**New strategy for rapid isolation of stable cell lines from
DNA-transformed insect cells using fluorescence activated
cell-sorting**

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Abstract

A stably transformed insect cell expression system is superior to a baculovirus expression system, since the expression system is sustained and there is no cell lysis, but the isolation of cell lines producing recombinant proteins is time-consuming and laborious. In this study, we developed a technique for the rapid isolation and efficient cultivation of sorted cells in a 24 well deep plate Bioshaker, utilizing the fluorescence activated cell sorting (FACS) method. TnpXme11 cells, which stably expressed GFP_{uv}- β 1,3-*N*-acetylglucosaminyltransferase2 (GGT2), were transfected with a plasmid vector for the expression of a molecular chaperone (TnpXme11-hCNX6 cell line). The expression levels of GGT2 and the molecular chaperone fused with HcRed were analyzed by FACS. Two cell lines were established by single and double sorting. Sorting of the top 10% of the TnpXme11-hCNX6 cell population with the highest fluorescence yielded the TnpXme11-hCNX6-1 cell line. TnpXme11-hCNX6-2 cells were created in a similar fashion, as mentioned above, by a second sorting of the top 10% of the TnpXme11-hCNX6-1 cell population with the highest fluorescence. The cells thus isolated produced approximately 2-fold higher extracellular activity than that

29 before cell sorting. This procedure can be accomplished in only two weeks, including
30 transfection, isolation and analysis of high protein-producing cells, and is a
31 breakthrough strategy for the rapid isolation of a recombinant, stable insect cell line.

32 *Keywords:* Insect cell; Stable cell line; Protein expression; Fluorescence activated
33 cell-sorter; Screening.

34

1. Introduction

Insect cells are often used for recombinant protein production, due to their capacity for post-translational modification (Altmann et al., 1999). The baculovirus expression system (BES) is a well known system that utilizes insect cells, and only a few weeks are needed to construct recombinant baculoviruses and express recombinant proteins by using the bacmid system (Luckow et al., 1993). The BmNPV bacmid (Motohashi et al., 2005) was recently developed, which accelerated the rapid expression of recombinant proteins in silkworm larvae as well as silkworm-derived cell lines.

As an alternative, a stably transformed insect cell expression system is suitable for the expression of recombinant proteins (Douris et al., 2006; McCarroll and King, 1997). Protein expression in the stably transformed cell system is often higher than that in the BES, as the cell lines can be cultured for a longer period of time, as compared with BES, where the expression period is only 72-80 hrs post-infection. The stably transformed cell system is free from lytic cell death and causes less stress on the secretory pathway from the baculovirus infection. Moreover, living cells produce more correctly folded-proteins, as compared with BES. However, the isolation of cells producing

recombinant proteins requires several months of labor. Therefore, a key step in isolating cells for protein production is the rapid selection of cells that achieve the highest expression level.

Fluorescence-activated cell-sorting (FACS) technology allows the rapid isolation of polyclonal cell populations with the highest expression levels of recombinant proteins (Rieseberg et al., 2001; Sleiman et al., 2008). FACS technology can easily screen a million cells in a brief time, and its high-through-put screening allows the isolation of cell populations with high expression levels. Green fluorescent protein (GFP) has been used for monitoring protein expression and selecting cells expressing recombinant proteins. Yuk et al. reported the rapid selection of populations of CHO cells that could maintain a high level of recombinant protein expression, using GFP (Yuk et al., 2002).

In this study, we tried to isolate populations of insect cells expressing a high level of GFP-fused protein and to establish a rapid screening method using the cell sorter and the cultivation of a stably transformed expression cell line.

2. Materials and methods

2.1. Cell lines, media and cultivation

The Tn-5B1-4 and Tn-pXme11-hCNX6 cell lines were reported previously (Kato et al., 2005). The Tn-pXme11-hCNX6 cell line stably expresses the GFP_{uv}- β 1,3-*N*-acetylglucosaminyltransferase 2 (β 3GnT2) fusion protein (GGT2). Tn-5B1-4 and Tn-pXme11-hCNX6 cells were cultivated in Express Five medium (Invitrogen, San Diego, CA, USA), supplemented with 1% antibiotic-antimycotic (Invitrogen) and 30 mM glutamine.

Cells were cultivated in a 24 well deep plate Bioshaker (M·BR-024, TAITEC, Saitama, Japan) with working volumes of 1, 2, 4, and 8 ml per well. The agitation rates and the temperature were controlled at 200, 400, and 600 rpm and 27°C, respectively.

2.2. Plasmid Construction

Molecular chaperones were expressed under the control of the actin promoter. The genes encoding calnexin (CNX), calreticulin (CRT), ERp57, immunoglobulin

heavy chain binding protein (BiP, GRP78) and heat shock protein 70 (Hsp70) were amplified by PCR, using the primers shown in Table 1 and Human Brain Quick-Clone cDNA (Clontech, Palo Alto, CA, USA) as the template. Each amplified fragment was cleaved with *Sal* I and then inserted into the pHcRed1 vector (Clontech). Each chaperone gene fused with the HcRed1 gene was amplified by PCR, using the primers shown in Table I, and then inserted into the *Hind* III - *Eco*RI sites in pIB/His-V5. The resulting plasmids were named pIB/Bip-HcRed, pIB/ERp57-HcRed, pIB/CNX-HcRed, pIB/CRT-HcRed, and pIB/HSP70-HcRed.

2.3. *FACS and isolation of cell populations*

FACS and isolation of cell populations were performed using an EPICS ALTRA cell sorter (Beckman Coulter, Inc., Fullerton, CA, USA), equipped with an argon laser (488 nm) and a helium-neon laser (532 nm). GFP_{uv} and HcRed fluorescence were collected with 525 nm and 675 nm bandpass filters, respectively. The top 10% of cells with high-fluorescent intensity were sorted into a tube, using phosphate-buffered saline (PBS, pH 7.0) as a running buffer. The sorted cells were then washed with Express Five

95 medium and cultivated using a 24 deep well plate Bioshaker.

96 2.4. *Fluorescence microscopy*

97 Cell suspensions were collected from 4-6 day culture medium and immediately
98 observed using a confocal laser scanning microscope (TCS-LS, Leica Microsystem,
99 Heidelberg, Germany), equipped with argon (488 nm) and helium-neon (633 nm) lasers.

100 2.5. *SDS-PAGE*

101 To detect the expression of the recombinant proteins, cell supernatants and
102 lysates were subjected to SDS-PAGE on a 10 or 12% (w/v) polyacrylamide gel, using
103 the Mini-protean II system (Bio-Rad Hercules, CA, USA). The cell lysates were
104 prepared in lysis buffer (50 mM Tris-HCL, pH 8.0, 1% Triton X-100). For the detection
105 of the fluorescent GGT2 fusion protein on the SDS-PAGE gel, samples were suspended
106 in sample buffer (Aoki et al., 1996) and the fluorescent bands of the GGT2 fusion
107 protein were detected with a Molecular Imager FX (Bio-Rad).

108 2.6. *β 3GnT assay and protein concentration measurement*

The β 3GnT activity was measured in 50 mM Tris-HCl (pH 8.0), 15 mM MnCl_2 , 19 mM UDP-GlcNAc, 22 mM Gal β 1-4 GlcNAc β -pNP and 5 μ l enzyme solution (total volume 25 μ l). The reaction was started by the addition of the β 3GnT sample. At each sampling time, 5 μ l of the reaction mixture was added to 195 μ l of distilled water, and the solution was boiled for 5 min. After filtration with a 0.45 μ m nitrocellulose filter (Millipore, Bedford, Massachusetts, USA), the filtrates were analyzed by HPLC on a Mightysil RP-18 (H) GP 150 – 4.6 (Kanto Chem. Co. Inc.) column. The reaction products were eluted with 10% methanol and were detected at an absorbance of 300 nm. HPLC was performed at 40°C, with a flow rate of 1.0 ml per min. One unit of enzyme activity is defined as the amount of enzyme capable of catalyzing the transfer of 1 μ mol of GlcNAc per minute. Protein concentrations were determined by the Bradford method (Bio-Rad).

3. Results

3.1. Cultivation of insect cells using a 24-well deep plate bioshaker

To cultivate the cell populations rapidly isolated by FACS, a 24-well deep plate

Bioshaker was used for the suspension cultivation of Tn-5B1-4 cells. Shaking speeds and culture volumes were investigated. The cells precipitated when cultured in a shaker at 200 rpm in culture volumes of 1, 2, 4 and 8 ml, and the cells also did not grow at 600 rpm in culture volumes of 1 and 2 ml (data not shown). However, cell growth was confirmed at 400 rpm in culture volumes of 1 and 2 ml, but not in culture volumes of 4 and 8 ml (data not shown).

3.2. *Coexpression of GGT2 fusion protein with each chaperone*

Previously, Tn-5B1-4 cells stably expressing the GGT2 fusion protein (TnpXme11 cells) were isolated (Kato et al., 2004). A plasmid for the expression of each chaperone was constructed and then transfected into the TnpXme11 cells. In this study, five chaperones (Bip, ERp57, CNX, CRT, and HSP70) were studied, and HcRed was fused at the C-terminus of each chaperone. Bip, ERp57 and CRT each have an endoplasmic reticulum (ER) retention signal at the C-terminus. To maintain the three chaperones in the ER, the ER retention signal (-KDEL) was fused at the C-terminus of HcRed. The transfected cells were cultivated in the presence of 100 µg/ml Blasticidin

for three days. After 3 days of cultivation in a static culture, the cells were cultivated for 6 days in a suspension culture, using a 100-ml shaker flask with a working volume of 20 ml, and then fluorescence observations and β 3GnT assays were performed. The GFP_{uv} and HcRed fluorescence intensities were observed in TnpXme11 cells transfected with each plasmid for the expression of the chaperones (Data not shown). As compared to TnpXme11 cells, the intra- and extracellular β 3GnT activities increased in all chaperone expression plasmid-transfected cells (Fig. 1). The intracellular activity in TnpXme11 cells coexpressing Bip was enhanced by approximately 3.4-fold, as compared to that in TnpXme11 cells. The extracellular activity in TnpXme11 cells coexpressing CNX was approximately 2.9-fold higher than that in TnpXme11 cells.

3.3. Dual fluorescence analysis of chaperone-coexpressing cells sorted by FACS

TnpXme11 cells transfected with each plasmid for chaperone expression were analyzed by FACS. The GFP_{uv} fluorescence was increased in the chaperone-coexpressing cells (Fig. 2A, green areas). This increase in GFP_{uv} fluorescence corresponded to the increase in β 3GnT activity (Fig. 1). The HcRed

fluorescence derived from the expression of the chaperone was also increased in the chaperone-coexpressing cells (Fig. 2B, green areas). These results indicated that chaperone coexpression resulted in the increased expression of β 3GnT.

The top 10% of transformants with the highest GFP_{uv} fluorescence were separated by FACS sorting, and then the GFP_{uv} and HcRed fluorescence intensities of the sorted cell populations were analyzed, as shown Figs. 2A and B (red areas), respectively. The GFP_{uv} fluorescent intensity was higher in comparison with that of the parent cell line prior to FACS sorting, for all cell lines (Fig. 2A). However, the HcRed fluorescent intensity (chaperone-HcRed fusion protein) was similar or lower in comparison with that of the parent cell line prior to FACS sorting, for all cell lines (Fig. 2B). The data indicate that the sorted cell populations expressed a high level of GGT2, but a slightly lower level of each co-expressed chaperone.

3.4. Isolation of a cell line stably expressing a high level of GGT2 fusion protein by repeated cell sorting

The Tn-pXme11-hCNX6 cell line, which stably co-expresses GGT2 and h-CNX,

was isolated by the traditional transfection and isolation methods (Kato et al., 2005). This cell line produces around 20 and 8 mU/ml of β 3GnT activity in the culture supernatant and cells, respectively. This cell line was cultivated in a suspension culture for 4 days, and then the top 10% of the cell population with the highest GFP_{uv} fluorescence was isolated by FACS sorting (1st sorting). The separated cells were cultured in a 24 deep well plate Bioshaker in a culture volume of 2 ml for 4 days, with an agitation rate of 400 rpm. After cultivation in the 24 deep well plate Bioshaker, the cells were transferred into a 100 ml shaking flask with a culture volume of 20 ml, and were cultivated for 4 days at an agitation rate of 100 rpm. Again, the top 10% of the cell population with the highest GFP_{uv} fluorescence was isolated by FACS sorting (2nd sorting). After the second sorting, the separated cells were cultivated in the same manner as the first set of sorted cells, and then the GFP_{uv} fluorescence was analyzed.

Two cell lines, TnpXme11-hCNX6-1 and hCNX6-2, obtained from the 1st and 2nd sorting, respectively, were established and their protein expression was evaluated in suspension culture flasks. There was no difference in cell growth before and after cell sorting (Data not shown). The intracellular β 3GnT activities were 31.3 mU/ml

(TnpXme11-hCNX6-2), 27.3 mU/ml (TnpXme11-hCNX6-1) and 12.8 mU/ml (TnpXme11-hCNX6) (Fig. 3A). The maximum extracellular β 3GnT activity improved 2-fold in TnpXme11-hCNX6-2 and 1.8-fold in TnpXme11-hCNX6-1, as compared to before sorting (Table 2). The specific β 3GnT activity of TnpXme11-hCNX6-2 was 66.5 mU/mg protein, which was also 2-fold higher than that before sorting (data not shown). The fluorescent bands of the GGT2 fusion protein obtained from the TnpXme11-hCNX6-2 culture were denser than those before FACS sorting corresponding to the increase of β 3GnT activity, as revealed by SDS-PAGE (Fig. 3C, D). It indicates that GFP_{uv} fluorescence intensity of GGT2 is correlated with β 3GnT activity to a certain extent. Moreover, the amount of degraded GGT2 fusion protein in the culture supernatant of the final cell population (TnpXme11-hCNX6-2) was decreased, as compared to that of the TnpXme11-hCNX6 cells. Intracellular fluorescence intensity did not correspond to intracellular β 3GnT activity because of the accumulation of immature GGT2 fusion protein (Kato et al., 2003)

The protein expression ability of the TnpXme11-hCNX6-2 cell line was investigated through 35 passages, and then the intra- and extracellular β 3GnT activities

were assayed. The intra- and extracellular β 3GnT activities were maintained until 25 passages (Fig. 4). However, when the number of passages exceeded 30, both activities decreased.

4. Discussion

We have described the rapid isolation and selection of insect cell lines stably expressing high levels of recombinant proteins. The general protocol for the selection of stable insect cell lines involves transfection with expression vectors, followed by screening and isolation of antibiotic-resistant cell lines. The isolated cell lines were evaluated in a series of monolayer cultures in 96, 24, and 6 well plates and T flasks, and finally the best cell line was cultured in a suspension flask. All of these processes take at least 2-3 months (Fig. 5). This procedure for stably transformed cell isolation is slower than those of the bacterial and baculovirus expression systems. In this study, by using a fluorescent fusion protein and FACS, a stable cell line expressing a high level of recombinant protein was isolated in only two weeks (Fig. 5). Moreover, a series of sorting procedures enabled the isolation of a sub-population of cell lines with even

better expression, from the cell populations exhibiting high expression levels. This activity is approximately equal to that in the hemolymph of silkworms infected with BmNPV-GFP_{uv}-β3GnT2 (Park et al., 2007) (Table 2). This screening and isolation method is also applicable to the expression of transmembrane and intracellular proteins. This approach would be especially useful for the expression of transmembrane proteins, because their expression levels are higher in a stably transformed cell system than in the baculovirus expression system, and stably transformed cell lines can be maintained easily (McCarroll and King, 1997).

In this study, GFP_{uv}-fused protein was adopted as a protein of interest to detect recombinant protein expression easily and compare its expression level in the expression system described here with that in the others. As the FACS sorting criterion, the intracellular GFP fluorescence was used as a marker, because the GFP_{uv}-β3GnT2 fusion protein accumulates intracellularly when overexpressed in insect cells. In general, high accumulation levels of intracellular GFP_{uv}-β3GnT2 fusion protein lead to high extracellular secretion levels of the fusion protein. In general the fluorescence of GFP_{uv} is proportion to that of protein expression level, expect for intracellular degradation of

232 fusion protein. This work demonstrated that top 10% fluorescence increased 2-fold
233 higher expression of fusion protein. On the other hand, when fusion protein is not
234 desirable for target protein, coexpression of target protein and fluorescent reporter can
235 be applicable to this FACS method, but whether target protein level is correlated with
236 reporter fluorescent intensity is case by case.

237 The coexpression of chaperones has been performed in various expression systems,
238 and allows high expression levels of recombinant proteins (Hsu and Betenbaugh, 1997;
239 Kato et al., 2005; Tate et al., 1999). In a transformed cell system using insect cells, it
240 takes a long time to coexpress recombinant proteins and chaperones, because multiple
241 transfection and isolation processes are needed to insert more than one expression vector.
242 The cell screening and isolation method by FACS described in this report can facilitate
243 the evaluation of the effects of chaperone coexpression on recombinant protein
244 production and the isolation of high-producing cell populations. The fusion of the
245 recombinant protein with a fluorescent protein (GFP_{uv}) enables the cell screening and
246 isolation method by FACS described in this article. In other reports, the fluorescent
247 protein and the recombinant protein were expressed separately, using an internal

248 ribosomal entry site (IRES) in mammalian cells, and cell lines with high recombinant
249 protein production were isolated (Mancia et al., 2004). The expression level of each
250 chaperone (after sorting) in the high-level expression cell population was lower than
251 that before sorting. This indicates that chaperone overexpression is not necessary for the
252 enhancement of recombinant protein production. Indeed, in silkworm larvae, the
253 expression of CNX or Bip under the control of the ie-2 promoter, which has lower
254 activity than that of the polyhedrin promoter, is sufficient to enhance the production of
255 the GFP_{uv}- α 1,4-*N*-acetylglucosaminyltransferase fusion protein (Nakajima et al., 2009).
256 Various chaperones with moderate expression levels exist in the ER, and together, they
257 assist with protein folding. The coexpression of HAC1, which is an unfolded protein
258 response (UPR) pathway regulator that controls the expression of several ER-resident
259 chaperones and foldases, enhanced the production of recombinant proteins in yeast
260 (Gasser et al., 2006). In mammalian cells, the overexpression of XBP-1, which is a
261 mammalian ortholog of HAC1 that activates targets with an ER stress element (ERSE)
262 and a UPR element (UPRE), enhanced the secretion of interferon γ , erythropoietin,
263 human monoclonal antibody, secreted alkaline phosphatase, and human vascular

endothelial growth factor 122 (Ku et al., 2008; Ohya et al., 2008; Tigges and Fussenegger, 2006). These results suggested that a moderate increase in the expression of multiple chaperones in the ER could lead to the enhancement of recombinant protein production. However, these results are not applicable in general (Ohya et al., 2008).

Many factors that enhance the production of recombinant proteins have been found (Khan and Schroder, 2008; Ku et al., 2009; van Anken and Braakman, 2005). FACS is quite useful for the high-throughput selection, cell screening and isolation methods; moreover, the combination of FACS with the coexpression of other factors (chaperones, subunits, cofactors, transcriptional regulators) facilitates the efficient and time-saving screening of effective factors along with the rapid isolation of cell populations producing high levels of recombinant proteins.

5. Conclusion

In this report, we isolated stably transformed insect cells and analyzed the expression levels of recombinant proteins, using FACS and a 24 deep well plate Bioshaker, within two weeks. Moreover, repeated sorting of cell populations with high expression levels

allowed the isolation of populations with even better expression levels than those before cell sorting. This method is a breakthrough for the rapid screening of stable cell lines from DNA-transformed insect cells, and will also be applicable to the isolation of transformed mammalian cells expressing high levels of recombinant proteins.

References

- Altmann, F., Staudacher, E., Wilson, I.B., Marz, L., 1999. Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj. J.* 16, 109-123.
- Aoki, T., Takahashi, Y., Koch, K.S., Leffert, H.L., Watabe, H., 1996. Construction of a fusion protein between protein A and green fluorescent protein and its application to western blotting. *FEBS Lett.* 384, 193-197.
- Douris, V., Swevers, L., Labropoulou, V., Andronopoulou, E., Georgoussi, Z., Iatrou, K., 2006. Stably transformed insect cell lines: tools for expression of secreted and membrane-anchored proteins and high-throughput screening platforms for drug and insecticide discovery. *Adv. Vir. Res.* 68, 113-156.
- Gasser, B., Maurer, M., Gach, J., Kunert, R., Mattanovich, D., 2006. Engineering of

294 *Pichia pastoris* for improved production of antibody fragments. Biotechnol.
 295 Bioeng. 94, 353-361.

296 Hsu, T.A., Betenbaugh, M.J., 1997. Coexpression of molecular chaperone BiP improves
 297 immunoglobulin solubility and IgG secretion from *Trichoplusia ni* insect cells.
 298 Biotechnol. Prog. 13, 96-104.

299 Kato, T., Murata, T., Usui, T., Park, E.Y., 2003. Improvement of GFP_{uv}-beta 3GnT2
 300 fusion protein production by suppressing protease in baculovirus expression
 301 system. Biosci. Biotechnol. Biochem. 67, 2388-2395.

302 Kato, T., Murata, T., Usui, T., Park, E.Y., 2004. Comparative analysis of
 303 GFP_{uv}-beta1,3-N-acetylglucosaminyltransferase 2 production in two
 304 insect-cell-based expression systems. Protein Exp. Purif. 35, 54-61.

305 Kato, T., Murata, T., Usui, T., Park, E.Y., 2005. Improvement of the production of
 306 GFP_{uv}-beta1,3-N-acetylglucosaminyltransferase 2 fusion protein using a molecular
 307 chaperone-assisted insect-cell-based expression system. Biotechnol. Bioeng. 89,
 308 424-433.

309 Khan, S.U., Schroder, M., 2008. Engineering of chaperone systems and of the unfolded

310 protein response. Cytotechnol. 57, 207-231.

311 Ku, S.C., Ng, D.T., Yap, M.G., Chao, S.H., 2008. Effects of overexpression of X-box
 312 binding protein 1 on recombinant protein production in Chinese hamster ovary and
 313 NS0 myeloma cells. Biotechnol. Bioeng. 99, 155-164.

314 Ku, S.C., Lwa, T.R., Giam, M., Yap, M.G., Chao, S.H., 2009. Identification of HUGT1
 315 as a potential BiP activator and a cellular target for improvement of recombinant
 316 protein production using a cDNA screening system. Mol. Cells 27, 577-582.

317 Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., 1993. Efficient generation of
 318 infectious recombinant baculoviruses by site-specific transposon-mediated
 319 insertion of foreign genes into a baculovirus genome propagated in *Escherichia*
 320 *coli*. J. Virol. 67, 4566-4579.

321 Mancia, F., Patel, S.D., Rajala, M.W., Scherer, P.E., Nemes, A., Schieren, I.,
 322 Hendrickson, W.A., Shapiro, L.. 2004. Optimization of protein production in
 323 mammalian cells with a coexpressed fluorescent marker. Structure 12, 1355-1360.

324 McCarroll, L., King, L.A., 1997. Stable insect cell cultures for recombinant protein
 325 production. Curr. Opin. Biotechnol. 8, 590-594.

326 Motohashi, T., Shimojima, T., Fukagawa, T., Maenaka, K., Park, E.Y., 2005. Efficient
 327 large-scale protein production of larvae and pupae of silkworm by *Bombyx mori*
 328 nuclear polyhedrosis virus bacmid system. Biochem. Biophys. Res. Commun. 326,
 329 564-569.

330 Nakajima, M., Kato, T., Kanamasa, S., Park, E.Y., 2009. Molecular chaperone-assisted
 331 production of human alpha-1,4-*N*-acetylglucosaminyltransferase in silkworm
 332 larvae using recombinant BmNPV bacmids. Mol. Biotechnol. 43, 67-75.

333 Ohya, T., Hayashi, T., Kiyama, E., Nishii, H., Miki, H., Kobayashi, K., Honda, K.,
 334 Omasa, T., Ohtake, H., 2008. Improved production of recombinant human
 335 antithrombin III in Chinese hamster ovary cells by ATF4 overexpression.
 336 Biotechnol. Bioeng. 100, 317-324.

337 Park, E.Y., Kageshima, A., Kwon, M.S., Kato, T., 2007. Enhanced production of
 338 secretory beta1,3-*N*-acetylglucosaminyltransferase 2 fusion protein into
 339 hemolymph of *Bombyx mori* larvae using recombinant BmNPV bacmid integrated
 340 signal sequence. J. Biotechnol. 129, 681-688.

341 Rieseberg, M., Kasper, C., Reardon, K.F., Scheper, T., 2001. Flow cytometry in

342 biotechnology. *Appl. Microbiol. Biotechnol.* 56, 350-360.

343 Sleiman, R.J., Gray, P.P., McCall, M.N., Codamo, J., Sunstrom, N.A., 2008. Accelerated
344 cell line development using two-color fluorescence activated cell sorting to select
345 highly expressing antibody-producing clones. *Biotechnol. Bioeng.* 99, 578-587.

346 Tate, C.G., Whiteley, E., Betenbaugh, M.J., 1999. Molecular chaperones improve
347 functional expression of the serotonin (5-hydroxytryptamine) transporter in insect
348 cells. *Biochem. Soc. Trans.* 27, 932-936.

349 Tigges, M., Fussenegger, M., 2006. Xbp1-based engineering of secretory capacity
350 enhances the productivity of Chinese hamster ovary cells. *Metab. Eng.* 8, 264-272.

351 van Anken, E., Braakman, I., 2005. Endoplasmic reticulum stress and the making of a
352 professional secretory cell. *Crit. Rev. Biochem. Mol. Biol.* 40, 269-283.

353 Yuk, I.H., Wildt, S., Jolicoeur, M., Wang, D.I., Stephanopoulos, G., 2002. A GFP-based
354 screen for growth-arrested, recombinant protein-producing cells. *Biotechnol.*
355 *Bioeng.* 79, 74-82.

356

Table 1

Primers used in this study

	5' – 3'
Bip(F)	TATGTCGAC ATGAAGCTCTCCCTGGTGGCCGCGATG
Bip(R)	TATGTCGACCTACAACCTCATCTTTTCTGCAGTATCCTCTTCACC
CNX(F)	TATGTCGACATGGAAGGGAAGTGGTTGCTGTGTATGTTA
CNX(R)	TATGTCGACCTACAGCTCGTCCTTGGCCTGGCCGGGGAC
ERp57(F)	TATGTCGACATGCGCCTCCGCCGCCTAGCGCTGTTC
ERp57(R)	TATGTCGACTTAGAGATCCTCCTGTGCCTTCTTCTTCTTCTTGGG
Hsp70(F)	TATGTCGACATGGCCAAAGCCGCGGCGATCGGCATC
Hsp70(R)	TATGTCGACCTAATCTACCTCCTCAATGGTGGGGCCTGACCCAGA
Red(R)	TATAAGCTTCTACAGCTCGTCCTTGTTGGCCTTCTCGGGCAGGTCGCT
Red(Hsp-R)	TATAAGCTTTCAGTTGGCCTTCTCGGGCAGGTCGCT

360 **Table 2**

361 Extracellular β 3GnT activity in each expression system

	Baculoviruses	Hosts	Activity (mU/ml)	References
Baculovirus	rBmNPV/GFPuv- β 3GnT2	Tn-5B1-4 (+leupeptin)	2.09	Kato et al., 2003
expression system	rBmNPV/GFPuv- β 3GnT2	Silkworm	38.3	Park et al., 2007
	BmNPV/GFPuv- β 3GnT2 bacmid	Silkworm	56.1	Park et al., 2007
Stable transformed		TnpXme11	6.78	Kato et al., 2004
cell system		TnpXme11-hCNX6 (before sorting)	22.4	Kato et al., 2005
		TnpXme11-hCNX6-1 (1 st sorting)	43.1.	This study
		TnpXme11-hCNX6-2 (2 nd sorting)	46.4	This study

Figure legends

Fig. 1. Intracellular (A) and extracellular (B) β 3GnT activity in TnpXme11 cells

transfected with a plasmid for the expression of each chaperone. Transfected cells were cultivated for 6 days, and then the culture medium was centrifuged. Culture supernatants were recovered as extracellular fractions. Cells were washed with 50 mM Tris-HCl buffer (pH 8.0) and suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X-100. These suspensions were used as intracellular fractions. Error bars denote standard deviation (n=3).

Fig. 2. FACS analysis of GFP_{uv} fluorescence (A) and HcRed fluorescence (B) of

TnpXme11 cells transfected with a plasmid for the expression of each chaperone before sorting (Green areas) and after sorting (Red areas). TnpXme11 cells were transfected with a plasmid for the expression of each chaperone, and were cultivated for 3 days. Cells were analyzed by FACS and sorted. Sorted cells were cultivated using a 24 well deep plate Bioshaker and a shaking flask, and were re-analyzed by FACS. Gray areas indicate the GFP_{uv} fluorescence of TnpXme11 cells.

Fig. 3. Intracellular (A) and extracellular (B) β 3GnT activities of TnpXme11-hCNX6 (open triangles), TnpXme11-hCNX6-1 (open circles), and TnpXme11-hCNX6-2 (closed circles) cell cultures. GFP_{uv} fluorescence analysis of the intracellular (C) and extracellular (D) GGT2 of the TnpXme11-hCNX6-2 cell culture on SDS-PAGE gels. Intracellular and extracellular samples were prepared by the same methods as in Fig. 1, and were separated on a 12% SDS-PAGE gel. GGT2 bands were detected with a Molecular Imager FX (Bio-Rad). Error bars in (A) and (B) denote standard deviation (n=3). Arrows indicate full-length GGT2 fusion protein.

Fig. 4. Intracellular (A) and extracellular (B) β 3GnT activities during 35 passages. TnpXme11-hCNX6-2 cells were cultured, and cells after every 5 passages were cultivated using a shaking flask for 4 (open bars), 5 (grey bars) and 6 (black bars) days. Intracellular and extracellular fractions were prepared by the same methods as in Fig. 1, and the β 3GnT activity of each fraction was assayed. Error bars denote standard deviation (n=3).

Fig. 5. Schematic diagram of the isolation of insect cell lines stably expressing high levels of recombinant proteins, by the traditional method and the method described in

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