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Transfection of insect cell lines using polyethylenimine

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Abstract Insect cell lines have been widely used in recombinant baculovirus expression systems and transient gene expression studies. Critical to these applications have been the transfection of foreign DNA. This has been frequently done using labor intensive and cytotoxic liposome-based transfection reagents. In the current study we have optimized a new kind of polyethylenimine-based DNA transfection reagent on the *Spodoptera frugiperda* Sf9 insect cell line. A plasmid vector that transiently expresses green fluorescent protein (GFP) was effectively delivered into Sf9 cells. A transfection efficiency of 54% and cell viability of 85–90% were obtained for Sf9 cells. The developed transfection protocol has now been successfully used to transfect eight insect cell lines derived from *Bombyx mori*, *Trichoplusia ni*, *Helicoverpa zea*, *Heliothis virescens* and *S. frugiperda* with GFP and GUS with transfection efficiencies of at least 45%. This method provides high heterologous protein expression levels, transfection efficacy and cell viability, and could be used for transient gene expression in other lepidopteran cell lines.

Keywords In vitro · Insect cell lines · *Spodoptera frugiperda* · Transfection · Polyethylenimine (PEI) · Plasmid DNA · EGFP

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Introduction

Optimized transfection systems for introduction of expression cassettes into cultured insect cells have become increasingly important for generation of stably transformed cell lines and investigating and improving transient heterologous

protein expression using in vitro systems. Insect cell-based systems for the large-scale production of recombinant proteins have become indispensable for biotechnological, pharmaceutical and industrial applications. Additional continuous cell lines have been established from a large number of insect species, mainly from Lepidoptera and Diptera (Lynn et al. 1988) but those derived from *Spodoptera frugiperda* (IPLB-SF-21-AE; Vaughn et al. 1977 and Sf9 (Summers and Smith 1987)) and *Trichoplusia ni* (High Five™) have been the most widely used both commercially and in the research laboratory for baculovirus transient gene expression studies and transgenic cell lines. However, the large number of other insect cell lines represents a largely unexplored resource.

The transfection of an expression cassette into insect cells is the first step for obtaining transient or stable expression of heterologous proteins. A number of transfection methods, initially developed for mammalian cell lines have been adapted for insect cells but the conditions required for optimal transfection efficiency can vary greatly between vertebrate and invertebrate cell lines. Presently there are several transfection methods and techniques in use with lepidopteran cell lines (Trotter and Wood 1995; Keith et al. 2000; Gundersen-Rindal et al. 2001).

One of the first, and most widely used, transfection techniques is the calcium phosphate precipitation method of Graham and van der Eb (1973). The exact mechanism by which the DNA/calcium-phosphate complex penetrates into the cells has not been elucidated, but intact DNA is known to reach the nucleus. Typically 10% or fewer of the cells are successfully transfected with this method and it does not work at all for some cell types (Singer and Berg 1998). Alternative methods used for transfection of insect cells include DNA-adsorptive agents such as DEAE-dextran, poly-L-lysine, polyornithine, and polybrene (Walker 1989). The efficiency of transfection using these agents may be improved with the use of carrier DNA or with osmotic shock using DMSO or glycerol (Walker 1989).

Two additional methods have also been used for delivery of DNA into cells: liposome-mediated transfection (Bangham 1992; Zhu and Huang 1994) and electroporation (Andreason and Evans 1988; Shigikawa and Dower 1988). In many cases, these provide improved transfection efficiency. Liposome-mediated transfection (lipofection), first reported by Felgner et al. (1987) and Felgner and Holm (1989), employs a cationic lipid, *N*-[1-(2, 3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA), to form liposomes. The liposome complexes sequester the DNA with 100% efficiency (Trotter and Wood 1995). Lipofection efficiencies of 34%–45% have been obtained with *Bombyx mori (*Bm5), *S. frugiperda* (Sf21), and *Lymantria dispar* (IPLB-LdEp, IPLB-LdEIta, IPLB-Ld652Y) cell lines (Trotter and Wood 1995; Keith et al. 2000; Gundersen-Rindal et al. 2000).

Neumann et al. (1982) first used electroporation as a method for delivering DNA into tissue culture cells. This method induces the formation of nanometer size reparative pores in cell membranes by subjecting cells to a brief high-voltage electric field. These pores allow DNA to penetrate the cell. This technique is often more efficient for transfection of cell types that are generally resistant to any other transformation method. Electroporation has been used for delivery of DNA into the *Helicoverpa zea* and *Musca* insect embryos (Leopold et al. 1996) and in a *S. frugiperda* cell line with *Autographa californica* nucleopolyhedrovirus (AcMNPV) DNA. This method yielded 100 times more progeny recombinant virus when compared to the calcium phosphate precipitation method (Mann and King 1989).

Unfortunately, in spite of this diversity of gene delivery methods, their application with insect cells can be difficult due to toxicity of the transfection reagents, the complexity of the procedures, low transfection efficiency, or poor reproducibility of results. Therefore the search for new alternative agents for in vitro DNA introduction into the insect cells and the development of novel, simple, and highly efficient transfection methods for insect cell lines are of great interest.

Two main parameters can be optimized and investigated in a transfection procedure—the level of heterologous protein expression and the transfection efficiency. Evaluation of these parameters can be done more conveniently using transfection marker or reporter genes such as β-galactosidase (β-gal) (Alam and Cook 1990) luciferase (de Wet

et al. 1987) and CAT (Gorman et al. 1982), each of which has easily detectable expression levels. However, the green fluorescent protein (GFP) is especially useful since it is not toxic to most cell types and is easily detected by the presence of fluorescence without addition of substrates. Parallel to using GFP, the Typhoon variable mode scanning system for GFP expression evaluation along with flow cytometry has become popular means for gene transfer assessment because it allows protein expression level measurement, i.e. transfection efficiency, directly in living cells, excluding the necessity of cell fixation and sample preparation stages (Han et al. 2002). In this procedure, starting from 24 h after transfection, the tissue culture plate containing transfected cells is simply placed onto the scanning bed of the Typhoon 9200 and scanned using green (532 nm) for measurement of GFP-fluorescence.

In the current study we investigated the transfection of insect cells in vitro using the *S. frugiperda* Sf9 cell line and polyethylenimine-mediated transfection of reporter plasmid vector pBAC-5-GFP, expressing enhanced GFP (EGFP). The Sf9 cell line (Summers and Smith 1987), a clone of IPLB-SF-21-AE (Vaughn et al. 1977), was used because it has better growth characteristics compared to many other available lepidopteran cell lines and is susceptible to several baculoviruses, including *Plutella xylostella* NPV, *Anticarsia gemmatalis* NPV, *Rachoplusia ou (Anagrapha falcifera)* NPV, *Galleria melonella* NPV, and *Helicoverpa armigera* MNPV. Alternatively, some cell lines have limited virus susceptibilities, such as the *B. mori* lines BMN (Maeda 1989) and Bm5 (Je et al. 2001), susceptible only to *B. mori* NPV, and the *L. dispar* line, IPLB-Ld652Y (Goodwin et al. 1978) that only supports *L. dispar* NPV (Lynn et al., 1988; Godman et al. 2001; Harrison and Bonning 1999; Rahman and Gopinathan 2004).

For the transfection of Sf9 cell we used the commercial polyethylenimine reagent ExGen500, belonging to a new class of non-viral and non-liposomal gene delivery agents. The purpose of the current research is the development of the transfection protocol providing the greatest possible number of transfected cells with the maximal heterologous protein expression level.

Materials

Equipment

1. Incubator, Model No. BOD10A14, REVCO.[1]
2. Laminar flow hood class II biosafety cabinet, Model Faster.[2]
3. Thermoblock GRANT, Model QBT1.[3]
4. Microscope, inverted, florescent, Leica Model DM IL-FLUO.[4]
5. Filter system GFP (BP 470/40 nm EX, BP 525/50 nm EM), Cat. No.11513852.[4]
6. Gilson pipetman, Models P-20, P-200, P-1000.[5]
7. Pipet-aid, Model No. 4-000-100.[6]
8. VibroGene vibromixer (STUART SCIENTIFIC), Model SA4.[7]
9. Benchtop (swinging bucket) centrifuge, Model No. 5804R, Eppendorf.[8]
10. Centrifuge rotor, Type A-2-DPW, Eppendorf.[8]
11. Levy Hemocytometer, Cat. No. 3500.[9]
12. Cooled charge-couple device (CCD) camera, Leica Model DFC320 R2.[3]
13. Leica Image Manager IM50 v4.0 software.[10]
14. Variable mode imager (Molecular Dynamics) Model Typhoon 9200.[11]
15. Photoshop 6.0 software.[12]

[1] GS Laboratory Equipment, 275 Aiken Rd., Asheville, NC 28804, USA.

[2] Faster, 46 Via Vespucci, Ferrara 44100, Italy.

[3] Grant Instruments (Cambridge) Limited, 29 Station Rd., Shepreth, Royston, Herts SG8 6PZ, England.

[4] Leica Microsystems Wetzlar GmbH, 17–37 Ernst-Leitz Str., Wetzlar 35578, Germany.

[5] Rainin Instrument Co., Inc., P.O. Box 4026, Mack Rd., Woburn, MA 01888–4026, USA.

[6] Drummond Scientific Co., 500 Parkway, P.O. Box 700, Broomall, PA 19008, USA.

[7] Stuart Scientific Co., Staffordshire, UK.

[8] Eppendorf AG, 1 Barkhausenweg, Hamburg 22339, Germany.

[9] Hausser Scientific Co., 935 Horsham Rd., Suite C, Horsham, PA 19044–1286, USA.

[10] Leica Microsystems Imaging Solution Ltd. PO Box 86, 515 Coldhams Lane, Cambridge CB1 3XJ, UK.

[11] Amersham Biosciences Corp., P.O. Box 1327, 800 Centennial Ave., Piscataway, NJ 08855–1327, USA.

[12] Adobe Systems Incorporated, San Jose, CA 95110, USA.

16. ImageQuant™ v5.2 software (Molecular Dynamics).[11]
17. Microsoft Excell2003 software.[13]

Culture medium and reagents

1. Complete medium
   - 90% v/v Grace's insect medium (Supplemented) 1X, Cat. No. 11605–094.[14]
   - 10% v/v Fetal bovine serum (heat inactivated), Cat. No. 16140–071.[14]
2. Complete medium plus added antibiotics
   - 89% v/v Grace's insect medium (Supplemented) 1X, Cat. No. 11605–094.[14]
   - 10% v/v Fetal bovine serum (heat inactivated), Cat. No. 16140–071.[14]
   - 1% v/v Antibiotic-antimycotic solution (100X) stabilized (10,000 U/ml penicillin-10 mg/ml Streptomycin-25 μg Amphotericin B), Cat. No. A5955.[15]
3. ExGen500 Transfection reagent, 10 μM in PEI, Cat. No. R0511.[16]
4. Transfection buffer
   - 150 mM NaCl, sterile endotoxin free, Cat. No. S-3014.[15]

Plastic and glassware

1. Culture flasks (Greiner), 25 cm^2, 75 cm^2, Cat. No. 690160 and 658170.[17]
2. Glass serological pipets (Kimble), 5 ml, 10 ml, Cat. No. 72105–5110 and 72105–10110.[18]
3. 50 ml screw capped polypropylene tube, Cat. No. 2098.[19]
4. 24-well tissue culture plate, (Costar) Cat. No. 3524.[20]
5. 1,000-μl disposable pipette tip, Cat. No. P3290–1800[21]
6. 200-μl disposable pipette tip, Cat. No. P3290–1510.[22]
7. 0.22-μm Acrodisc Supor filter, Cat. No. 4612.[23]
8. 1.5-ml Eppendorf tubes, Cat. No. 0030120086.[8]

Cell lines

The Sf9 cell line, obtained from Guido Caputo, Great Lakes Forestry Center, Ontario, Canada, was maintained in our laboratory by weekly subculturing in Grace's insect cell culture medium containing 10% v/v heat inactivated FBS at 26°C following the procedure described in Lynn (2002) for firmly attached cell lines. The number of passages exceeded 500 at the time of these studies.

Procedures

Preparation of plasmid DNA for transfection

The reporter plasmid pBAC-5-GFP was used for this study to transfect Sf9 cells was described in detail by Slack et al. (2001). Plasmid DNA was produced and prepared via ultracentrifugation in CsCl density gradients as described previously by Slack and Lawrence (2003). The isolated plasmid DNA was separated from ethidium bromide (EtBr) by extraction with equal volume of 20X-SSC-saturated isopropanol and shaking vigor-

[13] Microsoft Corp., One Microsoft way, Redmond, WA 9052–6399, USA.

[14] Life Technologies, 3175 Staley Rd., Grand Island, NY 14072, USA.

[15] Sigma, P.O. Box 14508, St. Louis, MO63178, USA.

[16] Fermentas Inc., 7520 Conneley Drive, Suite A, Hanover, MD 21076, USA.

[17] Greiner, GmBH D-72636 Frickenhausen, West Germany.

[18] Kimble/Kontes, Vineland, NJ, USA.

[19] Falcon brand, Beckon Dickenson and Co, Lincoln Park, NJ 07035, USA.

[20] Costar, 205 Broadway, Cambridge, MA 02139, USA.

[21] ISC BioExpress, 420 North Kays Dr., Kaysville, UT 84037, USA.

[22] Fisher Scientific, 3970 Johns Creek Court Suite 500, Suwanee, GA 30024, USA.

[23] Pall Gelman Laboratory, 600 South Wagner Rd., Ann Arbor, MI 48103–9019 USA.

ously for 10 s. After the layers separated, the upper isopropanol/EtBr layer was removed and additional isopropanol was added at least two more times until both layers were colorless. The volume of the plasmid DNA (lower layer) was adjusted to a total volume of 5 ml with doubly deionized H_2O and precipitated in two volumes of 100% ethanol (10 ml), mixed and placed at –20°C for 10 min. The precipitated DNA pellet was washed with 1 ml of 70% v/v ethanol and dried in air for 30 min. Then the DNA was dissolved in 1× TE, pH 8.0 to a final concentration of 500 ng/μl. The DNA concentration was determined using a spectrophotometer.

Cell preparation and transfection protocol

Several modifications were made to the procedure described in the product literature for the ExGen500 transfection reagent for transfection of adherent mammalian cells. These include optimizing the cell density and incubation temperature required for insect cells. Additionally, the DNA:ExGen500 ratio was experimentally optimized for Sf9 cells as described in detail in the following subsection.

1. Incubate a confluent 25-cm^2 T-flask of Sf9 cells at 4°C for 20 min. Remove the medium and replace with 6 ml of fresh FBS/Grace's medium with added antibiotic-antimycotics. Suspend the cells by gently bumping the T-flask followed by flushing with medium from the pipet. Count cells using a Levy hemocytometer and inverted microscope. Dilute the suspended cells in 50 ml sterile tube in complete Grace's with added antibiotic-antimycotics to a density 7×10^4 cells/ml. Uniformity in the cell seeding density between experiments is critical for reproducibility of transfection efficiency results.
2. Using a 10-ml serological pipette, seed 1 ml of suspended cells to each well of a 24-well tissue culture plate. Allow 10–15 min for cells to attach at 22°C.
3. In 1.5-ml sterile Eppendorf tubes for each transfection dilute 1.0 μg (2.0 μl) of DNA into 100 μl of 150 mM NaCl, vortex gently and spin down briefly for 5–10 s.
4. Place tubes with DNA into the wells of solid-state thermoblock and incubate at 72°C for 5–10 min for DNA sterilization. Allow the DNA solution to cool down to room temperature.
5. Add the 3.3 μl of polyethylenimine reagent ExGen500 to each tube with DNA solution (not the reverse order) and vortex-mix the solution immediately for 10 s, spin down briefly and incubate at room temperature for 10 min for generation/forming of DNA/polyethylenimine complexes.
6. After the incubation, for each transfection add 100 μl of DNA/polyethylenimine complexes by dropping slowly and evenly onto the cells to distribute the complexes. Seal the tissue culture plate and gently rock it back and forth and from side to side to achieve even distribution of the complexes. Centrifuge the culture plate for 5 min at 280 g.
7. Incubate transfected cells at 26°C in a plastic sealed container with damp paper towel to maintain humidity for 120 h.

Optimization of DNA:ExGen500 ratio

Sf9 cells were transfected using ExGen500 with various DNA/ExGen500 ratios to determine the conditions providing for greatest transfection efficiency with minor cells damage. The method modified for insect cells as described above was used with three concentrations of plasmid DNA (0.5, 1, and 2 μg) and three quantities of ExGen500 (6, 8, and 10 eq). One equivalent (eq) represents the amount of ExGen500 required to neutralize the negative charge of the DNA phosphate groups. One microgram of DNA is 3 nmol of phosphate and 1 μl of ExGen500 is 5.47 μM in nitrogen residues. The required amount of ExGen500 depends on the amount of DNA and the number of equivalents needed and was calculated according to the following formula:

$$\text{Number of equivalents} = \frac{\mu l\, Ex\, Gen\, 500 \times 5.47}{\mu g DNA \times 3}$$

Thus, for 0.5 μg of DNA, 6, 8, and 10 eq correspond to 1.64, 2.2, and 2.74 μl of ExGen500,

respectively; for 1 μg of DNA—3.3, 4.4, and 5.5 μl; and for 2 μg of DNA—6.6, 8.8, and 11.0 μl of transfection reagent.

Visualization of GFP and determination of transfection efficiency

Digital image analyses were used to determine the efficiencies of Sf9 cells transfections (Stewart et al. 2000). GFP-transfected Sf9 cells were visualized directly in 24-well plates using a UV-light source and GFP S filter cube on a Leica DMIL Fluo inverted microscope. For this study, visible light and UV/fluorescent images for each transfection were captured at 10 × magnification using a Leica DFC R2 CCD camera system. The captured images were normalized and level adjusted with Adobe Photoshop 6.0 software to achieve an equivalent background for each image. Successfully transfected cells were then counted from digital images. At least four images were captured for each transfection and at least ~2.5×10^3 cells were counted in each image. A visible light image of the same microscope field was also captured for each fluorescent image to determine the total cell numbers for calculating the percentage of transfected cells.

Determination of expression levels in transfected cells based on quantification of GFP-fluorescence

GFP-fluorescence was measured using laserscanning technology with the Typhoon™ 9200 variable mode imager (Han et al. 2002). A 24-well plate with transfected cells was placed onto the scanning bed with the scanning focus set to 3 mm above the scanning bed. The plate was scanned using laser (532 nm) excitation source and the resulting fluorescence emitted (526 nm, short pass filter) was recorded using the Typhoon™ PMT at resolution 100 μm (600 V). PMT readings were converted to 16-bit digital images by the Typhoon™ software and the background fluorescence was subtracted by adjusting the grey scale range with ImageQuant™ software. The adjusted images were saved as 8-bit TIFF files and the expression levels of transfected cells, expressed in relative fluorescent units (rfu) (Slack and Lawrence 2003) were determined using ImageQuant™ software.

Results and discussion

ExGen500 belongs to the new class of non-viral and non-liposomal agents for introduction of foreign DNA into cells and consists of 10 mM linear polyethylenimine (PEI) in water with molecular weight of 22 kDa (or 5.47 mM in nitrogen residues). The polyethylenimine molecules possess a highly positive potential, making them superior for DNA condensing and for delivering agents across the cell membrane (Godbey et al. 1999; Mislick and Baldeschwieler 1996). Every third atom of ExGen500 crystal matrix is nitrogen of an amino group that can be protonated, making these molecules effective ''proton sponges'' (Boussif et al. 1995). This characteristic provides neutralization of negative pH of endosomes and protects DNA from liposomal degradation thus facilitating the particles penetration and release of ExGen500/DNA complexes into cytoplasm followed by DNA transfer into the cell nucleus without degradation. The mechanism of action of this reagent is based on the interaction of linear polyethylenimine molecules of ExGen500 with DNA due to the difference in the molecular charges. This results in the formation of small (30–100 nm), stable, high-diffusion particles that attach to the cell surface and are taken into the cells by endocytosis. The ''proton sponge'' effect of ExGen500 neutralizes the pH of endosomes, stimulating mass accumulation of protons and passive inflow of chlorides. This results in rapid osmotic swelling, causing endosomes to rupture and release the ExGen500/DNA complexes into the cytoplasm. The DNA then migrates into the cell nuclei without degradation (Demeneix et al. 1998). ExGen500 is effective for transfection of many different cell types, including many difficult-to-transfect cells such as stem cells, differentiated cells, and primary cultures. It can transfer oligonucleotides and DNA up to 10 kb into the cell nucleus, exhibits minimal cytotoxicity, and provides high reproducibility of results (Boussif et al. 1995; Zou et al. 2000).

The reporter plasmid construct (pBAC-5-GFP) expresses the enhanced green fluorescent protein (EGFP) gene, a variant of GFP under the control of the *Autographa californica* (AcMNPV) viral promoter gene, *gp64*. The level of transfection with plasmid DNA pBAC-5-GFP was determined by measuring the GFP fluorescence of transfected Sf9 cells and the percentage of GFP-positive cells. GFP-fluorescence was monitored using fluorescent microscopy and the Typhoon fluorescent imaging system (Fig. 1). Fluorescent microscopy confirmed the presence of GFP-specific fluorescence (Fig. 1A), while the imaging system permitted the detection of the total expression levels of transfected cells (Fig. 1B). The levels of GFP-fluorescence were measured every 24 h after the

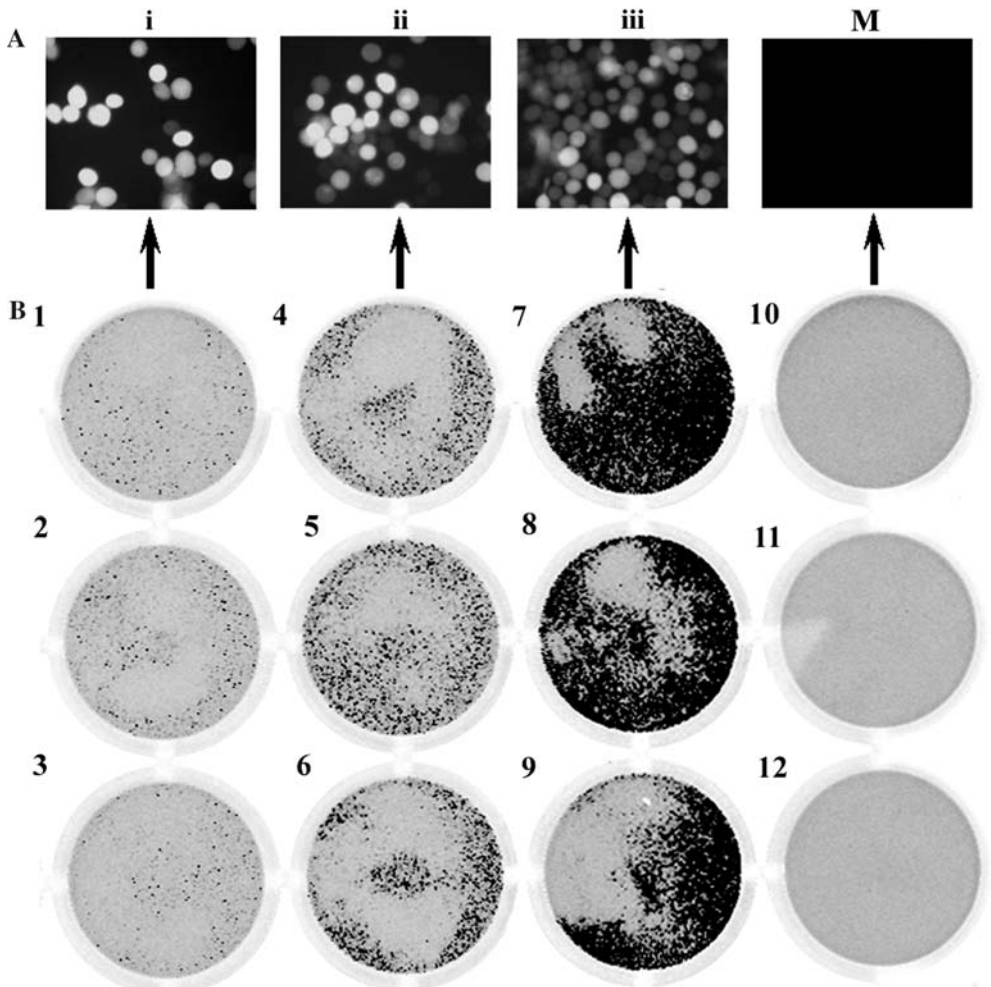

Fig. 1 Imaging of GFP-specific fluorescence. (**A**) Sf9 cell were transfected with the pBAC-5-GFP plasmid and visualized at 72 h post transfection using an inverted fluorescent microscope and GFP S filter system under UV-light. For transfection were used 0.5 μg (i), 1.0 μg (ii) and 2.0 μg (iii) of plasmid DNA and 6 eq of transfection reagent ExGen500. Mock transfected cells (M) also included and represent Sf9 cells transfected with plasmid DNA in the absence of ExGen500. (**B**) The same Sf9 cells were scanned for GFP-specific fluorescence using Typhoon 9,200 variable mode imaging system at the resolution of 25 μm. Each well is 16 mm in diameter. For transfection were used the following ratios of DNA:ExGen500: wells 1, 2, 3–0.5 μg plasmid DNA and 6 eq (1), 8 eq (2), 10 eq (3) ExGen500; wells 4, 5, 6–1.0 μg plasmid DNA and 6 eq (1), 8 eq (2), 10 eq (3) ExGen500; wells 7, 8, 9–2.0 μg plasmid DNA and 6 eq (1), 8 eq (2), 10 eq (3) ExGen500; wells 10, 11, 12-mock, Sf9 cells transfected with plasmid DNA in the absence of ExGen500 (10), Sf9 cells transfected in the absence of plasmid DNA (11), mock Sf9 cells

transfection for 5 days and revealed maximal GFP fluorescence occurred at 72 h post transfection. This is consistent with previous results obtained with the gp64 promoter (Slack and Lawrence 2003; Slack et al. 2001; Chang et al. 1999). Inoculation of cells with plasmid DNA in absence of transfection reagent (i.e., a negative control) resulted in no fluorescent cells.

We have modified the Fermentas Inc. protocol for mammalian cells transfection using ExGen500 for use with Sf9 cells. In most transfection protocols, cells are seeded onto tissue culture plates using growth medium 12 to 24 h prior to transfection to permit strong attachment and adhesion of cells to the bottom of cell culture vessel, an essential feature to prevent loss of cells

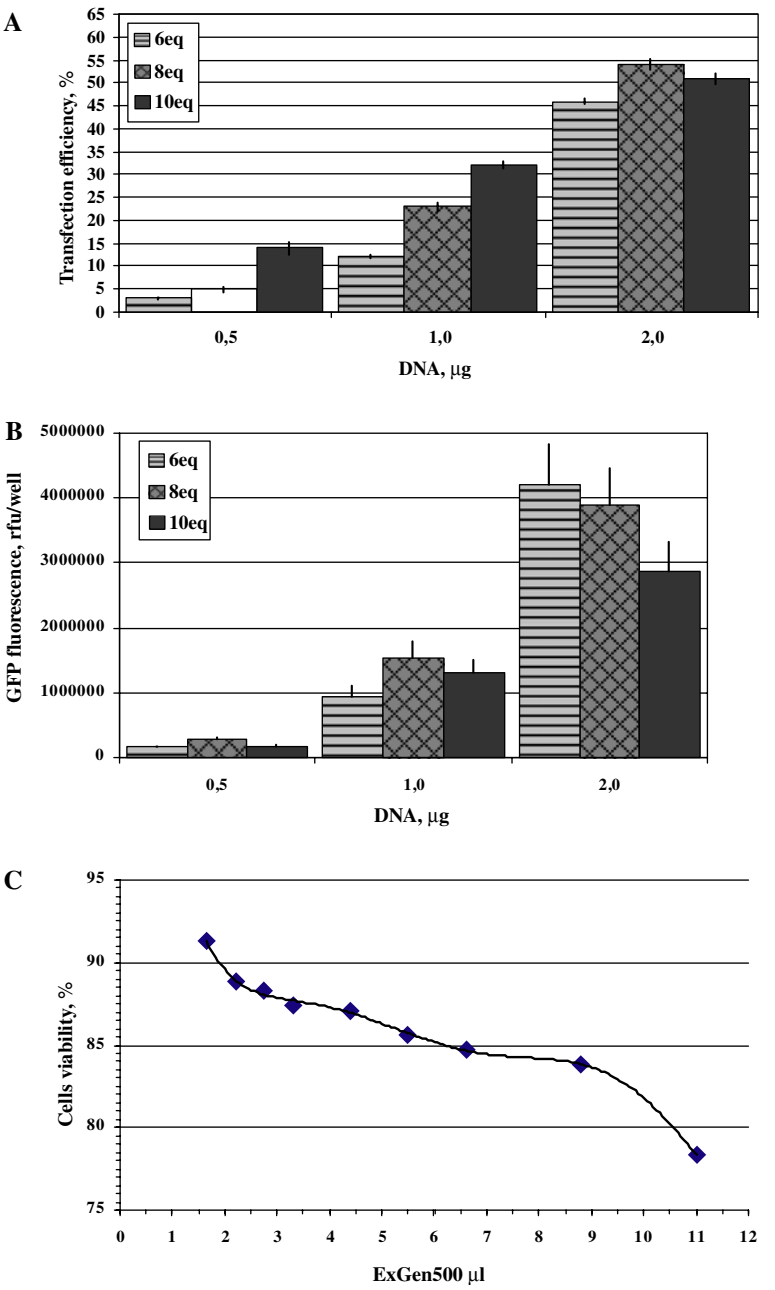

Fig. 2 Influence of different DNA:ExGen500 ratios on the efficiency of transfection (**A**), cellular expression level (**B**) and viability of Sf9 cells (**C**). (**A**) Sf9 cells were transfected using ExGen500 at the different DNA:ExGen500 ratios. At 72 h post transfection the percentage of transfected GFP-positive cells was determined. Experiments were done in triplicate. The error bars represent standard error. (**B**) For determination of cellular expression levels of transfected cells measured as pixel densities or rfu/pixel^2, transfected Sf9 cell were scanned for GFP-specific fluorescence using Typhoon 9,200 variable mode imaging system. Experiments were done in triplicate and the results were measured as pixel densities or rfu/pixel^2. The error bars represent standard error. (**C**) Toxicity of ExGen500 reagent was evaluated by Trypan Blue exclusion. Experiments were done in triplicate

during the changing of medium containing transfection reagent. This stage could be completely eliminated with ExGen500 because the reagent has very low toxicity so changing the medium is unnecessary.

The DNA:ExGen500 ratio was varied for Sf9 cell to determine optimal transfection conditions. For this purpose Sf9 cells were transfected with 0.5, 1.0, and 2.0 μg of plasmid DNA and 6, 8, and 10 eq of ExGen500:. The results of these tests are presented in Fig. 2. The transfection efficiency is improved by increasing the amount of DNA from 0.5 μg to 2.0 μg, as is shown by an increase in GFP-fluorescent cells from 3% to 54% (Fig. 2A). Thus, a functional relation appears between the transfection efficiency with the amount of DNA used, such that the number of GFP-fluorescent cells became maximal at 2.0 μg of DNA: 8 eq ExGen500 reagent and showed no further increase with increasing amounts of ExGen500 reagent. We believe that greater quantities of the transfection reagent may be toxic to the Sf9 cells (Fig. 2C). Additionally, while increasing the amount of DNA for transfection might increase the transfection efficiency further, this also would likely result in increased cytotoxicity. Therefore in the current work the maximum amount of DNA was limited to 2.0 μg since this concentration provided a sufficiently high percentage of transfection and acceptable cell viability level. As for the transfection reagent dose, increasing the ExGen500 from 6 eq to 10 eq at the constant amounts of DNA gave no apparent increase in GFP-positive cells number. Moreover, our data showed the greatest transfection efficiency at 10 eq ExGen500 reagent for 0.5 μg or 1.0 μg DNA and 8 eq for 2.0 μg (Fig. 2A). Figure 2B shows results of cellular expression levels with Sf9 cells transfected at the different DNA:ExGen500 ratios. The optimal level of cellular expression for GFP was observed at 8 eq ExGen500 reagent for 0.5, 1.0, and 2.0 μg DNA and reaches the maximal level at the ratio 2 μg DNA: 6 eq ExGen500 while the cell viability was above 80% with all of the concentrations of ExGen500 that were used (Fig. 2C).

The transfection protocol described here was successfully applied in the transfection of eight insect cell lines derived from *B. mori* (BMN), *T. ni* (TN-368 and IAL-TND1), *H. zea* (BCIRL-HzAM1), *Heliothis virescens* (IPLB-HvE6s), *Anticarsia gemmatalis* (UFL-AG-286) and *S. frugiperda* (IPLB-Sf21 and Sf-9) using both *GFP* and *GUS* marker genes. Transfection efficiency increased from 15% to 40% and from 24% to 52% for BmN and Sf21 respectively, and reached at least 45% in each of the other cell lines (data not shown).

In conclusion, we have shown a new, highly effective method for transfection of insect cell lines using polyethylenimine reagent ExGen500 from Fermentas Inc. that provides high levels of cellular expression of heterologous protein. We obtained transfection efficiencies of at least 45% and high cells viability of 85–90%. This transfection protocol for lepidopteran insect cell lines may also be appropriate for transient gene expression studies in different cell lines, and for comparison of different expression vectors and protein expression in stably transformed insect cell lines.

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