Developing Tni-FNL ET: A *Trichoplusia ni* Insect Cell Line for Easy Titration of Baculovirus

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ABSTRACT

Baculovirus titration methods include immunological, qPCR, flow cytometry, plaque, and mean tissue culture infection dose ($TCID_{50}$) assays, which vary in expense, labor, and length. $TCID_{50}$ assays are a common tool for determining viral titers and have advanced with techniques that allow the use of microplate readers for easier detection. This thesis work aimed to develop a $TCID_{50}$ assay for baculovirus titration using the novel Tni-FNL Easy Titer (Tni-FNL ET) cell line. The Tni-FNL ET titration assay was designed to attempt to improve upon the currently offered Sf-9 Easy Titer (Sf-9ET) assay with the use of a microplate reader and a shorter assay time.

Tni-FNL cells were stably transfected with plasmid DNA containing the mRuby3 gene under the control of the polyhedrin baculovirus promoter. Infection of the stable Tni-FNL cell line with baculovirus results in mRuby3 production due to the activation of polyhedrin promoter by viral gene products. It was observed that only plasmid DNA constructs containing a homologous region (hr) successfully produced detectable mRuby3 fluorescent protein. Polyhedrin promoter constructs with hr5 sequences were chosen to further develop the $TCID_{50}$ assay. Relative fluorescent measurements, detected by the BMG Labtech Omega FLUOstar microplate reader, two standard deviations above the mean of the control wells were considered positives when performing $TCID_{50}$ calculations. Calculations for viral titer were done using the Reed-Muench method. This new baculovirus titration method was able to yield the desired level of signal, as detected by a microplate reader after a 4-day incubation, to determine viral titers in the range of 10^5 to 10^{10}. Further assay optimization is needed to achieve consistent, usable titers.

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LISTS OF ABBREVIATIONS

AcMNPV	*Autographa californica multiple nuclear polyhedrosis virus*
BEVS	Baculovirus Expression System
BV	Budded Virion
eGFP	enhanced Green Fluorescent Protein
Hr	homologous region
MOI	Multiplicity Of Infection
ODV	Occlusion-Derived Virion
PEL	Protein Expression Laboratory
PFU	Plaque-Forming Units
PTM	Post-Translational Modification
qPCR	quantitative Polymerase Chain Reaction
Sf	*Spodoptera frugiperda*
Sf-9ET	Sf-9 Easy Titer
$TCID_{50}$	Mean Tissue Culture Infectious Dose
Tni	*Trichoplusia ni*
Tni-FNL ET	Tni-FNL Easy Titer

INTRODUCTION

The baculovirus expression vector system (BEVS) is an extensively utilized tool for expressing recombinant proteins in insect cells (Jarvis 2009). Within the Protein Expression Laboratory (PEL) at the Frederick National Laboratory for Cancer Research, the BEVS is used to optimize recombinant protein production for a wide range of eukaryotic proteins, including mammalian proteins. Insect cell lines, like Sf9 and High Five™, can perform similar post-translational modifications (PTMs) to mammalian cells, making them useful for producing more complex proteins (Jarvis 2009). The BEVS provides a safe and efficient method to produce large amounts of soluble protein for downstream applications, such as structural studies, activity assays (Bonsor *et al*. 2022), and vaccine development (Joshi *et al*. 2020; Hong *et al*. 2023). Baculovirus titration is a key step when using the BEVS to guarantee reproducible protein yields. This project proposes a new method of titrating baculovirus for use in recombinant protein expression.

Baculovirus Expression System

The BEVS works by infecting insect cells with a recombinant baculovirus that contains the foreign gene(s) of interest (Jarvis 2009). Under ideal conditions, infection is carried out with a specific multiplicity of infection (MOI) to ensure optimal protein production which can only be accomplished if the baculovirus has been properly titrated. Once the baculovirus enters cells and undergoes viral replication, the insect cell protein machinery is dedicated largely to viral proteins (Clem and Passarelli 2013). In this process, the foreign gene(s) of interest is expressed with the viral proteins (Jarvis 2009).

Common baculoviruses for the BEVS are *Autographa californica* (alfalfa looper) multiple nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* (silkworm) nuclear polyhedrosis virus (BmNPV) (Invitrogen c2006-2024). Baculoviruses typically have narrow host ranges (Clem and Passarelli 2013), however, AcMNPV is an exception and can infect 39 lepidopteran species (Song *et al*. 2016). *Spodoptera frugiperda* (Sf) and *Trichoplusia ni* (Tni) are the common lepidopteran species used for the BEVS with AcMNPV (Jarvis 2009).

Common Insect Cell Lines Used for the BEVS

Spodoptera frugiperda cell lines are derived from fall armyworm ovarian cells. The Sf21 insect cell line (IPLB-Sf-21-AE) is the parental *Spodoptera frugiperda* cell line. Sf9 insect cells are a clonal isolate from IPLB-Sf-21-AE and are commonly used for producing recombinant baculovirus stocks and recombinant proteins. Sf21 and Sf9 cells can be grown in suspension or adherent cell culture (Insect Cells c2024).

Trichoplusia ni cell lines are derived from cabbage looper ovarian cells and there are multiple isolates available for use. The ThermoFisher High Five™ cells (BTI-TN-5B1-4) are a clonal isolate derived from the parental *Trichoplusia ni* cell line and are specifically optimized for recombinant protein expression (ThermoFisher…c2006-2024). Tni-FNL cells were derived by PEL from the BTI-TN-5B1-4 cell line, received directly from Boyce Thompson Institute, and are also used for recombinant protein expression (Talsania *et al*. 2019). Both of these cell lines can be grown in adherent or suspension cell culture (ThermoFisher…c2006-2024; Talsania *et al*. 2019). *Trichoplusia ni* cell lines typically

produce higher yields of recombinant protein compared to Sf cell lines, making them a favorable choice for protein production (Wilde *et al*. 2014).

Baculovirus Replication

The baculovirus replication cycle occurs in a temporal fashion. There are four phases to its replication: immediate early, delayed early, late, and very late (Figure 1). Gene expression of each phase relies on the viral proteins expressed in the prior phase for transcriptional activation. In the immediate early and delayed early phases the virus is entering the cell and undergoing early gene expression that will prepare the cell for viral DNA replication. Once viral DNA replication begins in the late phase budded virions (BV) can assemble containing the viral genome and structural proteins (Figure 2). The very late phase concludes the replication cycle with high expression of polyhedrin and p10, the formation of occlusion-derived virions (ODV), and cell lysis to release the viral particles (Figure 3). Polyhedrin, the main protein that forms the occlusion bodies for ODVs, has a high transcription rate, making its promoter a commonly used tool for high production of recombinant proteins using the BEVS. Replacing the polyhedrin gene for recombinant protein expression prevents the formation of ODVs, making BVs the sole viral progeny in cell culture (Grose *et al*. 2021).

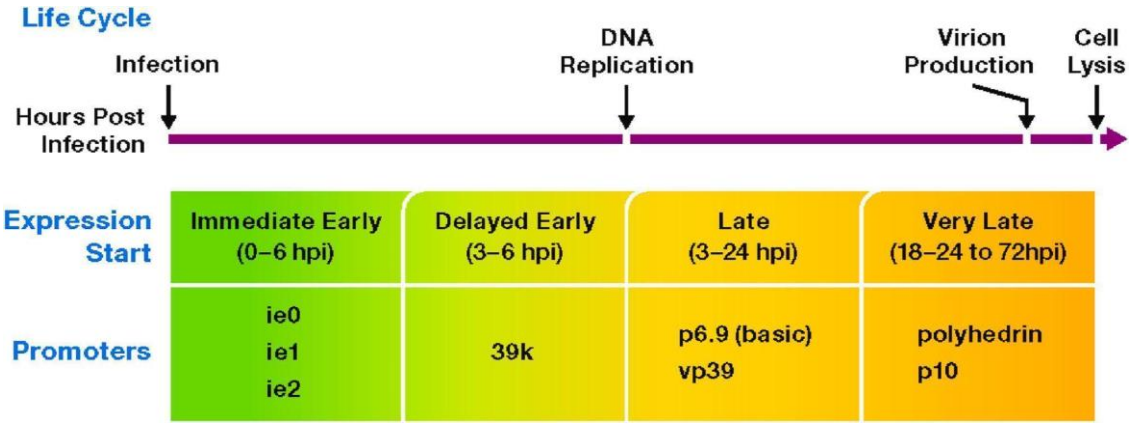

Figure 1. Baculovirus promoters and expression during the viral replication cycle. Key events during infection are put in chronological order and specific promoters for each stage are listed. From Grose *et al*. 2021.

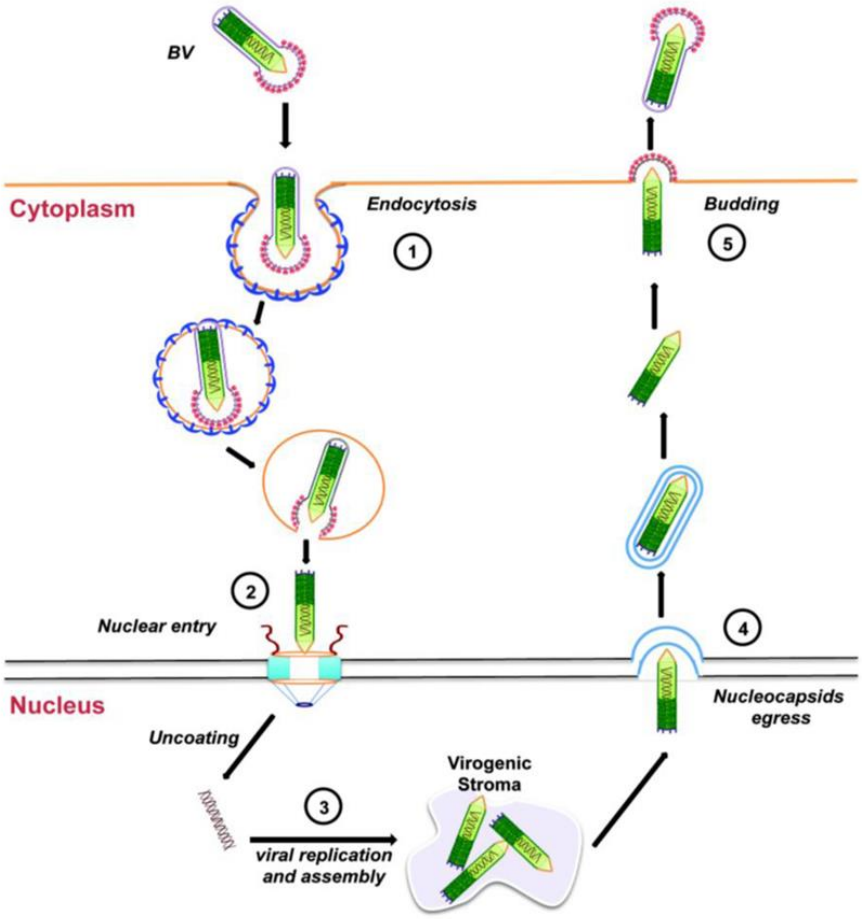

Figure 2. Diagram of the baculovirus replication cycle. The diagram shows infection by and production of BVs. From Au *et al*. 2013.

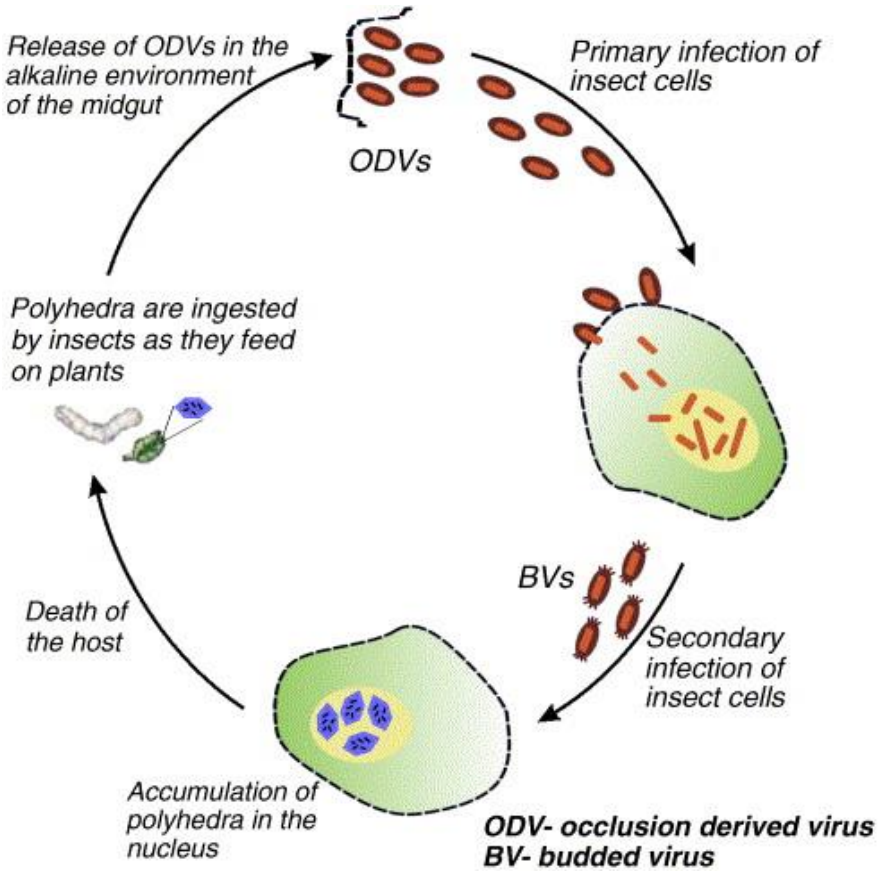

Figure 3. Life cycle of AcMNPV in insects. Insects ingest plant material that contains viral polyhedra, releasing ODVs that infect the epithelial midgut cells. The virus replicates producing BVs that infect other tissues. Polyhedra accumulates and is released by cell lysis causing death of the host. From Szewczyk *et al*. 2006.

Homologous Regions and Their Role in Transcription

Homologous regions (hrs) are found interspersed throughout the AcMNPV baculovirus genome and have been noted for their roles as origins of replication and enhancers in baculovirus replication (Kool *et al*. 1993). There are five main hrs and their presence and distribution are conserved between different strains of AcMNPV DNA (Cochran and Faulkner 1983). These sequences are aptly named, as the homology between them is 75% conserved and all have a 28-bp imperfect inverted repeat (Guarino *et al*.

1986a). AcMNPV is not the only baculovirus to have hrs; others such as *Bombyx mori* MNPV have hrs in their genomes as well (Kool *et al*. 1993).

Homologous regions enhance transcription of genes with certain baculoviral promoters. All five hrs were individually cloned upstream of the early 39K promoter controlling the CAT gene resulting in varying levels of enhancement of CAT activity. The degree to which each hr increases transcription varies based on both the hr and the promoter (Guarino *et al*. 1986a). Early viral transcription from the early p35 promoter is enhanced by hr5 by increasing transcription initiation, while the effect of hr5 on some late and very late promoters is seen to be non-beneficial to expression (Rodems and Friesen 1993).

The regulatory viral protein, IE-1, plays a major role in regulation of baculovirus gene expression (Guarino and Summers 1987). One of its roles specifically is binding to hrs to play a part in regulating transcription (Leisy *et al*. 1995; Rodems and Friesen 1995). IE-1 is seen to be required for hr-mediated transcriptional enhancement for certain combinations of hrs and promoters. Hr5-mediated enhancement of the early 35K promoter has been observed to not require the presence of IE-1, but the addition of IE-1 increases expression significantly. Hr5-mediated enhancement of the 39K promoter on the other hand requires the addition of IE-1 (Nissen and Friesen 1989). Hrs may be essential for the expression of certain genes during viral replication. They contain binding sites for IE-1 and host factors, allowing them to potentially recruite proteins to activate transcription (Guarino and Summers 1986b; Olson *et al*. 2003; Landais *et al*. 2006).

Importance of Infecting at Proper MOI Values

Infecting at appropriate MOI values is necessary for ideal protein expression. MOI is the ratio of infectious viral particles to cells**.** In order to infect at a chosen MOI an accurate titer is needed. Certain proteins may not express at all until a suitable MOI is found. For example, the recombinant antigenic glycoprotein GA733 fused to the human IgG Fc fragment was expressed in Sf9 cells at a range of MOIs from 0.05 to 3, and expression was not detected until an MOI of 1 (Moussavou *et al*. 2018).

MOI is particularly crucial for co-infections. Recombinant baculoviruses for the rabies glycoprotein (BVG) and matrix protein (BVM) were co-infected in Sf9 cells at various MOI ratios to attempt to produce rabies virus-like particles. Protein yield was assessed for each ratio to determine which was most successful. These experiments found the best MOI ratio for the specific conditions of BVG and BVM co-expression (Leme *et al*. 2023). If the co-expression of BVG and BVM were to be repeated by this group with the preferred MOI ratio, but new recombinant baculovirus stocks used, it is important that the titers of the new stocks are accurate to accomplish the same results.

Baculovirus Titration Methods

Current baculovirus titration options include plaque assays, flow cytometry fluorescence-based titer determination assays, standard immunological assays combined with light microscopy, qPCR, and $TCID_{50}$ assays (Table 1).

Plaque assays determine the number of plaque-forming units (PFU) on a monolayer of infected cells. Plaques are caused by the lysing of an infected cell. This lysis releases viral particles that are transmitted to neighboring cells, spreading the infection. Baculovirus

titration by a plaque assay is done first by adherently growing the insect cell line of choice in cell culture plates. These adherently grown insect cells are infected with serial dilutions of baculovirus, and an overlay media, such as agarose, is applied after infection. The cells are incubated for 7-10 days, and then the resulting number of plaques can be counted to determine the titer based on the dilution factor and plating volume (Jarvis 2009). This assay is laborious and is not favored if there is access to newer technologies.

Proteos offers a flow cytometry fluorescence-based titer determination assay service. Baculovirus stock is sent to Proteos where they serially dilute it and infect insect cells. FITC-labeled anti-gp64 antibodies are added to the cells where they bind the AcMNPV envelope protein, gp64, on successfully infected cells. The number of infected cells is measured by flow cytometry. Proteos performs this assay in-house; materials are not sold by Proteos for individuals to perform this on their own. The cost as of January 2024 is $195.00 per sample, making it inaccessible for most. The timeline given for receiving the baculovirus titer data is 3-5 days (Proteos c2020). If individuals were to perform this assay on their own it would require the acquisition of expensive reagents and equipment, including fluorescent-tagged antibodies and a flow cytometer.

Takara Bio currently offers two baculovirus titration kits. The first is their BacPAK™ Baculovirus Rapid Titer Kit that can produce baculovirus titers in 48 hours. This kit uses a primary monoclonal antibody that binds gp64 on cells that have been infected with serial dilutions of virus, similar to the Proteos assay. A secondary HRP-conjugated antibody binds the primary antibody, staining the infected cells. Light microscopy is used to count the number of infected foci. The cost as of January 2024 is $110.80 per assay (Takara c2024).

The second kit Takara Bio offers is the BacPAK™ qPCR Titration Kit that can produce baculovirus titers in 4 hours. This kit involves DNA purification and qPCR using green intercalating dyes. Viral and control DNA are serially diluted across a plate for qPCR. The viral titer is determined from qPCR data by comparing Ct values to a standard curve generated from the control samples. The cost as of January 2024 is $2.90 per reaction, making it significantly cheaper than the rapid titer kit (Takara C2024). The expensive downside is this assay requires access to a qPCR machine.

Many baculovirus titration methods take advantage of the baculovirus gp64 envelope protein. gp64 is a fusion envelope protein present on BVs and plays a key role in transmitting baculovirus infection. It grants viral entry into cells by mediating membrane fusion with the plasma membrane in a pH-dependent manner. This fusion envelope protein is the major protein present on the outside of infectious BVs, making it an attractive target for titration methods (Monsma *et al*. 1996). Methods utilizing gp64 to measure titer require continual purchase of assay kits. It is also important to note that gp64 methods measure total viral particles, not infectious virions.

The method of titration this project aims to develop is a $TCID_{50}$, mean tissue culture infectious dose, assay. A $TCID_{50}$ assay is an endpoint dilution assay performed in a 96-well microplate. From the assay a $TCID_{50}$ value is determined, which is the dilution of a virus required to infect 50% of a cell culture. The plate layout has a serial dilution of a virus across the plate with the same concentration of cells across all wells. This assay is commonly performed using cytopathic effects (CPE) as the indicator of infection; however, CPEs can only be utilized if the virus of interest causes cell death. CPEs must also be visualized manually using a fluorescent microscope without the addition of other reagents,

such as those for a viability assay. $TCID_{50}$ assays that can be executed using a microplate reader to measure a change in fluorescence or absorbance caused by viral infection have benefits over CPE assays with an easier detection (BMG Labtech 2021). This assay relies on infection to produce a response, meaning its titer is based on infectious virus particles only, unlike gp64-based assays.

Sf-9ET Stable Cell Line and $TCID_{50}$ Assay

The inspiration for the Tni-FNL Easy Titer (ET) cell line was the Sf-9 Easy Titer (Sf-9ET) stable cell line created by PEL. The Sf-9ET cell line was derived from Sf9 cells and designed for easy titration of baculovirus in a $TCID_{50}$ assay. They were created by stable transfection with a plasmid containing the gene for eGFP under the control of the baculovirus polyhedrin promoter, as well as the gene for neomycin resistance (neo) for selection by geneticin. The neo gene is under the control of the ie1 promoter, which is active without the presence of a baculovirus infection, to allow for continual geneticin resistance (Hopkins and Esposito 2009; Guarino and Summers 1986c). When Sf-9ET cells are infected with baculovirus, early viral transcription factors activate the polyhedrin promoter, resulting in eGFP expression. The presence of eGFP fluorescence serves as the indicator of infection. The cells were single-cell cloned to have a clonal population with desired eGFP output following baculovirus infection (Hopkins and Esposito 2009).

To perform this assay, Sf-9ET cells in a 96-well plate are infected with serial dilutions of virus and incubated for 7 days. At the end of 7 days, the plate is scored for the presence of eGFP expression using a fluorescence microscope. This is done by individually viewing each well and searching for the presence of clusters of green cells by eye, making

it potentially unreliable. There were attempts to score the assay using a plate reader, but the high autofluorescence of the media in the green channel was a barrier to this option. Viral titer is calculated using the Reed-Muench method, taking into account the dilution scheme and number of positive wells (Hopkins and Esposito 2009). This assay uses relatively affordable materials and is comparable in length to some currently offered titration methods. The downside to this method is the combination of low eGFP output and the high media autofluorescence making the detection aspect more difficult.

Table 1. Comparison of Baculovirus Titration Methods.

Titration Method	Typical Assay Length	Specialized Equipment	Citations
Plaque assay	7-10 days	None	Jarvis 2009
Flow cytometry fluorescence-based assay	24 hours, 3-5 days if sent out to a company	Flow cytometer, gp64 fluorescent antibody	Takara c2024; Proteos c2020
Standard Immunological Assay	2 days	Light microscope, gp64 primary antibody, HRP-conjugated secondary antibody	Takara c2024
qPCR	Less than 24 hours	qPCR machine, viral nucleic acid purification kit, qPCR reagents	Takara c2024
Sf-9ET cell line ($TCID_{50}$)	7 days	Sf-9ET cells, fluorescence microscope	Hopkins and Esposito 2009
Tni-FNL ET cell line ($TCID_{50}$)	4 days	Tni-FNL ET cells, microplate reader OR fluorescence microscope	

PEL developed the novel Sf-9ET cell line and $TCID_{50}$ assay with the goal of making a more accessible baculovirus titration method. The Sf-9ET work served as a starting point to create the Tni-FNL ET cells. The choice of Tni-FNL cells over Sf9 cells to develop an improved cell line and assay is purposeful. Tni-FNL cells produce greater yields of protein than Sf9 cells, while not significantly propagating baculovirus (Wilde *et al.* 2014). This will allow for increased fluorescent protein output, giving hope to decrease the length of the assay, and make detection simpler and automated. The decreased virus propagation in Tni-FNL cells will make the amount of virus present in the assay remain consistent.

Baculovirus titers are required to infect at specified MOI values to achieve optimal and reliable protein expression. Some methods are cost-prohibitive, leading laboratories to skip titrating their baculoviral stocks all together. The importance of baculovirus titration when using the BEVS creates a need for inexpensive and timely methods to be developed. The Tni-FNL ET assay could provide a relatively affordable method to determine baculoviral titers.

MATERIALS AND METHODS

Designing the Expression Plasmid Constructs

Expression plasmids were designed to contain the mRuby3 gene (Bajar *et al*. 2016) under the control of the selected baculovirus promoters: hr5-p6.9, polyhedrin, and hr5-polyhedrin (Figures 4-7). A puromycin resistance gene was present in all plasmids under the control of the ie1 promoter. One of the polyhedrin promoter constructs was designed with hr5 upstream of the ie1 promoter, as opposed to upstream of the polyhedrin promoter (Figure 7).

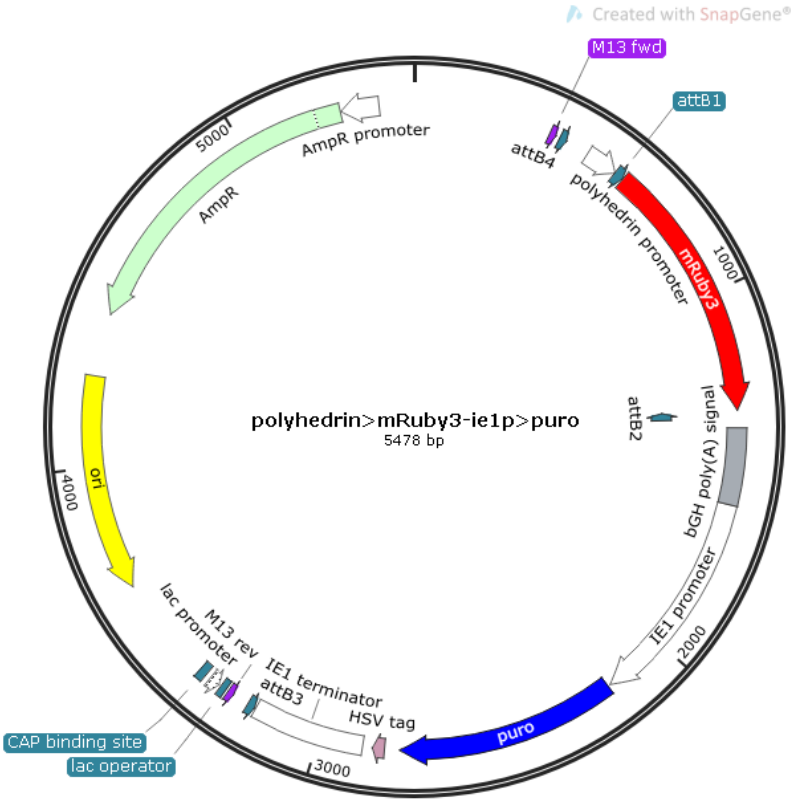

Figure 4. Expression clone design for mRuby3 under control of the polyhedrin promoter with no hr5 element. Ampicillin resistance is used for the selection of *E. coli* colonies during plasmid construction/propagation. The puromycin resistance gene controlled by the ie1 promoter allows for selection of Tni-FNL cells that have been transfected with the plasmid by the addition of puromycin.

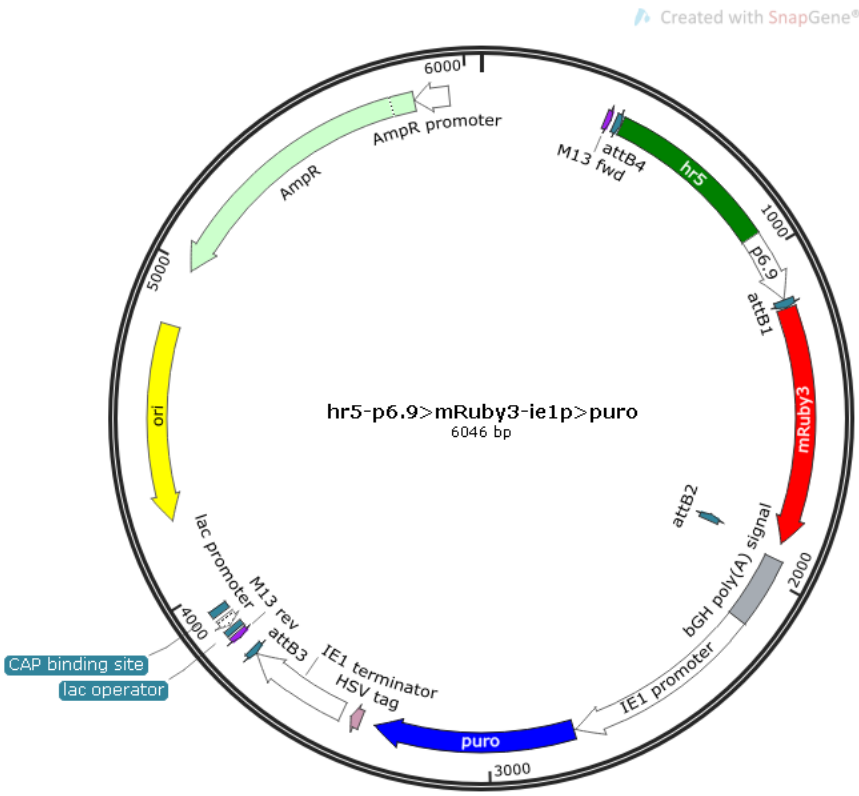

Figure 5. Expression clone design for mRuby3 under control of the hr5-p6.9 promoter. Ampicillin resistance is used for the selection of *E. coli* colonies during plasmid construction/propagation. The puromycin resistance gene controlled by the ie1 promoter allows for selection of Tni-FNL cells that have been transfected with the plasmid by the addition of puromycin.

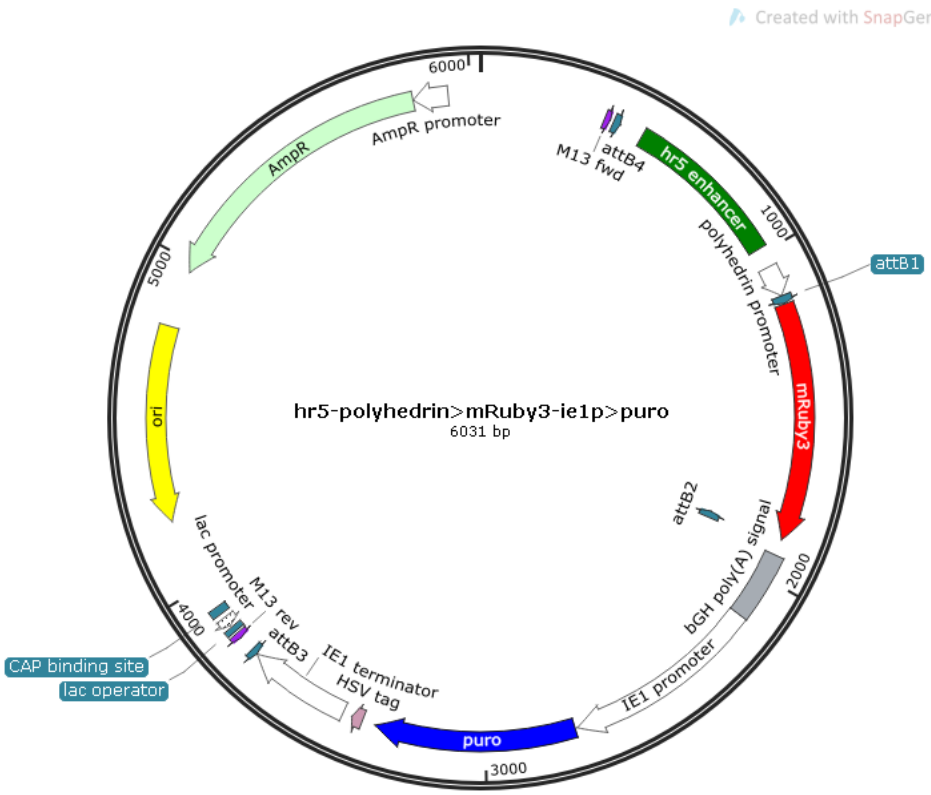

Figure 6. Expression clone design for mRuby3 under control of the hr5-polyhedrin promoter. Ampicillin resistance is used for the selection of *E. coli* colonies during plasmid construction/propagation. The puromycin resistance gene controlled by the ie1 promoter allows for selection of Tni-FNL cells that have been transfected with the plasmid by the addition of puromycin.

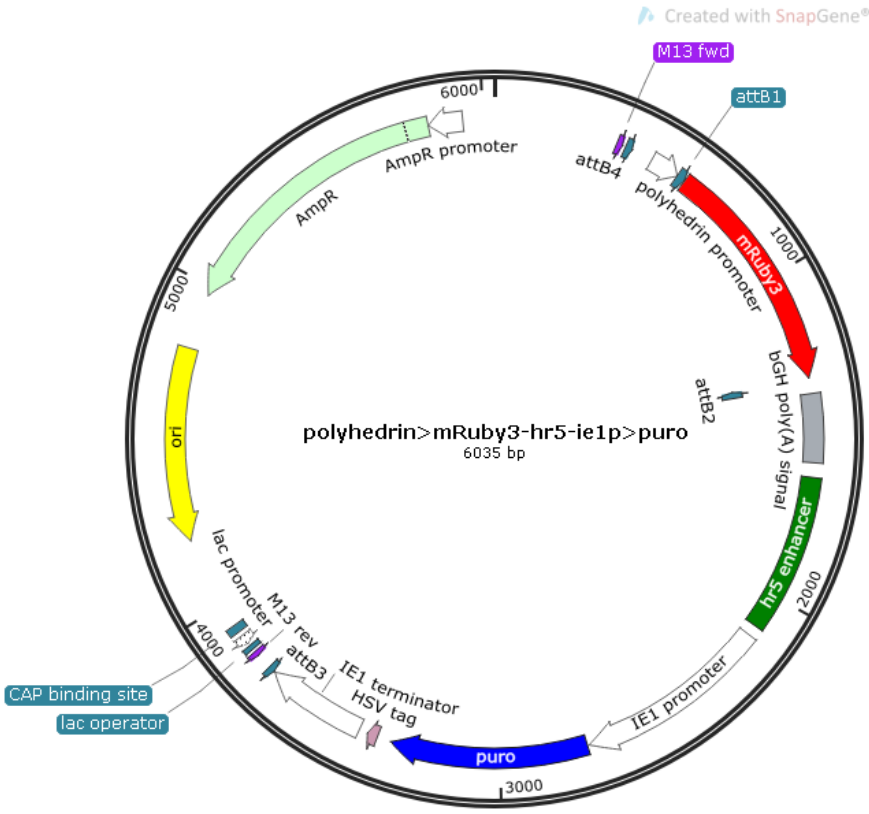

Figure 7. Expression clone design for mRuby3 under control of the polyhedrin promoter with an hr5 element upstream of the ie1 promoter. Ampicillin resistance is used for the selection of *E. coli* colonies during plasmid construction/propagation. The puromycin resistance gene controlled by the ie1 promoter allows for selection of Tni-FNL cells that have been transfected with the plasmid by the addition of puromycin. An hr5 sequence is upstream of the ie1 promoter.

Cloning the Expression Plasmid Constructs

The expression plasmids were constructed with Gateway cloning protocols. The multisite LR recombination protocol previously described was followed for the construction of the clones (Wall et al., 2013) (Figure 8). Entry clone plasmid inserts were generated by PCR, or ordered from ATUM (ATUM, Newark, CA) and Gene Universal (Gene Universal, Newark, DE) and used in multisite LR reactions with the LR Clonase II enzyme mix (ThermoFisher Scientific™, Waltham, MA) to construct the expression

clones. The LR reactions were transformed into chemically competent DH10B *E. coli* cells and individual colonies selected. Expression clones were purified using the QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and analyzed by agarose gel electrophoresis to verify the supercoiled size. Correctly sized plasmids were digested with the BsrGI restriction enzyme, then analyzed by agarose gel electrophoresis to confirm the presence of the inserts (New England Biolabs, Beverley, MA). One verified plasmid was prepared using the QIAGEN Plasmid Maxi Kit protocol to purify DNA for transfection (Qiagen Inc., Valencia, CA).

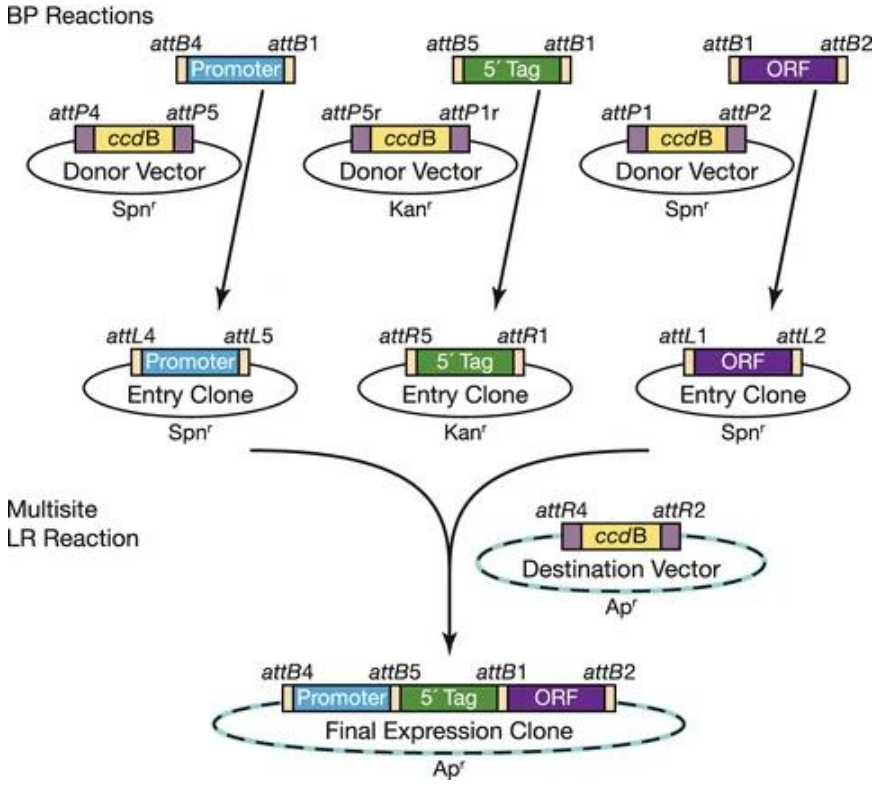

Figure 8. Construction of a multisite expression clone utilizing Gateway Recombination reactions. The representation begins with a PCR product and donor vector which produces an entry clone. Entry clones are then assembled in a multisite reaction into a destination vector to produce a final expression clone. Wall *et al*. 2013.

Creating the Stably Transfected Cell Lines

Tni-FNL cultures were set at a density of 1.0 x 10^6 cells/ml in 50 ml culture sizes using Sf-900 III serum-free media (ThermoFisher Scientific™, Waltham, MA). Cultures were transfected with 50 μg plasmid DNA using the Cellfectin™ II Reagent (ThermoFisher Scientific™, Waltham, MA), then were grown at 27°C. Puromycin was added 24 hours post-transfection at a concentration of 4 μg/ml in culture (ThermoFisher Scientific™, Waltham, MA). The first subculture was done at 1 x 10^6 cells/ml once cultures reached densities of around 6 x 10^6 cells/ml. Cell density and viability were monitored for the first couple weeks with daily cell counts to assess the need for subculturing and puromycin addition. Once selection was complete, indicated by maintaining a high cell viability, cells were subcultured on a normal schedule and puromycin was added only at subculturing at a concentration of 4 μg/ml in culture. Cells were usable for $TCID_{50}$ assay testing after the third passage.

Tni-FNL ET $TCID_{50}$ Baculovirus Titration Plate Setup

The protocol for the Tni-FNL ET titration plate setup was based on the Sf-9ET protocol (Hopkins and Esposito 2009). Black 96-well microplates with clear wells were used for the assay. Prior to plating, the baculovirus stock of interest was diluted in insect media in a sterile tube. Media was distributed in 100-μl volumes to columns 2-10. A 125-μl volume of the diluted baculovirus stock was added to each well in column 1 and serially diluted 1:5 across the plate, stopping at column 9. Tni-FNL ET cells were distributed across columns 1-10 in 100-μl volumes. Row 10 served as a control, receiving cells and no virus (Figure 9). The final volume in the wells was 200 ul.

The plate was put shaking at 27°C in a container with a slightly damp paper towel underneath the plate and an unsecured lid on top to prevent evaporation. This maintained the original volumes and therefore concentrations in each well.

After a 96-hour incubation the plate was set stationary for 45 minutes to allow the cells to settle the bottom of the well. The BMG Labtech Omega FLUOstar microplate reader was used to measure the relative fluorescence from columns 1-10. Raw data is exported and used for scoring the plate.

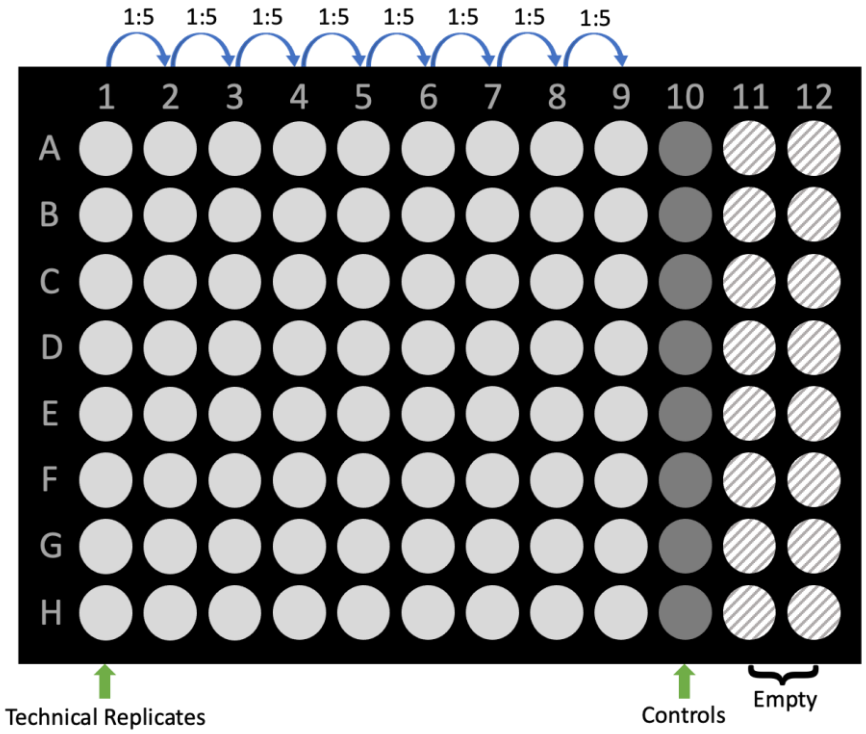

Figure 9. Layout of the $TCID_{50}$ assay plate. Light gray wells indicate where baculovirus is present. Dark gray wells indicate the control column where only cells are present. Striped wells are empty. The arrows on top of the plate indicate the 1:5 serial dilution of baculovirus across the plate.

Calculating $TCID_{50}$ Assay Titers

The Reed-Muench method is used for $TCID_{50}$ calculations when performing the Sf-9ET titration assay, and that method was used for the Tni-FNL ET titration assay as well. A well was marked as positive for infection if the relative fluorescence measurement was 2 standard deviations about the average of the control wells. The number of infected and non-infected wells was calculated for each column in the 96-well microplate, and then the "accumulated" infected or non-infected wells was calculated and displayed in a table (Table 2). From this, a ratio of infection was calculated for each dilution and given as a percentage.

The proportionate distance (PD) was calculated with the ratios from Table 2 using the first equation below. Once the PD was calculated the $logID_{50}$ could be found with the next equation. From $logID_{50}$ the ID_{50} could be determined, referred to as the endpoint dilution. The end dilution is the dilution at which 50% of the cell cultures would be infected. ID_{50} was input into the last equation to calculate the viral titer ($TCID_{50}$/ml) (Lei *et al*. 2021).

$$PD = \frac{\%positive\ above\ 50\% - 50\%}{\%positive\ above\ 50\% - \%positive\ below\ 50\%}$$

$$logID_{50} = log(dilution\ with > 50\%\ positive) + [PD \times (-\ log(dilution\ factor))]$$

$$\text{Viral titer} = \frac{1}{ID50} / volume\ in\ each\ well$$

For the example shown in Figure 10 and Table 2, the calculations are as follows:

$$PD = \frac{60\% - 50\%}{60\% - 17\%} = 0.2326$$

$$logID_{50} = log(10^{-6.2}) + [0.2326 \times (-\ log(10^{0.7})] = -6.36$$

$$Viral\ titer = \frac{1}{10^{-6.36}} / 0.2\ ml = \mathbf{1.15 \times 10^{7}\ TCID_{50}/ml}$$

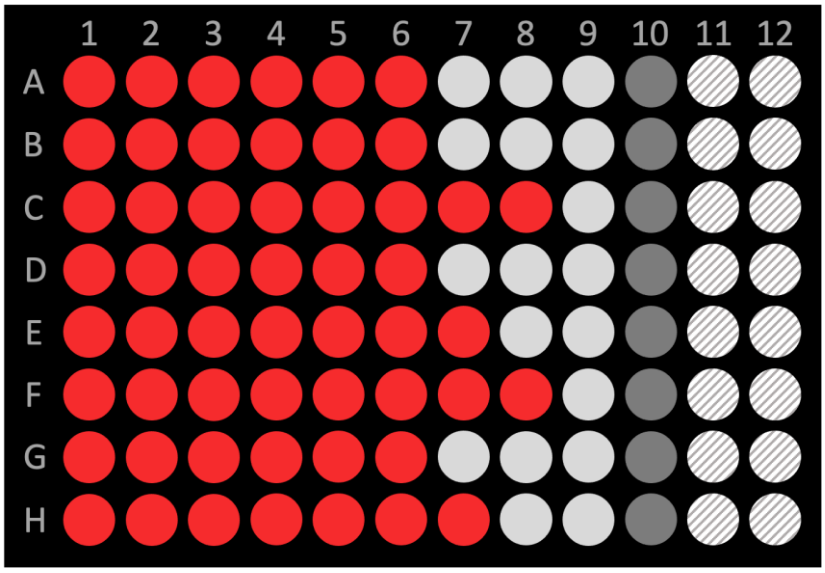

Figure 10. Example of a $TCID_{50}$ assay microplate with wells scored positive and negative for infection. Red indicates a positive well; light gray indicates a negative well. Table 2 shows how this translates to the numbers used to calculate viral titer using the Reed-Muench method.

Table 2. Example table layout for Reed-Muench calculations. The numbers are based on the example $TCID_{50}$ assay plate in Figure 10.

Column	Virus Dilution	Infected Wells	Non-Infected Wells	Accumulated Infected Wells (A)	Accumulated Non-Infected Wells (B)	Ratio (%) A/(A+B)
1	$10^{-2.0}$	8	0	54 ↑	0	100%
2	$10^{-2.7}$	8	0	46	0	100%
3	$10^{-3.4}$	8	0	38	0	100%
4	$10^{-4.1}$	8	0	30	0	100%
5	$10^{-4.8}$	8	0	22	0	100%
6	$10^{-5.5}$	8	0	14	0	100%
7	$10^{-6.2}$	4	4	6	4	60%
8	$10^{-6.9}$	2	6	2	10	17%
9	$10^{-7.6}$	0	8	0	18 ↓	0%

Once viral titer is determined a correction factor may need to be applied that is predetermined based on the final optimized assay. This will occur if the signal was not sufficient to work with a high enough virus dilution.

Correction factors in this project were determined by dividing a qPCR titer by the $TCID_{50}$ assay titer. The correction factors calculated were used for assessment of consistency across the assay, with the assumption that qPCR titers have consistent error values, more so than for direct comparison to the magnitude of the qPCR titer. $TCID_{50}$ and qPCR titers are determined with greatly differing methods, but qPCR titers were the readily available option for comparison. qPCR-determined titers tend to be higher than $TCID_{50}$ assay titers as they measure total viral particles as opposed to infectious virions.

Total Cell DAPI Staining

Total live cell DAPI staining was done with protocol from Biotium (Biotium c2024). Cells were spun down in 2 x 10^5 cell aliquots for 8 minutes at 10,000 RPM. The supernatant was discarded, and the cells resuspended in 200 µl of DAPI-dye-containing media at 10 µg/ml. The entire 200 µl volume was plated and diluted 1:2 down a 96-well microplate. The plate was imaged in the red and blue channels on a fluorescent microscope.

RESULTS

Expression Plasmid Design Choices

The following choices had to be made when designing expression plasmids to test for the Tni-FNL ET cell line: RFP or GFP to serve as the indicator of infection, which baculoviral promoter would control the fluorescent protein expression, and what gene would be used for selection.

eGFP was chosen in the Sf-9ET cell line as a reporter to indicate baculovirus infection. This choice was undesirable for the Tni-FNL ET cells, as insect media has high autofluorescence in the green channel (data not shown). If a GFP was chosen there would be significant background fluorescence from the media when read on a plate reader resulting in a lower signal. RFP protein, mRuby3, was selected for all expression plasmid constructs to be tested.

The polyhedrin promoter controls eGFP expression in Sf-9ET cells. For Tni-FNL ET cells, two baculoviral promoters were selected for experimentation with different purposes in mind. The very late stage polyhedrin promoter was chosen for its known strength in recombinant protein production. The late stage p6.9 was chosen because of its earlier activation after baculovirus infection. If the p6.9 promoter was able to have a sufficient fluorescent protein output it could allow for a shorter assay. An hr5 enhancer was placed upstream of the p6.9 promoter to enhance its expression.

Sf-9ET cells have the neo gene controlled by the ie1 promoter to be under selection by geneticin. Antibiotic kill curves were set up in Tni-FNL cells with geneticin and puromycin, administering amounts within and surpassing the suggested working concentrations, to determine which is best for selection. Tni-FNL cells exhibited resistance

to geneticin (Figures 11 and 12). The puromycin resistance gene under the control of ie1 promoter was chosen for all expression plasmid constructs to be tested.

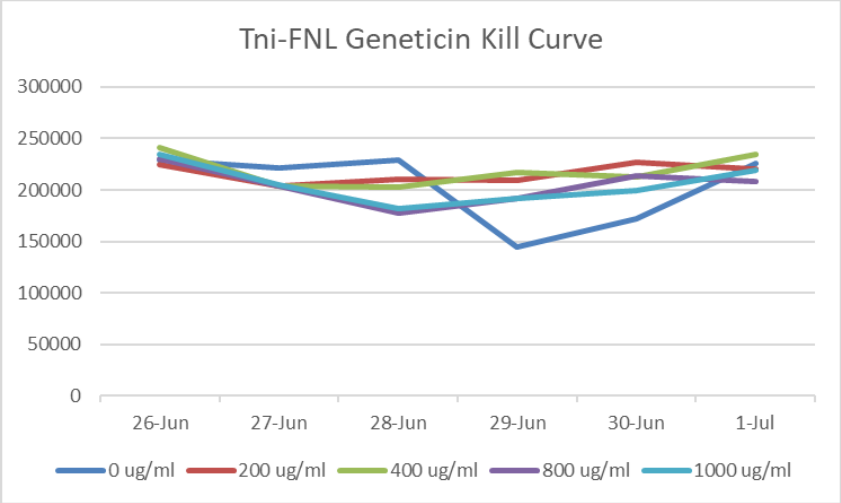

Figure 11. Kill curve of Tni-FNL cells with increasing concentrations of Geneticin™ (Gibco™, Carlsbad, CA). The cell viability was determined by using the alamarBlue™ Cell Viability Reagent (Invitrogen™, Carlsbad, CA) and reading the absorbance values on the BMG Labtech Omega FLUOstar microplate reader (Durham, NC, USA).

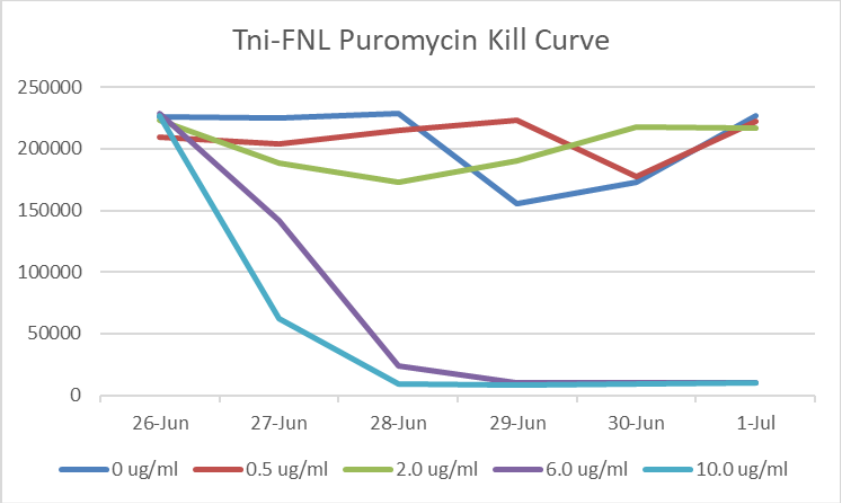

Figure 12. Kill curve of Tni-FNL cells with increasing concentrations of puromycin dihydrochloride (Gibco™, Carlsbad, CA). The cell viability was determined by using the alamarBlue™ Cell Viability Reagent (Invitrogen™, Carlsbad, CA) and reading the absorbance values on the BMG Labtech Omega FLUOstar microplate reader (Durham, NC, USA).

Assessing the Tni-FNL Stable Cell Lines for mRuby3 Output

Unexpected Results from the Hr5-p6.9 and Polyhedrin Promoter Constructs

The initial plasmid DNA constructs designed were the hr5-p6.9 and polyhedrin promoter constructs which were cloned and stably transfected into Tni-FNL cells. These constructs were assessed for relative mRuby3 output by infecting them at an MOI of 3 with the same recombinant baculovirus. After 3 days the cells were plated in 96-well microplates, viewed under a microscope, and measured on a microplate reader to observe mRuby3 fluorescence. mRuby3 fluorescence could be seen from hr5-p6.9, and nothing was detected for the polyhedrin construct (Figure 13).

To confirm this outcome, Tni-FNL cells were stably transfected with the polyhedrin construct once more. The test infection was repeated and analyzed in the same method as before. The results remained the same, confirming that the polyhedrin promoter construct fails to produce detectable fluorescence in Tni-FNL cells.

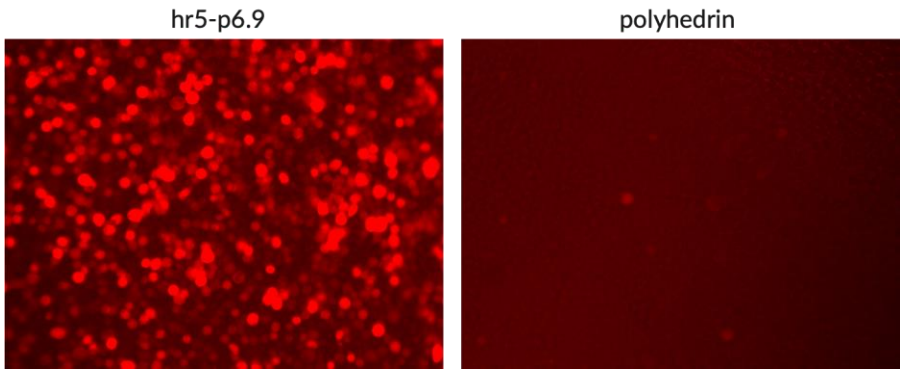

Figure 13. Fluorescent microscope images comparing the hr5-p6.9 and polyhedrin promoter constructs. Images were taken in the RFP channel of cells plated from test baculovirus infection flasks.

The Sf-9ET cell line was created with a polyhedrin promoter construct and successfully produces eGFP. To rule out any Tni-FNL specific issues with the lack of

mRuby3 production from the polyhedrin construct, Sf9 cells were transfected with the polyhedrin plasmid DNA. Results were the same in Sf9 cells (data not shown).

Determining Why the Polyhedrin Construct is Failing

Several new expression plasmids were cloned to determine why the polyhedrin construct is not performing as expected. Plasmids were designed with delayed early, late, and very late baculoviral promoters to assess if something unexpected was occurring in the very late stage that was causing the polyhedrin construct to fail. Constructs with and without hrs were made, including a p6.9 construct and a hr5-polyhedrin construct to compare directly to the original hr5-p6.9 and polyhedrin constructs.

The new plasmid constructs were each stably transfected into Tni-FNL cells and the stable cell lines were infected with the same recombinant baculovirus in small scale cultures. Only constructs containing hrs were successful in producing detectable mRuby3 fluorescence after baculovirus infection (Figure 14). From this data, it was decided to move forward with the hr5-polyhedrin promoter cell line. The fluorescence from the stable cells with this construct was the brightest from all of the constructs tested thus far.

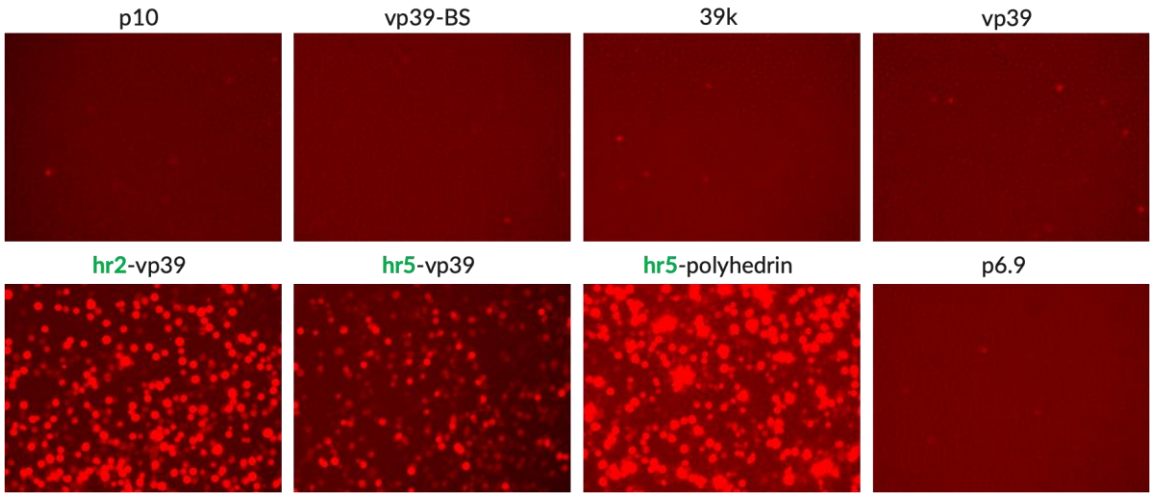

Figure 14. Fluorescent microscope images of the new experimental constructs. Images were taken in the RFP channel of cells plated from test baculovirus infection flasks. The same number of cells has been imaged for each construct. The construct transfected for each stable cell line is labeled above its corresponding image.

Designing a New Polyhedrin Construct with a Downstream Hr5

Data from another ongoing PEL project revealed that the hr5 placement upstream of the polyhedrin promoter may negatively impact the strength of the promoter. The project was comparing the levels of mNeonGreen fluorescent protein output from Tni-FNL cells infected with recombinant baculoviruses containing different baculoviral promoters, some of which included hr sequences. Recombinant baculoviruses were made for each construct and used to infect small scale Tni-FNL cultures at MOI 3. After 3 days relative fluorescence measurements were taken on a plate reader from the whole cells and the clarified lysates. Among the constructs tested there was a polyhedrin and a hr5-polyhedrin virus. The polyhedrin baculovirus outperformed the hr5-polyhedrin baculovirus (Figure 15).

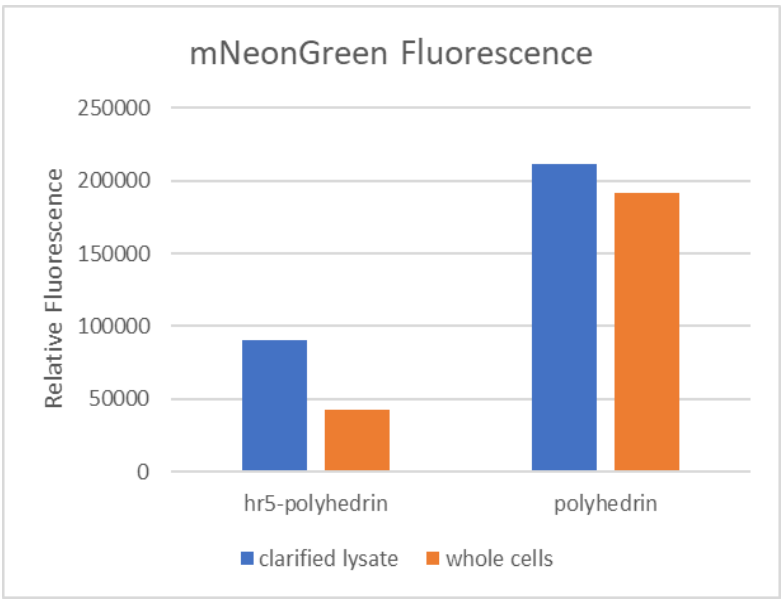

Figure 15. mNeonGreen fluorescent protein output from hr5-polyhedrin and polyhedrin promoter recombinant baculoviruses. mNeonGreen recombinant baculoviruses were used to infect Tni-FNL cells and the relative fluorescence of the whole cells and the clarified lysate was measured using the BMG Labtech Omega FLUOstar microplate reader.

Based on this data, a new plasmid was designed: polyhedrin>mRuby3-hr5. The hr5 region was placed downstream of the polyhedrin promoter as an enhancer of the ie1 promoter. This is the same placement used in the Sf-9ET plasmid construct.

The Hr5-polyhedrin and Polyhedrin>mRuby3-hr5 Constructs Perform Similarly

Small cultures of the hr5-p6.9, hr5-polyhedrin, and polyhedrin>mRuby3-hr5 stable cells were infected with the same baculoviral stock at an MOI of 3 to compare. After 3 days the cells were plated and serially diluted 1:2 down a 96-well microplate. Relative fluorescence measurements were taken on the plate reader and the values for the different cell amounts charted (Figure 16). The hr5-polyhedrin and polyhedrin>mRuby3-hr5 constructs had notably higher fluorescence than the hr5-p6. Despite efforts to redesign the

hr5-polyhedrin plasmid to move the hr5 downstream, the hr5-polyhedrin and polyhedrin>mRuby3-hr5 constructs had similar levels of mRuby3 output.

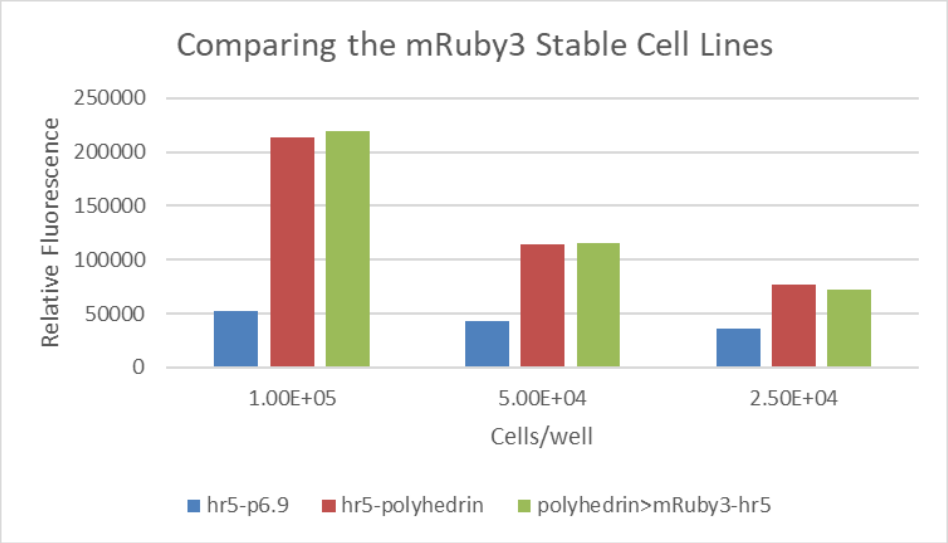

Figure 16. Comparison of mRuby3 outputs between the hr5-p6.9, hr5-polyhedrin, and polyhedrin>mRuby3-hr5 stable cell lines after baculovirus infection. 50 ml cultures were infected with baculovirus at MOI 3. After 3 days, cells were plated and serially diluted 1:2 down the plate and measurements taken on the BMG Labtech Omega FLUOstar microplate reader.

Not All Cells are Producing Detectable Levels of mRuby3

When the stable cells were viewed under a fluorescent microscope following baculovirus infection to visualize mRuby3, it was observed by eye that many cells had none or little fluorescence. To confirm this, following baculovirus infection the hr5-polyhedrin promoter stable cells were live-DAPI stained and imaged with a microscope using the red and blue channels (Figure 17). It was clear not all of the hr5-polyhedrin stable cells were expressing detectable levels of mRuby3.

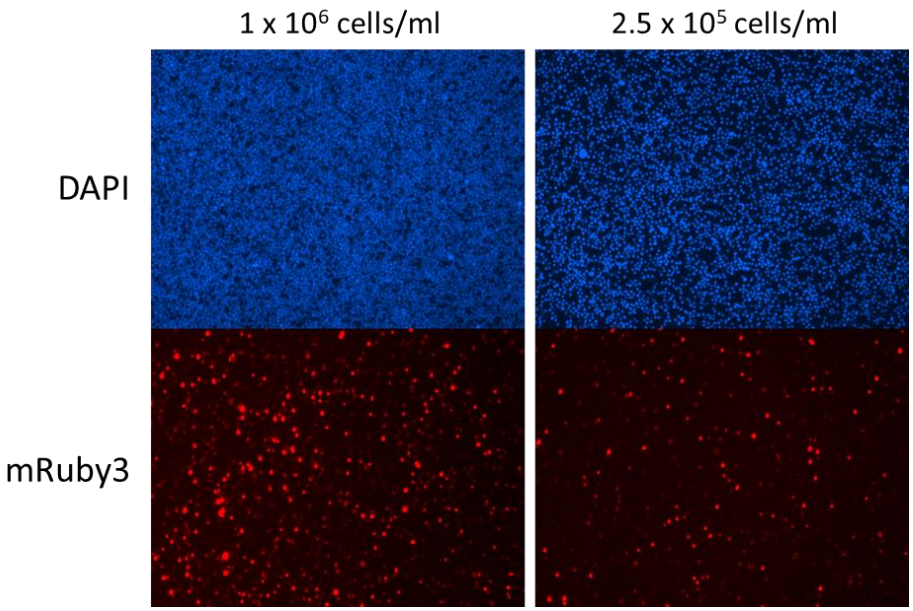

Figure 17. Fluorescent microscope images of infected hr5-polyhedrin stable cells after live total DAPI staining. Images taken in the blue and red channels of cells at concentrations of 1 x 10^6 cells/ml and 2.5 x 10^5 cells/ml to visualize cells producing, or failing to produce, detectable levels of mRuby3. Images in the blue and red channels were taken cells in the same placement.

Assay Development

The Sf9-ET protocol was used as a starting point in assay development for the Tni-FNL ET cell line. For the initial test, the Sf-9ET protocol was followed as written for virus dilutions and cell amounts. The Sf-9ET protocol instructs a pre-plating virus dilution of 1:500 and serial dilution of 1:5 across the plate. The cell amount used is 7.5 x 10^4 cells per well. Black plates with clear wells are used to prevent background fluorescence. To attempt to achieve the goals of a shorter assay time and easier detection, the incubation time was shortened from 7 days to 3 days to start, and detection was done with a microplate reader as opposed to a fluorescence microscope.

Viruses with qPCR-determined titers of 5–6 x 10^9 were used, if not specified otherwise, for the initial optimization steps to assess general signal of positive wells being

achieved. The Sf-9ET assay was able to produce a 10^9 viral titer when titrating a virus that had a 10^9 qPCR-determined titer. The Sf-9ET titer was ~2.5x less than the qPCR titer (data not shown). This was kept in mind when optimizing the Tni-FNL ET assay.

The titer range potential of 10^5 to 10^{10} for this assay was followed when assessing the level of signal achieved for different experiments. The signal of positive wells for a 10^5 titer virus would be expected to be present in columns 1-3, and the signal of a 10^{10} titer virus would extend through column 10.

The Hr5-p6.9 Promoter Construct Does Not Have Sufficient mRuby Output

Initial tests were carried out with the hr5-p6.9 construct before the polyhedrin construct was determined to need an hr and a new plasmid was created. From these tests, the first alteration made was in pre-plating virus dilution. The 1:500 pre-plating virus dilution was tested in the hr5-p6.9 cells and there were no positive wells after the 3-day incubation. Plates were prepared without a pre-plating virus dilution and signal was then present but much below what was needed (Figure 18). In all tests, cells were clustering around the edges of the well, as visualized with a fluorescence microscope. The plate reader reads straight through the center of the well and will not be able to detect the fluorescence efficiently from cells around the edge. The mRuby3 output from the hr5-p6.9 construct was insufficient for this assay.

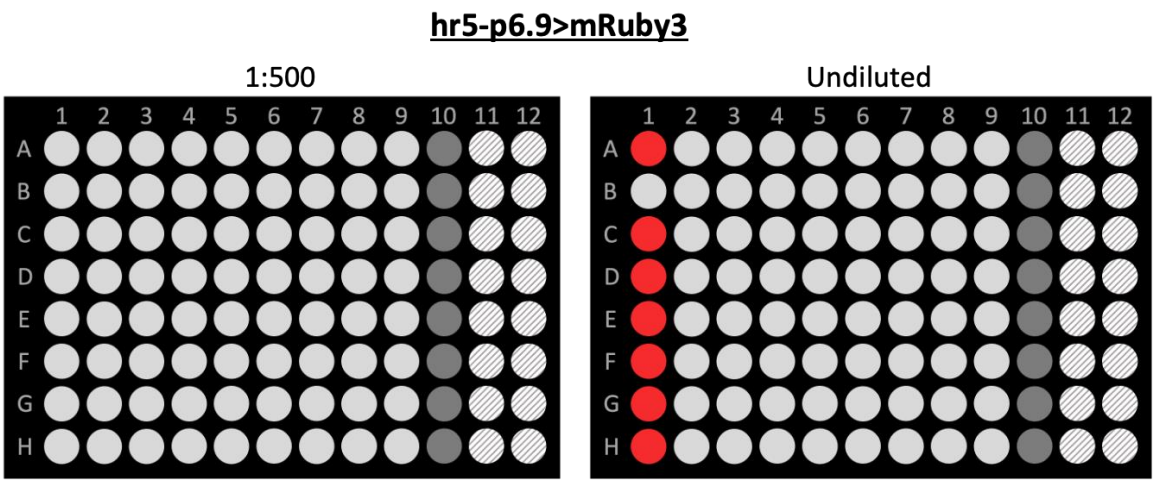

Figure 18. $TCID_{50}$ plate scoring of mRuby3 reporter from hr5-p6.9 tests. Red indicates a positive well; light gray indicates a negative well. The 1:500 plate labeled plate followed the 1:500 pre-plating virus dilution in the Sf-9ET protocol. The undiluted plate had undiluted virus plated into the first column.

Shaking the Plates Increases mRuby3 Output from Tni-FNL ET Cells

Shortly after the initial hr5-p6.9 tests, the hr5-polyhedrin construct was made and ready for use. $TCID_{50}$ assay plates were set using the hr5-polyhedrin construct with undiluted virus plated in the first column, based on the initial tests with the hr5-p6.9 construct. This showed a mild improvement in signal from the hr5-p6.9 tests. Another plate was prepared in the same way and set to shake as opposed to sitting stationary for the 3-day incubation. The plate that was shaking had improved signal, around 2x that of the stationary plate (Figure 19). All future plates were set shaking the entirety of the incubation period. After the incubation period, plates must be placed stationary for about 45 minutes before taking measurements to allow the cells to lay down. The plate reader cannot accurately take readings on cells in suspension. Even with the addition of shaking, cells were still noticeable clustering around the edges of the wells.

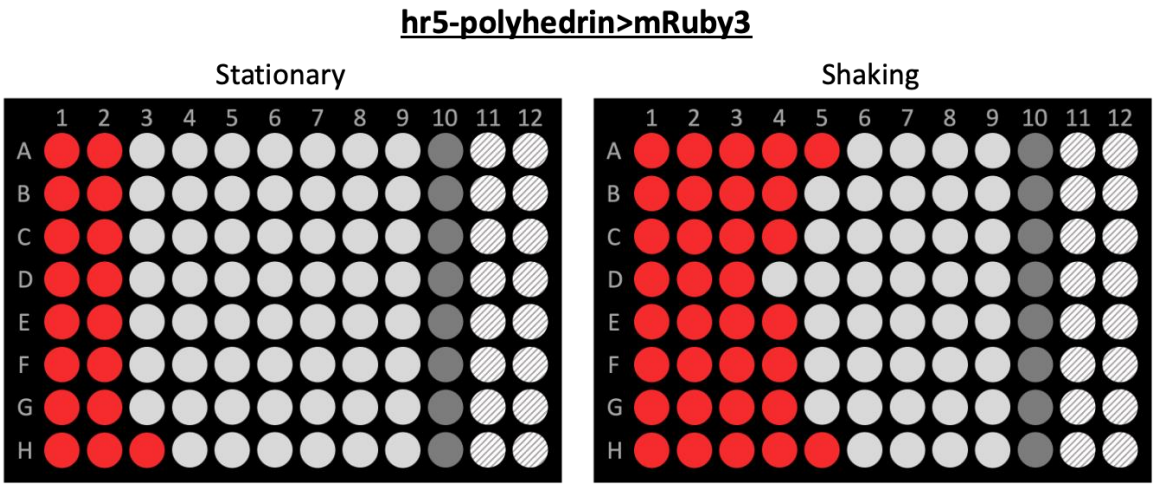

Figure 19. $TCID_{50}$ plate scoring of mRuby3 reporter from hr5-polyhedrin plates stationary versus shaking. Red indicates a positive well; light gray indicates a negative well.

Decreasing the Cells Per Well Amounts Improves Assay Performance

The cell concentration in the wells was decreased from the 7.5 x 10^4 cells/well stated in the Sf-9ET assay. Sf9 cells are smaller than Tni-FNL cells, making it logical to decrease cells per well when working with a larger cell size. The other benefit from decreasing the cell amount, but not the virus amount, was an increased MOI. The following cell amounts were tested: 5 x 10^3, 1 x 10^4, and 2 x 10^4 cells/well. The best results were from 5 x 10^3 cells/well (Figure 20).

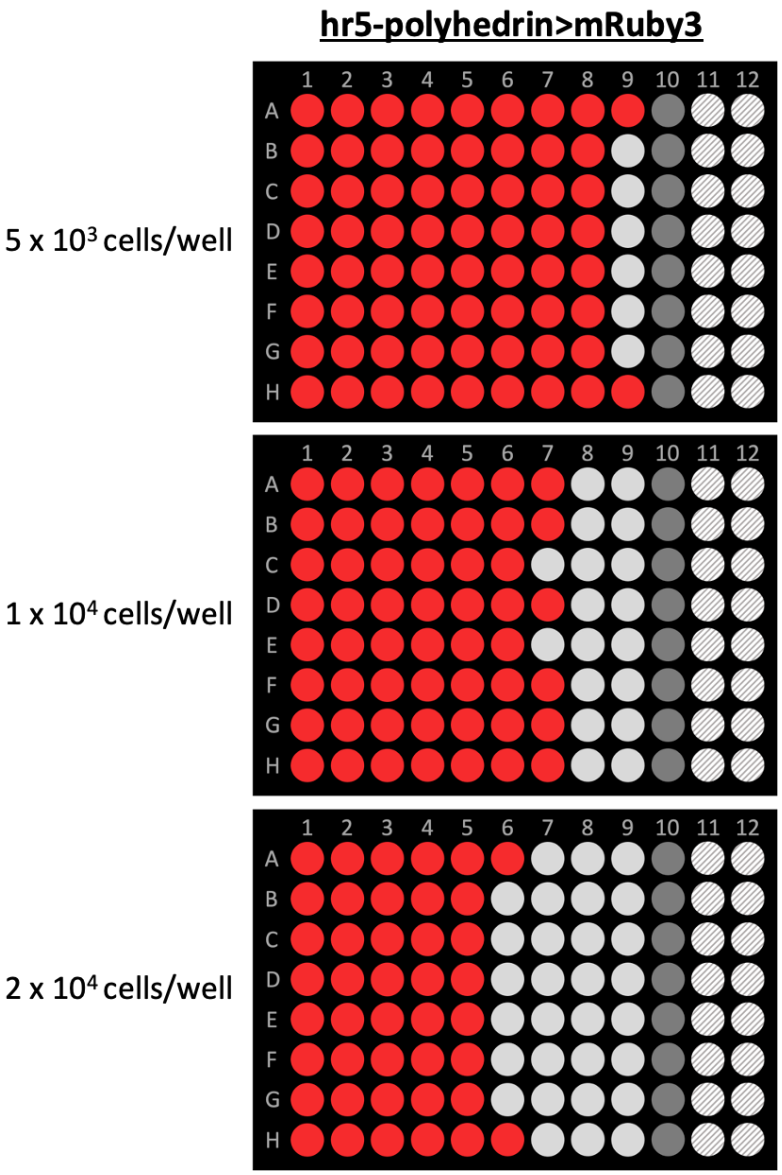

Figure 20. $TCID_{50}$ plate scoring of mRuby3 reporter from hr5-polyhedrin cells per well comparison tests. Red indicates a positive well; light gray indicates a negative well. 5 x 10^3 cells/well, 1 x 10^4 cells/well, and 2 x 10^4 cells/well were separately used in the $TCID_{50}$ assay protocol with the hr5-polyhedrin stable cells.

A 1:100 Pre-plating Virus Dilution Does Not Gives Sufficient Signal

A pre-plating dilution would be desirable to decrease the need for a correction factor. Once the signal improved from using undiluted virus, shaking the plates, and

optimizing the cells per well amount, a pre-plating virus dilution of 1:100 was performed to assess the effect on signal.

The polyhedrin>mRuby3-hr5 stable cells were available at this point and were used alongside the hr5-polyhedrin stable cells for these tests to compare. The 1:100 virus dilution was tested on 5 x 10^3, 1 x 10^4, and 2 x 10^4 cells/well amounts again to make sure that changing the virus dilution would not change the optimal cells/well amount. It was still observed that plates with 5 x 10^3 cells/well had the highest signals. Two viruses with 4.4 x 10^8 and 5.42 x 10^9 titers as determined by qPCR, were used for these tests. Plates were measured at 3-day (data not shown) and 4-day incubation times to assess if a 4-day incubation could improve signal (Figure 21). The 3-day incubation did not suffice, and even with the 4-day incubation it was clear a lesser dilution was needed. Titers from the $TCID_{50}$ assay plates at day 4 were compared to the qPCR titers with correction factors to assess consistency between the rounds and the viruses tested (Table 3). 1:100 was too high of a pre-plating dilution to achieve the signal needed to fit the desired titer range of the assay.

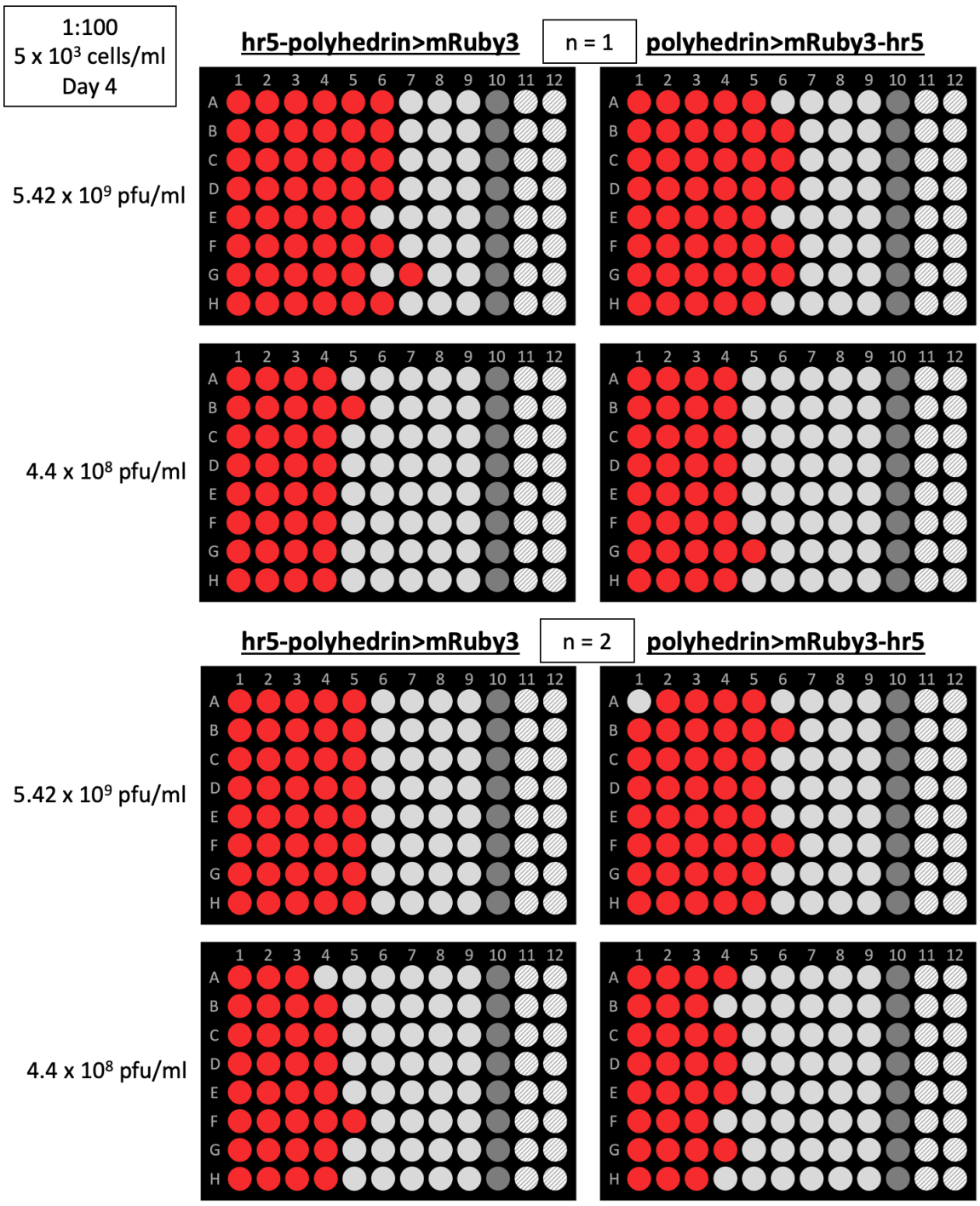

Figure 21. $TCID_{50}$ plate scoring of mRuby3 reporter from 1:100 pre-plating dilution tests. Red indicates a positive well; light gray indicates a negative well. These results are from plates with 5 x 10^3 cells/well with scoring done on day 4 measurements.

Table 3. Results from 1:100 pre-plating dilution data at day 4 incubation time. The construct used, hr5-polyhedrin or polyhedrin>mRuby3-hr5 is designated. The correction factors are color coded to match the cells per well amounts.

Construct	Virus Used	qPCR Titer	Cells/well	$TCID_{50}$ Assay Titer	Correction Factor*	Round
hr5-polyhedrin	R771-X59-636	4.40E+08	5.00E+03	3.18E+05	1383.65	1
			5.00E+03	2.82E+05	1560.28	2
	R711-X70-635	5.43E+09	5.00E+03	6.14E+06	884.36	1
			5.00E+03	1.56E+06	3480.77	2
	R771-X59-636	4.40E+08	1.00E+04	4.20E+05	1047.62	1
			1.00E+04	6.34E+04	6940.06	2
	R711-X70-635	5.43E+09	1.00E+04	3.86E+06	1406.74	1
			1.00E+04	1.41E+06	3845.61	2
	R771-X59-636	4.40E+08	2.00E+04	1.76E+05	2505.69	1
			2.00E+04	4.02E+04	10945.27	2
	R711-X70-635	5.43E+09	2.00E+04	6.30E+05	8619.05	1
			2.00E+04	3.68E+05	14755.43	2
polyhedrin>mRuby3-hr5	R771-X59-636	4.40E+08	5.00E+03	3.18E+05	1383.65	1
			5.00E+03	1.75E+05	2517.16	2
	R711-X70-635	5.43E+09	5.00E+03	5.04E+06	1077.38	1
			5.00E+03	1.64E+06	3302.92	2
	R771-X59-636	4.40E+08	1.00E+04	2.82E+05	1560.28	1
			1.00E+04	9.22E+04	4772.23	2
	R711-X70-635	5.43E+09	1.00E+04	4.42E+06	1228.51	1
			1.00E+04	3.52E+05	15426.14	2
	R771-X59-636	4.40E+08	2.00E+04	5.62E+04	7829.18	1
			2.00E+04	6.22E+04	7073.95	2
	R711-X70-635	5.43E+09	2.00E+04	1.09E+06	4963.44	1
			2.00E+04	3.18E+05	17075.47	2

*Correction factor = qPCR titer/$TCID_{50}$ assay titer

This stage of testing served additionally as an experiment to determine what humidity setup to use for future plates. Initial tests with fewer plates could be carried out with the humidity chamber described in the $TCID_{50}$ protocol, but in rounds of testing with multiple variables being assessed there were not enough containers to accommodate the plates. This resulted in some inconsistencies between the rounds of testing, as well as some plates drying out that could not be measured. A humidified incubator with a plastic cover lightly secured overtop of all the plates was the method used moving forward to accommodate the number of plates.

Different Microplate Well Shapes Do Not Improve Signal

In an attempt to improve signal without decreasing the pre-plating virus dilution from 1:100, different plate types were tested using the hr5-polyhedrin stable cells. U-bottom and v-bottom plates were used to perform $TCID_{50}$ assays to see if they would lessen the clustering of cells around the edges of the well, and instead have cells settle more in the bottom of the wells. This did not prove to be the case as cells were still noticeably clustering on the edges and signal was not improved (Figure 22). The u-bottom and v-bottom plates were completely clear as opposed to black with clear wells like the standard flat well plates being used for all other testing.

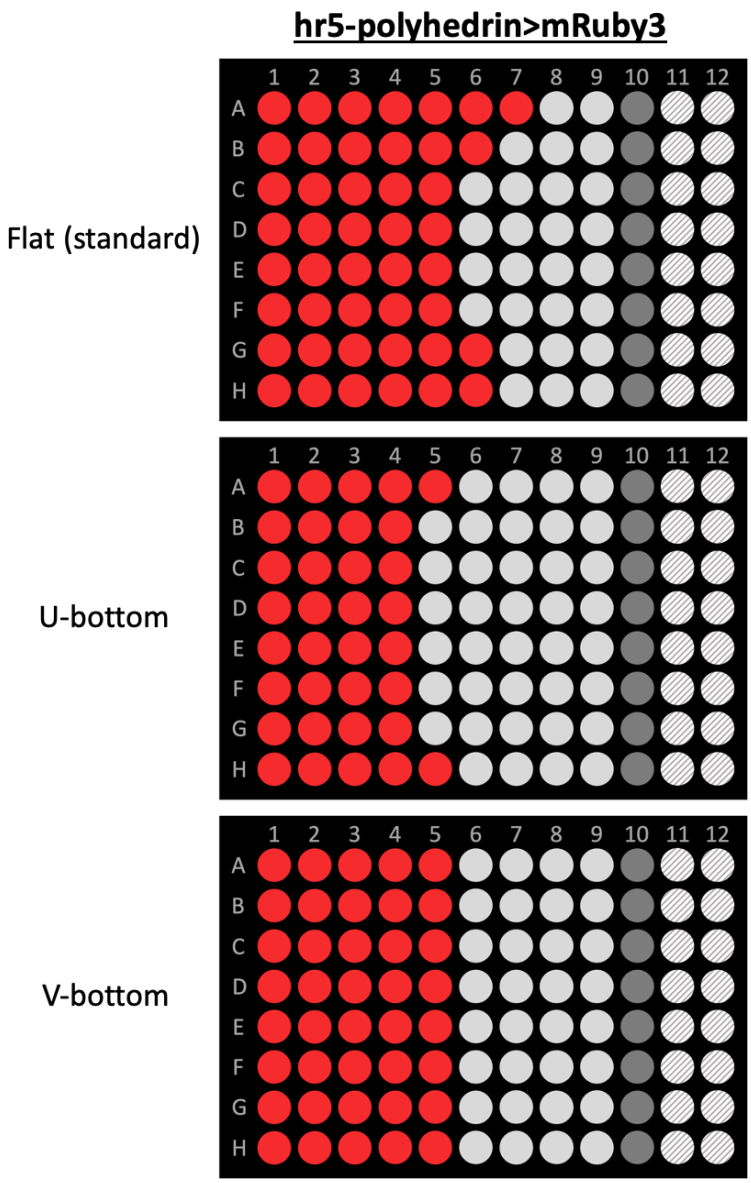

Figure 22. $TCID_{50}$ plate scoring of mRuby3 reporter from a standard flat well plate compared to u-bottom and v-bottom plates. Red indicates a positive well; light gray indicates a negative well.

A 1:10 Pre-plating Virus Dilution Gives the Desired Range of Signal

A 1:100 pre-plating virus dilution produced a signal that was too low for the assay and experimenting with different plate types produced no improvement. To increase signal to be suitable for the desired range of the assay, a 1:10 pre-plating dilution was tested.

The hr5-polyhedrin and polyhedrin>mRuby3-hr5 stable cells were used for these tests to compare. The 1:10 pre-plating virus dilution was tested with 5 x 10^3 cells/well, and relative fluorescence measurements were taken at 3-day and 4-day incubation times. Two viruses, with 4.4 x 10^8 and 5.42 x 10^9 qPCR titers, were used for these tests. The 3-day incubation time again did not suffice (data not shown). The 4-day incubation gave signals that fit in the desired range for the assay based on the qPCR titers, which were used for a rough comparison here as the Sf-9ET assay can produce titers on the same log as qPCR. A virus with a 10^8 titer would be expected to have signal that carries to columns 6-7, and 10^9 titers would be expected to have signal through columns 7-8 (Figure 22). Titers determined from the $TCID_{50}$ assay plates measured at day 4 were compared to the qPCR titers to calculate correction factors. The mean of the correction factors across all three rounds, with the elimination of a single outlier, is 2701.10 and the standard deviation is 2194.96 (Table 4). There is still notable inconsistency between rounds. A 1:10 pre-plating virus dilution combined with a 4-day incubation provides enough signal on the plate to theoretically allow for the desired assay range.

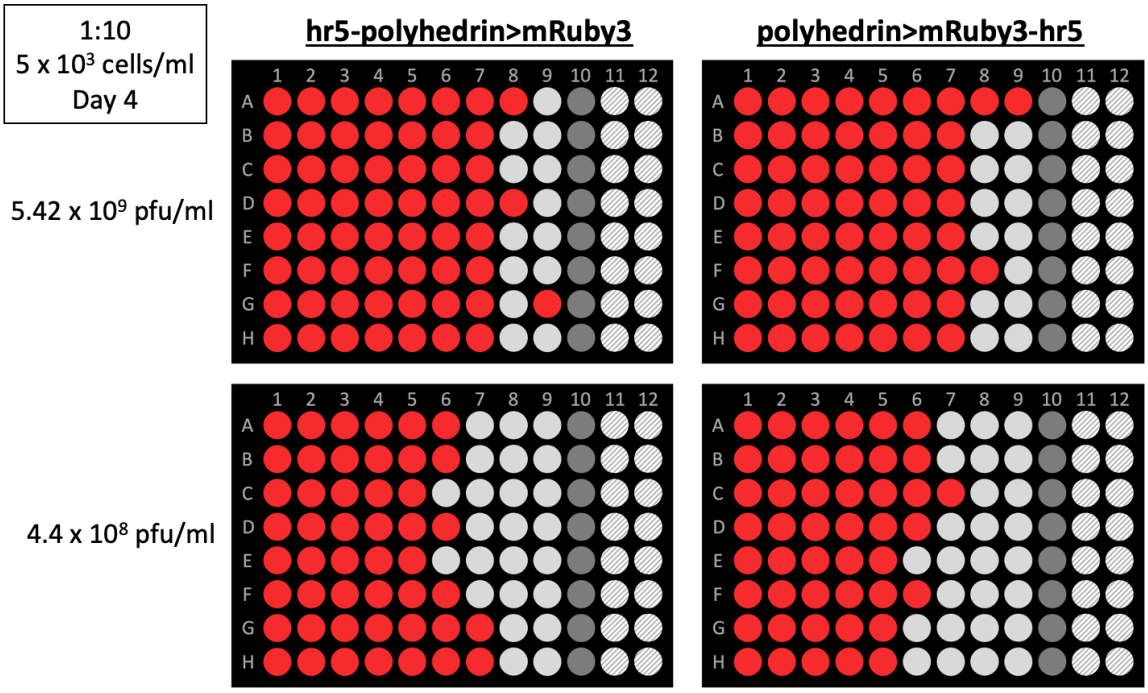

Figure 23. $TCID_{50}$ plate scoring of mRuby3 reporter from 1:10 pre-plating dilution tests. These results are from round 2. The plates had 5 x 10^3 cells/well and scoring was done on day 4 measurements.

Table 4. Results from 1:10 pre-plating dilution data at day 4 incubation time. The construct used, hr5-polyhedrin or polyhedrin>mRuby3-hr5 is designated. Correction factors are calculated to assess the differences between the qPCR titers and the $TCID_{50}$ assay titers. The correction factors are colored based on the values.

Construct	Virus Used	qPCR Titer	$TCID_{50}$ Assay Titer	Correction Factor*	Round
hr5-polyhedrin	R771-X59-636	4.40E+08	2.32E+05	1896.55	1
			7.08E+05	621.47	2
			8.80E+04	5000.00	3
	R711-X70-635	5.43E+09	2.38E+06	2281.51	1
			4.00E+06	1357.50	2
			1.34E+06	4064.37	3
polyhedrin>mRuby3-hr5	R771-X59-636	4.40E+08	1.59E+05	2760.35	1
			5.08E+05	866.14	2
			5.30E+04	8301.89	3
	R711-X70-635	5.43E+09	3.54E+06	1533.90	1
			5.28E+06	1028.41	2
			5.02E+05	10816.73	3

*Correction factor = qPCR titer/$TCID_{50}$ assay titer

The Assay is Producing Inconsistent Results

To assess how the assay optimized to this point performs across more virus samples, plates were set up for 6 viruses and incubated for 4 days. The polyhedrin>mRuby3-hr5 stable cell line was used solely for this as its design reflected the Sf-9ET construct and its performance was indistinguishable from the hr5-polyhedrin stable cells. The plates were set up with the conditions determined above to give the desired level of signal: 1:10 pre-plating dilution, 5 x 10^3 cells/well, and 4-day incubation time. All 6 viruses had an altered humidity setup as described earlier in the results, in a humidified incubator with a plastic cover lightly secured overtop of all the plates. Three of the viruses had separate plates prepared to be put in the humidity setup as described in the $TCID_{50}$ assay protocol to assess the difference the altered humidity setup may have on results.

The results from the three plates that follow the original protocol humidity setup have higher signal than the plates from the same viruses in the altered humidity setup. The mean correction factor across all three rounds, with a single outlier value removed, of the 6 viruses in the altered humidity set up is 8380.45, and the standard deviation is 4368.68. The mean correction factor, across all rounds, of the three viruses with the humidity setup as described in the $TCID_{50}$ protocol is 3535.52 and standard deviation is 2525.41 (Table 5).

Table 5. Results from tests comparing several viruses. A 1:10 pre-plating dilution is used and a 4-day incubation time. Correction factors are calculated to assess the differences between the qPCR titers and the $TCID_{50}$ assay titers. The correction factors are colored based on the values.

Virus Used	qPCR Titer	$TCID_{50}$ Assay Titer	Correction Factor*	Round
R775-M80-624	6.30E+09	5.70E+05	11052.63	1
		6.34E+05	9936.91	2
		5.42E+05	11623.62	3
R771-X81-636	6.56E+09	4.00E+06	1640.00	1
		1.58E+06	4141.41	2
		1.05E+06	6235.74	3
R805-X07-008	7.71E+09	1.00E+06	7710.00	1
		7.98E+05	9661.65	2
		1.58E+06	4867.42	3
R801-X97-636	3.60E+09	2.08E+06	1730.77	1
		4.54E+05	7929.52	2
		6.14E+05	5863.19	3
R801-X97-636**	3.60E+09	6.08E+06	592.11	1
		2.82E+06	1276.60	2
		3.08E+06	1168.83	3
R716-X20-635	1.23E+10	2.54E+06	4842.52	1
		1.37E+06	8991.23	2
		6.90E+05	17826.09	3
R716-X20-635**	1.23E+10	1.40E+07	878.57	1
		3.52E+06	3494.32	2
		2.72E+06	4522.06	3
R775-X82-636	4.76E+08	3.18E+04	14968.55	1
		3.54E+04	13446.33	2
		6.48E+03	73456.79	3
R775-X82-636**	4.76E+08	7.72E+04	6165.80	1
		7.72E+04	6165.80	2
		6.30E+04	7555.56	3

*Correction factor = qPCR titer/$TCID_{50}$ assay titer

**Plates with humidity setup described in the $TCID_{50}$ assay protocol

DISCUSSION

This project was able to achieve the goal of creating a working Tni-FNL ET stable cell line. Creating a corresponding $TCID_{50}$ assay protocol for baculovirus titration was a partial success but is incomplete and will require future work. The assay was able to be optimized to achieve the goals of utilizing a microplate reader for detection and decreasing the assay time from 7 days, as needed for the Sf-9ET protocol, to 4 days. It was not able to achieve a high enough signal to avoid the need for a correction factor, which could be interpreted as an inability to outperform the Sf-9ETs, which do not require a correction factor. The ability of Sf9 cells to propagate virus, combined with a 7-day incubation, likely plays a role in the Sf-9ET $TCID_{50}$ assay not requiring a correction factor. Over the course of the 7 days the Sf-9ET cells are able to build up a higher signal with the help of an increasing MOI due to virus propagation. The Sf-9ET cells are also single-cell cloned, giving them the advantage of all cells being capable of producing desired levels of eGFP (Hopkins and Esposito 2009). The current Tni-FNL ET cells will need to be modified to overcome the need for a higher signal.

The live-cell total DAPI staining showed the lack of detectable mRuby3 expression amongst many of the cells after baculovirus infection. This is not entirely unexpected, but it is undesired as it is lowering the overall signal. These cells are not single-cell clones and contain varying copy numbers of the mRuby3 gene. Baculovirus infection is also not completely efficient at delivering the same number of virions to each cell, adding to the inconsistency.

Unexpected findings through this project include unexpected antibiotic resistance of Tni-FNL cells and a requirement to have an hr in the plasmid DNA construct. Antibiotic

kill curves demonstrated that Tni-FNL cells were resistant to geneticin. This was surprising, but after searching, the same finding had been briefly mentioned in reference to a different *Trichoplusia ni* cell line, Tn-5B1-4 (Breitbach and Jarvis 2001). This is noted for any future stable cell line work.

The necessity for an hr became clear when stable cell lines that had constructs with and without hr sequences were infected with baculovirus. Homologous regions allow the baculoviral transcription factor, IE-1, to bind to initiate transcription (Nissen and Friesen 1989; Guarino and Summers 1986b; Olson *et al*. 2003). Without the hr regions the baculoviral promoter upstream of the mRuby3 gene in the plasmid appears to not be recognized by the baculoviral machinery to allow transcription.

After confirming the need for an hr region it was seen that the hr5-polyhedrin and polyhedrin>mRuby3-hr5 promoter constructs had the highest mRuby3 outputs. The hr5 region in the hr5-polyhedrin plasmid was placed upstream of the promoter without knowing the effect it might have on expression. Homologous regions are known to enhance expression in certain cases from baculoviral promoters, therefore the placement seemed inconsequential, and if anything, beneficial (Rodems and Friesen 1993). The data received from another research project in the laboratory led to the design of the polyhedrin>mRuby3-hr5 plasmid. The plasmid still contains an hr5 due to its necessity, but the downstream placement after the mRuby3 sequence was hoped to be enough to negate a potential dampening effect on mRuby3 expression. The polyhedrin>mRuby3-hr5 construct did not prove to remedy the problem, which could be potentially due to differences in expressing protein from a recombinant baculovirus versus from a gene in a stable cell line with baculovirus infection. The proteins tested in the two projects differ as

well, mNeonGreen versus mRuby3. It could also be the case that when the hr5 is in relatively close proximity its effect will be the same whether it is upstream or downstream of the polyhedrin promoter, and the mRuby3 sequence could be not enough distance to separate it from polyhedrin.

Future Direction

The need for inexpensive, timely baculovirus titration methods makes this work important to continue researching to reach an end product. There are several areas that need improvement moving forward to develop a successful Tni-FNL ET assay based on the results of these preliminary studies.

mRuby3 can be Replaced with a Brighter RFP to Improve Signal

While a desired level of signal was able to be achieved, there is room for improvement to avoid needing a correction factor. A brighter RFP, such as tdTomato could allow for a stronger signal to solve this issue (Campbell *et al*. 2002; Shaner *et al*. 2004). qPCR is the titration method used within PEL but comparing the $TCID_{50}$ assay titers to the qPCR titers to calculate a correction factor is not ideal as they are significantly different methods of titration. If a correction factor is required even with future optimization, comparisons will likely need to be made to the Sf-9ET assay.

The Tni-FNL ET Cells Should be Single-Cell Cloned to Improve Consistency and Signal

To achieve consistent mRuby3 output across all cells and improve signal the Tni-FNL ET cell line will need to be single-cell cloned. Tests have already been in progress

utilizing a protocol from Fu *et al*. (2018) with some promising results. Single-cell cloning would assist in increasing signal and consistency for the assay.

A Consistent, Reproducible Humidity Setup is Necessary

It was a clear problem when performing optimization for this assay that there was not a singular best humidity setup utilized. This affected the results between replicates that otherwise had the same variables. Future tests will be done with some experimental setups. An option is to put 1X PBS on outer wells of the plate, which means that the number of wells in each column that could be utilized would decrease from eight to six, but there would be protection from evaporation of the inside wells. Another potential option is using breathable plate seals with the hard plastic plate cover on top to keep the seal in place over a 3-to-4-day incubation period. These options may help overcome the inconsistencies in humidity between different incubators.

The Stability of the Tni-FNL ET Cells Needs to be Assessed

For further testing of the assay, it needs to be established how long the Tni-FNL ET cells can be passaged before viral titers cease to be reliable. The Tni-FNL ET cells are likely to have decreased performance at a high passage. The passage number at which the Tni-FNL ET cells fail to be reliable in the $TCID_{50}$ assay will need to be determined.

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