Fluorescent labeling for clonal selection of Marc 145 cells secreting high levels of recombinant protein PBD-1

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Abstract Despite the powerful impact gene expression markers like the green fluorescent protein (GFP) or enhanced GFP (EGFP) exert on linking the expression of recombinant protein for selection of high producers in recent years, there is still a strong incentive to develop more economical and efficient methods for isolating mammalian cell clones secreting high levels of recombinant proteins. Here we present a new method based on the co-expression of EGFP that allows clonal selection in standard 96-well cell culture plates. The genes encoding the EGFP protein and the related protein are linked by an internal ribosome entry site and thus are transcribed into the same mRNA in an independent translation process. Since both proteins arise from a common mRNA, the EGFP expression level correlates with the expression level of the therapeutic protein in each clone. By expressing recombinant porcine β -defensin 1 in Marc 145 cells,

we demonstrate the robustness and performance of this technique. The method can be served as an alternative to identify high-producer clones with various cell sorting methods.

Keywords High producer · Mammalian cell culture · Recombinant protein · Porcine β -defensin 1

Abbreviations

Marc 145	African Green Monkey kidney cell line
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence-assisted cell sorting
G418	Geneticin (antibiotic used for screening)
IRES	Internal ribosome entry site
RFU	Relative fluorescence unit

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Introduction

The selection of high-producer clones through transfection and selection is usually regarded as a vital step for generating cell lines for producing recombinant proteins with high efficiency (Browne and Al-Rubeai 2007). In general, a subcloning stage with the use of 96-well plates is required for successful transfection of cells. An effective method for screening at this stage is valuable, as it helps to pinpoint good producers as early as possible. In addition, analyzing the product titre in the cell culture supernatant by ELISA is commonly adopted at this stage to identify clones secreting high levels of the recombinant. However, the process of randomly picking a large number of clones (e.g. by limited dilution and individually assaying them by ELISA) is boring, time-wasting, and reportedly not always very effective (Borth et al. 2001; Zeyda et al. 1999). Moreover, this method can not take factors like differences in either cell density or medium volume between wells into consideration. Apart from the above-mentioned weakness, this approach may fail to accurately predict the clones with high specific productivity.

High-throughput screening method based on fluorescence-activated cell sorting (FACS) or magnetic bead-based cell separation is, in many cases, faster than the previous method (Caroll and Al-Rubeai 2005; De Maria et al. 2007), but it requires, especially in the case of FACS, not only expensive equipment but also highly-skilled operators. Therefore, such a method seems to be in use in very a limited area such as labs which are in frequent need of cell sorting. Moreover, unless the target molecule itself shows fluorescence, labelling or coexpression of a fluorescent or detectable molecule is an urgent requirement (Bailey et al. 2002; Mosser et al. 1997). The enhanced green fluorescent protein (EGFP) has been recommended in the past as an important coexpressed indicator protein for application in FACS-based cell separation and screening of cultures in multiwell plates (Meng et al. 2000; Hunt et al. 1999, 2002). In this approach, the recombinant cells express both the fluorescent and the target protein, and the titres are assumed to be linked with each other. However, the genes for EGFP and the target protein have mostly been cotransfected into the cells via individual plasmids so that no physical linkage between the expression of the two proteins exists (Hunt et al. 1999; Hellweg et al. 2001).

In this study, we selected Pig β defensins-1 (PBD-1) as the object protein, because antimicrobial peptides are

an essential part of innate defense mechanisms of animals against the attack by pathogens. Pig β defensins are an important component of these defense mechanisms in epithelial cells and constitute a family of small cationic peptides (4–8 ku) with widespread antimicrobial activity (Qi et al. 2009; Sang and Blecha 2009). However, only few β -defensins, porcine defensin 1 (PBD-1) and -2 (PBD-2), have been described as up to current requirement (Zhang et al. 1999; Veldhuizen et al. 2007). These defensins were found primarily in tongue mucosa but low mRNA levels were also found in other epithelial tissues including the intestine. Therefore, recombinant β defensins became an intensive technological requirement.

Considering that the African Green Monkey kidney (Marc 145) cell line is widely used in the vaccine production fields, we try to establish a simple fluorescence-based microplate screening method for cell subpopulations based on the true coexpression of the fluorescent and the target protein. For this purpose, the two proteins were brought into the Marc 145 cell line via an eukaryotic expression vector. The method combines the efficiency of EGFP/fluorescence-based screening with the limited dilution method to select high-producing subclones and does not require any special equipment except a plate reader equipped with fluorescence option.

Materials and methods

Materials

24-well and 96-well plates from Corning (Corning, NY, USA) and BD Falcon (Heidelberg, Germany; Microtest 96) were used for transfecting and screening. Lipofectamine 2000 reagent from Invitrogen (Carlsbad, CA, USA), G418 from Promega (Madison, WI, USA) were also necessary. In addition, High-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 15 % fetal bovine serum (FBS; Hyclone, Logan, UT, USA), and 100 U/mL penicillin and 100 μ g/mL streptomycin (Sigma, St. Louis, MO, USA) were essential for this experiment.

Cell culture

Marc 145 cells (ATCC, Manassas, VA, USA) were maintained in growth medium (High-glucose DMEM

medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/100 µg/mL streptomycin and 10 % foetal calf serum) in an atmosphere of 5.0 % CO₂ and at 37 °C (incubator: Sanyo, Osaka, Japan).

Prior to the transfection experiments, the adherent Marc 145 cells were harvested by digesting with 0.25 % trypsin and 0.02 mM EDTA solution (Invitrogen), then split 1:3 and seeded on to 24-well plates.

Plasmid construction, transfection and single-cell cloning

The total RNA was extracted from an adult Landrace pig's liver and the PBD-1 cDNA was obtained by reverse transcription and amplified by PCR. The primers were: 5'-CGGAATTCATGAGACTCCACCGCTC-3' and 5'-CGGGATCCCTTCTGAGCCATATCTGTG-3' (synthesized by Shanghai Shengong Bioengineering Co., Ltd, Shanghai, China). The site of the restriction endonuclease *EcoRI* was included in the up-stream primer as well as that of *BamHI* in the down-stream primer (underlined). The PCR cycling parameters were listed as follows: 94 °C for 40 s; 59 °C for 30 s; 72 °C for 30 s for 35 cycles; 72 °C for 10 min. Then, the PCR products were ligated into the vector pMD-18T (Takara, Otsu, Shiga, Japan) and cloned in *E. coli* strain DH5α followed by sequencing from both sides.

Subsequently, the recombinant pMD-18T- PBD-1 plasmids were digested by both *EcoRI* and *BamHI* (TaKaRa) to obtain the PBD-1 full-length gene simultaneously. The pIRES2-EGFP (BD Biosciences Clontech), which is designed for the efficient selection (by flow cytometry or other methods) of transiently or stably transfected mammalian cells expressing EGFP and the protein of interest, was also digested by both above mentioned restriction endonucleases. The PBD gene was then ligated into the pIRES2-EGFP vector. The reconstructed pIRES2-EGFP-PBD-1 plasmid was transfected into *E. coli* DH5α and amplified through them. The product from the above amplification process was again confirmed by enzyme digestion analysis and sequencing and kept for gene transfer.

Lipofectamine 2000 reagent (Invitrogen) was adopted for gene transfection using the provided protocol. The Marc 145 cells were seeded in 96-well plates at the density of 1×10^4 cells/100 µL for each well. Twenty-four h later (at a confluency of about 80–85 %), the cells were transfected by Lipofectamine 2000 with the pIRES2-EGFP-PBD-1 plasmid, and the

ratio of plasmid mass to lipofectamine 2000 volume was 1:3. The transiently transfected cells were grown first in growth medium supplemented with 800 µg/mL G418 for selecting stably transfected clones. Non-transfected cells lack of the resistance to this antibiotic. One week later, the selection pressure was increased to 1,000 µg/mL G418. The transfected Marc 145 cells were transferred to 96- well plates at an average density of less than one cell per well in 100 µL of selection medium for screening high-producing clones, (procedure of “limited dilution” (Puck 1958). The basis for this method is a dilution of the cell suspension to a point were statistically less than one cell per well (here 0.9) are plated. Wells were checked for cell number and those containing a number of cell ($\neq 1$) were excluded for the subsequent measurements. In our hands the method was faster and easier than isolation of the clones by using cloning cylinder (Freimark et al. 2010). Following dilution, the cells were further incubated for 2–3 weeks. A total of 20 plates were screened in this study.

Fluorescence measurement and Elisa assay and fluorescence-activated cell sorting (FACS) analysis

After 2–3 weeks the experiment was terminated, at which point the expression level of the object protein of interest was measured by ELISA and correlated to the EGFP fluorescence value obtained at the same time. The fluorescence intensity of the clones was monitored with a Mithras LB940 microplate reader (Berthold Technologies, Bad Wildbach, Germany) and the emission was measured at 488 nm. Autofluorescence of EGFP was measured with Mithras LB940 (Berthold Technologies) as described by Takeda et al. (2006) and Freimar et al. (2010).

At the same time, the PBD-1 concentration in the cell culture supernatant was determined with an ELISA kit (US Biological, Swampscott, MA, USA) following the manufacturer's instructions. The ELISA standard curve was prepared by a serial dilution of the PBD-1 standard protein for obtaining concentrations ranging from 25 to 800 ng/mL. The absorbance at 450 nm was measured by a microplate OD reader. The levels of PBD-1 were calculated from the OD450 values in the descending portion of the ELISA standard curve for the PBD-1 standards.

For further analysis of the correlation between the PBD-1 levels and EGFP expressed in transfected Marc 145 cells, these cells whose cell culture supernatant

Table 1 List of antibodies

Primary antibodies	Species	Dilution	Supplier	Secondary antibodies	Dilution	Supplier
PBD-1	Mouse	200	ABbiotec	goat anti-mouse IgG	400	ABbiotec
EGFP	Mouse	200	ABbiotec	goat anti-mouse IgG	400	ABbiotec

had been determined by ELISA were dissociated into a single-cell suspension (non-transfected Marc 145 cells were used as a control). Flow cytometric analysis was carried out on a FACSCalibur employing the antibodies listed (Table 1). Cells were incubated in a 1:200 mouse primary polyclonal antibody PBD-1 or EGFP

Fig. 1 Construction of the eukaryotic expression vector of the *PBD-1* gene. **a** Schematic representation of the construction of the eukaryotic expression vector of the *PBD-1* gene. **b** Agarose electrophoresis of PCR products. M, DL 2000 marker

for 30 min on ice with shaking, respectively. Then the cells were washed with PBS and centrifuged at 2000 RPM for 5 min. The pellet was resuspended in 1.5 mL of PBS and 1:400 Cy3-labeled goat anti-mouse secondary antibodies and incubated for 30 min on ice. After 3 washes on PBS, the pellet was resuspended on cytometry buffer and analyzed using a FACSCalibur with a 633 nm (red) laser light. The data were analyzed with FlowJo software (Tree Star, Portland, OR, USA).

Southern blot analysis

Genomic DNA from the stably *PBD-1*-transfected cells (three culture wells were randomly selected) was digested with either *Pst*I or *Sac*I overnight, resolved by gel electrophoresis and transferred to Hybond-N+Nylon membrane (GE Healthcare, Shanghai, China). Hybridisation and detection of the probe was performed using the DIG High Prime DNA Labelling

Fig. 2 The screening of stable transfected cell clones. **A–D**, top panels, bright field images; lower panels, fluorescent images. a, one cell; b, two cells; c, one clone cluster; d, stable cell line. Scale bar, 100 μ m

Table 2 Background and autofluorescence experiments

Plate type	Background and autofluorescence
<i>BD Falcon microtitre plate</i>	
Empty	12,723 ± 218
Control cells	13,386 ± 1,251
EGFP-expressing cells	23,809 ± 2,802
SBR	0.78
<i>Corning microtitre plate</i>	
Empty	3,218 ± 6
Control cells	3,482 ± 124
EGFP-expressing cells	15,083 ± 2,065
SBR	3.33
Cells without liquid	3,320 ± 14
PBS	3,322 ± 101
Antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin in PBS)	3,290 ± 66
DMEM medium with 100 U/mL penicillin G, 100 µg/mL streptomycin and 10 % serum	42,055 ± 1,517
G418 (800 µg/mL in PBS)	3,343 ± 57
Selection medium (DMEM medium plus 800 µg/mL G418)	43,119 ± 1,601

Plates were measured with their respective lid in the fluorescence reader with the filter combinations 485/535 and the optics position “bottom”. For estimation of the SBR, 40 000 cells expressing EGFP were plated per well containing 100 µL of liquid, the cells were allowed to attach before the fluorescence signal was measured. Fluorescence intensities are given as mean ± SD of 96 wells. The signal to background ratio (SBR) was calculated according to: $SBR = \frac{S-B}{B}$ where S is the fluorescence of EGFP-expressing cells and B the autofluorescence of non-transfected cells under otherwise identical conditions. Values are given as RFU and represent average values of ten independent wells of 96-well plate ± SD

and Detection Starter Kit II (Roche, Mannheim, Germany), and Image Quant 350 imager and associated software (GE Healthcare), according to manufacturer’s instructions. The GFP and PBD-1 probes encompassed the coding sequences of EGFP (Invitrogen) and PBD-1, respectively.

Western blot analysis

Stable Marc 145 transfectants (1.0×10^5) were resuspended into 1 ml of growth medium in 24-well plates. On the following day, the plates were transferred to an incubator fixed at 37 °C for 14 days. At the selected time points, an aliquot of growth medium was mixed

with an equal volume of lysis buffer 2× supplemented with 5 % β-mercaptoethanol. The samples were boiled for 10 min and separated on a 12 % SDS-PAGE (stacking gel: pH6.7, 75V and separating gel: pH8.9, 120V). The proteins were transferred onto polyvinylidenedifluoride (PVDF) membrane (Millipore Co., Billerica, MA, USA). The conditions of the transfer to membrane were 200 mA for 1h. The membrane was blocked with 5 % non-fat dried milk in PBS with 0.05 % Tween 20 (PBST) for 1 h at room temperature, washed three times with PBST and incubated with mouse anti-PBD-1 antibody (1:100) or mouse anti-EGFP antibody (1:100) or mouse anti-GAPDH antibody (1:100) at room temperature for 1 h. After washing with PBS containing 0.05 % Tween-20 (PBST) solution, the membrane was reacted with peroxidase-conjugated antimouse IgG at room temperature for 1 h and treated with enhanced chemiluminescence (ECL) solution (Amersham Biosciences, Uppsala, Sweden), followed by exposure to X-ray film. Bands were analysed using the Image J software and normalised to GAPDH bands.

Statistical analysis

Data were presented as mean ± standard deviation. Statistical analysis was conducted by one-way ANOVA, and the difference between groups was determined by further analysis with LSD-test (SPSS for window, version 13.0). And $P < 0.05$ was considered statistically significant.

Results and discussions

Gene constructs

The eukaryotic expression vector pIRES2-EGFP-PBD-1 was constructed, as schematically shown in Fig. 1. In the pIRES2-EGFP-based gene constructs used here, the genes encoding for the recombinant target protein and EGFP are linked by an internal ribosome entry site (IRES) sequence. Consequently, both proteins arise from a common mRNA and the EGFP expression level should closely correlate with the relative expression level of the co-expressed protein of interest (Rees et al. 1996). Furthermore, the use of pIRES2-EGFP for transgene expression, apart from giving good product titres, also makes the screening strategy developed in this contribution possible.

Fig. 3 a Correlation between EGFP and PBD-1 expression in a number of high-producer clones. Prior to measurements, clones exhibiting high EGFP fluorescence were transferred to 24-well plates and cultured for 10 days in protein-free medium. The PBD-1 concentration in the cell culture supernatant was analysed by ELISA and the EGFP fluorescence signal was measured optically. The EGFP fluorescence is given as relative fluorescence unit (RFU) after deduction of BFplate (background fluorescence of transparent microtitre plate). Data shown are representative of results in three independent experiments. **b** The fluorescence signal of EGFP-expressing Marc 145 cells was analyzed by flow cytometry with a FACScan flow cytometer. Histograms show fluorescence intensity versus cell number for each well indicated. Untransfected Marc 145 cells were used as a control

Stable transfection of Marc 145 cells

When Marc 145 cells exceeded 80 % confluent, they were transfected by Lipofection 2000 (Invitrogen) mediated gene transfer with a mammary gland special expression construct (pIRES2-EGFP- PBD-1). Stably transfected clones were selected by G418 (Promega) at 1,000 µg/mL for the first 5 weeks and surviving cells were maintained in 500 µg/mL G418. The expression of EGFP in the selected clones was verified by fluorescent microscope (Fig. 2).

Correlation between EGFP and PBD-1 expression

The plate reader used in this study provides us with a choice between multiple and single reading of the wells. Multiple reading is often adopted to compensate for an unequal distribution of cells in the wells.

However, the results from the “multiple reading mode” were not significantly different from single measurements (data not shown) by our experiment. Therefore, all further measurements were done in the single reading mode (shown in Table 2), which agreed with previous reports (Freimark et al. 2010).

Possible background fluorescence effects were of concern for the assay development. For cell and tissue culture of mammalian cells, especially when microscopic observation of the cells is necessary, microplates with a clear bottom have to be used. Special microplates, characterized by low background fluorescence and minimal light scattering, are designed for fluorescence measurements.

To further investigate the correlation of PBD-1 and EGFP production, transfected cells were subjected to limited dilution down to 1 cell/well in 96-well plates. When the EGFP expression was analyzed for 1920

Fig. 4 Time course of PBD-1 and EGFP concentration during spinner flask batch culture of the clone 9/D6. The PBD-1-expressing cell line 9/D6 was cultured into a spinner flask. The cells were inoculated at a density of 5×10^5 cells/mL in 100 mL DMEM medium on day 0 and cultured for 14 days. The PBD-1 concentrations were measured by ELISA and are indicated by *black triangles*. The EGFP production was measured optically and the values obtained were converted into µg/L EGFP produced using a standard curve established with pure EGFP. The EGFP concentrations are indicated by *black squares*. Data shown are representative of results in three independent experiments

($96 \times 20 = 1,920$) clones, approximately 0.3 % of the screened clones were high producers (EGFP fluorescence). Clones at the highest fluorescence levels were then isolated and expanded in 24-well plates. The product concentration in the cell culture supernatant was assessed after 10 days of culture by ELISA. In parallel, the EGFP fluorescence was measured in the plate by the plate reader. Figure 3 shows that the EGFP signals and the amount of recombinant protein of interest clearly correlate.

One of the PBD-1-producing clones (9/D6) was subsequently adapted to suspension culture by an exchange of the culture medium and cultivated in AP10 riptide bioreactor (AmProtein, Hangzhou, China). The production of PBD-1 and EGFP was followed for 336 h (Fig. 4). We observed the high-level expressing of EGFP in Marc 145 cells (Fig. 5), and the stable insertion of the EGFP and PBD-1 genes in Marc 145 cell genome were further analyzed by

Fig. 5 High-producing Marc 145 cell clones under fluorescence microscope. **a–c** present three typical PBD-1-producing cell clones; *upper panel*, bright field images; *lower panel*, fluorescent images. Scale bar 100 µm

Fig. 6 **a** Southern blot analysis of genomic DNA from stably PBD-1-transfected cells. **b** Representative Western blots for PBD-1 and EGFP. GAPDH was used as a loading control. These subclones were lysed by lysing buffer after 10 days of culture

and then were analyzed by Western blot. **c** Graphs representing the analysis of the Western blots for the analysed proteins PBD-1 and EGFP

Southern blot (Fig. 6A). Figs. 6B shows the Western blot result of EGFP and PBD-1 proteins expressed in stably PBD-1-transfected cells. Quantitative analysis of grey values representing a good general correlation between PBD-1 and EGFP production (Fig. 6C). At the end of the culture, the PBD-1 level had reached 330.8 $\mu\text{g/L}$. Thus this clone had already performed in the same range as previously described ones based on the CHO-DHFR system (101–520 $\mu\text{g/L}$ (Ferrara et al. 1993). We expect a further improvement in productivity once the process is established in the bioreactor. The similar yield of EGFP may result from identities in translation efficiency of the PBD-1 by the transfected Marc 145 cells. Moreover, EGFP is known as a stable protein with a half-life of more than 30 h in animal cells (Cubitt et al. 1995), and the PBD-1 has a half-life of about 30 h (Rouveix and Giroud 2000). Final concentration of the protein of interest therefore correlates with EGFP as result of the co-transcription. Further investigation of these effects is currently under way in our laboratory.

Conclusions

The adoption of a recombinant dicistronic expression plasmid to express the protein of interest along with EGFP requires a fast screening for “high producers” via the EGFP fluorescence. The fast screening method requires efficient detection of good producer clones at an early stage. It does not merely rely on FACS, or the availability of an antibody specific for the therapeutic protein to be expressed. Furthermore, it can be easily adopted to conduct the experiment in any laboratory anytime at the stage of the cell line development process. Clones with unstable transgene expression can be identified by the decline in EGFP fluorescence. A possible caveat of the method concerns a putative cytotoxic effect of EGFP at very high concentrations, as it has been indicated (Browne and Al-Rubeai 2007), especially when one is looking for very superior producers. However, in view of the many EGFP over-expressing cells and organisms that have been investigated (Gubin et al. 1997), some of them expressing

much higher EGFP amounts than it has been described. There is still much potential for higher productivity before EGFP cytotoxicity becomes a problem.

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Conflict of interest The authors have declared no conflict of interest.

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