recombinant virus-containing medium can be used to further amplify the virus (see Amplification of Recombinant Baculoviruses section).

Long-term Storage of Recombinant Baculoviruses

Infective virus particles in high-titer stock solutions can be stored for several weeks without a significant decrease in the virus titer. However, a 10-fold decrease in the virus titer during 6 months storage at 4ºC should be expected. For ultimate storage, viral DNA should be isolated from a low-passage virus and stored at –80ºC. Whenever necessary, infectious virus particles can be obtained by transfecting the baculovirus DNA into insect cells.

Recombinant Baculovirus DNA Isolation:

- Take the baculovirus-containing medium and remove the insect cell debris by spinning it 10,000 x g for 10 min at 4ºC.
- Transfer the supernatant to ultracentrifuge tubes and pellet the virus particles by spinning at 40,000 x g at 4ºC for 1 hr (e.g., Sorvall SS34; 18,000 rpm).
- Decant the supernatant and resuspend the virus particles in TE Buffer (3 x 10^{10} virus equivalents per ml).
- Layer the virus-suspension on top of a 10-40% sucrose step gradient.
- Spin the tubes at 40,000 g for 1 hr at 4ºC. Harvest the virus particles at the interphase.
- Dilute 5-fold with TE buffer and re-pellet the virus particles at 40,000 g for 1 hr at 4ºC.
- Resuspend the virus pellet in TE and add proteinase K (100 µg/ml) and SDS (0.5%).
- Shake carefully and incubate solution for 30 min at 37ºC.
- Extract once with one volume phenol, once with one volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1[v/v]) and once with one volume of chloroform/isoamyl alcohol (24:1 [v/v]). The baculovirus DNA is relatively sensitive to shearing, so extract by inverting the tubes just fast enough to thoroughly mix the phases. Do not vigorously shake!
- Add 0.1 volumes of 3 M sodium acetate (pH 4.5). Invert tubes several times and add 2.5 volumes of pure 100% ethanol.
- Incubate at –20ºC overnight.
- Spin down precipitated DNA, wash twice with 75% ethanol, and once with 100% ethanol.
- Air-dry pellet and resuspend the baculovirus DNA in TE buffer (0.1 µg/µl).
- Measure OD_{260}, digest the virus DNA with BamHI and run the fragments on a 0.6% agarose gel to check the purity of the DNA. A characteristic restriction fragment pattern should be visible.[3,4]
- The baculovirus DNA can be stored frozen for many years and whenever needed, it can be thawed and transfected into fresh insect cells. This guarantees that a recombinant virus doesn't get lost over time due to subsequent amplification passages or titer decreases during long-term storage of infective virus particles.

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BD BaculoGold™ Linearized Baculovirus DNA

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INTRODUCTION

The use of insect cells and lytic baculoviruses for expression of full-length mammalian proteins has been the recent method of choice for many disciplines.[1] *Autographa californica* nuclear polyhedrosis virus (AcNPV) infects the clonal tissue culture line Sf9 derived from *Spodoptera frugiperda* cells. Expression of the highly abundant polyhedrin gene is non-essential in tissue culture and its strong promoter can be used for the synthesis of foreign gene products. The polyhedrin promoter is maximally expressed very late in infection when the lytic virus is already killing the host cells, giving a reasonable chance for high levels of expression even for certain toxic proteins. Many post-translational modifications are made correctly in insect cells and proteins unable to be expressed in *E. coli* have been successfully expressed in the insect cell system.

The BD BaculoGold™ Linearized Baculovirus DNA from BD Biosciences Pharmingen™ provides a tool for recombination efficiencies close to 100%.[2] The principle of this technique lies in the construction of a modified type of baculovirus DNA, which contains a lethal deletion. This DNA does not code for viable virus. Only co-transfection of insect cells with the viral DNA and a complementing transfer vector construct reconstitutes viable virus. Essentially, 99% of all virus plaques are derived from plasmid-rescued viruses which contain and express the foreign gene from the plasmid. Since the BD BaculoGold baculovirus contains a lacZ gene that is replaced by

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recombination with the plasmid containing the foreign gene, all recombinants will produce colorless plaques on X-gal plates. The small portion of non-recombinant virus plaques (usually less than 1%) will stain blue on X-gal plates. If preferred, the virus may be amplified from a single plaque from a plaque assay.

BD BaculoGold Linearized DNA

51-21100D	BD BaculoGold Linearized DNA	2.5 µg
51-21484P	pVL1392-XylE Control Vector	5 µg

NOTE: Each vial contains materials sufficient for five transfections when used as described in this protocol.

Selection of a Transfer Vector

All polyhedrin gene locus-based baculovirus transfer vectors can be used to rescue the lethality of the BD BaculoGold DNA. Transfer vectors from BD Biosciences Pharmingen include pVL1392, pVL1393 (provided with the BD BaculoGold Transfection Kit), pAcSG2, pAcGP67, pAcUW21, pAcUW51, pAcMP2 and pAcMP3, and pAcGHLT(GST fusion transfer vectors) and pAcHLT (6xHis fusion transfer vectors). Other vectors on the market may also be used, but have not been tested for compatibility with the BD BaculoGold DNA.

Co-transfection Using BD BaculoGold

NOTE: TNM-FH medium contains 10% fetal calf serum.

- Seed 2 x 10^6 Sf9 cells (*Sf9* insect cells, live, Cat. No. 554763); onto a 60 mm tissue culture plate. Initial cell density should be 50-70% confluent.
- Allow the insect cells to attach firmly to plate (approximately 15 min).
- Remove the culture medium from the plate and add 1 ml of Transfection Buffer A. Make sure that all areas of the plate are covered with Transfection Buffer A, to prevent the cells from drying out.
- Mix 0.5 µg of BD BaculoGold Baculovirus DNA and 2 µg of recombinant plasmid DNA containing your gene, in a sterile 1.5 ml Eppendorf tube.
- Let mixture sit for 5 min before adding 1 ml Transfection Buffer B.
- Mix well.
- Add 1 ml of the Transfection Buffer B/DNA solution drop-by-drop to the insect cells on the tissue culture plate. After every two or three drops, gently rock the plate back and forth to mix the newly added solution with the Transfection Buffer A. During this procedure, a fine precipitate should form making the solution slightly milky.

- Incubate the plate at 27ºC for 4 hr.
- After 4 hr, remove the transfection solution from the plate and add 3 ml of TNM-FH insect medium. Rock the plate back and forth several times before once again removing all the medium. Add 3 ml fresh TNM-FH medium and incubate the plates at 27ºC for 4 to 5 days. To maintain a humid environment, place a moist paper towel in a dish in the bottom of the 27˚C incubator.
- After 4 days, collect the supernatant and infect more cells for amplification or, if preferred, start with a single virus clone by picking a single plaque from a plaque assay plate.

Positive Control

NOTE: Set up at the same time as the co-transfection.

- Seed 2 x 10^6 cells on a 60 mm tissue culture plate.
- After cells have attached, remove media and replace with 1 ml of fresh media.
- Prepare co-transfection positive control by mixing 0.5 µg BD BaculoGold DNA and 2 µg pVL1392-XylE Control Vector DNA in a microcentrifuge tube.
- Let mixture sit for five min before adding 1 ml of Transfection Buffer B.
- Mix well, by gently vortexing or by flicking the tube.
- Add the 1 ml of Transfection Buffer B/XylE Positive Control DNA solution drop-by-drop to the co-transfection plate. After every two or three drops, gently rock the plate back and forth to mix the newly added solution with the Transfection Buffer A. During this procedure, a fine precipitate should form making the solution slightly milky.
- Continue co-transfection protocol as above.

Negative Control

NOTE: Set up at the same time as the co-transfection.

- Seed 2 x 10^6 cells on a 60 mm tissue culture plate.
- After cells have attached, remove media and replace with 3 ml of fresh media.

Amplification of Recombinant Baculoviruses

After the co-transfection or after the plaque assay, the recombinant baculovirus must be amplified to obtain a high titer stock solution. To this end, freshly seeded insect cells should be infected at a multiplicity of infection (MOI) of >1. This is usually done by infecting 5 x 10^6 cells per 10 cm plate (approximately 60% confluent) with 500 µl of transfection supernatant or 100 µl of the plaque pick-up viral supernatant in 15 ml of TNM-FH medium.

The cells should be incubated at 27ºC for 3 days before harvesting the medium. At 24h past infection, virus-infected cells can be visualized by fluorescence microscopy. The medium will contain at least 10^7 virus particles per ml and should be used for another round of amplification. Two rounds of amplification usually give a virus titer of 2 x 10^8/ml virus particles.

Plaque Assay for Insect Cells

- Seed 7 x 10^6 Sf9 cells/10 cm plate (70-80% confluent), or 2-2.5 x 10^6 Sf9 cells/60 mm plate (70-80% confluent).
- Let cells attach for 5 min at room temperature.
- Make serial dilutions (10^{-4} – 10^{-7}) of your co-transfection virus supernatant. Add 1 ml of diluted viral supernatant to the medium of each plate.
- Let infection occur for 1 hr at room temperature. Rock every 15 min for 1 hr..
- In the meantime, prepare a 2% solution of Plaque Assay Agarose, Cat. No. 554766, in sterile water. Heat mixture in microwave oven to 60ºC and dissolve agarose completely. Solution should stay clear (don't overheat).
- After cooling down to 42ºC, add 1 volume of 2X Grace's Medium (LTI, Cat No. 11667) prewarmed to 42ºC). Mix well. The final agarose concentration should be 1%.
- If you will be amplifying your virus from a single plaque, or if color selection is required, add X-gal to the agarose solution to a final concentration of 250 µg/ml. Colorless plaques will be the recombinant virus, blue plaques (less than 1%) will be the non-recombinant BD BaculoGold Linearized Baculovirus DNA.
- Aspirate medium containing the virus inoculum. Overlay cells with 4 ml of the 1% agarose solution by pipetting carefully from one side of the plate. Remove all bubbles using a pipette.
- Let plates sit undisturbed until agarose is completely hardened, approximately 10-15 min.
- Plates should be kept in a humid atmosphere for 5-7 days at 27ºC until visible plaques develop. Plaques can be used for screening to identify the recombinant virus, to determine the virus titer or for virus amplification.
- To amplify virus from a single plaque, mark the plaque by making a dot or circle on the tissue culture plate. Harvest the plaque by taking a plug of the agarose containing the plaque using a sterile pasteur pipet. Elute the virus particles by rotating the agar plug in 700 µl of TNM-FH medium at 4ºC overnight. 100 µl of this