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Purification of Recombinant Baculovirus by End-Point Dilution and gp64 Screening

By BROOKS HAYES and THERA MULVANIA

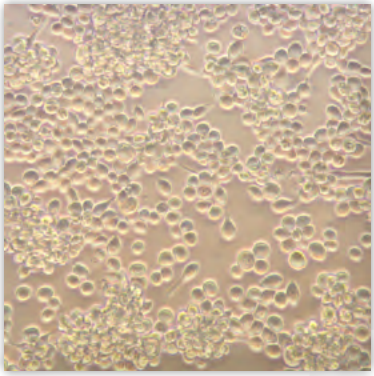
Introduction

The baculovirus expression vector system (BEVS) has emerged as a powerful tool for the production of recombinant proteins used as therapeutics, reagents, and diagnostics. In order to maximize the system's efficiency and thereby reduce costs, optimizing production parameters is imperative.

A critical factor in optimization is the production of a high-quality baculovirus stock with a high-titer, pure clonal population of recombinant virus that is stable over time. Baculovirus stocks may contain alternate varieties of infectious virus due to cross-contamination, outgrowth of non-recombinant virus,[1] and excision of inserts attributable to some recombinant virus production technologies.[2] Since the advent of the BEVS, the "gold standard" for production of pure baculovirus stocks has been plaque purification. Briefly, plaque purification involves infecting a monolayer of cells with dilutions of virus before applying an agarose overlay to the monolayer. After a 5–7 day incubation, isolated plaques can be picked, virus eluted from the agarose plug, and amplified.[3] The drawbacks of plaque purification are: (1) it is time and labor-intensive; (2) the results hinge greatly on the health of the cells and the cell density at infection; (3) identification and picking of isolated plaques is challenging; and (4) the integrity of the procedure is easily compromised by virus diffusion and mass flow of virus-containing liquid beneath the agarose overlay.

An alternative method for baculovirus stock purification is end-point dilution (EPD). Individual cultures of cells, typically in 12 or 96-well plates, are inoculated with highly diluted virus stocks such that only a fraction of the cultures receive an infectious virus dose. As infection of cultured insect cells with baculovirus follows Poisson distribution,[4] the virus should be diluted such that 10% or fewer of the cultures become infected in order to be 95% confident that infected cultures have received only one infectious virus unit.[3] Viral supernatants from infected cultures resulting from inoculation by this method can then be amplified or subjected to further rounds of EPD purification. The EPD procedure has been used in association with BEVS for as long as plaque purification, but has never achieved the same widespread level of usage. The primary benefit of EPD purification over plaque purification lies in the fact that the individual virus clones are separated by the plastic barrier of the well, whereas individual clones are not separated by any physical barrier within the monolayer of a plaque purification culture. The lack of a physical barrier between viral clones in the cell monolayer of a plaque purification undermines confidence that cross-contamination between clones has not occurred.

Typically, infected cultures of an EPD purification have been identified by the observation of cells exhibiting a cytopathic effect, characterized by inclusions of polyhedrin detectable in the nucleus[5] or an increase in cell diameter.[6] As it has been demonstrated that polyhedrin expression is not required for infection of insect cells in culture,[7] most modern recombinant baculovirus (rBV) production technologies generate viruses with the polyhedrin (*polh*) gene knocked out, rendering the polyhedrin screening strategy obsolete. In *polh*-negative baculovirus-infected insect cells, the cytopathic effect is generally nothing more than an increase in cell diameter,



Spodoptera frugiperda cells (Sf9)

by an average of around 3 µm.[6] This cell diameter increase can be subtle and easily overlooked, especially in a culture whose infection has been initiated by a single infectious unit and may not yet have spread to a majority of the cells.

A modification to the EPD assay allows for less subjective, automated screening for wells receiving an infectious virus dose. We have developed a flow cytometric screening assay that utilizes a fluorescently labeled anti-gp64 antibody to identify cells expressing cell membrane-associated gp64, indicating productive infection by baculovirus. With a modern modification to the procedure, EDP purification requires less time and labor, eliminates the effects of viral mass flow and diffusion, and omits the subjectivity of discerning cytopathic effects that are often subtle.

Materials and Methods

Cell Lines and Culture Conditions

Spodoptera frugiperda cells (Sf9) were routinely passaged in ESF 921 protein-free insect cell culture media (Expression Systems LLC). Cultures were maintained in a New Brunswick shaker incubator (Eppendorf) at 27°C, shaking at 135 rpm.

Plasmid Constructs

The red fluorescence-expressing gene, mCherry, was polymerase chain reaction (PCR)-amplified from viral DNA isolated from a mCherry-expressing baculovirus (kind gift from Taro Ohkawa, PhD, UC Berkeley) and ligated with SmaI digested pVL1393 (Expression Systems) to produce pVL-mCherry. The enhanced green fluorescent protein (EGFP) gene was PCR-amplified from pEGFP-N1 (Clontech) and ligated with SmaI-digested pVL1393 to produce pVL-EGFP.

Recombinant Baculovirus Generation

Plasmids pVL1393, pVL-EGFP, and pVL-mCherry were co-transfected with BestBac 2.0 (Expression Systems) following manufacturer's protocol, generating viruses rBV, rBV-EGFP, and rBV-mCherry respectively. The rBVs were titered using the flow cytometric screening assay referred to earlier.[8]

Flow Cytometry

Cells were stained for gp64. The MACSQuant® flow cytometer (Miltenyi Biotec) was used to analyze, by excitation with a 488 nm laser, three fluorescent indicators of protein expression. Uninfected control cultures were used to set gates. Flow cytometry data was analyzed with MACSQuantify™ software (Miltenyi Biotec) and FlowJo analysis software (Tree Star, Inc.).

Endpoint Dilution and gp64 Screening Methodology

The basis of the EPD purification strategy is dilution of the virus such that only a small number of cultures become infected—fewer than 10% of cultures to be 95% confident that each infected culture receives only one infectious dose. In order to meet this criteria, the virus stock must be diluted to a point were at most, one in every ten cultures becomes infected when inoculated with a given volume of virus. One can start by estimating the dilution of virus, based on the infectious titer of the virus stock, theoretically containing one infectious unit (IU) per ten volumes of virus being added to wells. For example, if 50 µL of diluted virus will be added to each well at a dilution that will theoretically infect one in ten wells, then the virus stock should be diluted to 2 IU/mL (as shown in formula 1).

$$\frac{1\,IU}{\frac{50\,\mu L}{well} * 10\,wells} = \frac{1\,IU}{500\,\mu L} = \frac{2\,IU}{mL} \qquad (1)$$

The initial dilution determined from the virus titer is simply an informed starting point. There will always be errors involved in titer determination, and calculations based on infectious titer do not always accurately predict how a virus infection will behave in practice. It is important to prepare additional dilutions, more and less dilute than the calculated theoretical dilution. Two-fold to log dilutions are generally appropriate. The number and magnitude of additional dilutions is left up to the researcher; more dilutions increase the likelihood of achieving the goal of arriving at a dilution infecting 10% or fewer wells.

After virus dilutions have been prepared, a 96-well tissue culture-treated plate is seeded with 100 µL Sf9 cells per well at a density of 1×10^5 cells/mL. It is convenient to divide the plate in two so that each plate can accommodate two different virus dilutions, four rows of 12 wells per dilution. One well is reserved as a negative control receiving no

virus, and one well as a positive control inoculated with an infectious dose of virus, leaving 46 wells for each virus dilution. Plates are incubated at 27°C in a humidified chamber for 5–6 days.

After incubation, the cells in each well are screened for cell surface gp64 expression. Plates are centrifuged at 400 g for 5 min and the supernatants are transferred to a new 96-well plate with a multi-channel pipettor, retaining the cells in the original plate. It is important to maintain the same well orientation in both plates. The supernatant in well A1 of the new plate should correspond to the cells in well A1 of the original plate. The cells in the original plate are then incubated with 50 µL phycoerythrin conjugated anti-gp64 antibody (ACV1-PE, Expression Systems) diluted 1:200 with phosphate buffered saline (PBS) containing 1% bovine serum albumin (Sigma-Aldrich) and incubated at 4°C.

After a 20 min incubation, wells are washed with 150 µL PBS and plates are centrifuged at 400 g for 5 min. Before re-suspending the cells in 200 µL PBS, the supernatant is aspirated and wells washed two additional times with 200 µL PBS. The cells can now be analyzed for cell-associated PE fluorescence using a flow cytometer equipped with the appropriate laser and detector. Wells containing cells exhibiting a fluorescence shift above background have received an infectious dose of virus. Now the viral supernatant in the corresponding well of the supernatant plate can be amplified or subjected to another round of EPD purification, provided that <10% of the wells contain gp64-positive cells for that dilution. Before initiating additional rounds of purification or virus amplification, the selected virus supernatant should be screened to confirm that the desired recombinant virus has been isolated. This can be accomplished by a PCR screen for an expression cassette, a Western blot, or ELISA assay for expression of the protein of interest, or any other assay confirming the identity of the virus isolate.

To ensure a high level of confidence that the final virus stock is a pure clonal population, the EDP purification procedure should be repeated at least two more times for a total of three purification cycles. The viral supernatant picked from one round of purification can be used directly for the next round of purification. However, as the volume of supernatant will be less than 150 µL, determining the viral titer is not practical. In this case, more dilutions should be tested to ensure that at least one dilution generates a statistically desirable outcome. Alternatively, the viral supernatant can be amplified, the virus titer determined, and then subjected to the same purification procedure described previously.

To illustrate the potential of EPD to purify a particular infectious baculovirus from a mixed population of baculoviruses, rBV-mCherry (a baculovirus expressing the mCherry fluorescent protein under transcriptional control of the baculovirus polyhedrin promoter) was mixed with rBV-EGFP, which expresses EGFP under transcriptional control of the polyhedrin promoter. This mixture of two recombinant baculoviruses was subjected to EPD purification to isolate a pure mCherry-expressing virus population. This example demonstrates that the gp64 screen for baculovirus-infected cultures can be supplemented with a simultaneous flow cytometric screen for the recombinant protein of interest, if the desired virus expresses a fluorescent or fluorescently tagged protein. EPD purification of baculoviruses expressing other recombinant proteins that lend themselves to rapid detection (*e.g.*, by means of a chromogenic reaction with a substrate)—as is the case with β-Galactosidase—can also be simultaneously screened for cultures expressing gp64 as well as the protein of interest. This eliminates the need for an intermediary screening step between rounds of EPD purification to determine that the isolated virus expresses the protein of interest.

Results and Discussion

Figure 1 (following page) presents typical data gathered after gp64 screening of EPD purification plates. Assuming a virus titer of 2×10^9 IU/mL, a series of dilutions were prepared to bracket the dilution of virus stock predicted to infect one in ten wells inoculated with 50 µL. Twelve wells of a 96-well plate were inoculated with each dilution in triplicate and incubated for six days before screening for gp64 expression. Figure 1 displays the percentage of gp64-positive cells in each well for every dilution. Negative control wells that received no virus inoculum were used to set gates and determine the threshold beyond which a well is scored positive for gp64 expression. Figure 2 (following page) illustrates the number of wells per dilution that were determined to contain gp64-positive cells and therefore to have received an infectious dose of virus. Inoculation with virus dilutions 4×10^{-9} through 4×10^{-10} all resulted in infection of less than 10% of the wells and therefore meet the standard criteria for selection. Though each of these dilutions fit the selection criteria, consideration must still be taken for which wells are selected for further work.

If two or more virus dilutions result in a lower than 10% infection rate, a well from the most dilute sample should be picked for the greatest confidence of clonal virus isolation. The probability that the infection was initiated from a single infectious unit is higher for wells inoculated with the more dilute virus, even if the higher dilution happens to yield

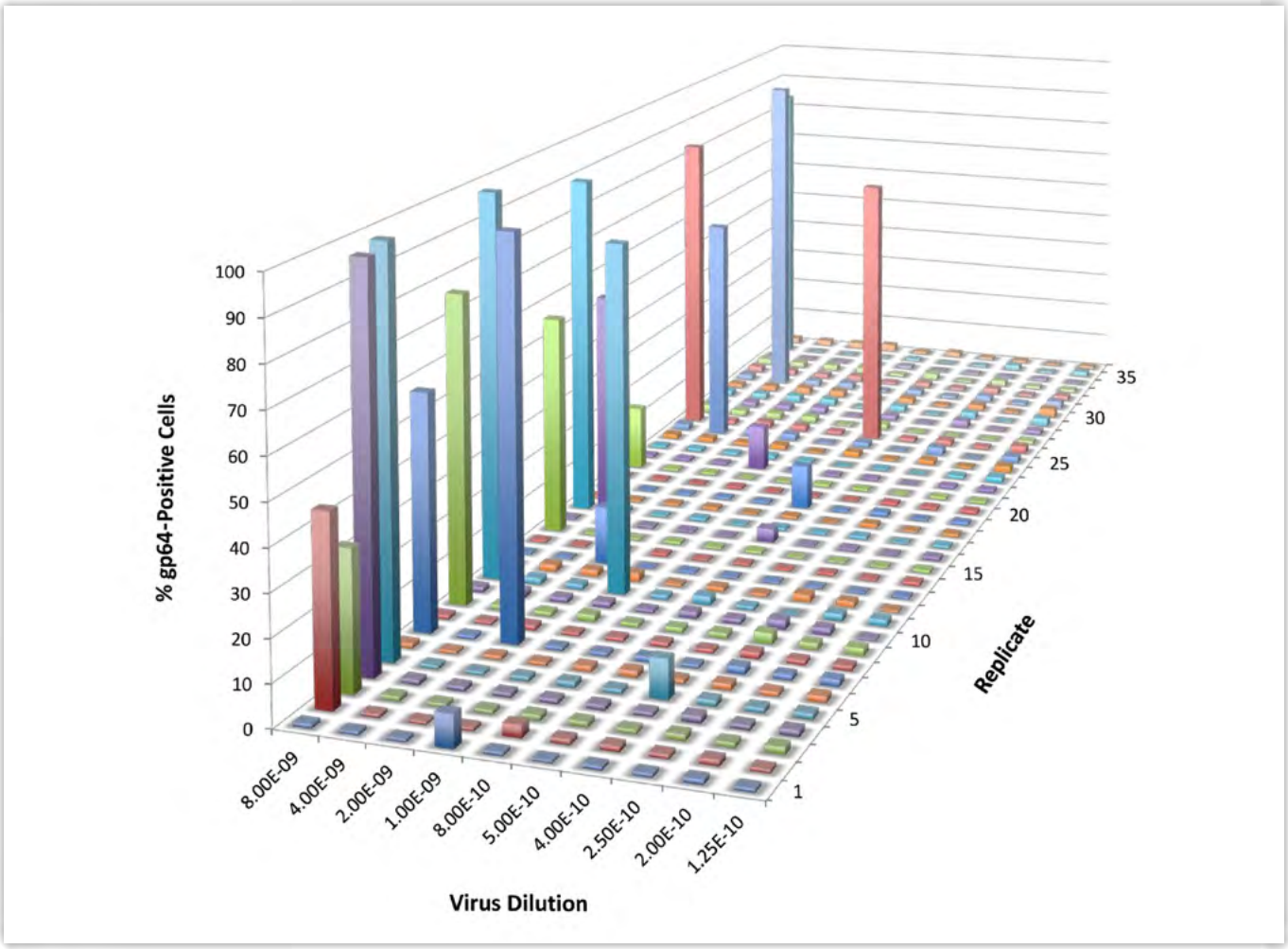


FIGURE 1. Virus-infected wells as determined by staining for gp64 expression. Three replicate 96-well plates were inoculated with dilutions of rBV indicated on the x-axis and incubated at 27°C for six days. The cells in each well were then stained for gp64 as described in the text. The percentage of gp64-positive cells in each well was determined by flow cytometry. Each replicate per dilution is displayed along the z-axis.

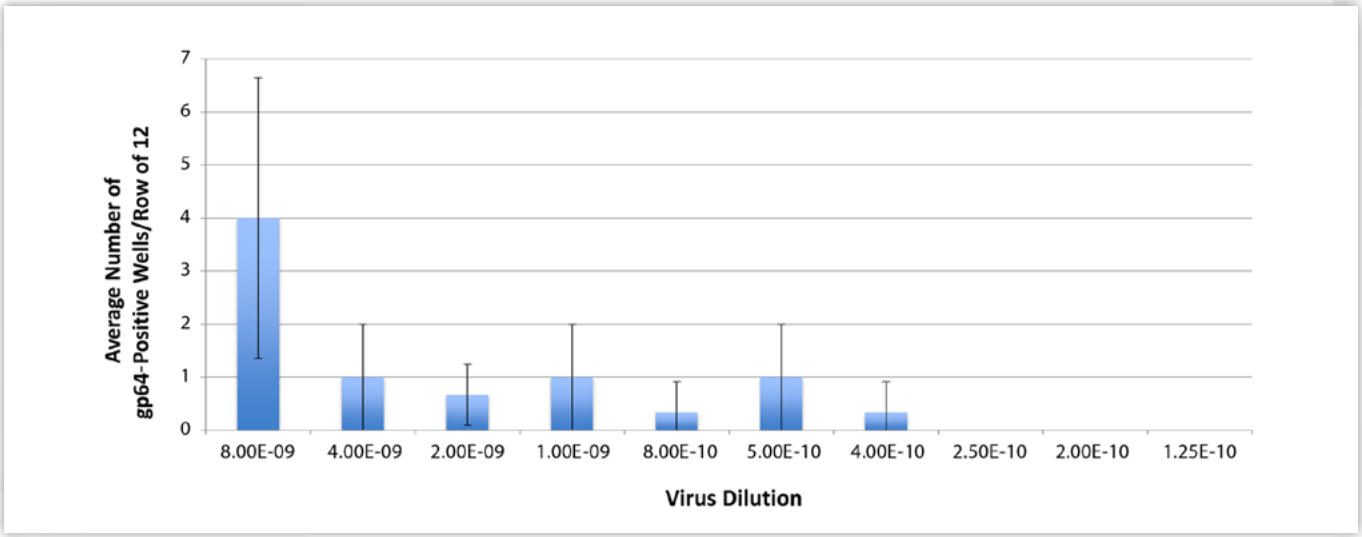


FIGURE 2. Frequency of gp64-positive wells per virus dilution. Three replicate sets of 96-well plates were inoculated with log virus dilutions and stained for gp64-expressing cells after six days of incubation. Virus dilutions as indicated on the x-axis were used to inoculate 12 wells each of a 96-well plate.

more infected wells than the lower dilution. In the data depicted in Figure 2, the 4×10^{-10} and 8×10^{-10} virus dilutions infect the same percentage of inoculated wells. However, the 4×10^{-10} dilution is the appropriate selection for the highest confidence in isolation of a clonal virus population.

Figure 1 illustrates one of the primary benefits of the gp64 screen over screening for cytopathic effect. A number of cultures contained fewer than 20% gp64-positive cells per well, but exhibit staining well above background. These wells have undoubtedly received an infectious dose of virus, however the infection has only spread to a small minority of the cell population. These lower level infections could be attributable to the micro-environment within the culture where the primary infection was established, the phenotype of the initial infecting virus, or the cells may begin to out-grow the virus infection. If only 10–20% of cells in a culture have been infected, it is likely that some of these low level infections could go unnoticed by the researcher screening for a cytopathic effect in the form of cell swelling. Overlooking the more slowly progressing infections may mislead the researcher into believing that they have selected wells from dilutions infecting 10% or fewer of cultures when in reality they have not. Without screening wells for cells expressing gp64, or another protein expressed only in baculovirus infected cells, one cannot be confident that all wells receiving an infectious dose of virus have been accounted for. The gp64 screen for baculovirus infection is an extremely sensitive and objective method, allowing the investigator to confidently identify even low-level infections of any recombinant baculovirus.

To visually demonstrate the ability of EPD purification to separate one virus population from the other, a virus stock was created by mixing together two virus stocks, each of which contains a gene for a unique fluorescent protein. rBV-mCherry was of a titer approximately 10-fold higher than that of rBV-EGFP resulting in a final stock with a 10:1 rBV-mCherry to rBV-EGFP ratio. Log dilutions of this stock were used to infect three replicate 96-well plates as described for the previous experiments. Figure 3 displays representative flow cytometric dot plots from EPD purification of rBV-mCherry from contaminating rBV-EGFP baculovirus. Lower dilutions of the virus stock are not adequate to isolate rBV-mCherry from rBV-EGFP as demonstrated by the dot plot in Figure 3A where the cells in the analyzed well contained

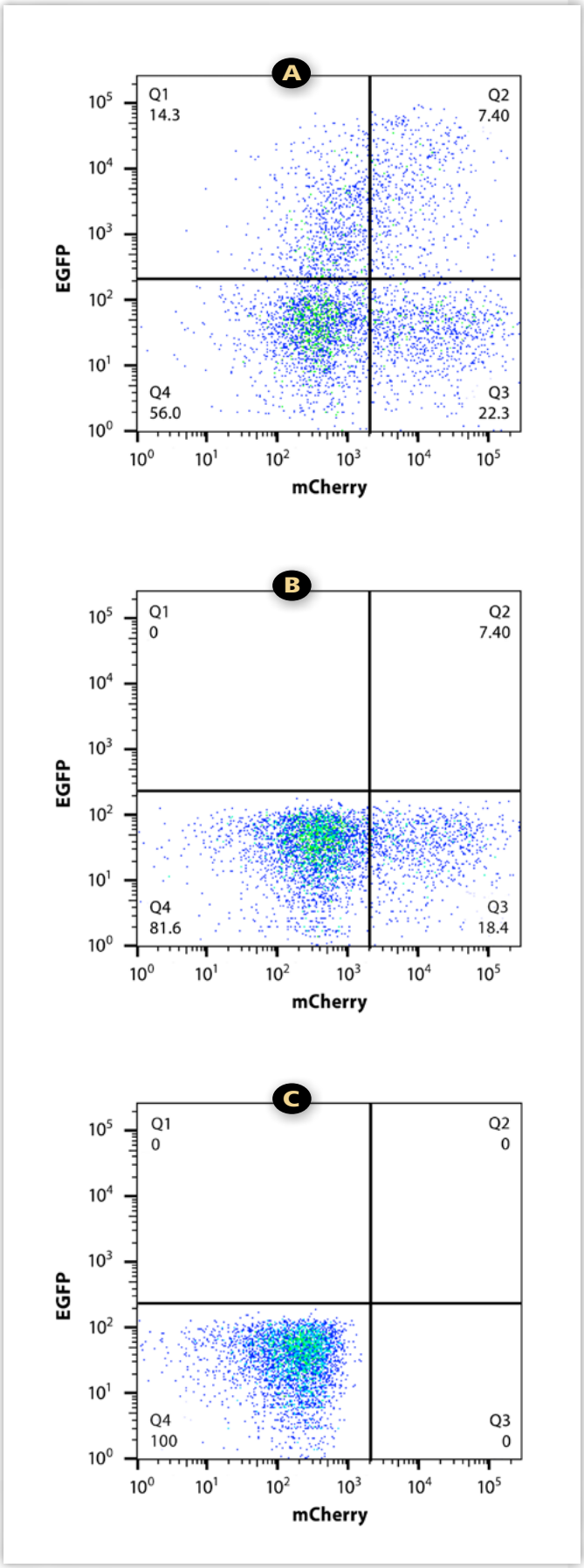


FIGURE 3. Flow cytometric analysis of dilutions of a mixed virus stock containing mCherry and EGFP-expressing baculoviruses. (A) representation of wells from a 1×10^{-3} dilution that received infectious doses of both rBV-mCherry and rBV-EGFP viruses; **(B)** a dilution of 1×10^{-6} that resulted in infection with rBV-mCherry only; and **(C)** a dilution of 1×10^{-7} that did not result in infection with either virus construct.

a mixed population of cells infected with rBV-EGFP (Q1), rBV-mCherry (Q3), and cells infected with both viruses (Q2). At higher virus stock dilutions, analyzed wells contain cells that are expressing only mCherry, indicating that in these wells, rBV-mCherry has been isolated from rBV-EGFP, as illustrated in Figure 3B. Figure 3C depicts a typical dot plot from an EPD purification well inoculated with highly diluted virus stock that did not receive an infectious dose of either virus.

The results of flow cytometric fluorescence data collected from three replicate EPD purification plates, where each row was inoculated with a dilution of the virus stock containing a mixed population of rBV-mCherry and rBV-EGFP baculoviruses, are summarized in Figure 4. There is no separation of rBV-mCherry from rBV-EGFP until the 1×10^{-5} dilution. However, more than 10% of analyzed wells inoculated with this dilution of virus have been infected, therefore none of these wells are appropriate to pick for a clonal population of mCherry-expressing baculovirus. Inoculation with the 1×10^{-6} dilution of virus stock results in only 5.6% of wells containing mCherry-expressing viruses. Therefore, any of these wells are appropriate to select for further rounds of EPD purification or amplification.

The possibility remains that the virus supernatant picked from a well inoculated with virus infecting less than 10% of cultures still contains a contaminating virus population. However, as in the case of the culture depicted in Figure 3B, the vast majority of infected cells were infected with rBV-mCherry and are producing a proportionally large amount of progeny rBV-mCherry virus. If contaminating rBV-EGFP cells are present in the population, far less rBV-EGFP progeny virus is being produced by these cells. The high ratio of rBV-mCherry progeny virus to rBV-EGFP progeny virus will increase the probability that rBV-mCherry can be fully isolated from contaminating rBV-EGFP during the second round of EPD purification. The probability of rBV-mCherry isolation is similarly increased with even further rounds of EPD purification.

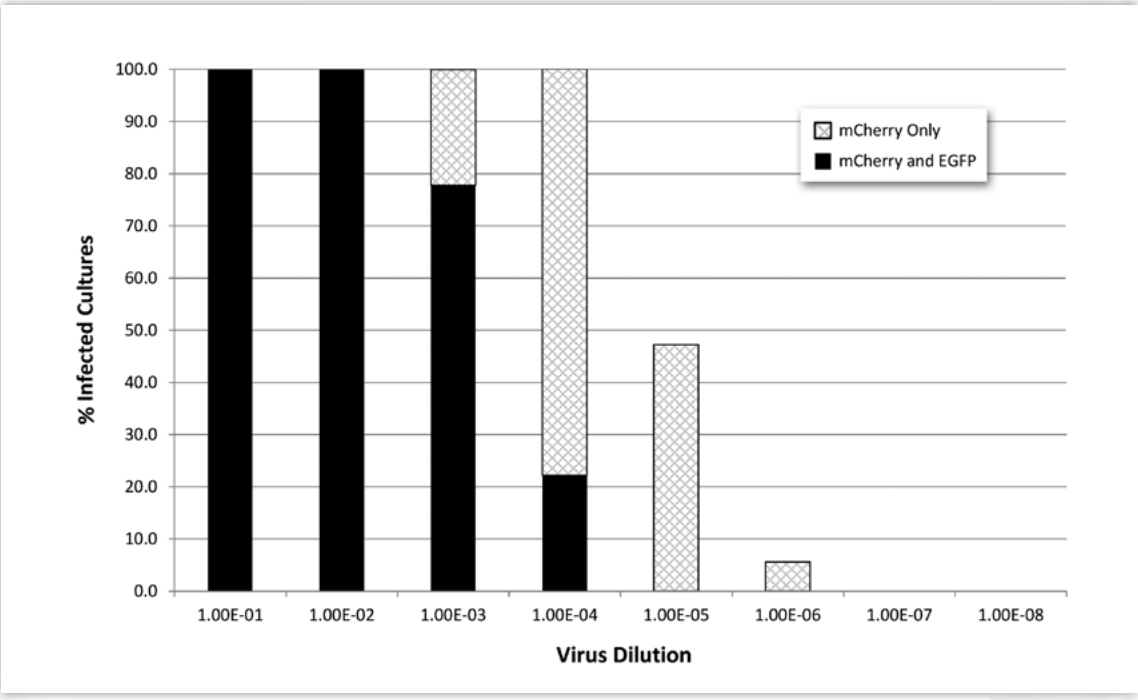


FIGURE 4. Characterization of cultures infected with various dilutions of a mixed virus stock. The columns represent the percentage of infected cultures per dilution. The ■ proportion represents those cultures expressing both mCherry and EGFP. The ▨ proportion represents cultures expressing only mCherry, and therefore were infected with only rBV-mCherry.

Conclusion

The end-point dilution purification technique offers a number of advantages over the more conventional plaque purification method, all leading to a higher probability of obtaining a clonal virus isolate. End-point dilution infections are established in 96-well plates, where individual virus isolates are physically separated by the walls of the wells, preventing the type of cross-contamination that is possible in plaque purification cultures. The 96-well

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plate format of the EPD purification also lends itself to identification of infected cultures by means of screening cells for gp64 expression. With the exception of some unusual recombinant baculoviruses that require special cell lines or helper plasmids for infection of insect cells, all recombinant baculoviruses express gp64 and are therefore amenable to the gp64 screen. The sensitivity and objectivity of the flow cytometric gp64 expression screen is critical to accurately identifying all infected cultures and determine which dilutions infect 10% or fewer inoculated wells. Additionally, it allows for the possibility of screening for an expressed protein of interest by directly analyzing the supernatant and/or infected cells, thus eliminating an expression step that would be required when screening from plaques. Finally, analysis of EPD purification plates can be automated for high-throughput screening if a flow cytometer outfitted with robotics for sampling 96-well plates is available.

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