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Denise Freimark, Valérie Jérôme, Ruth Freitag

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A GFP-based method facilitates clonal selection of transfected CHO cells

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Technical Report ((4783 words))A GFP-based method facilitates clonal selection of transfected
CHO cells*Denise Freimark, Valérie Jérôme and Ruth Freitag**

Chair for Process Biotechnology University of Bayreuth, Germany

Key words: growth factor, high producer, mammalian cell culture, recombinant protein production, transfection

*corresponding author: Chair for Process Biotechnology, University of Bayreuth, 95440 Bayreuth, Germany, phone: 0049 921 55-7371, Fax: 0049 921 55-7375, e-mail: bioprozesstechnik@uni-bayreuth.de

Abbreviations:

BSA: Bovine serum albumin

CHO: Chinese hamster ovary

EGFP: Enhanced green fluorescent protein

FACS: Fluorescence assisted cell sorting

G418: Geneticin (antibiotic used for screening)

hIGFIA: Human insulin-like growth factor 1A

IRES: Internal ribosome entry site

hVEGFA: Human vascular endothelial growth factor A

Abstract

The identification of highly expressing clones is a crucial step in the development of cell lines for production of recombinant proteins. Here we present a method based on the co-expression of enhanced GFP that allows clonal selection in standard 96-well cell culture plates. The genes encoding the EGFP protein and the protein of interest are linked by an internal ribosome entry site and thus are transcribed into the same mRNA but are translated independently. Since both proteins arise from a common mRNA, the EGFP expression level correlates with the expression level of the therapeutic protein for each clone. By expressing recombinant growth factors in CHO cells, we demonstrate the robustness and performance of this technique. The method is an alternative to the identification of high-producer clones using various cell sorting methods, as it can be performed with standard laboratory equipment.

Introduction

A key step in the generation of cell lines for the efficient production of recombinant proteins is the selection of high producer clones following transfection and selection [1]. Most often, successfully transfected cells pass through a subcloning stage using 96-well plates. A robust method for screening at this level is valuable, as it helps to focus on good producers early on. Analysis of the product titre in cell culture supernatant by ELISA is commonly used at this stage to identify clones secreting high levels of recombinant protein. However, the process of randomly picking a large number of clones produced, e.g. by limited dilution, and assaying them individually by ELISA is tedious, time consuming, and reportedly not always very effective [2,3]. Moreover, this method does not account for differences in either cell density or media volume between wells. Apart from the already mentioned tediousness, this approach may therefore not accurately predict the clones with high specific productivity.

High throughput screening methods based, e.g., on fluorescence-activated cell sorting (FACS) or magnetic bead-based cell separation are faster [4,5], but require, especially in case of FACS, not only expensive equipment but also highly skilled operators. Hence such methods tend to be restricted to labs in frequent need of cell sorting. Moreover, unless the target molecule itself shows fluorescence, labelling or co-expression of a fluorescent or otherwise detectable molecule is required [6,7]. The enhanced green fluorescent protein (EGFP) has been suggested in the past as co-expressed indicator protein for applications in FACS-based cell separation, but also for the screening of cultures in multiwell plates [8-10]. In this approach the recombinant cells express both the fluorescent and the target protein, the titres are assumed to be linked. However, mostly the genes for EGFP and the target protein were co-transfected into the cells via individual plasmids, e.g. [9,11]. Hence no physical connection between the expression of the two proteins existed.

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3 In this contribution we describe a simple fluorescence-based microplate screening method for
4 cell subpopulations based on the true co-expression of the fluorescent and the target protein.
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6 For this purpose, the two proteins were introduced into the CHO cells via a dicistronic
7
8 construct. The method combines the efficiency of green fluorescent protein/fluorescence-
9
10 based screening with the limited dilution method to select high-producing subclones and does
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12 not require special equipment beyond a plate reader equipped with fluorescence option.
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21 **Materials and Methods**

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23 *Materials* 96-well plates used for screening were from Nunc, Wiesbaden, Germany (96 F
24 TC Nunclon) and BD-Falcon, Heidelberg, Germany (Microtest 96). Chemicals were from
25 established suppliers such as Sigma Aldrich and used as received. High quality water was
26 produced by a Millipore unit. Basal medium RPMI 1640 with or without phenol red was from
27 PAA (Pasching, Austria), serum-free media ProCHO-AT, ProCHO4 and ProCHO5 were
28 from Lonza, Verviers, Belgium, and supplements (L-Glutamine, Pen/strep, G418 solution,
29 foetal calf serum) were from PAA or Carl Roth, Karlsruhe, Germany.
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41 *Cell culture* Chinese Hamster Ovary cells (cell line CHO-K1 (CCL-61, ATCC)) were
42 maintained in growth medium (R10: RPMI 1640 medium supplemented with L-glutamine (2
43 mM), penicillin/streptomycin (0.1 mg/mL) and 10 % foetal calf serum) in an atmosphere of
44 5.0 % CO₂ and at 37 °C (incubator: Forma Steri-Cult or Forma Direct-Heat, ThermoFisher
45 Scientific, Dreieich, Germany).
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53 Prior to the spinner (IIBS, Chur, Switzerland) experiments, the adherent cells were adapted to
54 cultivation in suspension. For this purpose the cells were plated in **serum- and protein-free**
55 ProCHO-AT medium at a density of 1×10^5 cells per well into a 6-well plate. After two days
56 the medium was exchanged for ProCHO4 and the cells were incubated for another 2 to 5
57 days. ProCHO4 is a special medium for inducing adaptation to suspension in CHO cells.
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3 Afterwards cells were recovered by centrifugation (5 min, 200 g) and resuspended in the
4 **serum-free** production medium ProCHO5. Spinner flasks were filled with 100 mL ProCHO5
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6 medium and inoculated at a cell density of 1×10^5 cells/mL. Spinners were placed in the
7
8 incubator (37 °C, 5 % CO₂) and stirred at 50 rpm.
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12 *Plasmid construction*

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15 The coding regions of the human growth factors VEGFA and IGF1A were amplified by RT-
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17 PCR as follows, using total human RNA from Saos-2 and HEK293 as template. The total
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19 RNA was prepared using the RNeasy kit from Qiagen (Hilden, Germany) according to the
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21 manufacturer's protocol. For RT-PCR the OneStep RT-PCR kit from Qiagen was used
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23 according to the manufacturer's protocol. The following forward and reverse primers were
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25 used: forward primer 5'-tcaccaccatcactcttctggtatgaactttctgctgcttgggtgc-3' and reverse primer
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27 5'- tccccgggggatcaccgcctcggtgtcacatct -3' for hVEGFA, and forward primer 5' -
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29 tataccatggcaatgggaaaaatcagcagttcttccaac- 3' and reverse primer 5' -
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31 tccccgggggactacatcctgtagttcttgttcc- 3' for hIGF1A. The PCR products were purified and
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33 subcloned into pGEMT-Easy (Promega, Mannheim, Germany). The resulting plasmids,
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35 namely pGEMT-hVEGFA and pGEMT-hIGF1A, were digested with XcmI/SmaI and
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37 NcoI/SmaI, respectively, and the genes subcloned into the target vector pTriEx2neo
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39 (Novagen, Darmstadt, Germany) to construct the monocistronic plasmids (pTriExneo-
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41 hVEGFA, pTriExneo-hIGF1A). These monocistronic plasmids were digested with
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43 Eco47III/EcoRI and the genes, containing an IRES down stream of the growth factor
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45 encoding gene, were subcloned in the MCS of pEGFP-N1 (Clontech, Heidelberg, Germany)
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47 to construct the dicistronic plasmids (pEGFP-N1-hVEGFA and pEGFP-N1-hIGF1A). The
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49 resulting expression plasmids were confirmed by colony PCR, restriction analysis and
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51 sequencing (MWG, Martinsried, Germany).
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3 *Transfection, selection, and single-cell cloning* CHO cells in a 6-well-plate (about 80 - 85
4 % confluent) were transfected with the linearized (restriction enzyme: ApaLI) plasmid vector
5 pEGFP-N1-hVEGFA (3 μ g) or pEGFP-N1-hIGF1A (3 μ g), respectively, and 10 μ l
6 ExGen500 (PEI-based reagent, Fermentas, St. Leon-Rot, Germany) transfection reagent
7 according to the manufacturer's protocol. After 48 h, the cells were collected by trypsinisation
8 and the transfection efficiency was roughly estimated via the EGFP fluorescence by flow
9 cytometry in a Cytomics FC500 (Beckman Coulter, Krefeld, Germany). For selection of
10 stably transfected clones, the transiently transfected cells were grown first in growth medium
11 supplemented with 500 μ g/ml G418. Non-transfected cells lack the resistance to this
12 antibiotic. After one week, the selection pressure was increased to 800 μ g/ml G418. To screen
13 for high producing clones, the transfected CHO cells were transferred into 96-well plates at an
14 average density of less than one cell per well in 100 μ L of selection medium (procedure of
15 "limited dilution" [12]). The basis for this method is a dilution of the cell suspension to a
16 point where statistically less than one cell per well (here 0.6) are plated. Wells were checked
17 for cell number and those containing a number of cell \neq 1 were excluded for the subsequent
18 measurements. In our hands the method was faster than isolation of the clones by cloning
19 cylinder [13]. Following dilution, the cells were further incubated for 2 - 3 weeks. For each
20 growth factor, at least twenty plates per transfection were screened.

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46 *Fluorescence measurement* The EGFP fluorescence in the 96-well plates was measured with
47 a plate reader (GeniosPro, Tecan, Crailsheim, Germany), top reading mode, using the
48 standard fluorescein filter combination (Ex 485 (20)/Em 535 (25)). For measurement, the
49 medium was quickly removed under sterile conditions. Afterwards, each well was quickly
50 refilled with 100 μ L of fresh selection medium using a multi-channel-pipette. The complete
51 analysis required less than 5 minutes per plate. The signal to background ratio (SBR) was
52 calculated according to:
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$$SBR = \frac{S - B}{B} \quad (1)$$

where S is the fluorescence of EGFP expressing cells and B the autofluorescence of non-transfected cells under otherwise identical conditions.

The limit of detection (LOD) was calculated according to:

$$LOD = \frac{25000}{(F_{(25000)} - B)} * 3 * SD_B \quad (2)$$

where $F_{(25000)}$ is the measured RFU (relative fluorescence value) value of 25,000 EGFP-expressing cells, B the measured autofluorescence of non-transfected cells (blank) and SD_B the standard deviation of blank.

In case of the spinner experiments, the EGFP concentrations were determined spectrophotometrically (Beckman Coulter, Krefeld, Germany) at 475 nm. The concentration was calculated using an extinction coefficient of $55,000 \text{ M}^{-1} \text{ cm}^{-1}$ [14].

ELISA The concentration of hVEGFA and hIGF1A in cell culture supernatants were determined using the specific ELISA development kits from Peprotech (London, UK) and from R&D systems (Wiesbaden, Germany) according to the manufacturer's instructions. Briefly, ELISA plates were coated overnight with 100 μL of a solution containing the capture antibody (4.0 $\mu\text{g}/\text{mL}$ mouse anti-hIGF1 or 0.5 $\mu\text{g}/\text{mL}$ rabbit anti-hVEGFA antibody). Then, the plates were blocked with 300 μL of blocking solution (1.0 % BSA in PBS). After that, 100 μL of either standard or sample, diluted appropriately in diluent (0.05 % Tween-20, 1.0 % BSA in PBS), was added and incubated for 2 hours at room temperature. 100 μL of the detection antibody (0.08 $\mu\text{g}/\text{mL}$ biotinylated goat anti-hIGF1 or 0.25 $\mu\text{g}/\text{mL}$ biotinylated goat anti-hVEGF) was added and incubated for 2 hours at room temperature. 100 μL of a solution containing avidin peroxidase 1/2000 in diluent was added and incubated for 30 minutes at room temperature. After each of the previous steps, the plates were washed six times in

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3 washing solution (0.05 % Tween-20). Finally, 100 μ L of the substrate solution (100 μ g/mL
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5 3,3', 5, 5' tetramethylbenzidine, 60 μ g/mL H_2O_2) was added and incubated for 30 minutes in
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8 the dark at room temperature for colour development. The reaction was stopped with 1 M
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10 H_2SO_4 , and the absorbance was read at 450 nm on a microplate reader (Genios Pro). Each
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12 sample/standard was measured in triplicate. The standard curves ranges were 0.016 – 2 ng/mL
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14 and 0.3 – 20 ng/ml for hVEGFA and hIGF1A, respectively. Data points, which fell in the
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16 range of the standard curve, were used for calculation of the hVEGFA and hIGF1A
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18 concentrations in the samples.
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26 **Results and discussions**

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28 In the pEGFP-N1-based gene constructs used here, the genes encoding for the recombinant
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30 target protein and for EGFP are linked by an internal ribosome entry site (IRES).
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32 Consequently, both proteins arise from a common mRNA and the EGFP expression level
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34 should closely correlate with the relative expression level of the co-expressed protein of
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36 interest. The direct use of the pTriEx2-neo-based constructs for expression of the transgene
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38 was less efficient, which is not surprising given the much weaker promoter and the larger size
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40 of pTriEx2-neo [15,16]. Moreover, the use of pEGFP-N1 for transgene expression besides
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42 good product titres also makes possible the screening strategy developed in this contribution
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50 *Determination of optimal screening parameters*

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53 The plate reader used in this study offers a choice between multiple and single reading of the
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55 wells. Multiple reading is often suggested to compensate for an unequal distribution of cells in
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57 the wells. In our hand, however, the results obtained with the “multiple reading mode” were
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3 not significantly different from single measurements (data not shown). Thus, all further
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5 measurements were done in the single reading mode.
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8 Possible **background fluorescence** effects were of concern for the assay development. For cell
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10 and tissue culture of mammalian cells, especially when microscopic observation of the cells is
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12 necessary, microplates with a clear bottom have to be used. Special microplates exist for
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14 fluorescence measurements, which are characterised by low **background fluorescence** and
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16 minimal light scattering. However, these plates are significantly more expensive than plates
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18 completely made of transparent material. Since in a typical screening experiment thousands of
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20 clones need to be evaluated, costs are an issue. Thus, transparent microtitre plates from two
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22 suppliers (Nunc and Falcon) were investigated for **background fluorescence** (BF_{plate}) at the
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24 FITC filter combination (485/535). Due to technical limitations, readings took place from the
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26 top (“top reading mode”) rather than through the bottom of the plate. Results are summarized
27
28 in Table 1. Since sterile conditions had to be maintained during measurements, plates were
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30 covered with either a lid or a sterile plastic foil. Both examined types of transparent
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32 microplates exhibited a rather high **background fluorescence**, if compared to values given by
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34 the suppliers for the special fluorescence plates. However, the Falcon plates showed a five
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36 times higher **background fluorescence**, than the Nunc plates. For both types of plates, the
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38 **background fluorescence** was higher when a lid was used instead of the plastic foil.
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47 Next, the fluorescence contribution of the cell culture medium (BF_{medium}) and of non-
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49 transfected cells (AF_{cells}) was estimated using the Nunc plates covered with plastic foil, Table
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51 1. Plates containing 100 μL of the indicated liquid were analyzed. Clearly, the composition of
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53 the cell culture medium affects drastically the background fluorescence and leads to a 17 to
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55 23-fold increase of the **background fluorescence** compared to the original BF_{plate} . Such a high
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57 **background fluorescence** would make the intended measurements impossible, therefore it was
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59 decided to remove the cell culture medium and replace it by PBS or to leave the wells free of
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3 liquid during the measurements (duration ca. one minute per plate). For the estimation of the
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5 AF_{cells} , non-transfected cells at 384 to 50,000 cells/well were plated in the 96-well plates and
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7 grown overnight in R10 medium. The medium was removed and the fluorescence measured.
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10 The detected fluorescence values were in the range of BF_{plate} , indicating that the contribution
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12 of the non-transfected cells to the measured fluorescence is low and does not depend on the
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14 number of cells per well.
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18 One of the reasons that fluorescence-based assays are usually performed in black plates with
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20 clear bottom is to reduce crosstalk between wells. In order to investigate the intensity of the
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22 crosstalk effect in case of the transparent plates, EGFP-expressing cells (30,000 cells/well)
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24 were plated either in adjacent wells or in such a way that the inoculated well was surrounded
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26 by eight wells containing no cells. Plates were incubated overnight and the EGFP
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28 fluorescence was measured. The results show that the contribution of crosstalk was below
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30 10 % in case of the adjacent wells and thus negligible (data not shown).
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38 *Limit of detection and detection range*

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40 The signal to background (SBR) ratio was determined for both the Nunc and the Falcon plates.
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42 For this purpose, EGFP-expressing cells and non-transfected cells, both at 80 % confluency
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44 (ca. 40,000 cells/well) were measured. Plates were covered with plastic foil. The Nunc plates
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46 showed a significantly better SBR ratio, Table 1. Nunc plates were thus used in the
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48 subsequent measurements. For the estimation of the limit of detection (LOD), two-fold serial
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50 dilutions of transfected cells (down to 384 cells/well) were inoculated into the well together
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52 with appropriate numbers of non-transfected cells to keep the total number of cells/well at
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54 50,000, and incubated overnight. This mixture of transfected and non-transfected cells was
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56 expected to simulate an environment found in transfection studies where, depending on the
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58 transfection and selection efficiency, only a few cells out of a high cell number might be
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3 modified. When the fluorescence was measured the next day, the fluorescence signal
4 displayed a good linearity ($r^2 = 0.9932$, $y = 0.08362 x + 42.12439$, errors of the individual
5 data points $< 10 \%$) with a limit of detection of approximately 3000 cells per well ($S/N > 3$).
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10 When a generation time of 24 h is assumed, this would correspond to an incubation time of
11 approximately 10 days in order to reach a detectable amount of cells starting from one cell per
12 well. While the detection limit is still fairly high – using special fluorescence measurement
13 plates and bottom reading as few as 250 EGFP expressing cells can be detected according to
14 the manufacturer – it was in our hand sufficient (see below) to identify high producing clones
15 and could certainly be improved, e.g., by using a plate reader capable of performing in the
16 bottom reading mode.
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30 *Screening for high producers*

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33 In order to test the method for clone screening, CHO cells were transfected with pEGFP-
34 hVEGFA or pEGFP-hIGF1A. Flow cytometry measurements performed 48 h post
35 transfection showed an average transfection efficiency of ca. 40 % as judged by the EGFP
36 fluorescence. Subsequently, the transfected cells were cultivated for two weeks under
37 selection pressure before being subjected to limited dilution into 96-well plates. During that
38 time, expression of EGFP was measured via fluorescence measurements by plate reader at
39 days where medium had to be changed. After the measurement each well was filled with 100
40 μL fresh selection medium using a multi-channel-pipette. 3840 clones were analysed per
41 screening experiment. Out of these, on average nine IGF1A clones and seven VEGFA clones
42 were considered high producers, i.e. their fluorescence was a least three standard deviations
43 higher than the median value. This number of clones corresponds to a percentage of 0.23 %
44 (IGF1A) and 0.18 % (VEGFA) and thus is in the same range as values reported in the
45 literature (0.2 %, e.g. [17]). After 2-3 weeks the experiment was terminated at which point
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3 the expression level of the growth factor of interest was measured by ELISA and correlated to
4 the EGFP fluorescence value obtained at the same time. As shown in Figure 1 for hVEGFA,
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6 most of the 260 clones analyzed exhibited low fluorescence intensity and a low growth factor
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8 production. Correlation between EGFP fluorescence and hVEGFA expression was weak
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10 below a hVEGFA concentration of 1.0 ng/mL. However, this range was of little interest. On
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12 the other hand, clones showing the highest fluorescence readings consistently tended to be
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14 also good hVEGFA producers.
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20 In order to further investigate the correlation of growth factor and EGFP production,
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22 experiments were repeated for both growth factors. Transfected cells were subjected to
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24 limited dilution down to one cell per well in 96-well plates. When the EGFP expression was
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26 analysed for 1920 clones, approximately 0.3 % of the screened clones were high producers
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28 (EGFP fluorescence). Clones displaying the highest fluorescence levels were then isolated
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30 and expanded in 24-well plates. The product concentration in the cell culture supernatant was
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32 assessed after nine days of culture by ELISA. In parallel, the EGFP fluorescence was
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34 measured in the plate using the plate reader. Figure 2 shows that the EGFP signals and the
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36 amount of recombinant protein of interest clearly correlate. However, although regardless of
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38 the expressed growth factor, the intensity of the EGFP fluorescence was in the same range for
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40 all the investigated clones, the quantity of secreted hVEGFA was ten-fold higher than that of
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42 hIFG1A. The reasons for this discrepancy can only be speculated upon (e.g., stability of the
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44 growth factor in the cell culture medium, different translation/secretion rate, ...) and are
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46 currently under investigation.
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54 One of the hVEGFA-producing clones (11/F1) was subsequently adapted to suspension
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56 culture by an exchange of the culture medium and cultivated in the batch spinner mode. The
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58 production of hVEGFA and EGFP was followed for 312 h, Figure 3. At the end of the culture
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60 the hVEGFA-level had reached 72 µg/L (1.6 nM) corresponding to a yield Q_{VEGFA} of 134 pg

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3 10^{-6} cells h^{-1} . This clone thus already performs in the same range as previously described ones
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5 based on the CHO-DHFR system (101 to 520 $\mu\text{g/L}$ [18]). We expect a further improvement in
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7 productivity once the process is established in the bioreactor. Again, we observe that in spite
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9 of the good general correlation between hVEGFA and EGFP production, the final EGFP
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11 concentration reached in the spinner was six times higher than the hVEGFA concentration
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13 198 $\mu\text{g/L}$ (10.6 nM). This corresponds to a yield Q_{EGFP} of 368 $\text{pg } 10^{-6}$ cells h^{-1} . The higher
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15 relative yield of EGFP may be due to differences in translation efficiency or the stripping of
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17 the hVEGFA by the CHO cells. Moreover, EGFP is known to be a stable protein with a half-
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19 life of more than 30 h in animal cells [19], while the growth factor has a half-life of minutes
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21 to a few hours [20]. In spite of the co-transcription, final protein concentrations therefore need
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23 not necessarily to correlate. Further investigation of these effects is currently under way in our
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25 laboratory.
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35 Conclusions

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37 The use of a recombinant bicistronic plasmid to express the protein of interest along with
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39 EGFP allows for a fast screening for “high producers” via the EGFP fluorescence. The
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41 screening can be performed in standard transparent microtitre plates and less than 3000 EGFP
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43 expressing cells can be detected. The new screening method allows an efficient detection of
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45 good producer clones at an early stage of the process development. As it does not rely on
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47 fluorescence-activated cell sorting, nor does it require the availability of an antibody specific
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49 for the therapeutic protein to be expressed, it can be easily implemented in any laboratory and
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51 stage of the cell line development process. Clones with unstable transgene expression can be
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53 identified by the decline in EGFP fluorescence. A possible *caveat* of the method concerns a
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55 putative cytotoxic effect of EGFP at very high concentrations, as e.g. indicated in [1], i.e.
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57 especially when one is looking for very superior producers. However, in view of the many
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3 EGFP over-expressing cells and organisms that have been investigated [21] – some of them
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5 expressing much higher EGFP amounts than described herein – there is still much
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7 productivity increase possible before EGFP cytotoxicity becomes a problem.
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Figure legends

Figure 1: Productivity versus GFP fluorescence in hVEGFA-EGFP clones.

The transfected CHO cells were transferred in 96-well plates at an average density of one cell per well (process of limited dilution) in 100 μ L selection medium and further incubated for 2 weeks. EGFP fluorescence was measured by microplate reader (Ex 485nm/Em 535 nm). The amount of hVEGFA in the cell supernatant was analyzed via ELISA. EGFP fluorescence: relative fluorescence unit (RFU) minus BF_{plate} (correlation coefficient = 0.56, $P < 0.0001$; $n=260$ hVEGFA-EGFP clones).

Figure 2: Correlation between EGFP and growth factor expression in a number of high producer clones.

Prior to measurements, clones exhibiting high EGFP fluorescence were transferred into 24 well plates and cultured for nine days in protein-free medium. The growth factor concentration in the cell culture supernatant was analyzed by ELISA and the EGFP fluorescence signal was measured optically. The EGFP fluorescence is given as relative fluorescence units (RFU) after deduction of BF_{plate} . A: hVEGFA, B: hIGFA, bars: growth factor concentration, v: EGFP fluorescence.

Figure 3: Time course of hVEGFA and EGFP concentration during spinner flask batch culture of the clone 11/F1.

The human VEGFA expressing cell line 11/F1 was cultured in a spinner flask. The cells were inoculated at a density of 5×10^5 cells/mL into 100 mL of ProCHO5 medium on day 0 and cultured for 13 days. The hVEGFA concentrations were measured by ELISA and are indicated by black squares. The EGFP production was measured optically and the values obtained were converted into $\mu\text{g/L}$ of EGFP produced using a standard curve established with pure EGFP. The EGFP concentrations are indicated by white squares.

Table 1. Background and autofluorescence experiments

Plate type	with lid	with plastic foil
Falcon Microtest		
empty	12431 ± 181	8147 ± 209
control cells	n.d.	11,778 ± 1146
EGFP-expressing cells	n.d.	22,707 ± 2582
SBR	n.d.	0.93
Nunclon D		
empty	4002 ± 79	3347 ± 74
control cells	n.d.	3504 ± 167
EGFP-expressing cells	n.d.	14,159 ± 3514
SBR	n.d.	3.04
Cells without liquid		3310 ± 4
PBS		3276 ± 38
RPMI 1640 medium		34,408 ± 919
RPMI 1640 medium without phenol red		31,815 ± 504
R10 medium		41,594 ± 1655
R10 medium without phenol red		38,833 ± 1705
Selection medium		43,133 ± 1027
Selection medium without phenol red		38,833 ± 820
Serum		11,689 ± 534
Glutamine		3208 ± 32
Antibiotics		3278 ± 36
G418 (selection agent)		3290 ± 53

Plates were measured with their respective lid or with a plastic foil in the fluorescence reader with the filter combinations 485/535 and the optics position “top”. For estimation of the signal to background ratio (SBR),

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3 40,000 cells expressing EGFP were plated per well, the cells were allowed to attach before the fluorescence
4 signal was measured. Fluorescence intensities are given as mean \pm standard deviation of 96 wells.
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6 Serum: 10 % FCS in PBS; glutamine: 4 mM in PBS; antibiotics: 100 U.ml⁻¹ penicillin G, 100 μ g.ml⁻¹
7 streptomycin in PBS; G418, 1 mg.ml⁻¹ in PBS. Values are given as relative fluorescence unit (RFU) and
8 represent average values of ten independent wells of 96-well plate \pm standard deviations. Selection medium: R10
9 plus 1 mg ml⁻¹ G418. The fluorescence was measured for wells containing 100 μ L of liquid.
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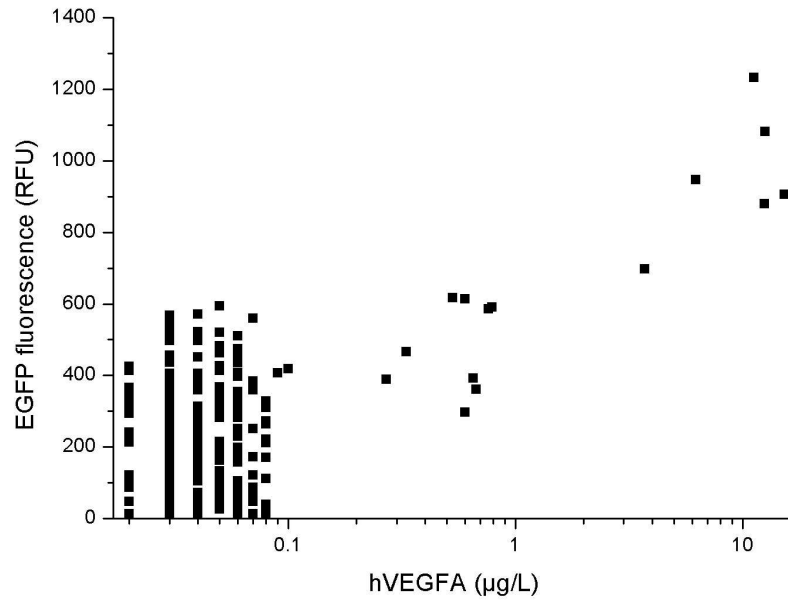


Fig. 1 (Freimark et al.)
288x200mm (150 x 150 DPI)

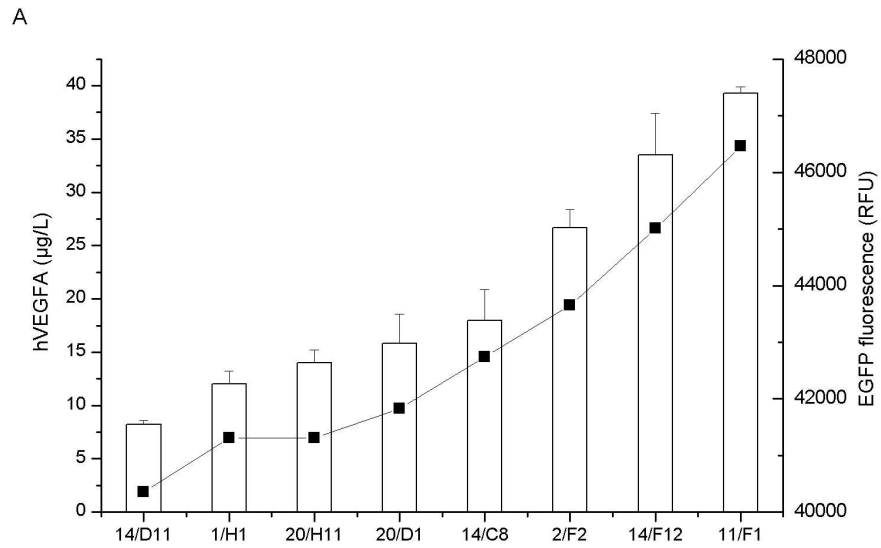


Fig. 2A (Freimark et al.)
288x200mm (150 x 150 DPI)

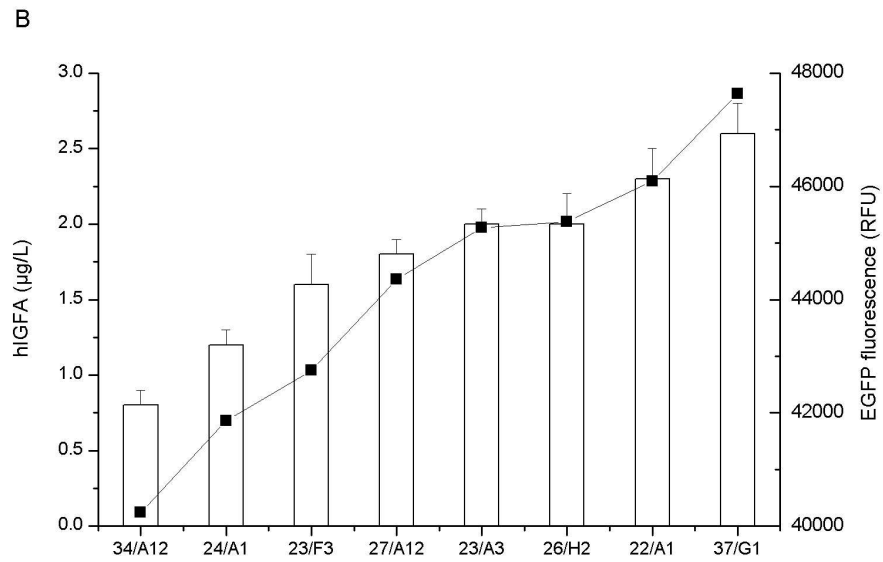


Fig. 2B (Freimark et al.)
288x200mm (150 x 150 DPI)

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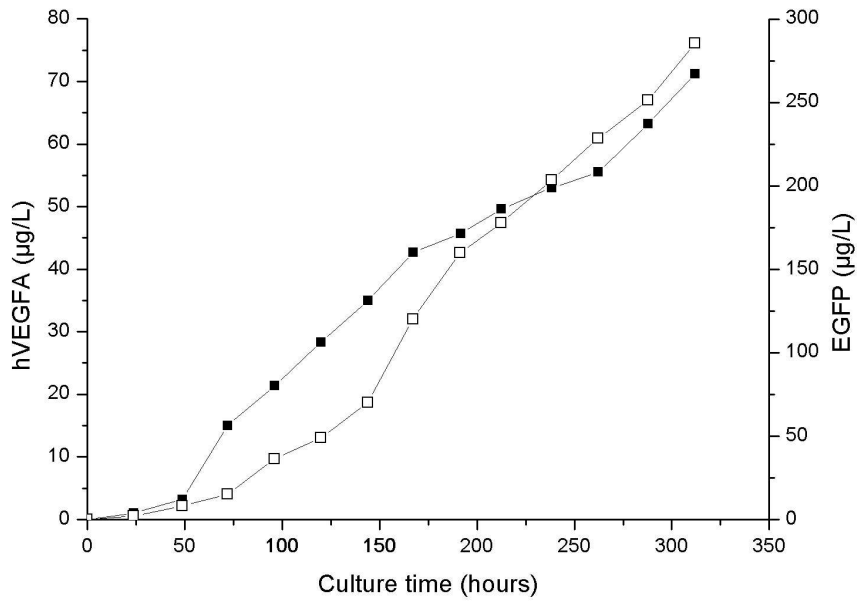


Fig. 3 (Freimark et al.)
288x200mm (150 x 150 DPI)

Review