Comparison of Colorimetric Assays to Use for the Investigation of the Mitogenic Activity of vFGF in Cell Cultures

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Abstract

Fibroblast growth factors (FGFs) are part of a large family of polypeptide growth factors that play a large role in the development and regulation of organisms using intracrine, paracrine and endocrine cell signaling mechanisms. FGFs are known to be found in both vertebrate and invertebrate organisms. Most recently they have also been identified in viruses, such as baculoviruses. A sequence analysis of the baculovirus genomes has found that they encode for a viral fibroblast growth factor homolog (*vfgf*) early on in their stage of infection (Katsuma et al. 2006). Although there have been studies from other labs that have revealed a great deal about vFGF function, to date, nothing has been published on the potential mitogenic activity of vFGFs. To test the effects of vFGFs on cell mitogenic activity, the best method to assess cell viability and cell proliferation needed to be used, which is colorimetric assays. The goal was to find the most accurate *in-vitro* colorimetric assay that measures cell proliferation with insect Sf9 cells and mammalian NIH/3T3 cells. Such assays included the resazurin assay, the crystal violet assay, and the CCK-8 assay. To accurately measure cell proliferation, I used a multi-well plate reader that measured the dyes' absorbance at specific wavelengths varying from 450nm to 590nm, depending on the reagent. From the data collected, I created a variety of standard curve graphs and produced figures that compared each reagent's sensitivity. After comparing the slopes, confidence intervals and correlation coefficients from each standard curve graph, CCK-8 and crystal violet were found to be the best *in-vitro* colorimetric assays to measure cell proliferation with both Sf9 and NIH/3T3 cells.

Introduction

What are FGFs and vFGFs?

- Fibroblast growth factors (FGFs) are cytokines found in many multicellular organisms that are essential to embryonic development as well as postnatally maintaining stability in response to injury and the regulation of metabolism through hormone cell signaling.
- FGF genes are found in the genomes of at least two DNA virus families, Baculoviridae and Iridoviridae.
- We have studied two virus encoded FGFs (vFGFs) from the baculoviruses *Choristoneura fumiferana* Multiply-embedded Nuclear Polyhedrosis Virus (*Cf*MNPV) and *Autographa californica* Multiply-embedded Nuclear Polyhedrosis Virus (*Ac*MNPV).
- Colorimetric assays can be used to test the effects of vFGFs on cell mitogenic activity

What are colorimetric assays?

- Colorimetric assays measure the survival and proliferation of cells. They have become more common in cell culture research as they provide for a simple, cost and time efficient methods compared to radiometric approaches.
- Two common *in-vitro* colorimetric assays that are used to measure cell proliferation are the resazurin assay, crystal violet assay and most recently the Cell Counting Kit (CCK-8) assay utilizing WST-8.
- The **Resazurin** assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and dihydro-resorufin. This reduction causes the resazurin to change from the non-fluorescent blue form to the fluorescent red form (Vega-Avila and Pugsley 2011).
  - **Shown in figures 1, 3 and 4**
- The **CCK-8** assay uses a highly water-soluble tetrazolium salt (WST-8). WST-8 is reduced by dehydrogenases in cells which produce a water-soluble formazan dye that shows up as an orange-colored product.
  - **Shown in figures 1,2,4,7-12, and 15**
- The **Crystal Violet** assay is the cheapest and most used dye in Gram-staining procedures used to differentiate bacteria. The crystal violet works by staining the DNA of cells which can then give the relative density of cells that have adhered to multi-well dishes.
  - **Shown in figures 5-7 and 13-15**

Why is this research important?

- Although all 3 colorimetric assays are efficient at measuring cell proliferation and cell viability, the goal was to find the best reagent that has a higher detection sensitivity and better reproducibility compared to the others.
- It is also important to identify at least two independent approaches for measuring cell proliferation to support our hypothesis that vFGFs act as mitogens.
- The last goal was to create a standard curve graph for each cell line that will help to quantify the amount of cell proliferation when measuring the mitogenic activity of cells after adding vFGF in future experiments.

Acknowledgments

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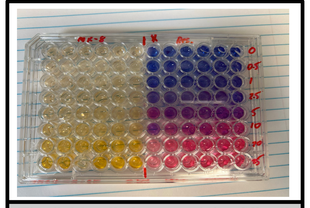
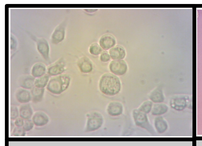
Sf9

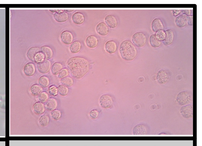
Phase One



(Fig. 1) 96-well plate containing Sf9 cells with CCK-8 (columns A-F) and resazurin (columns G-L)

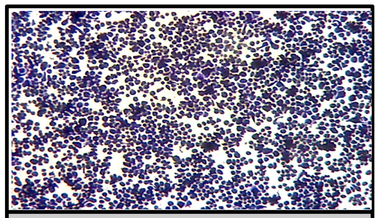


(Fig. 2) Sf9 cells after 23h in CCK-8 (100x)

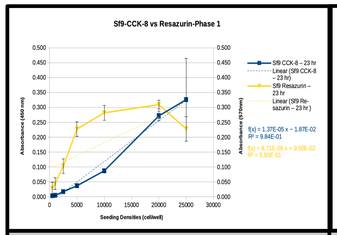


(Fig. 3) Sf9 cells after 23h in resazurin (100x)

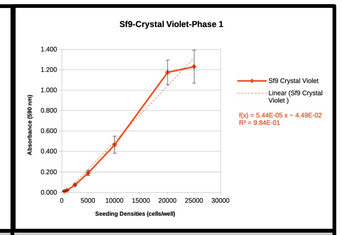
Phase Two



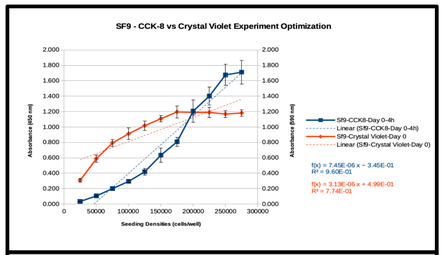
(Fig. 6) Sf9 cells stained with crystal violet at 100x (125k cells per well)



(Fig. 4) Phase 1 CCK-8 and resazurin standard curve graphs



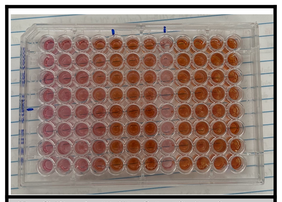
(Fig. 5) Phase 1 crystal violet standard curve graphs



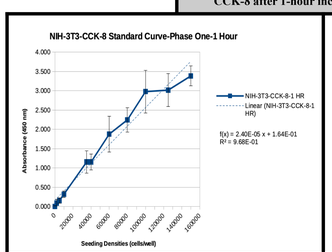
(Fig.7) Phase 2 comparison of CCK-8 and crystal violet standard curve graphs

NIH/3T3

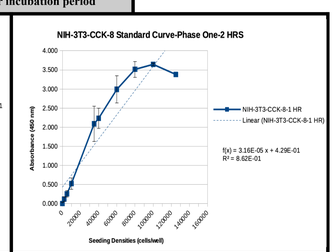
Phase One



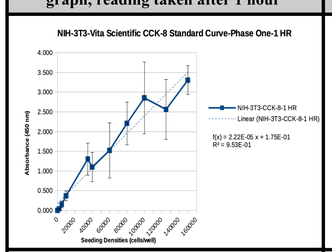
(Fig. 8) Plate 1 with Vita-Scientific (top four rows excluding wells D11 and D12) and ApexBio's (bottom four rows including wells D11 and D12) CCK-8 after 1-hour incubation period



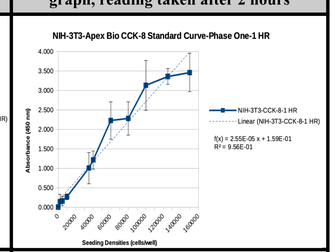
(Fig. 9) Plate 1 CCK-8 standard curve graph, reading taken after 1 hour



(Fig. 10) Plate 1 CCK-8 standard curve graph, reading taken after 2 hours

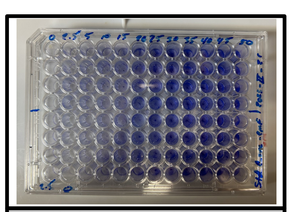


(Fig. 11) Plate 1 Vita-Scientific CCK-8, reading taken after 1 hour

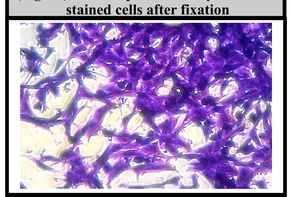


(Fig. 12) Plate 1 Apex Bio CCK-8, reading taken after 1 hour

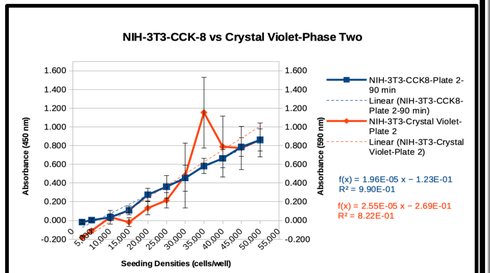
Phase Two



(Fig. 13) 96-well plate with crystal violet-stained cells after fixation



(Fig. 14) NIH/3T3 cells stained with crystal violet at 100x (35k cells/well)



(Fig.15) Plate 2 comparison of CCK-8 and crystal violet standard curve graphs

Methods

Sf9

Phase 1
- Seeding density: 0-25,000 cells per well
- Incubation period: 2 hours at 27°C
- Absorbance readings taken at 2h; 3h; 23h; 26h before cells divided and reached different densities
- Amount of replicates: 6 per CCK-8 and resazurin assay, 12 for crystal violet

Phase 2
- Seeding density:0-275,000 cells per well
- Incubation period: 2 hours at 27°C
- Absorbance readings taken at 2h; 3h; 4h before cells divided and reached different densities
- Amount of replicates: 8 per assay

Standard Curve Criteria:
- Sensitivity (slopes)
  - We interpret greater slope (signal vs. cell number) to mean higher sensitivity
- Reproducibility (confidence intervals)
  - We interpret smaller confidence intervals (among replicates) to mean greater reproducibility.

NIH/3T3

Phase 1
- Seeding density: 0-150,000 cells per well
- Incubation period: 3 hours at 37°C in 5% CO_2
- Absorbance readings taken at 1h; 2h; before cells divided and reached different densities
- Amount of replicates: 8 per assay

Phase 2
- Seeding density: 0-50,000 cells per well
- Incubation period: 2 hours at 37°C in 5% CO_2
- Absorbance readings taken at 30 min; 1h; 90 min; 2h before cells divided and reached different densities
- Amount of replicates: 8 per assay

Reagent Absorbance Wavelengths
- **CCK-8**
  - Absorbance: 450nm
- **Resazurin**
  - Absorbance: 570nm
- **Crystal Violet**
  - Absorbance: 590nm

Results and Discussion

- Figure 1 shows a 96-well plate that was incubated at 27°C for 23 hours in CCK-8 and resazurin. The plate was seeded with Sf9 cells with densities ranging from 500-25,000 cells per well. We encountered pipetting issues in column G and row H which is shown in the color development of those wells. (These wells were not included in our calculations.) As seen in figure 1, there seems to be a smoother color development as the densities get higher in CCK-8 compared to resazurin. This was a sign that resazurin had a harder time being absorbed by cells at higher densities, which is reflected in figure 4.

- Figures 2 and 3 show Sf9 cells in CCK-8 and resazurin after 23 hours. There were more granulations present in the cells that were sitting in resazurin, which is a sign of stress, as seen in figure 3.

- Figure 4 shows phase one's standard curves from the CCK-8 and resazurin assays after a 23-hour incubation period. The slope of the resazurin assay starts positive with the lower densities but then curves downward as we get above 20k, which is not ideal for future experiments. **CCK-8 shows better response linearity across the range of cell numbers we expect to observe in our proliferation assays.** It also has great reproducibility, with 95% confidence intervals that are very small compared to resazurin.

- Figure 5 shows phase one's crystal violet standard curve with a line that is very similar to that of CCK-8. Despite them looking similar, the slope value is higher than CCK-8's, which means that its sensitivity is greater. **The resazurin 95% confidence intervals were greater than those observed for CCK-8 and crystal violet, which we interpreted as resazurin assays having lower reproducibility. Therefore we eliminated resazurin from further assays.**

- Figure 6 shows the staining of individual Sf9 cells with crystal violet. The image shows the reagent's high sensitivity due to its intense staining.

- Figure 7 shows phase two's comparison of CCK-8 and crystal violet with higher cell densities. Both show great reproducibility and high sensitivity with the lower range of cell densities, until 175k, where the signal plateaus for crystal violet. With a correlation coefficient of 0.96, **CCK-8 assay has greater linearity than crystal violet. Therefore, CCK-8 is usable with both low and high cell density ranges, whereas crystal violet is best within a lower range of cell densities.**

- Figure 8 is an image of a 96-well plate with two companies' CCK-8 reagent: Vita-Scientific and Apex Bio. After a 1-hour incubation period, we noticed that the **NIH/3T3 cells were metabolizing the reagents a lot quicker than Sf9 cells**, as seen in the dark color development. Due to this, the absorbance readings came out with high values, as shown in figures 9 and 10.

- Figures 9 and 10 show phase one's standard curve graphs of both companies CCK-8. They show **good reproducibility with the smaller cell densities**, but turns poor after 35k. Although the absorbance values are very high, CCK-8 shows great sensitivity, with slopes that don't plateau until 100k after a 2-hour incubation period. A lot of overflow data was observed, so we ran a second trial after optimizing the conditions for the CCK-8 assay. Due to time restraints, we decided not to go ahead with a crystal violet assay for phase one.

- Figures 11 and 12 compare the sensitivity of each company's CCK-8 reagent. With a larger slope value, **Apex Bio's reagent wins over Vita Scientific.**

- Figure 13 shows the color gradient of crystal violet in a 96-well plate as the cell densities get higher. Figure 14 is an image of crystal violet-stained NIH/3T3 cells.

- Figure 15 shows phase two's standard curve graphs for CCK-8 and crystal violet. After changing the absorbance reading time to every 30 minutes, we saw a big change in the absorbance values as they were now within a reliable range. Despite CCK-8 having greater linearity, **crystal violet's slope value is higher, indicating better sensitivity**. **Both reagents show good reproducibility with the lower cell densities**, but become poor after 30k with large 95% confidence intervals.

- After comparing the data and standard curve graphs from each *in-vitro* colorimetric assay, we concluded that CCK-8 and crystal violet were the best assays to measure cell proliferation. With its lower sensitivity and reproducibility, resazurin comes last and will no longer be used in further experiments.

- We also have standard curve graphs for each cell line and assay that will be used as references to study the effects of vFGF on cell mitogenic activity.

- We are currently in the experimentation process of adding vFGF to insect SF9 cells, mammalian NIH/3T3 cells, and fish EPC cells.

References

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