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Efficient production of antibody Fab fragment by transient gene expression in insect cells

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[**Key words:** Insect cell; Transient gene expression; Recombinant protein production; Fab fragment; High Five cell]

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Abstract

Transient gene expression allows a rapid production of diverse recombinant proteins in early-stage preclinical and clinical developments of biologics. Insect cells have proven to be an excellent platform for the production of functional recombinant proteins. In the present study, the production of an antibody Fab fragment by transient gene expression in lepidopteran insect cells was investigated. The DNA fragments encoding heavy-chain (Hc; Fd fragment) and light-chain (Lc) genes of an Fab fragment were individually cloned into the plasmid vector pIHAneo, which contained the *Bombyx mori* actin promoter downstream of the *B. mori* nucleopolyhedrovirus (BmNPV) IE-1 transactivator and the BmNPV HR3 enhancer for high-level expression. *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells were co-transfected with the resultant plasmid vectors using linear polyethyleneimine. When the transfection efficiency was evaluated, a plasmid vector encoding an enhanced green fluorescent protein (EGFP) gene was also co-transfected. Transfection and culture conditions were optimized based on both the flow cytometry of the EGFP expression in transfected cells and the yield of the secreted Fab fragments determined by enzyme-linked immunosorbent assay (ELISA). Under optimal conditions, a yield of approximately 120 mg/L of Fab fragments was achieved in 5 days in a shake-flask culture. Transient gene expression in insect cells may offer a promising approach to the high-throughput production of recombinant proteins.

Introduction

Currently, novel biologics that include therapeutic antibodies are typically selected from a large pool of lead candidate proteins during early-stage preclinical and clinical developments. This type of selection requires high-throughput production of a large number of recombinant candidate proteins in sufficient quantity and quality. Transient gene expression using plasmid vectors provides rapid production of recombinant proteins, and has recently been used for the production of diverse lead proteins for the early-stage preclinical and clinical developments of biologics. Mammalian cells such as human embryonic kidney 293 cells and CHO cells commonly serve as the host cells for transient gene expression due to the requirement for post-translational processing and modifications and the complexity of target proteins (1–3). Insect cells have recently been recognized as an excellent platform for the production of biologically active recombinant proteins (4–6). Insect cells are easier to be cultivated than mammalian cells, and can be grown to a high cell density in suspension with a serum-free medium. They can also produce considerable amounts of recombinant proteins through post-translational processing and modifications that are similar to those performed in mammalian cells (4). While insect cells have been widely used in the baculovirus–insect cell system for recombinant protein production (4–8), only a few studies have reported transient gene expression using insect cells as host cells (9–11).

In the present study, the production of an antibody Fab fragment by transient gene expression in lepidopteran insect cells was investigated. An inexpensive transfection reagent, polyethyleneimine (PEI), was used to co-transfect *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells with plasmid vectors separately carrying the heavy-chain (Hc) and light-chain (Lc) genes of the Fab fragment. Factors that affect the expression levels of

Fab fragments, such as the Hc/Lc gene ratio, the DNA:PEI ratio, and culture conditions, were optimized by evaluating the transfection efficiency and the yield of secreted Fab fragments. Under optimal conditions, a high volumetric productivity of approximately 120 mg/L of Fab fragments was achieved within 5 days in a shake-flask culture.

MATERIALS AND METHODS

Insect cell line and culture media The lepidopteran insect cell line *Trichoplusia ni* BTI-TN-5B1-4 (High Five; Thermo Fisher Scientific, Waltham, MA, USA) was used in the present study. The cells were maintained at 27 °C in T-flasks in a non-humidified incubator (12). The culture medium used for routine maintenance was Express Five serum-free medium (Thermo Fisher Scientific), supplemented with 16.5 mM L-glutamine and 10 mg/L gentamicin. The cells were adapted to serum-free media that included COSMEDIUM 009 (COSMO BIO, Tokyo, Japan) and PSFM-J1 (Wako Pure Chemical Industries, Osaka, Japan), both of which were supplemented with 10 mg/L gentamicin. Cell density was determined by microscopically counting the number of cells with a Bürker-Türk hemocytometer, while cell viability was judged by trypan blue dye exclusion (12).

Plasmid vectors The transient expression of the Fab fragment of 3A21 mouse anti-bovine RNaseA (13) was examined in the present study. The plasmids containing the Hc and Lc genes of the 3A21 Fab fragment were kindly provided by Dr. Y. Kumada of the Kyoto Institute of Technology. The DNA encoding the *Drosophila* immunoglobulin heavy chain binding protein (BiP) signal peptide and the Hc or Lc gene of the 3A21 Fab fragment was amplified via PCR from the plasmids using primers

including the BiP signal sequence (14). The amplified DNA fragments were separately cloned into the plasmid pIHAneo (14) between the *Sac*I and *Xba*I sites for the Hc gene and the *Xba*I and *Sac*II sites for the Lc gene to give pIHAneo/Hc and pIHAneo/Lc, respectively. The pIHAneo utilizes the *Bombyx mori* cytoplasmic actin promoter, from which foreign gene expression is remarkably stimulated with the *B. mori* nucleopolyhedrovirus (BmNPV) IE-1 transactivator and the BmNPV HR3 enhancer for high-level expression (14,15).

To determine transfection efficiency, the plasmid pXINSECT-EGFP was also co-transfected. This plasmid carries the enhanced green fluorescent protein (EGFP) gene downstream of the BmNPV IE-1 transactivator, the BmNPV HR3 enhancer, and the *B. mori* actin promoter.

Transfection and cell culture Linear PEI (Mw 40,000; Polysciences, Warrington, PA, USA) was used as a transfection agent. PEI was prepared at a concentration of 1 g/L in 150 mM NaCl, pH 7.0 and sterilized by filtration through a 0.20 μm membrane filter.

In a static culture, High Five cells in the exponential growth phase were inoculated into 6-well plates with 2 ml of fresh Express Five medium at a cell density of 2×10^5 cells/cm^3 1 h before transfection. Per 10^5 cells, 1 μg of plasmids (pIHAneo/Hc and pIHAneo/Lc:pXINSECT EGFP = 9:1 (w/w)) and 2 μg PEI were prepared in 100 μl of 150 mM NaCl, pH 7.0 and incubated at room temperature for 5 min. The DNA/PEI complex was added to the cells, and the cells were statically incubated at 27 °C. At the appropriate time, culture supernatants were separated from the cell suspensions via centrifugation and stored at −20 °C for subsequent analyses.

In a shake-flask culture, cells in the exponential growth phase were suspended at a predetermined cell density in fresh medium. Fifteen ml of the cell suspension was transferred into a 100-ml screw-capped Erlenmeyer flask. Various amounts of plasmids and DNA were prepared in 300 μl of 150 mM NaCl, pH 7.0, and incubated at room temperature for 5 min. The DNA/PEI complex was added to the cells, which were then incubated at a predetermined temperature on a rotary shaker (90 rpm). At the appropriate time, aliquots of the cell suspension were sampled to the measure the cell density, and the culture supernatants were separated via centrifugation and stored at −20 °C for subsequent analyses.

Analyses Culture supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel under non-reducing conditions, followed by transfer to a poly(vinylidene difluoride) (PVDF) membrane. Immunoreactive proteins were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (H + L) (Promega, Madison, WI, USA), which was polyclonal antibody binding to both Hc and Lc of mouse IgG, and stained using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Promega).

The concentration of the Fab fragments secreted into the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). The anti-RNaseA Fab fragment was purified from the culture supernatant with NHS-activated Sepharose 4 (GE Healthcare, Little Chalfont, UK) coupled with bovine RNaseA according to a recommended protocol, and was used as a standard. ELISA plates were coated with bovine RNaseA as the antigen, and peroxidase-conjugated goat anti-mouse IgG (Exalpha Biologicals, Watertown, MA, USA) was used. The detections were carried out using the ELISA POD substrate TMB kit (Nacalai Tesque, Kyoto, Japan) according to the

manufacturer's protocol. The absorbance for each well was measured at 405 nm using a microplate reader and then converted to the Fab concentration by interpolating the value on a standard curve.

On day 2 after transfection, the cell suspension was removed, and the numbers of green fluorescent cells and total cells were determined using a flow cytometer (Guava easyCyte 5HT, Merck Millipore, Darmstadt, Germany) equipped with a blue laser with excitation at 488 nm. Fluorescence emission was collected using a 525/30 band-pass filter.

RESULTS AND DISCUSSION

Effect of Hc/Lc gene ratio on Fab production High Five cells inoculated into 6-well plates with a serum-free medium Express Five were co-transfected using various ratios of pIHAneo/Hc to pIHAneo/Lc. At 72 h post-transfection, the culture supernatants were analyzed by western blotting under non-reducing conditions. Specific protein bands were detected at an electrophoretic mobility of approximately 50 kDa in the culture supernatants of the cells transfected with both the Hc and Lc genes at Hc:Lc gene ratios (w/w) ranging from 9:1 to 1:9 (Fig. 1a). The electrophoretic mobility coincided with the molecular weight of the 3A21 Fab fragment. Specific protein bands at approximately 25 kDa were observed not only in the culture supernatants of cells transfected with both the Hc and Lc genes, but also in the supernatant of the cells transfected with the Lc gene alone (0:10). This result indicates that the Lc monomer was secreted alone from High Five cells. Specific bands coinciding with the Lc homodimer were also detected in the culture supernatants of both the cells transfected with the Lc gene alone and those transfected with both the Hc and Lc genes. In contrast,

← Fig. 1

no specific band was identified in the culture supernatant of the cells transfected with the Hc gene alone (10:0), suggesting that High Five cells did not secrete Hc alone. These observations are consistent with those reported for mammalian cells (16). Samples of the culture supernatant of transfected High Five cells were also analyzed by ELISA (Fig. 1b). When the cells were transfected with both the Hc and Lc genes, strong ELISA signals were confirmed. The results of western blotting and ELISA suggest that the transfected High Five cells secreted 3A21 Fab fragments with antigen-binding activity. The results also show that High Five cells transfected with a relatively low Hc/Lc gene ratio (4:6 to 2:8) efficiently secreted Fab fragments. This trend is slightly different from that reported for mammalian cells (16).

High Five cells were adapted to different serum-free media, COSMEDIUM 009 and PSFM-J1. Each of the adapted cells were co-transfected with pIHAneo/Hc and pIHAneo/Lc at a ratio (w/w) of 1:1 and incubated in a static culture with a corresponding serum-free medium. After 6 days, the Fab fragment concentration in each culture supernatant was measured by ELISA. A higher level of Fab fragment productivity was obtained from cells that had been adapted to PSFM-J1 (56 mg/L) than cells adapted to COSMEDIUM 009 (39 mg/L) and Express Five (43 mg/L). Therefore, PSFM-J1 was selected for use in further experiments.

Optimization of transfection and culture conditions in a shake-flask culture

Compared with a static culture, a shake-flask culture generally leads to higher levels of cell density and product yield. High Five cells were inoculated in Erlenmeyer flasks with PSFM-J1, and the cells were transfected with 0.5 μg DNA/(10^5 cells) (pIHAneo/Hc:pIHAneo/Lc:pXINSECT-DEST38-EGFP = 27:63:10) at different DNA:PEI ratios (w/w). At 48 h post-transfection, the cells were analyzed via flow

cytometry. The culture supernatant was analyzed via ELISA at 24 and 72 h post-transfection (Fig. 2). The transfection efficiency was defined as the percentage of EGFP-positive cells to total cells. When cells were transfected at a DNA:PEI ratio of 1:0.5, the transfection efficiency was almost 0%. The transfection efficiency increased with a decrease in the DNA:PEI ratio, but the increase was insignificant at ratios of 1:2 and below (Fig. 2a). A similar trend was observed with the Fab fragment concentration in the culture supernatant (Fig. 2b). Since the positive electric charge of PEI negates the negative charge of DNA, DNA/PEI complexes with a positive charge may integrate easily into cells via endocytosis.

← Fig. 2

Next, cells were transfected with varying amounts of DNA at a DNA:PEI ratio (w/w) of 1:2 (Fig. 3). Although the maximum transfection efficiency was obtained when cells were transfected with 1.0 μg DNA/(10^5 cells) (Fig. 3a), the maximum Fab fragment yield was achieved with 0.5 μg DNA per 10^5 cells (Fig. 3b).

← Fig. 3

To investigate the effects of culture temperature, cells were transfected with 0.5 μg DNA (pIHAneo/Hc:pIHAneo/Lc:pXINSECT-DEST38-EGFP = 27:63:10) using 1.0 μg/(10^5 cells) PEI and cultured at 24, 27, and 30 °C. The transfection efficiency was determined on day 2 post-transfection, and no significant differences in the transfection efficiency were observed among all temperatures (data not shown). Fig. 4 shows the time course of viable cell density and Fab fragment concentration. At 27 °C, 1 μg PEI per 10^5 cells did not significantly inhibit the growth of High Five cells by comparison with untransfected cells (Fig. 4a). Maximum cell density reached more than 4×10^5 cells/cm^3 within 6 days with the exception of cells cultured at 24 °C. The viability remained high (> 95%) at all temperatures until day 6 post-transfection (data not shown). The specific growth rate of transfected cells at the exponential growth phase increased with temperature. The Fab fragment concentration increased in an almost linear fashion

← Fig. 4

from day 2 to day 5 (Fig. 4b). As the culture temperature decreased, the Fab fragment concentration increased along with the specific production rate. Mild hypothermia improved the recombinant protein production in CHO cells, and an increase in yield was correlated with a block of cells in the G1 phase of the cell cycle, an increase in cell size, higher steady-state levels of mRNA, reduced consumption of nutrients, and decreased accumulation of inhibitory waste products (17). Improvement in the Fab production by High Five cells at 24 °C might have been the result of the same developments.

Finally, the effects of initial cell density were evaluated. Cells inoculated at a density of 2–20 × 10^5 cells/cm^3 in Erlenmeyer flasks were transfected with 0.5 μg/(10^5 cells) DNA (pIHAneo/Hc:pIHAneo/Lc:pXINSECT-DEST38-EGFP = 27:63:10) using 1.0 μg /(10^5 cells) PEI and cultured at 27 °C. The transfection efficiencies determined on day 2 post-transfection approximated at all the initial cell densities (data not shown). Fig. 5 shows the time course change in viable cell density and Fab fragment concentration. ← Fig. 5 The maximum cell density increased with the initial cell density, but the period of the exponential growth phase decreased (Fig. 5a). The highest Fab fragment concentration was obtained when the initial cell density was 1 × 10^6 cells/cm^3 (Fig. 5b), while the specific productivity decreased with the initial cell density.

Fab fragment production under optimal conditions High Five cells were transfected and cultured under the optimized conditions determined above. Cells were inoculated into Erlenmeyer flasks with 15 ml of PSFM-J1 at a cell density of 1 × 10^6 cells/cm^3, transfected with 0.5 μg/(10^5 cells) DNA (pIHAneo/Hc:pIHAneo Lc = 3:7) using 1 μg/(10^5 cells) PEI, and maintained at 24 °C in a shake-flask culture. Fig. 6 ← Fig. 6 shows the time course of viable cell density and Fab fragment concentration. Again, transfection did not significantly inhibit the growth of High Five cells as compared with

untransfected cells (Fig. 6a). The Fab fragment concentration reached approximately 120 μg/ml on day 5 post-transfection and then decreased on day 7 (Fig. 6b). Consequently, under optimal conditions, a high yield of approximately 120 mg/L of Fab fragments was obtained within 5 days in a serum-free shake-flask culture.

It is difficult to compare the efficiency of transient gene expression systems since the volumetric productivity through transient gene expression depends considerably on the target recombinant protein. Reportedly, the volumetric productivity of a recombinant protein in transient gene expression using mammalian 293 and CHO cells in a shaking or bioreactor culture ranges from 1 to 80 mg/L (1). The volumetric productivity in transient expression in a shaking culture of insect cells, such as High Five, *Spodoptera frugiperda* Sf9, and *Drosophila melanogaster* Schneider 2 cells, varies from 0.3 to 100 mg/L (9–11). In the present study, optimized protocols were developed for transient gene expression in High Five cells with PEI, which allowed a high yield of approximately 120 mg/L of Fab fragments after 5 days in a serum-free shake-flask culture. Therefore, transient gene expression in insect cells may be an efficient way for the high-throughput production of recombinant proteins, which include antibody molecules.

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Figure captions

FIG. 1. Effect of the ratio of the heavy chain (Hc) gene to the light chain (Lc) gene on the transient expression of Fab fragments of 3A21 mouse anti-bovine RNase A in High Five cells. Cells at a density of 2×10^5 cells/cm^3 were transfected with 1 μg/(10^5 cells) of plasmids at different ratios of pIHAneo/Hc to pIHAneo/Lc (w/w) using 2 μg/(10^5 cells) of polyethyleneimine (PEI) in Express Five serum-free medium. Transfected cells were incubated at 27 °C for 3 days in a static culture. Western blot analysis (a) and enzyme-linked immunosorbent assay (ELISA) (b) of the culture supernatants. Bars represent the means ± S.D. obtained from three independent experiments. 0:0, untransfected cells.

FIG. 2. Effect of the DNA:PEI ratio. High Five cells at a density of 2×10^5 cells/cm^3 were transfected with 0.5 μg/(10^5 cells) of DNA (27% pIHAneo/Hc, 63% pIHAneo/Lc, and 10% pXINSECT-DEST38-EGFP) at different ratios of DNA to PEI (w/w) in PSFM-J1. Transfected cells were incubated at 27 °C in a shake-flask culture. The percentages of enhanced green fluorescent protein (EGFP)-positive cells were measured by flow cytometry on day 2 post-transfection (a). The Fab fragments in the culture supernatants on days 2 and 5 post-transfection were measured by ELISA (b). Bars represent the means ± S.D. obtained from three independent experiments.

FIG. 3. Effect of DNA amount. Cells at a density of 2×10^5 cells/cm^3 were transfected with different amounts of DNA (27% pIHAneo/Hc, 63% pIHAneo/Lc, and 10% pXINSECT-DEST38-EGFP) using PEI at a ratio of DNA to PEI (w/w) of 1:2 in PSFM-J1. Transfected cells were incubated at 27 °C in a shake-flask culture. The percentage of EGFP-positive cells was measured by flow cytometry on day 2 post-transfection (a).

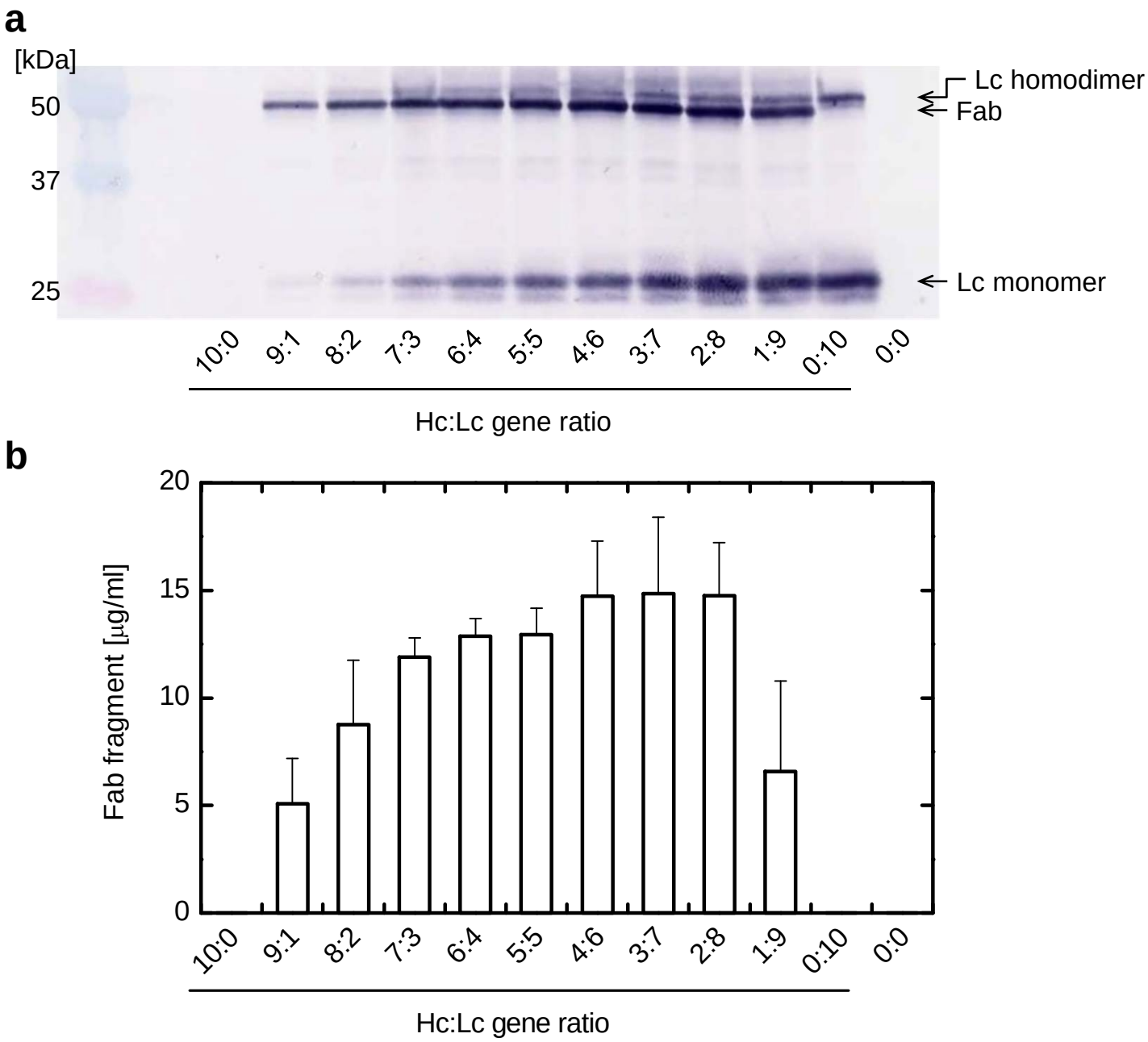
The Fab fragments in the culture supernatants on days 2 and 5 post-transfection were measured by ELISA (b). Bars represent the means ± S.D. obtained from three independent experiments.

FIG. 4. Effect of temperature. Cells at a density of 2×10^5 cells/cm^3 were transfected with 0.5 μg/(10^5 cells) of DNA (27% pIHAneo/Hc, 63% pIHAneo/Lc, and 10% pXINSECT-DEST38-EGFP) using 1 μg/(10^5 cells) of PEI in PSFM-J1. Transfected cells were incubated in a shake-flask culture at 24 (triangles), 27 (open circles), and 30 (squares) °C. (a) Density of viable cells. Growth of untransfected cells (closed circles) at 27 °C is also shown. (b) Concentration of Fab fragments in the culture supernatant. Bars represent the means ± S.D. obtained from three independent experiments.

FIG. 5. Effect of initial cell density. Cells at a density of 2×10^5 (circles), 5×10^5 (triangles), 1×10^6 (squares), and 2×10^6 (diamonds) cells/cm^3 were transfected with 0.5 μg/(10^5 cells) of DNA (27% pIHAneo/Hc, 63% pIHAneo/Lc, and 10% pXINSECT-DEST38-EGFP) using 1 μg/(10^5 cells) of PEI in PSFM-J1. Transfected cells were incubated in a shake-flask culture at 27 °C. (a) Density of viable cells. (b) Concentration of Fab fragments in the culture supernatant. Bars represent the means ± S.D. obtained from eight (a) or three (b) different determinations.

FIG. 6. Cell growth and Fab fragment production under optimized conditions. Cells at a density of 1×10^6 cells/cm^3 were transfected with 0.5 μg/(10^5 cells) of DNA (30% pIHAneo/Hc and 70% pIHAneo/Lc) using 1 μg/(10^5 cells) of PEI in PSFM-J1. Transfected cells were incubated at 24 °C in a shake-flask culture. (a) Density of viable cells. Growth of untransfected cells (closed circles) is also shown. (b) Concentration

of Fab fragments in the culture supernatant. Bars represent the means ± S.D. obtained from eight (a) or three (b) different determinations.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37
25
Lc homodimer
Fab
Lc monomer
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1:9
0:10
0:0
Hc:Lc gene ratio
b
Fab fragment [μg/ml]
20
15
10
5
0
10:0
9:1
8:2
7:3
6:4
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1:9
0:10
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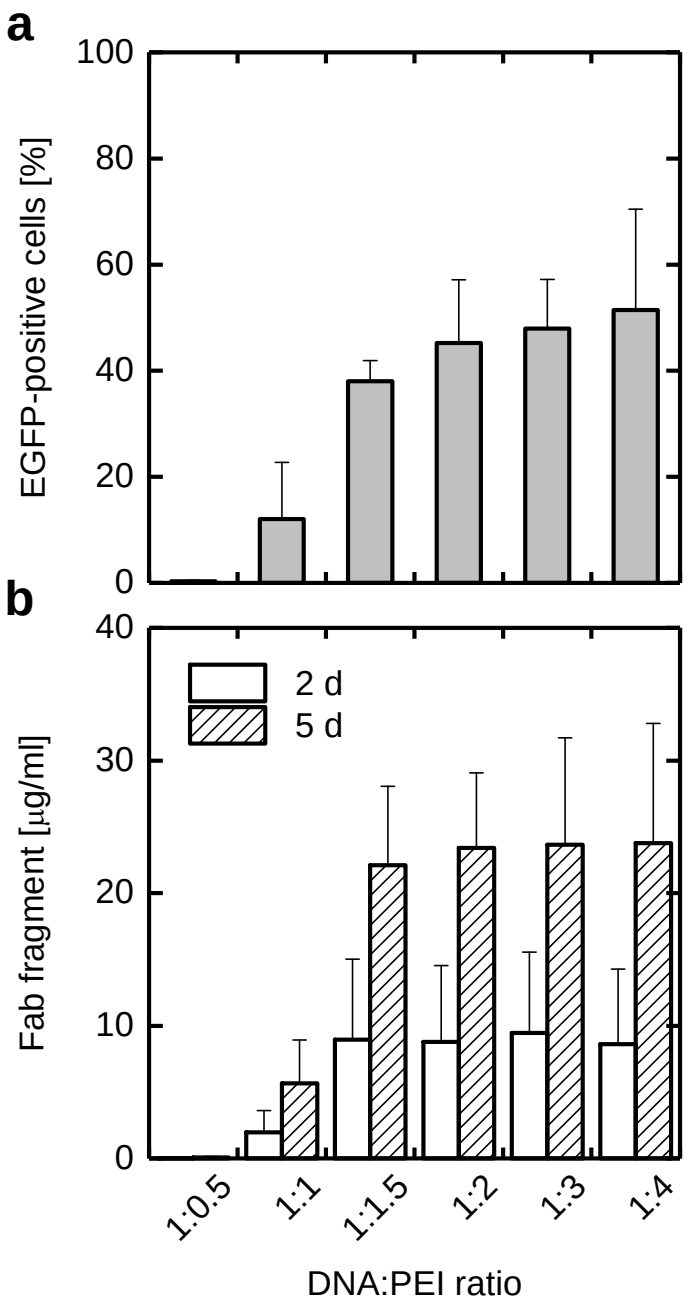
Hc:Lc gene ratio

Fig. 1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0
EGFP-positive cells [%]
b
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Fab fragment [μg/ml]
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1:1.5
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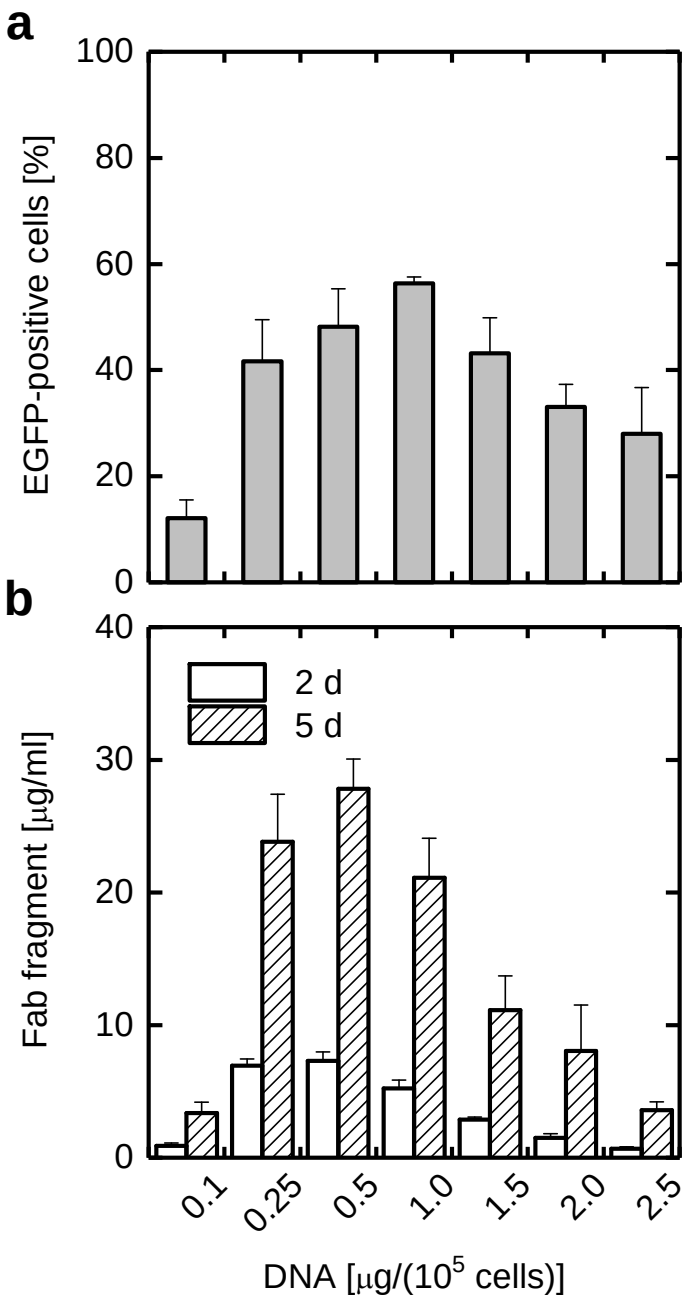
1:3
1:4
DNA:PEI ratio

Fig. 2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100
80
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EGFP-positive cells [%]
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Fab fragment [μg/ml]
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0.25
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1.0
1.5
2.0
2.5
DNA [μg/(10^5 cells)]

Fig. 3

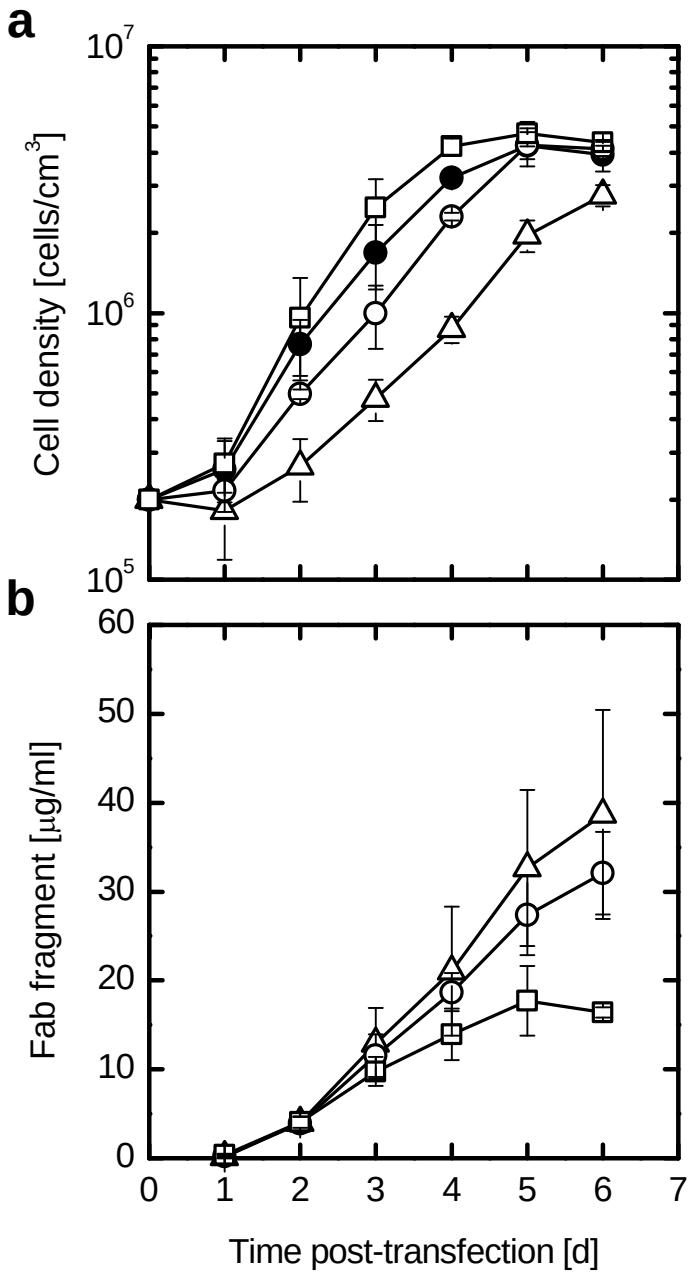
a
b
Cell density [cells/cm3]
Fab fragment [µg/ml]
Time post-transfection [d]
10^7
10^6
10^5
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7

Fig. 4

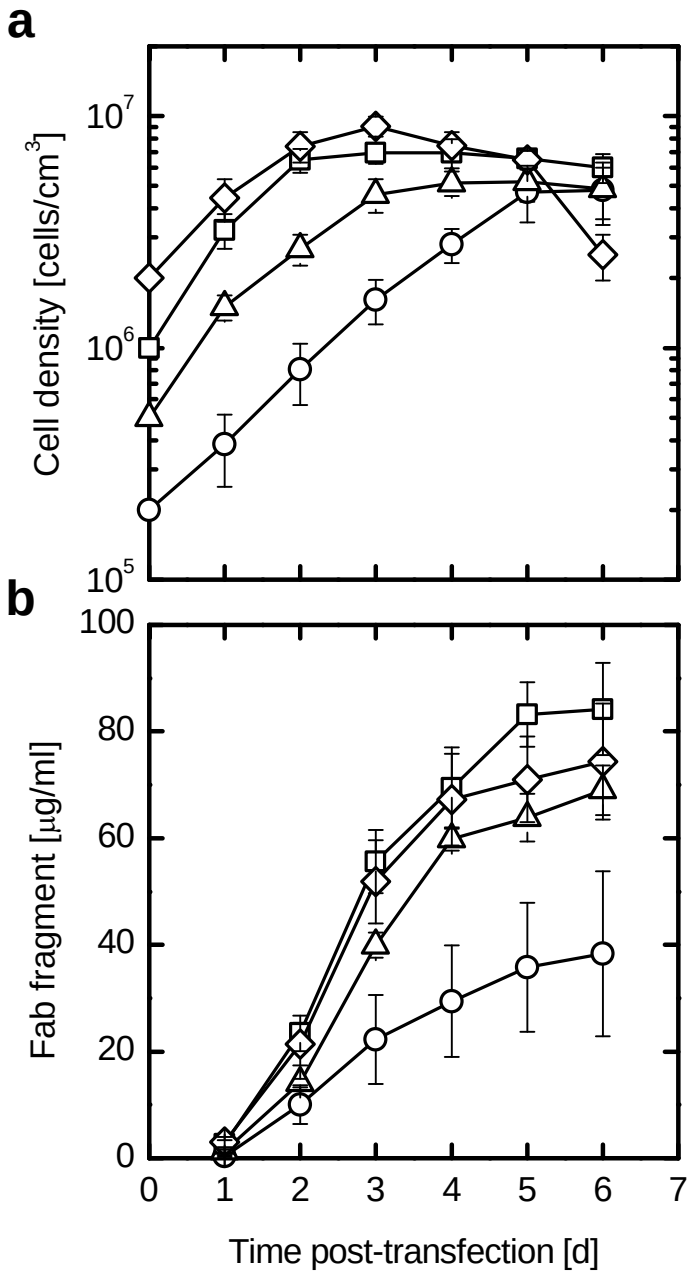
a
b
Cell density [cells/cm3]
10^7
10^6
10^5
Fab fragment [µg/ml]
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Time post-transfection [d]

Fig. 5

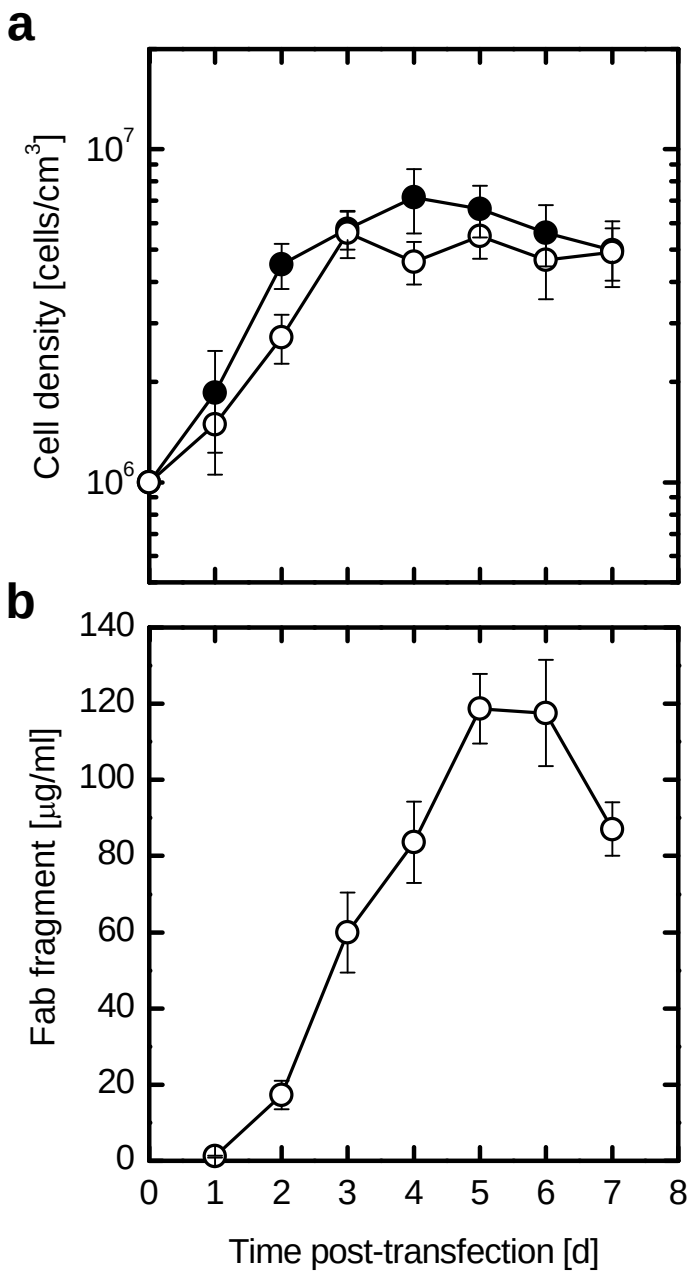
a
b
Cell density [cells/cm3]
10^7
10^6
Fab fragment [µg/ml]
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8
Time post-transfection [d]

Fig. 6