optimem	4 μl/100 μl	26.6 μl/670 μl	267 μl/6.7 ml
Cells (1.1×10^6/mL)	2.8 mL	18. 66 mL	186.6 mL