Rapid and Gentle Method for the Isolation of DNA from Nuclear Polyhedrosis Viruses

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The baculoviruses are a large family of double-stranded DNA viruses that infect holometabolous insects (4). They are now widely used as eukaryotic expression vectors and as biopesticides. Use of baculoviruses for the expression of foreign genes requires the isolation of pure viral DNA for transfection in permissive insect cell lines. The genotypic identity of a baculovirus is also a prerequisite for its use as a biopesticide and in molecular studies. Several methods such as characterization of viral proteins by polyacrylamide gel electrophoresis, enzyme-linked immunosorbent assay (ELISA), serological characterization and biochemical identification of nucleic acids have been investigated (3), but restriction enzyme digestion patterns of the viral DNA is the most sensitive and reliable method for this purpose because no two clones of viruses have the same restriction endonuclease band pattern (9).

Baculovirus DNA from polyhedra is usually isolated from occluded virus after dissolving the polyhedra either in dilute alkali, followed by isolation of polyhedra-derived virus (PDV), or directly in strong alkali (7). The yield of viral DNA is poor when dilute alkali is used, and the DNA isolated by using strong alkali often renders it unsuitable for transfection. This requires extensive dialysis to make the DNA pure for restriction enzyme digestion and for transfection.

Uses of chaotropic agents like guanidine hydrochloride and guanidinium isothiocyanate (GIT) in isolating nucleic acids from different organisms and even tissues rich in nucleases are well documented. Earlier we described a procedure for rapid isolation of pure plasmid and phage DNA using GIT (2). Viral DNA isolated by using chaotropic agents alone (5,6) requires extensive dialysis to ensure purity for restriction enzyme digestion. Here we describe a simple and rapid procedure for the direct isolation of pure nuclear polyhedrosis virus (NPV) DNA from polyhedra and extracellular virus (ECV) using GIT in combination with ammonium acetate.

Table 1. Comparison of Tissue Culture Infectious Dose$_{50}$ ($TCID_{50}$) and pfu/mL of the Extracellular Virus (ECV) Generated by Transfection of 1 µg of Viral DNA Isolated by the GIT Method (1) and by CsCl-Ethidium Bromide Gradient Centrifugation (2)

DNA Isolation Method	Amount of DNA Used	$TCID_{50}$	pfu/mL
By GIT	1 µg	$10^{-6.536}$	2.4×10^8
CsCl, EtdBr-purified DNA	1 µg	$10^{-5.5}$	3.2×10^7

Autographa californica NPV (AcNPV) E 2 (obtained from Max. D. Summers, Texas A & M University) was propagated by infecting and transfecting *Spodoptera frugiperda* IPLB-Sf-21 AE or Sf9 cells maintained at 27°C in TNM-FH medium (Sigma Chemical, St. Louis, MO, USA), supplemented with 10% fetal calf serum (Life Technologies, Gaithersburg, MD, USA) as described by Summers and Smith (10). Virus derived from *Spodoptera litura* (SlNPV) was propagated in the larvae of *S. litura* by the standard method (8).

Tissue culture dishes were seeded with 1×10^6 cells in a final volume of 2 mL of complete medium and incubated at 27°C for 1 h to allow the cells to attach. The medium was then aspirated out and the viral inoculum, 0.1 plaque-forming units (pfu) per cell, was added to the cell monolayer. After 1 h, the medium was replaced by fresh medium and was again incubated at 27°C.

The ECV was isolated from 48-h post-infected culture. The medium was first centrifuged at 4000× *g* for 10 min at 4°C in Beckman Instruments GS-6R low-speed centrifuge (Fullerton, CA, USA). The supernatant was further centrifuged at 34 000 rpm at 4°C for 75 min in a Beckman Instruments ultracentrifuge using a VTi 50 rotor to pellet the virus.

Occluded virus (OV) was isolated from a 6-day-old infected culture. The cells were scraped out of the culture plate using a rubber policeman and homogenized in a Potter Elvehjem homogenizer (New York, NY, USA) (glass-glass, Piston B) in 0.1% (wt/vol) sodium dodecyl sulfate (Sigma Chemical), and the polyhedra were pelleted by centrifugation for 2–3 min at 12 000 rpm in a microcentrifuge. The pelleted polyhedra were washed with distilled water and then with acetone by centrifugation. The pellet was finally air-dried and stored at -20°C.

Occluded SlNPV from infected dead larvae of *S. litura* was isolated af-

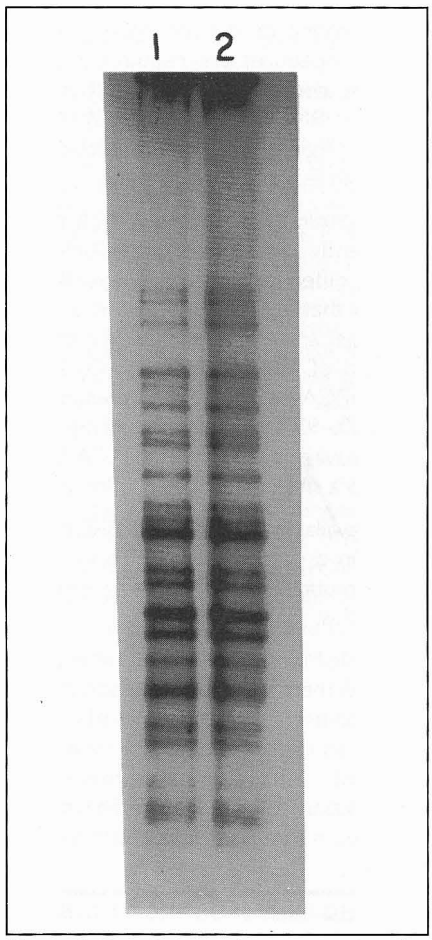

Figure 1. Restriction endonuclease digestion and kinase treatment of SlNPV DNA. Comparison of *Eco*RI-digested, kinased (using [γ-^{32}P]ATP) SlNPV DNA isolated from polyhedra derived from infected larvae by the GIT method (lane 2) and by the GIT method followed by CsCl-EtdBr gradient centrifugation (lane 1).

ter homogenization in water, as described above, and filtration through two layers of cheese cloth. The filtrate was centrifuged at 4°C for 10 min at 10000× *g* in a Sorvall® RC 5B centrifuge (Du Pont, Wilmington, DE, USA). The polyhedral pellet was washed repeatedly with acetone until the supernatant became colorless, and then it was dried under vacuum.

ECV, pelleted from 12 mL of infected culture, were suspended in 200 µL of 10 mM Tris-HCl/0.1 mM EDTA (Merck, Bombay, India), pH 8.0. To this and to the dried polyhedra (5 mg), 200 µL of GIT solution (4.0 M GIT [Fluka Chemie AG, Bucks, Switzerland]; 0.05 M Tris-HCl, pH 7.6; 0.01 M EDTA; 0.148 M 2-mercaptoethanol [Sigma Chemical]; 2% [wt/vol] *N*-lauroyl sarcosine, sodium salt [Sigma Chemical] containing 0.2 M NaCl [Merck]) were added to dissolve OV and ECV completely. Equal volumes of 5 M ammonium acetate (Merck) were added to both the solutions, mixed gently and centrifuged in a microcentrifuge at 8000 rpm for 5 min at room temperature, and the supernatant was collected. The pellets were extracted once by centrifugation with 100 µL of 2.0 M GIT containing 2.5 M ammonium acetate as before. The supernatants were pooled and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (1:1:1/24) and once with chloroform alone. The viral DNA was finally precipitated with an equal volume of isopropanol at -70°C for 20 min and was collected by centrifugation at 12000 rpm in a microcentrifuge. The DNA pellet was washed with 70% ethanol, dried and dissolved in 10 mM Tris-HCl/50 mM NaCl/0.1 mM EDTA, pH 8.0.

Transfection of insect cell line Sf9 with AcNPV DNA was done following the method of Summers and Smith (10). The transfection supernatant was collected 96 h post-transfection, and the titer of the ECV was determined by end-point dilution (10).

The yield of viral DNA by this method varied from 1.3 to 1.5 µg per mg of polyhedra, while 3.7 to 4.0 µg of DNA were obtained per mL of ECV. The absorbance ratio of the DNA at 260 nm and 280 nm was found to be 1.8 in all the cases. This high yield facilitates the isolation of recombinant viral DNA directly from 2 mL of transfection supernatant from 35-mm culture plates. One microgram of viral DNA isolated from ECV by this method, when used for transfection, yields 2.4×10^8 pfu/mL (Table 1), which is about 8-fold higher than the titer obtained from transfection done by using DNA isolated with conventional methods (5–7).

Figure 1 shows restriction endonuclease digestion and kinase treatment of baculovirus DNA isolated by this and conventional methods.

Strongly associated basic proteins

from baculovirus DNA are usually removed by proteinase K digestion to avoid shearing of the DNA. The high solubility of polyhedra and ECV in GIT solution, and the increased transfection efficiency of DNA isolated by this method, suggest that these chaotropic agents dissociate proteins from DNA without shearing it. Chaotropic agents are also known to remove low molecular weight impurities such as polyamines (1) that are attached to DNA by salt linkage. Ammonium acetate prevents alcohol precipitation of low molecular weight impurities along with DNA.

This method should be very useful for isolating sufficient quantities of pure baculovirus DNA without proteinase K digestion and dialysis, even from a single infected larva or the virus propagated in a 35-mm plate.

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Long Primers for RAPD Mapping and Fingerprinting of Grape and Pear

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The random-amplified polymorphic DNA (RAPD) technique (10,12) has been widely used in plants for the construction of genetic maps in species such as *Arabidopsis* (9), bananas (4) and slash pine (7), and for genotype identification and taxonomic studies (2,5). RAPD markers are detected by the use of short oligonucleotides of arbitrary sequence as primers for the amplification of segments of the target genome. Generally, 10-mer primers with 50%–80% G+C content are preferred (8). However, complex banding patterns were also generated with primers as short as 5 bases (1). There are few reports on the use of long primers (over 12 bases) (10,11). The potential value of long primers (17–24 bases) for generating RAPD polymorphisms was investigated in this study. We compared the use of both short and long primers in RAPD assays of two plant species: grape and pear.

DNA was extracted from leaves according to the methods of Lodhi et al. (6) for grape and of Doyle and Doyle (3) for pear. RAPD amplification was performed in a reaction volume of 25 µL containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton® X-100, 120 µM each dNTPs, 0.4 µM primer, 100–200 ng genomic DNA and 0.5 unit of *Taq* DNA polymerase. Amplifications were performed on a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) for *(i)* 35 cycles of 30 s at 94°C, 1 min at 35°C and 1 min 45 s at 72°C, followed by an 8-min extension at 72°C for grape, or *(ii)* 40 cycles of 1 min at 94°C, 2 min at 35°C and 2 min at 72°C, followed by an 8-min extension at 72°C for pear. Primers (10-mers) were purchased from Operon Technologies (Alameda, CA, USA), National Bio-Sciences (Plymouth, MN, USA), Genosys Biotechnologies (The Woodlands, TX, USA), University of British Columbia (Vancouver, B.C., Canada) and the New York State Center for Advanced Technology in Biotechnology (Cornell University, Ithaca, NY, USA). Long (17-mer to 24-mer) primers were provided by Dr. Sheng-Zhi Pang (Dept. of Plant Pathology, Cornell University, Geneva, NY, USA).

Typical gels displaying the amplification products generated from grape or pear DNA using long primers are shown in Figure 1. In general, long primers generated more DNA fragments, a wider range of DNA fragment sizes

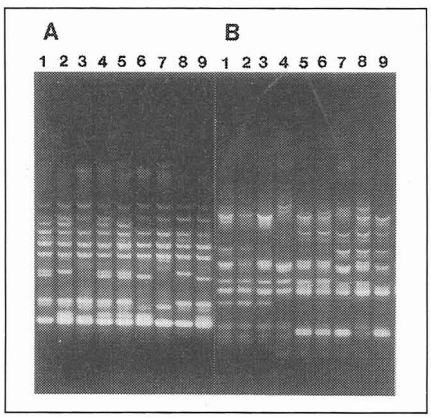

Figure 1. RAPD profiles of grape DNA generated with 21-mer GY169 (CTAAGCTGCTTTTGTTTGAGC) (Panel A) and pear DNA with the 18-mer GY107 (GTTCAGGGCTGTTTATAG) (Panel B). Each panel includes DNA from 9 individuals from the crosses Horizon x Ill. 547-1 (Panel A) and Bartlett x NY10353 (Panel B), respectively. Amplification products were separated by electrophoresis in 2% agarose gels (1% agarose/1% NuSieve® GTG® agarose; FMC BioProducts, Rockland, ME, USA), visualized by staining with ethidium bromide and photographed on a transilluminator using Polaroid Type 55 film (Cambridge, MA, USA).