Evaluation of Sf9 RV-Free rhabdovirus-free cells and ESF AdvanCD™ chemically defined cell culture medium for the amplification of all-in-one CRISPR baculovirus vectors for mammalian cell genome editing

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BACKGROUND: BACULOVIRUSES AND CRISPR DELIVERY IN MAMMALIAN CELLS

Genome editing via CRISPR/Cas9 requires simultaneous delivery of multiple components to mammalian cells. This task cannot be easily accomplished using widespread viral vectors such as adeno-associated viral vectors (AAVs) and lentiviruses (LVs) which are characterised by a fixed genetic cargo capacity (4-4.5 kb for AAVs and 8-9 kb for LVs). Thanks to their extensive DNA cargo capacity (>45 kb), baculoviruses (BV) are optimally suited to deliver all the required components on a single virion, so far representing one of the only viral platforms which can efficiently deliver all-in-one CRISPR constructs to mammalian cells independently from their complexity, design or size. Additionally, BVs are intrinsically safe as they are replication deficient in the mammalian host and lack genetic elements which trigger viral genome integration [1,2].

We and others have demonstrated that BV-mediated delivery of all-in-one CRISPR toolkits can be used efficiently in a range of cultured mammalian cells, with high transduction and gene editing efficiencies [2-6]. Given their potential for gene editing applications in mammalian cells, higher manufacturing standards are now required to minimise batch-to-batch variability and improve quality of baculovirus vectors. Here, we test two Expression Systems products which are designed for this purpose:

ESF AdvanCD™ is a chemically defined medium which supports high cell densities and holds the potential to support higher viral titre production combined with the reproducibility of a chemically defined composition.

Sf9 RV-Free cells are a clonal isolate of Sf9 cells free from *S. frugiperda* adventitious rhabdovirus (Sf-RV). Sf-RV was recently discovered in Sf21 and Sf9 derivates and its nature has not been yet fully elucidated [7,8]. While the typical rhabdovirus bullet-shaped morphology cannot always be observed [7,9], RNA or proteins with transfection-like properties are invariantly detected in the supernatant of Sf-RV+ cells. For its properties, Sf-RV cannot be easily removed from baculovirus preparations and its impact on subsequent mammalian cell transduction is currently not known.

MONITORING VIRAL AMPLIFICATION PARAMETERS

To assess the compatibility of the new media formulation with the amplification of all-in-one CRISPR BVs, viral amplification parameters were monitored across two different cell lines (Sf9 and Sf9 RV-Free) and two media composition (ESF 921 and ESF AdvanCD™). The baculovirus harbours a 17.5 kb construct encoding Cas9, EGFP, gRNA and a DNA donor for human Actin C-terminal tagging with mCherry. The construct additionally encodes a polH VSV-G cassette for BV pseudotyping which confers a broad mammalian cell tropism compared to standard gp64 pseudotyping [3,6].

MONITORING VIRAL AMPLIFICATION PARAMETERS

A starter virus was prepared by transfection of Sf9 RV-Free cells. The clarified viral supernatant was harvested and titrated by qPCR 5 days post-transfection. The starter virus was then used to inoculate Sf9 cultured in ESF 921 and ESF AdvanCD media or Sf9 RV-Free cultured in ESF AdvanCD medium at MOI 5 (genomes copies/cell). It must be noted however, that titre estimation via qPCR is known to overestimate viral titres between 1 and 2 orders of magnitude compared to traditional plaque-forming assays (Takara, BacPAK™ Baculovirus Rapid Titer Kit User Manual). As a reference, an MOI of 5 genome copies/cell thus is converted to 0.05-0.5 pfu/cells.

Sf9 RV-Free cells and ESF AdvanCD medium support higher viable cell densities without affecting viral amplification parameters

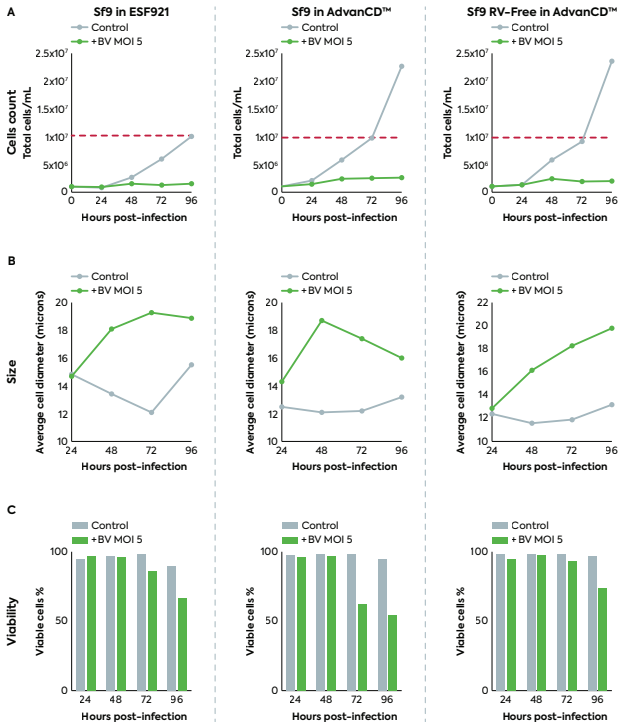
Uninfected cells (grey bars) reached higher cell densities when cultured in ESF AdvanCD by day 4 post-seeding (up to 2.3 x 10^7 cells/mL, >95% viability). Over the course of 4 days, infected cells (green bars) showed similar proliferation arrest dynamics across all tested condition and cell diameters increased from 13-15 µm (uninfected cells) to 18-20 µm (infected cells) (**Figure 1A-B**). Viability during infection showed a similar trend for Sf9 cells, regardless of the culture medium, with a sharp decrease by 72-96 hrs post-infection (**Figure 1C**).

These results show that the chemically defined composition of ESF AdvanCD medium does not interfere with normal baculovirus amplification and can substitute conventional media (e.g. ESF 921) for baculovirus vector production with a clear advantage in batch-to-batch reproducibility.

When compared to their parental cell line cultured in ESF AdvanCD, infected Sf9 RV-Free cells exhibited a delay in cell size increase and cell death which continued to rise throughout the rest of the viral amplification (**Figure 1B,C**), suggesting that viral harvesting could have been extended beyond 4 days post-inoculation. With the exception of these observations, infected Sf9 RV-Free cells were indistinguishable from the parental Sf9 cells indicating that BV amplification is not compromised in this new clonal cell line.

These results suggest that Sf9 RV-Free cultured in ESF AdvanCD medium Sf9 RV-Free can be effectively used for manufacturing RV-free baculovirus stocks.

Figure 1: Comparison of viral amplification parameters in Sf9 or Sf9 RV-Free cells cultured in ESF 921 or AdvanCD media.



Insect cells were seeded at 1x10^6 cells/mL in 50 mL flasks and inoculated with an all-in-one CRISPR baculovirus vector at MOI 5 (5 baculovirus genome copies per cell). Sf9 cultured in ESF 921 or in AdvanCD and Sf9 RV-Free in AdvanCD were monitored for cell proliferation (A), average cell diameter (B) and viability (B) over the course of 4 days post-infection. Grey bars/lines = uninfected cells, green bars/lines = infected cells. Data obtained through automated cell counting. Viability was estimated by trypan blue staining of dead cells.

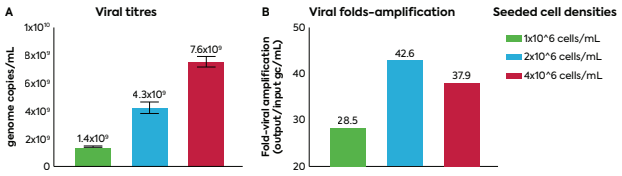
SF RV-FREE CELLS AND ESF ADVANCD MEDIUM SUPPORT HIGH-TITRE VIRAL PRODUCTION

Given the ability of ESF AdvanCD medium to support higher viable cell density, we sought to explore if altering the seeding cell density could promote harvesting of higher titre viral stocks without altering the size of the culturing vessel.

For this purpose, Sf RV-Free cells were seeded in ESF AdvanCD medium at 1x10^6, 2x10^6 or 4x10^6 cells/mL in 50 mL flasks and inoculated with an all-in-one CRISPR BV at MOI 50 (50 genomes copies/cell). At 4 days post-infection, viral titres were quantified via qPCR in clarified viral supernatants and measured as genome copies per mL of culture (gc/mL). With identical MOIs and culturing vessel, Sf RV-Free cells cultured in ESF AdvanCD media produced markedly higher viral titres at initial seeding densities equal or greater than 2x10^6 cells/mL (**Figure 2A**), with a maximum of 7.6x10^9 gc/mL.

The increased viral recovery was not only due to higher seeding densities, as the amount of recovered virus did not linearly correlate with the number of seeded cells. Indeed, the viral fold-amplification, calculated as the output gc/mL (harvested) over the input gc/mL (used for the inoculum), was markedly improved for higher initial cell seeding densities (**Figure 2B**). At 1x10^6 cells/mL, the virus was amplified 28.5 times, while higher fold-amplifications were obtained for higher seeding densities (42.6 and 37.9 folds-amplification).

Figure 2: Higher viral titre recoveries upon increased seeded cell densities.



(A) Viral titres recovery at 4 days post infection. Cells were seeded at the indicated seeding densities in 50 mL flasks and inoculated with an all-in-one CRISPR BV at MOI 50 (genome copies/cell). qPCR data from viral DNA extracted from clarified viral supernatants, mean and SD of technical triplicates. (B) Viral folds-amplification calculated as gc/mL at day 4 (harvest) over the gc/mL at day 0 (inoculum).

ASSESSMENT OF TRANSDUCTION AND GENE EDITING EFFICIENCIES IN HUMAN CELLS

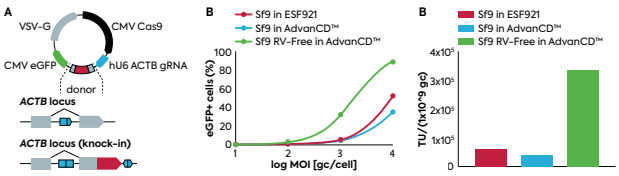
The tested all-in-one CRISPR BV delivers encodes Cas9, guide RNA and DNA donor to elicit human ACTB C-terminal tagging with a fluorescent mCherry cassette [3]. The construct is further equipped with a CMV eGFP cassette to facilitate monitoring of transduction efficiencies in mammalian cells, and a polH VSV-G cassette to enhance mammalian cells tropism and endosomal escape [3,6] (**Figure 3A**). Upon transduction in mammalian cells, transduction efficiencies can be monitored via eGFP detection, while mCherry, whose expression is only restored if correctly integrated in frame at the ACTB locus, reports on gene editing efficiencies (knock-in). The details of the construct, including the validation of the gene editing strategy and its full sequence, have been previously described [3,6].

Baculovirus particles produced in Sf9 RV-Free cells cultured in ESF AdvanCD medium display higher transduction and gene editing efficiencies in mammalian cells

To assess the transducing power of viral stocks produced in different cell lines/media, human embryonic kidney cells (HEK293T) were transduced with serially diluted virus, normalised for their genome copies number.

Transduction efficiencies were monitored via flow-cytometry at 72 hours post-transduction and transducing units (TU) were calculated. The viruses produced in Sf9 RV-Free cells cultured in ESF AdvanCD medium displayed markedly higher transduction efficiencies at all tested dilutions, (**Figure 3B**), with transducing titres between 5 and 10 times higher than Sf9 RV+ cells cultured in either ESF 921 or ESF AdvanCD media (**Figure 3C**).

Figure 3: Viruses derived from Sf9 RV-Free cells display higher transduction efficiencies in mammalian cells.

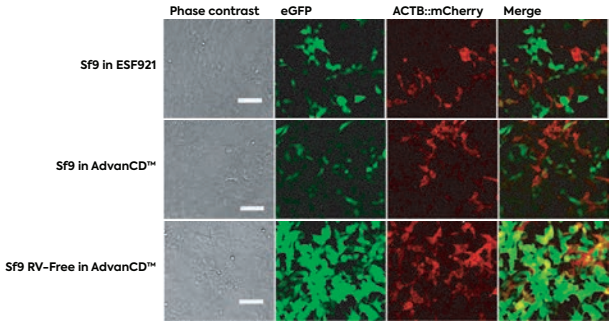


(A) Schematic representation of the construct used to generate all-in-one CRISPR BV for human ACTB C-terminal tagging with mCherry, described in Aulicino et al. 2022 and Mattioli et al. 2024. **(B)** Flow-cytometry analysis of HEK293T cells transduced with all-in-one CRISPR BV in (A) produced under the indicated conditions. Clarified BV stocks were normalised to 4x10^9 gc/mL, serially diluted and used to transduce 2x10^4 HEK293T at the indicated MOI (gc/cell). HEK293T were analysed at 72 hrs post-transduction. Lines = non-linear regression curve fitting, symbols = mean + SD of 3 independent replicates. **(C)** Estimation of transducing units (TU) per 1x10^9 gc obtained from data in (B).

ASSESSMENT OF TRANSDUCTION AND GENE EDITING EFFICIENCIES IN HUMAN CELLS (CONT.)

To confirm the ability of the tested viral stocks to elicit gene editing events, transduced cells were imaged at 72 hours post-transduction (**Figure 4**). In all conditions, mCherry+ cells with an ACTB like subcellular localisation could be detected, indicating successful gene editing events in a subset of the transduced cells. While no striking differences could be detected between cells transduced with viruses produced in Sf9 cells cultured in ESF 921 or ESF AdvanCD medium, cells transduced with viruses produced in Sf9 RV-Free cells cultured in ESF AdvanCD medium displayed much higher transduction efficiencies (eGFP+) with overall higher per-cell transgene expression levels. Higher gene editing efficiencies (ACTB::T2A::mCherry+ cells) were observed in cells transduced with viruses produced in Sf9 RV-Free cells (**Figure 4 bottom panel**).

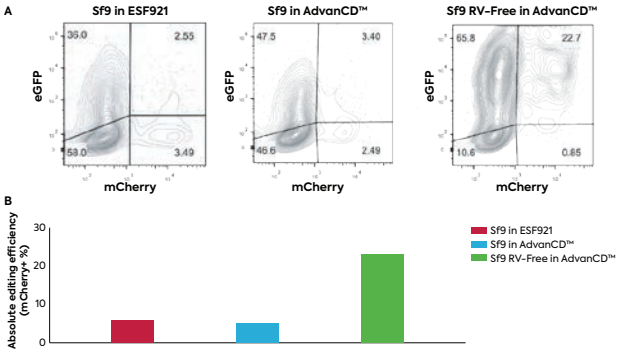
Figure 4: BVs produced in Sf9 RV-Free cells cultured in ESF AdvanCD medium display higher transduction and gene editing efficiencies in mammalian cells.



Live cell imaging of HEK293T at 72 hours post-transduction at 1x10⁴ gc/cell with all-in-one CRISPR BVs produced in the indicated culturing conditions. eGFP expression indicates transduction, mCherry expression indicates successful gene editing events, with consequent ACTB C-terminal tagging (knock-in). Scale bars are 50 µm.

To monitor transduction and gene editing efficiencies, transduced HEK293T were further analysed at 72 hours post-transduction via flow-cytometry. At the tested MOI, transduction (eGFP+ cells) and gene editing efficiencies (mCherry+) were overall comparable for BVs produced in Sf9 cultured in ESF 921 or ESF AdvanCD medium, with an average 5% knock-in. On the other hand, BVs produced in Sf9 RV-Free cells displayed superior transduction efficiencies, with up to 1 order of magnitude higher transgene expression and up to 4 times higher gene editing efficiencies (23%) (**Figure 4 A-B**).

Figure 4: BVs produced in Sf9 RV-Free cells cultured in ESF AdvanCD medium display higher transduction and gene editing efficiencies in mammalian cells.



(A) Representative flow-cytometry plots of HEK293T at 72 hrs post-transduction at 1x10⁴ gc/cell with all-in-one BVs produced in the indicated culturing conditions. Y-axis = eGFP fluorescence intensity (FITC-A, A.U.), X-axis = mCherry+ cells (knock-in)(PE-CF594-A, A.U). (B) Absolute editing efficiencies. Histogram of flow-cytometry data in (A). Mean + SD of n=3 independent replicates.

DETECTION OF SF-RHABDOVIRUS PROTEIN CONTAMINANTS IN CELL AND VIRAL SUPERNATANTS

Sf-rhabdovirus is characterised by a negative RNA strand virus encoding five proteins (N, P, M, G and L) [8]. Using negative strand specific oligos, Sf-rhabdovirus has been shown to be present in virtually all Sf21 and Sf9 stocks to date [8] occasionally resulting in the production of rhabdovirus particles with a characteristic "bullet-shaped" nanostructure [7]. More commonly however, Sf-rhabdovirus protein products, alongside its genome, appear to be jointly released in the supernatant in absence of a structurally identifiable virion but retaining the ability to infect insect cells [10] probably through an exosome mediated entry pathway.

To monitor the levels of Sf-rhabdovirus contamination, supernatants from insect cells were analysed by SDS-PAGE and Coomassie staining (**Figure 5A**). Protein bands previously associated to Sf-rhabdovirus [9] with a molecular weight of ~60 kDa (N protein) and ~35 kDa (N,G,P proteins and fragments) were readily detected in the supernatant of Sf21 and Sf9 cells, but they were absent in Sf9 RV-Free cells (**Figure 5A**).

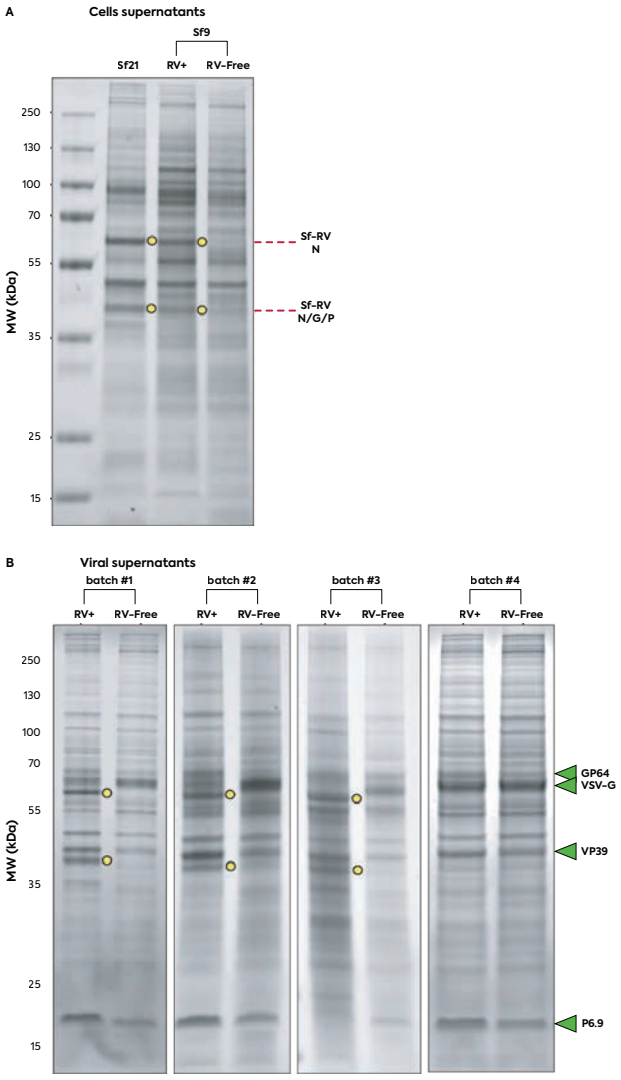
To investigate the impact of Sf-rhabdovirus protein contaminants in BV stocks, we analysed the supernatant of four different viral batches produced in either Sf9 or Sf9 RV-Free cells cultured in ESF AdvanCD. GP64, VSV-G, VP39 and P6.9 were readily identifiable by SDS-PAGE and Coomassie staining in all viral preps (with the exception of batch#3 RV+ cells in which P6.9 was not stained). Sf-rhabdovirus ~60 and ~35 kDa proteins were detected in RV+ cells batches 1-3 but they were absent in batch #4. (**Figure 5B**). The intensity Sf-rhabdovirus protein bands additionally varied from batch to batch, adding unpredictability to the BV harvest overall protein content.

VSV-G band intensity additionally appeared to decrease in BV produced in Sf9 RV+ cells in anticorrelation with the presence of rhabdovirus proteins, suggesting defects in BV pseudotyping levels which can explain the different performances noted on mammalian cells transduction levels (**Figure 5B**).

Occasionally, as in batch#4, Sf9 RV+ cells can release negligible amounts of Sf-rhabdovirus proteins, resulting in the production of BVs which are similar in VSV-G pseudotyping levels to those produced in Sf9 RV-Free cells.

These experiments suggests that Sf-rhabdovirus contamination levels vary across batches and can alter the protein content and the performance of BV stocks, particularly in regard to mammalian cell applications.

Figure 5: Detection of Sf-rhabdovirus proteins in insect cells and viral supernatants.



(A) Supernatants from Sf21, Sf9 and Sf9-RVFree at 2x10^6 cells/mL. (B) Viral supernatants from Sf9 or Sf9 RV-Free cells inoculated with the all-in-one CRISPR BVs. Viral batches were inoculated with the same all-in-one CRISPR BV stock produced in Sf9RV-Free cells one week apart from each other. In all cases, cellular and viral supernatant were clarified to remove cellular debris (800xRCF for 5 minutes), filtered (0.22 µm) and concentrated 50 times (17000 x RCF for 90 minutes). Pellets from 2 mL of concentrated supernatant were resuspended in 40 µL PBS, supplemented with 13.3 µL Laemmli buffer and boiled at 95°C for 5 minutes. 20 uL per lane were loaded on a SDS-GEL and stained with colloidal Coomassie blue.

CONCLUSIONS

Chemically defined ESF AdvanCD cell culture medium

Chemically defined media have the obvious advantage of enabling standardised BV production, with minimal batch-to-batch variability. In the baculovirus-insect cells system, an ideal medium should not only consistently support proliferation of insect cell stocks, but it should not impair baculovirus amplification parameters. For the development of baculovirus vectors tailored for gene delivery to mammalian cells, said medium should additionally not interfere with BV-mediated gene delivery into the mammalian host.

Our results confirm that ESF AdvanCD media is a viable substitute of ESF 921 for baculovirus vector manufacturing and does not alter BV amplification parameters or its transduction properties in mammalian cells. Under all tested conditions, from virus amplification to transduction properties, ESF AdvanCD performed as well as the ESF 921 medium. ESF 921 and ESF AdvanCD media, supported amplification of all-in-one BV vectors (23 kb synthetic constructs) and were compatible with efficient delivery in mammalian cells.

One key difference however, is the ability of ESF AdvanCD medium to support higher viable cell density in the uninfected stocks. This feature can effectively be used to significantly reduce the volume of cell culture, and consequently the cost of BV manufacturing by increasing the recovered viral titres without altering the culturing vessel volume. In addition, its chemically defined composition will enable minimal batch-to-batch variation, with important implications for scaling up and increasing consistency of BV manufacturing processes.

Sf9 RV-Free cells

Although the presence of adventitious Sf-rhabdovirus has been confirmed in most Sf21 and Sf9 cell lines (and it is virtually present in all of them) [7,8,11], this contamination has been shown to have only marginal implications for biological products derived from the baculovirus-insect cells expression systems, providing that downstream purification steps can physically eliminate Sf-RV from biological products (e.g., recombinant proteins, AAVs etc.).

Isolating Sf-rhabdovirus free baculovirus particles, on the other hand, is technically more challenging as both viral species are secreted in the supernatant and appear to have similar densities on sucrose gradient cushion [9].

While Sf-rhabdovirus has been considered incapable of infecting mammalian cells, more recent results demonstrated that it can sometimes permanently infect cultured mammalian cells *in vitro* and produce persistent, pantropic infections *in vivo* [11]. The implications of these findings for baculovirus-mediated gene delivery *in vitro* and *in vivo* however, have not been tested.

Only a handful of Sf9 RV- cell lines have been so far described and, to our knowledge, none of them has been tested for manufacturing of baculovirus particles aimed at mammalian cells gene delivery.

Our results show that, while Sf9 RV-Free cells display similar proliferation and viral amplification parameters to their parental cell line, the deriving baculoviruses display up to 10 times higher transduction efficiencies in mammalian cells, with a concomitant improvement of gene editing efficiencies when all-in-one CRISPR BVs are deployed.

The presence of Sf-rhabdovirus additionally adds batch variability in BV manufacturing with variable amount of Sf-rhabdovirus proteins detected across several batches. The levels of Sf-rhabdovirus proteins also appear to diminish VSV-G pseudotyping levels, which in turn could explain the different performance of BVs on mammalian cells and, more generally, for all those applications based on BV-display technology (e.g., vaccine development or enveloped VLP production).

Taken together, these findings suggest that the adventitious Sf-rhabdovirus could be a long ignored detrimental factor and the adoption of Sf9 RV-Free cells greatly improved the performance of baculoviruses on cultured mammalian cells. In our tests, baculovirus produced in Sf9 RV-Free cells cultured in ESF AdvanCD medium outperformed BVs produced in any other condition, and hold the potential to significantly expand BV-gene delivery applications both *in vitro* and *in vivo*.

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