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Reproducible transfection in the presence of carrier DNA using FuGENE6 and Lipofectamine2000

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Abstract We have examined transfection conditions of chinese hamster ovary cells using FuGENE6 and immortalized gonadotrope cell line LβT2 cells using Lipofectamin 2000 and to obtain reproducible and reliable transfection. The experiments were performed with fluorescent protein expression vectors, pEYFP-C1 and pECFP-C1, or secreted-type alkaline phosphatase vector, pSEAP2, as reporter genes. The number of cells that received reporter plasmid increased in proportion to the amount of DNA and reached a plateau at a large amount. Co-transfection using two fluorescence vectors with a small amount of DNA demonstrated that every transfected cell received both vectors without discrimination. The results further indicate that there is a hierarchy of DNA receptiveness among competent cells. Simultaneously, we observed that a reliable transfection took place at the high dose of DNA. That is, the addition of carrier DNA makes possible a reliable delivery of a small amount of DNA of interest to the competent cells. Similar results were also obtained by pSEAP2 vector. Co-transfection of pEYFP-C1 and pECFP-C1 with various ratios at adequate amounts demonstrated that the fluorescence intensities by each vector are proportional to each amount of vector used with comparable efficiency. In addition, we observed that the variation of the assay using fluorescent vectors or secreted alkaline phosphatase vectors were small enough within the ± 25% (SD, $n = 4$), showing that the internal marker often used to normalize the data is not essential, since the vectors used allow us to exclude cell-harvest and cell-lysis. Thus, the present study demonstrates that the addition of carrier DNA during transfection provides reproducible and reliable results.

Keywords Carrier DNA · Transfection · Lipofectamine 2000 · FuGENE6

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Introduction

Delivery of DNA and RNA into animal cells has contributed to the recent development of bioscience and molecular cell biology. In the past, several transfection techniques such as calcium phosphate [1], DEAE-dextran [2], lipofection [3], electroporation [4] and viral vector [5] were developed as useful tools of DNA delivery to animal cells. These currently available methods have both advantages and disadvantages. Moreover, they have sometimes caused problems in respect to reproducibility or reliability. Chemical reagents provide a relatively easy method without special apparatus, although they have low transfection efficiencies and cytotoxicities. Several investigators in recent years have attempted to improve transfection efficiency and reliability and to reduce cytotoxicities [6–9]. Newly developed synthetic cationic lipids, such as Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA) and FuGENE6 (Roche Diagnostics GmbH, Mannhein, Germany) allow us to achieve high-efficiency transfections and

low cytotoxicities with easy handling. Estimation of time for complex formation, procedures for transfection, ratio of transfectant and DNA, expression time, and amount of DNA are examined in detail for Lipofectamine 2000 [10] and FuGENE6 [11] to obtain improved results.

Although optimizations for Lipofectamine 2000 and FuGENE6 have been performed, several subjects remain to be examined. These include: Whether the cell number that received the transgene is proportional to the amount of DNA used; Whether different DNA species are delivered to the same cell with similar efficiency; and How to obtain reliable and consistent transfection? Generally, β-galactosidase (β-gal) [12], chloramphenicol acetyl transferase (CAT) [13, 14] , and luciferase [12, 13] have been used as reporter proteins in transfection experiments. The measurements of these reporter proteins are usually required as an internal marker to detect the recovery of the cell lysate [12, 13]. On the other hand, green fluorescent protein (GFP) expression vector and secreted alkaline phosphatase (SEAP) vector do not need to recover lysate of the cells for their assay. Therefore, these vectors offer several benefits for transfection assay.

This study was aimed to discover a correlation between DNA amount and transfection efficiency and the condition of reproducible and reliable DNA delivery, using vectors that produce fluorescent protein and secreted type enzymes. Delivery of DNA to the competent cells was in a dose-dependent manner at low amounts and reached a maximum, followed by accumulation to the competent cells. Different DNA species were similarly delivered to the every competent cell in proportion to their ratio. Addition of carrier DNA enables reliable and consistent transfection even at low amounts of DNA.

Materials and methods

Reporter vectors

Reporter vectors, pEYFP-C1, pECFP-C1 and pSEAP2-Promoter, were purchased from CLONTECH (Inc., Palo Alto, CA), and pcDNA3.1 was purchased from Invitrogen. Purification of DNA was carried out using a Nucleobond PC Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocols. Concentration of DNA was measured with UV absorption at 260 nm and diluted with distilled water to the concentration used.

Cell culture and transient transfection

The pituitary tumor derived gonadotrope cell line, LβT2, was kindly supplied by Dr. P. L. Mellon and was maintained in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT) and antibiotics (SIGMA-ALDRICH Corp., St. Lous, MO) in humidified 5% CO_2-95% air at 37°C. Chinese hamster ovary cell line (CHO) was obtained from the RIKEN Cell Bank (Tukuba, Japan), and was maintained in monolayer cultures in F12 (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and antibiotics in humidified 5% CO_2-95% air at 37°C.

All cells were seeded at 1×10^4 cells in 0.1 ml DMEM or F12 medium per well in a 96-well plate for 24 h prior to transfection. LβT2 cells and CHO cells were transfected using 0.2 μl/well of Lipofectamine2000 and 0.3 μl/well of FuGENE6, respectively, according to the manufacturer's protocols.

Transfection assay

Cells expressing fluorescent proteins were photographed by a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a Pixela Color CCD camera (Osaka, Japan) and the specific filter sets for EYFP or ECFP. The intensity of fluorescence was measured after replacing the medium with PBS (-) using Wallac1420 ARVOsx (PerkinElmer, Boston, MA) equipped with a filter set for excitation at 485 nm and emission at 535 nm. Quantification of the fluorescent protein images were performed using the program, Quantity One (Bio-Rad Laboratories, Richmond, CA).

Then, the cells in the 96 well plate were dispersed by 0.025% trypsin, and the number of fluorescence-emitting cells and total cells were counted using a cell counting chamber. The transfection efficiency was expressed as a percentage of the number of fluorescence cells in the total number of cells counted. The cell viability was counted with 0.2 % trypan blue staining and expressed as a percentage of the number of living cells in the total number of cells counted.

The assay of SEAP activity was performed using an aliquot of culture medium (5μl) 24 h and 48 h after the transfection for CHO cells and LβT2 cells, respectively, with the Phosphalight Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols.

Results and discussion

Relation of DNA amount and transfection efficiency

To accomplish the transfection quantitatively, it is important to deliver DNAs reproducibly to the recipient cells. Hence we have examined a correlation between DNA amount and number of cells that received DNA by the

transfection of CHO cells using FuGENE6 reagent (Fig. 1A and B).

The result showed that the fluorescence value increased in a dose-dependent manner (Fig. 1A), though the increase was not necessarily proportional to the DNA amount at high dosage. In contrast, the proportion of fluorescence-emitting cells reached a plateau of about 12% at 20 ng DNA/well (Fig. 1B), while the fluorescence intensity still continued to increase (Fig. 1A). The simultaneous measurement with trypan blue staining showed that FuGENE6 alone (without DNA) did not affect the cell vaiability. In addition, the cell vaiability was not affected by transfection at high amount DNA (160 ng/well) (Fig. 1C). A comparable experiment was carried out for LβT2 cells with the Lipofectamine 2000 reagent, since the FuGENE6 reagent is hardly effective for this cell line. The dose dependency to the fluorescence intensity similar to that of CHO cells was observed with a plateau at more than 80 ng DNA/well (Fig. 1D). However, the proportion of fluorescence-emitting cells reached the plateau of 20% at about 10 ng DNA/well (Fig. 1E). The same cell viabilities as those of CHO cells were also observed (Fig. 1F).

10 ng of DNA contains about 2×10^9 DNA molecules, providing an excess number of molecules for each 10^4 cells/well. Therefore, the observation that fluorescence intensity continues to increase after the maximal level of transfection efficiency indicates the accumulation of the excess DNA molecules in the recipient cells. In addition, we realized that only a limited number of cells are competent to receive DNAs.

Delivery of different DNA species in the same cells

The linear increase of fluorescence intensity at low amounts of DNA created some doubt that DNAs are randomly delivered to cells. To explore this question, we performed a dual transfection with two fluorescence vectors, whose fluorescence products are distinguishable from each other by different fluorescence filter sets. The distinguishable detection of EYFP and ECFP from each other using specific filter sets is indicated in Fig. 5A and B. This dual transfection at the same DNA amount transfected into the same cells simultaneously was observed from the emission of two different fluorescence colors (Fig. 2A and B).

Interestingly, there was a difference in the intensities among transfected cells (Fig. 2B). The images suggest that the two DNA vectors were delivered unequally to recipient cells. To confirm this difference in delivery, we have

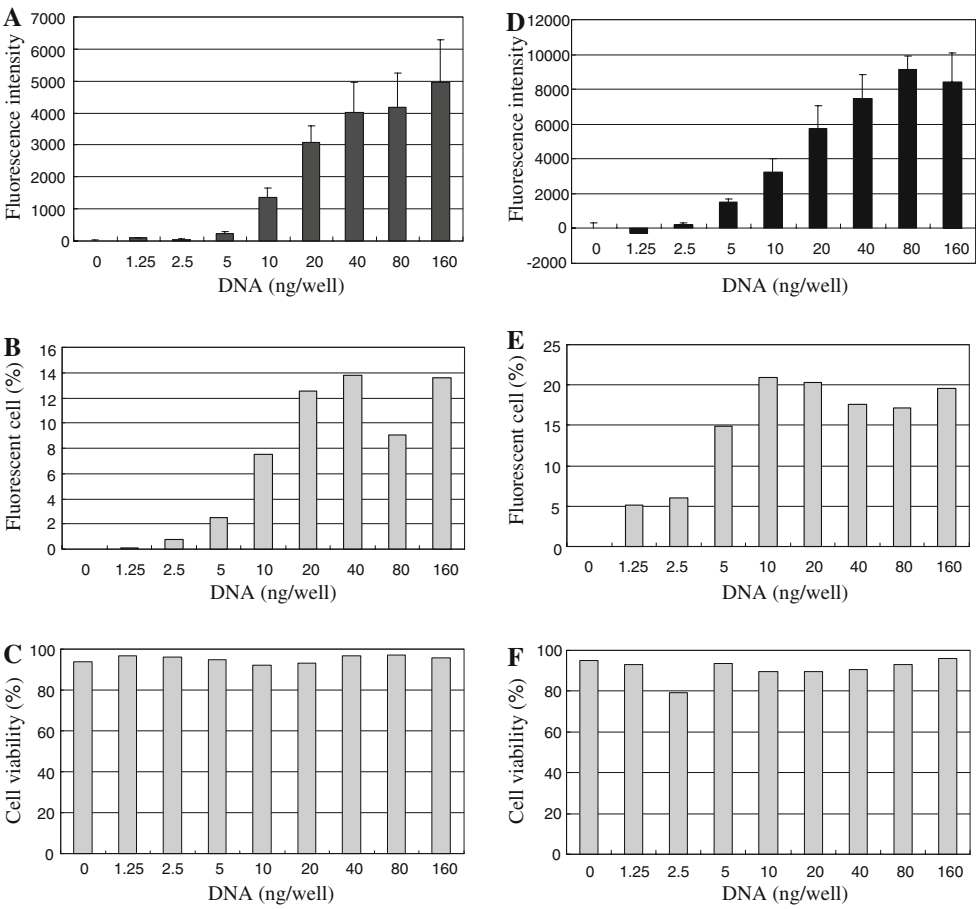

Fig. 1 Correlation between fluorescence intensity and transfection efficiency. Transient transfections were performed using FuGENE6 for CHO cells (**A**, **B** and **C**) and Lipofectamine 2000 for LβT2 cells (**D**, **E** and **F**) with an increasing amount of pEYFP-C1 for the constant number of cells (1×10^4 cells/well in a 96-well plate, $n = 4$). The fluorescence intensities (**A** and **D**) were measured with Wallac1420 ARVOsx and indicated as the mean ± SD for quadruplicate samples. After the measurement of fluorescence intensities, cells were removed by trypsin, and the ratios of the number of fluorescence cells and total cells were calculated (**B** and **E**). The cell viabilities were also counted by trypan blue staining (**C** and **F**)

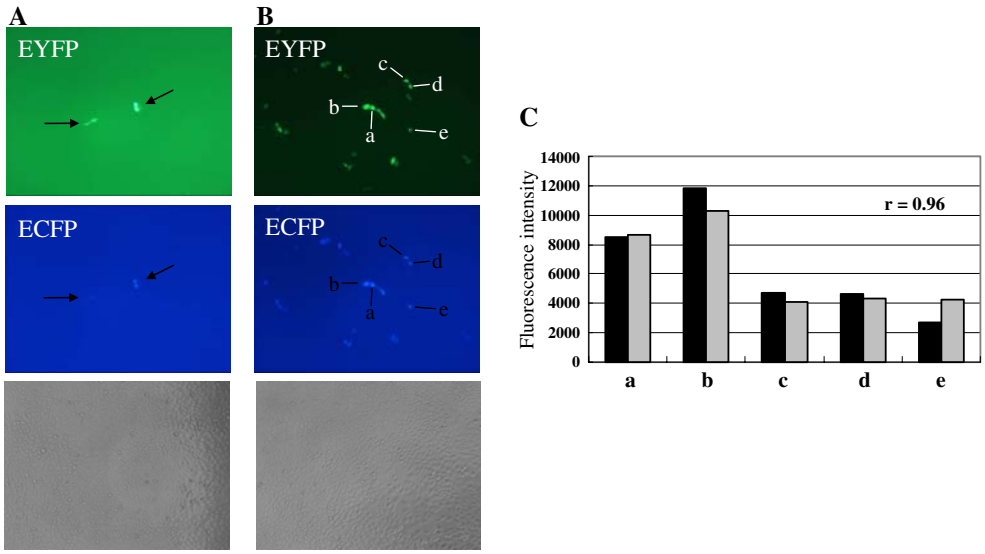

Fig. 2 Co-transfection of pEYFP-C1 and pECFP-C1 DNAs at small amounts. A small amount of pEYFP-C1 and pECFP-C1 DNAs (**A**: 1.25 ng each/well, **B**: 2.5 ng each/well) were co-transfected to CHO cells. Fluorescent proteins were observed with a specific filter set for each fluorescent protein under the Leica fluorescence microscope. Arrows (**A**) indicate the cell producing both fluorescent proteins. In A and B, upper panel; EYFP, middle panel; ECFP, lower panel; bright field. **C**: two fluorescence intensities in each cell (marked as a ~ e in **B**) were quantified by Quantity One. Solid and shaded bars represent EYFP intensities and ECFP intensities, respectively

estimated the intensity of two fluorescent proteins in each cell. As shown in Fig. 2C, there was a good relation between the two intensities in each cell with a coefficient of 0.96. Therefore, an equivalent delivery of vectors occurred during the transfection. This difference in the intensities might indicate the presence of a hierarchical competency among the recipient cells.

Improvement of transfection by carrier DNA

The results described above show that DNAs in excess amounts are accumulated into the competent cells (Fig. 1). This suggests that an adequate amount of DNA might function as a carrier DNA to deliver a small amount of DNA interest to all competent cells. The carrier DNA was often employed to obtain good transfection results. To ascertain the relevance of carrier DNAs, we performed the dual transfection of pEYFP-C1 vector (2.5 ng/well) by adding 20 or 40 ng of non-fluorescence vector, pcDNA3.1, as a carrier DNA to CHO cells (Fig. 3). Only a small number of cells emitted fluorescence by pEYFP-C1 vector without carrier DNA (Fig. 3A). In contrast, the transfection in the presence of 20 or 40 ng carrier DNA notably increased the number of fluorescence-emitting cells (Fig. 3B and C). These results indicate that the carrier DNA makes it possible to transfect a small amount of DNA interest to competent cells uniformly and consistently.

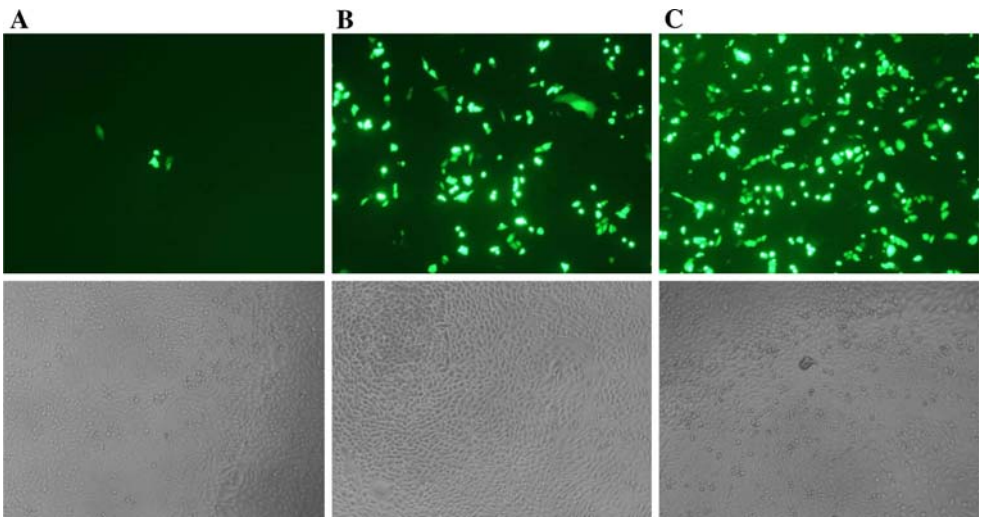

Fig. 3 Addition of carrier DNA. Transfection of pEYFP-C1 (2.5 ng) to CHO cells was performed in the absence (**A**) or presence of pcDNA3.1 (**B**: 20 and **C**: 40 ng, respectively) as a carrier DNA. Upper panel; EYFP and lower panel; bright field

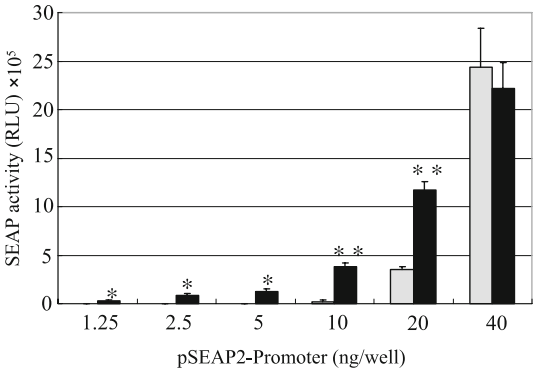

Fig. 4 SEAP assay for transfection with carrier DNA. CHO cells were transiently transfected with pSEAP2-Promotor (1.25 ng, 2.5 ng, 5 ng, 10 ng, 20 ng, 40 ng) without or with carrier DNA (20 ng pcDNA3.1). SEAP activity was assayed and expressed as RLU (relative luminescence unit). The RLUs were plotted as the mean ± SD of quadruplicate samples. Shaded and solid bars represent without and with carrier DNA (20 ng), respectively. $*P < 0.01$ and $**P < 0.001$

We performed a similar transfection experiment with pSEAP2-Promoter vector to CHO cells, in the presence or absence of 20 ng of pcDNA3.1 as carrier DNA (Fig. 4). In the absence of pcDNA3.1, only low SEAP activity was detected at lower than 10 ng reporter vector/well. However, the SEAP activity value was increased proportionally to the amount of carrier DNA (Fig. 4). The transfections of 40 ng pSEAP2-Promoter with or without carrier DNA gave almost the same RLU value, indicating that the transfection efficiency reached a plateau with 40 ng of DNA. Similar observations were obtained for LβT2 cells (data not shown). These results further suggest that we can perform a consistent and reproducible transfection by the addition of a complementing amount of carrier DNA sufficient to deliver DNA molecules to all competent cells.

Quantitative delivery by carrier DNA

Further confirmation for the effect of carrier DNAs was performed by transfection by altering the DNA amount or the proportion of the two different fluorescence vectors. As shown in Fig. 5, fluorescent proteins of EYFP and ECFP are distinguishable from each other using specific filter sets (Fig. 5A and B). Use of pEYFP-C1 at a constant amount of

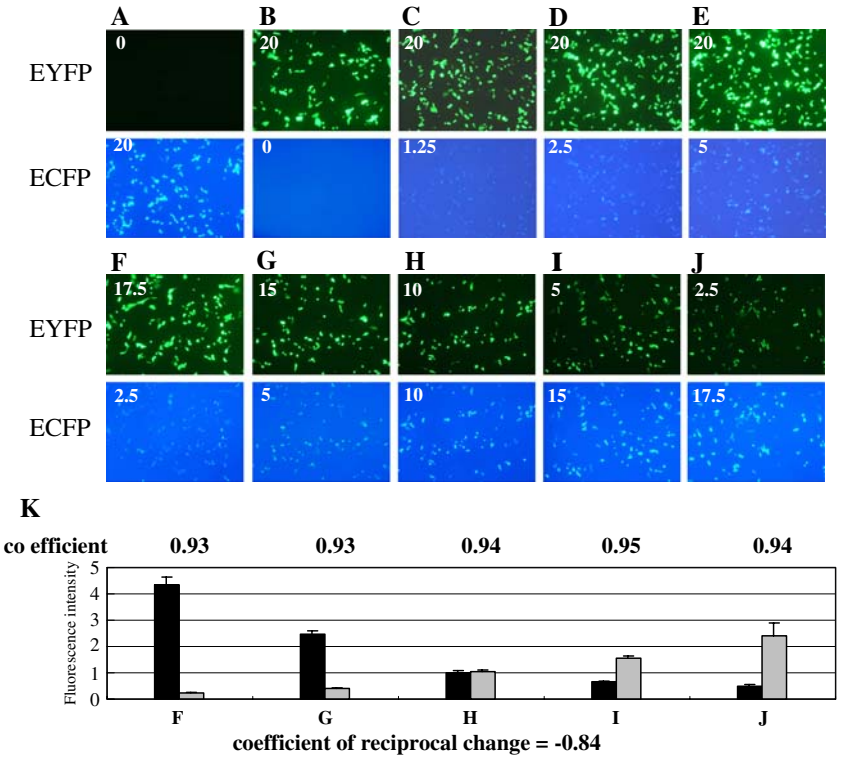

Fig. 5 Co-transfection of pEYFP-C1 and pECFP-C1 DNAs. Co-transfection to CHO cells was performed with pEYFP-C1 and pECFP-C1 by changing their ratio. Expression of each fluorescent protein was detected with a specific filter set under the Leica fluorescence microscopy. Each 20 ng/well of pECFP-C1 (**A**) and pEYFP-C1 (**B**) alone was transfected. C–E: Co-transfection was performed with an increasing amount of pECFP-C1 DNA (**C**: 1.25, **D**: 2.5 and **E**: 5 ng/well) together with a constant amount (20 ng/well) of pEYFP-C1. Co-transfection (**F**–**J**) was performed by changing the proportion of pEYFP-C1 and pEYFP-C1 as indicated in the figure at a constant total amount (20 ng/well). Upper panel; EYFP and lower panel; ECFP. The quantification of each fluorescence intensity of F - J and the analysis of coefficient of correlation between two intensities are indicated in graph K. Solid and shaded bars represent EYFP and ECFP intensities, respectively. $n = 10 \pm$ SEM

20 ng gave almost the same population of fluorescence produced by the pECFP-C1 vector from 1.25 ng to 5 ng to those of pEYFP-C1 (Fig. 5C–E), although fluorescence of ECFP at a small amount was weak in the photograph due to the small amount of expression level. Thus, a small amount of pECFP-C1 was satisfactorily delivered to the cells that received the carrier DNA, pEYFP-C1. Similarly, transfection was performed by altering the proportion of the two vectors with a constant amount of total DNA (20 ng/well) (Fig. 5F–J). The results show that delivery of the two vectors and the cooperative expression of two fluorescent proteins took place in any proportion. These data were quantified by their intensities of fluorescence, and the coefficients of correlation between intensity by EYFP and ECFP in each cell were estimated in Fig. 5K. Each coefficient had a sufficiently high value of more than 0.93. In addition, the coefficient of reciprocal change of intensity by EYFP and ECFP was -0.84 indicating that these two vectors were well delivered at any proportion. Thus, these results suggest that diverse DNAs are quantitatively delivered and expressed into the recipient cells.

Most commonly, the reporter gene assays are performed by co-transfection with target DNAs of interest and other reporter genes as internal markers to normalize the expression data of interest. The classical transfection reagents, such as calcium phosphate and DEAE-dextran, were toxic for cells, and removal of these cells from a dish for transfection assay resulted in unreliable and unreproducible measurements. The normalization by internal marker gene was essentially important to avoid experimental variation by cell toxicities and cell harvest. As demonstrated in this study, FuGENE6 to CHO cells and Lipofectamine 2000 to LβT2 cells could give a reliable and low variation of expression under the designed condition including a carrier DNA. Notably, the assays for fluorescent protein intensity and/or for enzyme activity secreted in medium by pSEAP2-Promoter also enable us to avoid an internal marker. In fact, the variations of the assay using fluorescent vectors or secreted alkaline phosphatase vectors were small enough and within the ± 25% (SD, $n = 4$) in this study (Fig. 4) and our current transfection experiments are still performing with only small variations of the measurements [15–18].

In conclusion, this study demonstrated that the addition of carrier DNA improves the reliable and reproducible transfection of interested genes to the recipient cells and allows the elimination of an internal marker. Further, the improvement by carrier DNA provides a consistent transfection of a small amount of target genes to all competent cells. We are currently able to use several types of transfectant and cell lines, therefore, in such case, the availability of carrier DNA in the transfection assay is of value to be examined.

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