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Modification of a System Based on the Use of Selection and Sorting Markers for the Screening of Stable Transfectants

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Abstract—The p-hook technique, which is based on protein domain expression on the cell surface, has been modified and used for the selection and highly sensitive and efficient sorting of stably transfected CHO-S cells. The model protein EYFP served as both the target protein produced by the cell and the expression marker in this study. Chimeric proteins TiBP-PDGFR and CBD-PDGFR, which enabled the binding of protein-producing cells to titanium carriers and cellulose supports, respectively, have been constructed. Combined selection with the use of the chimeric CBD-PDGFR protein exposed on the cell membrane resulted in the production of a clonal line series characterized by a high and stable level of model protein synthesis. The level of model protein expression (inferred from fluorescence) in three clones from the selected series was more than 30 times higher than the average level for the original cell pool. The expression stability in EYFP-positive clones was preserved at late (20th and 35th) culture passages. The use of the chimeric CBD-PDGFR selection and sorting marker developed in this study enables the rapid selection of highly productive stable transfectants and thus reduces the costs associated with the creation of a productive cell line collection.

Keywords: adhesion, CHO cells, selection, sorting, titanium-binding domain, cellulose-binding domain, expression, exposed chimeric proteins

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INTRODUCTION

Higher eukaryote cell lines are used for the production of full-length recombinant proteins. The advantages of the producers derived from these cells are usually not limited to the high quality (correct folding and glycosylation) of the produced protein; they also include high productivity and more straightforward purification of the product due to its secretion into the culture medium [1, 2]. At the same time, the modification of higher eukaryote cells to obtain producing lines is associated with higher costs than in the case of bacteria and yeast due to the expensive consumables and equipment, as well as higher time and labor costs, of the process.

Recombinant protein production in CHO (rCHO) cell lines uses vector systems, which can integrate the gene of interest into a random site in the genome, and selection markers, such as resistance to antibiotics (geneticin and puromycin). At this, highly productive transfectants are selected by means of immunohistochemical screening, since they do not have phenotypic differences from or selective advantages as compared to transfected cells with a low transgene expression [3].

Enhanced selection efficiency for highly productive transfectants can be achieved with the use of selection and sorting markers (SSMs), which enable an increased growth rate of the studied transfectants or the expression of another phenotypic feature amenable for differentiated assessment and selection.

DHFR or *GS* genes are true SSMs of the first type, as the expression level of these genes affects survival and growth rate of the transfectants. The drawbacks of their use include the limited range of cell lines amenable for manipulation and a decrease of strain stability and productivity in the generation series that is proportional to the round of amplification and selection marker concentration [3, 4].

The *GFP* gene is a basic sorting marker that can be used in combination with the FACS method to select

Abbreviations: SSM—selection and sorting markers; bp—base pairs; PCR—polymerase chain reaction; BSA—bovine serum albumin; CBD—cellulose-binding domain; CHO—Chinese hamster ovary (cells); DHFR—dihydrofolate reductase; EYFP—enhanced yellow fluorescent protein; FACS—fluorescence-assisted cell sorting; GS—glutamate synthetase; PDGFR—platelet derived growth factor receptor; TiBP—metal (titanium)-binding domain.

highly productive transfectants according to the intensity of cell fluorescence [5, 6]. The possibility of their use in any cell line, including lines not amenable for sorting (in contrast to gs– or dhfr– lines) is an indisputable advantage of the technique. However, the *GFP* gene cannot be used as a true SSM that would allow for both the selection and sorting of recombinant protein producers, and the use of an additional selection marker (which confers antibiotic resistance, for example) therefore becomes necessary [7, 8].

Cell surface-exposed protein domains obtained by cell display technology, which confer a capacity for adhesion to a certain carrier to the transfected cells, are classified as SSMs of the second type [9–15]. This technique does not interfere with the cell metabolism and has potential for use in the selection of various cell lines, particularly for cells with the highest expression level of recombinant target genes [9, 10].

One of the first techniques that used SSMs of this type incorporated the p-hook method, a selection technology based on enhanced adhesion of membrane-exposed proteins to an adsorbent surface [9]. A PDGFR receptor fragment served as an anchor for these proteins, and an antibody domain that was exposed on the membrane and capable of binding to different carriers served as a "molecular hook." The target protein gene expression was monitored by beta-galactosidase gene coexpression. Enrichment of transitory pool by transgene-expressing cells was registered. However, experience showed that a low level of differentiation between clones of high and low productivity was a major drawback of this technique. The current implementation of the p-hook method essentially represents a selection technique but not a sorting technique, since it only provides a new method for the selection of random transfectants.

A relatively low degree of protection from the nonproductive selection of lines characterized by high background adhesion to the carrier is another drawback of the p-hook technique. Efficient elimination of the background requires multiple repetitions of selection rounds that are based on that transgene expression level and not related to sorting; this increases the time of selection, but not its quality.

The goal of the present work was to modify the p-hook method and transform it into a full-value selection and sorting technique for stably transfected CHO-S cells by virtue of coexpression of genes coding for surface-exposed cellulose-binding or titanium-binding domains. The possibility of using the technique to suppress the development of cell lines originating from false background selection was also studied.

EXPERIMENTAL CONDITIONS

Construction of the EYFP-IRES-iggHATiBP-mucPDGFR Gene

The aforementioned gene was constructed in the pUC57 vector derived from the pCI-neo vector (Promega, United States). The iggTiBP+mucPDGFR construct in a temporary transport vector pUC57 was synthesized by Sintol (Russia) at the first stage. The vectors pUC57-iggHA+mucPDGFR (recipient) and pEYFP (Clontech, United States) (donor) were treated with *Bgl*II/*Bsr*GI restriction endonucleases (endonucleases and nucleic acid-modifying enzymes used were from Fermentas, Lithuania). Restriction product isolation in 1% agarose gel (SE-2 horizontal electrophoresis chamber, Helicon, Russia) was followed by ligation of the 800-bp fragment that contained the *EYFP* gene to other components of the pUC57-EYFP-iggHA+mucPDGFR construct. The EYFP-iggHATiBP-mucPDGFR fragment was excised from this construct at the *Xba*/*Not* sites and transferred into the pCI-neo vector. The PCR product of the IRES gene (IRES FMDV from the pVITROneo vector (InvivoGen, United States)) was excised at the *Nhe*/*Eco*RV sites and the final gene construct *EYFP-IRES-iggHATiBP-mucPDGFR* was obtained by ligation.

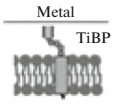
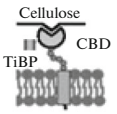
Construction of the EYFP-IRES-iggHATiBP-CBD-mucPDGFR Gene

The *EYFP-IRES-iggHATiBP-CBD-mucPDGFR* gene was derived from the pCI-EYFP-IRES-iggHATiBP-mucPDGFR vector created earlier. The PCR product of the *CBD* gene (the gene was synthesized by Sintol (Russia)) in the pUC57 vector was treated by *Sac*II and *Xho*I restriction endonucleases, the restriction products were fractionated in an agarose gel, and the 460-bp fragment that contained the *CBD* gene was excised and ligated to obtain the final *EYFP-IRES-iggHATiBP-CBDmucPDGFR* gene construct. Correct assembly of the expression vector was verified by extended restriction analysis. The nucleotide sequences of both genes and the adjacent DNA fragments were confirmed by sequencing (Evrogen, Russia).

CHO-S Cell Line Cultivation

CHO-S cells (Invitrogen, United States) were cultivated in 125- and 250-mL Erlenmeyer flasks in a Multitron Cell shaker–CO_2-incubator (Infors HT, Switzerland) at 125 rpm in an atmosphere of 5% carbon dioxide at 37°C and 95% humidity. Power CHO-2 CD serum-free nutrient medium (Lonza, Switzerland) supplemented with 4 mM alanyl glutamine, 16 μM thymidine and 0.1 mM hypoxanthine was used (the reagents were from Invitrogen). The cells were reinoculated in fresh medium every 2 to 3 days and grown to a density of 0.3×10^6 cells/mL. Cell counting and cell viability analysis were performed after staining with Trypan Blue (Panreac, Spain) in a Goryaev chamber with a CKX41 microscope (Olympus, Japan).

Table 1. Scheme of activity of chimeric proteins anchored on the membrane by the PDGFR domain and capable of binding to various carrier types

Construct identifier	Sequence of elements in the construct	Scheme of action	Adhesion to carrier
TiBP-EYFP+	CMVpr_EYFP_IRES_iggTiBP_PDGFR_polyA		Metals, TiO_2, Si
CBD-EYFP+	CMVpr_EYFP_IRES_iggTiBP_CBD_PDGFR_polyA		Cellulose

Transfection of CHO-S Cell Lines

The procedure employed the FreeStyle MAX lipophilic agent (Invitrogen) according to the manufacturer's instructions. The CHO-S cell culture was grown to a density of 0.5×10^6 cells/mL one day before transfection. The optical density of the culture was assessed on the day of transfection, and the cells were pelleted by centrifugation at 200 g for 10 min at room temperature. The supernatant was decanted, and the cells were resuspended in FreeStyle™ CHO Expression Medium (Invitrogen) supplemented with 8 mM alanyl glutamine (Invitrogen) to a final density of 1.0×10^6 cells/mL. The subsequent transfection in 6-well plates was performed according to the instructions of the manufacturer (FreeStyle CHO-S Cells, Invitrogen, Cat. # R800-07).

Manipulations with the Transfected Pool

Two types of vectors containing the Ti-binding domain with or without CBD (CBD-EYFP+ and TiBPEYFP+, respectively) were used for the transfection of CHO-S cells in CD-CHO medium (Invitrogen). The cells were pelleted by centrifugation after transfection, the CD-CHO medium used for the transfection was removed, and the cells were resuspended in fresh serum-free Power-CHO medium (Lonza) and left for another 4 h. The adhesion of transfected cells to carriers was registered 12–16 h after the addition of modified DNA. Cellulose fibers (sterilized filter paper (Plf-roll, Russia) of 4 × 8 mm in size) or titanium rings of 1 cm in diameter were used for cell sorption; the carrier was placed into a well with fresh Power-CHO medium, into which the cells of the transfected pool were subsequently transferred. Since the EYFP protein gene served as both the target gene and the marker gene in the present work, the fluorescent cells in the suspension and on the carrier were counted after transfer to a fresh medium (see below), and the result served as an indicator of cell sorption.

Flow Cytometry

Fluorescence intensity was assessed on a Beckman Coulter Navios flow cytometer (Germany). Measurement parameters were as follows: wavelength 488 nm, emission 500 nm, number of events 10 000. The results were calculated in the FlowJo 10.0.Br1 software. The autofluorescence was measured in the original nontransfected culture at the same growth stage. The background values obtained were subtracted from the fluorescence of EYFP-positive pools.

RESULTS AND DISCUSSION

Chimeric genes were constructed in order to expose cellulose- or metal carrier-binding protein fragments on the cell membrane surface [16, 17]. These genes included sequences encoding a cellulose-binding and/or metal-binding domain fused to a transmembrane fragment sequence of the PDGFR protein and the *Myc* tag sequence. The target EYFP gene structure was linked to the synthetic gene by the IRES element and served as a marker of target and marker protein coexpression. Table 1 gives data on the mechanism underlying cell adsorption to carriers mediated by the aforementioned chimeric proteins.

The *Myc* tag fragment was inserted between the transmembrane domain and the carrier-binding site in the *CBD-PDGFR* sequence of the vector construct for contrast enhancement in the selection system and enhancement of the difference between transfectants and nontransformed cells. The binding domain was located at a distance from the membrane. Cells characterized by more active expression of the construct acquired an additional advantage upon sorption, because a longer chimeric protein exposed on the parts of the membrane that did not contact the carrier directly interacted with the sorbent in this case. Adhesion of the pool of cells expressing the (TiBP-EYFP+)—(metal-binding sequence) construct referred to as *TiBP* in published studies [17] and its use as the binding domain was studied in experiments with metal surfaces.

EYFP Expression in Transiently Transfected Cells

The initial analysis of sorption capacity on carriers involved cells that expressed a gene construct with a transmembrane fragment of the PDGFR protein

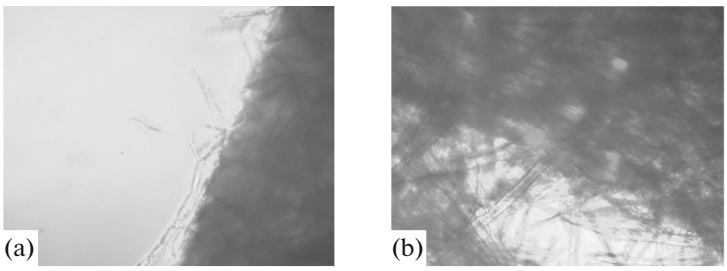


Fig. 1. Adsorption of CHO-S cells transfected by the EYFP+ (a) or CBD-EYFP+ (b) vector on a cellulose support.

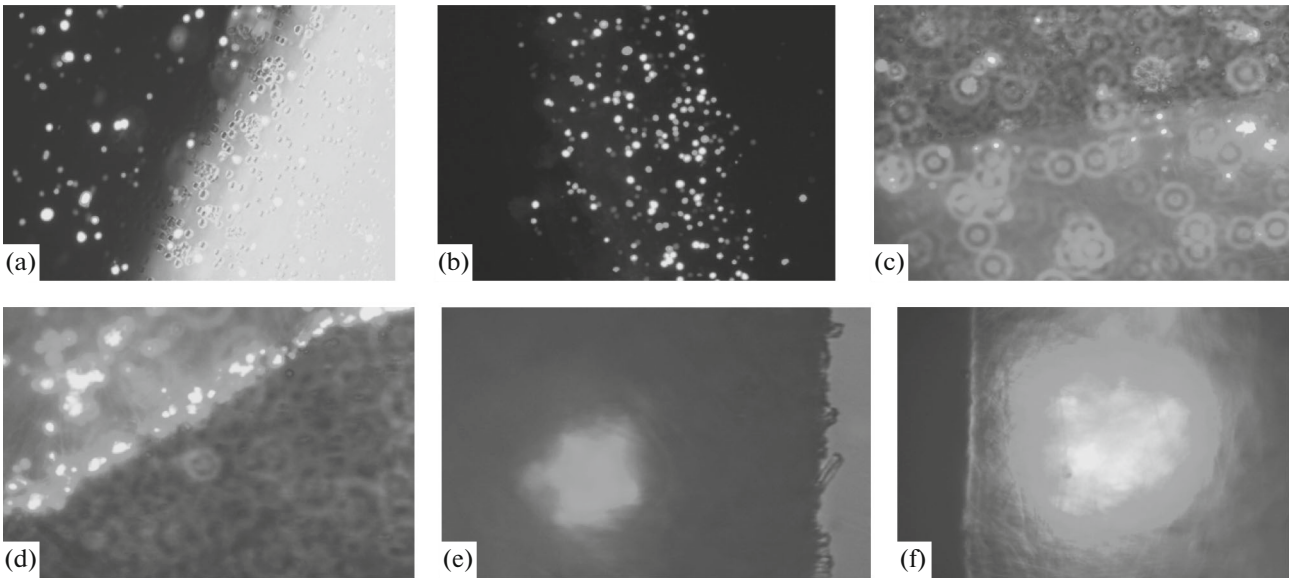


Fig. 2. Adsorption of transfected CHO-S cells on metal and cellulose supports: (a) part of a titanium ring (darker color) immersed in a suspension of p-EYFP-transfected cells; (b) part of a titanium ring immersed in a suspension of TiBP-EYFP-transfected cells; (c) cells transfected by the EYFP vector and the edge of a cellulose support (lighter color); (d) the edge of a cellulose support immersed in a CBD-EYFP+ cell suspension; (e) and (f) colonies grown on a cellulose support in the CBD-EYFP+ cell pool in the presence of geneticin (600 μg/mL).

directly fused to CBD. No difference in expression could be observed visually when the experimental CBD-EYFP+ constructs and the control EYFP+ constructs were compared in this experiment at a transfection efficiency of 10–45%. The cells that expressed the CBD-containing construct showed better sorption on the carrier than the cells of the original CHO-S strain, since transfectants that expressed the CBD-EYFP+ target constructs were found to adsorb to cellulose fiber, whereas the cells expressing control EYFP+ constructs were not adsorbed (there were no fluorescent cells on the fragment of the carrier) (Fig. 1). The data obtained provided a preliminary indication of the possibility of selection using a CBD-containing marker.

Cell Adsorption on Titanium Rings

Only one metal-binding sequence (TiBP-EYFP+) of all of the constructs used in this work enabled cell adhesion to metal. It is evident from Fig. 2b that selective sorption of cells expressing the construct named above differed significantly from the sorption of control reference samples (Fig. 2a). This difference was observed only upon the use of chimeric protein constructs in which the metal-binding element was located in the immediate vicinity of the PDGFR anchor but not in the case of these two coding sequences being separated by the CBD (Table 1). Neither the transfer of titanium rings with adsorbed cells to fresh medium nor the decantation of unbound cell suspension allowed for pool enrichment by transfectants. No noticeable or reproducible changes of the number of cells transfected by the TiBP-EYFP+ vector and characterized by a higher expression level than the original pool were observed at several subsequent repetitions of decantation of the nonbound cell suspension. The difference in the binding of cells with a high transgene expression and nontransfected cells could not be registered visually on incubation day 2.

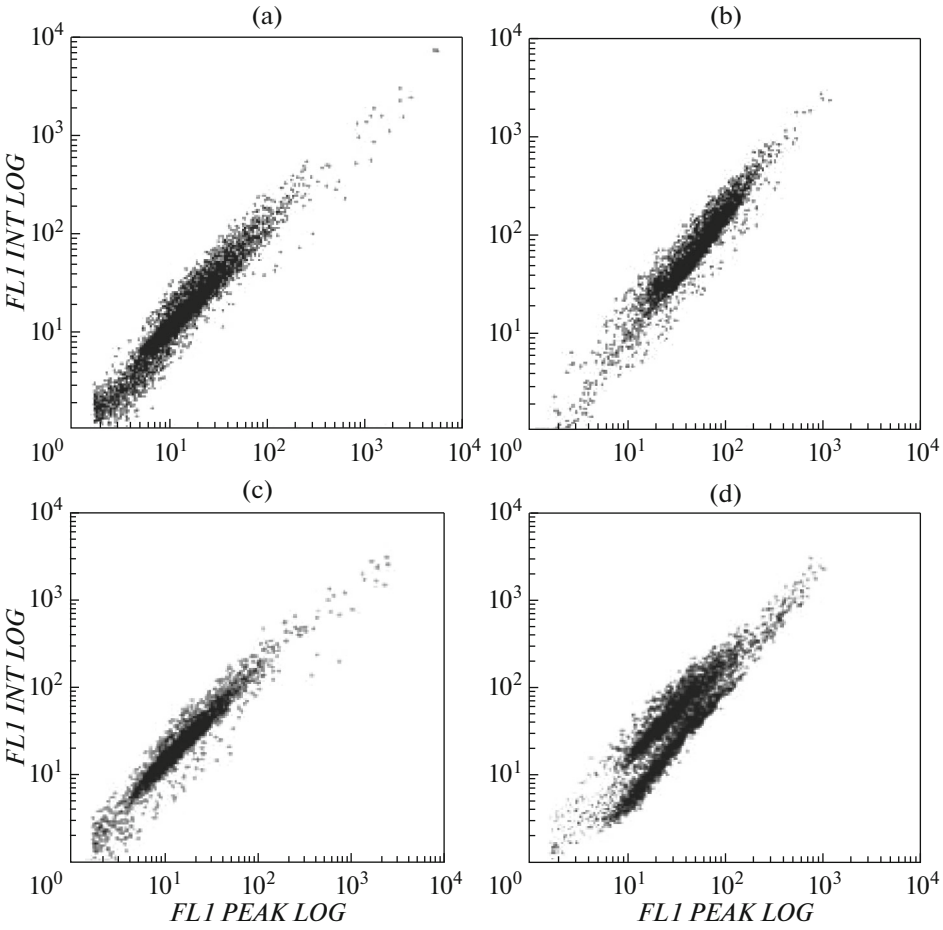


Fig. 3. Fluorescence intensity detected by flow cytometry in stably transfected cell lines adsorbed on cellulose supports: (a) original transfected pool; (b) a clone with the highest fluorescence intensity; (c) and (d) homogenous and heterogeneous cell suspensions with medium fluorescence intensity, respectively.

This could be due to active cadherin expression at a high cell titer in a serum-free medium [18] and the resulting enhancement of nonspecific background sorption. Adhesion could also be prevented by globular proteins released into the medium by the pool [16].

Cell Sorption on Cellulose Supports

Strong and stable binding of transfected cells to cellulose fibers was detected if the transfection construct contained CBD (Figs. 2c and 2d). A large part of the bound cells, or 15–30% of the total number of cells used for the transfection, remained on the carrier after the transfer of the carrier with adsorbed cells to fresh medium. This parameter was comparable to the parameter of transfection efficiency, which usually was in the range of 10–40%. The difference in the number of transfected cells in the original pool and the carrier-bound cell pool was preserved even after transgenic vector elimination and the cease of fluorescent

Table 2. Decrease of the share of fluorescent cells in transfected and control CHO-S pools before and after the transfer of immobilized cells to fresh medium

Transfection construct	Before transfer		After transfer	
	suspension density, $\times 10^6$ cells/mL	share of fluorescent cells among all viable cells, %	suspension density, $\times 10^6$ cells/mL	share of fluorescent cells among all viable cells, %
Control (EYFP+)	2.20	12.2	2.20	14.30
CBD-PDGFR+	1.20	9.1	1.32	4.16
TiBP-EYFP+	1.98	9.5	2.31	4.30

protein production by the transfected cells, and this could also be observed during microscopy (data not shown). The transfer of the carrier with the cells to fresh medium revealed a problem with the study of selection with the use of cellulose fibers as a part of nontransformed cells that apparently possessed an elevated capacity for adhesion bound to the carrier along with EYFP+ cells (Table 2). The pool of cells adsorbed on the carrier became enriched with stable transfectants for two weeks of cultivation, but the ratio of wild-type cells and modified cells was lower than that in the original transitory pool.

We managed to achieve a decisive improvement of selection specificity due to an increase of the geneticin concentration in the medium to 600 μg/mL and more harsh triplicate washes of the carrier prior to transfer to a fresh growth medium once each 2 to 3 days (Figs. 2e and 2f).

Six transfection series were performed, and no less than 500×10^3 transfected cells in 2 mL of cell suspension with a single cellulose carrier as the sorbent were thus obtained. One to five fluorescent colonies were picked from the cellulose support in each case (Figs. 2e and 2f) and used for further studies.

As mentioned above, the colonies selected by carrier binding at a high geneticin concentration continued growing and showed a stable and high EYFP expression. Subsequent experiments showed that cells of these colonies retained a capacity for growth and selection after transfer to a medium that did not contain the antibiotic. The obtained lines showed neither preferential growth on the inner surface of the culture flask nor aggregation, and the cell density in the suspension during cultivation remained at a level characteristic of the original CHO-S strain. Fluorescence analysis in the selected cultures showed that the cells were stable transfectants, i.e., clones that did not become split with regard to EYFP protein expression, a trait not related directly to adhesion.

Characterization of the model protein expression level in the selected clones and the assessment of clone heterogeneity and stability involved clone isolation, removal from the support, and cultivation in suspension. The fluorescence intensity in the cultivated cells was measured by flow cytometry at passages 20 and 35 (Fig. 3). The cell pool at day 4 posttransfection was used for control and normalization of EGFP protein gene expression. Some clones exhibited considerable heterogeneity, which is clearly illustrated by the images obtained (Figs. 3b and 3c). The heterogeneity of monoclonal CHO-EYFP lines is in agreement with published data [19]: Figure 3 shows the only nonhomogeneous population in the collection (detected by flow cytometry analysis).

CONCLUSIONS

Thus, the technology that we modified and used combines selection on a carrier, differential selection of cells based on instrumental procedures for the detection of a protein encoded by a coexpressed gene, and clone selection methods.

This technology, the combined selection for binding to a cellulose support and resistance to high concentrations of geneticin in particular, allowed for the selection of cell lines with stable (over 20–35 passages) and high expression of the model protein from the 500×10^3 transfectants that received the transgene. Cytometry at the 35th passage showed that the model protein expression was more than 30 times higher than the average level for the original pool. The modified p-hook technique allowed the isolation of genetically modified cells via the strong and specific interaction between cellulose and a chimeric protein exposed on the membrane. The selection of transfected cells was possible on posttransfection days 10–15 already. The harsh selection conditions did not affect the viability of the obtained cell lines and did not exert a toxic effect on cell proliferation. The use of the selective system described above enables efficient selection of cell lines characterized by stable expression of the target protein and minimal changes in the metabolism as compared to the original strain. Cell line stability is enhanced due to the selection of genetically stable transfectants in the transfectant population and the self-destruction of colonies that split due to unstable expression (a decrease in the colony number during cultivation and the elution of some colonies from the carrier is a sign of such self-destruction).

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