Expression of Class I Histone Deacetylases in Insect Cells

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Abstract

Histone deacetylases (HDACs) have become one of the leading areas of research for cancer, neurodegenerative diseases, diabetes, obesity, and inflammation. Although HDACs are currently expressible in mammalian cultures and yeast, it is important to explore other cost effective options. Here it is shown that class I HDACs are expressible in insect cells. As well as expressing full length domains for class I HDACs, predicted active domains have also been expressed. This information can be utilized in many areas for future research including identifying unique sites to allow development of specific inhibitors for each HDAC, and developing a better understanding of the specific role of each HDAC.

Acknowledgements

I would like to acknowledge my advisor Professor Kristen Wobbe for her guidance while I worked on my master's thesis off campus. I would also like to thank Blue Sky Biotech for providing me with the financial support and a lab to conduct my research. In particular I would like to thank Paul Wengender, Norman Garceau, Bill Hermans, and Denis Kole all from Blue Sky for offering any form of assistance or information that I needed during my research. Finally I would like to thank Professor William Hobey for taking on the role of offering me guidance throughout the many stages of my research and helped me coordinate between my off campus research and on campus requirements.

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1.0 Introduction:

DNA packaging is important in the regulation of transcription. When cells are in a resting state DNA is compacted tightly into a DNA-protein complex called chromatin. This structure is tightly put together to protect it from the binding of transcription factors and allow the compaction of the long genome into the smaller nuclear compartment. Chromatin's main subunit is the nucleosome which is made up of an octamer of core histones consisting of a H3-H4 histone tetramer and two H2A-H2B histone dimers, all wrapped in 146 bp DNA (De Ruijter et al., 2003; Hassig and Schreiber, 1997). When gene transcription is activated the chromatin relaxes and transcription factors are able to bind the DNA. Changes to the chromatin structure by acetylating the core histones can facilitate gene expression. The acetylation is carried out by histone acetyltransferases (HATs); this decreases the interaction between DNA and histone proteins. Histone deacetylases (HDACs) remove acetyl groups, increasing DNA and histone interactions. The nature of the DNA-histone interaction will determine how well transcription factors can bind to the DNA.

1.1 Class information

Currently, eleven HDACs have been identified and placed within separate classes based on phylogenetic characteristics. Class I consists of HDACs 1, 2, 3, and 8 and is similar to the yeast protein Rpd3; Class II consists of HDACs 4, 5, 6, 7, 9, and 10 and is related to the yeast protein Hdalp; and HDAC 11 has recently been placed into its own class, Class IV (Wang et al., 2005). There is also a Class III that consists of sirtuin deacetylases, which are related to the yeast protein Sir2 (Gregoretti et al., 2004). The

separation of HDACs into these separate classes is based on sequence similarities. For example, the DNA sequence similarity for class I HDACs is 75% for HDAC1 and 2, 50% for HDAC1 and 3, and finally 51% similarity between HDAC2 and 3.

Although eleven HDACs have been identified, only HDAC8 has been crystallized. As scientists try to characterize the other ten HDACs, they will be using the information on HDAC8s structure, as well as possible structures and functions of the other HDACs and applying them to one another. For example, because the classes have already been distinguished based on a phylogenetic analysis then HDAC8 can be compared to HDACs 1, 2, and 3 because of the many similarities. When attempting to use information that is already known about one HDAC and apply it to another it is best to know whether those HDACs are "paralogs" or "orthologs" of one another. It is expected that paralogs will be functionally different, while orthologs will have similar functions and characteristics (Gegoretti et al., 2004).

The information currently known about the separate classes of HDACs identifies the first class as being found exclusively in the nucleus and expressed in various immune tissues, while Class II HDACs appear to be located in both the nucleus and cytoplasm and are more tissue specific and associated with muscle function (Blanchard and Chipoy, 2005; Gregoretti et al., 2004). Although there appear to be separate functions for each class of HDACs as well as each HDAC itself, there is interaction between HDACs from different classes. This can be seen with HDAC3, a class I HDAC, which interacts with many class II HDACs including HDACs 4, 5, and 10 (De Ruijter et al., 2003). HDAC3 has both a nuclear import signal and a nuclear export signal, while HDAC1, 2, and 8 only have a nuclear import signal. These signals allow HDAC3 to be recruited by class II

HDACs and form oligomers with them with the assistance of the co-repressors: silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressors (N-CoR) (De Ruijter et al., 2003).

1.2 Acetylation effects

As previously mentioned histone acetylation and deacetylation have been shown to be linked to transcriptional regulation (Hassig and Schreiber, 1997). Acetylation weakens the histone-DNA interactions by neutralizing the positive charge on the lysine residues. This reduces the attraction between the histone tail and the negatively charged DNA. When the DNA-histone interaction weakens there is more access to DNA binding sites by transcription factors and possibly DNA replication factors. Histone deacetylases can suppress transcription and reduce DNA replication by deacetylating chromatin and allowing ionic interaction between the histone lysines and the phosphates on DNA (Fig. 1).

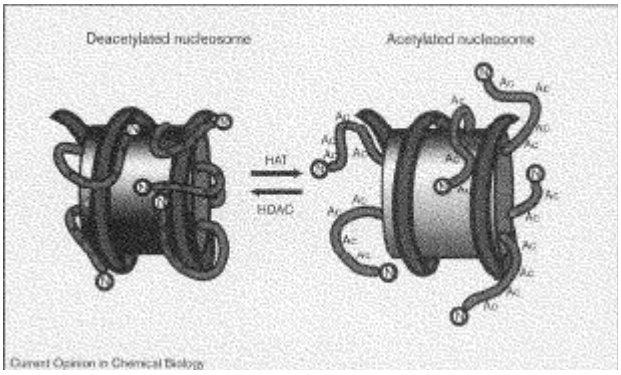


Figure 1: Acetylation and Deacetylation of Histones
The acetylated nucleosome shows histone tails that still have acetyl groups attached and are relaxed away from the nucleosome core. This relaxed formation makes it easier for transcription factors to bind (Hassig and Schreiber, 1997).

1.3 HDAC complexes

HDACs appear in many different protein complexes. The mSin3A-HDAC and the nucleosome-remodeling histone deacetylase (NuRD)-HDAC complexes, as well as

interaction with co-repressor Ying-Yang 1 (YY1) are a few examples (Ayer, 1999; Cress and Seto, 2000).

mSin3A was originally identified by its association with Mad-Max, a transcriptional repressor (Ayer, 1999). mSin3A exists as the central part of a complex it forms with HDACs, with other proteins building from this complex to form a 'core repressor complex'. It has been shown that mSin3A will associate with HDAC1 and 2 *in vivo* to form a complex that will allow for repression of transcription by Mad-Max. However, mSin3A can suppress transcription without binding to HDACs (Cress and Seto, 2000). A model has been developed termed the 'targeted repression' model which involves the mSin3A-HDAC complex being tied to a promoter by a transcriptional repressor, such as Mad-Max (Ayer, 1999). Besides its association with Mad-Max, the mSin3A-HDAC complex also interacts with two other co-repressors SMRT and N-CoR, though there is no definitive information on the nature of that interaction.

HDACs 1 and 2 also interact with the NuRD-HDAC complex. This complex does not contain any of the subunits of the mSin3A-HDAC complex (Ayer, 1999). Instead it associates with other proteins that assist in HDAC activity and chromatin-remodeling activity. There is a possibility that the co-repressor YY1 binds to HDACs and targets the NuRD complex. YY1 is a repressor that negatively regulates transcription (Cress and Seto, 2000). It can bind HDAC2 to DNA and this complex then acts as a repressor of transcription. HDAC1 and 3 can also repress transcription when they are targeted to promoters of various repressors. YY1 is expected to interact with various HDACs, but it has not been co-purified in complex at this point.

1.4 Inhibitors

The information that HDACs are important in controlling gene expression has spiked interest in them as target sites for treatment of cancer, diabetes, obesity, inflammation, and neurodegenerative diseases.

1.4.1 Areas for use

Inhibitors that are developed for specific HDACs can be used as cancer treatment because they can cause cell arrest and reverse the transformations of some cells. In certain types of cancer, tumor suppressor genes have been turned off or silenced. HDAC inhibitors are able to target specific regulatory pathways and reactivate silenced suppressor genes (Cress and Seto, 2000).

HDAC inhibitors are also of interest for diabetes, obesity, and inflammatory diseases research. HDACs, specifically class I, have an effect on inflammation. HDAC inhibitors appear to have an effect on a specific transcription factor, NF-κB, which reduces inflammatory mediator production (Blanchard and Chipoy, 2005). Not only do HDAC inhibitors affect NF-κB, they also reduce cytokine and NO production, both of which contribute highly to inflammatory diseases. HDAC inhibitors can act as both an activator of a pro-inflammatory gene or as a suppressor of pro-inflammatory genes based on its molecular target.

1.4.2 Types

Different HDAC inhibitors can act in millimolar concentrations while others are much more sensitive and can inhibit at nanomolar concentrations. Currently all inhibition done on HDACs is reversible except for that done by trapoxin and depudesin, both of which are cyclic tetrapeptides (De Ruijter et al., 2003).

1.4.2.1 Short Chain Fatty Acids

The first discovered HDAC inhibitor was the short chain fatty acid, butyrate (Fig. 2) (Hassig and Schreiber, 1997).

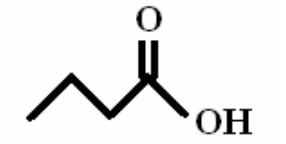


Figure 2: Butyric acid structure

Butyrate, in millimolar concentrations, inhibits both class I and class II HDACs, with the exception of HDAC6 and HDAC10 (Blanchard and Chipoy, 2005). It can also have side effects on cell membrane, cytoskeleton, and other enzymatic activities.

1.4.2.2 Hydroxamic acids

Hydroxamic acids are a class of weak acids which includes Trychostatin A (TSA) (Fig. 3) and suberoylanilide hydroxamic acid (SAHA) (Fig. 4). Currently the most potent inhibitor of HDACs is TSA which can inhibit HDACs in nanomolar concentrations. It was discovered to have proliferation-inhibitory properties with cancer cells making it an attractive drug candidate (De Ruijter et al., 2003). However, TSA is a fermentation product of *Streptomyces* that takes many steps with a low yield making it costly and inefficient as a cancer therapeutic agent.

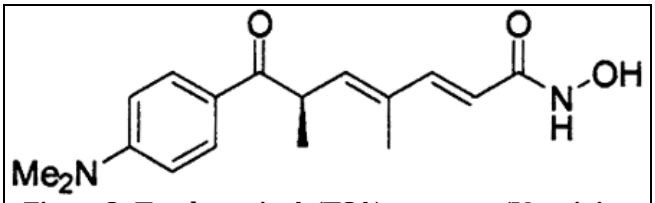


Figure 3: Trychostatin A (TSA) structure (Vannini et al., 2004)

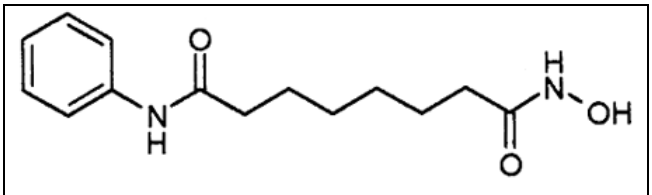


Figure 4: Suberoylanilide hydroxamic acid (SAHA) structure (Vannini et al., 2004)

SAHA has also attracted a lot of attention as a possible inhibitor to be used in clinical work. It has also recently finished being tested in Phase II clinical trials with positive results. (Wang et al., 2005) SAHA is an

inhibitor of both class I and class II HDACs in nanomolar amounts and inhibits by binding to the zinc-containing pockets of HDACs (Blanchard and Chipoy, 2005).

1.4.2.3 Cyclic tetrapeptides/epoxides

Cyclic tetrapeptides are recognized by their complicated structure of a 12 atom cyclic tetrapeptide backbone as can be seen below (Hassig and Schreiber, 1997). The structure also consists of various hydrophobic side chains that project out and can bind or disrupt interactions of HDACs with other proteins. A current cyclic tetrapeptide that is being studied is apicidin (Fig. 5).

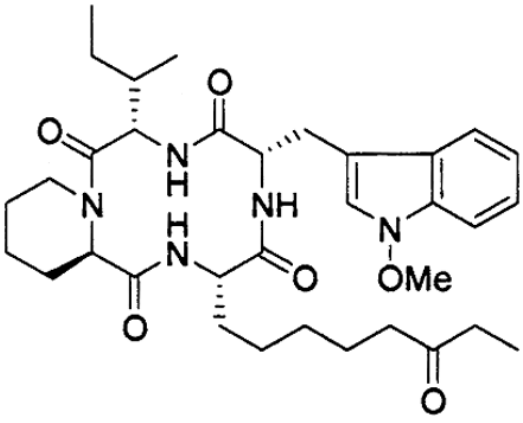


Figure 5: Apicidin structure (Vannini et al., 2004)

Apicidin is a potent inhibitor that shows anti-protozoal effects on certain parasites and anti-proliferative effects on specific cancer cells (Kim, 2004). Studies performed on its effects on various cancer cells established anti-invasive and anti-angiogenic traits providing evidence that it may act as treatment for tumors and will be tested in phase I and II clinical trials.

1.4.2.5 Benzamides

Benzamides are the final identified class of HDAC inhibitors. MS-275 is an example of a benzamide inhibitor that has an affinity for HDAC1, but also inhibits HDACs 3 and 8 (Fig. 6) (Simonini et al., 2006).

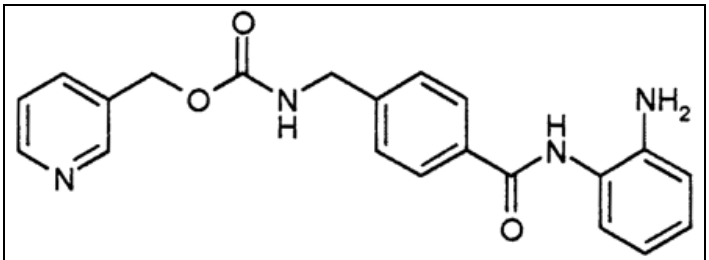


Figure 6: MS-275 structure (Vannini et al., 2004)

MS-275 acts as a potent inhibitor of leukemia tumor cells in micromolar concentrations where it induces differentiation and apoptosis of cells. It exists as a possible treatment and phase I clinical trials were recently completed establishing that there is a longer half-life than was expected from original trials and an optimum oral dose was determined (Minucci and Pelicci, 2006; Ryan et al., 2005).

1.5 Goals

The goal of my research will be to assist in the cloning and production of both full length (FL) and catalytic or active domains (AD) of all class I HDACs in insect cells. Once expressed, each HDAC will be purified and then tested for activity using a commercial activity assay. If the activity results are positive these proteins can be used for future research in the identification of unique inhibitor binding sites for individual HDACs. To summarize the various steps of the research a flow chart was constructed (Fig. 7).

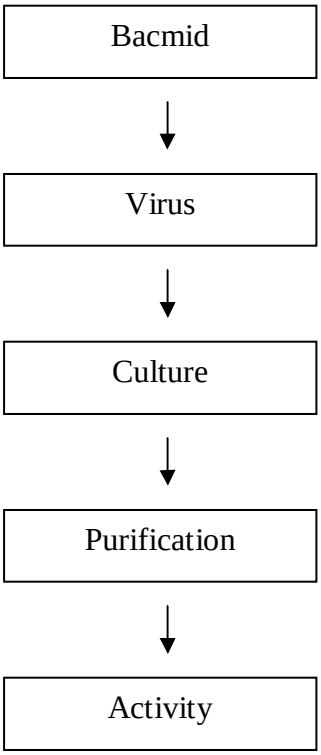


Figure 7: Research Flow Chart
A flow chart that depicts the multiple steps of obtaining the HDAC protein and testing activity

2.0 Methodology:

2.1 Virus Production

HDAC DNA was obtained in bacmid form from the Molecular Biology department at Blue Sky Biotech. In total seven constructs were obtained, HDAC1 AD, 1FL, 2AD, 3AD, 3FL, 8AD, and 8FL. All full length constructs would produce a protein with molecular weight of around 50kD and the active domain constructs, a protein of roughly 30kD. 10µL of bacmid DNA was pipetted into 750µL of Opti-Mem, which is an ion free medium, and gently mixed. 45µL of Cell Fectin was pipetted into 750µL of Opti-Mem and gently mixed. The two separate mixtures were allowed to rest for five minutes, followed by gentle mixing of the two. This mixture was incubated at room temperature for 30 minutes. The solution for each HDAC construct was then pipetted into 36mL of SF9 insect cells at a density of around 1.1 X 10^6 cells/mL. The insect cell medium is HyQ SFX Insect Medium and is supplemented with 1mg/mL gentamycin. Cultures were grown at 27°C at 120rpm for five days. The transfection was monitored by sampling on the fourth day. A 1.5mL cell suspension sample was taken for each virus stock for western analysis, as well as a Cedex sample. The Cedex provides cell counts, viability analysis, and information on the overall health of the cells, including cell diameter. For information on the Cedex refer to the Innovatis website (http://www.innovatis.com). When the viability had dropped below 70% and the cell size had begun to fall the virus was harvested by centrifugation at 1600 rpm for six minutes. The supernatant was then filtered through a 0.45 micron filter. Standard fetal bovine

(Invitrogen) was added at a concentration of 3% to protect the virus for storage for extended periods of time. The virus stocks were stored at 4° C.

2.2 Viral Infections

To test the expression of the virus stocks in insect cells, three types of cells were used, SF21, SF9, and High 5. All cultures are inoculated at a density of around 7.5 X 10^5 cells/mL. Cultures are allowed to grow for 24 hours at 27°C at 120rpm. The media used for SF21 and High5 insect cells was Insect Express (Cambrex) and was supplemented with 1mg/mL gentamycin. Media used for SF9 insect cells was HyQ SFX Insect Medium (Fisher) supplemented with 1mg/mL of gentamycin. After the growth period of 24 hours, cultures are infected with varying dilutions of the different virus stocks.

For expression tests of HDAC3 AD and FL and HDAC2 AD 50mL SF21 insect cell cultures were infected with virus stocks at dilutions of 1:100, 1:1000, and 1:10000 and allowed to grow for 72 hours. Cedex and 1.5mL cell suspension samples were collected every 24 hours in each of the three dilutions, as well as from a control flask. A separate expression test of HDAC1 AD, 1FL, and 8AD occurred in 250mL SF21 insect cell cultures infected with virus stocks at a dilution of 1:800. Cultures were allowed to grow for 72 hours after which a 1.5mL cell suspension sample was taken. A final expression test of the three different insect cell lines occurred. 50mL cultures of SF21, SF9, and High 5 insect cells were infected with the HDAC2 AD virus stock at a dilution of 1:2500. Cultures were grown for 72 hours with 1.5mL cell suspension and Cedex samples being collected every 24 hours. All cell suspension samples were analyzed on a 12% polyacrylamide gel and a western blot was performed to measure expression.

Using dilutions that would produce optimum expression in HDAC3 AD, 3FL, 2AD, and 8FL 800mL cultures of SF21 insect cells were infected with the different virus stocks. For HDAC3 AD this was a dilution of 1:5000, 3FL was 1:5000, 2AD was 1:5500, and 8FL was 1:800. All of the cultures were grown and sampled as previously described. When the cells had reached optimum expression where the viability had begun to dip and the cell size was high, around 72 hours, the cultures were harvested and a 1.5mL cell suspension sample was collected. The cultures were centrifuged at 2500rpm for 15 minutes and were re-suspended and washed with 1X phosphate buffered saline (PBS) and transferred to 50mL conical tubes and centrifuged again at 2500rpm for 15 minutes. Cell suspension samples were analyzed using a 12% polyacrylamide gel and a western blot.

2.3 Purification

The cell pellet, roughly 8.0 grams, obtained from the expression runs was weighed and 200µL of protease inhibitor cocktail (PIC) (American Bioanalytical) was added. The pellet was then resuspended in 40mL of 1X 'WX'. 'WX' is a proprietary lysis buffer of Blue Sky Biotech. The resuspended cells were then lysed by sonication three times for 30 seconds on ice with a one to two minute period in-between for the sample to cool. After sonication the sample was centrifuged at 15,000 rpm for 30 minutes. A 5mL pre-charged Ni Sepharose 6 Fast Flow column (Amersham Biosciences) was prepped by adding 5mL of 1X WX three times to the column to clean it and adjust it for the buffer that will be used. The supernatant is loaded on the column. The column was then washed two times with 5mL of 1X WX with 20mM imidazole. The column was eluted twice with 3mL of 1X WX and 300mM imidazole. Finally the column is

stripped using 5mL of 1X WX and 500mM imidazole. Western blot and coomassie stain were used to analyze the 1.5mL samples collected from the whole cell, flow through, wash, each elution, and strip.

2.4 Protein Gels and Western Blots

All cell suspension samples collected were analyzed using 12% SDS-Page polyacrylamide gel (Sambrook and Russell, 2001) and then performing a western transfer onto 0.45 micron nitrocellulose membrane (Pierce Biotechnology Inc.). Each cell suspension sample was first centrifuged at 14000rpm for five minutes. The supernatant was then discarded and the cell pellet was prepped using 200µL of Cytobuster (Novagen) and 0.25µL of PIC to bust open the cells and release the protein. The mixture was then placed on an orbital vortexer for 20 minutes after which a 50µL cell suspension sample was combined with 150µL dH_2O and 100µL SDS-Page loading dye and labeled the whole cell fraction. The resuspended cells were then centrifuged at 14000rpm for 10 minutes to spin down cell debris. A 100µL sample was collected from the supernatant and combined with 100µL dH_2O and 100µL SDS-Page loading dye and labeled the soluble fraction. The whole cell and soluble fractions were boiled on a heating block for 5 minutes, allowed to cool, and then run on a 12% gel and a western transfer was performed.

After the transfer is completed the membrane is blocked with a 5% milk solution using 1X tris buffered saline-tween (TBST) for 30 minutes and then placed in a solution of 1:1000 anti-His primary antibody (Molecular Probes) overnight at 4° C. The western is then washed three times for five minutes each with 1X TBST and placed in a solution of 1:2000 anti-mouse AP secondary antibody (Promega) for four hours. It was then

washed three times again with 1X TBST and is developed using Western Blue developer (Promega).

2.5 Protein Concentration

To measure the concentration of protein obtained through purification a bicinchoninic acid (BCA) assay was performed. The assay kit (Pierce) provides a protein standard and a developing buffer. First different concentrations of the protein standard are prepared at 0, 0.2, 0.4, 0.6, 0.8, and 1.0 µg/mL. The HDAC samples are prepared in three separate dilutions of 100% HDAC sample, a 50% HDAC sample, and a 20% HDAC sample. All dilutions were prepared using purified water. All samples were run in triplicate. When loading the samples into a 96-well plate 20µL of protein sample was added with 200µL of the developing buffer and thoroughly mixed. The plate was allowed to incubate at 37° for 30 minutes. After the incubation period the plate is allowed to cool to room temperature and then read using a microplate reader at 570nm and the data is graphed.

2.6 Activity Assay

An activity kit (Epigentek) that uses colorimetric detection to measure deacetylation was used. The assay kit obtained provides 8-well strips. The wells are first washed with 150µL of 1X wash buffer. Then 50µL of a biotinylated HDAC substrate is added to each well and incubated at room temperature for 45 minutes during which time the biotinylated substrate will bind to the well. The wells are then washed twice with 150µL wash buffer to remove excess substrate. After washing, 28µL of assay buffer and 2µL of the HDACs were added to the wells and allowed to incubate at 37° for 60 minutes. The kit provided TSA as an inhibitor. The wells are washed with 150µL wash

buffer after the incubation period and 50µL capture antibody is added and incubated on a rocking platform for 60 minutes at 100rpm. Another wash occurs and 50µL detection antibody is added and incubated for 30 minutes at room temperature. There is one final wash phase that consists of four washes with 150µL and 100µL of the developing solution is added. It is allowed to develop for up to 10 minutes away from light and the development progress can be monitored by the amount of blue that appears in each well. After 10 minutes 50µL of stop solution is added and the plate can be read on a microplate reader at 450nm and charted graphically. Blank wells with no substrate or HDAC and control wells of only substrate are also assayed. In addition, an active HDAC (UpState) was used as a positive control.

3.0 Results:

3.1 Virus Production

Using previously constructed and verified clones of HDAC 3FL, 3AD, and 2AD viral stocks were produced by transforming SF9 insect cells with bacmid DNA.

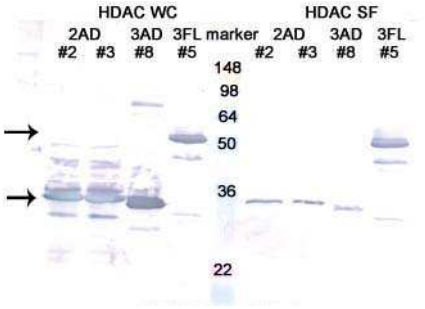
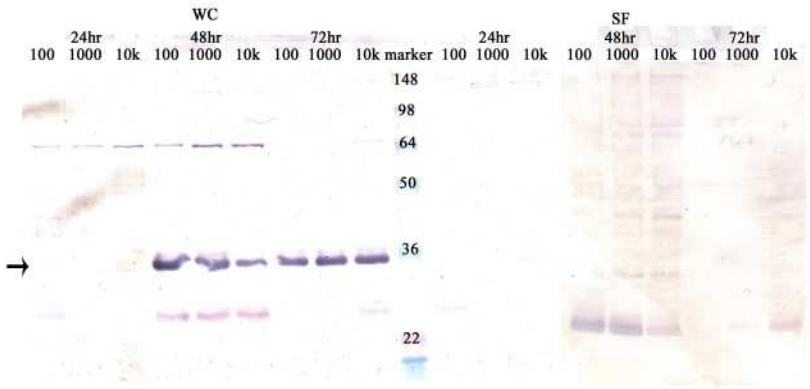


Figure 8: HDAC Expression during Viral Stock Production
Western blot of HDAC2 AD, 3AD, and 3FL viral stocks produced in SF9 insect cells. Arrows represent the expected size of the HDAC protein. WC: whole cell, SF: soluble fraction

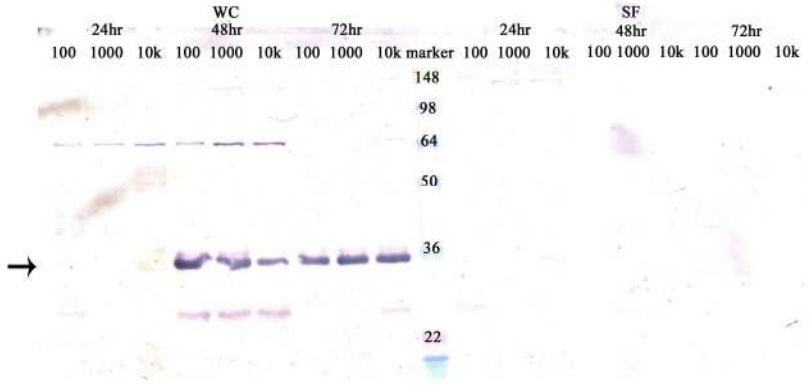
All transfections were monitored on westerns using whole cell lysates and soluble fractions from lysed cells. Each transfection showed expression for the corresponding HDAC (Fig. 8) so the viruses that were harvested from these cells were then used for subsequent expression testing and scale-ups. Expression in the soluble fraction is weaker than that in the whole cell most likely due to some of the protein remaining in the cell fragments after lysing.

3.2 Expression Test

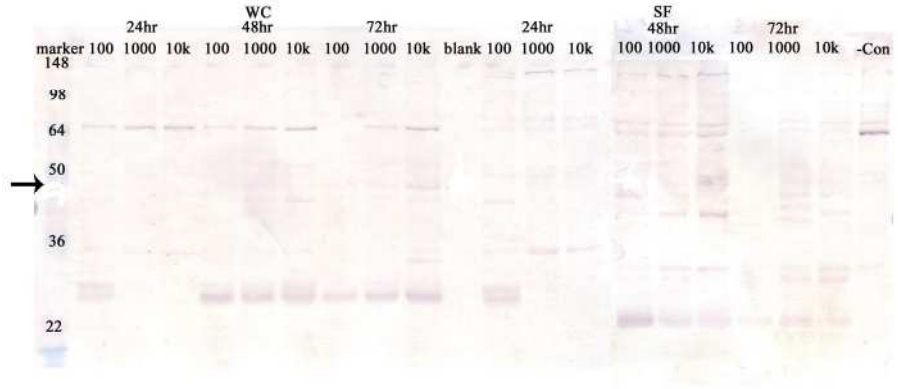
For this set of viral stocks an expression test was performed. Each viral HDAC construct was used to infect 50mL SF21 insect cell cultures in dilutions of 1:100, 1:1000, and 1:10000. The cultures were allowed to grow for 72 hours with samples being taken every 24 hours (Fig. 9).



A.



B.



C.

Figure 9: HDAC Expression Tests
Western blots for different HDAC constructs with data for every 24 hours at dilutions of 1:100, 1:1000, and 1:10000 in both whole cell (WC) and soluble (SF) fragments. HDAC2 AD expression (A), HDAC3 AD expression (B), and HDAC3 FL expression (C). Arrows represent expected protein size.

There appears to be some expression for each construct. Expression is significantly higher in the whole cell samples, but it is not uncommon for protein levels to be significantly lower in the soluble fraction.

When comparing the westerns from the transfected cells with those of the expression tests, the transfected cells appear to have better expression of the HDAC protein. To determine if this was due to differences in the insect cell lines a test was run of a single dilution of 1:2500 of HDAC2 AD in SF9, SF21, and High-5 cells over a period of 72 hours. HDAC2 AD was chosen because it had showed strong expression in SF9 insect cells and faint expression in SF21s.

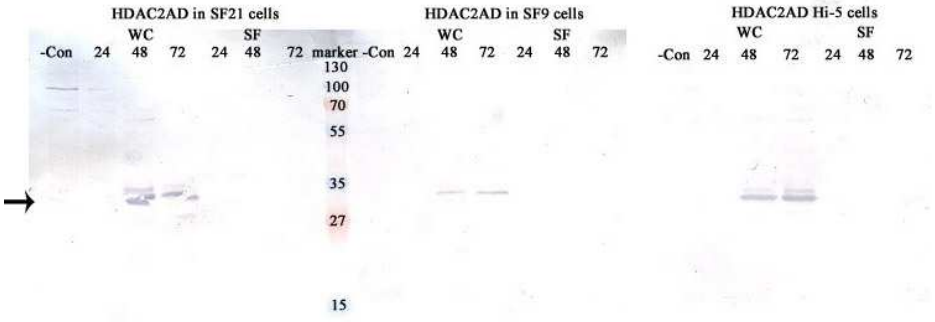


Figure 10: Insect Cell Expression Test
Western blot of HDAC2 AD in SF21, SF9, and High-5 insect cells at a dilution of 1:2500 with data from 24 hour intervals. Arrow represents expected protein size.

The results (Fig. 10) appear to show that there is no discernable difference between expression levels in the different cell lines. These westerns also verified expression of HDAC2 AD which had shown faint expression in the expression test.

Additionally an expression test was done for HDAC constructs already in P1 virus stocks, HDACs 1AD, 1FL, and 8AD. Cultures were grown in a 250mL volume of SF21 insect cells and infected at a dilution of 1:800 for each virus stock.

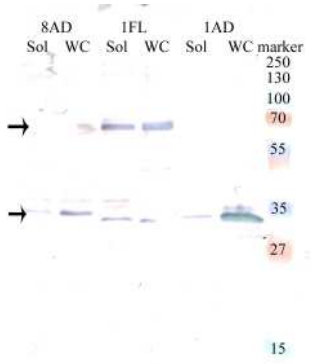


Figure 11: HDAC1 AD, 1FL, and 8AD Expression Test
Western blot that verifies expression of HDAC8 AD, 1FL, and 1AD in SF21 insect cells in both whole cell and soluble fractions.

Figure 11 verifies the expression of HDACs 1AD, 1FL, and 8AD in SF21 insect cells.

3.3 Expression (Large Scale)

Using the data obtained from the expression tests, larger scale infections were performed in 800mL volume. In addition to scaling up HDACs 2AD, 3AD, and 3FL, HDAC8 FL was also run. HDAC2 AD was infected at a dilution of 1:5500, 3AD was infected at a dilution of 1:5000, 3FL at a dilution of 1:5000, and 8FL was infected at 1:800. After 72 hours the cells were harvested and tested for presence of the HDAC protein (Fig. 12).

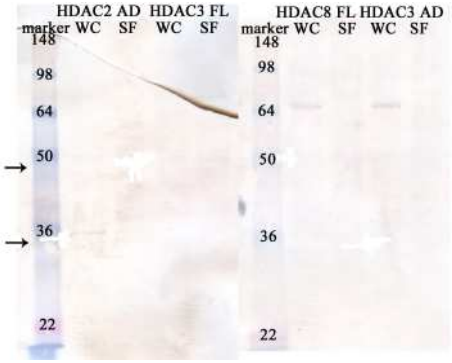


Figure 12: HDAC Scale-Ups
Western blot for HDAC2 AD, 3FL, 8FL, and 3AD 800mL scale-ups in SF21 insect cells.

The western results are not conclusive. However, the information obtained from the Cedex appears to confirm that the cells were infected, so it is possible that the cells are expressing the HDAC protein and it is not highly visible because the antibody did not

bind. Following this reasoning the harvested cells were used in further protein purification stages.

3.4 Purification

Each HDAC construct included an N' terminal polyhistitidine tag to allow for purification on a Ni column. HDACs 2AD, 3AD, 3FL, and 8FL were all extracted from the large scale infections and subjected to column chromatography. Samples were collected throughout the purification and analyzed by coomassie stain and western blotting (Fig. 13).

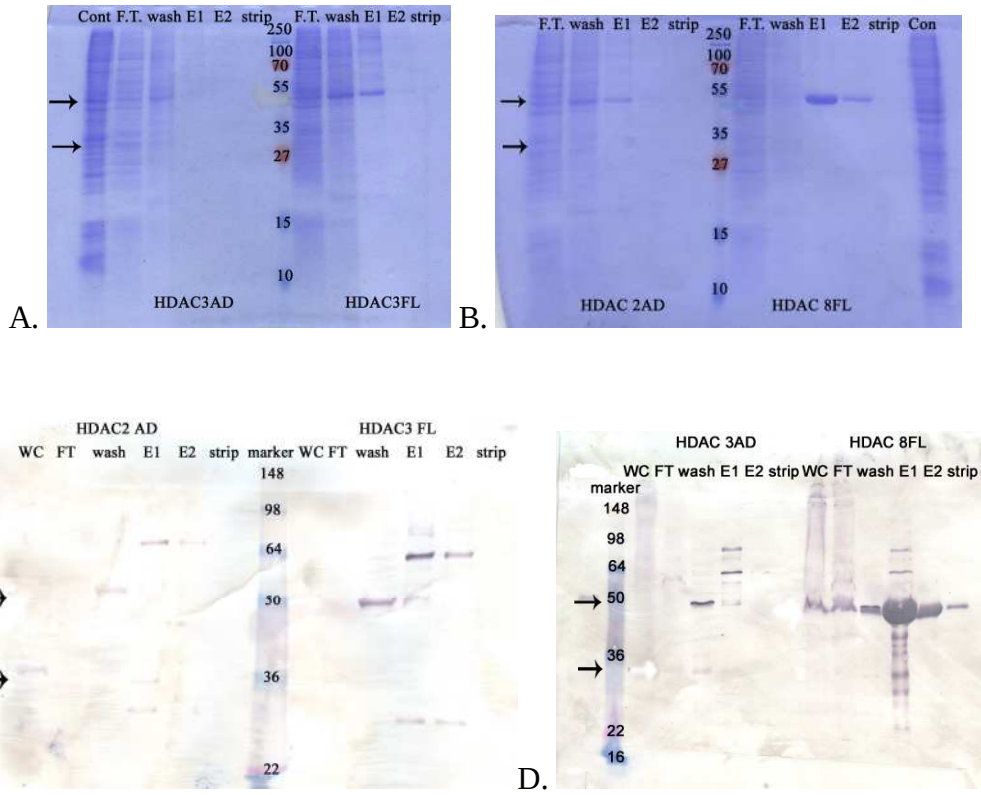


Figure 13: HDAC Purification Results
Coomassie stains (A, B) and western blots (C, D) that show the results of the HDAC purification. Samples collected from whole cell (WC), flow through (FT), wash, elution 1 (E1), elution 2 (E2), and strip were analyzed for each HDAC construct. HDAC3 FL purification (A, C), HDAC3 AD purification (A, D), HDAC2 AD purification (B, C) and HDAC8 FL purificatoin (B, D).

The western results appear positive for HDAC8 FL and HDAC3 FL, but not necessarily for HDAC2 AD and 3AD. Using the elution 1 samples collected, protein concentrations were calculated using the BCA assay.

3.5 Activity Assay

Using the protein concentrations obtained from the BCA assay 100ng and 2500ng HDAC samples were prepared for use in a commercially purchased HDAC activity assay. For the first test of the assay these samples were run in duplicate along with substrate only and a commercial source of active HDAC8. In addition to the purified proteins mentioned previously, the activity of other purified proteins, HDACs 1AD, 1FL, 8AD, and 8FL was also tested (Fig. 14).

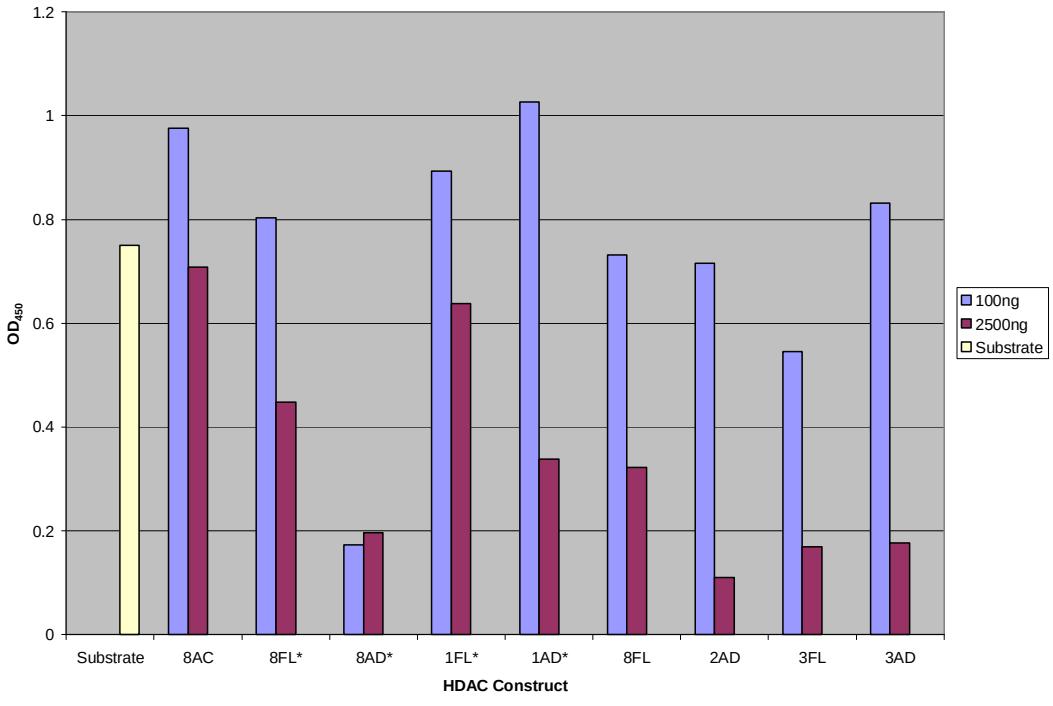


Figure 14: Activity Assay Using Two Concentrations
Each sample was run in duplicate and an average of the two readings is plotted. HDACs were tested in 100ng and 2500ng samples. The HDAC proteins obtained through purification were tested against substrate only and an active HDAC8 available commercially (8AC). HDAC samples marked with a '*' were produced previously and also tested in this assay.

Deacetylation of the biotinylated substrate will result in a decrease in OD. It appears that all HDACs, except the commercially purchased HDAC8, have deacetylation activity. This is more obvious in the samples that are treated with the higher concentration of HDAC. The commercially purchased HDAC, which is supposed to be active, does not appear to be active in this assay.

To determine if the deacetylation observed in the first assay was due to HDACs the assay was repeated using the higher concentration of purified protein and a specific inhibitor, TSA. This should show substantial reduction in deacetylation if it is indeed due to HDAC activity (Fig. 15).

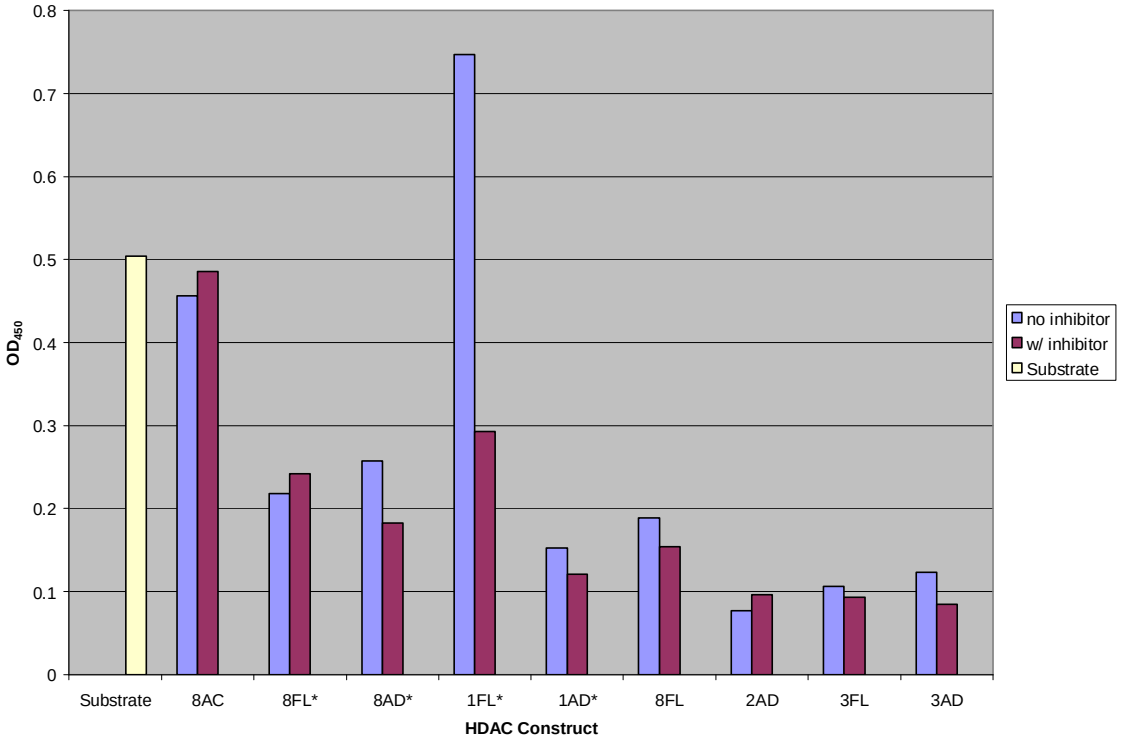


Figure 15: Activity Assay w/ Inhibitor
Data obtained from the activity assay is charted graphically with HDACs without an inhibitor and with an inhibitor. Samples were run in duplicate and the average was taken for analysis.

Again the commercially purchased HDAC8 does not appear to be active in this assay as there is no significant reduction in signal upon its addition. Additionally, although the other HDACs appear to be active in this assay, there is no inhibition by TSA. The TSA is present in 200X molar excess if it is assumed the protein is 100% HDAC. This should be enough to inhibit all HDAC activity. The odd data for HDAC1 FL may be due to an abnormal sample that was significantly higher OD then its duplicate without inhibitor, the OD readings of the duplicate samples were 0.972 and 0.523.

This assay was repeated (Fig. 16). The major difference in this assay is that the purchased HDAC8 appears to show some minimal activity with less than 50% of the signal compared to the substrate alone. However, this activity is not inhibited by TSA. Only one sample, 1FL, shows a decrease in deacetylation when the inhibitor is added, but it is not clear if this is a significant, particularly as it is contrary to the previous data.

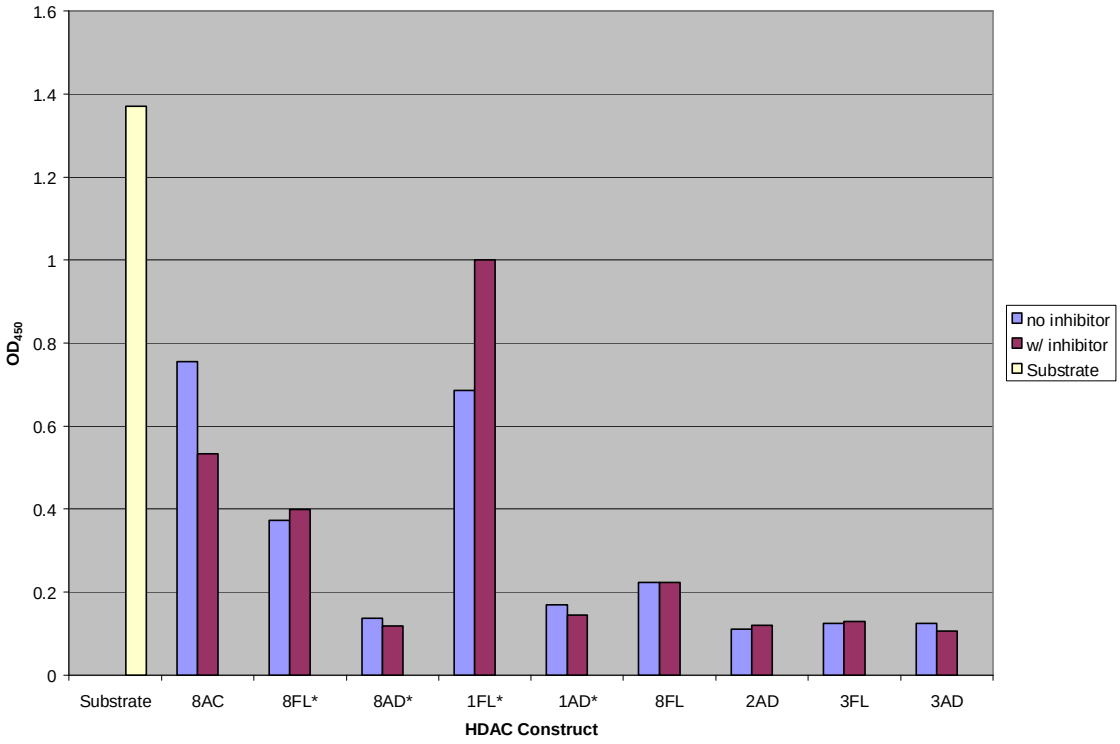


Figure 16: Activity Assay w/ Inhibitor
Data obtained from the activity assay is displayed graphically for HDAC constructs with both an inhibitor and no inhibitor. All samples were run in duplicate and the average is being used for analysis.

Because of lack of materials only one more assay was able to be performed. The final assay repeats the first assay by testing the HDACs in two separate concentrations, this time using 1000ng and 2500ng samples (Fig. 17).

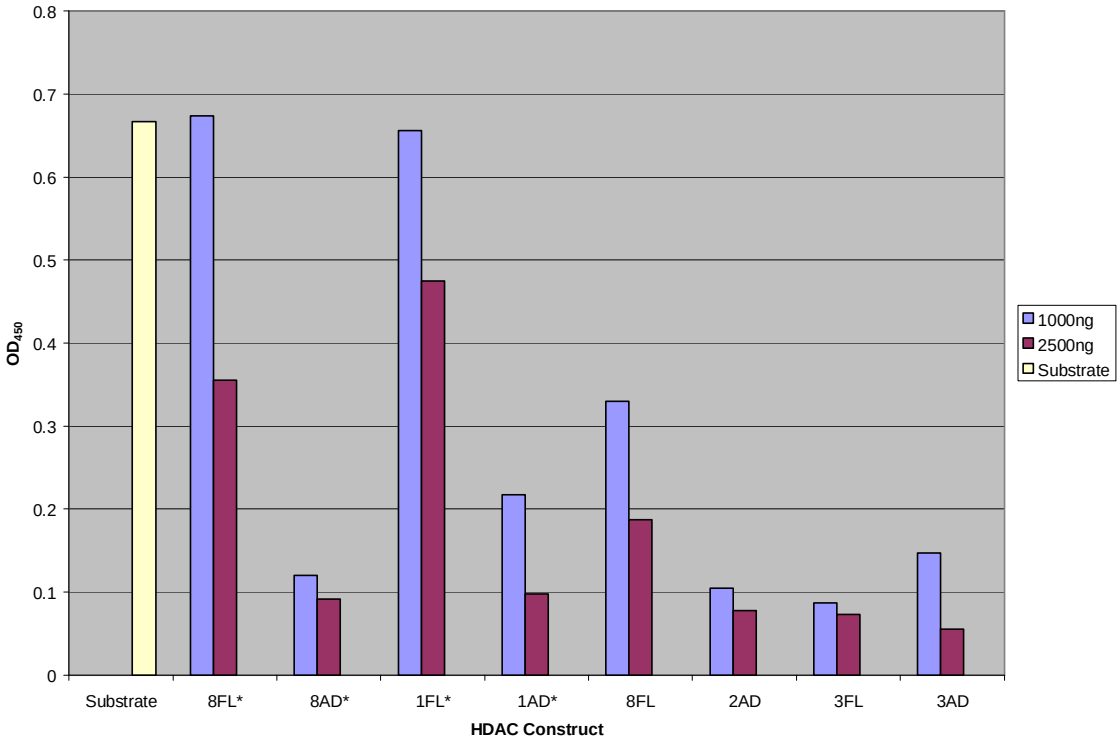


Figure 17: Activity Assay Using Two Concentrations
Data obtained from the activity assay is plotted graphically to display these differences. All samples are run in duplicate and the average is taken for analysis.

For several samples, 8FL, 1AD, 1FL, 8FL, there is a clear difference between the two concentrations. For several others, 2AD, 3FL, 3AD, 8AD, the OD is very low and not significantly changed by increasing the protein concentration. This suggests that even the lower concentration has saturated the system. In general these are the active domain constructs. Since they are smaller, the same amount of protein would be expected to have a higher concentration of enzyme. However, since the protein samples were not purely active domain constructs it is difficult to extrapolate activity analysis from this data.

4.0 Discussion:

Histone deacetylases have become a target area for research in many scientific fields, particularly cancer. To perform the large amount of research on HDACs that is currently occurring it is important to find a way to produce them in bulk. The ability to express class I HDACs in insect cells appears to be a viable option. Not only were full length HDACs expressed, but predicted catalytic or active domains of HDACs were also expressed. To be able to show expression of possible active domain HDACs is also important in the understanding of the possible structure of this area.

There is a definite variation in expression levels between the different HDAC constructs. One way to separate the data is between the full length and the active domains. The active domain constructs appear to have a lower expression level than the full length versions. This could be due to a decrease in stability.

An important part of obtaining the HDAC proteins was to verify that they were active. Inactive HDACs would not be beneficial for clinical research. To test the activity an activity assay was performed. The assay measured the acetylated substrate via a colorimetric assay. Thus, the lower the average OD value for each HDAC construct, the more active that HDAC would appear to be. Unfortunately, the results of the activity assay are not conclusive for HDAC activity. Although the purified HDAC samples all showed some activity in the assay, none of them were inhibited by TSA, a specific HDAC inhibitor. It is possible there was no inhibition because the TSA was inactive. However, there is no way to determine if this is true based on the data. A commercially purchased, and supposedly active HDAC8, also showed no significant activity in this assay. If this enzyme showed activity it could have been used to show the efficiency of

the inhibitor. However, because it showed no activity there was no way to determine if the inhibitor was active. Thus, this data only demonstrates that the purified protein samples, which all clearly have multiple proteins, do contain an enzymatic activity that can deacetylate the substrate in the assay. Further experiments must be done to demonstrate that the activity present in the assay is in fact due to the cloned HDACs.

At the end of the testing all HDAC constructs had been expressed, purified, and activity tested except for one. A summary of the various steps can be seen in Table 2.

Construct	Cloned	Expressed	Purified	Activity	Active
1AD	X	X	X	X	Yes
1FL	X	X	X	X	Yes
2AD	X	X	X	X	Yes
2FL					
3AD	X	X	X	X	Yes
3FL	X	X	X	X	Yes
8AD	X	X	X	X	Yes
8FL	X	X	X	X	Yes

Figure 18: Summary of Completion

Although all the constructs that were tested for activity appeared to be active there were varying degrees of activity. If another activity assay was purchased then it could be attempted to determine actual activity values. Also, it would be beneficial to test more concentrations of HDACs, in particular HDAC3, to better view the differences between active domains and full length domains. In addition to measuring actual activity values an attempt at cloning HDAC2 FL could also be performed to continue this research. Finally, to better examine the inhibitor data, an active inhibitor should be purchased from a separate source and used when repeating the activity assay.

The ability to produce HDACs in a cost effective manor is important because of the amount of research that is currently being conducted using these enzymes. However,

being able to express class I HDACs in insect cells is not the only thing provided here. Expression of possible active domain constructs for class I HDAC has also been established. This data can be used in further studies in the understanding of what makes each HDAC unique. If unique domains for each HDAC can be identified, then the development of inhibitors that are specifically designed for those domains becomes a possibility. By creating HDAC specific inhibitors a better understanding can begin of what each HDACs role is and what the effects are of inhibiting it. This data can be applied to developing treatments for specific cancers or other diseases where HDACs play a role.

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Data Run Sheet

Experiment **HDAC viral stock** **Date: 10-14-2005** HDAC2AD #2
Flask Vol.: **36ml/125ml Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
10/13/05	P_{20}	14:00	—	Inoculated @ 7.0×10^5 cells/ml						
10/14/05	P_{20}	10:30	0.00	11.41×10^5	97.6	11.69×10^5	14.22			
10/14/05	P_{20}	10:30	0.00	Infected w/ bacmid #2						
10/18/05	P_{20}	10:30	96.0	47.73×10^5	98.5	48.48×10^5	16.55			
10/19/05	P_{20}	13:00	122.50	52.67×10^5	94.0	56.02×10^5	18.14			1.5 mL sample
10/20/05	P_{20}	10:30	142.00	31.50×10^5	57.1	55.13×10^5	18.42			Harvested

Data Run Sheet

Experiment **HDAC viral stock** **Date: 10-14-2005** HDAC2AD #3
Flask Vol.: **36ml/125ml Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
10/13/05	P20	14:00	—	Inoculated	@ 7.0×10^5	cells/mL				
10/14/05	P20	10:30	0.00	11.41×10^5	97.6	11.69×10^5	14.22			
10/14/05	P20	10:30	0.00	infected w/	bacmid	#3				
10/18/05	P20	10:30	96.0	44.73×10^5	98.3	45.49×10^5	17.22			
10/19/05	P20	13:00	122.50	47.09×10^5	91.7	51.34×10^5	18.11			1.5 mL sample
10/20/05	P20	10:30	142.00							Harvested

Data Run Sheet

Experiment **HDAC viral stock** **Date: 10-14-2005** HDAC3FL #5
Flask Vol.: **36ml/125ml Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21** 9

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
10/13/05	P20	14:00	—	inoculated	@ 7.0×10^5 cells/mL					
10/14/05	P20	10:30	0.00	11.41×10^5	97.6	11.69×10^5	14.22			
10/14/05	P20	10:30	0.00	infected w/ bacmid #5						
10/18/05	P20	10:30	96.0	28.95×10^5	97.2	29.79×10^5	17.42			
10/19/05	P20	13:00	122.50	30.67×10^5	94.6	32.42×10^5	20.27			1.5 mL sample
10/20/05	P20	10:30	142.00							Harvest

Data Run Sheet

Experiment **HDAC viral stock** **Date: 10-14-2005** HDAC3AD #8
Flask Vol.: **36ml/125ml Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
10/13/05	P_{20}	14:00	—	Inoculated ~~cells~~ @		7.0×10^5 cells/mL				
10/14/05	P_{20}	10:30 9:00	0.00	11.41×10^5	97.6	11.69×10^5	14.22			
10/14/05	P_{20}	10:30 9:00	0.00	Infected cells w/ bacmid #8						
10/18/05	P_{20}	10:30 9:00	96.0	29.16×10^5	98.7	29.55×10^5	17.42			
10/19/05	P_{20}	13:00	122.50	30.69×10^5	92.3	33.24×10^5	17.58			1.5mL sample
10/20/05	P_{20}	10:30	142.00							Harvested

Data Run Sheet

Experiment: HDAC 2AD Scout **Date: 11-09-2005** SF1 Control
Flask Vol.: 500ml/2L Shake Flask
Medium: Insect Express (Cambrex) plus 5 mg/L Gentamicin
Cell Line: BS-SF21

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-07-05	P45	11:00	—	Inoculated @ ~7.0 $\times 10^5$ cells/mL						
11-08-05	P45	11:00	0.00	13.37×10^5	99.0	13.50×10^5	16.11			
~~11-10-05~~	~~P45~~	"	"	Infected w/ media as control						
11-09-05	P45	11:00	24.0	27.65×10^5	98.8	27.98×10^5	16.60	—	—	1.5 mL sample
~~11-10-05~~	~~P—~~	~~11:00~~	48.0							

Data Run Sheet

Experiment: **HDAC 2AD Scout** **Date: 11-09-2005** SF1 **1:100**
Flask Vol.: **500ml/2L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line: **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-09-05	P	11:00	0.00	Inoculated @		1.4×10^5 cells/mL				
"	"	"	"	Infected w/ P4 viral stock # @ 1:100						
11-10-05	P	11:00	24.0							
11-07-05	P_{45}	11:00	—	Inoculated @ ~7.0×10^5 cells/mL						
11-08-05	P_{45}	11:00	0.00	13.37×10^5	99.0	13.50×10^5	16.11			
"	"	"	"	Infected w/ P4 viral stock #2 @ 11:00						
11-09-05	P_{45}	11:00	24.0	13.68×10^5	99.0	13.81×10^5	21.31	—	—	1.5mL sample
11-10-05	P_{45}	11:00	48.0	11.24×10^5	63.6	17.69×10^5	17.62	—	—	"
11-11-05	P_{45}	11:00	72.0	3.57×10^5	23.4	15.28×10^5	13.33	—	—	"

Data Run Sheet

Experiment **HDAC 2AD Scout** **Date: 11-09-2005** SF1 **1:1000**
Flask Vol.: **500ml/2L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-07-05	P_{45}	11:00	~~0:00~~	Inoculated	@ ~	7.0×10^5	Cells/mL			
11-08-05	P_{45}	11:00	0.00	13.37×10^5	99.0	13.50×10^5	16.11			
"	"	"	"	Infected w/ P_1 viral stock #2 @ 1:1000						
11-09-05	P_{45}	11:00	24.0	15.88×10^5	98.2	16.17×10^5	21.55	—	—	1.5 mL sample
11-10-05	P_{45}	11:00	48.0	17.60×10^5	89.0	19.78×10^5	20.21	—	—	"
11-11-05	P_{45}	11:00	72.0	7.33×10^5	29.3	25.03×10^5	17.27	—	—	"

Data Run Sheet

Experiment **HDAC 2AD Scout** **Date: 11-09-2005** SF1 **1:10K**
Flask Vol.: **500ml/2L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-07-05	P_{45}	11:00	—	Inoculated	@ ~	7.9×10^5	cells/mL			
11-08-05	P_{45}	11:00	0.00	13.37×10^5	99.0	13.50×10^5	16.11			
"	"	"	"	Infected w/ P_4 viral stock #2 @ 1:10k						
11-09-05	P_{45}	11:00	24.0	22.14×10^5	99.1	22.34×10^5	18.65	—	—	1.5 mL sample
11-10-05	P_{45}	11:00	48.0	23.08×10^5	97.7	23.63×10^5	22.27	—	—	"
11-11-05	P_{45}	11:00	72.0	12.87×10^5	40.7	31.63×10^5	19.73	—	—	"

Data Run Sheet

Experiment **HDAC Scout (Internal-Erin B.)** **Date: 10-24-2005** HDAC 3AD 100
Flask Vol.: **36ml/125ml shake flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
10/24/05	P_{41}	14:00	–	Inoculated @ $\approx 7.5 \times 10^5$ cells/mL						
10/25/05	P_{41}	14:00	0.0							
"	"	"	"	Infected w/ P_4 viral stock @ 1:100						
10/26/05	P_{41}	15:00	25.0	13.24×10^5	94.4	14.02×10^5	20.87	–	–	1.5mL Sample
10-27-05	P_{41}	14:00	48.0	12.16×10^5	63.4	19.19×10^5	19.44	–	–	1.5 mL sample
10-28-05	P_{41}	14:30	72.5	3.52×10^5	26.3	13.38×10^5	15.45	—	—	"

Data Run Sheet

Experiment **HDAC Scout (Internal-Erin B.)** **Date: 10-24-2005** HDAC 3AD 1000
Flask Vol.: **36ml/125ml shake flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
10/24/05	P_{41}	14:00	—	Inoculated @ ~7.5 x 10^5	cells/mL					
10/25/05	P_{41}	14:00	0.0							
"	"	"	"	Infected w/	P_1 viral	stock @ 1:1000				
10/26/05	P_{41}	15:00	25.0	19.68 x 10^5	96.6	20.31 x 10^5	20.34	—	—	1.5mL sample
10/27/05	P_{41}	14:00	48.0	19.51 x 10^5	90.7	21.52 x 10^5	21.59	—	—	"
10/28/05	P_{41}	14:30	72.5	10.39 x 10^5	37.7	27.54 x 10^5	20.14	—	—	"

Data Run Sheet

Experiment **HDAC Scout (Internal-Erin B.)** **Date: 10-24-2005** HDAC 3AD 10K
Flask Vol.: **36ml/125ml shake flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
10/24/05		14:00	–	Inoculated @ ~7.5		x 10^5 cells/ml				
10/25/05		14:00	0.0							
"	"	"	"	Infected w/ P1 viral	stock @ 1:10K					
10/26/05	P41	15:00	25.0	21.65 x 10^5	98.1	22.08 x 10^5	17.18	—	—	1.5mL sample
10/27/05	P41	14:00	48.0	26.37 x 10^5	95.6	27.60 x 10^5	21.81	—	—	"
10/28/05	P41	14:30	72.5	19.17 x 10^5	63.8	30.04 x 10^5	20.94	—	—	"

Data Run Sheet

Experiment **HDAC 3FL Scout** **Date: 11-09-2005** SF1 1:100
Flask Vol.: **500ml/2L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-07-05	P_{45}	11:00	—	Inoculated @ ~		7.0×10^5 cells/mL				
11-08-05	P_{45}	11:00	0.00	13.37×10^5	99.0	13.50×10^5	16.11			
"	"	"	"	Infected w/ P_1 viral stock #			5 @	1:100		
11-09-05	P_{45}	11:00	24.0	13.24×10^5	99.0	13.37×10^5	21.24	—	—	1.5mL sample
11-10-05	P_{45}	11:00	48.0	11.96×10^5	83.4	14.35×10^5	24.78	—	—	"
11-11-05	P_{45}	11:00	72.0	4.87×10^5	33.8	14.39×10^5	20.99	—	—	"

Data Run Sheet

Experiment **HDAC 3FL Scout** **Date: 11-09-2005** SF1 **1:1000**
Flask Vol.: **500ml/2L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-07-05	P_{45}	11:00	—	Inoculated	@ "	7.0×10^5	cells/mL			
11-08-05	P_{45}	11:00	0.00	13.37×10^5	99.0	13.50×10^5	16.11			
"	"	"	"	Infected w/ P_4 viral stock #5 @ 1:1000						
11-09-05	P_{45}	11:00	24.0	17.69×10^5	98.9	17.88×10^5	20.42	—	—	1.5 mL sample
11-10-05	P_{45}	11:00	48.0	19.13×10^5	96.4	19.85×10^5	23.19	—	—	"
11-11-05	P_{45}	11:00	72.0	10.15×10^5	52.8	19.24×10^5	24.27	—	—	"

Data Run Sheet

Experiment **HDAC 3FL Scout** **Date: 11-09-2005** SF1 **1:10K**
Flask Vol.: **500ml/2L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-07-05	P_{45}	11:00	–	Inoculated	@ ~	7.0×10^5 Cells				
11-08-05	P_{45}	11:00	0.00	13.37×10^5	99.0	13.50×10^5	16.11			
"	"	"	"	Infected @ w/ P_4 viral stock #5 @ 1:10K						
11-09-05	P_{45}	11:00	24.0	23.52×10^5	98.8	23.82×10^5	17.51	—	—	1.5 ml sample
11-10-05	P_{45}	11:00	48.0	24.62×10^5	98.8	24.92×10^5	22.34	—	—	"
11-11-05	P_{45}	11.00	72.0	18.75×10^5	75.3	24.92×10^5	25.19	—	—	"

Data Run Sheet

~~Experiment#~~ ~~**HDAC 2AD expression scout**~~ ~~**Date: 02-14-2006**~~ ~~Control~~

Flask Vol.: **50ml/250mL shake flask**

Medium: **HyQ SFX-Insect (Hyclone) plus 5mg/L Gentamicin**

Cell Line **BS-SF9**

Date 2006	Pass#	Time	Run Time hours	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
2-13-06	P18	8:00	—	inoculated	@ 7.5	$\times 10^5$ cells/mL				
2-14-06	P18	8:00	0.00	12.42×10^5	98.5	12.60×10^5	14.83			
"	"	"	"	infected w/ 1 mL media						
2-15-06	P18	8:30	24.50	22.50×10^5	98.2	22.91×10^5	14.57			1.5 mL sample
2-16-06	P18	8:00	48.00	46.54×10^5	99.0	47.02×10^5	14.35			"
2-17-06	P18	8:30	72.50	92.91×10^5	98.7	94.13×10^5	13.91			"

Data Run Sheet

~~Experiment#~~ ~~**HDAC 2AD expression scout**~~ ~~**Date: 02-14-2006**~~ ~~2500~~

Flask Vol.: **50ml/250mL shake flask**

Medium: **HyQ SFX-Insect (Hyclone) plus 5mg/L Gentamicin**

Cell Line **BS-SF9**

Date 2006	Pass#	Time	Run Time hours	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
2-13-06	P18	8:00	—	Inoculated @ 7.5 x 10^5 cells/mL						
2-14-06	P18	8:00	0.00	12.42×10^5	98.5	12.60×10^5	14.83			
"	"	"	"	Infected w/ P1 viral stock @ 1:2500						
2-15-06	P18	8:30	24.50	17.44×10^5	98.0	17.80×10^5	16.22			1.5 mL sample
2-16-06	P18	8:00	48.00	18.21×10^5	96.1	18.95×10^5	18.29			"
2-17-06	P18	8:30	72.50	13.44×10^5	64.4	20.88×10^5	18.70			"

Data Run Sheet

~~Experiment~~ ~~**HDAC 2AD expression scout**~~ ~~**Date: 2-14-2006**~~ ~~**Control**~~

Flask Vol.: **50ml/250mL Shake Flask**

Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**

Cell Line **BS-SF21**

Date 2006	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
2-13-06	P18	8:00	—	Inoculated @ 7.5×10^5 cells/mL						
2-14-06	P18	8:00	0.00	16.97×10^5	98.9	17.15×10^5	17.59			
"	"	"	"	infected w/ 1 mL media						
2-15-06	P18	8:30	24.50	40.32×10^5	98.6	40.91×10^5	17.11			1.5 mL sample
2-16-06	P18	8:00	48.00	48.46×10^5	99.1	48.91×10^5	17.68			"
2-17-06	P18	8:30	72.50	51.69×10^5	98.5	52.48×10^5	18.21			"

Data Run Sheet

~~Experiment~~ ~~**HDAC 2AD expression scout**~~ ~~**Date: 2-14-2006**~~ ~~**2500**~~

Flask Vol.: **50ml/250mL Shake Flask**

Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**

Cell Line **BS-SF21**

Date 2006	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
2-13-06	P18	8:00	—	Inoculated @ 7.5×10^5 cells/ml						
2-14-06	P18	8:00	0.00	10.97×10^5	98.9	17.15×10^5	17.59			
"	"	"	"	infected w/ P1 viral stock @ 1:2500						
2-15-06	P18	8:30	24.50	23.45×10^5	99.0	23.69×10^5	20.75			15 mL sample
2-16-06	P18	8:00	48.00	25.42×10^5	94.8	26.81×10^5	21.50			"
2-17-06	P18	8:30	72.50	16.64×10^5	43.6	38.17×10^5	19.54			"

Data Run Sheet

Experiment **HDAC 2AD expression scout** **Date: 2-14-2006** **Control**
Flask Vol.: **50ml/250mL Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-Hi-5**

Date 2006	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
2-13-06	P39	8:00	—	inoculated @ 7.5×10^5 cells/mL						
2-14-06	P39	8:00	0.00	15.72×10^5	99.0	15.88×10^5	16.45			
"	"	"	"	infected w/ 1 mL media						
~~2-15-06~~	~~P39~~	~~8:30~~	~~24.50~~	~~40.32×10^5~~	~~98.0~~	~~4091×10^5~~				
2-15-06	P39	8:30	24.50	34.62×10^5	97.7	35.44×10^5	15.84			1.5 mL sample
2-16-06	P39	8:00	48.00	41.41×10^5	97.4	42.52×10^5	15.54			"
2-17-06	P39	8:30	72.50	42.89×10^5	91.1	47.08×10^5	12.64			"

Data Run Sheet

Experiment **HDAC 2AD expression scout** **Date: 2-14-2006** **2500**
Flask Vol.: **50ml/250mL Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-Hi-5**

Date 2006	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
2-13-06	P39	8:00	—	Inoculated @ 1.75 x 10^5 cells/mL						
2-14-06	P39	8:00	0.00	15.72 x 10^5	99.0	15.88 x 10^5	16.45			
"	"	"	"	Infected w/ P1 viral stock @ 1:2500						
2-15-06	P39	8:30	24.50	23.06 x 10^5	97.9	23.56 x 10^5	17.76			1.5 mL sample
2-16-06	P39	8:00	48.00	30.57 x 10^5	93.6	32.56 x 10^5	17.86			"
2-17-06	P39	8:30	72.50	39.59 x 10^5	71.6	41.32 x 10^5	16.03			"

Data Run Sheet

Experiment **HDAC Scale-Up** **Date: 1-09-2006** HDAC 3FL #5
Flask Vol.: **800ml/2.8L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
1/9/06	P8		—	Inoculated @ 7.5 $\times 10^5$ cells/mL						
1/10/06	P8	8:30	0.00	11.76×10^5	98.4	11.95×10^5	18.116			
"	"	"	"	Infected w/ P. viral stock #5 @ 1:5,000						
1/11/06	P8	9:00	24.50	19.62×10^5	98.4	19.94×10^5	18.36			
1/12/06	P8	9:00	48.50	19.49×10^5	98.0	19.88×10^5	20.09			
1/13/06	P8	9:30	73.00	20.04×10^5	97.4	20.57×10^5	19.67			
				Harvest						

Data Run Sheet

Experiment **HDAC Scale-Up** **Date: 1-09-2006** HDAC 2AD #2
Flask Vol.: **800ml/2.8L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
1/9/06	P8		—	Inoculated @ lower density						
1/10/06	P8	8:30	0.00	8.76×10^5	97.2	9.02×10^5	17.88			
"	"	"	"	Infected w/ P1 viral stock #2 @ 1:5555						
1/11/06	P8	9:00	24.50	14.41×10^5	96.4	14.95×10^5	18.86			
1/12/06	P8	9:00	48.50	15.62×10^5	95.4	16.38×10^5	22.24			
1/13/06	P8	9:30	73.00	~~14.39×10^5~~ 13.14×10^5	87.7	14.98×10^5	22.12			Harvest

Data Run Sheet

Experiment **HDAC scale-up** 3AD **Date: 11-14-2005** ~~SF21~~ HDAC 3AD
Flask Vol.: **800ml/2.8L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

1:5,000 @ 48hr.

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-14-05	P38		—	Inoculated @ 7.5×10^5 cells/mL						
11-15-05	P38	~~16:00~~ 07:00	07.00	15.93×10^5	99.2	16.06×10^5	16.27			
11-15-05	P38	16:00	0.00	Infected w/ P4 viral stock #8			@ 1:5,000			
11-16-05	P38	14:30	22.5	29.41×10^5	98.4	29.90×10^5	17.02			labelled as 2AD
11-17-05	P38	14:30	46.5	29.76×10^5	98.3	30.28×10^5	20.48			
11-18-05	P38	8:30	64.5	29.54×10^5	96.8	30.51×10^5	21.37			Harvest

Data Run Sheet

Experiment **HDAC scale-up** 8 FL **Date: 11-14-2005** ~~SF21~~ HDAC 8FL
Flask Vol.: **800ml/2.8L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

1:800 @ 48hrs.

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
11-14-05	P_{38}		—	Inoculated flask @ $\sim 7.5 \times 10^5$						
11-15-05	P_{38}	16:00	0.00	15.93×10^5	99.2	16.06×10^5	16.27			
11-15-05	P_{38}	16:00	0.00	Infected w/ P_1 viral stock #1 @ 1:800						
11-16-05	P_{38}	14:30	22.5	28.68×10^5	98.7	29.06×10^5	16.40			labeled as 3FL
11-17-05	P_{38}	14:30	46.5	33.13×10^5	98.6	33.59×10^5	19.15			
11-18-05	P_{38}	8:30	64.5	32.64×10^5	98.4	33.17×10^5	20.45			Harvest

Data Run Sheet

Experiment# ~~HDAC scale-up~~ **Date: 12-06-2005** HDAC 3AD

Flask Vol.: **800mL/2.8L Shake Flask**

Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**

Cell Line **BS-SF21**

1:5,000

Date 2004	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex)) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
12/6/05	P_{22}	10:30	0.00	12.81×10^5	98.4	13.02×10^5	14.33			
12/6/05	P_{22}	10:30	0.00	infected @ 1:5,000 w/ P_1 stock #						
12/7/05	P_{22}	10:30	24.00	21.13×10^5	98.1	21.53×10^5	15.22			
12/8/05	P_{22}	2:00	51.50	21.69×10^5	97.2	22.32×10^5	18.23			
12/9/05	P_{22}	1:00	74.50	21.38×10^5	89.7	23.83×10^5	18.60			

Data Run Sheet

Experiment# HDAC scale-up **Date: 12-06-2005** HDAC 8FL
Flask Vol.: **800mL/2.8L Shake Flask**
Medium: **Insect Express (Cambrex) plus 5 mg/L Gentamicin**
Cell Line **BS-SF21**

Date 2005	Pass#	Time	Run Time (hours)	Viable Cells (Cedex) (cells/ml)	Viability (%)	Total Cells (Cedex)) (cells/ml)	diameter (micrometers)	Subcultured 1:X	New Pass#	Comments
12/6/05	P_{22}	10:30	0.00	10.76×10^5	98.2	10.96×10^5	14.30			
12/6/05	P_{22}	10:30	0.00	Infected @ 1:800 w/ P_1 stock #1						
12/7/05	P_{22}	10:30	24.00	19.58×10^5	98.8	19.82×10^5	14.98			
12/8/05	P_{22}	2:00	51.50	21.86×10^5	97.3	22.46×10^5	18.17			
12/9/05	P_{22}	1:00	74.50	19.80×10^5	91.7	21.69×10^5	18.67			