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HIV infection, and tobacco smoking, two areas of major health concern which continue to grow worldwide, both of which have detrimental health effects on the population. The point of intersection where these two health concerns cross is the focus of this study.

Here, a possible link between HIV infectivity levels and tobacco smoking at the point of cellular gene expression differences seen in the presence of tobacco smoke is described. This research presents evidence of increased HIV infectivity in the presence of tobacco smoke. qRT-PCR analysis is used to confirm micro array gene expression results. Twelve genes were identified to be overexpressed or under-expressed as a result of exposure to tobacco smoke extract (TSE). The expression of those twelve genes was then knocked down individually using siRNA technology in T-cells. HIV infectivity levels were then measured using a novel luciferase assay system in those cell lines which were under expressing the gene of interest. Dual gene knockdown cell lines were also used in the study.

The results show that in the eight genes whose expression is up regulated as a result of TSE (which could be increasing HIV infectivity), upon the knockdown in expression of those genes, six of the eight show a significant decrease in HIV infectivity in T-cells. In the four genes whose expression is down regulated as a result of TSE (which could be increasing HIV infectivity), after the knockdown in expression of those

genes, three of the four show a significant increase in HIV infectivity in T-cells.

Together, these results shed light on the specific genes whose expression is altered due to TSE and how these gene regulation changes may affect HIV viral infectivity levels.

ELUCIDATION OF THE CELLULAR GENES, PATHWAYS, AND BIOCHEMICAL
MECHANISMS INVOLVED IN HIV STIMULATION BY
AQUEOUS TOBACCO SMOKE EXTRACT

by

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CHAPTER I
INTRODUCTION

HIV and Smoking

Tobacco smoking and HIV/AIDS are the only two international major causes of death that continue to grow [1]. Where these two factors cross should present a major area of public health concern. Cigarette smoking has many negative biological effects on the body, but for those individuals who are exposed to HIV it may be especially harmful. Smoking is known to increase the risk of various types of cancers, decrease the ability of the body to heal wounds, cause oral and gum diseases, and chronic inflammatory disease of the lungs leading to decreased lung function. With chronic inflammation of the lungs comes emphysema and bronchitis, which can lead to smoker's leukocytosis, which is the body's immune response by increasing the white blood cell count. A study by the United States Food and Drug Administration indicated that constituents of cigarette smoke may induce HIV production in chronically infected cells [2]. Individuals infected with HIV are about three times more likely to smoke than those who are not, and HIV infection is more common among smokers than non-smokers. HIV infected war veterans who currently smoke have a decreased quality of life, an increase in mortality, chronic obstructive pulmonary disease, and bacterial pneumonia compared to non-smoking HIV infected war veterans [3].

Background

Previous clinical studies measuring the relevance of tobacco smoking on HIV seroconversion (detectable amounts of anti-HIV antibodies in the blood serum) indicate that tobacco smoking is associated with an increased risk of acquiring HIV infection. Briefly, Siraprapasiri et. al. did a cross sectional study of men attending a sexually transmitted diseases clinic located in Chiang Mai, Thailand. 124 HIV negative and 26 HIV positive men were included in the study where they used a multivariate analysis, concluding that smoking was associated with HIV seropositivity (95% CI = 1.2 – 10.5) [4]. Burns et. al. looked at the seroconversion rate of 202 homosexual men enrolled with three primary care physicians in Washington D.C. and New York over a period of 6 years (1982-1988). The study included those found to be anti-HIV antibody positive at the time of enrollment (n=84), those who seroconverted during the observation period (n=47), and those who stayed anti-HIV antibody negative at the end of the study (n=71). The researchers found that those who were initially anti-HIV antibody negative were more likely to seroconvert if they smoked (p=0.03), the study also found that there was no difference between smoking status and the progression to AIDS (p=0.31) [5]. Studies looking at the association of tobacco smoking and the progression to AIDS show a different outcome. Nine published studies were found that tested whether smoking increased the time it took patients to progress to AIDS [5-13]. However, most of these studies were done over a short period of time in more developed countries, and were performed before the use of antiretroviral therapies were the standard care in these cases. Due to this it may be the case that smoking does not have an effect in the development of

AIDS, which may be because the immune mechanisms that smoking alters are more relevant in acquiring HIV infection rather than in the progression to AIDS [14].

Since the first use of Highly Active Anti-Retroviral Therapy (HAART) in 1996, studies have shown a 60%-80% decrease in new AIDS illnesses. This type of aggressive treatment is used to suppress the replication of HIV, and therefore the progression of the disease. HAART therapy uses a combination of three different drugs, of which can include Protease Inhibitors (PI), Nucleoside Reverse Transcriptase Inhibitors (NRTI's), and Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI's).

Protease inhibitors prevent viral replication by inhibiting the activity of HIV-1 protease which is essential in the life cycle of the virus. HIV-1 protease cleaves viral polyproteins to make mature viral proteins needed to form a fully infectious HIV virion. Protease inhibitors are peptidomimetic and contain a non-scissile hydroxyethylene bond (-CH₂-CHOH-) rather than a normal hydrolysable peptide bond (-CO-NH-), and bind in the active pocket of the HIV-1 protease enzyme which will inactivate them preventing cleavage of the HIV polyproteins. When the HIV-1 protease is stopped the virions are unable to replicate and infect more cells.

Nucleoside reverse transcriptase inhibitors are competitive inhibitors and work by introducing faulty versions of nucleotides that the reverse transcriptase of HIV uses in the conversion of viral RNA into DNA. There are 3 phosphorylation steps that convert the 2'3'-dideoxynucleoside into its 5'-triphosphate derivative. Due to the absence of the 5'-OH group in NRTI's when it is incorporated into the forming DNA strand it will act as a chain terminator in the reverse transcription reaction.

Non-Nucleoside Reverse Transcriptase Inhibitors act as a non-competitive inhibitor of reverse transcriptase. This class of drugs allosterically binds a hydrophobic pocket of the enzyme close to the catalytic site causing a conformational change in the structure of the enzyme. The binding of the NNRTI affects the catalytic activity and blocks HIV-1 replication by inhibiting the ability of the reverse transcriptase to transcribe the viral RNA. When the NNRTI binds the hydrophobic pocket, it causes the p66 domain to become hyper extended due to the rotamer conformation of amino acid residues Tyr-181, and Tyr-188 [15].

HAART can be very effective in the treatment of HIV and has even been proven to reduce the amount of active virus so it is undetectable by blood testing techniques. However, some of the benefits provided by HAART can be severely decreased in those who smoke. Feldman et. al. assessed the association of cigarette smoking with the effectiveness of HAART in low income women. The study included 400 women who were nonsmokers and 524 women who smoke cigarettes all of which were on an HAART regimen, and lasted up to 7.9 years. The study looked at seven variables in HAART women who were smokers or nonsmokers. The variables were viral response (HIV-1 RNA levels dropped to <80 copies/ml), viral rebound (HIV-1 RNA increased to >1000 copies/ml after initial viral response), immunologic response (time for CD4+ lymphocyte counts to increase by 100 cells beyond pre-HAART levels), immunologic failure (CD4+ lymphocyte counts dropped below pre-HAART levels), AIDS incidence, total deaths, and death from AIDS. The results indicated that smokers using HAART had poorer viral responses, poorer immunologic responses, a greater risk of viral rebound, and more

frequent immunologic failure than nonsmokers on HAART. Smokers on HAART also had a higher risk of death and higher risk of developing AIDS than nonsmokers on HAART. In fact only one variable, risk of death due to AIDS, had no significant difference between smokers and nonsmokers [16]. The data suggests that treatment of HIV positive individuals with HAART will be less effective in those who smoke cigarettes than those who do not.

In trying to determine the biochemical association between tobacco smoking and the increase in HIV infectivity, most of the research has focused on epidemiological studies rather than the molecular interactions between the two. In order to rectify this, this research was aimed at filling this gap by studying the interactions between tobacco smoke and HIV infection at a cellular and genetic level.

Preliminary studies show that an aqueous tobacco smoke extract (TSE) used at nicotine concentrations consistent with plasma nicotine concentrations found in smokers, can enhance HIV infectivity. Although, nicotine alone has no effect on HIV infectivity at this concentration level. The studies that will be proposed here will explore the ramifications of the discovery that water soluble components of tobacco smoke, other than nicotine, are able to induce up to 100% increase in the level of HIV production by T-cells in vitro. The proposal will try to deduce the cellular genes and mechanisms behind this increase in HIV production. This could potentially reveal a novel viral activation mechanism, for which inhibitors could be discovered, leading to a new way to possibly inhibit the spread of HIV.

In order to elucidate the cellular genes, pathways, and biochemical mechanisms involved in HIV stimulation by TSE, the specific aims were to:

1. Use micro arrays to assess the effect of tobacco smoke extract on the genetic expression profile of Human T-cells.
2. Confirm results using quantitative real-time PCR analysis of the expression of specific genes that were identified by the micro array.
3. For the genes that are up-regulated as a result of tobacco smoke extract, siRNA will be used to silence the expression of that particular gene in human T-cell's. If the knockdown of this gene causes a decrease in HIV production, this would indicate a role for that gene in the stimulation of HIV infectivity resulting from overexpression due to the tobacco smoke extract.
4. For the genes that are down-regulated as a result of tobacco smoke extract, siRNA will be used to silence the expression of that particular gene. If the knockdown in expression of that gene in turn mimics the effect seen with the tobacco smoke extract, by leading to an increase in HIV production, this would indicate a role for that gene in the stimulation of HIV infectivity by tobacco smoke extract.
5. In order to determine if there is a combinatorial effect caused by gene expression levels on HIV production, siRNA will be used to knockdown the expression of two genes simultaneously in human T-cells. HIV production levels will then be compared to the levels in cells with only a single gene altered.

CHAPTER II
PRELIMINARY STUDIES

Preparation of Aqueous Tobacco Smoke Extract (TSE)

A side arm 125ml Erlenmeyer flask was used and 40ml sterile PBS at room temperature was added to the flask. A 10ml pipette was inserted into a rubber stopper which capped the flask so that the end of the pipette was submerged into the PBS. 5 Natural American Spirit cigarettes (Blue Pack: Full-Bodied Taste, manufactured by Santa Fe Natural Tobacco Company) were inserted into the end of the pipette with the filter still attached. The purpose for using American Spirit cigarettes is to rule out the possibility of a chemical additive from certain cigarettes as the constituent that enhances HIV infectivity. American Spirit cigarettes are all natural and are made with no additives in the production process. The cigarette was lit and vacuum was applied to the side arm, pulling the cigarette smoke through the PBS. The nicotine concentration in the TSE using this method was 100 μ M, measured by the bromination of nicotine method [17]. This TSE solution will be used throughout all of the studies. When diluted 100-fold in culture media (10 μ l in 1ml) it gives a working nicotine concentration of 1 μ M. Plasma nicotine levels of smokers measured one hour after smoking average around 0.1 μ M, with some individuals being as high as 0.25 μ M[18]. This solution can be refrigerated for a

couple months and it does not lose any of its potency or the ability to stimulate HIV replication.

Use of TMZ-bl (JC53) Cell Line as Quantitative Comparison of Viral Titer

The T2M-bl cell line is from the NIH AIDS Research and Reference Reagent Program (Catalog #8129) and is used to measure HIV replication levels. The T2M-bl line comes from the JC53 HeLa cervical cancer cell line, which has been engineered to express the CD4 HIV receptor and the CCR5 HIV co-receptor so that it can be infected by the virus (JC53-bl). Then the cell line was further modified by introducing separate integrated copies of the luciferase and b-galactosidase genes, which are controlled by the HIV-1 long terminal repeat (LTR) promoter/enhancer. The T2M-bl cell line is readily infected by HIV and produces luciferase in proportion to the amount of replicating HIV.

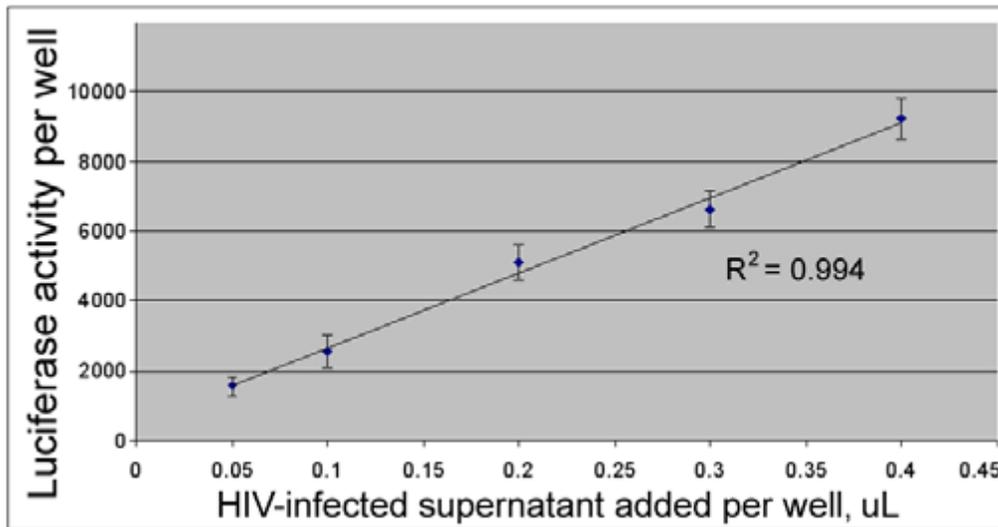


Figure 1. Standard Curve for Luciferase Activity Based on Concentration of HIV Added. Increasing the dose of viral stock solution increases luciferase production in a linear direction, indicating the usefulness of this assay in measuring HIV replication.

TSE Does Not Activate HIV LTR Signaling

In order to use the TZM-bl cell line as an effective measurement of viral titers as a result of incubation with TSE, it needed to be determined if TSE alone will activate the HIV LTR enhancer region. The expression vector pHIV-lacZ (NIH AIDS Research and Reference Reagent program Catalog #151) was used to construct a vector which had four copies of the NF- κ B site in the HIV LTR region. Hela cells were plated in a 24-well plate at a concentration of 8.0×10^4 cells/well and were transfected with the vector and pGL-2 (a transfection efficiency control) and treated with PBS, TSE ($2 \mu\text{l}/1 \text{ml}$ cells), or TNF- α , used as a positive control.

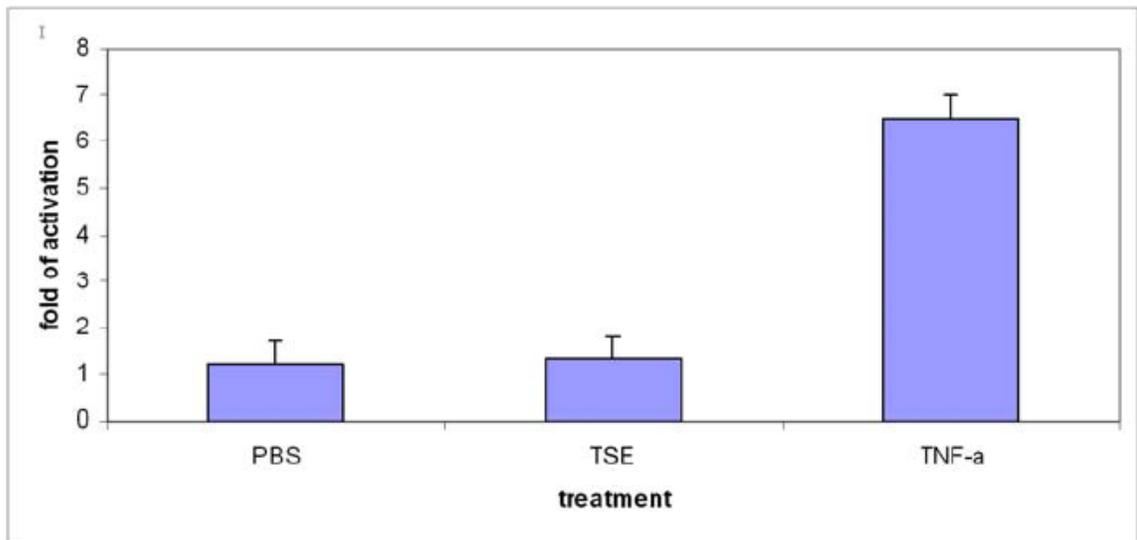


Figure 2. TSE Does Not Activate the HIV LTR. Activation of the HIV LTR by the negative control (PBS) and TSE are the same, indicating that TSE dose not cause activation of the HIV LTR. The positive control (TNF- α) shows activation due to the four copies of the NF- κ B site in the HIV LTR of the expression vector transfected into the cells.

Nicotine Ruled Out as the Constituent Responsible for the Enhancement of HIV Infectivity

Previous research has shown that nicotine can stimulate the production of HIV by in vitro infected alveolar macrophages [19], and the pretreatment of microglia with nicotine (300 μ M) would also stimulate HIV production [20]. However, nicotine concentrations at the level tested (300 μ M), would not be found in the plasma of living humans which have a maximum nicotine concentration of 0.25 μ M an hour after smoking. To this end the HIV infectivity enhancement of nicotine at normal levels was tested. TZM-bl cells were plated onto a 96-well plate at a concentration of 1.2×10^4 cells/well, the cells were then incubated with increasing doses of TSE (0.075 μ l-2.5 μ l/100 μ l cells), or

increasing concentrations of nicotine alone 0.075 μ M-2.5 μ M. The cells were then infected with HIV-1 (MOI=0.1). 48 hours post infection, the luciferase activity was measured. At doses of TSE as low as 0.15 μ lTSE/100 μ l cells there is an increase in HIV infectivity, which nicotine alone shows no increase at normal human plasma concentrations.

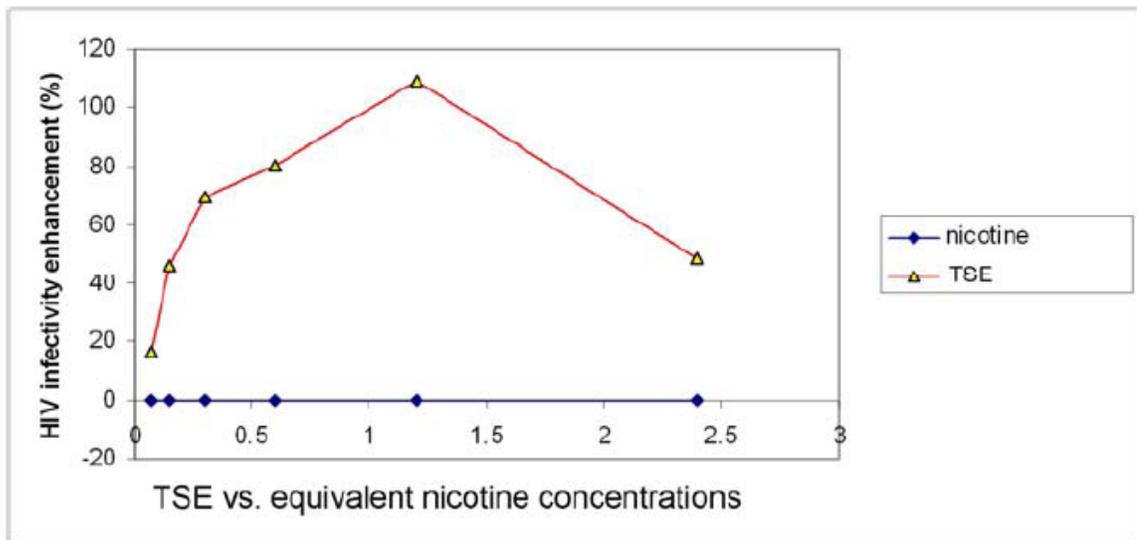


Figure 3. Nicotine Alone is Not Responsible for the Stimulation of HIV Infectivity As Seen when Treated with TSE. TZM-bl luciferase activity was measured to determine if nicotine alone increased HIV infectivity. It should be noted that because the measured concentration of nicotine in TSE is 100 μ M, 1 μ L of TSE added to 100 μ L media is a 100-fold dilution, thus equivalent to 1 μ M nicotine.

CHAPTER III
CELL TOXICITY OF TSE

Use of MTT Assay to Measure Cell Proliferation

Cells are plated on a 96-well plate at a concentration of 1.2×10^4 cells/well and incubated at 37 deg. C and 5% CO₂. After 48 hours 20 μ l of MTT reagent (Thiazolyl Blue Tetrazolium Bromide) is added to each well. The plate is placed on a shaking table at 150 rpm's for 5 minutes to ensure mixing of MTT into the media. The plate is then incubated at 37 deg. C, 5% CO₂ for 3-4 hours to allow MTT to be metabolized by the cells. After incubation the metabolic product of MTT, formazan, produced by the living cells will have formed and appear as dark purple crystals in the bottom of the plate. 200 μ l of DMSO is added to each of the wells and placed on the shaking table at 150 rpm's for 5 minutes in order to dissolve the crystals. The plates are then wrapped in foil and placed in a dark room at room temperature for 6 hours. The plates are then read on a micro plate reader with the optical density at 450nm measured and the background of 550nm subtracted from the reading.

Toxicity of TSE on TZM-bl Cells

TZM-bl cells were plated on a 96-well plate at a concentration of 1.2×10^4 cells/well and incubated at differing concentrations of TSE that range from 0.15 μ l-10 μ l

per 200 μ l growth media. Forty-eight hours after incubation, cell proliferation was measured using the MTT method. At about 1 μ l TSE/200 μ l cells there was a ~ 20% increase in proliferation. At 5 μ l TSE/200 μ l cells and above there is an anti-proliferative effect. The data shown is from three separate experiments which all show similar results. Based on this, no more than a 100 fold dilution 2 μ lTSE/200 μ l cells will be used in the experiments to stimulate HIV production.

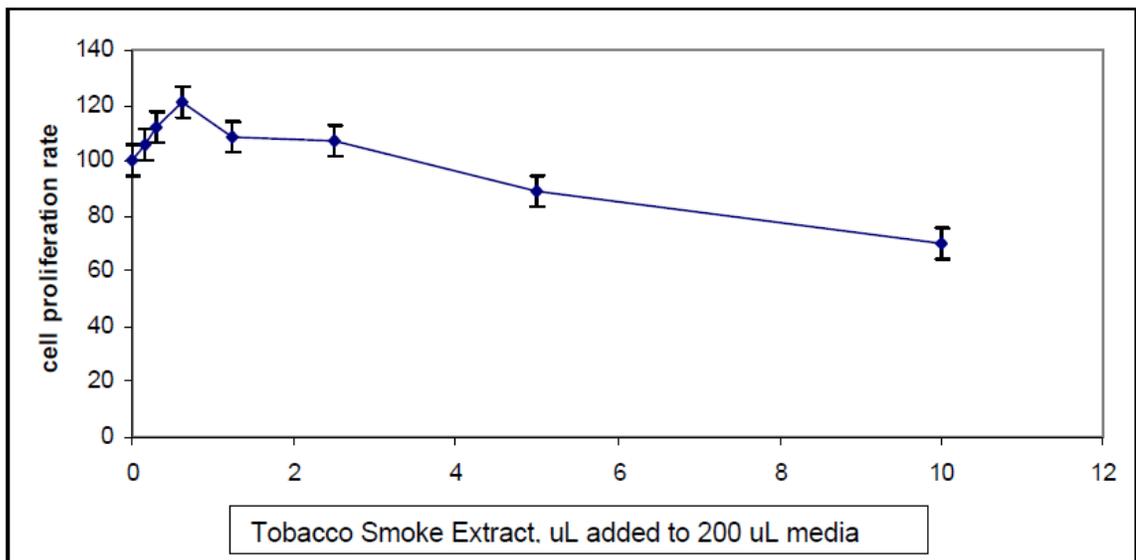


Figure 4. Effect of TSE on Cell Proliferation Using TZM-bl Cells. TSE shows an increase in cell proliferation at 2.25 μ lTSE/200 μ l cells and less. Below 5 μ lTSE/200 μ l cells there is a decrease in proliferation due to the toxicity of the TSE.

Use of Calcein AM and EthD-1 to Measure Cell Viability in the Presence of TSE

Calcein acetoxymethyl ester (Calcein AM) is a hydrophilic compound that easily permeates intact live cells and is non-fluorescent. Upon permeating a live cell it is hydrolyzed by intercellular esterases turning it into Calcein, a strongly fluorescent, hydrophilic compound that is well retained in the cell cytoplasm and not influenced by cellular pH [21,22]. Calcein AM was used to measure toxicity levels in Jurkat cells at different concentrations of TSE.

Ethidium homodimer-1(EthD-1) is a weakly fluorescent compound that enters cells with a damaged membrane and upon binding to nucleic acids becomes highly fluorescent. EthD-1 cannot enter intact cells through the plasma membrane thereby causing no fluorescent signal in living cells. EthD-1 was used to measure the dead or dying Jurkat cells as a result of different concentrations of TSE.

Toxicity of TSE on Jurkat Cells

Jurkat cells were mixed with 1xPBS at a concentration of 1.0×10^6 cells/ml, then 50ul of cell solution was added to a 96well plate (5.0×10^4 cells/well). TSE solution was added to wells in the following amounts, 0.1ul, 0.2ul, 0.3ul, 0.4ul, 0.5ul, 1.0ul, 1.5ul, 2.0ul, 2.5ul, and no TSE was used as the positive control. 70% EtOH was added as a negative control. The plates were then incubated for 6 hours at 37°C and 5.0% CO₂. Dye solutions were prepared according to manufactures recommendations, 2uM Calcein AM,

and 4 μ M EthD-1 both diluted in 1xPBS. Following the 6 hour cell incubation 50ul of the diluted dye solution was added to each well. Fluorescence was measured using the Biotek fluorescence microplate reader. Calcein was measured at ex322nm/em435nm, and EthD-1 measured at ex528nm/em617nm.

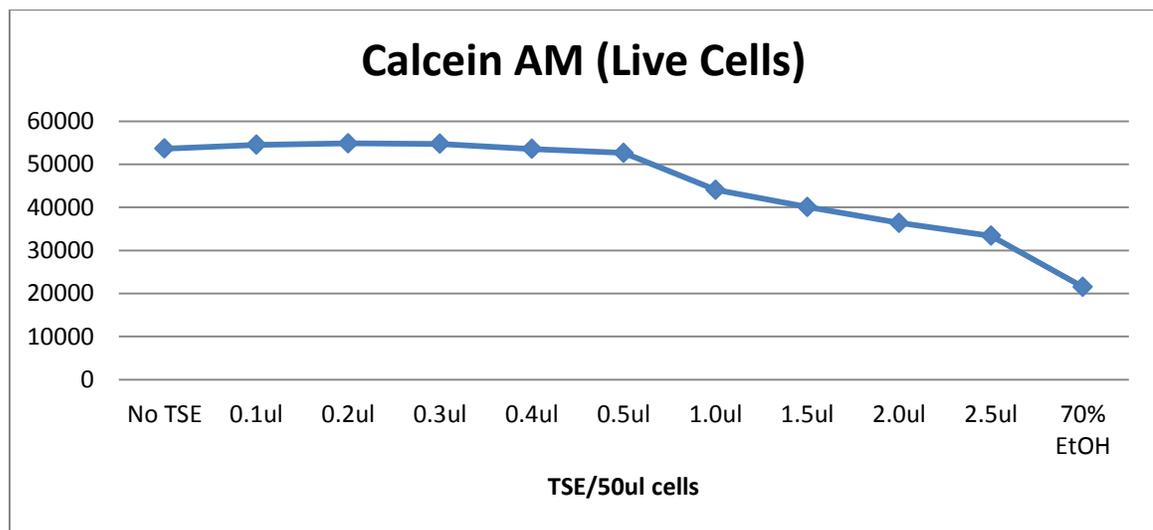


Figure 5. Calcein Fluoresce Levels in Jurkat Cells with Increasing Concentrations of TSE

Jurkat cell viability was measured using Calcein AM with increasing concentrations of TSE. TSE dilutions above 0.5ul/50ul cells (100 fold TSE dilution) cause a steady decrease in the number of live cells in the sample as seen by the decrease in Calcein fluorescence.

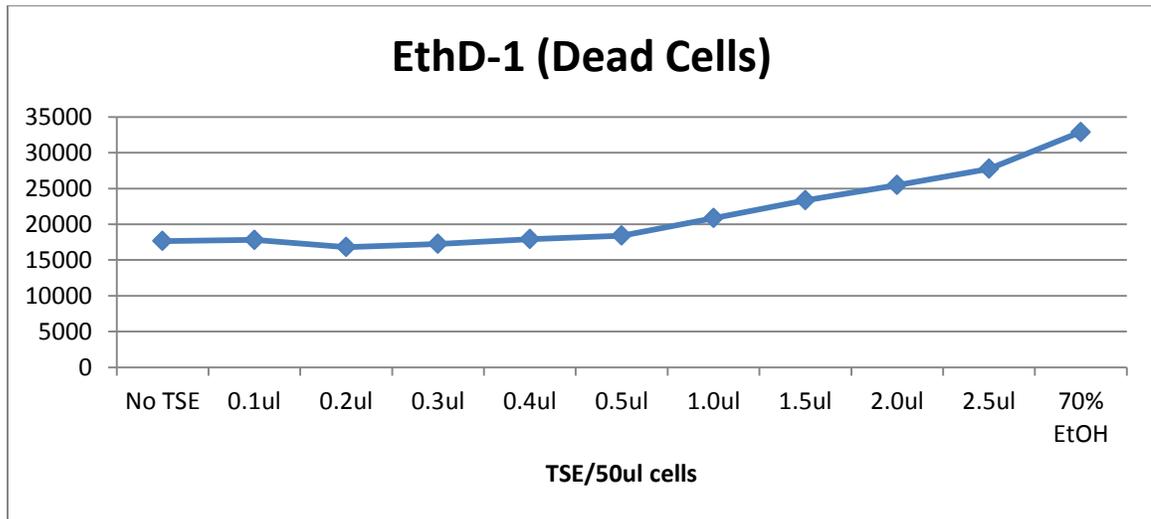


Figure 6. Ethidium Homodimer-1(EthD-1) Fluorescence with Increasing Concentrations of TSE.

EthD-1 becomes highly fluorescent when bound to nucleic acids once inside dead or dying cells with compromised cell membranes. At concentrations above 0.5ulTSE/50ul cells (100 fold TSE dilution) there is a steady increase in the number of dead Jurkat cells as seen by the increase in EthD-1 fluorescence.

The Calcein AM assay indicates a decrease in live Jurkat cells at TSE concentrations of 1.0ul TSE/50ul cells and greater. The EthD-1 assay shows an increase in the number of dead Jurkat cells at TSE concentrations of 1.0ulTSE/50ul cells and greater. Based on this, similar to the TZM-bl cells, no more than a 100-fold dilution of TSE will be used in the stimulation of HIV production in Jurkat cells.

CHAPTER IV

EFFECT OF TSE ON HIV INFECTIVITY

Enhancement of HIV Infectivity by TSE in TZM-bl Cells

TZM-bl cells were plated in a 96-well plate at a concentration of 1.2×10^4 cells/well and incubated with increasing concentrations of TSE (0.15 μ l-10 μ lTSE/200 μ l cells). The cells were then infected with HIV-1, multiplicity of infection - MOI= 0.1 (1 infectious viral particle per 10 cells). 48 hours post infection, luciferase activity was measured. The results show the % HIV infectivity enhancement based on the amount of TSE added to each well. There is an increase in HIV infectivity up to a concentration of 5 μ lTSE/200 μ l cells, with the highest value exceeding 100% increase at 2.5 μ lTSE/200 μ l cells. Beyond 5 μ lTSE/200 μ l cells there is a decline due to the cellular toxicity effect of the TSE on the TZM-bl cells. The data is the average of three separate experiments.

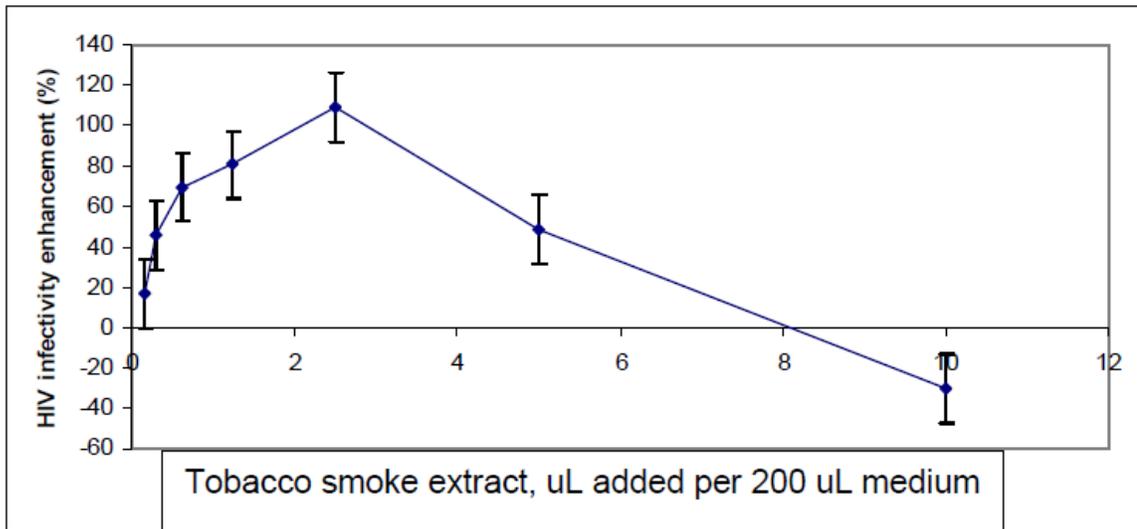


Figure 7. HIV Infectivity Enhancement Control Based on the Concentration of TSE Added to Each Well. TSE concentrations from 0.15 μ L-10 μ L TSE/200 μ L cells were used. TZM-bl luciferase values were measured and reported as % enhancement over control. The % enhancement decline beyond 5 μ L TSE/200 μ L cells is due to the cellular toxicity of the TSE on the TZM-bl cells.

Enhancement of HIV Infectivity by TSE in Jurkat T-cells

Human T-cells (Jurkat) were plated on a 24-well plate at a concentration of 1.0×10^6 cells/well, then were infected with HIV-1 (MOI=0.1). The cells were then treated with TSE (2 μ L/200 μ L cells), or sterile PBS (2 μ L/200 μ L cells) as a control. 48 hours post infection 100 μ L of culture supernatant was added to TZM-bl cells to test for relative viral titers. Luciferase activity from TZM-bl cells was measured and HIV production in the T-cells treated with TSE was increased by 51% compared to T-cells treated with PBS.

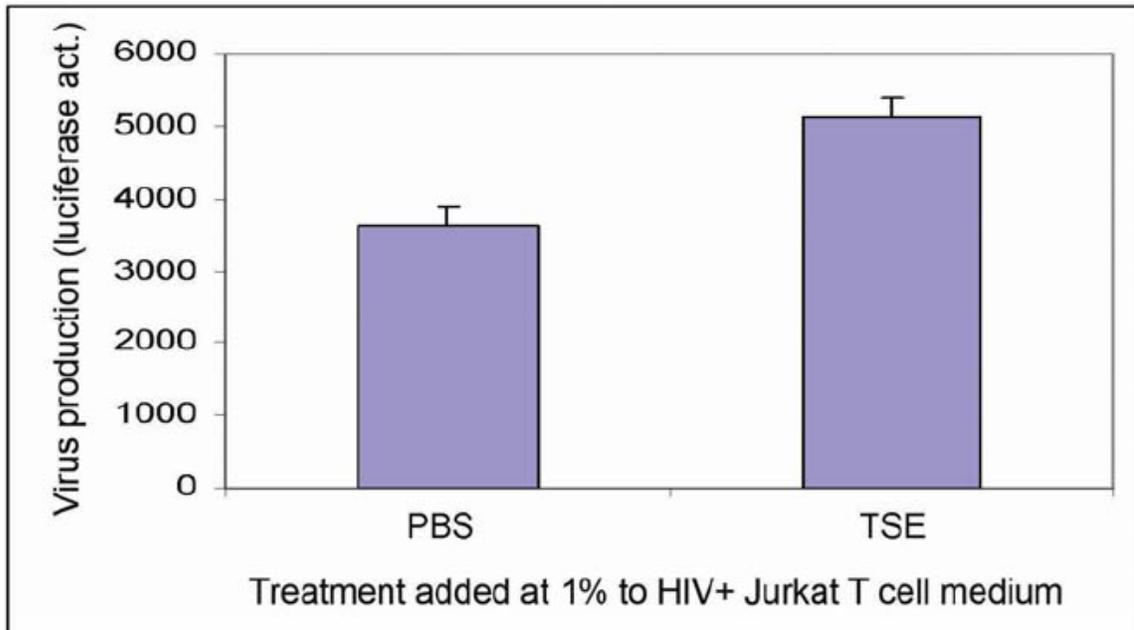


Figure 8. HIV Stimulation in Human T-cells Treated with TSE or PBS (control).

TZM-bl luciferase activity was measured following the addition of 100 μ l of supernatants from Jurkat cell cultures treated with TSE or PBS following HIV infection. Cells treated with TSE show a 51% increase in HIV production over cells treated with PBS as measured by luciferase activity.

CHAPTER V

TSE INDUCED GENE EXPRESSION PATTERNS

Regulation of Cellular Gene Expression by TSE

Since human T-cells are the primary target of HIV, the gene expression profile from Jurkat cells was used instead of other cell types. Jurkat cells were treated with TSE or sterile PBS overnight, and then total cellular RNA was extracted and purified using the RNeasy Kit (Qiagen Valencia, CA). The samples were sent to the MicroArray Core at Wake Forest University, Winston-Salem, NC and run on an Affymetrix HG-U133 Plus 2.0 chips. The results showed that TSE affected the expression of more than 12 genes (mRNA) based on what was seen with the control PBS RNA. In order to narrow down the results, 12 genes were chosen that were all altered by more than 3 times that of the control set. Table 1 list the genes that were most significantly up-regulated by TSE. Table 2 list the genes that were most significantly down-regulated by TSE. Therefore, these are the 12 genes that will be focused on in the following studies.

Table 1. Up-Regulated Genes

Up-regulated Genes		
Gene Name	Gene Function	NCBI GenBank Accession Number
TAX1BP1	Indirectly binds with viral LTR	NM_006024
CD59, Protectin	Incorporates into newly formed HIV particles and protects the virus from compliment mediated destruction	NM_000611
Coatomer Protein Complex β -2	Essential for Golgi budding and vesicular trafficking	NM_004766
Thioredoxin-Related Transmembrane Protein 1	an adaptor protein involved in clathrin-mediated endocytosis and HIV-mediated down-regulation of CD4	NM_030755
Calmodulin 1	Binds HIV gp160 and mediates gp160-enhanced apoptosis	NM_006888
HSP90 beta 1	Responsible for P-TEFb-mediated Tat transactivation	NM_003299
Sjogren Syndrome Antigen B	Enhances HCV replication	NM_003142
DDX3	Required for HCV replication	NM_001356

Table 2. Down-Regulated Genes

Down-regulated Genes		
Gene Name	Gene Function	NCBI GenBank Accession Number
PHGPx	Over-expression of PHGPx will inhibit NF- κ B activation	NM_002085
MHC, HLA-E	Antigen presentation to NK cells. Down regulation will protect virus from MHC 1 cytotoxic T-lymphocytes	NM_005516
Stearoyl-CoA Desaturase	Required for the assembly of VLDL particles	AA_678241
Thioredoxin Interacting Protein	Binds and inhibits activity of thioredoxin	NM_006472

qRT-PCR Analysis of Gene Expression

Jurkat cells were plated on a 24well plate at a concentration of 1.0×10^6 cells/ml, 1ml of cells were added to each well along with 10ul TSE solution (100 fold dilution). Cells were then placed in an incubator at 37°C and 5% CO₂. Cells were collected at the following time points following incubation, 0 hour, 3 hours, 7 hours, 24 hours, 48 hours, and 72 hours. Upon collection, cells were pelleted and stored at -80°C. Cell pellets were then thawed and mRNA isolation was performed using Sigma GenElute Direct mRNA isolation kit (appendix). RNA samples were then quantified and all samples diluted to 100ng/ul. mRNA samples (200ng) were run through one reverse transcriptase cycle using random hexamers in preparation for real time PCR analysis. RT-PCR reaction was setup using manufactures recommendations (appendix). Sigma SYBR Green Jumpstart Taq was used along with appropriate primer set for each gene. The $\Delta\Delta C_t$ method was

performed using the ABI 7500 Fast PCR system, and β -actin was used as an endogenous control. GAPDH expression was also measured as a positive control. Negative controls containing no RT sample were also performed in tandem. Zero hour fluorescence reading was normalized to 1 on relative quantitation scale.

The RT-PCR cycle used was as follows:

95°C x 120secs	} 50x
95°C x 15secs	
55°C x 60secs	
Fluorescence read	

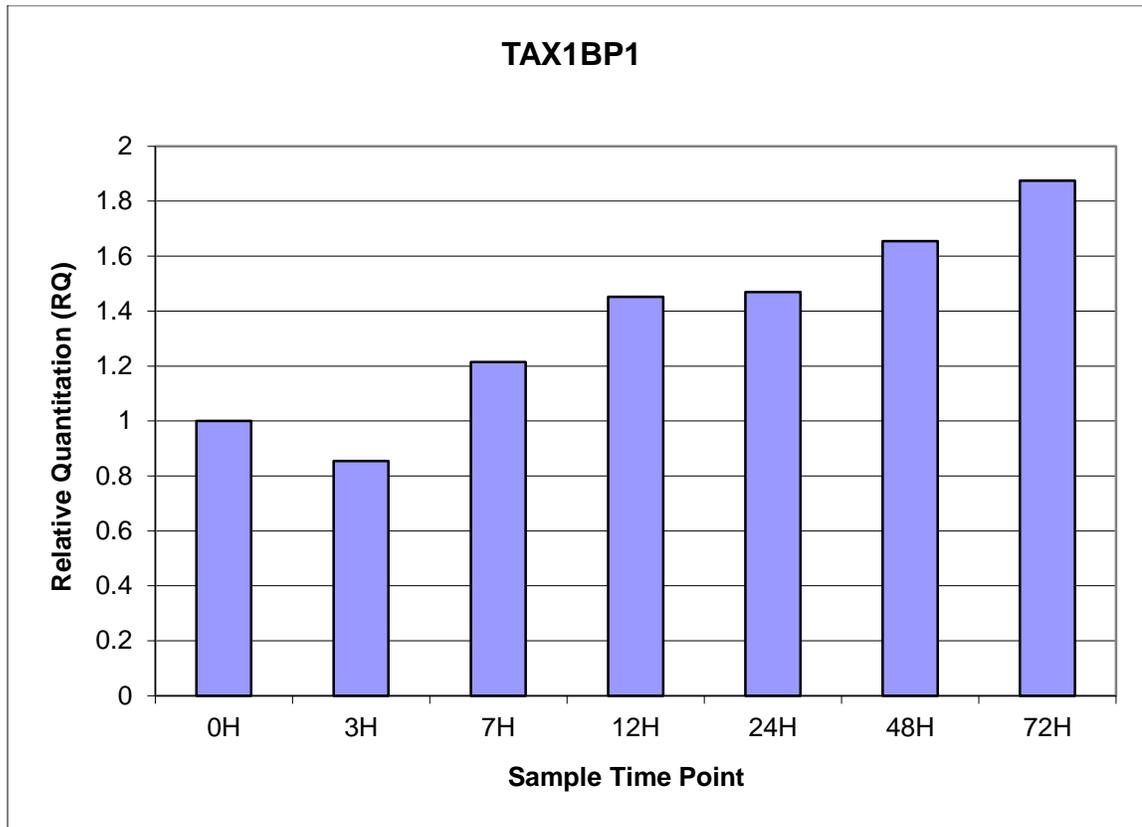


Figure 9. TAX1 Binding Protein 1 RNA Expression in Jurkat Cells after 1% TSE Incubation.

Seven hours post 1% TSE incubation in Jurkat cells, the expression of TAX1BP1 starts to increase up to a maximum of 1.87x (at the 72 hour time point) that of the expression levels without TSE.

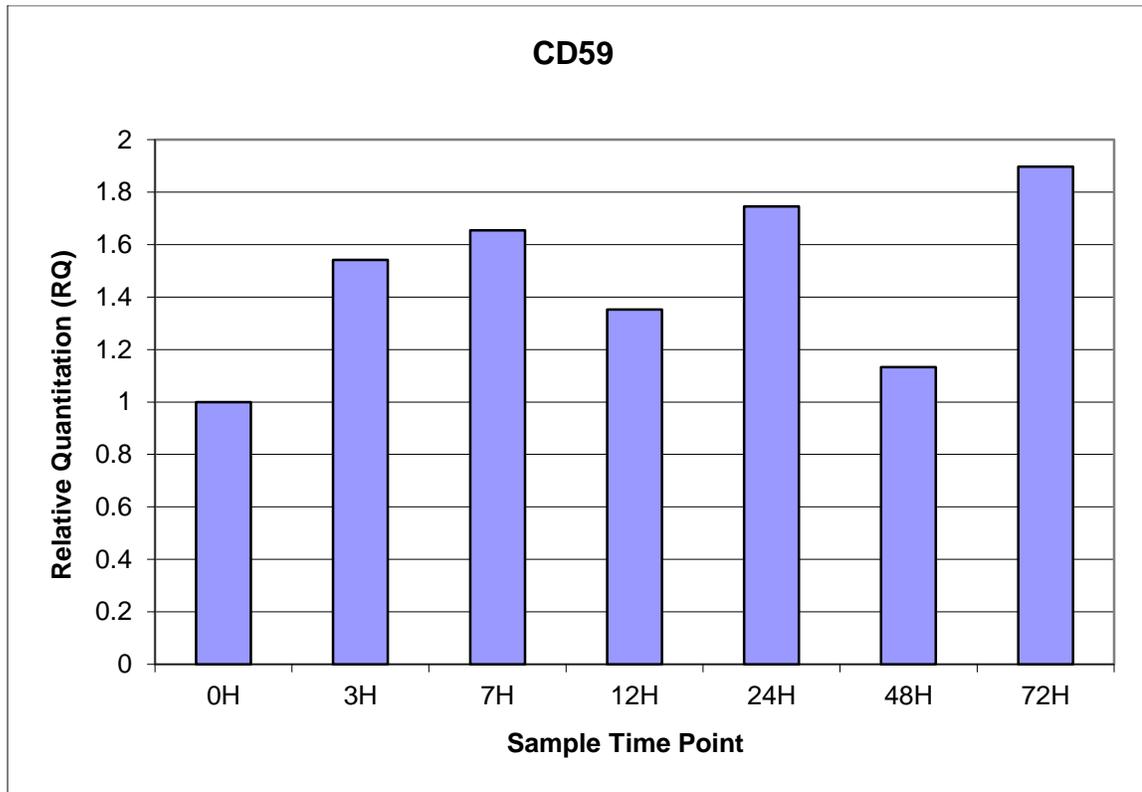


Figure 10. CD59 (Protectin) RNA Expression in Jurkat Cells after 1% TSE Incubation.

There is an increase in the expression of CD59 RNA following the incubation with TSE, the highest expression levels were seen at the 72 hour time point with levels of 1.90x greater than Jurkat expression without TSE.

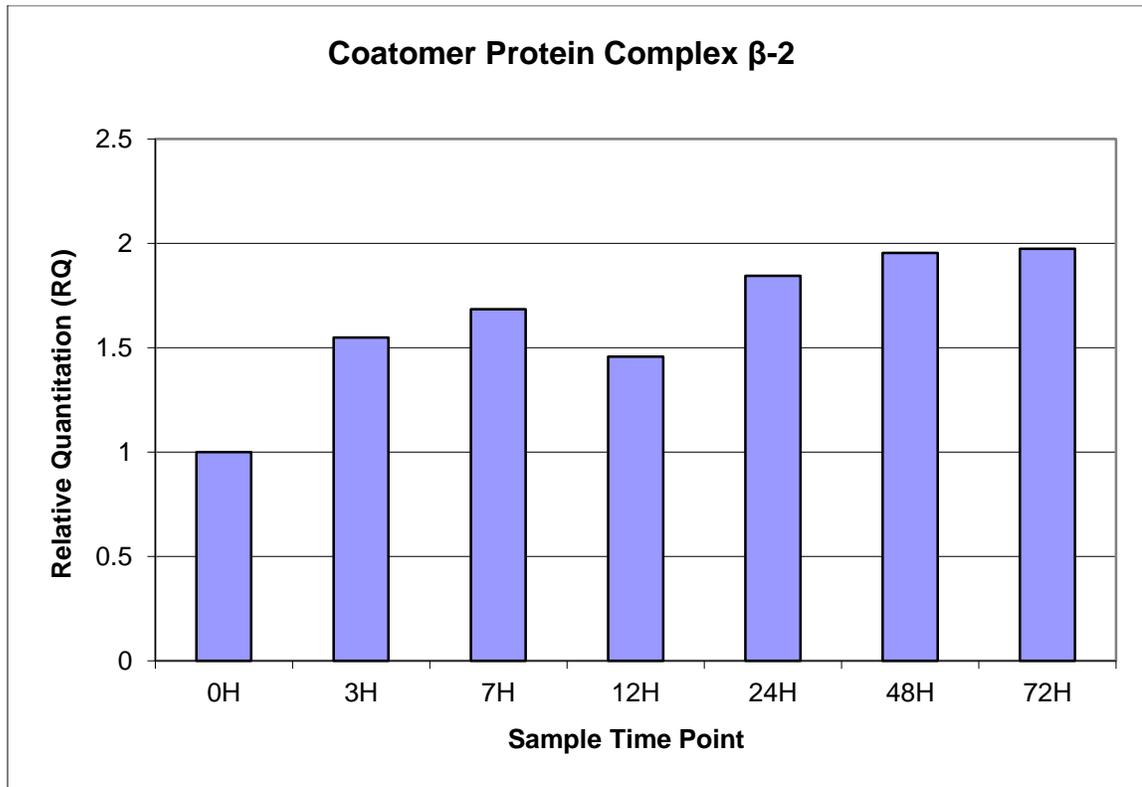


Figure 11. Coatomer Protein Complex β -2 RNA Expression in Jurkat Cells after 1% TSE Incubation.

There is an increase in the expression of coatomer protein complex β -2 RNA following the incubation with TSE, the highest expression levels were seen at the 72 hour time point with levels of 1.97x greater than Jurkat expression without TSE.

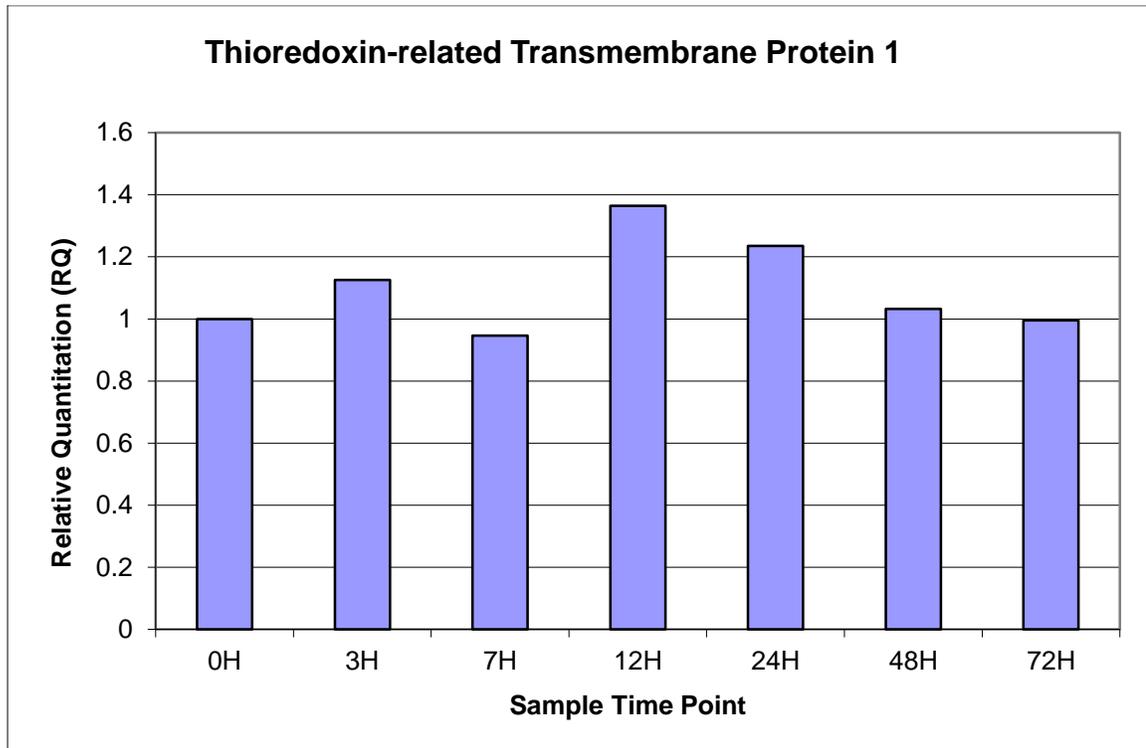


Figure 12. Thioredoxin-related Transmembrane Protein 1 RNA Expression in Jurkat Cells after 1% TSE Incubation.

There is an increase in the expression of thioredoxin-related transmembrane protein 1 RNA following the incubation with TSE, the highest expression levels were seen at the 12 hour time point with levels of 1.36x greater than Jurkat expression without TSE.

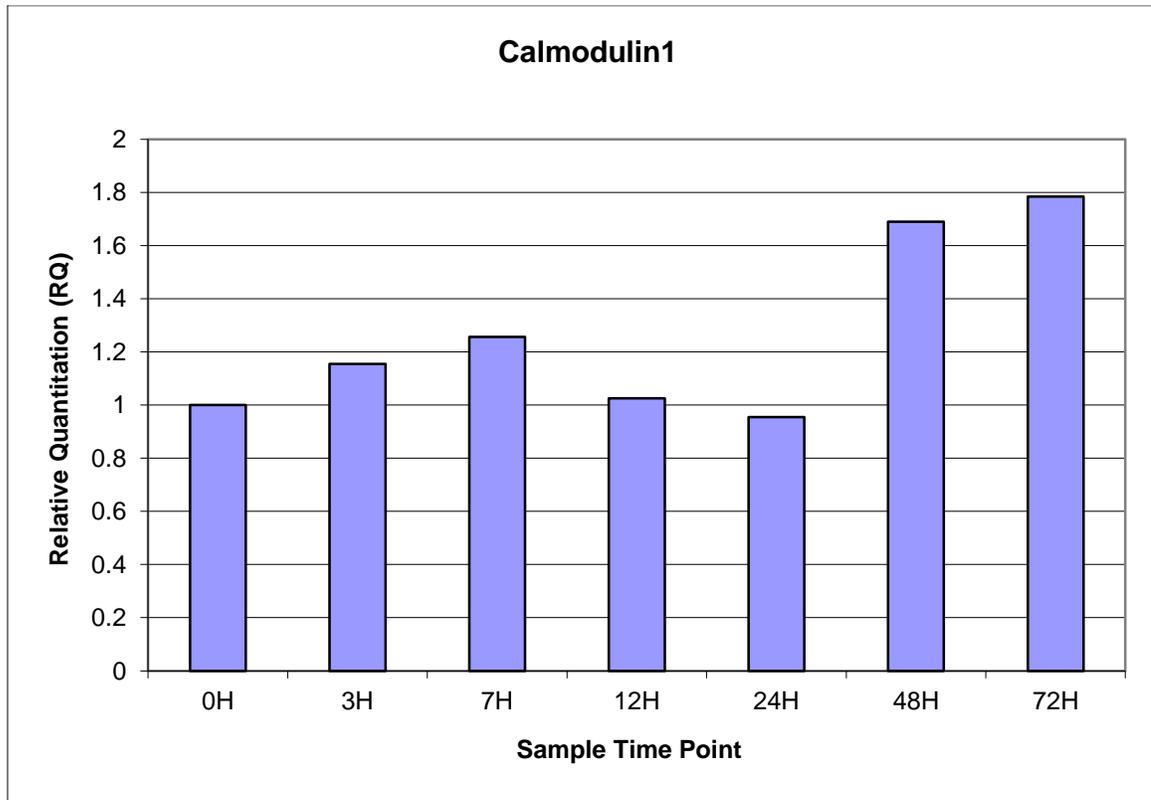


Figure 13. Calmodulin 1 RNA Expression Levels in Jurkat Cells after 1% TSE Incubation.

There is an increase in the expression of calmodulin 1 RNA following the incubation with TSE, the highest expression levels were seen at the 72 hour time point with levels of 1.78x greater than Jurkat expression without TSE.

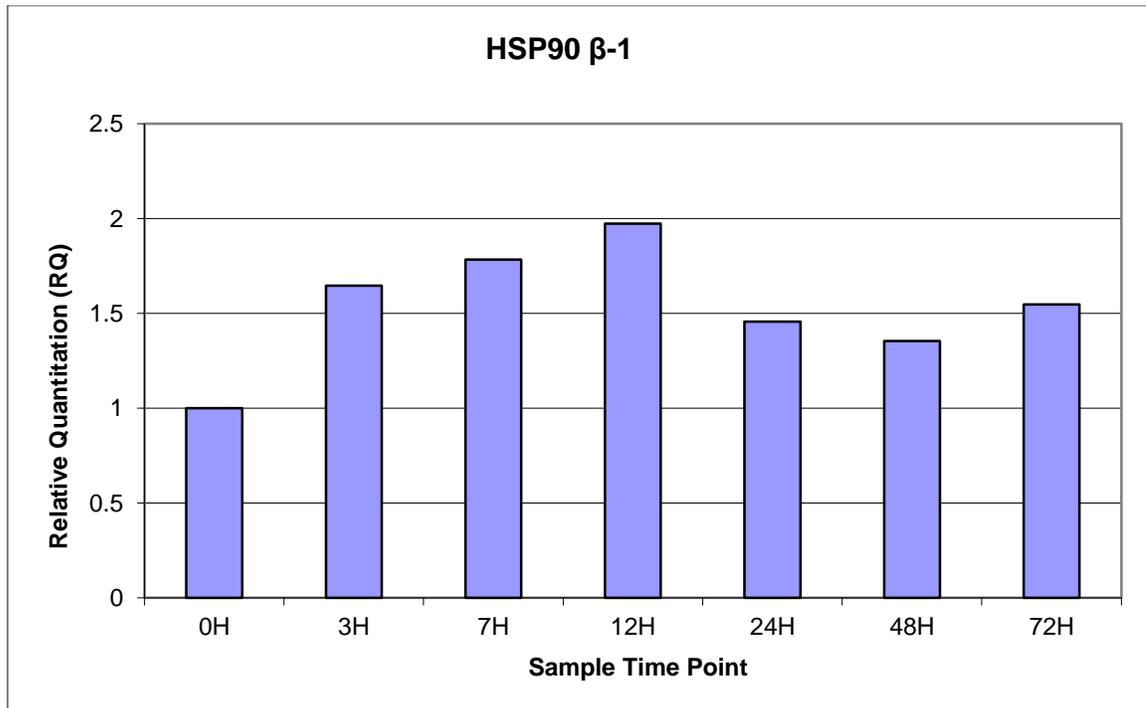


Figure 14. Heat Shock Protein 90 β -1 RNA Expression Levels in Jurkat Cells after 1% TSE Incubation.

There is an increase in the expression of HSP90 β -1 RNA following the incubation with TSE, the highest expression levels were seen at the 12 hour time point with levels of 1.97x greater than Jurkat expression without TSE.

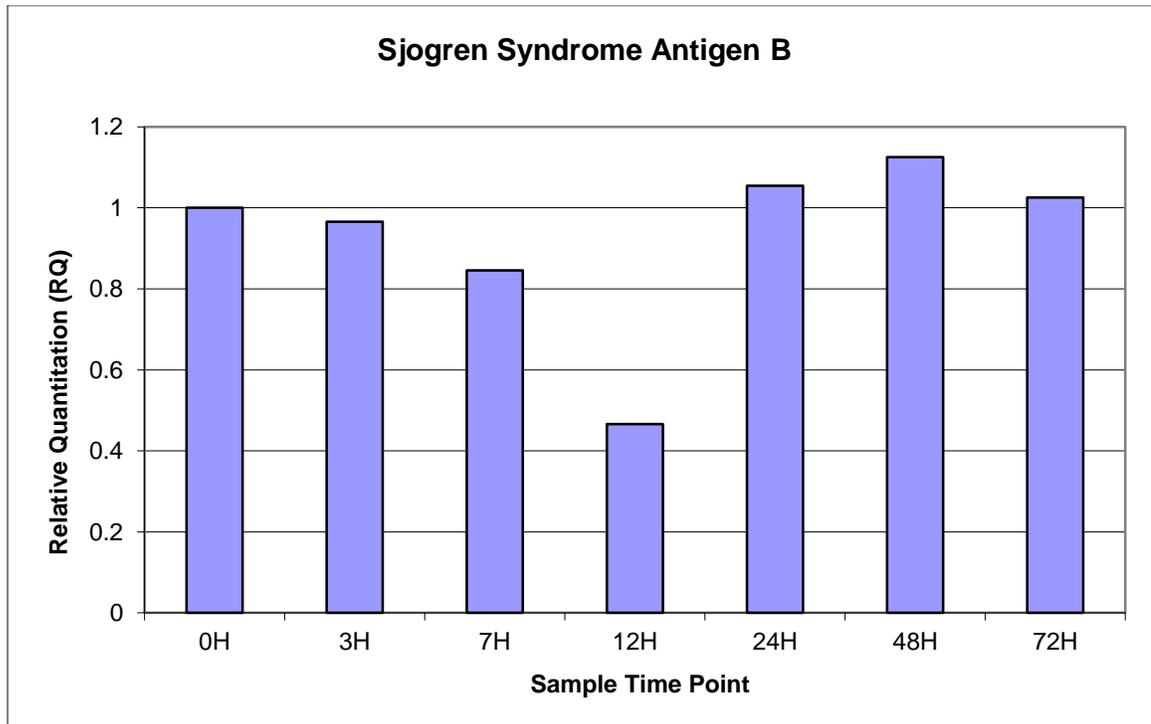


Figure 15. Sjogren Syndrome Antigen B RNA Expression Levels in Jurkat Cells after 1% TSE Incubation.

There is a slight increase in the expression of sjogren syndrome antigen B RNA following the incubation with TSE, the highest expression levels were seen at the 48 hour time point with levels of 1.13x greater than Jurkat expression without TSE.

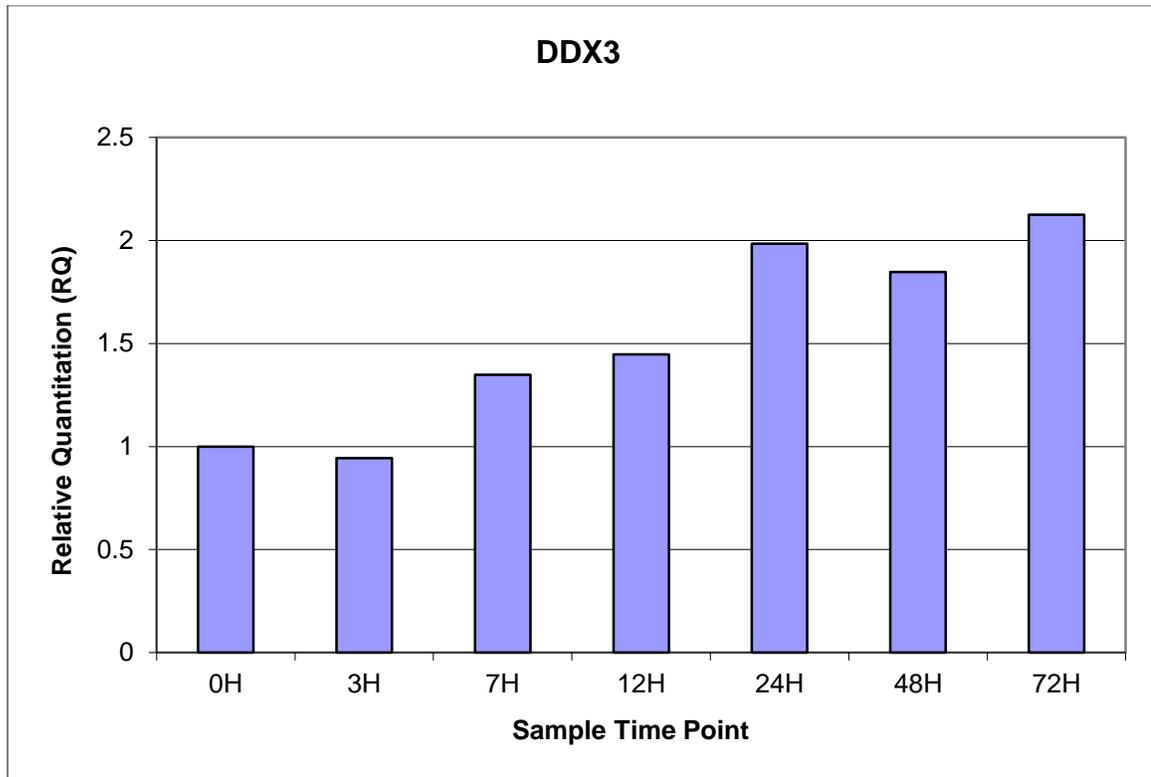


Figure 16. DEAD Box RNA Helicase 3 RNA Expression Levels in Jurkat Cells after 1% TSE Incubation.

There is an increase in the expression of DDX3 RNA following the incubation with TSE, the highest expression levels were seen at the 72 hour time point with levels of 2.13x greater than Jurkat expression without TSE.

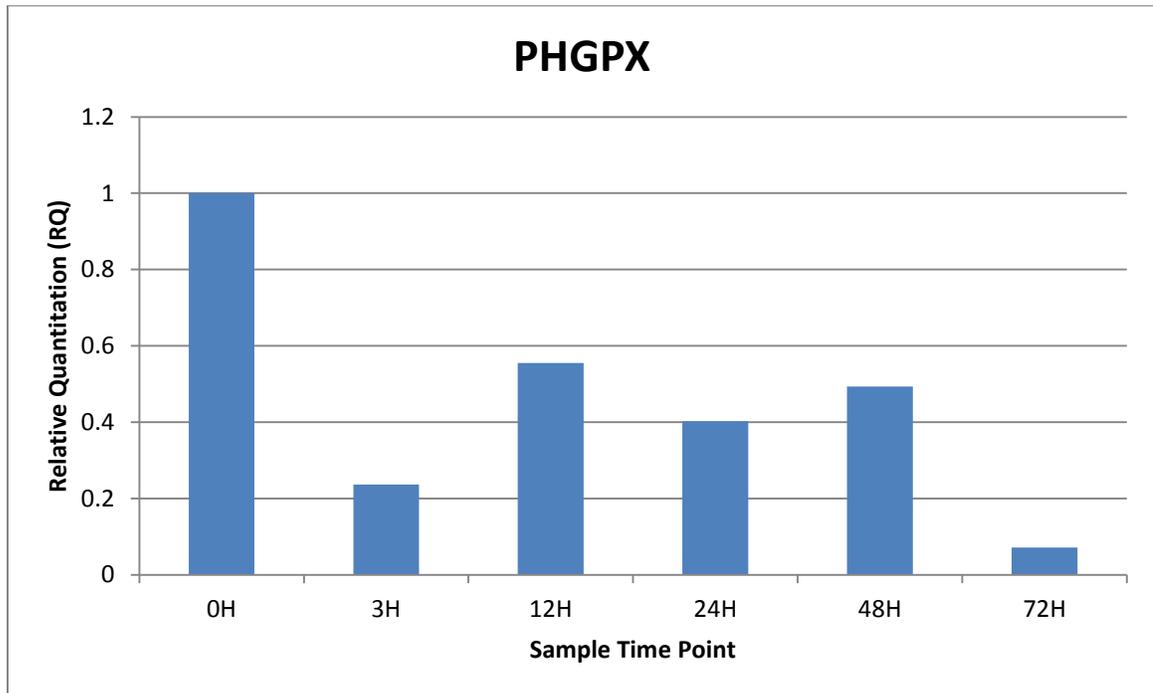


Figure 17. Phospholipid Hydroperoxide Glutathione Peroxidase RNA Expression Levels in Jurkat Cells after 1% TSE Incubation.

There is a decrease in the expression of PHGPx RNA following the incubation with TSE, the lowest expression levels were seen at the 72 hour time point with levels of 7% that of Jurkat expression without TSE.

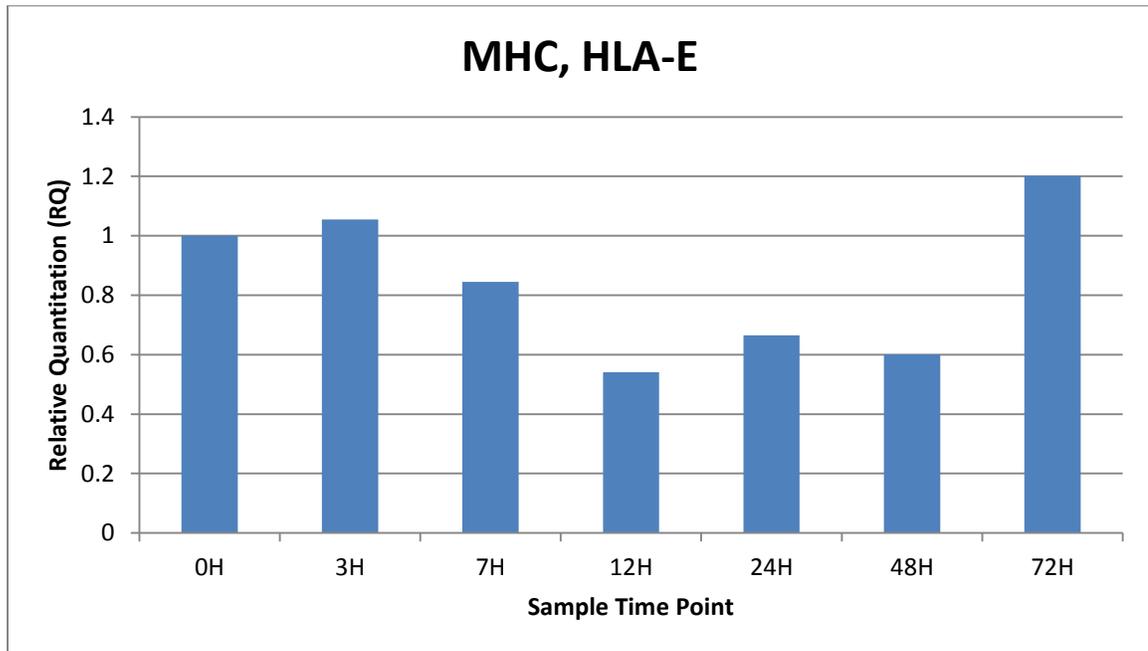


Figure 18. Human Leukocyte Antigen E RNA Expression Levels in Jurkat Cells after 1% TSE Incubation.

There is a decrease in the expression of HLA-E RNA following the incubation with TSE, the lowest expression levels were seen at the 12 hour time point with levels of 54% that of Jurkat expression without TSE.

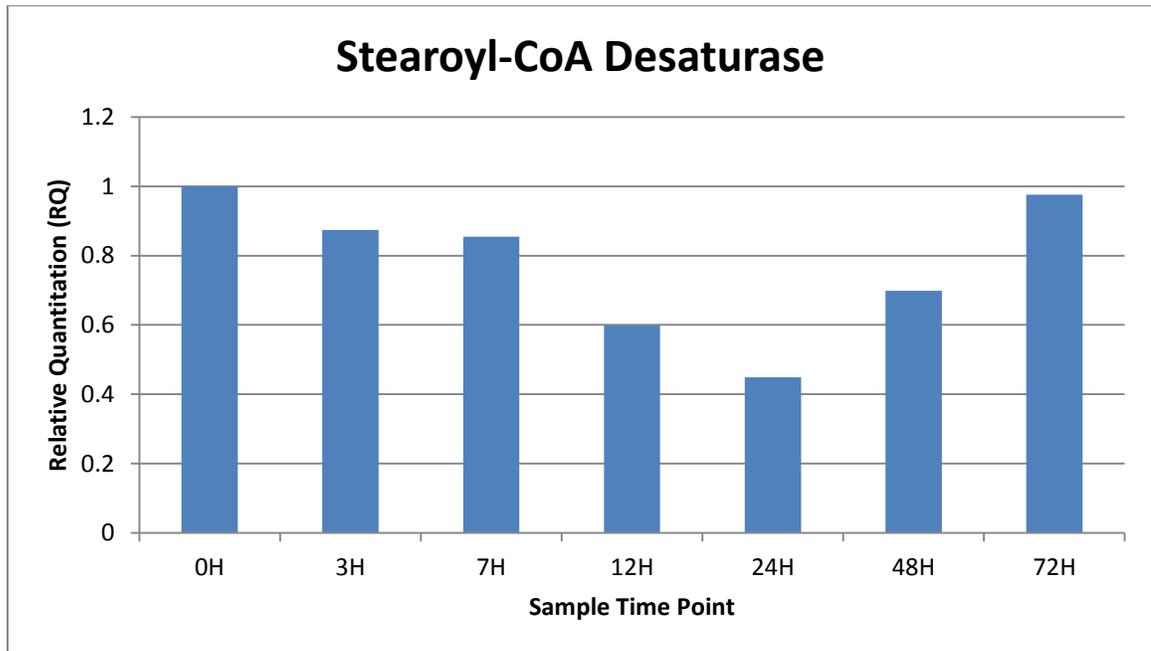


Figure 19. Stearoyl-CoA Desaturase RNA Expression Levels in Jurkat Cells after 1% TSE Incubation.

There is a decrease in the expression of stearoyl-CoA desaturase RNA following the incubation with TSE, the lowest expression levels were seen at the 24 hour time point with levels of 45% that of Jurkat expression without TSE.

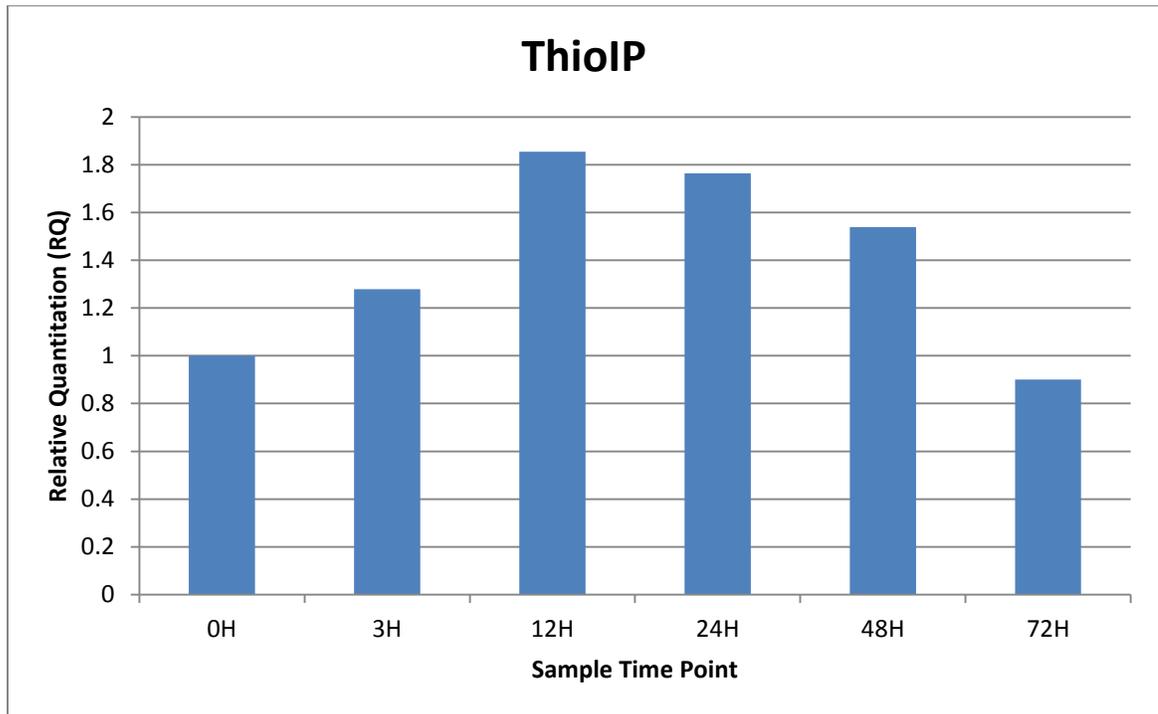


Figure 20. Thioredoxin Interacting Protein RNA Expression Levels in Jurkat Cells after 1% TSE Incubation.

There is an overall decrease in the expression of thioredoxin interacting protein RNA following the incubation with TSE, the lowest expression levels were seen at the 72 hour time point with levels of 90% that of Jurkat expression without TSE.

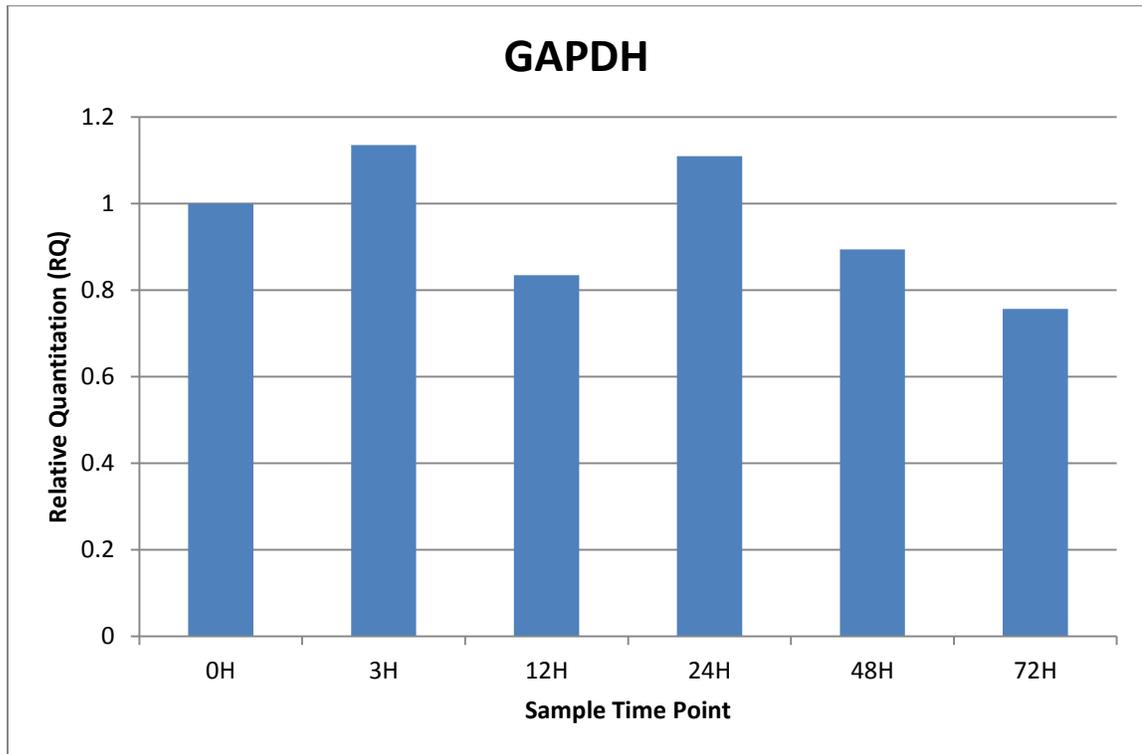


Figure 21. Glyceraldehyde 3-Phosphate Dehydrogenase RNA Expression in Jurkat Cells after 1% TSE Incubation.

GAPDH expression levels were used as a positive control for qRT-PCR assay.

CHAPTER VI

HIV INFECTIVITY DUE TO GENE KNOCKDOWN

Jurkat siRNA Transfection

Silencer Select pre-designed siRNAs (Ambion) targeting each of the 12 genes in question were used in transfection. Jurkat cells were diluted to a concentration of 5.0×10^5 cells/ml then 400ul of cells were added to wells of a 24 well cell culture plate (2.0×10^5 cells/well). Lipofectamine 2000 was used to transfect siRNA into cells. Transfection complexes were formed by adding 3ul of Lipofectamine 2000 to 47ul Opti-MEM I reduced serum media without serum. In a separate micro-centrifuge tube 10ul gene specific 0.5nM siRNA (final cell culture concentration = 10nM) was added to 40ul Opti-MEM I reduced serum media without serum. These two 50ul samples were then added together and allowed to sit for 5 minutes at room temperature. Following the 5 minute incubation 100ul of transfection complex solution was added to Jurkat cells. Cells were then incubated at 37°C and 5% CO₂ for 48 hours with new media exchanged at 24 hours post-transfection. Following incubation, cells were pelleted and stored at -80°C for RT-PCR analysis or growth media was exchanged in preparation for HIV infectivity assay.

qRT-PCR Analysis of Gene Knockdown

Cell pellets obtained in siRNA transfection were thawed and total RNA isolation was performed using Sigma RNA isolation kit. RNA samples were then quantified and

all samples diluted to 100ng/ul. Total RNA samples (200ng) were run through one reverse transcriptase cycle using random hexamers in preparation for real time PCR analysis. qRT-PCR reaction was setup using manufactures recommendations. Sigma Sybr Green Jumpstart Taq was used along with appropriate primer set for each gene. The $\Delta\Delta C_t$ method was performed using the ABI 7500 Fast PCR system, and β -actin and GAPDH as endogenous controls. Zero hour fluorescence reading was normalized to 1 on relative quantitation scale.

The qRT-PCR cycle used was as follows:

95°C x 120secs	} 50x
95°C x 15secs	
55°C x 60secs	
Fluorescence read	

HIV Infectivity as a Result of Gene Knockdown in Jurkat Cells

Forty-eight hours post transfection of jurkat cells, media was removed from cells and replaced with RPMI 1640 growth media. Cells were then infected with HIV-1 (MOI=0.1) and incubated at 37°C and 5% CO₂ for 48 hours. After the 48 hour incubation period, the jurkat cells were then pelleted and 100ul culture supernatant was added to 1.2 x 10⁴ cells/100ul TZM-bl cells plated in 96-well cell culture plate. The TZM-bl cells were incubated at 37°C and 5% CO₂ for 48 hours. Following the TZM-bl cell incubation, the growth media was removed from the wells containing cells. Cells attached to plate surface were washed with 1x PBS solution two times, then 20ul of 1X luciferase cell culture lysis reagent (Promega) was added to each well. The plate was rocked to ensure complete coverage of cell layer in each well. The plate was then incubated at 37°C and

5%CO₂ for 5 minutes. 100ul luciferase assay reagent was added to each well using multi-channel pipet to reduce pipetting time. The plate was immediately placed inside the Biotek luminescence microplate reader, and luminescence measured in each well.

TAX1BP1

Chin et. al. found that Tax1 binding protein 1 (TAX1BP1) interacts with the oncoprotein Tax, a transcriptional regulator from the Human T-cell leukemia virus type 1 (HTLV-1) [23]. They show that TAX1BP1 is a nuclear receptor coactivator that will colocalize with Tax to the nucleus, where it can bind to HTLV-1 long terminal repeat (LTR). The Viral LTR controls viral gene expression through the presence of enhancer, promoter, transcription initiation, and transcription termination sites. It is fair to say that if TAX1BP1 is overexpressed in cells treated by TSE, and that it can bind to the viral LTR, it may be possible that TAX1BP1 could have some role in activating HIV infectivity.



Figure 22. siRNA Induced TAX1 Binding Protein 1 mRNA Knockdown in Jurkat Cells.

Left to Right: TAX1 Binding Protein 1 mRNA levels in Jurkat control cells (no siRNA), TAX1BP1 siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and TAX1BP1 siRNA transfected cells.

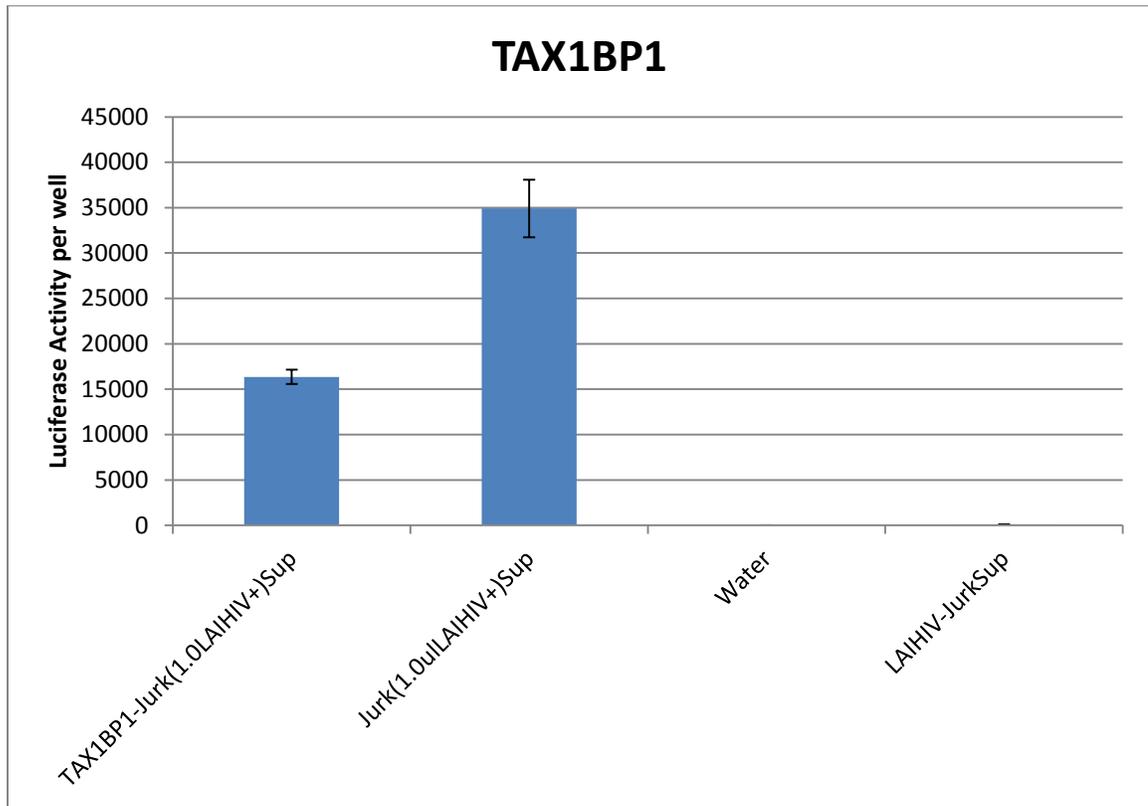


Figure 23. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of TAX1 Binding Protein 1 Gene Expression Knockdown.

Jurkat cells transfected with TAX1BP1 siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 53.2% reduction ($p < 0.05$, statistically significant) in HIV infectivity as a result of TAX1BP1 expression knockdown.

CD59, Protectin

CD59 is a compliment regulatory glycoprotein that incorporates into the cellular membrane, and will bind C8 and C9, thus inhibiting the formation of the C5b-9 membrane attack complex [24]. This protects cells containing CD59 in the membrane from autologous complement mediated destruction, or destruction originating from the same cell being destroyed. If CD59 is overexpressed in cells exposed to TSE, then it

could be more readily incorporated into virus particles being made from the infected cells, which would in turn increase the infectivity of the viral infection.

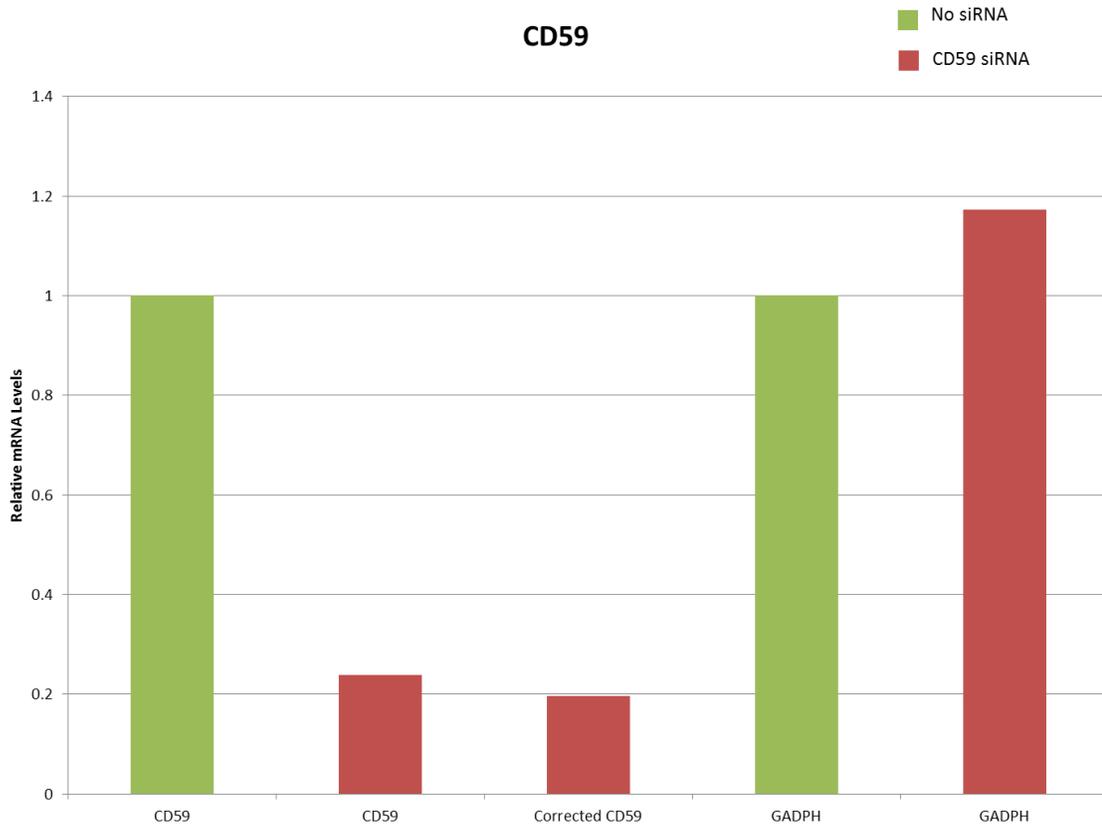


Figure 24. siRNA Induced CD59 mRNA Knockdown in Jurkat Cells.

Left to Right: CD59 mRNA levels in Jurkat control cells (no siRNA), CD59 siRNA transfected cells, GAPDH housekeeping gene mRNA levels in Jurkat control cells, and CD59 siRNA transfected cells.

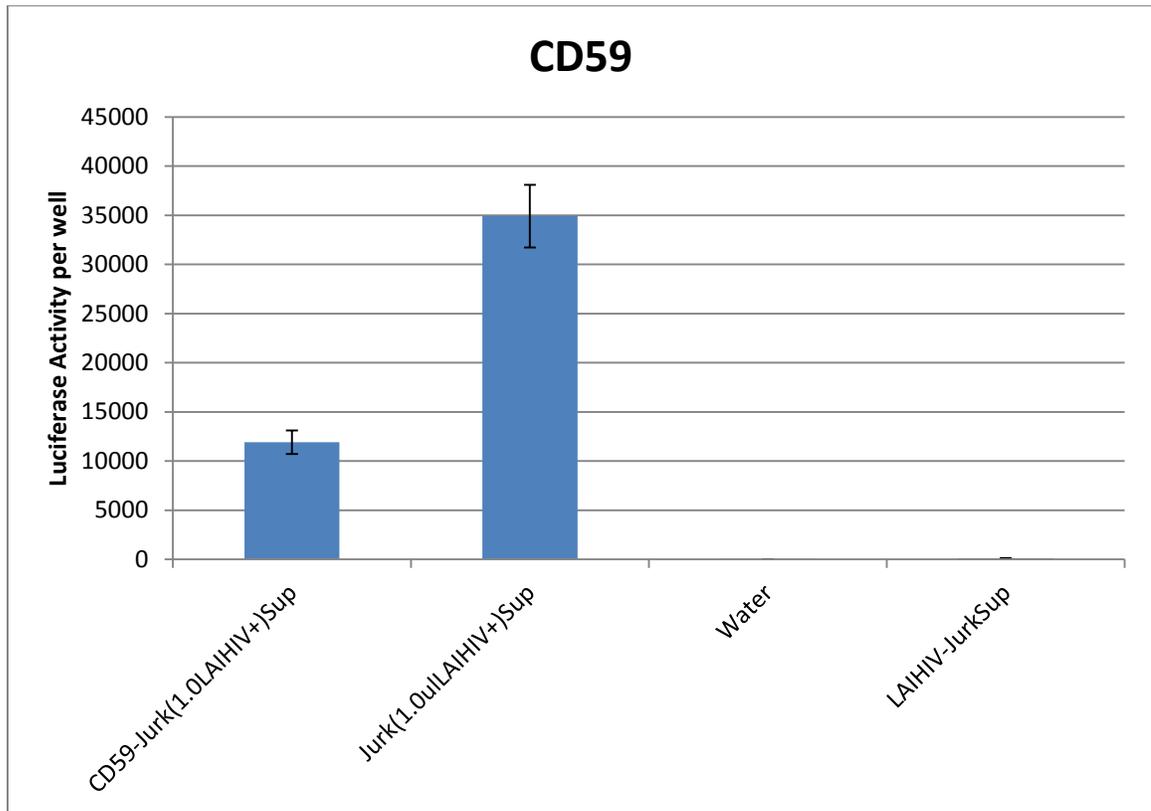


Figure 25. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of CD59 Gene Expression Knockdown.

Jurkat cells transfected with CD59 siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 65.9% reduction ($p < 0.05$, statistically significant) in HIV infectivity as a result of CD59 expression knockdown.

Coatmer Protein Complex β -2

Nef is a dimerization protein from HIV that has been shown to down regulate the expression of the CD4 cell surface receptors in HIV infected cells through a post-translational mechanism that is not fully understood yet [25, 26]. Co-immunoprecipitation assays as well as in vitro studies have shown that there is an interaction between Nef and Coatmer Protein Complex β -2 [27]. Coatmer Protein Complex β -2 is an essential component in the protein trafficking through the golgi [28]

and has also been detected in the endosomal fraction of cells [29]. Both of these areas happen to be sites where the CD4 ends up as a result of Nef induced down regulation [30]. This suggests that Coatamer Protein Complex β -2 might be one of the cellular mediators in the down regulation of CD4 by Nef, and that the up regulation of Coatamer Protein Complex β -2 as a result of exposure to TSE might play a role in the increased infectivity of HIV.

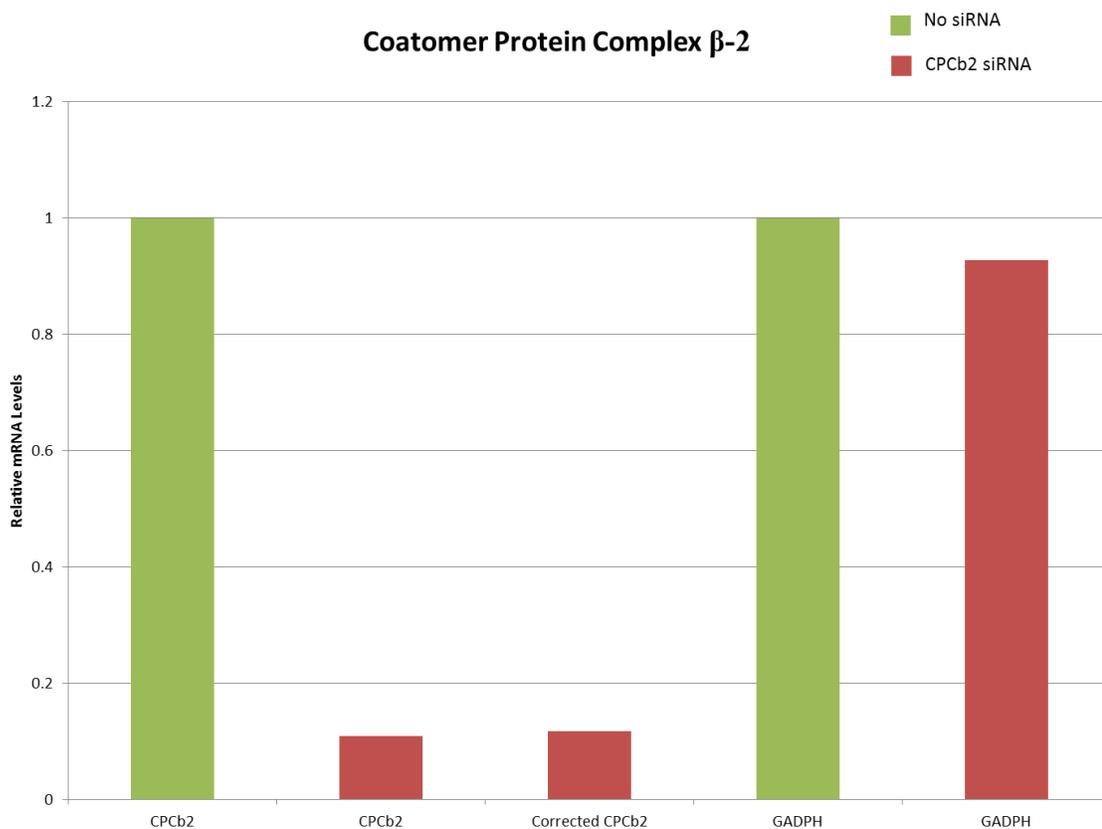


Figure 26. siRNA Induced Coatamer Protein Complex β -2 mRNA Knockdown in Jurkat Cells.

Left to Right: Coatamer Protein Complex β -2 mRNA levels in Jurkat control cells (no siRNA), CPC β 2 siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and CPC β 2 siRNA transfected cells.

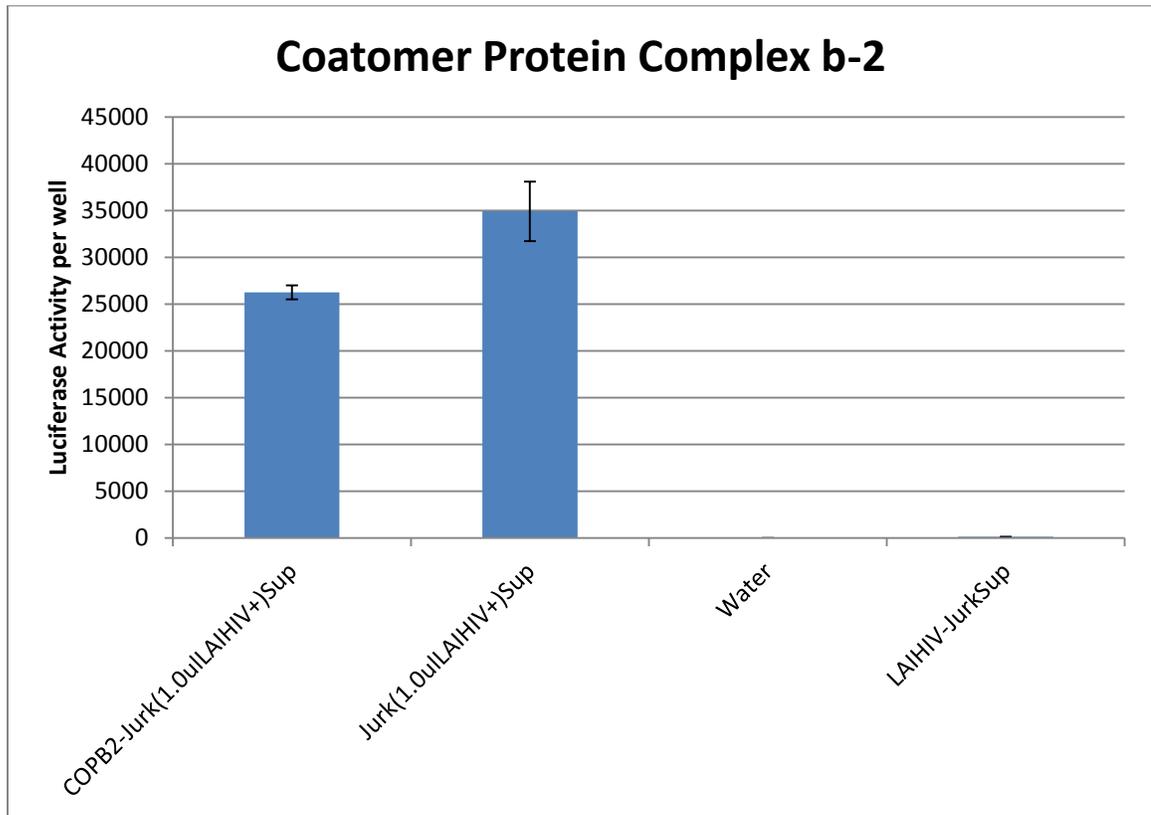


Figure 27. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of Coatomer Protein Complex β 2 Gene Expression Knockdown.

Jurkat cells transfected with CPC β 2 siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 24.8% reduction ($p > 0.05$, not statistically significant) in HIV infectivity as a result of CPC β 2 expression knockdown.

Thioredoxin-related Transmembrane Protein 1

Thioredoxin-related transmembrane protein 1 is a major component of clatherin-associated adaptor protein complex that is involved in the clathrin-mediated endocytosis, and has been shown to be involved in the down regulation of CD4 and MHC class I molecules by the HIV protein Nef [31]. Just like Coatomer Protein Complex β -2, the up regulation of Thioredoxin-related transmembrane protein 1 due to the TSE may aid in the

increase in HIV infectivity due to an association with the down regulation of CD4 and MHC class I by the HIV protein Nef.

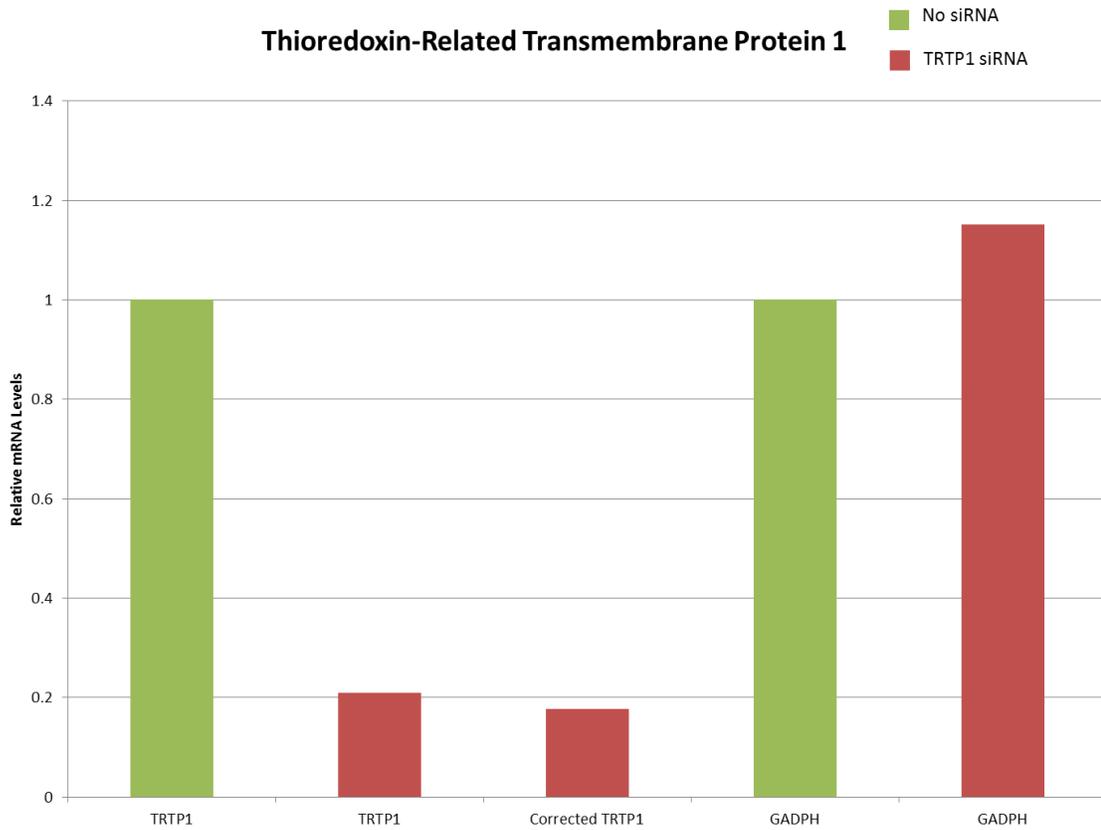


Figure 28. siRNA Induced Thioredoxin-Related Transmembrane Protein 1 mRNA Knockdown in Jurkat Cells.

Left to Right: Thioredoxin-related transmembrane protein 1 mRNA levels in Jurkat control cells (no siRNA), TRTP1 siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and TRTP1 siRNA transfected cells.

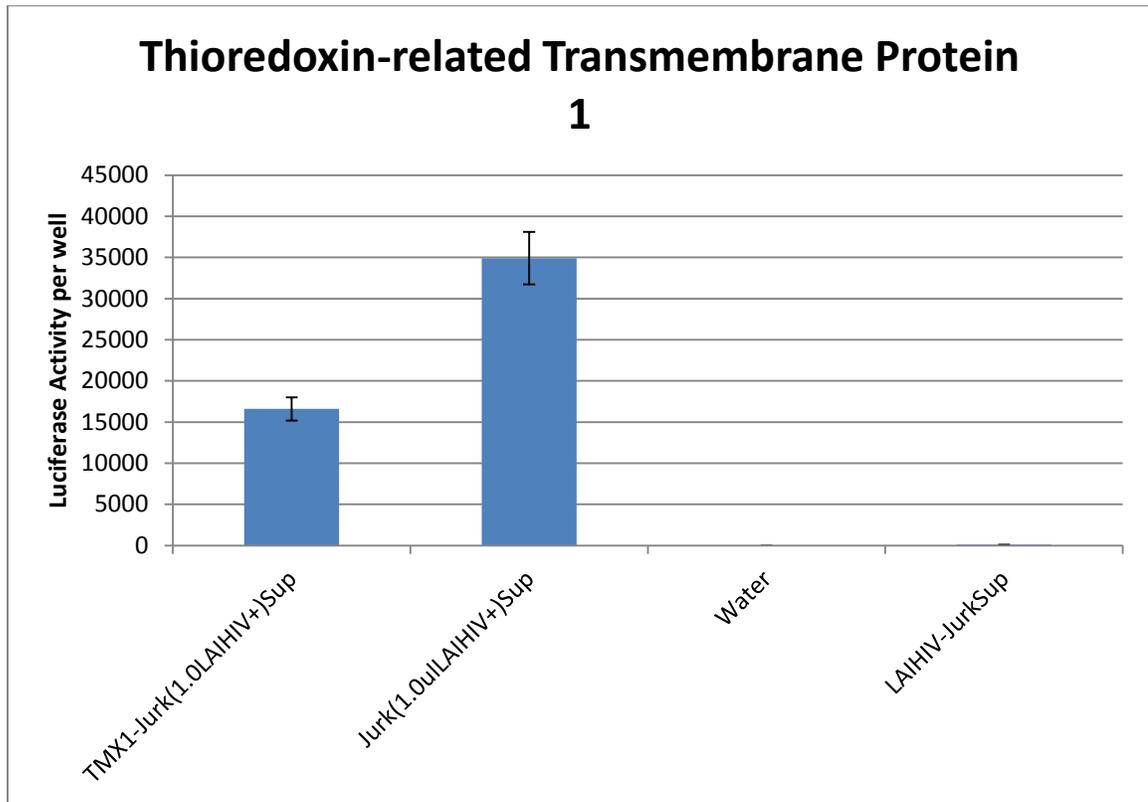


Figure 29. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of Thioredoxin-Related Transmembrane Protein 1 Gene Expression Knockdown. Jurkat cells transfected with TRTP1 siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 52.5% reduction ($p < 0.05$, statistically significant) in HIV infectivity as a result of TRTP1 expression knockdown.

Calmodulin 1

Upon HIV infection, there is eventually an accelerated loss of CD4+ T-lymphocytes, which is a key factor in the progression of the disease. One mechanism in this rapid loss of CD4+ T cells is through apoptosis [32]. Although not fully understood it is suggested that HIV related apoptosis is from the FAS pathway. When FAS ligand binds FAS it recruits signaling molecules which activate caspase 3, which then activates caspase 6, and eventually DNA fragmentation factor resulting in cleavage of the DNA

[33]. The HIV glycoprotein gp160 contains two calmodulin binding sites on its c-terminal end. Expression of gp160 in Jurkat T cells results in increased sensitivity to FAS mediated apoptosis. This increase in sensitivity is blocked in the presence of the two calmodulin antagonists tamoxifen and trifluoperazine. In addition, the mutation of the two calmodulin binding sites on gp160 eliminates the gp160 dependent FAS-mediated apoptosis [34]. This indicates that the gp160 induced apoptosis seen in HIV infection of T cells is dependent on calmodulin up regulation, and binding to the c-terminal domain of gp160. This clearly demonstrates the need for the up regulation of calmodulin as seen with exposure to TSE in the increase in HIV infectivity.

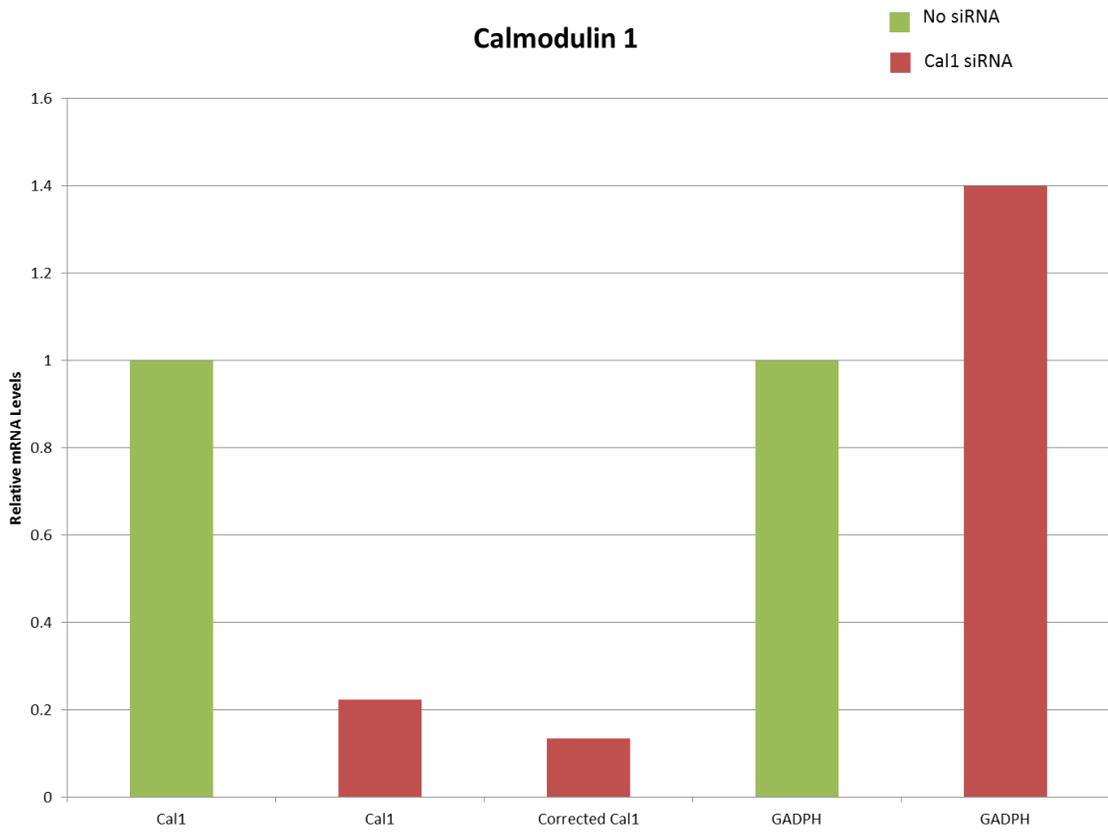


Figure 30. siRNA Induced Calmodulin 1 mRNA Knockdown in Jurkat Cells. Left to Right: Calmodulin 1 mRNA levels in Jurkat control cells (no siRNA), Cal1 siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and Cal1 siRNA transfected cells.

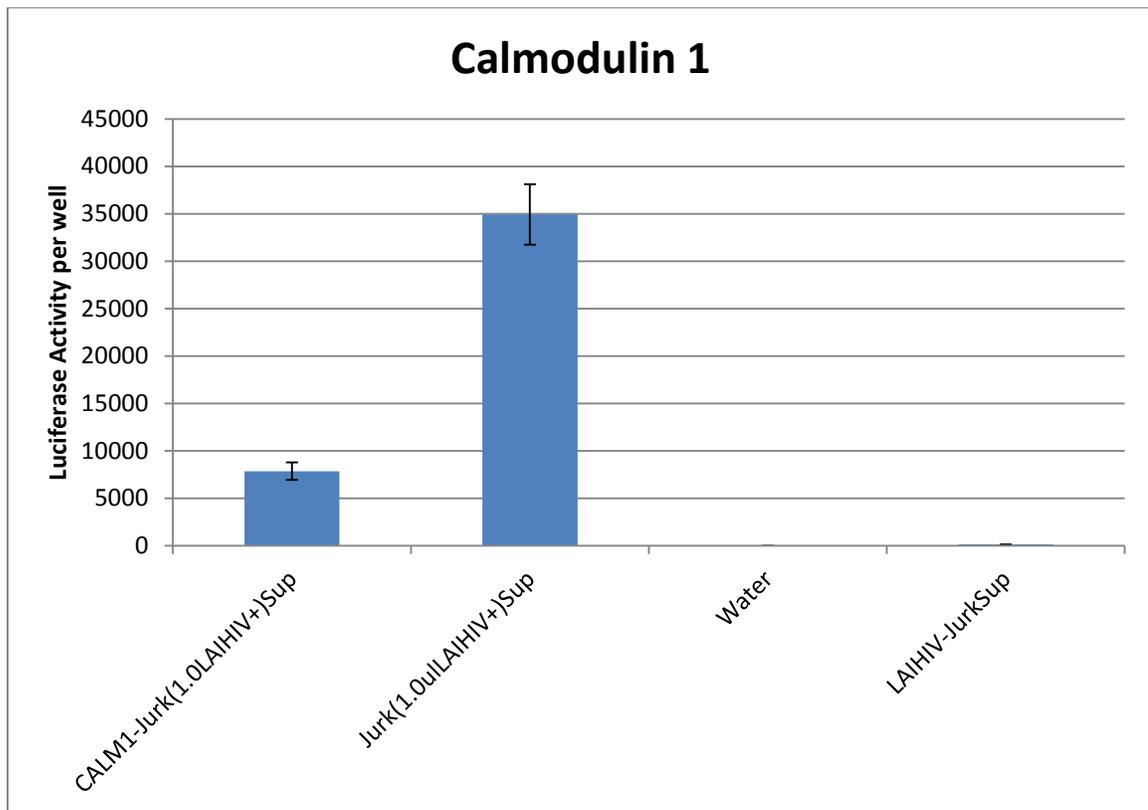


Figure 31. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of Calmodulin 1 Gene Expression Knockdown.

Jurkat cells transfected with Cal1 siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 77.5% reduction ($p < 0.05$, statistically significant) in HIV infectivity as a result of Cal1 expression knockdown.

HSP90 β -1

Activation of HIV transcription requires the activity of human transcription elongation factor (P-TEFb) which then interacts with Tat and then phosphorylates the C-terminal domain of RNA polymerase II. It is believed that the Cdk9/cyclin T1 dimer is the active form of P-TEFb, which interacts with Tat [33]. Cdk9 is the catalytic subunit that eventually phosphorylates RNA polymerase II. In order for Cdk9 to interact with cyclin T1 it must first bind with the kinase-specific chaperone complex Hsp90/Cdc37.

Upon inactivation of the Hsp90/Cdc37 complex by geldanamycin, there was a significant increase in the levels of Cdk9 unable to bind to cyclin T1. Effectively breaking the chain which allowed for activation of RNA polymerase II [36]. This shows a need for the presence of Hsp90 in the HIV transcriptional activation cascade which indicates the importance of this protein and possibly its up regulation by TSE in the increase in HIV infectivity.

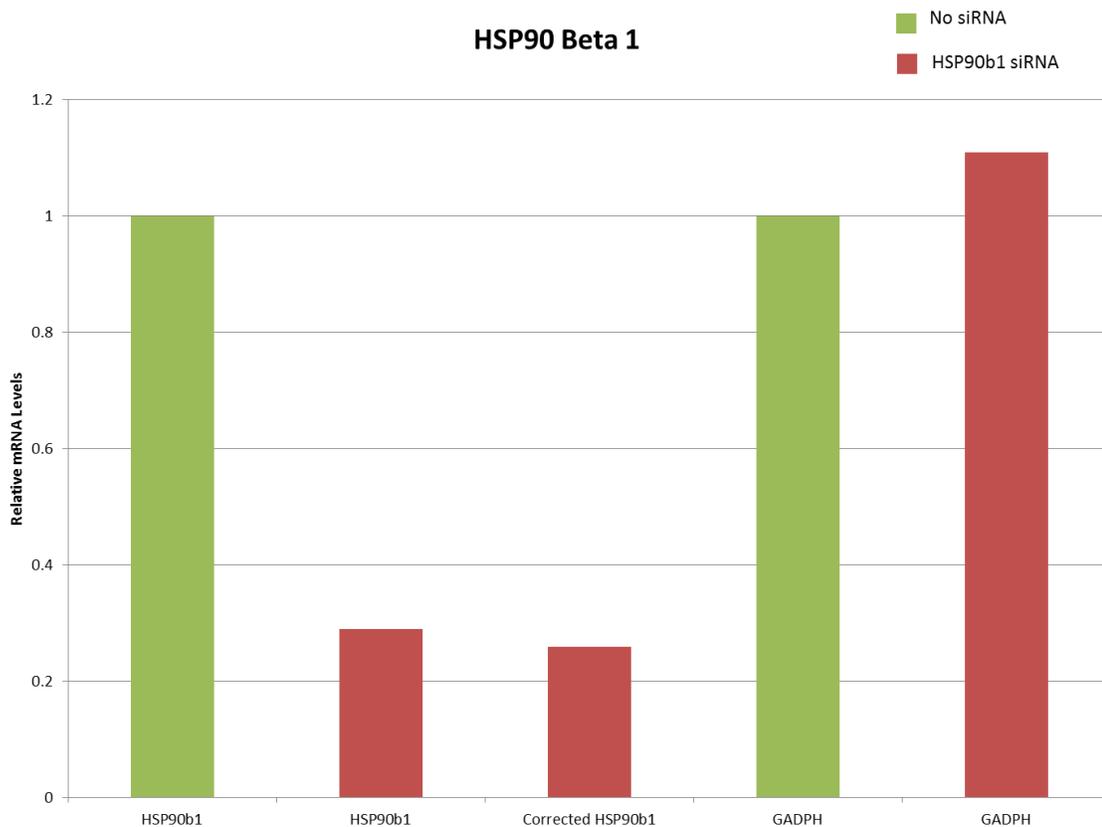


Figure 32. siRNA Induced Heat Shock Protein 90 β 1 mRNA Knockdown in Jurkat Cells.

Left to Right: Heat shock protein 90 β 1 mRNA levels in Jurkat control cells (no siRNA), HSP90b1 siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and HSP90b1 siRNA transfected cells.

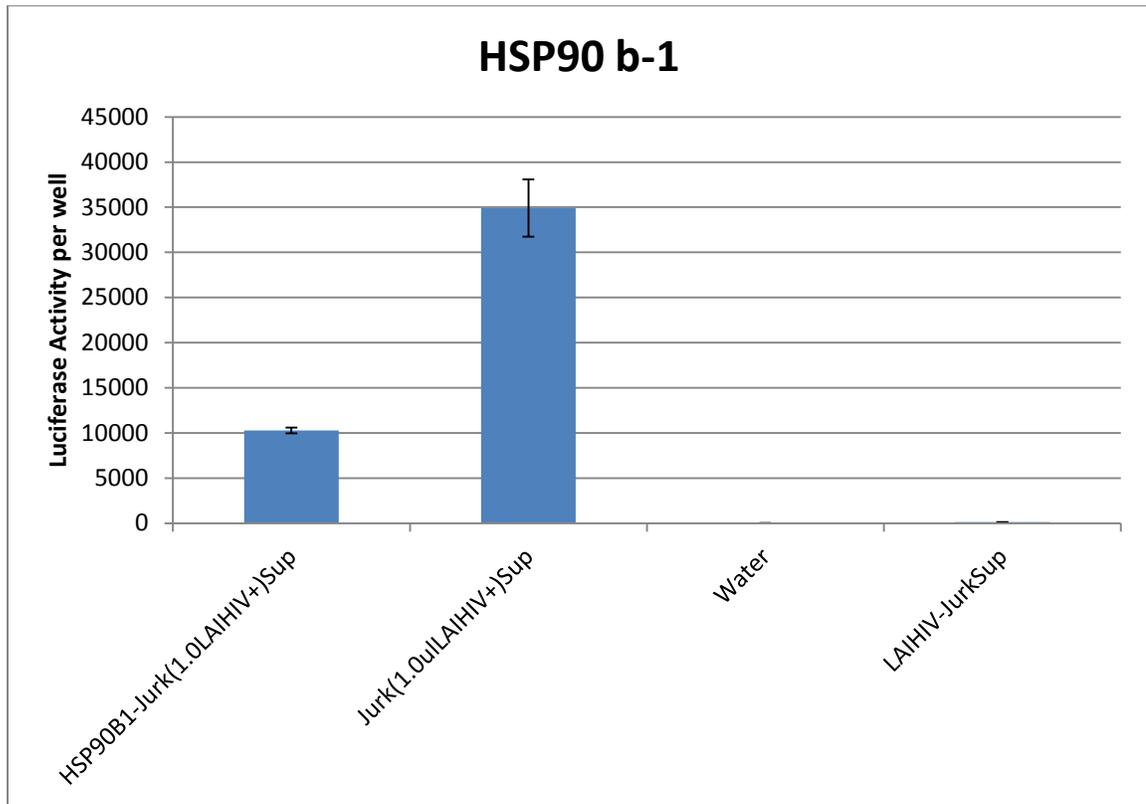


Figure 33. TzM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of Heat Shock Protein 90 β -1 Gene Expression Knockdown.

Jurkat cells transfected with HSP90b1 siRNA were infected with HIV, then cell supernatant was added to TzM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 70.6% reduction ($p < 0.05$, statistically significant) in HIV infectivity as a result of HSP90b1 expression knockdown.

Sjogren Syndrome Antigen B

Sjogren Syndrome Antigen B, also known as La protein, is involved in RNA protection and metabolism. It has been shown to protect the 3'-UUU(OH) segments of newly RNA polymerase III transcribed RNA. It also acts as an RNA chaperone and binds to RNA's from the Hepatitis C virus [37]. Since both HIV and Hepatitis C virus are both positive-sence RNA viruses, Sjogren Syndrome Antigen B may also play a role in the increase of HIV infectivity from TSE.

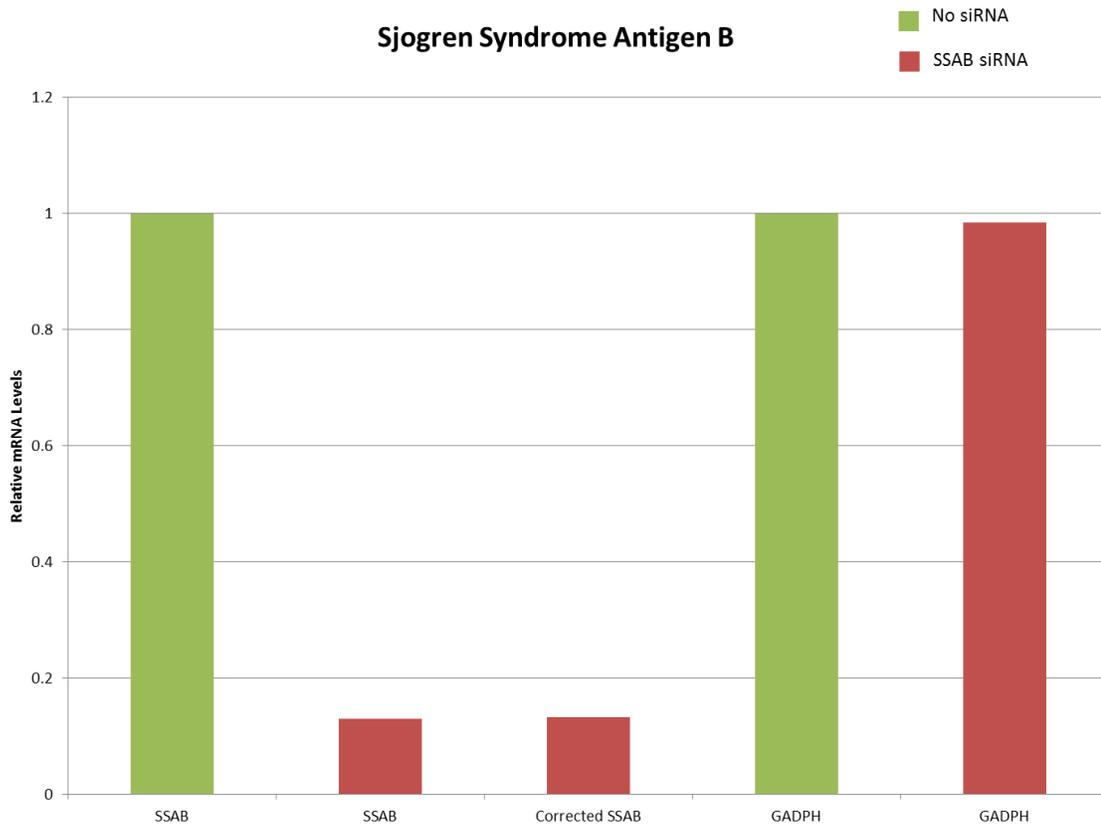


Figure 34. siRNA Induced Sjogren Syndrome Antigen B mRNA Knockdown in Jurkat Cells.

Left to Right: Sjogren Syndrome Antigen B mRNA levels in Jurkat control cells (no siRNA), SSAB siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and SSAB siRNA transfected cells.

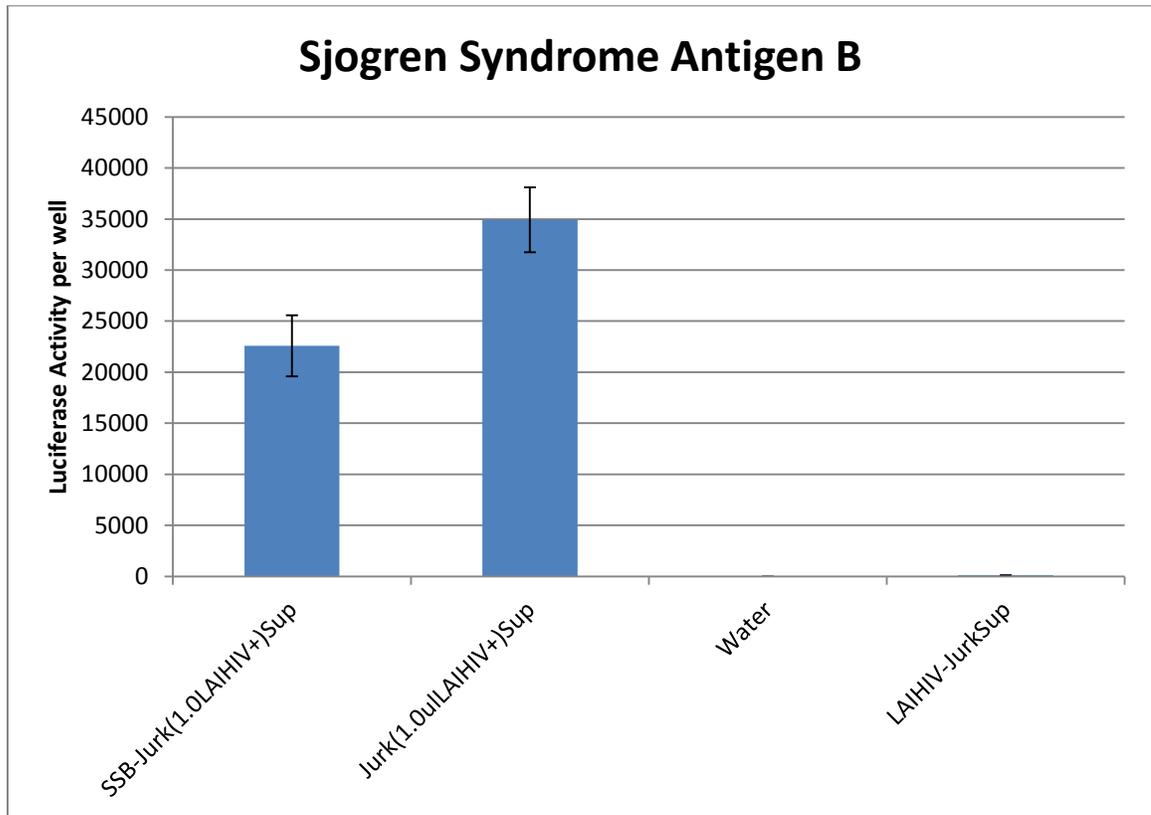


Figure 35. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of Sjogren Syndrome Antigen B Gene Expression Knockdown.

Jurkat cells transfected with SSAB siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 35.3% reduction ($p > 0.05$ not statistically significant) in HIV infectivity as a result of SSAB expression knockdown.

DDX3

DEAD-box RNA helicases are involved in RNA transcription, translation, RNA splicing, RNA transport, and RNA degradation. DDX3 is a DEAD-box RNA helicase that has been implicated in the replication of HIV. DDX3 is a nucleo-cytoplasmic shuttling protein that will localize near the nuclear pores. Knockdown of DDX3 suppressed the export of HIV RNA's to the cytoplasm causing inefficient viral

replication [38, 39]. The up regulation of DDX3 from TSE may also play a role in the increase of HIV infectivity.

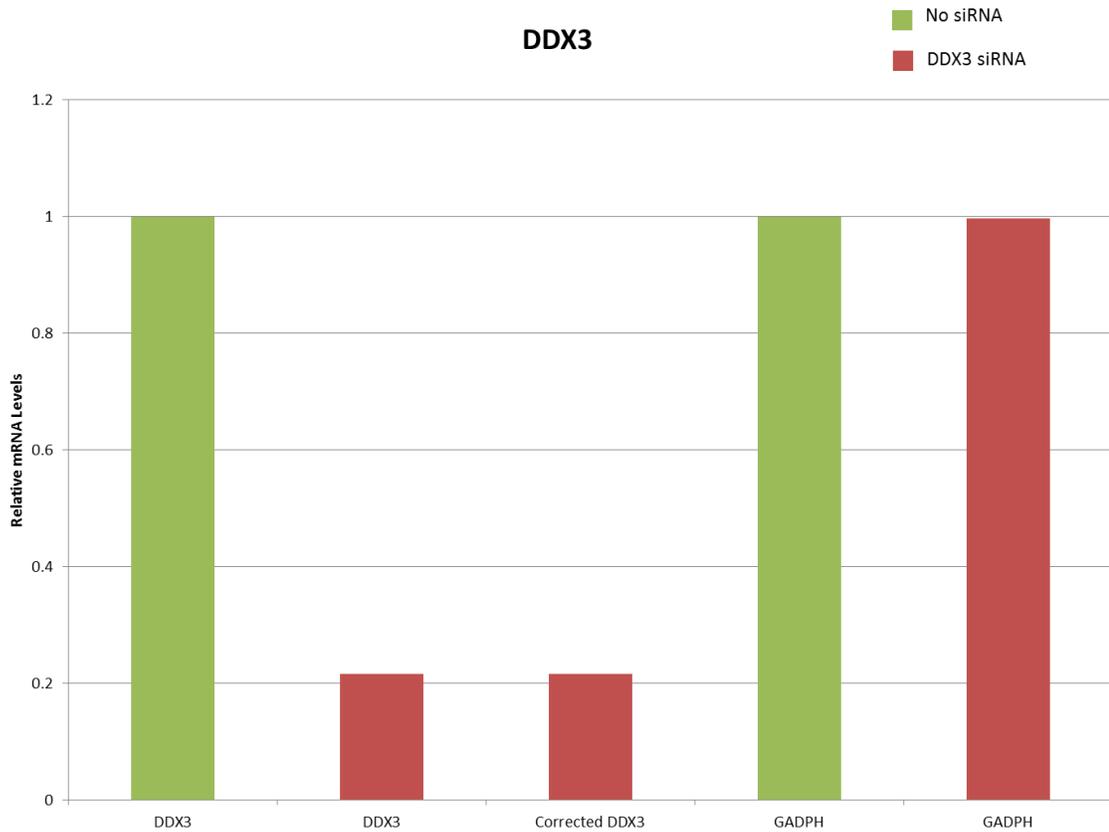


Figure 36. siRNA Induced DDX3 mRNA Knockdown in Jurkat Cells. Left to Right: DDX3 mRNA levels in Jurkat control cells (no siRNA), DDX3 siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and DDX3 siRNA transfected cells.

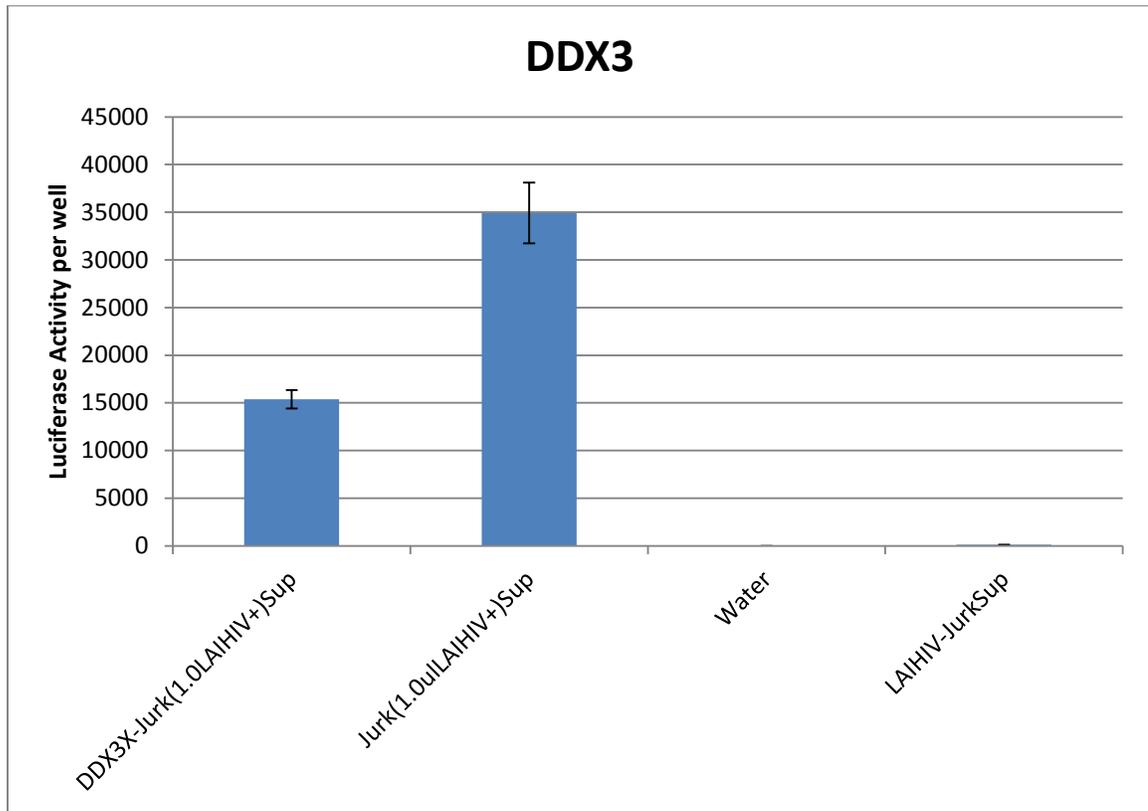


Figure 37. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of DDX3 Gene Expression Knockdown.

Jurkat cells transfected with DDX3 siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 56.0% reduction ($p < 0.05$, statistically significant) in HIV infectivity as a result of DDX3 expression knockdown.

Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx)

Research has shown that overexpression of glutathione peroxidases (GPx) specifically PHGPx is sufficient to inhibit NF- κ B activation by IL-1 [40]. HIV transcription is dependent on host transcription factors like NF- κ B for activation. The HIV LTR contains NF- κ B binding sites needed for efficient HIV transcription. The lack of NF- κ B in infected HIV T cells does not completely inhibit HIV transcription but in the

absence of TAT, the transcripts are prematurely terminated. By down-regulating the expression of PHGPx in infected cells, it may allow more NF- κ B to be activated causing an increase in HIV infectivity.

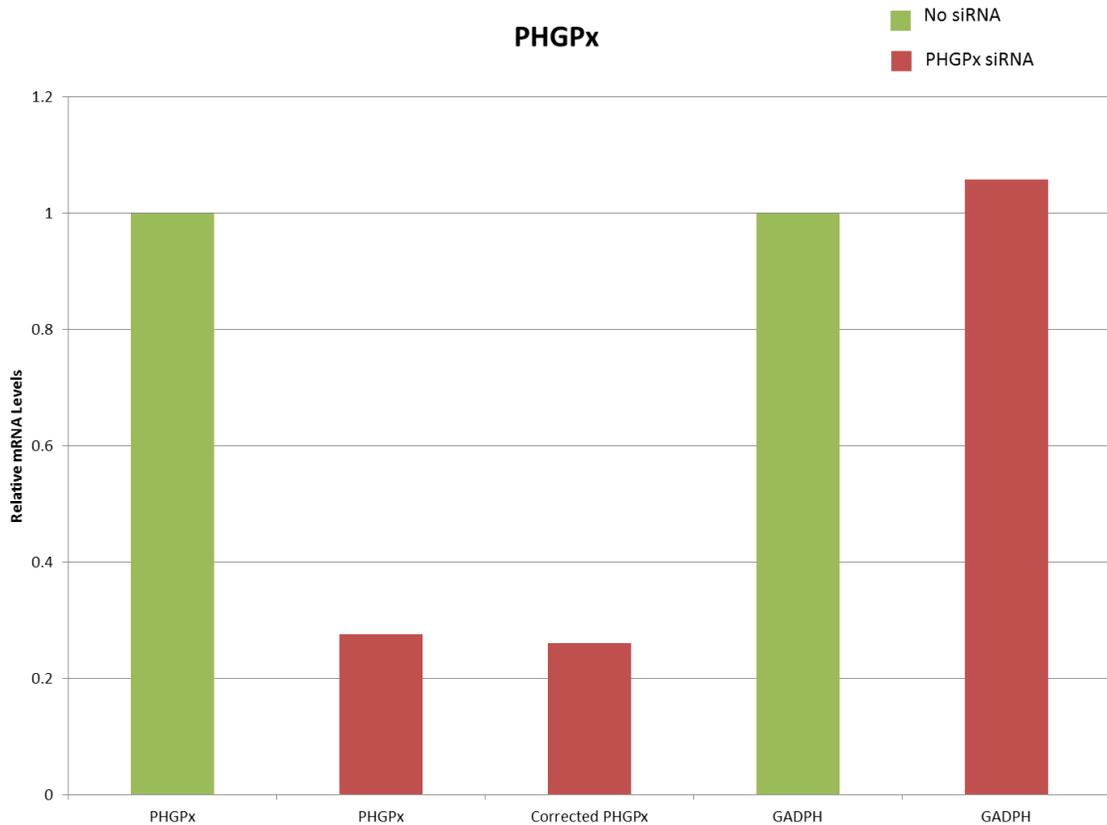


Figure 38. siRNA Induced PHGPx mRNA Knockdown in Jurkat Cells.

Left to Right: PHGPx mRNA levels in Jurkat control cells (no siRNA), PHGPx siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and PHGPx siRNA transfected cells.

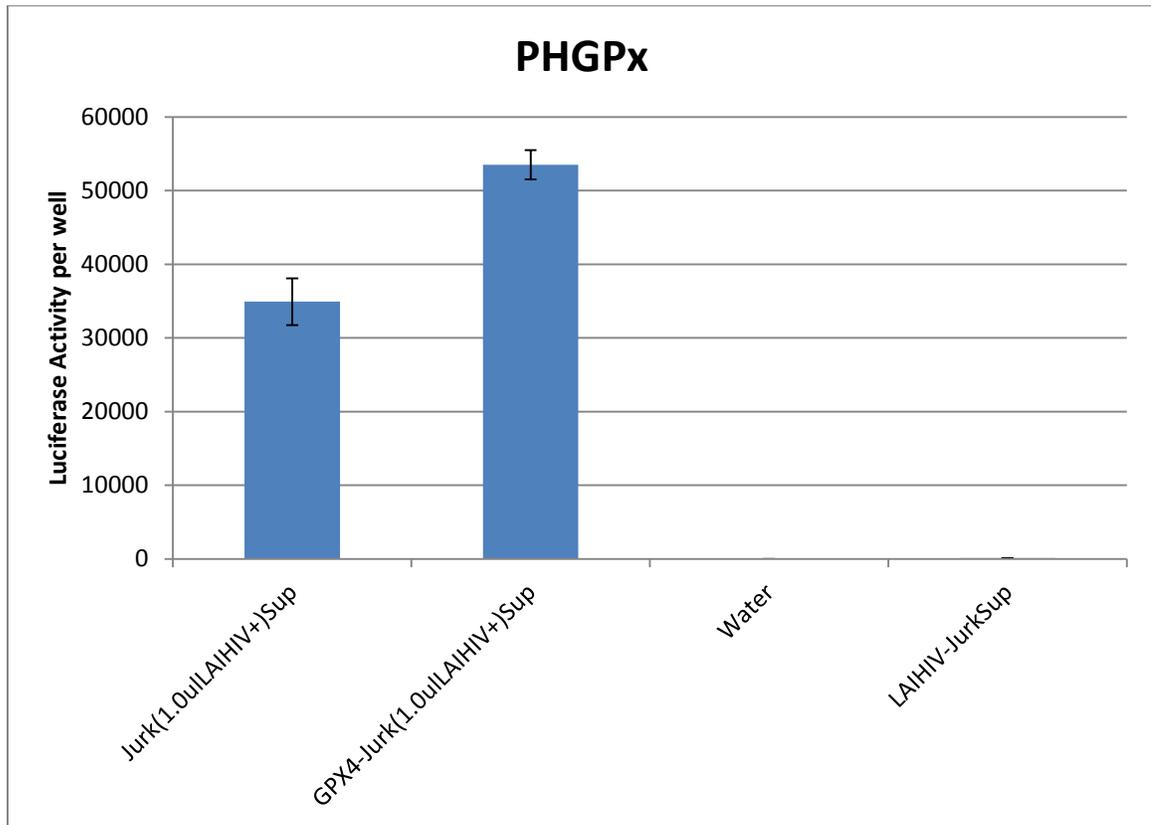


Figure 39. TzM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of PHGPx Gene Expression Knockdown.

Jurkat cells transfected with PHGPx siRNA were infected with HIV, then cell supernatant was added to TzM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 53.2% increase ($p < 0.05$, statistically significant) in HIV infectivity as a result of PHGPx expression knockdown.

MHC, HLA-E

MHC class I is the gene product of one of 6 human leukocyte antigen (HLA) genes (HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G). Each gene product is slightly different in structure, but they have the same function, to present fragments of proteins on the cell surface in order to alert the immune system. The HIV Nef protein has been shown to down regulate the expression of MHC class I molecules from HLA-A, and

HLA-B genes [41]. The MHC molecules from HLA-C and HLA-E then signal the natural killer (NK) cells to lyse those cells expressing small amounts of MHC. By the down-regulation of MHC from the HLA-E gene, the cell is then better able to prevent recognition from the NK cells and can continue to produce HIV viral particles, thereby causing the HIV to be more infective.

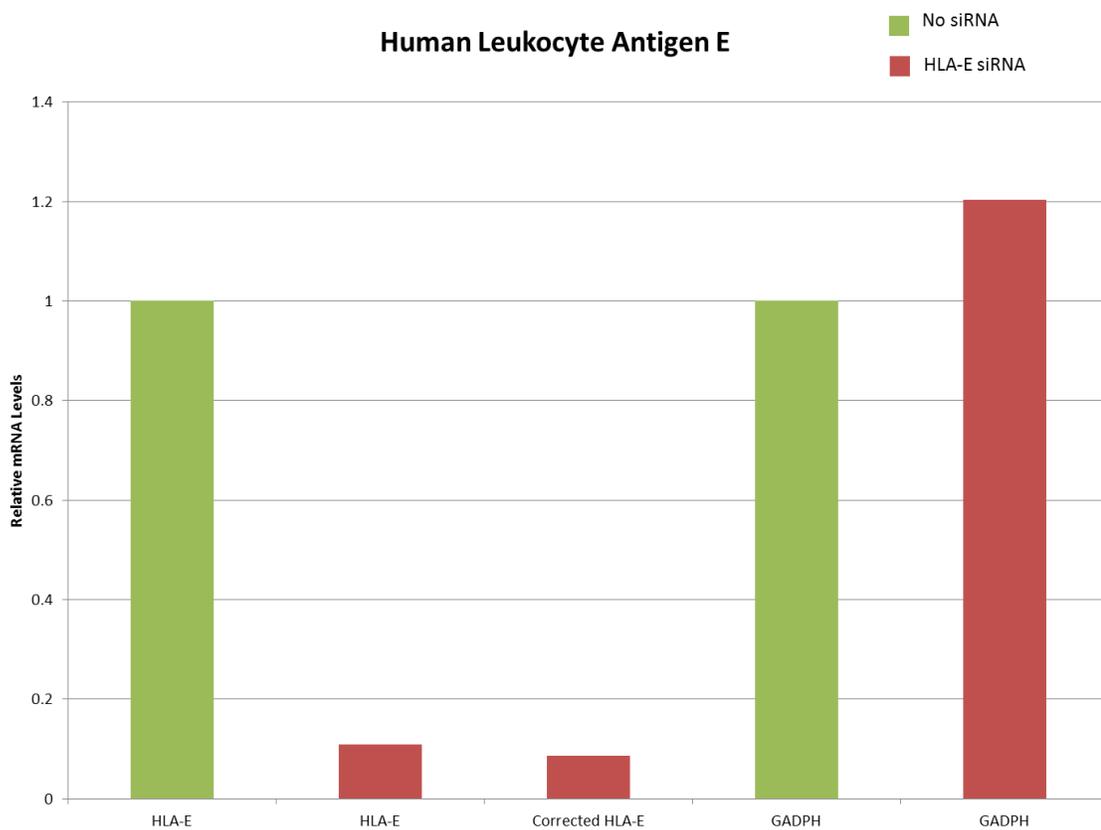


Figure 40. siRNA Induced Human Leukocyte Antigen E mRNA Knockdown in Jurkat Cells.

Left to Right: Human Leukocyte Antigen E mRNA levels in Jurkat control cells (no siRNA), HLA-E siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and HLA-E siRNA transfected cells.

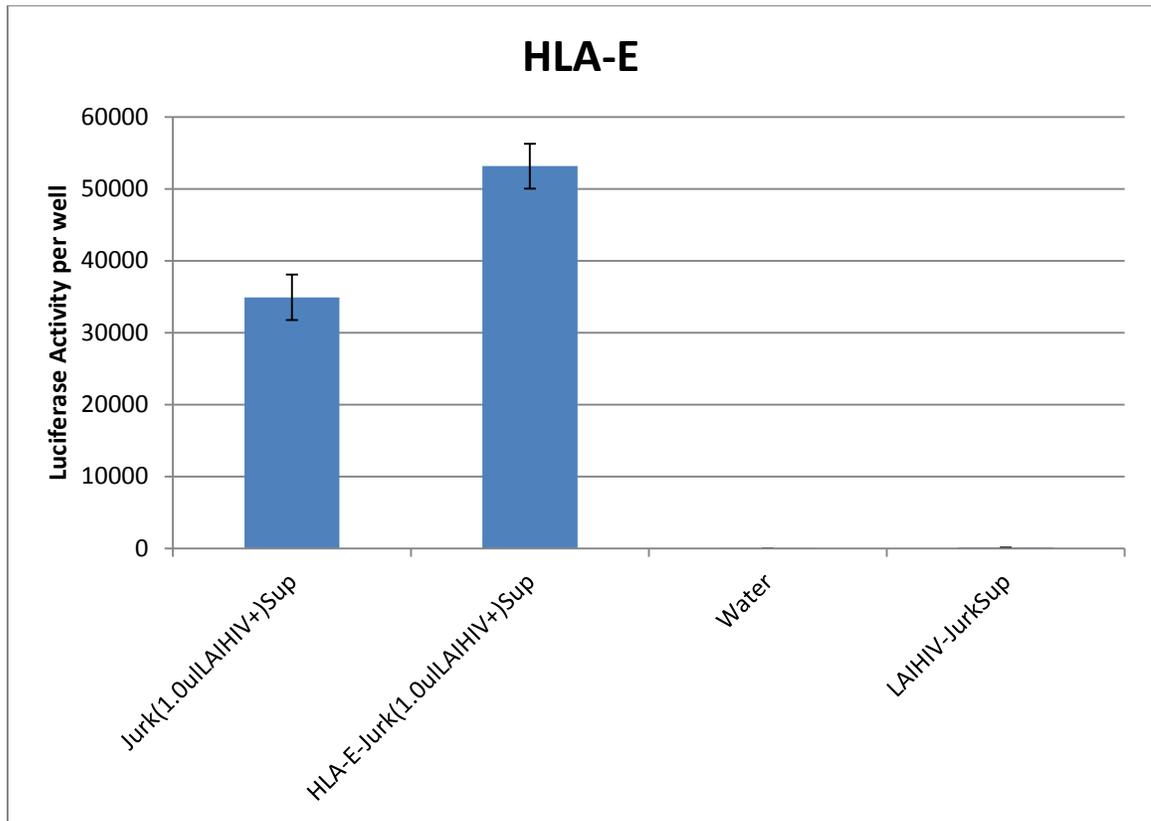


Figure 41. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of Human Leukocyte Antigen E Gene Expression Knockdown.

Jurkat cells transfected with HLA-E siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 52.3% increase ($p < 0.05$, statistically significant) in HIV infectivity as a result of HLA-E expression knockdown.

Steroyl-CoA Desaturase

Steroyl-CoA Desaturase is a key enzyme in fatty acid metabolism. The absence or down regulation of Steroyl-CoA Desaturase causes a state of lipodystrophy [42].

Lipodystrophy is the partial or complete absence of adipose tissue caused by the inability of the body to completely metabolize fatty acids. This causes triglycerides to accumulate in the liver, heart, muscle, and other tissues [43]. In individuals infected with HIV, this

ensures the infected cells have a steady supply of cholesterol for the newly forming virions. Nef will bind to cholesterol and deliver it to the cell membrane where the new virus particles are assembled. By down regulating the expression of Stearoyl-CoA Desaturase it allows the HIV infected cells a more abundance of cholesterol, which may increase the infectivity of the virus.

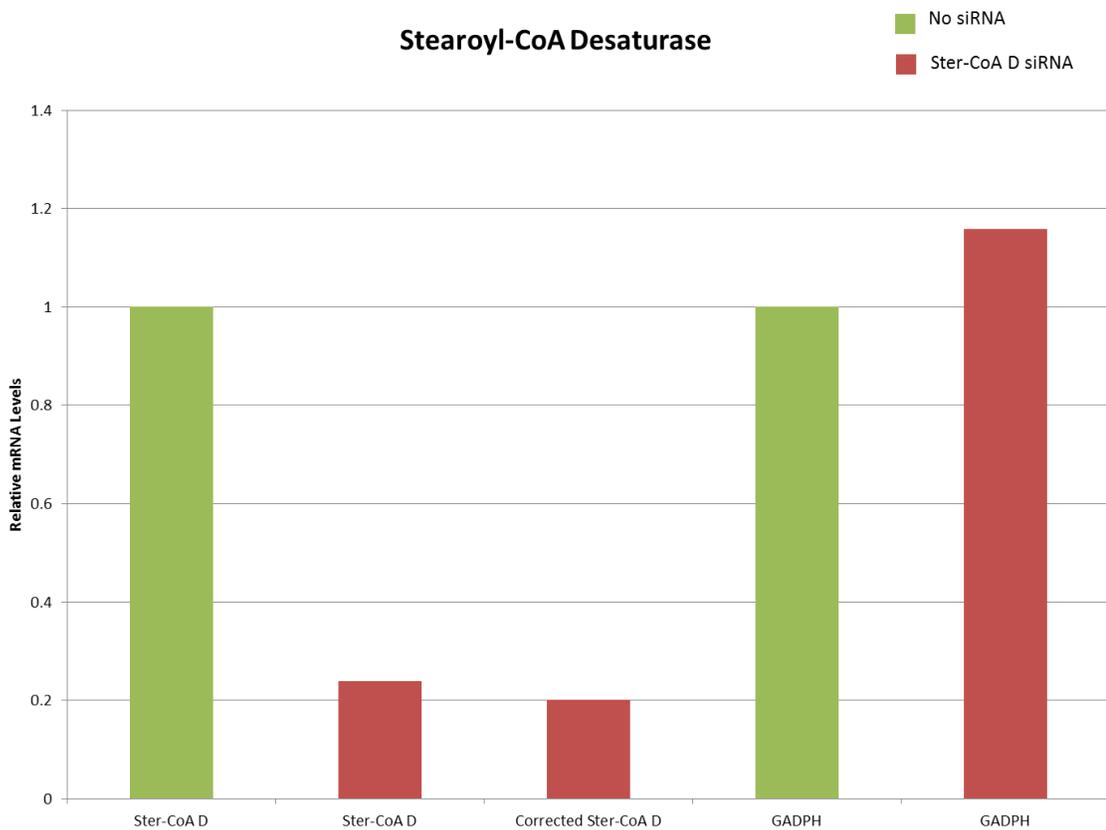


Figure 42. siRNA Induced Stearoyl-CoA Desaturase mRNA Knockdown in Jurkat Cells.

Left to Right: Stearoyl-CoA Desaturase mRNA levels in Jurkat control cells (no siRNA), Ster-CoA D siRNA transfected cells, GAPDH housekeeping gene mRNA levels in Jurkat control cells, and Ster-CoA D siRNA transfected cells.

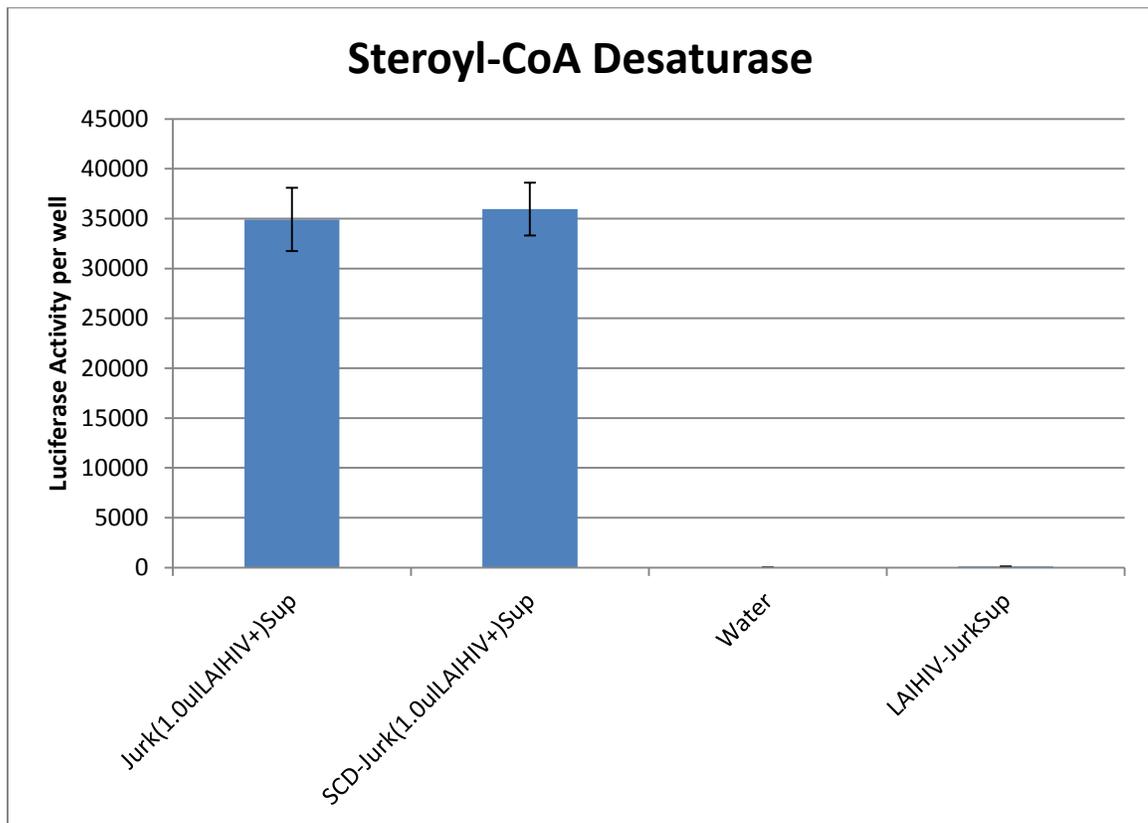


Figure 43. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of Steroyl-CoA Desaturase Gene Expression Knockdown.

Jurkat cells transfected with Ster-CoA D siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 3.0% increase ($p > 0.05$ not statistically significant) in HIV infectivity as a result of Ster-CoA D expression knockdown.

Thioredoxin Interacting Protein

Thioredoxin Interacting Protein is an inhibitor of the reduction ability of Thioredoxin, by interacting with the catalytic center of the reduced form of Thioredoxin [44]. The action of TSE down regulates the expression of Thioredoxin Interacting Protein which will increase the reducing activity of Thioredoxin. Significantly increased levels of Thioredoxin, is an indicator in patients infected with HIV. Patients with high plasma

thioredoxin levels also tend to have lower overall CD4+ cell counts [45]. This indicates that by down-regulating the expression of Thioredoxin Interacting Protein the TSE is increasing the infectivity of the HIV by presenting it with a better environment to replicate in.

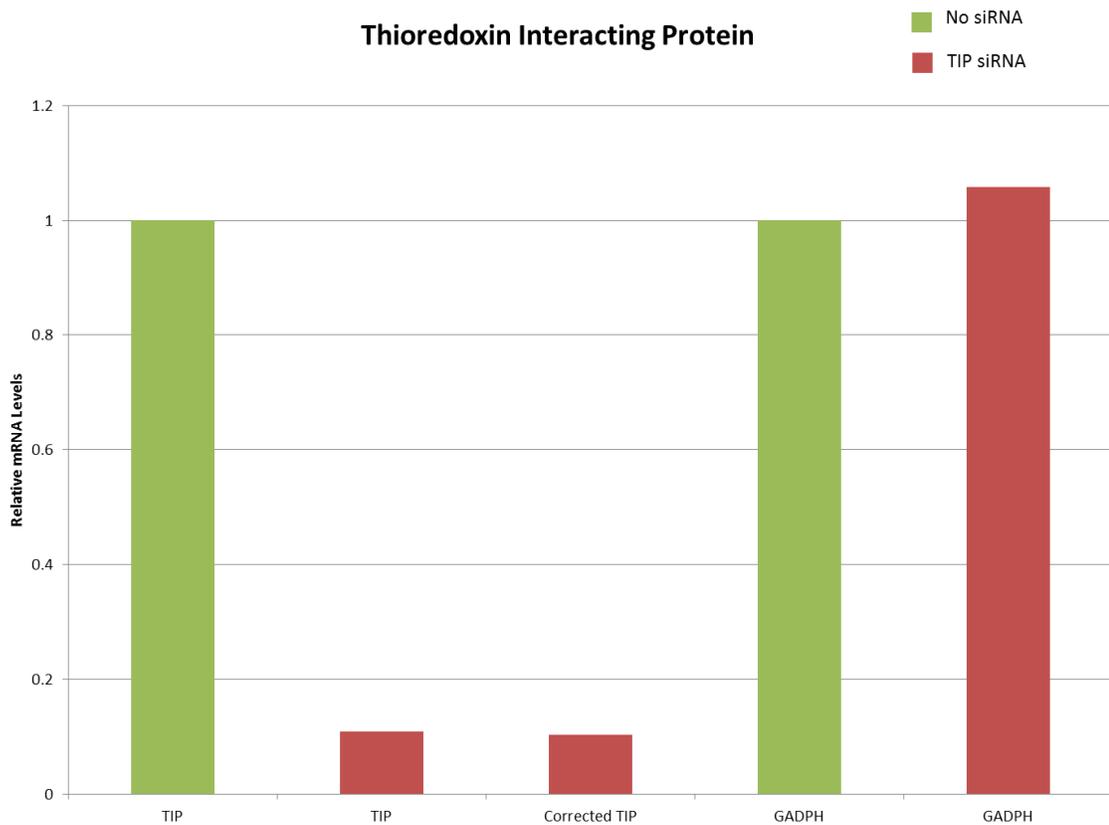


Figure 44. siRNA Induced Thioredoxin Interacting Protein mRNA Knockdown in Jurkat Cells.

Left to Right: Thioredoxin interacting protein 1 mRNA levels in Jurkat control cells (no siRNA), TIP siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and TIP siRNA transfected cells.

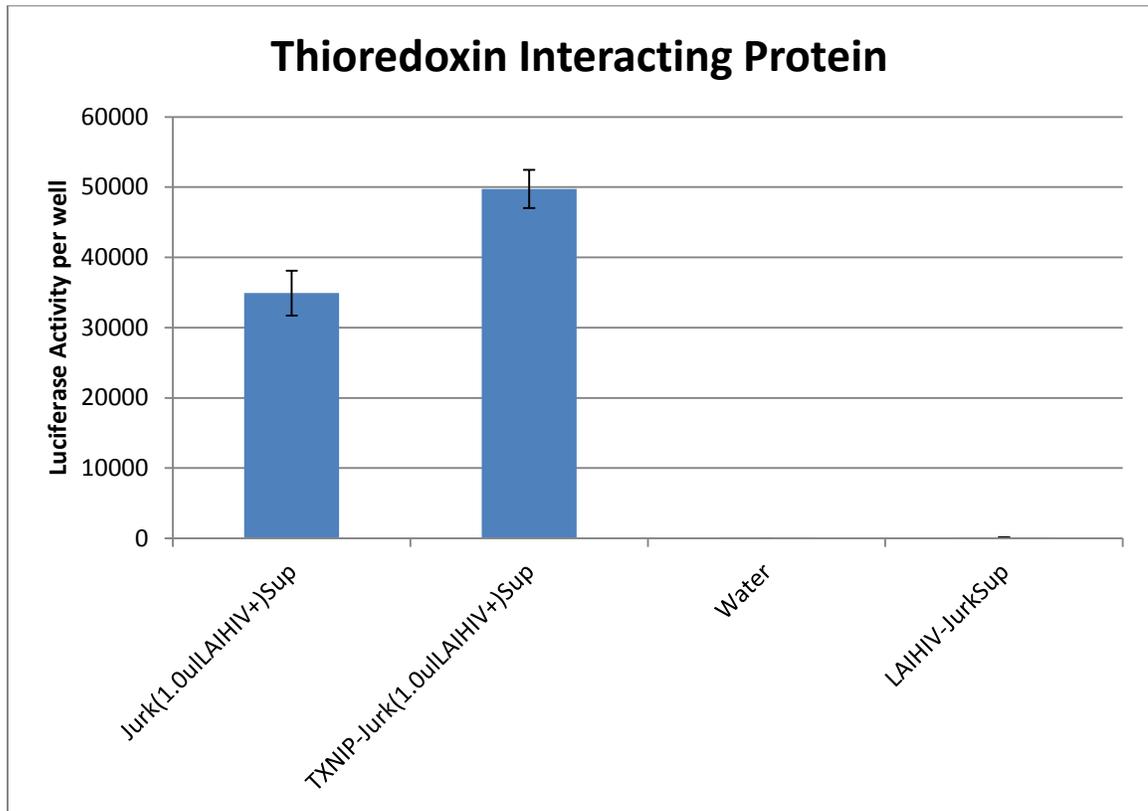


Figure 45. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of Thioredoxin Interacting Protein Gene Expression Knockdown.

Jurkat cells transfected with TIP siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 42.4% increase ($p > 0.05$ not statistically significant) in HIV infectivity as a result of TIP expression knockdown.

Dual Knockdown of DDX3 and Thioredoxin-related Transmembrane

Protein 1

DDX3 is a RNA helicase which has been shown to act as a nucleo-cytoplasmic shuttling protein which localizes near the nuclear pores. When the expression of DDX3 is knocked down the result is suppressed export of HIV RNA's to the cytoplasm [36, 37]. Thioredoxin-related transmembrane protein 1 has been shown to play a role in the HIV Nef protein mediated down regulation to CD4 and MHC class I proteins from the cell

surface. The down regulation of CD4 from the cell surface enhances the release of fully infectious HIV-1 virions thereby promoting HIV infectivity [46-49]. The down-regulation of HLA MHC class I allows the HIV infected cell to evade the host immune response [50]. With DDX3 and TRTP1 being utilized by HIV in different pathways, and the up-regulation of both genes showing the possibility of enhancing HIV infectivity, these 2 genes were selected in order to create a dual-knockdown cell sample and then HIV infectivity measured using the luciferase assay.

Jurkat cells were transfected with DDX3 and TRTP1 siRNA/Lipofectamine 2000 complexes simultaneously according to previously outlined procedure. Forty-eight hours post-transfection a cell sample was used in qRT-PCR quantitation of DDX3 and TRTP1 gene expression. A second cell sample was used to measure HIV infectivity using the previously used luciferase assay in TZM-bl cells.

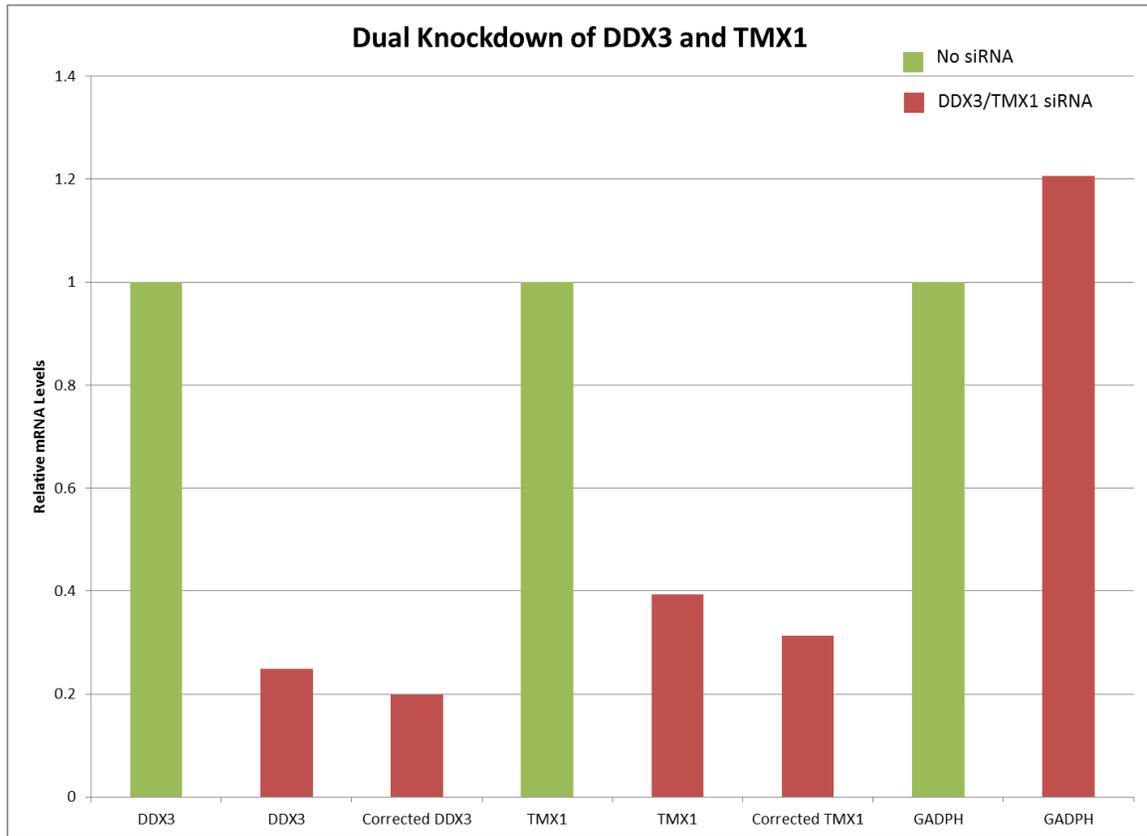


Figure 46. siRNA Induced DDX3 and Thioredoxin-Related Transmembrane Protein 1 mRNA Dual Knockdown in Jurkat Cells.

Left to Right: DDX3 mRNA levels in Jurkat control cells (no siRNA), DDX3/TMX1 siRNA transfected cells, Thioredoxin-related transmembrane protein 1 mRNA levels in Jurkat control cells, TRTP1 siRNA transfected cells, GADPH housekeeping gene mRNA levels in Jurkat control cells, and TRTP1 siRNA transfected cells.

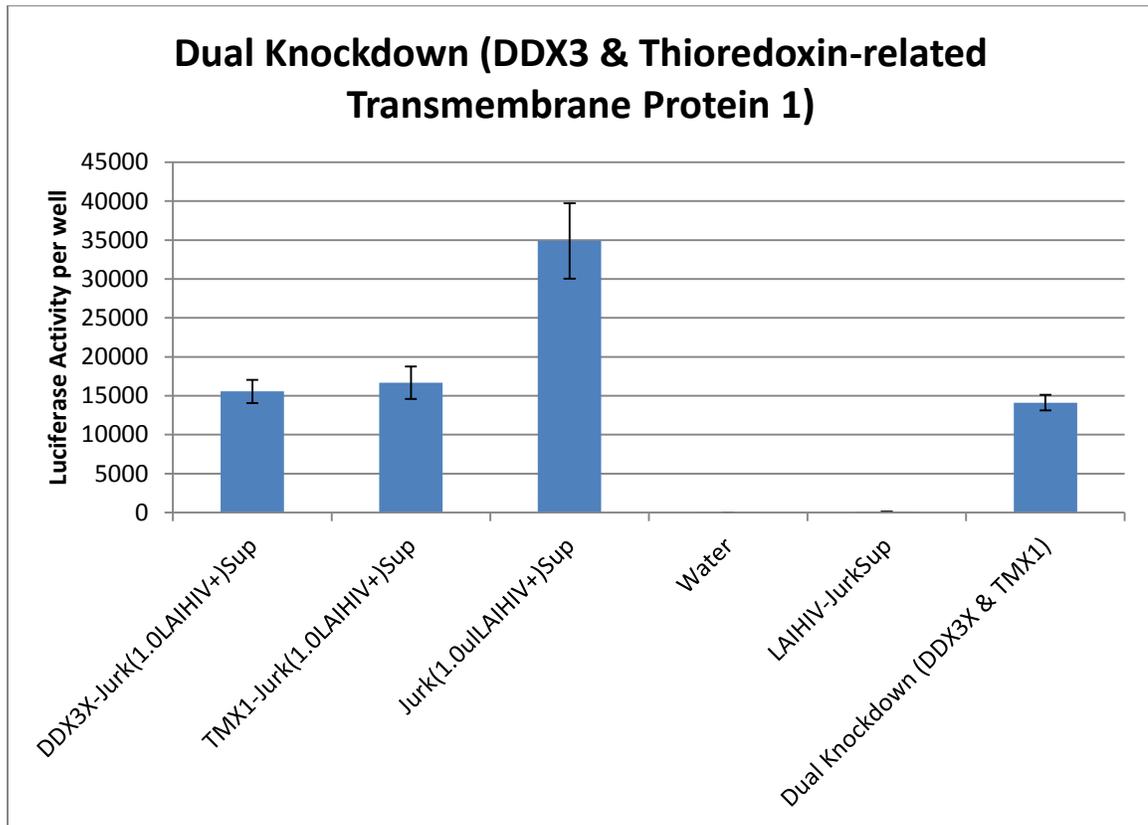


Figure 47. TZM-bl Luciferase Activity Indicating HIV Infectivity Levels as a Result of DDX3 and Thioredoxin-Related Transmembrane Protein 1 Dual Gene Expression Knockdown.

Jurkat cells transfected with DDX3 and TRTP1 siRNA were infected with HIV, then cell supernatant was added to TZM-bl cells and luciferase activity measured 48 hours post supernatant addition. Luciferase activity indicates a 55.4% decrease ($p < 0.05$, statistically significant) in HIV infectivity as a result of DDX3 expression knockdown alone, and a 52.2% decrease ($p < 0.05$, statistically significant) in HIV infectivity as a result of TRTP1 expression knockdown alone. When the expression of both DDX3 and TRTP1 have been decreased by siRNA, there is a 59.6% reduction in HIV infectivity. While the reduction in HIV infectivity as a result of knockdown in each gene individually is statistically significant, the reduction in infectivity as a result of the dual knockdown of DDX3 and TRTP1 is not statistically significant ($p > 0.05$) in relation to the individual knockdown levels.

CHAPTER VII

DISCUSSION

The HIV viral epidemic has impacted almost all nations on the planet, along with the prevalence of tobacco smoking, so determining if a link exists between the two should be of top priority. In the U.S., tobacco smoking prevalence among those infected with HIV is believed to be 50%-70%, and the rate in substance abusers is even higher at 75%, compared to the general U.S. population who are HIV negative with a smoking rate of 20.8% [3, 51-52].

Very few studies have looked at a possible link between tobacco use and the effect on HIV infectivity in respect to non-nicotine tobacco constituents, primarily due to the shortened life expectancy of those living with HIV. With the recent developments of new anti-retroviral therapies, however, life expectancy has continued to rise. With the current continued use of these treatments in developing countries where the rate of HIV incidence is at its highest, the life expectancy of those living with HIV will also continue to rise. This introduces the need to evaluate and address other health concerns, like tobacco smoking, as a way to further extend life expectancy as well as increase the quality of life of those living with HIV/AIDS . This study has filled a void at the cellular/genetic level determining what genes are altered by tobacco constituents and how the gene expression patterns may affect the infectivity level of the HIV virus.

qRT-PCR analysis was used to confirm micro array results which indicate that there are eight genes whose expression is increased in the presence of a 1% TSE solution whose concentration mimics the plasma concentration levels seen in smokers. The results also indicate that there are four genes whose expression levels were decreased as a result of the TSE solution. It is important to note that these studies were performed in Jurkat T-cells, as T-cells are the primary target of the HIV virus. Gene expression studies have been performed on other cell types which show differing expression patterns. Maunders et.al. exposed tracheobronchial epithelium cells to cigarette smoke for one hour and genetic expression patterns were measured [53]. Gumus et. al. looked at expression differences in oral leukoplakia cells [54], and Rock et. al. examined TSE induced gene expression differences in HIV infected microglial cells [55]. The patterns of gene expression due to tobacco smoke vary among cell type making the study of gene expression changes in T-cells that much more crucial.

The changes in gene expression as a result of tobacco smoke, outlined in this research take a multi-pronged approach by examining the pathways in which they affect the infectivity of HIV. There are combinations of proteins which act within the same cellular pathways, for example, the down regulation of PHGPx and Thioredoxin Interacting protein. Both of which influence the NF- κ B pathway. It has been widely shown that the HIV LTR contains NF- κ B binding sites which are required for efficient transcription of HIV genes. NF- κ B, a pleiotropic transcription factor, in its dormant state exists as a dimer bound by I- κ B inhibitory proteins found in the cytoplasm. Upon cellular

stimulation, notably by the production of reactive oxygen species (ROS), I- κ B becomes phosphorylated inducing ubiquitination and the subsequent degradation of the inhibitory protein. After the degradation of I- κ B, NF- κ B will relocate to the nucleus in order to activate transcription. PHGPx, a glutathione peroxidase, has been shown to inhibit the (oxidative) cytosolic activation of NF- κ B. This in turn will inhibit HIV gene transcription. Thioredoxin Interacting Protein has been shown to bind the reduced form of Thioredoxin at the catalytic center, inhibiting its reduction potential [44]. Reduction in levels of Thioredoxin have been linked to the reduction of activated NF- κ B. Taken together, the results reported here show that tobacco smoke induces the down regulation of both PHGPx, and Thioredoxin Interacting Protein in jurkat cells, which in turn will increase levels of activated NF- κ B, which is beneficial in the transcription of HIV genes.

Other tobacco-induced gene expression changes affect independent pathways, from protecting infected cells or HIV viral particles from the host's immune responses, to enhancing vesicular trafficking within a viral infected cell. The gene expression changes shown here contribute in one way or another to the enhanced infectivity of HIV.

In conclusion, the results presented in this research have exhibited a link between the non-nicotine induced increase in HIV infectivity and the gene expression changes in jurkat cells as a result of tobacco smoke. This work provides a solid base on which further studies can be built. The need for more in depth dual and triple knockdown cell lines will begin to delve into the extent in which these genes work together to enhance viral infectivity. They may also provide insight into cellular targets for which agonists

and/or antagonists can be designed to decrease the level of HIV infectivity to an accepted level in those living with the virus. In addition, the effect on infectivity of other viruses with similar cellular pathways can be examined when these genes are either over or under expressed.

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APPENDIX A

CELL MEDIA PREPARATION

To make 500 mL of RPMI1640 Medium for Jurkat Cells

Starting with RPMI 1640, 1X Medium w/o L- Gln,

RPMI1640	435mL
10% FBS(fetal Bovine Serum)	50 mL
1% of 200 mM L-Glutamine	5 mL of 200 mM solution

1. Remove a 50 mL aliquot of FBS from the freezer and thaw in warm water.
2. Open a 500 mL bottle of fresh RPMI under sterile conditions
3. Remove 65 mL of media.
4. Add 5 mL of 200 mM L-glutamine from the -20 freezer
5. Add 50 mL (56.8 mL) of FBS
6. Mix well, label bottle with initials, date, contents, etc.
7. The media need not be filtered unless any of the reagents are not sterile (everything we purchase is cell culture grade)
8. Place 1 mL of newly prepared media in the incubator overnight to check sterility before storing cell, if it turns cloudy contamination has occurred.
9. Media will last about 2 months in the refrigerator.

APPENDIX B

PROCEDURE FOR COUNTING CELLS

Procedure:

1. Percuss the cells vigorously to dissociate the cells from each other.
2. Open the cell flask and remove less than 100 μL of the media (fill the tip only).
3. Place the sample in the centrifuge tube (if more than one flasks of cells is being counted be sure to label the tubes).
4. Place 50 μL of trypan blue in the empty tube.
5. To save plastic, reuse the pipette tip that was just used for the trypan blue and remove 50 μL of cells/media from the first bullet tube, being sure to mix the trypan blue and the cells well in the tube. (there is now a dilution factor of 2)
6. Take the mixture in the tube to the microscope.
7. Place a cover slip over the hemocytometer slide and insert 20 μL of the cell media/TB mixture in the small groove in-between the cover slip and the slide.
8. Place slide on the microscope and bring into focus the grid and cells.
9. Bring into focus the gridlines, use the 0.25 magnification which will narrow the sight to one field. Count a total of 4 different fields.

10. Counting cells:

A method is needed so that there is consistency to the count. If cells placement is on top of a gridline count only cells on left and upper gridline and ignore cells on bottom and right gridline or vice versa. Either way, be consistent.

Additionally, always move the slide in the same direction either clockwise or counter clockwise to count cells. Again, either way, be consistent.

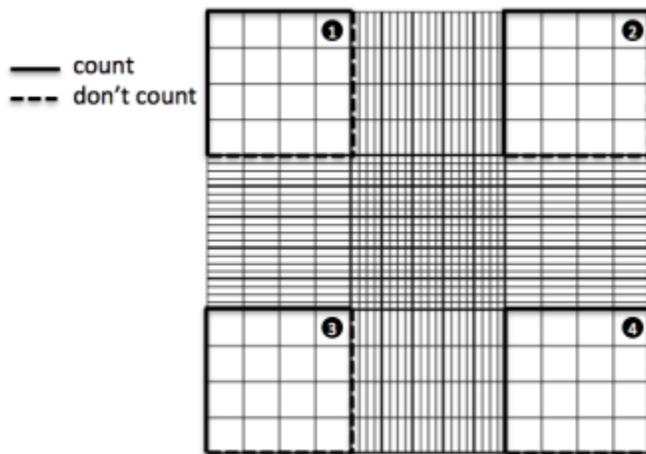
If there is difficulty focusing in on the gridline start with the lowest magnification (0.10) and graduate up in magnification power. 0.25 seems to work best. At this power one can see one field (4x4 squares) fully, making counting easy.

The number between squares would be within 5% if the cells are evenly distributed. Keep an eye out for dead cells (dark blue) if there are numerous dead cells it is time to start troubleshooting.

11. Using the counter, count number of cells in each 4x4 grid being sure to count all four of the 4x4 fields.

12. Take the total number of cells counted, and calculate the total number of cells in culture:

$$(\text{total \# of cells counted}/4) \times 2 (\text{dilution factor}) \times 10^4 \text{ cells/mL}$$



Cell senescence is characterized by changes in morphology and decreased growth rate.

APPENDIX C
FREEZING CELLS

1. Spilt cells into a T-75 in 15 mL of media and grow until cell count is 8×10^5 or 9×10^5 cells/mL.

2. Prepare media with 10% DMSO + 90% FBS.

3. Calculate the number of cryovials (externally threaded) that will be needed to freeze cells. Each cryovial should hold 2×10^6 cells

CALCULATION: (vol. media in T-75) x cell count (cells/mL) = # cells

cells/ 2×10^6 = # vials needed

4. Cryovials should be sterile and endotoxin free or autoclaved with drying to destroy endotoxin.

5. Prepare the appropriate amount of STERILE freezing medium to have 1 mL per cryovial.

The freezing medium is 7.5% DMSO in complete RPMI (with FBS) or the alternative mentioned above.

CALCULATION: # tubes x 1mL/tube = mL of media needed.

6. Keep media at 4°C (on ice) during procedure.

7. Fill cryocontainer with 100% isopropanol and be sure it is already cooled.

8. While medium is warming, label the cryotubes with the cell type, generation, cell number, date, your initials. Place clear tape over the labels to be sure that they don't get washed off in the liquid N₂.

9. Be sure all supplies are ready and that all labeled tubes are under hood with caps off and

ready to have cells added to them before putting cells in freezing medium (this is necessary because DMSO is toxic to cells and should only be combined with cells for the minimum amount of time).

10. Spin cells in Falcon tube at 1500 rpm for 5 min. (check to be sure pellet has formed)

11. Discard supernatant from cells and add the appropriate volume of freezing medium (calculated in step 5)
12. Pipet gently up and down to resuspend cells.
13. Aliquot 1 mL of cell mixture into each cryotube, replace lids.
14. Place in -20°C for 2 hours.
15. Move the vials to -80°C freezer and allow them to cool overnight.
16. Move cells from cryostorage device to liquid nitrogen in 4 -5 hr (although cells can survive up to 5 weeks at -80°C , better results are had with a shorter time).

APPENDIX D

THAWING CELLS

Note: Vials that have been stored in liquid N₂ can explode on warming if the vials are leaky. For this reason it is wise to wear face protection and gloves.

Be sure to test media for bacterial contamination by placing 5 mL in incubator for 24 hr.

Procedure

1. Turn on water bath to 37° C.
2. Prepare one T25 flask and one 15 mL falcon tube with 5 mL media each.
3. Remove cell vial from liquid N₂. Check that cap is securely fastened. Transfer immediately to 37° C water bath.
4. Swirl gently (bottom half only) in bath until barely thawed. Ensure lid is not submerged under surface of water bath. Be sure you have recovered any information recorded on the vial.
5. Submerge vial in beaker of ETOH before placing vial under hood (water bath is a major source of contamination). Be sure the cap is on tight. Change your gloves.
6. Under sterile hood, suck cells up in 1 mL pipette and transfer to falcon tube with 5 mL media already present. Rinse pipette up and down to facilitate complete transfer.
7. OR: Transfer cells immediately into 15 mL of media in T-25 flask place in incubator for 24 hr and then go to step 8.
8. Spin @1500 rpm (no more than 800 rcf) for 5 min, should see pellet of cells (if media is cloudy, spin longer).

9. Remove media and discard.
10. Add < 5 mL of media, gently rinse up and down in pipette to resuspend pellet. (If the cells have been frozen for a long period of time (decreased cell viability) best to suspend the cells in a smaller volume of media. You can always add more media in 24 – 48 hr)
11. Gently transfer into a T-25 falcon flask
12. Label the flask with the date, the cell type, and your initials.
13. Place in CO₂ incubator at 37.0 °C, 5.0% CO₂, 72% relative humidity.
14. Check cell count every 24 – 48 hours
15. After 20 passages, cells should be discarded.

APPENDIX E

GENELUTE DIRECT MRNA ISOLATION

Preparation Instructions

Before beginning the procedure, do the following:

1. Thoroughly mix reagents. Examine for precipitation. If any reagent forms a precipitate, warm at 55-65°C until the precipitate dissolves and allow to cool to room temperature before use.
2. Dissolve the proteinase K in the 40% glycerol. Use 0.5ml of 40% glycerol for 5 mg proteinase K (10 prep package) or 2.5 ml of 40% glycerol for 25mg proteinase K (70 prep package). Let sit at room temperature for a few minutes, and mix thoroughly before use. This product is stable at room temperature in its dry form, but store the proteinase K solution at 2-8°C.
3. Add the proteinase K solution to a sufficient volume of Lysis Solution for that day's use. The amount of Lysis Solution containing proteinase K required per preparation is 0.5 ml for cultured cells and 1.0 ml for tissue. Add 20µl of proteinase K solution per ml of Lysis Solution and mix thoroughly.
4. Transfer approximately 120µl of Elution Solution per preparation into a microcentrifuge tube and heat to 65°C in a heating block.
5. Ensure that the oligo(dT) beads are at room temperature and vortex thoroughly before use.

Procedure

1. **Harvest cells or prepare tissue.** For best yields of intact RNA, use only rapidly growing cells before they reach their maximum density or harvest tissue immediately from a freshly sacrificed animal.

- a. **Suspension cell cultures:** Count cells. Pellet up to 10⁷ cells for 5 minutes at 300xg.
Remove the culture medium completely and discard. Continue with step 2.
 - b. **Attached cell cultures:** Release attached cells with trypsin and pellet before lysis. To release cells with trypsin, see the protocol under Trypsin in the technical information within the Tissue Culture section of the Sigma Catalog. Count cells. Pellet up to 10⁷ cells and proceed as for suspension cultures.
 - c. **Mammalian tissue:** Quickly slice and weigh a piece of fresh or frozen tissue. Use 10 to 40mg per preparation. Transfer to an appropriate vessel for homogenization and continue with step 2. Note: Tissue may be flash-frozen in liquid nitrogen and stored at -70°C for several months before preparing RNA. However, do not allow frozen tissue to thaw before disruption in Lysis Solution.
2. **Lyse and homogenize cells/tissue.** This is a critical step that must be accomplished quickly and thoroughly.
 - a. **Pelleted cells:** Vortex the pellet to loosen the cells. Add 0.5 ml Lysis Solution containing proteinase K. Vortex or pipette thoroughly until all clumps disappear. Transfer the lysed cells into a GenElute filtration column (blue insert with a 2ml receiving tube) and spin for 2 minutes. Discard the blue filtration column, and continue to step 3 with the homogenized lysate (flow-through liquid).
 - b. **Mammalian tissue:** Add 1 ml of Lysis Solution containing proteinase K and homogenize immediately until no visible pieces remain. Continue with step 3.
 3. **Digest with proteinase K.** Incubate homogenized lysate at 65°C for 10 minutes to degrade nucleases and other proteins. After incubation, remove the tube(s) from the heating block, and proceed with steps 4 and 5.
 4. **Prepare mRNA for binding.** Add 32µl of the 5M NaCl solution to the digested cell lysate, or 64µl of 5 M NaCl to the digested tissue lysate. Note that the sodium chloride and oligo(dT) beads (step 5) may be added while the lysate is still warm.

5. **Bind mRNA to oligo(dT) beads.** Mix the oligo (dT) beads thoroughly by vortexing and inverting until resuspended and homogenous. Add 25 μ l of the resuspended oligo(dT) beads to the lysate-NaCl mixture, cap tube, and mix thoroughly by vortexing. Incubate the lysate/bead mixture at room temperature for 10 minutes. No mixing or rocking is necessary. During this incubation, the poly(A) tails of mRNA will hybridize with the oligo(dT) on the beads.
6. **Collect beads: mRNA complex.** Pellet the oligo(dT) beads: mRNA complex by centrifuging for 5 minutes at maximum speed. Carefully remove and discard the supernatant, leaving behind approximately 50 μ l to avoid disturbing the pellet.
7. **Release and rebind mRNA (optional).** For a more highly enriched mRNA preparation, bound material may be released from the beads into fresh lysis solution; mRNA is then rebound to the same beads. Add 0.5ml of Lysis Solution and 32 μ l of 5 M NaCl solution. Vortex thoroughly to resuspend the pellet. A series of short bursts on a vortex mixer are usually more effective than one long burst. Invert tube and inspect to verify complete release of pellet. Incubate suspension at 65°C for 5 minutes. Remove from heat and incubate at room temperature for 5 minutes. Repellet the bead: mRNA complex for 2 minutes. Remove and discard all but ~50 μ l of the supernatant as above. Note: This step may be omitted if rRNA contamination is not a concern, or if purification will be repeated as described in the note at the end of the procedure.
8. **First wash.** Resuspend the pellet in 350 μ l of Wash Solution by vortexing or pipetting. A series of short bursts on a vortex mixer will help release the pellet. Transfer the suspension into a GenElute spin filter-collection tube assembly by pipetting. Make sure that all suspension is expelled from the pipette tip and that the pellet is completely removed from tube. Spin for 1-2 minutes at maximum speed. Discard the flow-through liquid, but retain the collection tube.
9. **Second wash.** Pipette 350 μ l of Low Salt Wash Solution into the column. Spin for 1-2 minutes. Discard the flow-through liquid, but retain the collection tube.

10. **Third wash** .Pipette another 350µl of Low Salt Wash Solution into the column.
Spin for 1-2 minutes.
11. **First elution**. Transfer the spin filter into a fresh collection tube. Discard the flow-through liquid and the original collection tube. Pipette 50µl of preheated Elution Solution (65°C) onto the spin filter ensuring that it contacts the bead: mRNA complex. Incubate for 2-5 minutes at 65°C. Spin for 1-2 minutes. Save the flow- through liquid; it contains most of the purified mRNA.
12. **Second elution**. To maximize recovery of mRNA, pipette an additional 50µl of preheated Elution Solution (65°C) onto the bead: mRNA complex. Incubate for 2-5 minutes at 65°C. Spin for 1-2 minutes. The poly(A)+mRNA is now in the flow-through eluate (90-100µl total), and is ready for immediate use or storage at-70°C. Keep the mRNA on ice whenever it is thawed for use. Note: If a highly enriched preparation is desired, repurify the mRNA by adding 400µl of Lysis Solution, 32µl of 5 M NaCl solution, and a fresh 25µl aliquot of oligo(dT) beads to the 100µl eluted mRNA. Vortex and incubate 10 minutes at room temperature. Pellet the beads for 2minutes, then wash and elute as in steps 8 through 12 above.

APPENDIX F

SYBR GREEN JUMPSTART TAQ POLYMERASE

Optimizing Primer Concentrations

1. Prepare and dispense diluted primers (Fig 1).
 - a. Prepare 60 μL of 8 μM working solutions of both forward (fwd) and reverse (rev) primers in the first tubes of 2 separate 8-tube strips.
 - b. Dispense 30 μL of water into tubes 2-5.
 - c. Transfer 30 μL of the 8 μM primer solution from tube 1 into tube 2. Mix thoroughly by pipetting up and down at least 5 times.
 - d. Repeat transfer and mixing from tube 2 to 3, 3 to 4, and 4 to 5.
 - e. Using a multichannel pipettor, transfer 5 μL from the strip-tubes containing diluted fwd primer into the first 5 wells down columns 1-5 of a 96-well PCR plate. After adding fwd primer, PCR mix and template, final concentrations of fwd primer will be 1000, 500, 250, 125, 62.5 nM.
 - f. Similarly transfer 5 μL from the strip-tubes containing diluted rev primer into the first 5 wells across rows A-E. After adding PCR mix and template, final concentrations of rev primer will be 1000, 500, 250, 125 and 62.5 nM
2. Prepare qPCR master mix:

Add reagents below in an appropriate sized DNase-free tube. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.

Volume	Reagent	Final Concentration
520 μ L	2x SYBR Green JumpStart <i>Taq</i> ReadyMix	1.25 units <i>Taq</i> DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM MgCl ₂ , 0.2 mM dNTP, stabilizers
(7 μ L)	Rox Reference Dye	1x
q.s. to 676 μ L	Water	
676 μ L	Total Volume	

- Aliquot 26 μ L master mix into all wells in the PCR plate that contain primers (A1-E5)
- Mix Thoroughly and transfer 18 μ L from each of wells A1 through E5 to wells A8 through E12.
- Add 2 μ L template DNA (10-50 ng genomic DNA or 0.1-1 ng plasmid) to one set of reactions (columns 1-5) and 2 μ L of water to the other columns (8-12).
- Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.
- Perform Thermal cycling:

Typical cycling parameters for 100 bp – 600 bp fragments:

Initial Denaturation	95 °C	2 min
50 cycles:		
Denaturation	95 °C	15 sec
Annealing, extension, and read fluorescence	55 °C or 5°C below lowest primer T_M	1 min
Fluorescent Read		

- Evaluate fluorescence plots (DRn) for reactions containing target nucleic acid (columns 1-5). Primer combinations with the lowest C_t and the highest fluorescence will give the most sensitive and reproducible assays.

B. Procedure for Routine Analysis

1. Preparation of a reaction master mix is highly recommended to give best reproducibility. Mix all reagents but template in a common mix, using ~10% more than needed. Once template is diluted into the reaction vessel, master mix is aliquoted into the proper tube or plate for thermocycling.

Volume*	Reagent	Final Concentration
25 μ L	2 x JumpStart <i>Taq</i> ReadyMix	1.25 units <i>Taq</i> DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM MgCl ₂ , 0.2 mM dNTP, stabilizers
0.5 μ L	Rox Reference Dye	1 x
0.5mM	Forward Primer	Optimal Conc. from Sec. A
0.5mM	Reverse Primer	Optimal Conc. from Sec. A
1.0 μ L	Template DNA	100 ng
q.s. to 50 μ L	Water	
50 μ L	Total Volume	

* Volume for 50 mL reaction, however component volumes may be scaled to give the desired reaction volumes.

Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.

2. Perform Thermal cycling

Typical cycling parameters for 100 bp – 600 bp fragments:

Initial Denaturation	95 °C	2 min
50 cycles:		
Denaturation	95 °C	15 sec
Annealing, extension, and read fluorescence	55 °C	1 min
Fluorescent Read		

APPENDIX G

LUCIFERASE ASSAY

Protocol for Preparing Cell Lysates

1. Add 4 volumes of water to 1 volume of 5X lysis buffer. Equilibrate 1X lysis buffer to room temperature before use.
2. Carefully remove the growth medium from cells to be assayed. Rinse cells with PBS, being careful to not dislodge attached cells. Remove as much of the PBS rinse as possible.
3. Add enough 1X Cell Culture Lysis Reagent buffer (CCLR) to cover the cells (e.g., 400 μ l/60mm culture dish, 900 μ l/100mm culture dish or 20 μ l per well of a 96-well plate).
4. Rock 96-well plate several times to ensure complete coverage of the cells with lysis buffer.
5. Store the supernatant/cell lysate at -70°C or proceed to Section 4.

Protocol for Plate-Reading Luminometers

1. Program the luminometer for the appropriate delay and measurement times (1 second delay, and 1 second measurement)
2. Add 100 μ l of Luciferase Assay Reagent to each well.
3. Place the plate, containing cell lysate and Luciferase Assay Reagent per well, into the luminometer. The well is read, then the plate is advanced to the next well for a repeat of the delay-read process.
4. Measure the light produced for a period of 10 seconds. The light intensity of the reaction is nearly constant for about 1 minute and then decays slowly, with a half-life of approximately 10 minutes. The typical delay time is 2 seconds, and the typical read time is 10 seconds. The assay time may be shortened significantly to decrease the total read time if sufficient light is produced. For example, the total read time for all samples in a 96-well plate can be less than 5 minutes.