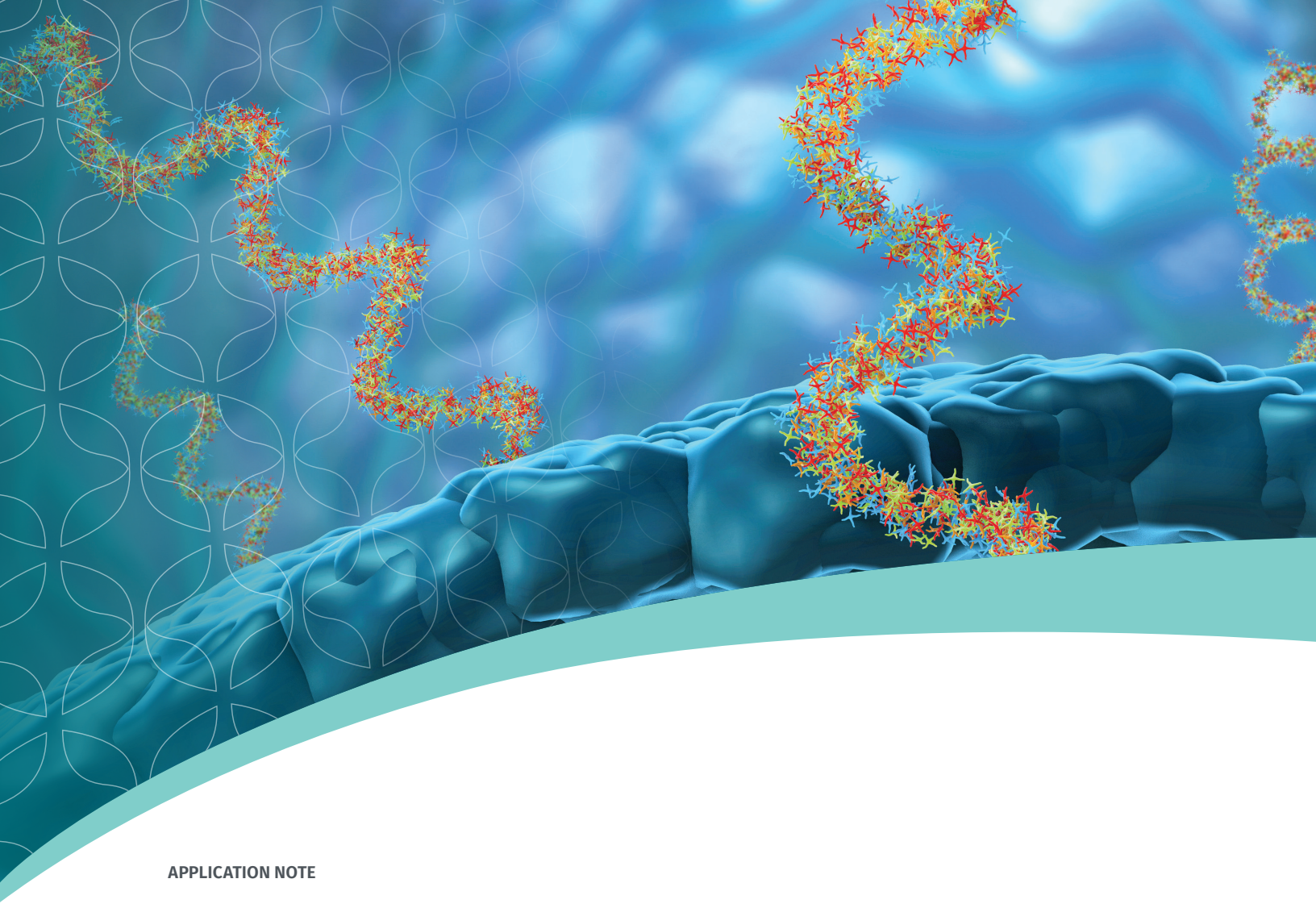

APPLICATION NOTE

Generation of recombinant baculovirus stocks by suspension cotransfection

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Expression Systems
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Introduction

The baculovirus-insect cell expression vector system (BEVS), has been a reliable platform for recombinant protein production for over 25 years. In this system, a recombinant baculovirus expressing the gene of interest is generated and propagated in insect cells, typically the *Spodoptera frugiperda* cell lines such as Sf9 or Sf21. Recombinant baculovirus expression vectors can be produced by utilizing homologous recombination between linearized baculovirus DNA deleted in a portion of the essential viral gene orf1629 and a transfer plasmid encoding both the gene of interest and the remaining portion of orf1629. One such system is BestBac™ linearized baculovirus DNA paired with a transfer vector containing the regions of homology, such as pVL1392 or pVL1393. The recombination of the linearized DNA and the transfer vector occurs in the insect cell after introduction by liposome-mediated cotransfection.

Traditionally, recombinant baculovirus was generated by transfecting insect cells in 6-well plates, yielding only 2–3 mL of low-titer virus that required one or two amplification rounds to obtain usable stocks. Here, we demonstrate that using suspension culture for cotransfection can produce up to 100 mL of Passage 0 virus stocks directly. This approach uses the same amount of DNA and transfection reagents as the adherent method but takes advantage of Sf9 cell growth in suspension, resulting in higher volumes, increased titers, reduced costs, and significant time savings.

Methods

Cotransfections were performed in suspension cultures at varying volumes using deep well blocks and Optimum Growth® Flasks from Thomson Instrument Company. Both Sf9 cells in ESF 921 and Sf9 RV-Free cells in ESF AdvanCD™ cell culture media were used to propagate recombinant baculoviruses. For all conditions, the cotransfection mixture contained 200 µL Transfection medium, 0.5 µg BestBac™ 1.0 linearized DNA, 2 µg pVL1393 transfer vector, and 6 µL Expres^2 TR transfection reagent. Cotransfection mixtures were incubated at room temperature for 20 minutes then added to the cell culture. The cell cultures were seeded at 1x10^6 viable cells/mL at volumes of 20 mL and 50 mL in 125 mL flasks, 100 mL in 250 mL flasks, and 25 mL per well in deep well blocks.

Cultures were incubated at 27°C in a shaker incubator at 135 rpm with a 25 mm throw in ambient air. Samples were analyzed daily for viable cell density, viability, and diameter using a Vi-CELL BLU cell viability analyzer. To track recombinant baculovirus infection, surface gp64 expression was analyzed by incubating a 50 µL culture sample with anti-gp64-PE and detecting fluorescence with a MACSQuant flow cytometer.

Suspension cotransfections were compared with results from adherent cotransfections. Briefly, the cotransfection mixture was applied to 0.9 x10^6 cells in the well of a 6-well plate, allowed to incubate for 4 hours, and then 3 mL of cell culture media was added. The culture was incubated for 4 to 5 days at 27°C in a static incubator and the supernatant harvested.

Levels of infectious baculovirus in all harvested samples were determined using an Expression Systems Baculovirus Titer Kit.

Results and Conclusion

Cotransfections were performed both in adherent cultures and in suspension cultures to determine relative efficacy of each method. Suspension cotransfection outperformed adherent methods. Data collected over a three-year period was compiled for comparison including both direct side-by-side experiments and independent cotransfections leading to a broad representation of both methodologies. Cotransfections were performed by multiple operators. On average, suspension cotransfections produced infectious baculovirus titers nearly two orders of magnitude higher than adherent cotransfections, **Figure 1**.

Figure 1. Adherent and Suspension Cotransfections

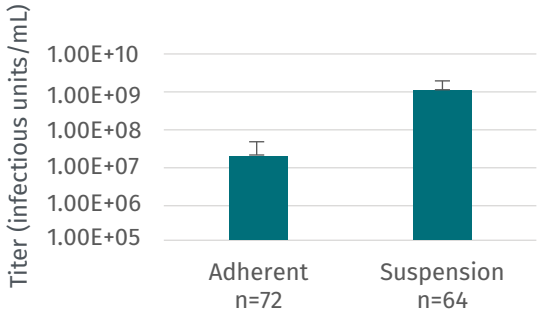
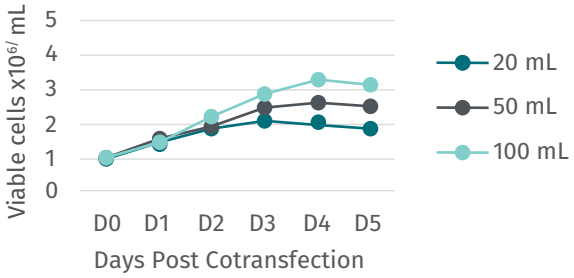


Figure 2. Cell Growth Following Cotransfection



Thomson Optimum Growth® Flasks were seeded in ESF 921 at 1x10⁶ cells/mL and received the cotransfection mixture on Day 0. 20- and 50-mL cultures used 125 mL flasks and the 100 mL cultures used 250 mL flasks. Cultures were performed in duplicate and averaged results are presented.

To better demonstrate the mechanism by which suspension cotransfection yields higher infectious baculovirus titers, cultures were assessed for cell growth, infection status, and baculovirus yield. While Sf9 cell growth in shake flasks is effective for the production of recombinant baculovirus, the culture characteristics in different flasks can affect the final yield. Lower volume cultures may have been impacted by evaporation as the humidity was not controlled. Flask choice (for instance, those with low evaporation caps), humidity control, and environment can impact yield and should be taken into consideration. When low volume cultures were performed using low evaporation caps, cell growth more closely matched that of higher volume cultures (data not shown).

In **Figure 2**, the viable cell counts following addition of the transfection mixture on day 0 is presented. All flasks received the same amount of transfection mixture; therefore, the dose added per culture volume decreases as the volume increases. As expected, with the lower volume and higher dose of DNA, proportionally more cells become infected initially and cell growth is reduced (**Figure 2**). Cell growth plateaus approximately 4 days post-cotransfection, a consequence of arrested cell growth of the entire culture. This indicates the majority of cells are producing baculovirus (**Figure 2** and **Figure 4**).

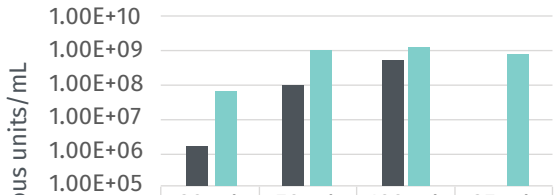
All culture volumes and vessels demonstrated robust production of recombinant baculovirus at 4 and 5 days post-cotransfection. (**Figure 3**). The relatively lower yields in the 20 mL cultures may be a result of evaporation in the low humidity environment where the experiments were performed. Low-evaporation caps have been incorporated into the process, and cell growth improved (data not shown). The improved cell growth may further increase recombinant baculovirus yield.

The higher titers in suspension culture reflect more efficient virus spread and infection. Multiple events are required for recombinant baculovirus production in insect cell culture. The cotransfection complex is taken up by the insect cell via endocytosis. Then, the DNA complex is transported to the nucleus, where DNA replication and homologous recombination occurs. Finally, packaging into the baculovirus capsid, transport through the cytoplasm, and budding through the cell membrane into the cell culture supernatant occurs. Only a small proportion of the cells in culture produce infectious baculovirus one day following cotransfection (**Figure 4**). As the newly formed recombinant baculovirus bud from the cells, these viruses infect nearby cells until all cells in the culture become infected (**Figure 4**).

Many published protocols suggest harvesting recombinant baculovirus after the culture viability drops below approximately 70%. Baculovirus infection is a lytic process with generation of budded virus beginning approximately 18 hours post-infection. Insect cells infected by baculovirus increase in diameter due to the reorganization of the insect cell nuclei while viral DNA is propagated. The harvest time can therefore be determined

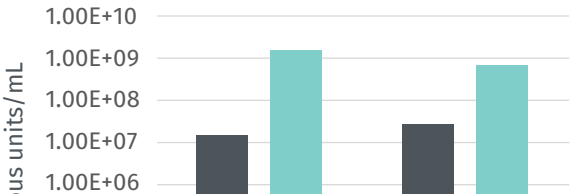
Figure 3. Recombinant Baculovirus Titers Produced from Cotransfection

A. ESF 921 Medium



	20 mL 125 mL Flask	50 mL 125 mL Flask	100 mL 250 mL Flask	25 mL Deep Well Block
Day 4	1.46E+06	9.87E+07	5.50E+08	2.17E+08
Day 5	7.12E+07	1.18E+09	1.42E+09	8.89E+08

B. ESF AdvanCD™ Medium

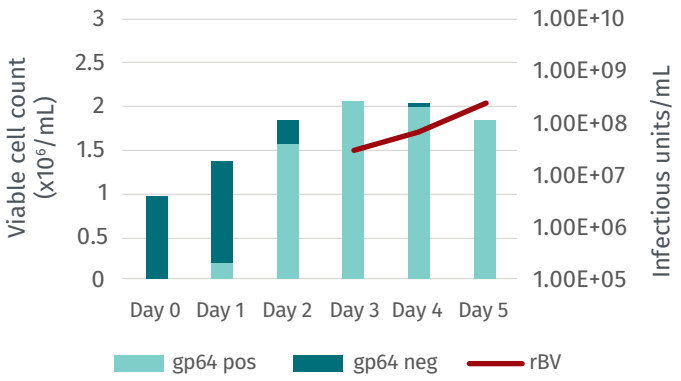


	100 mL 250 mL Flask	25 mL Deep Well Block
Day 4	1.21E+07	2.41E+07
Day 5	1.49E+09	7.24E+08

Culture vessels were seeded in ESF 921 or ESF AdvanCD™ at 20, 50, and 100 mL in 125 or 250 mL flasks, and 25 mL per well of deep well block. 200 µL of transfection mixture comprised of BestBac™ linearized DNA, transfer vector, transfection reagent, and transfection medium were added to each culture. Samples were taken 4- and 5-days post-cotransfection and analyzed for infectious titer.

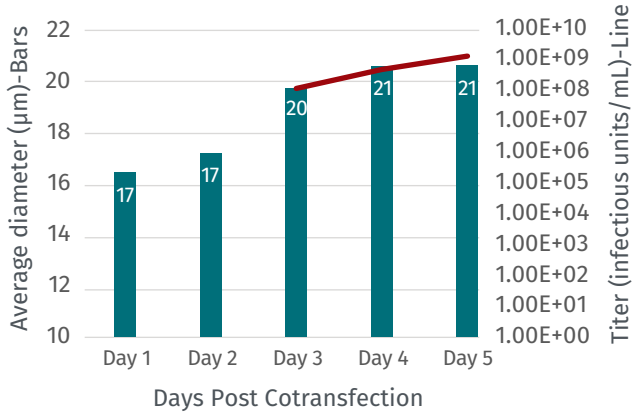
by culture viability or by the average diameter of the cells. Peak baculovirus yield appears to occur two days after a significant increase in the average cell diameter, which guides the optimal harvest (**Figure 5**). As the infection progresses, the biphasic nature of the baculovirus creates a shift from production of budded virus to expression of proteins under the p10 and polyhedrin promoters. Accordingly, harvesting later in the infection, after the culture viability has dropped, does not significantly increase the infectious baculovirus yield as demonstrated by amplification of a virus stock.

Figure 4. Baculovirus Spread Through Culture Leads to High Titers



A 20 mL Shake flask culture was inoculated with 200 µL transfection complex. Spread of recombinant baculovirus was followed by staining with an antibody against gp64. Viable cells are represented in bars with uninfected, or gp64 negative cells represented in dark teal. Infected cells positive for gp64 are represented in light teal. As the infection spreads, the proportion of light to dark increases. The resulting infectious baculovirus titer produced is shown in red.

Figure 5. Increased Cell Diameter and Infectious Baculovirus Over Time



Cotransfection was performed in ESF 921 in 100 mL volume. Culture was monitored daily for average cell diameter and the production of infectious baculovirus from 3 days post-infection.

Capitalizing on the characteristics of recombinant baculovirus generation and spread within a cell culture allows for the production of high titer infectious recombinant baculovirus stocks in suspension culture. The low amount of recombinant baculovirus produced in the first round of viral production can efficiently spread in a suspension culture environment, leading to multiple rounds of infection. This yields a high titer virus stock after only 4-5 days with the added benefit of a larger volume. The useful volume of high titer baculovirus can then be used for expression of recombinant protein, eliminating time spent amplifying baculovirus from a low yield adherent cotransfection.

References:

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Mulvania T, Hayes B, Hedin D. A Flow Cytometric Assay for Rapid, Accurate Determination of Baculovirus Titers. *BioProcess J*, 2004; 3(3): 47-53.

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Cotransfection Protocol

Protocol Notes

1. Insect cell cultures are sensitive to the effects of evaporation. When working with low culture volumes in a low humidity environment, consider using a humidified incubator or low evaporation flask caps.
2. It is always advisable to titrate reagents when working in a dynamic system such as cell culture. While the data shown here demonstrates successful cotransfections with small amounts of transfection mixture, optimal results may be obtained by increasing the cotransfection complex volume.
3. DNA quality will impact the efficiency of the cotransfection. The use of Transfection Medium can help increase the efficiency of DNA uptake but cannot compensate for transfer plasmid DNA of poor quality.
4. Recombinant baculovirus is produced most efficiently when using healthy cells in a state of logarithmic growth at the time of cotransfection.
5. Incubation conditions in a 27±1°C in a shaker incubator with a 1-inch (25 mm) throw:
6. Filtration of the supernatant after harvest can remove large cell debris and slow aggregation in the harvested virus stock.

Required materials

Culture volume	Culture Vessel	Shake Speed
25 mL	6-Well Block	175 rpm
50 mL	6-Well Block	225 rpm
20 mL to 50 mL	125 mL Erlenmeyer Flask	135 rpm
100 mL	250 mL Erlenmeyer Flask	135 rpm

- BestBac™ Linearized DNA (Expression Systems PN 91-001 or 91-002)
- Transfer Vector containing your Gene of Interest compatible with recombination at the polyhedrin locus (pOET, pTriEx, pBAC, pBacPAK, pVL, and pAc).
- Transfection Reagent such as Expres² TR (Expression Systems PN 95-055)
- Transfection Medium (Expression Systems PN 95-020)
- Insect Cell Culture Medium (ESF 921 Expression Systems PN 96-001, ESF AF Expression Systems PN 99-300, ESF AdvanCD™ Expression Systems PN 54-018)

- When using ESF AdvanCD™, Virus Stabilization Additive (Expression Systems PN 95-010)
- Sf9 Cells (in ESF 921 94-001, in ESF AF 94-006, in ESF AdvanCD™ 94-030)
- 1.0-1.5 mL Polypropylene tubes
  (**NOTE**: Do not use polystyrene tubes as DNA will adhere!)
- Culture vessel:

Culture Volume	Culture Vessel
25 mL to 50 mL	6-Well Block
20 mL to 50 mL	125 mL Erlenmeyer Flask
100 mL	250 mL Erlenmeyer Flask

Procedure

1. Add 200 µL Transfection Medium to polypropylene tube
2. Add DNA to transfection medium
   a. 0.5 µg BestBac™ DNA
   b. 2 µg Transfer Vector
   c. Alternatively, 1 µg of bacmid DNA can be used in place of BestBac™ and transfer vector.
3. Add 6 µL Expres² TR transfection reagent and mix by flicking tube or vortex gently for 2-3 seconds.
4. Incubate mixture at room temperature for at least 20 minutes, no more than 30 minutes.
5. While the transfection mixture is incubating, seed desired suspension culture. Recommended volume ranges from 20 to 100 mL.
6. The culture media determines the seed density of the culture:

Culture Media	Seed Density
ESF 921	$1x10^6$/mL
ESF AF	$1x10^6$/mL
ESF AdvanCD	$0.75x10^6$/mL

7. At the end of the incubation period, transfer the transfection mixture to the culture vessel using a 1 mL pipette tip. Add the complex directly into the cell culture solution. Do not let the mixture contact the culture vessel directly.
8. Incubate at 27°C shaking at 135 rpm for shake flasks, 175-225 rpm for well plates, for 4-5 days.
   a. Staining for gp64 expression can identify when all cells in the culture are infected.
   b. Monitor culture for viable cell density, viability, and average diameter to identify best day of harvest.
   c. Harvest when the culture shows signs of widespread infection and the viability is between 85-90%, though it is acceptable to harvest between 70-90% viability.
9. Harvest supernatant by centrifugation and filter the supernatant using a 0.2-micron filter.
10. When using ESF AdvanCD™, add Virus Stabilization Additive equal to 10% of the culture volume prior to filtration.

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