Methods in Cell Science **24**: 155–163 (2002).

Purification of DNA for the transfection of a *Spodoptera frugiperda* cell line

Jeffrey M. Slack & Susan D. Lawrence
United States Department of Agriculture, Agriculture Research Service, Insect Biocontrol Laboratory, Beltsville, MD 20705, USA

Accepted in revised form 23 September 2002

Abstract. *Spodoptera frugiperda* (*Sf*-9) cells have been widely used in baculovirus expression systems, transient gene expression studies and transgenic cell lines. These applications commonly require the transfection of bacterial plasmid DNA. One of the most reliable methods of preparing transfection-quality plasmid DNA is cesium chloride (CsCl) density gradient centrifugation. However, the traditional CsCl DNA purification is a long and laborious process. We have made a series of modifications to the traditional method that makes it faster, safer and easier. In the current study we demonstrate that DNA prepared by our modified CsCl method was also better for the transfection of Sf-9 cells than DNA prepared by the traditional CsCl method.

Key words: Cesium chloride, Density gradient, Plasmid, *Spodoptera frugiperda*, Transfection

1. Introduction

The *Sf*-9 cell line is a clonal isolate from a cell line that was generated from the ovarian tissues of *Spodoptera frugiperda* insect larvae [9]. This lepidopteron cell line has been widely used in recombinant baculovirus expression systems [4]. The engineering of recombinant baculoviruses commonly requires the transfection of bacterial plasmid DNA [4]. *Sf*-9 cells have also been used for transient gene expression studies [8] and for the production of transgenic insect cell lines [6]. These applications also require the transfection of bacterial plasmid DNA.

The traditional method of obtaining transfection-quality DNA has been through CsCl density gradient centrifugation [5]. The routine preparation of DNA by this method is difficult due to long preparation time, hazardous waste generation and safety concerns. Consequently, we have developed a modified CsCl purification method that eliminates or reduces many of the undesirable steps of the traditional CsCl purification method.

Our changes begin at the alkaline lysis extraction of plasmid DNA from bacteria. Alkaline lysis in the traditional CsCl purification, requires high-speed centrifugations and the use of several types of centrifuge rotors. In our alkaline lysis protocol, we employ short time, low speed centrifugations with a single bench top swinging bucket centrifuge. Consequently, our modified CsCl method saves time and materials during the alkaline lysis steps.

Also in our modification, we reduce hazardous waste by reducing CsCl solution volumes and the concentration of ethidium bromide (EtBr). We have also saved time by doubling the relative g force during the centrifugation. Thus, a 36 hour centrifugation time is reduced to 12 hours.

Perhaps the most unpleasant step of the CsCl DNA purification is the collection of the plasmid DNA band. This traditionally involves puncturing of centrifuge tubes with syringe needles. We have eliminated this time consuming and hazardous procedure by simply removing the top off the centrifuge tube and then collecting the DNA band using an elongated plastic pipette tip. We have also eliminated the use of messy mineral oil from the traditional protocol.

Following the collection of the DNA band, EtBr extraction is required. Traditionally, this employs the use of isoamyl alcohol or isobutyl alcohol partitioning. In our modified CsCl method we have substituted these unpleasant smelling solvents with isopropanol. We also perform all the consecutive extractions in a single tube. The upper EtBr/Isopropanol layer is easily removed. The traditional method involves the cumbersome transfer of the lower DNA/CsCl solution to a fresh tube after each extraction. Our method generates less EtBr-contaminated plasticware than the traditional method.

Finally, the traditional CsCl purification protocol employs over-night dialysis to remove CsCl salts. We instead employ ethanol precipitation followed by washing with 70% v/v ethanol. This also significantly reduces DNA preparation time.

In this study, the bacterial plasmid, pBRNX-EGFP [3] was purified by either the traditional CsCl method or by our modified CsCl method. We compared the

yield and quality of DNA. We also transfected *Sf*-9 cells with DNA from each purification. pBRNX-EGFP is a plasmid that expresses the gene for the enhanced green fluorescent protein (EGFP). EGFP is a variant of the green fluorescent protein (GFP) [2] (In this study, we refer to EGFP as GFP). The 'transfection quality' of pBRNX-EGFP DNA was determined by measuring the amount of GFP produced after the transfection of *Sf*-9 cells. Plasmid DNA purified by our modified CsCl method was better for the transfection of *Sf*-9 cells than equivalent material purified by the traditional method.

2. Materials

A. Equipment:

1. Orbital shaker, Model No. 4535.[1, *]
2. Incubator, Model No. BOD10A14, REVCO.[2]
3. Laminar flow hood, Model No. 1200.[1]
4. Bench top (swinging bucket) centrifuge, Model No. 5804R, Eppendorf.[3]
5. Preparative ultracentrifuge, Model No. L8M.[4]
6. Preparative centrifuge, Model No. RC 26 Plus, Sorvall.[5]
7. Bench top ultracentrifuge, Model Optima™ Max 130,000.[4]
8. Centrifuge rotor, Type SLA-3000, Sorvall, Cat. No. 15-700-500.[6]
9. Centrifuge rotor, Type SS-34, Sorvall, Cat. No. 15-700-510.[6]
10. Centrifuge rotor, Type SW-28, Beckman, Cat. No. 342204.[4]
11. Centrifuge rotor, Type 80Ti, Beckman, Cat. No. 341963.[4]
12. Ultem floating spacer (for 8.9 ml Optiseal™ tube), Cat. No. 361670.[4]
13. Centrifuge rotor, Type TLA-110, Beckman, Cat. No. 366735.[4]
14. Ultem floating spacer (for 4.7ml Optiseal™ tube), Cat. No. 361676.[4]
15. Pipetman, Models P-20, P200, P-1000, Gilson.[7]
16. Pipet-aid, Cat. No. 4-000-100.[8]
17. Inverted microscope, Model TE300.[9]
18. Levy Hemocytometer, Cat. No. 3500.[10]
19. Endow GFP filter cube (HQ 470/40 nm EX, Q495LP BS, HQ525/50 nm EM), Cat. No. 41017.[11]
20. Cooled charge-couple device (CCD) camera, Model LAS 1000.[12]
21. Variable mode imager (Molecular Dynamics) Model Typhoon 9200.[13]
22. Electrophoresis power supply (BioRAD) PowerPack 300.[14]
23. Agarose gel box Wide Mini-sub Cell GT, Cat. No. 165-5004.[14]
24. 20-well agarose gel comb, Cat. No. 170-4447.[14]
25. UV/Visible Spectrophotometer Model Ultrospec 3000.[15]
26. Photoshop 5.0 software.[16]
27. ImageQuant™ v5.2 software (Molecular Dynamics).[13]
28. Microsoft Excel2000 software.[17]
29. Gloves powder free latex, Cat. No. EV-2050-L.[18]
30. Goggles, Visorgogs™ (Jones & Co.).[19]
31. 10 inch slip joint pliers, Cat. No. 00945380000.[20]

B. Culture mediums and reagents:

1. Complete media
   - 90% v/v Grace's insect medium (Supplemented) 1X, Cat. No. 11605-094.[21]
   - 10% v/v Fetal bovine serum (heat inactivated), Cat. No. 16140-071.[21]
2. Complete media plus added antibiotics
   - 88% v/v Grace's insect medium (Supplemented) 1X, Cat. No. 11605-094.[21]
   - 10% v/v Fetal bovine serum (heat inactivated), Cat. No. 16140-071.[21]
   - 1% v/v 250 g/ml w/v Fungizone (Amphotericin B), Cat. No. 15290-018.[21]
   - 1% v/v 10,000 U/ml Penicillin G – 1 mg/ml Streptomycin, Cat. No. 15140-148.[21]
3. FBS/Graces salts
   - 10% v/v Fetal bovine serum (FBS) (heat inactivated), Cat. No. 16140-071.[21]
   - 90% v/v Grace's insect medium 1X, Cat. No. 11590-056.[21]
4. 1 Kb DNA ladder, Cat. No. 15615-016.[21]
5. Luria-Bertani (LB) media
   - 10 g/L w/v Bacto™ yeast extract, Cat. No. 0127-12-9.[22]
   - 5 g/L w/v Bacto™ Tryptone, Cat. No. 0123-17-3.[22]
   - 10 g/L w/v NaCl, Cat. No. S-3014.[23]
6. $CaPO_4$ transfection buffer (pH 7.1)
   - 140 mM NaCl, Cat. No. S-3014.[23]
   - 125 mM $CaCl_2$, Cat. No. C-5080.[23]
   - 25 mM HEPES, Cat. No. H-8651.[23]
7. STET (Sodium Tris EDTA Triton) buffer (pH 7.8)
   - 100 mM NaCl, Cat. No. S-3014.[23]
   - 1 mM EDTA (Disodium salt), Cat. No. E-1644.[23]
   - 10 mM Tris-HCl (Trizma HCl), Cat. No. T-3253.[23]
8. Suspension buffer (pH 8.0)
   - 50 mM Glucose (Dextrose), Cat. No. G-7528.[23]
   - 25 mM Tris-HCl (Trizma HCl), Cat. No. T-3253.[23]
9. Lysis buffer
   - 0.2 N NaOH, Cat. No. S-8045.[23]
   - 1% w/v SDS (Sodium dodecyl sulphate), Cat. No. L-4390.[23]

10. Neutralization buffer
    - 3 M Potassium Acetate, Cat. No. P-9333.[23]
    - 2 M Acetic Acid, Cat. No. A-6283.[23]
11. TE (Tris EDTA) buffer (pH 8.0)
    - 1 mM EDTA (Disodium salt), Cat. No. E-1644.[23]
    - 10 mM Tris-HCl (Trizma HCl), Cat. No. T-3253.[23]
12. 20X SSC (Saline Sodium Citrate) (pH 7.0)
    - 3 M NaCl, Cat. No. S-3014.[23]
    - 300 mM Sodium Citrate, Cat. No. S-4641.[23]
13. 20X SSC-saturated isopropanol (isopropanol will partition to the top)
    - 250 ml isopropanol, Cat. No. A451-4.[6]
    - 250 ml 20X SSC.
14. 50X TAE (Tris Acetate EDTA) buffer (pH 8.0)
    - 2M Tris base (Trizma Base), Cat. No. T-6791.[23]
    - 1 M Acetic Acid, Cat. No. A-6283.[23]
    - 50 mM EDTA (Disodium salt), Cat. No. E-1644.[23]
15. DNA sample loading buffer
    - 10X TAE buffer.
    - 7% w/v Sucrose, Cat. No. S-7903.[23]
    - 10% w/v Glycerol, Cat. No. G-6279.[23]
    - 0.5% w/v Bromophenol blue dye, Cat. No. B-5525.[23]
16. CsCl (Ultrapure), Cat. No. US75822.[24]
17. Ethidium bromide, Cat. No. E-8751.[23]
18. Ethanol 200 Proof, Cat. No. 64-17-5.[25]
19. Mineral oil, Cat. No. M-5904.[23]
20. Isopropanol, Cat. No. A451-4.[6]
21. Isoamyl alcohol (Biotech Research Grade), Cat. No. BP1150-500.[6]
22. Agarose, Cat. No. 15510-027.[21]
23. Lysozyme (Egg White), Cat. No. 0663-10G.[26]
24. Ampicillin Sodium Salt, Cat. No. BP1760-25.[6]

C. Plastics, glassware and disposables:
1. 2 L Erlenmeyer flask Pyrex Brand, Cat. No. 4980-2L.[27]
2. 250 ml Erlenmeyer flask Pyrex Brand, Cat. No. 4980-250.[27]
3. Pyrex baking dish 2 L 28 × 18 × 4 cm, Cat. No. 232-R.[27]
4. 500 ml screw capped polypropylene centrifuge bottle, Cat. No. 3120-9500PP.[28]
5. 28 ml Oakridge Type polycarbonate centrifuge tube, Cat. No. 3118-0028PC.[28]
6. SW-28 thick wall polycarbonate ultracentrifuge tube, Cat. No. 336091.[4]
7. 50 ml screw capped polypropylene tube, Cat. No. 2098.[29]
8. 15 ml screw capped polypropylene tube, Cat. No. 430791.[27]
9. 8.9 ml polyallomer Optiseal™ centrifuge tube, Cat. No. 361623.[4]
10. 4.7 ml polyallomer Optiseal™ centrifuge tube, Cat. No. 361623.[4]
11. Tissue culture flask (Greiner), Cat. No. 658170.[30]
12. 24-well 16 mm diameter plate, Cat. No. 3524.[31]
13. Plastic sealed container, Cat. No. 3873-87.[32]
14. Serological pipette 10 ml, Cat. No. 7551.[33]
15. 1,000 µl disposable pipette tip, Cat. No. P3290-1800.[34]
16. 200 µl disposable pipette tip, Cat. No. P3290-1510.[34]
17. 200 µl disposable microcapillary pipette tip, Cat. No. 02-707-81.[6]
18. 96-well plate, Cat. No. 3072.[29]
19. 3cc syringe, Cat. No. 309585.[33]
20. 21 gauge syringe needle Cat No. 305165.[33]
21. Dialysis tubing (Cellulose membrane), Cat. No. D-9777.[23]
22. Dialysis Tubing Closures, Cat. No. 132 736.[35]
23. Filter paper 3MM, Cat. No. 3030-917.36

D. Cell lines:
1. DH5α bacterial strain.[21]
2. *Sf*-9 insect cell line, Cat. No. B825-01.[37]

E. Plasmid:
1. pBRNX-EGFP (4318 base pairs) [3].

3. Procedures

A. Bacteria growth:
1. In a 2 L Erlenmeyer flask, combine 500 ml of bacterial LB media with 100 mg of Ampicillin. Inoculate with DH5α bacteria that had been previously transformed with the plasmid pBRNX-EGFP. Shake the culture overnight at 37 °C at 200 rpm.
2. The next day, using a P-1000 pipetman, remove a 1 ml aliquot of media and measure the optical density at 595 nm (OD^{595}) on a UV/visible spectrophotometer. Continue shaking until the OD^{595} reaches or exceeds 1.2.
3. Transfer the culture to a 500 ml screw capped polypropylene centrifuge bottle and centrifuge at 4,000 ×*g* for 10 min at 4 °C (Sorvall, SLA-3000 Rotor).

B. CsCl density gradient DNA purification protocols, comparison of two methods:
1. Traditional CsCl DNA purification procedure based on Maniatis et al. (1982).
   a) Suspend the bacterial pellet in 100 ml of STET buffer and centrifuge at 4,000 ×*g* for 10 min at 4 °C (Sorvall, SLA-3000 rotor). Discard the supernatant and suspend the bacterial pellet in 9 ml of Suspension buffer. Transfer the suspension using a 10 ml serological pipette into a 50 ml polypropylene conical screw capped tube.
   b) Freshly prepare 1 ml of a 50 mg/ml

solution of lysozyme in Suspension buffer in a 15 ml screw capped polypropylene tube. Add the lysozyme solution to the bacterial suspension and incubate for 5 min at room temperature.

c) Using a 10 ml serological pipette, add 20 ml of Lysis buffer, mix gently and let stand on ice for 10 min.
d) Add 15 ml of Neutralization buffer, mix gently and let stand on ice for 10 min.
e) Transfer the mixture proportionally into two 38.5 ml, thick walled, polycarbonate tubes and centrifuge at 53,000 ×*g* for 20 min at 4 °C (Beckman, SW-28 rotor).
f) Transfer the supernatant proportionally into two 28 ml Oak Ridge type screw cap tubes.
g) Using a 10 ml serological pipette, add 13.5 ml of isopropanol, mix and let stand for 15 min at room temperature. Centrifuge at 12,000 ×*g* for 30 min at room temperature (Sorvall, SS-34 rotor).
h) Discard the supernatant and wash with 5 ml of 70% v/v ethanol using a 10 ml serological pipette. Centrifuge at 12,000 ×*g* for 5 min at room temperature (Sorvall, SS-34 rotor). Drain away the 70% v/v ethanol onto paper towels for 5 min.
i) Using a 10 ml serological pipette, add 6 ml of TE buffer into the tube and dissolve the crude DNA pellet by gentle agitation.
j) Add 6 g of CsCl, dissolve and then using a P-1000 pipetman, transfer the crude DNA solution to an 8.9 ml Optiseal™ polyallomer centrifuge tube.
k) Wearing latex gloves and using a P-1000 pipetman, add 500 µl of 10 mg/ml EtBr and mix. Fill the remaining tube volume with mineral oil.
l) Seal the polyallomer tube using the o-ring cap that comes with the tube. Place the tube into a fixed angle rotor along with an Ultem floating spacer and centrifuge 134,000 ×*g* for 36 hours at 22 °C (Beckman, 80 Ti rotor).
m) Wearing safety goggles and latex gloves, puncture near the top of the tube with a 21 gauge needle and then collect the DNA band by puncturing the side with a 3 cc syringe and a 21 gauge needle. Deposit the collected DNA into a 15 ml screw capped polypropylene centrifuge tube. Discard the remaining CsCl/EtBr into a hazardous waste disposal station.
n) Using a P-1000 pipetman, add one volume H_2O-saturated isoamyl alcohol to the collected DNA and extract EtBr by shaking vigorously for 10 s. Centrifuge at 4500 ×*g* for 5 min at 20 °C in a bench top swinging bucket centrifuge. Using a P-1000 pipetman, remove the lower DNA/CsCl solution and transfer it into a new 15 ml tube. Discard the remaining isoamyl alcohol layer into a hazardous waste disposal station. Repeat the H_2O-saturated isoamyl alcohol extraction two more times or more if EtBr staining persists in the lower DNA layer.
o) Transfer the DNA solution into 12,000 kDa dialysis tubing and dialyze for 2 hours against a 1 L volume of TE buffer. Repeat the dialysis against a 3 L volume of TE buffer for 15 hours at 6 °C. Drain the dialyzed DNA into a 15 ml screw capped tube.
p) Store dialyzed DNA at 4 °C.

2. Modified CsCl DNA purification method:
   a) Using a 10 ml serological pipette, suspend the bacterial pellet in 7 ml of Suspension buffer and transfer the suspension to a 50 ml polypropylene conical tube.
   b) Add 14 ml of Lysis buffer and shake vigorously for 10 s.
   c) Add 10.5 ml of Neutralization buffer and shake vigorously for 10 s.
   d) Centrifuge at 4,500 ×*g* for 5 min at 20 °C in a bench top swinging bucket centrifuge.
   e) Decant the supernatant into a new 50 ml polypropylene conical tube. Part of the insoluble debris will float as a 'plug' that can be gently pushed to one side with a 1,000 µl disposable pipette tip in order that the supernatant be released.
   f) Using a 10 ml serological pipette, add 19 ml of isopropanol, mix and let stand for 10 min at room temperature.
   g) Centrifuge at 4,500 ×*g* for 5 min at 20 °C in a bench top swinging bucket centrifuge. Discard the supernatant and invert the tubes onto paper towels for 5 min such that the supernatant residue drains away from the crude DNA pellet.
   h) Dissolve the DNA pellet in 3 ml of H_2O. Add H_2O using a P-1000 pipetman in such a manner that the pellet is dislodged from the bottom of the tube.
   i) After the DNA pellet has dissolved, dissolve 3.6 g of CsCl into the solution. Using a P-1000 pipetman, transfer the crude DNA/CsCl solution to a 4.7 ml Optiseal™ polyallomer centrifuge tube.
   j) Wearing latex rubber gloves and using a P-200 pipetman, add 50 µl of (10 mg/ml) EtBr and then fill the remaining volume of the tube with 1.1 g/ml CsCl solution such that mixing occurs.
   k) Seal the polyallomer tube using the o-ring cap that comes with the tube. Place the tube into a fixed angle centrifuge rotor along

with an Ultem floating spacer and centrifuge at 260,000 ×*g* overnight (12 hours) at 22 °C (Beckman-Coulter, TLA-110 rotor).

l) Wearing safety goggles and latex rubber gloves, remove the lid of the tube using pliers. Collect the DNA band using a P-1000 pipetman and a 1000 µl pipette tip inserted into a 200 µl microcapillary tip (Figure 1). Deposit the collected DNA into a 15 ml polypropylene screw capped tube. Discard the remaining CsCl/EtBr into a hazardous waste disposal station.

m) Using a P-1000 pipetman, add 800 µl of 20 X SSC-saturated isopropanol and shake vigorously for 10 s. Allow 30 s for the isopropanol/EtBr to partition to the top layer. Remove the upper isopropanol/EtBr layer and discard in a hazardous waste disposal station. Repeat the isopropanol extraction two more times or more if EtBr staining persists in the lower DNA/CsCl layer.

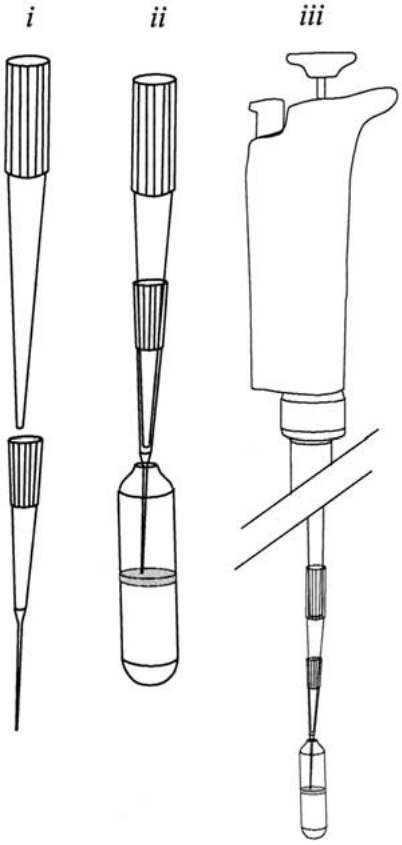

Figure 1. Collection of DNA bands using disposable plastic pipette tips. (i) A 1,000 µl disposable pipette tip (top) is inserted into a 200 µl disposable microcapillary tip (bottom). (ii) The stacked pipette tips then access the DNA band through the top of the CsCl gradient. The microcapillary tip minimizes disruption of the gradient during band collection and extends the reach of the 1,000 µl tip. (iii) This tip arrangement is mounted onto a P-1000 pipetman that can be used to collect the DNA band in one smooth step.

n) Add H_2O until the total volume is 5 ml, mix and then add 10 ml of ethanol. Mix and let stand at –20 °C for 10 min.

o) Centrifuge at 4500 ×*g* for 5 min at 20 °C in a bench top swinging bucket centrifuge. Discard the supernatant and invert the tubes onto paper towels for 5 min such that the supernatant residue drains away from the crude DNA pellet. Wash the pellet with 1 ml of 70% v/v ethanol and then dry the pellet in air for 30 min.

p) Dissolve the DNA pellet in 1 ml of sterile TE buffer and store at 4 °C.

C. Quantification of DNA:

1. Base quantification on a DNA standard of previously purified pBRNX-EGFP plasmid DNA. In a 96-well plate, make the 75/25 serial dilution series of a 500 ng/µl DNA standard across 12 wells. With a P-200 pipetman, add 100 µl of 500 ng/µl DNA standard to the first well. Fill the remaining 11 wells in one row with 25 µl of 1X TAE buffer and serially passage 75 µl of the DNA standard consecutively into each well. With a P-20 pipetman, add 10 µl of DNA sample loading buffer to each well. In the next row of wells, using a P-20 pipetman, add 10 µl of each DNA sample to be quantified into its own well along with 20 µl of 1X TAE buffer and 10 µl of DNA sample loading buffer.
2. Load 30 µl of each DNA standard and each DNA sample into the wells of a 0.8% w/v agarose gel. Fractionate plasmid DNA in the agarose gel at 100 V until the bromophenol blue dye reaches the middle of the gel. Stain the agarose gel in a Pyrex baking dish for 30 min in 500 ml of 1X TAE buffer and 1 mg/L EtBr.
3. Place the agarose gel onto the scanning bed of a Typhoon variable mode imaging system. Scan the gel using a green laser (532 nm) excitation source. Record fluorescence emitted (610 nm, 30 nm band pass filter) using the Typhoon photomultiplier tube (PMT) at 100 µm resolution (500V). PMT readings will be converted to 16-bit digital images by the Typhoon instrument. Use ImageQuant software to quantify pixels in each DNA sample lane and use Microsoft Excel to estimate DNA concentration from standards.

D. Culturing insect cells:

Carry out all cell culture manipulations in a laminar flow hood using sterile technique. Propagate *Sf*-9 cells in 75 cm^2 T-flasks at 28 °C in 11 ml of complete media. Passage cells weekly by bumping off cells to suspend them. Using a 10 ml serological pipette, remove 1 ml of sus-

pended cells, combine with 10 ml of complete media and transfer this dilution to a new 75 cm^2 T-flask.

E. Plasmid DNA Transfections (calcium phosphate precipitation):
   1. Remove the complete media from an *Sf*-9 cell confluent 75 cm^2 T-flask and replace with 10 ml of FBS/Graces salts using a 10 ml serological pipette. Suspend the cells by gently bumping the T-flask then pour the cells into a 50 ml sterile screw capped polypropylene tube. Count cells using a Levy hemocytometer and an inverted microscope. Dilute the suspended cells in FBS/Graces salts to a density of 1.5×10^6 cells/ml.
   2. Using a P-1000 pipetman, add 300 µl of suspended cells to each well of a 24-well tissue plate. Allow 1 hour for cells to attach at 22 °C.
   3. Dilute all DNA samples in TE buffer to a DNA concentration of 500 ng/µl. In a 96-well plate, make a 50/50 serial dilution series of each DNA sample. With a P-200 pipetman, add 200 µl of 500 ng/µl DNA sample to the first well of each row of a 96-well plate. Fill the adjacent 6 wells in each row with 100 µl of TE buffer and serially dilute 100 µl from each DNA sample across each row. In a new 24-well plate, combine 100 µl of each plasmid DNA dilution with 1,000 µl of $CaPO_4$ transfection buffer.
   4. Using a P-1000 pipetman, drip 330 µl of $CaPO_4$ transfection buffer/DNA mixture onto previously seeded cells. Mix the transfection solution with the already present 300 µl of FBS/Graces salts by swirling the 24-well plate for 10 sec. A cloudy precipitant will became visible. Incubate the transfected *Sf*-9 cells for 3.5 hours at 22 °C.
   5. Remove the transfection solutions by inverting the 24-well plates over a Pyrex baking dish and then using a 10 ml serological pipette, add 500 µl of complete media with added antibiotics.
   6. Incubate transfected cells at 28 °C in a plastic sealed container. In addition, add a damp paper towel to the container to maintain humidity.

F. Visualization and Quantification of the GFP:
   1. Visualize transfected *Sf*-9 cells directly in 24-well plates using an inverted fluorescence microscope. Visualize GFP fluorescence using a UV light source and an Endow GFP filter cube.
   2. Photograph using a CCD camera system.
   3. Quantify GFP by placing 24-well plates onto the scanning bed of a Typhoon variable mode imaging system. Place 3MM Whatman filter paper strips (0.5 cm × 15 cm) under the edges of the 24-well plates such that they lift the plates slightly and prevent scratching of the scanning bed. Set the scanning focus to 3 mm above the scanning bed and scan the plates using a green laser (532 nm) excitation source (Set the machine to press the plates during scans). Record fluorescence emitted (526 nm, short pass filter) using the Typhoon PMT at 100 um resolution (600V). PMT readings will be converted to 16-bit digital images by the Typhoon instrument.
   4. Use ImageQuant software to subtract background by adjusting the grey scale range (i.e. high range = 23,942, low range = 1,145). Save the image as an 8-bit TIFF file and use ImageQuant to quantify the pixel counts in each well. Use these data as relative fluorescence of GFP.

G. Calculations:

$$\text{Standard error} = \frac{\sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \overline{x})^2}}{\sqrt{n}}$$

n = the number of data points
x_i = the data point value
$\overline{x}$ = the average data point value

4. Results and discussion

CsCl density gradient purification of DNA is one of the foundation protocols of molecular biology [7]. The traditional CsCl DNA purification is a long and involved protocol. Our refinements to the protocol were implemented to simplify the routine purification of plasmid DNA for the transfection of *Sf*-9 cells. In addition to producing transfection-quality DNA, we were concerned with reducing preparation time, reducing hazardous waste and improving safety. Our modifications have significantly reduced DNA preparation time. The traditional CsCl density gradient method required nearly one week to complete whereas our modified CsCl method is easily completed within two days. The major time constraints of the traditional CsCl method are the long centrifugation time and the over-night dialysis. Actual labor for our modified CsCl method is less than 3 hours while the traditional CsCl method requires 6 to 8 hours of labor due to changes in tube types, manipulations with syringe needles and transferal to dialysis tubing.

We have reduced the volume of CsCl solution by 50% and the amount of EtBr by 90%. Waste reductions are also made in disposable items. The traditional CsCl purification protocol requires a total of 10 tubes while our modified CsCl purification requires only 4 tubes. Reducing the materials used becomes significant over the long term or when larger numbers of DNA preparations are required. Our substitution of disposable gel-loading tips

dramatically improves safety of the protocol. In addition, there is no need to dispose of hazardous syringe needles.

In this group of experiments, the traditional CsCl protocol produces plasmid DNA that appears cleaner. We observe apparent RNA contamination in the DNA that is prepared by our modified CsCl method (Figure 2A). Pre-treatment with RNAseH during the alkaline lysis step would likely have eliminated this problem. Our modified method produced some contaminating material in the wells of the agarose gel. This may be either protein or bacterial chromosomal DNA. In this study, the traditional method had a slightly higher DNA yield than did our modified method (Figure 2B). We did not observe significant differences in the amount of supercoiled DNA between preparations.

In spite of the appearance of lower quality on gels, our modified method produced DNA that was significantly better for transfection. pBRNX-EGFP plasmid DNA from both purifications was transfected onto *Sf*-9 cells and the amount of GFP produced in cells was monitored using fluorescence microscopy and Typhoon fluorescence imaging system (Figure 3). The fluorescence microscopy permitted the confirmation of GFP-specific fluorescence (Figure 3A), while the Typhoon fluorescence imaging system permitted the detection of total fluorescence per tissue culture plate well (Figure 3B).

Approximately 20% more GFP-specific fluorescence was detected in tissue culture plates that had been transfected with plasmid DNA from the modified CsCl purification method (Figure 4). We are uncertain as to why this difference occurred between DNA preparations. The observed contaminants in our purification may act as co-precipitants in the calcium phosphate transfection. We cannot preclude that the method of transfection may also be a factor. Other transfection methods such as liposome mediated ones may not reflect this difference. We routinely employ the calcium phosphate precipitation due to excellent reproducibility and due to the long-term stability of reagents.

We have purified DNA by our modified CsCl method for well over 100 preparations. Our yields range between 0.5 to 2 mg from 500 ml cultures. The plasmid type is often a major factor in yield. Plasmid DNA has a high level of stability when prepared by

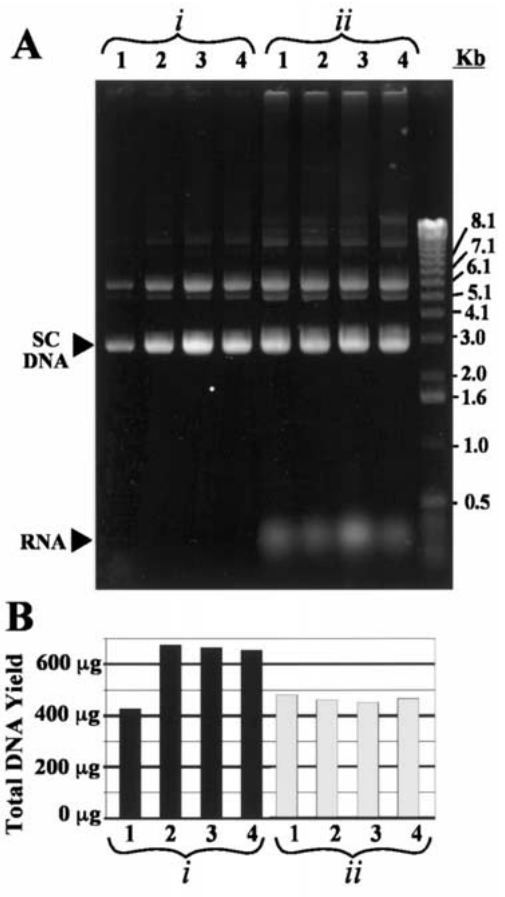

Figure 2. DNA yield from the traditional and modified methods of CsCl gradient purification. (A) DNA samples from the traditional CsCl method (i) and DNA from the modified CsCl method (ii) were separated on a 0.8% w/v agarose, 1X TAE gel and then stained with EtBr. DNA obtained from four separate purifications is shown for both methods. A DNA standard is included on the right most lane. The super coiled DNA (SC) and RNA are indicated (RNA) by the carats on the left side. (B) Total DNA yield of each preparation is shown on the bar graph.

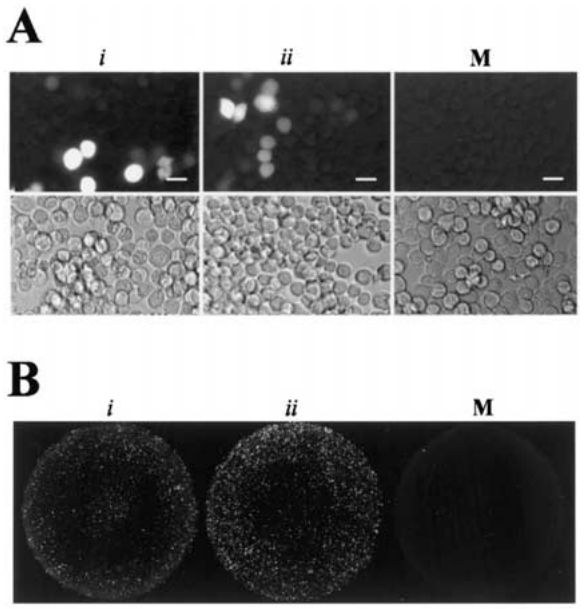

Figure 3. Imaging of GFP-specific fluorescence. (A) *Sf*-9 cells were transfected with the pBRNX-EGFP plasmid and were visualized at 120 hours post transfection using an inverted fluorescence microscope both under UV light (top panels) and visible light (bottom panels). Plasmid DNA was prepared either by the traditional CsCl method (i) or the modified CsCl method (ii). Mock transfected cells (M) are also included. The bars represent 10 µm. (B) The same cells were scanned for GFP-specific fluorescence using a Typhoon variable mode imaging system at a resolution of 25 µm. Each well is 16 mm in diameter.

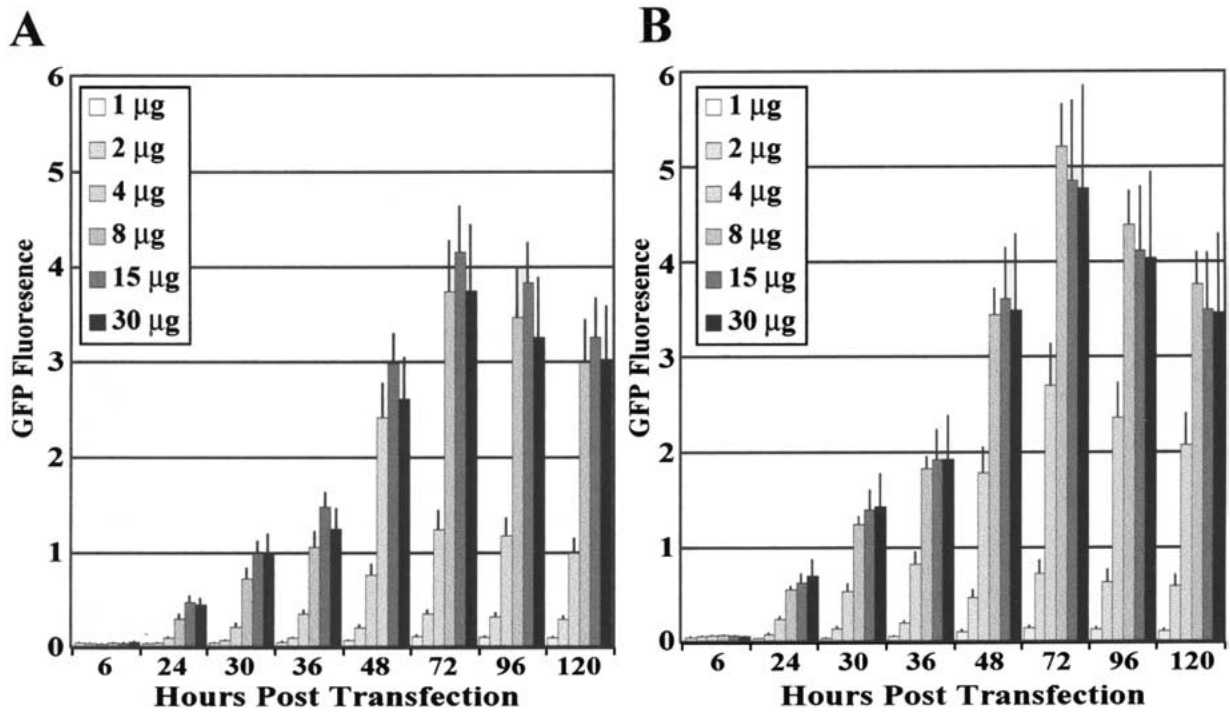

Figure 4. Comparison of pBRNX-EGFP reporter expression. *Sf*-9 cells were transfected with varying amounts of pBRNX-EGFP plasmid DNA that had been prepared by either the traditional CsCl method (Graph A) or the modified CsCl method (Graph B). Over a 120 hour time period transfected cells were scanned for GFP-specific fluorescence (See Procedures, Part F). For each purification method, the DNA from four plasmid purifications was transfected in triplicate onto 24-well plates of *Sf*-9 cells. Each bar represents the average fluorescence from 12 replicate transfections. Each vertical axis unit is 1000 ImageQuant counts/pixel. The error bars represent standard error.

our modified method. The DNA can be stably stored at 10 °C in H_2O for at least two years without apparent degradation. The DNA is also readily usable for cloning and for sequencing.

The amount of GFP fluorescence became maximal at 8 µg of DNA for both DNA preparations. For both DNA preparations, a plateau of fluorescence was reached at 8 µg and amounts of GFP fluorescence did not increase further with 16 µg or 32 µg of DNA. Thus, the 20% higher maximal fluorescence that was observed from our modified DNA purification was not due to discrepancies in DNA amounts. It should be noted that we were within the linear range of a serially diluted soluble GFP standard (Data not shown).

The time of maximal GFP fluorescence was at 72 hours post transfection for DNA from both purifications. This was unexpected since use of the same promoter in a different study had reported maximal expression at 36 hours post transfection [1]. However that study employed a *Lymantria dispar* cell line and produced a different transient protein product.

In recent years, many affinity column kits for plasmid DNA purification have been introduced. One of the major selling points of these kits is that they are easier alternatives to CsCl gradient purification. We have tried using one of these kits (Qiagen's MegaPrep™ endotoxin free plasmid purification kit). Similar DNA yields were produced and the DNA quality was excellent for transfection. However, we found that our modified CsCl method required less time and effort than that kit. Regardless of time and effort, kits have a major disadvantage that the user must blindly trust the DNA that comes out of the kit. In a cesium gradient, one can observe the quality and quantity of the DNA being purified. The DNA band can then be discriminantly collected with the knowledge that RNA, protein and other contaminants have been separated away. With kits one must worry about whether the RNAse treatment was fully effective, whether the plasmid DNA remained supercoiled, whether genomic DNA fragments were present and whether unknown kit contaminants such as resins have leached into the sample.

We conclude from this study that our modified CsCl DNA purification procedure produced DNA that was superior for the transfection of *Sf*-9 cells. With our modified protocol, the antiquated CsCl gradient purification was transformed into an easy and efficient way to purify plasmid DNA for transfection.

Acknowledgements

We thank Dwight Lynn for reviewing this manuscript and for his insightful comments.

Note

* Disclaimer: Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Notes on suppliers

1. ThermoForma, P.O. Box 649, Marietta, OH 45750, USA
2. GS Laboratory Equipment, 275 Aiken Rd., Asheville, NC 28804, USA
3. Brinkman Instruments, Inc., One Cantiague Rd., P.O. Box 1019, Westbury, NY 11590-0207, USA
4. Beckman-Coulter Inc., P.O. Box 3100, 4300 North Harbor Blvd., Fullerton, CA 92834 USA
5. Kendro Laboratory Products, 31 Pecks Ln., Newtown, CT 06470-2337, USA
6. Fisher Scientific, 3970 Johns Creek Court Suite 500, Suwanee, GA 30024, USA
7. Rainin Instrument Co., Inc., P.O. Box 4026, Mack Rd., Woburn, MA 01888-4026, USA
8. Drummond Scientific Co., 500 Parkway, P.O. Box 700, Broomall, PA 19008, USA
9. Nikon Instruments Inc., 1300 Walt Whitman Rd., Melville, NY 11747-3064, USA
10. Hausser Scientific Co., 935 Horsham Rd., Suite C, Horsham, PA 19044-1286, USA
11. Chroma Technology Corp., Brattleboro, VT 05301, USA
12. Fuji Medical Systems Inc., Stamford, CT 06912, USA
13. Amersham Biosciences Corp., P.O. Box 1327, 800 Centennial Ave., Piscataway, NJ 08855-1327, USA
14. Bio-Rad Laboratories, 1000 Alfred Nobel Dr., Hercules, CA 94547, USA
15. Amersham Pharmacia Biotech Ltd., Little Chalfont, Buckinghamshire, UK
16. Adobe Systems Incorp., San Jose, CA 95110, USA
17. Microsoft Corp., One Microsoft Way, Redmond, WA 98052-6399, USA
18. Midwest Scientific, 280 Vance Rd., Valley Park, MO 63088, USA
19. FISCO, 1808 3rd. Ave., South East, Rochester, MN 55904, USA.
20. Sears, White Oak S/C, 11255 New Hampshire Ave., Silver Spring, MD 20904, USA
21. Life Technologies, 3175 Staley Rd., Grand Island, NY 14072, USA
22. DIFCO Laboratories, Detroit, MI 48232-7058, USA
23. Sigma, P.O. Box 14508, St. Louis, MO 63178, USA
24. USB, 26111 Miles Rd., Cleveland, OH 44128, USA
25. The Warner-Graham Co., Cockeysville MD, 21030, USA
26. Amresco, Solon, OH 44139, USA
27. Corning Inc., Corning, NY 14831, USA
28. Nalgene Nunc Inc., P.O. Box 20365, 75 Panorama Creek Dr., Rochester, NY 14602-0365, USA
29. Becton Dickenson and Co., Lincoln Park, NJ 07035, USA
30. Applied Scientific, 154 West Harris Ave., South San Francisco, CA 94080, USA
31. Costar, 205 Broadway, Cambridge, MA 02139, USA
32. Rubbermaid, Wooster, OH 44691-6000, USA
33. Becton Dickenson and Co., Franklin Lakes, NJ 07417, USA
34. ISC BioExpress, 420 North Kays Dr., Kaysville, UT 84037, USA
35. Spectrum Laboratories Inc., 18617 Broadwick St., Rancho Dominguez, CA 90220-6435, USA
36. Whatman Inc., 9 Bridewell Place, Clifton, NJ 07014,USA.
37. Invitrogen Corp., P.O. Box 6482, 1600 Faraday Ave., Carlsbad, CA 92008, USA

References

1. Blissard GW, Wenz JR. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. J Virol 1992; 66(11): 6829–6835.
2. Chalfie M, Tu Y, Euskirchen G, Ward WW. Prasher DC. Green fluorescent protein as a marker for gene expression, Science 1994; 263(5148): 802–805.
3. Chang M-J, Kuzio J, Blissard GW. Modulation of translational efficiency by contextual nucleotides flanking a baculovirus initiator AUG codon. Virology 1999; 259(2): 369–683.
4. Kitts PA, Ayres MD, Possee RD. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. Nucleic Acids Res 1990; 18(19): 5667–5672.
5. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Laboratories, 1982.
6. Monsma SA, Oomens AG, Blissard GW. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J Virol 1996; 70: 4607–4616.
7. Radloff R, Bauer W, Vinograd J. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: The closed circular DNA in HeLa cells. PNAS 1967; 57: 1514–1521.
8. Slack JM, Blissard GW. Identification of two independent transcriptional activation domains in the *Autographa californica* multicapsid nuclear polyhedrosis virus IE1 protein. J Virol 1997; 71: 9579–9587.
9. Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). *In Vitro* 1977; 13: 213–217.

Author for Correspondence: Susan D. Lawrence, United States Department of Agriculture, Agriculture Research Service, Insect Biocontrol Laboratory, Bldg. 011A, Rm 214, Beltsville, MD 20705, USA
Phone: (301) 504-6144; Fax: (301) 504-5104
E-mail: lawrencs@ba.ars.usda.gov