A Simple and Reliable Reporter Assay to Test siRNA-Mediated Silencing of HIV-1 Gene Functions

Kylie R. Tager and William H. Jackson*, PhD

University of South Carolina Aiken, Department of Biology and Geology

The human immunodeficiency virus (HIV-1) infects and kills $CD4^+$ T-lymphocytes causing a progressive loss of host immune competence, which ultimately leads to AIDS. RNA interference, as mediated by short interfering RNAs (siRNAs) designed to target viral mRNAs and expressed endogenously, offer a potential gene therapy approach to inhibit HIV replication. However, a simple and reliable method to screen the silencing activity of particular anti-HIV siRNAs is useful prior to conducting more extensive experimentation to determine the downstream effects on viral replication. Here, a short hairpin RNA (shRNA) targeting HIV-1 Rev (Revsh8526) was designed and cloned into an expression plasmid under the control of the RNA Polymerase III H1 promoter. To test the ability of Revsh8526 to silence Rev, a chimeric β-galactosidase reporter plasmid containing Rev exon 2 (β-galactosidase-RevE2) was generated. The ability of Revsh8526 to target and inhibit reporter gene expression was shown by X-gal staining and ortho-nitrophenyl-β-galactoside (ONPG) assay. The results of these assays indicated Revsh8526 effectively targeted and significantly inhibited β-galactosidase expression as compared to a non-targeted shRNA. These results show the utility of the chimeric β-galactosidase reporter in initial screening of siRNA reagents.

Introduction

The human immunodeficiency virus (HIV) is a lentivirus that infects and destroys $CD4^+$ T-lymphocytes (Gallo et al., 1983; Popovic et al., 1984; Barre-Sinoussi et al., 1983; Levy et al., 1984; Daigleish et al., 1984), resulting in a gradual loss of immune competence and increased susceptibility to various opportunistic infections (Pantaleo et al., 1993; Douek et al., 2002). The eventual outcome of HIV infection, the Acquired Immunodeficiency Syndrome (AIDS), occurs when an infected individual has one or more defined opportunistic infections and a $CD4^+$ T -lymphocyte count of less than 200/µl (CDC, 1993). Although current drug treatments control HIV replication and the clinical manifestation of AIDS, they are not curative (Shafer and Schapiro, 2008) due to a failure to eliminate viral reservoirs in latently infected cells (Schnittman et al., 1989). Therefore, there continues to be a need to investigate alternative means to attack the HIV problem.

RNA Interference (RNAi), mediated by short hairpin RNAs (shRNA), provides a potential strategy to inhibit HIV replication. Short hairpin RNAs are single-stranded molecules that, when expressed in cells, are recognized and cleaved into short, double-stranded small interfering RNAs (siRNAs) by the ribonuclease, Dicer (Song and Rossi, 2017; Agrawal et al., 2003). One strand of the newly formed siRNA is selected as the guide strand and associates with Argonaute to form the RNA-induced Silencing Complex (RISC) (Song and Rossi, 2017), which binds to and cleaves the targeted viral mRNA, thereby silencing the gene (Reynolds et al., 2004). Importantly, shRNAs can be introduced into cells and expressed exogenously by plasmids containing RNA Polymerase III promoters (Brummelkamp et al., 2002).

A potential target of RNA interference is the HIV-I Regulator of Virion Expression (Rev). Rev is one of three small accessory proteins produced early in the HIV replication cycle that functions to regulate viral gene expression (Heaphy et al., 1990). Rev mRNA is generated following splicing of two exons located within the viral glycoprotein (Env) coding region. Once translated, the Rev protein is localized to the nucleus where it interacts with the Rev Response Element (RRE) found within all HIV-1 primary transcripts (Malim et al., 1990). Rev binding is cooperative and results in binding of multiple Rev monomers to partially spliced and unspliced viral mRNAs. In addition to binding the RRE, Rev interacts with the host nuclear export machinery (Fischer et al., 1999), which results in the export of partially spliced and unspliced viral mRNAs out of the nucleus before splicing is completed. This activity leads to a switch from early to late phase gene expression, which is associated with host take over and virion assembly (Pollard and Malim, 1998). Therefore, strategies designed to inhibit Rev function are hypothesized to negatively effect viral replication.

The ultimate test of anti-HIV siRNAs is the ability to downregulate viral replication. However, HIV replication analyses come with increased risk due to the use of HIV genomic clones that produce progeny virions. Such assays are also more complex as they require a multi-step process involving the use of multiple cell lines to produce and quantify viral progeny. This risk can be minimized by first screening siRNAs for their ability to inhibit target expression, then moving forward with those that show high potential. Screening can be accomplished by creating chimeric reporter plasmids that include the particular siRNA target site. Using such plasmids, siRNAs that effectively facilitate cleavage of the target sequence, will in turn, result in decreased reporter gene expression. A number of reporter genes, such as Firefly and Renilla luciferase, β-galactosidase, and green fluorescent protein, are available for this purpose. Here, we describe the creation of a chimeric β-galactosidase reporter plasmid, in which Rev exon 2 was inserted into the 3' untranslated region (UTR) of the reporter gene. Importantly, due to its location within the UTR, the Rev coding sequence does not affect β -galactosidase expression. This chimeric reporter plasmid, when transcribed in transfected cells, was shown to be a target for an anti Rev exon 2 targeted siRNA. RNAi-mediated silencing was measured using two β-galactosidase assays that rely on enzyme subtstrates that when cleaved produce colored byproducts that are readily measurable. Taken together, this system provides a means to rapidly test anti-HIV RNAi reagents to determine efficacy prior to the use of more complex assays measuring virus replication.

Methods and Materials

shRNA Design

The Rev mRNA sequence of the HIV-1 genomic clone NL43 (Accession number M19921) was analyzed using the Integrated DNA Technology RNAi Design Tool (idtdna.com) to identify potential siRNA substrates. One site, located at nucleotides 8526-8546, was selected for further analysis (Figure 1). A shRNA was designed to target this site using the pSUPER RNAi system protocol (oligoengine.com). For this, the Rev target sequence was used to generate both sense and antisense strands — the sense strand being identical to the Rev target sequence. The two strands were linked by a 9-nucleotide spacer sequence, after which the design was completed by converting the ribonucelotide sequence to DNA and adding terminal BglII and HindIII sites for cloning. Revsh8526 (GAT CCC CCT TGA GAG ACT TAC TCT TGA TTG TTC AAG AGA CAA TCA AGA GTA AGT CTC TCA AGT TTT TA) and its complementary oligonucleotide (AGC TTA AAA ACT TGA GAG ACT TAC TCT TGA TTG TCT CTT GAA CAA TCA AGA GTA AGT CTC TCA AGG GG) were synthesized (Integrated DNA Technologies) and 200 pMol of each was annealed to form a double-stranded DNA. The two oligonucleotides were annealed by mixing in Annealing Buffer (10mM Tris, 1mM EDTA, 50mM NaCl), heating to 90°C, then slowly cooling to 4°C over a 45 minute period.

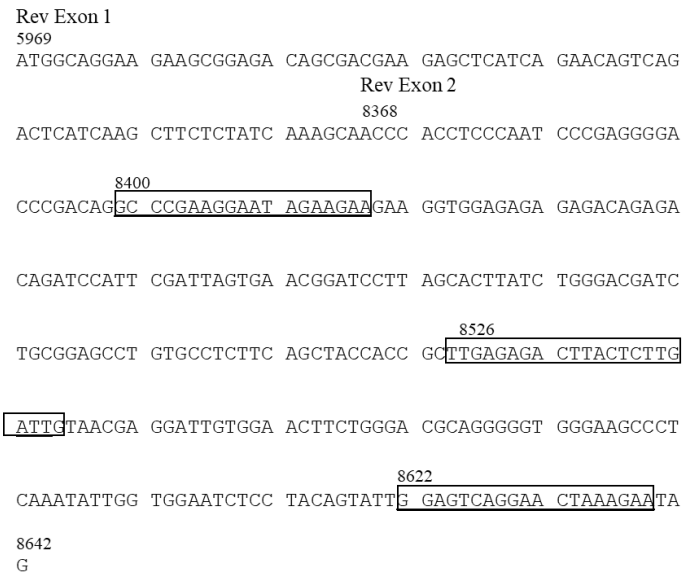

Figure 1. Rev exon 2 nucleotide sequence and siRNA targets. The HIV-1 NL43 Rev coding sequence was analyzed for potential shRNA target sites. Three potential sites are shown at nucleotides 8400-8418, 8526-8545, and 8622-8640. Rev 8526 was selected for analysis.

Recombinant plasmids

pH1Stuffer(-) contains the RNA Polymerase III H1 promoter and stuffer sequence from pSUPER.Retro-GPF/Neo (Brummelkamp et al., 2002). The 1.2 kb H1/stuffer sequence was amplified using Q5 Hot Start High Fidelity DNA Polymerase (New England Biolabs) and cloned into pCR4Blunt-TOPO (Invitrogen) generating a shRNA expression plasmid.

pH1.Revsh8526 was generated from pH1-stuffer(-). For this, pHI.Stuffer(-) was digested with *Bgl*II and *Hind*III to remove the stuffer sequence and produce restriction sites compatable with the annealed Revsh8526 oligonucleotides, which were subsequently ligated into the linearized plasmid. pH1.NTC was similarly generated from pH1Stuffer(-) by insertion of a non-templated control shRNA (NTC).

pCMV-β-gal.RevE2 contains the HIV-1 NL4 Rev exon 2 (RevE2) sequence within the 3' UTR of the β-galactosidase gene. The RevE2 sequence was amplified from the HIV-1 genomic clone, pNL4-3 (Adachi et al., 1986), using Q5 Hot Start High Fidelity DNA Polymerase (New England Biolabs) and primers designed for infusion cloning (RevE2IF forward: CTG GCG GCC GCT CGA GAC CCA CCT CCC AAT CCC G and RevE2IF reverse: TAG ATG CAT GCT CGA GCT ATT CTT TAG TTC CTG ACT CCA AT). The resulting Rev exon 2 fragment, corresponding to nucleotides 8369-8643 of pNL43, was cloned into the pCMV-β-gal *Xho*I site, located between the β-galactosidase stop codon and polyadenylation site.

Cells and Transfections

HeLa cells were cultured in Dulbecco's modification of Eagle's Medium (Corning) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin (Corning) and 1% L-glutamine (Corning). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator. Transfections were carried out 24 hours after initial plating using VIAfect Transfection Reagent (Promega) at a 4:1 reagent to DNA ratio.

Analysis of shRNA Activity Using a Chimeric β-Galactosidase Reporter Gene

Revsh8526-mediated silencing was analyzed using two chromogenic assays that allowed visualization and measurement of β-galactosidase activity. X-gal (5-brome-4-chloro-3-indolyl β-D-galactopyranoside) is a colorless substrate for beta-galactosidase that when cleaved forms 5-bromo-4-chloro-3-hydroxyindole, which dimerizes to form an insoluble blue product that can be visualized in cells using brightfield microscopy. X-gal staining is a rapid and relatively simple method to detect β-galactosidase expression in cells and was used in initial tests to visualize and quantify the ability of Revsh8526 to target RevE2 and reduce β-galactosidase expression. For this, HeLa cells were plated in 60 mm dishes ($5x10^5$/well) and co-transfected with DNAs corresponding to four treatments (Table 1). Each treatment included 0.5 µg pCMV-β-gal.Rev2 and either pH1.Revsh8526 or pH1.NTC at a 2:1 or 3:1 ratio. Treatments corresponding to a 2:1 ratio included 1.0 µg of either shRNA expression plasmid, while those corresponding to a 3:1 ratio included 1.5 µg of either shRNA expression plasmid. Total DNA in all treatments was brought to 2.0 µg by adding pH1Stuffer(-). In addition, each DNA mixture contained 0.05 µg pCMV-eGFP, which was used to monitor successful transfection. The transfected cells were stained with X-gal at 48 hours post-transfection.

Table 1. Plasmid combinations used in HeLa cell co-transfections.

	RevshRNA	Non-template control
2:1 ratio	0.5 µg pCMV-β-galRevE2 1.0 µg pH1Revsh8526 0.5 µg pH1Stuffer(-)	0.5 µg pCMV-β-galRevE2 1.0 µg pH1shNTC 0.5 µg pH1Stuffer(-)
3:1 ratio	0.5 µg pCMV-β-galRevE2 1.5 µg pH1Revsh8526	0.5 µg pCMV-β-galRevE2 1.5 µg pH1shNTC

50 ng pCMV-GFP was added to each treatment combination. If necessary, pH1Stuffer(-), which does not contain a shRNA, was added to bring all combinations to 2.0 µg of total DNA.

β-galactosidase expression in transfected cells was measured by X-gal staining based on the procedure of Lim and Chae (1989). For this, the culture medium was removed, and the cells washed two times with phosphate buffered saline (PBS). The cells were fixed by adding l mL PBS/0.2% glutaraldehyde/2% formaldehyde and incubated for 15 minutes at room temperature. Following removal of the fixing solution, the cells were washed three times with PBS prior to adding 1 mL X-gal stain (PBS/0.4 mM $K_4Fe(CN)_{63}H_20$/0.4 mM $K_3Fe(CN)_{63}H_20$/2 µM $MgCl_2$ with 16 µL/mL 2% X-gal in dimethyl formamide). The stained cells were incubated at room temperature for 24 hours to allow color formation, after which the staining solution was replaced with 1 mL of 70% glycerol. Then, X-gal positive (blue) cells were visualized and quantified using brightfield microscopy (200X).

An ONPG assay (Promega) was used to further examine Revsh8526-mediated silencing of β-galactosidase.RevE2. This assay utilizes ortho-nitrophenyl-β-galactoside (Lim and Chae, 1989), a colorless substrate of β-galactosidase that when cleaved forms a yellow product, ortho-nitrophenol. Briefly, HeLa cells were plated in 60 mm dishes ($5x10^5$ / well) and co-transfected with DNA combinations as described. Forty-eight hours post transfections, the cells were washed with PBS and lysed with 400 µL 1X Lysis Buffer. Lysates were transferred to 1.5 mL tubes and centrifuged at 14,000 rpm to remove cellular debris. The resulting supernatants were diluted in 1X Lysis Buffer, then 50 µl of each dilution was added to an equal volume of 2X Assay Buffer (ONPG) in a 96-well plate. After incubating at 37°C for 30 minutes, absorbance was read at 420 nm using a SpectaMax ABS plate reader.

Data Analysis

X-gal results were averaged from among the fields of view counted per treatment in each of three independent experiments, 15 fields in total. ONPG results were obtained from the mean absorbance of three replicates per sample. A Single Factor ANOVA analysis was used to determine if differences existed between treatments and a t-test was used to quantify observed differences.

Results and Discussion

Generating a shRNA/Reporter System to Analyze Anti-HIV Rev Activity

To determine the ability of shRNAs to target HIV-1 Rev, the HIV-1 Rev genomic sequence was analyzed for potential siRNA target sites using the Integrated DNA Technologies online siRNA analysis tool. The resulting analysis identified three potential sites (Figure 1). One of these,

located at nucleotides 8526-8545 was chosen for analysis. For this, a shRNA was designed and cloned into pH1Stuffer(-) creating pH1Rev8526, which placed the shRNA under control of the RNA Polymerase III H 1 promoter (Figure 2A). Similarly, a non-templated control (NTC) shRNA, designed using a randomly generated 22-nucleotide sequence, was synthesized and cloned into pH1Stuffer(-) creating pH1.NTC. Successful cloning was verified by sequencing (data not shown).

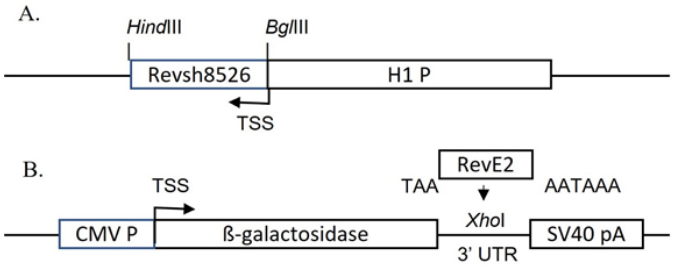

Figure 2. Plasmid construction. (A) pH1.Revsh8526 was cloned into pH1(-) Stuffer by insertion into the *Bgl*II and *Hind*III sites, placing shRNA expression under control of the RNA Polymerase III H1 promoter. pH1shNTC was similarly cloned by insertion of a non-tempated shRNA sequence. (B) The reporter plasmid, pCMV-ßgal expresses the ß galactosidase gene from the CMV promoter. To generate pCMV-ßgalRevE2, the HIV-1 Rev exon 2 sequence was amplified from pNL43 and cloned into the *XhoI* site located within the 3' UTR. Because of its location between the ß-galactosidase stop codon (TAA) and polyadenylation site (AATAAA), the RevE2 sequence is transcribed as part of the ß-galactosidase mRNA, but is not translated. TSS – transcriptional start site, TAA – Stop Codon, AATAAA – polyadenylation site, UTR – untranslated region.

The reporter plasmid, pCMV-β-gal expresses β-galactosidase under the control of the strong cytomegalovirus early intermediate promoter. To make the reporter gene a target for Revsh8526, the Rev exon 2 sequence was initially amplified from the HIV-1 genomic clone, pNL43. The resulting fragment was subsequently cloned onto the pCMV-β-gal *Xho*I site located within the β-galactosidase 3'UTR between the gene's stop codon and polyadenylation site (Figure 2B). β-galactosidase primary transcripts generated from this chimeric reporter plasmid, pCMV-β-gal.RevE2, will therefore include the Rev exon 2 sequence. Importantly, insertion of this sequence into the 3' UTR does not affect transcription or translation of the gene. However, shRNAs that target Rev exon 2, such as Revsh8526, are expected to induce cleavage of β-gal.RevE2 mRNAs and reduce overall expression.

Analysis of Chimeric Reporter Gene Expression Indicates Effective shRNA Targeting

To test the silencing ability of Revsh8526, a series of transfections were carried out in HeLa cells. Each series included treatments consisting of 2:1 and 3:1 ratios of shRNA (Revsh8526 or shNTC) to reporter target. Initial analysis of the ability of Revsh8526 to target Rev exon 2 and mediate silencing of β-galactosidase activity was measured by X-gal staining. Twenty-four hours post-transfection and prior to reporter analysis, GPF expression was observed to determine transfection uniformity. These observations showed equally high levels of GFP expression across all transfected cells (Figure 3). Importantly, because the relative amount of pCMV-eGFP was 10X less than the reporter plasmid and 20-25X less than the shRNA expression plasmids, GFP^+ cells were expected to contain both the reporter and shRNA plasmids. Forty-eight hours post-transfection, the cells were stained with X-gal to visualize β-galactosidase expression (Figure 3). Following X-gal staining, the relative levels of β-galactosidase expression were compared among treatments with Rev-targeted shRNA (Revsh8526) or non-targeted shRNA (shNTC). Initial observations indicated lower levels of β-galactosidase expression in treatments that included Revsh8526. These observations held for both the 2:1 (Figure 3A) and 3:1 (Figure 3B) ratios of shRNA to reporter plasmids. To quantify these

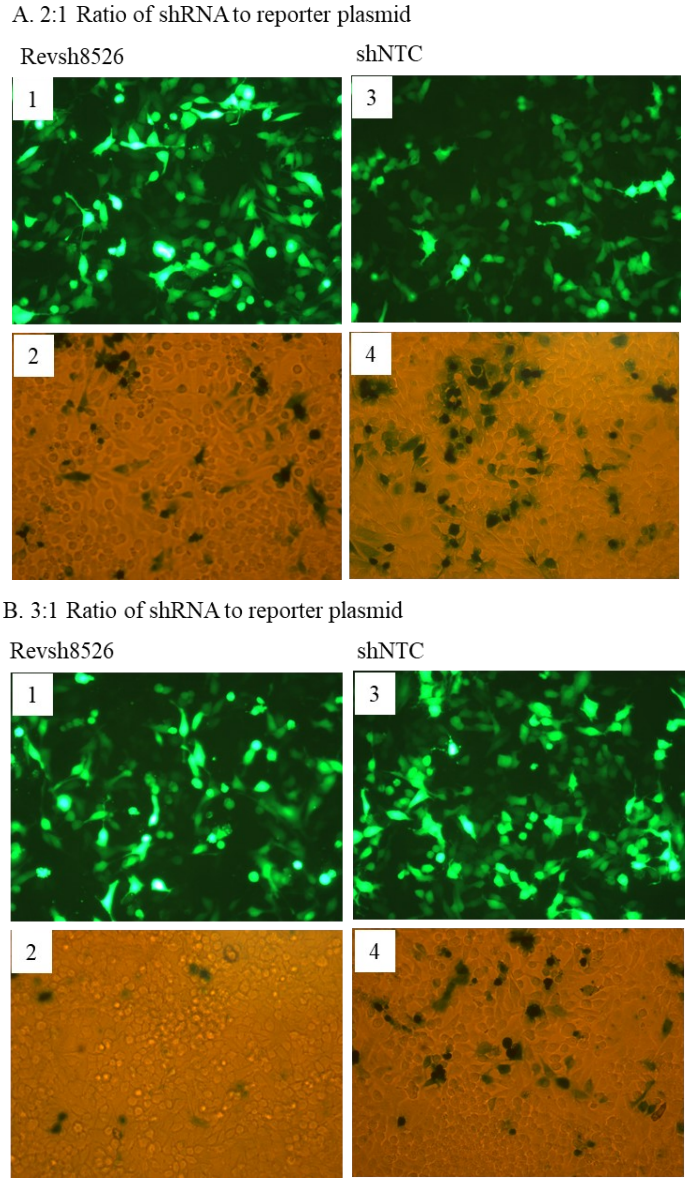

Figure 3. X-gal assay to quantify shRNA targeting of Rev exon 2. HeLa cell transfections were carried out at a (A) 2:1 or (B) 3:1 ratio of shRNA-expression plasmid (pH1Revsh8526 or pH1NTC) to pCMV-b-galRevE2. At 28 hours post-transfection, GFP was visualized by fluorescent microscopy. Forty-eight hours post-transfection ,the cells were stained with X-gal and positive (blue) cells were quantified using brightfield microscopy (200x). Shown are GFP^+ (1 and 3) and $X\text{-}gal^+$ (2 and 4) HeLa cells from representative fields of view.

results, the numbers of $X\text{-}gal^+$ cells were determined for each treatment. These results showed a significant decrease ($p<0.01$) in β-galactosidase expression in cells transfected with Revsh8526 at both the 2:1 and 3:1 ratio (Figure 4). An ONPG assay was used to verify these results and provide a less subjective measure of β-galactosidase expression. HeLa cells were transfected using a series of transfections as previously described for X-gal assays. After 48 hours, the cells were lysed, centrifuged to remove cellular debris, and diluted in Assay Lysis buffer. To measure β-galactosidase activity, three 50 µL replicate samples were taken from each lysate and transferred to a 96-well plate. Each lysate was mixed with 2X Assay Buffer and the plate was incubated at 37°C for 30 minutes before reading the absorbance at 420 nm. These results were in agreement with the X-gal results and indicated a significant decrease in β-galactosidase expression in treatments that included Revsh8526, as compared to shNTC, at both the 2:1 and 3:1 ratio of reporter plasmid to shRNA plasmid (Figure 5).

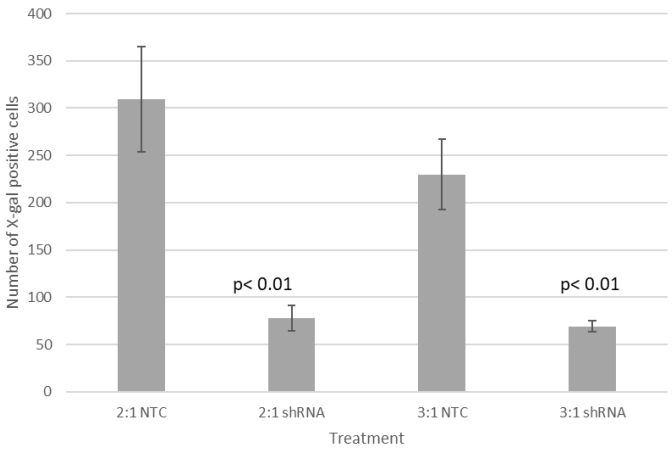

Figure 4. Revsh8526 decreases β-galactosidase activity. The number of X-gal positive cells across treatments indicates a significant decrease in X-gal expression in cells transfected with pH1Revsh8526 as compared to the non-template control at both a 2:1 shRNA to reporter ratio ($p<0.01$) and 3:1 shRNA to reporter ratio ($p<0.05$). NTC – non-targeted control, shRNA – Revsh8526.

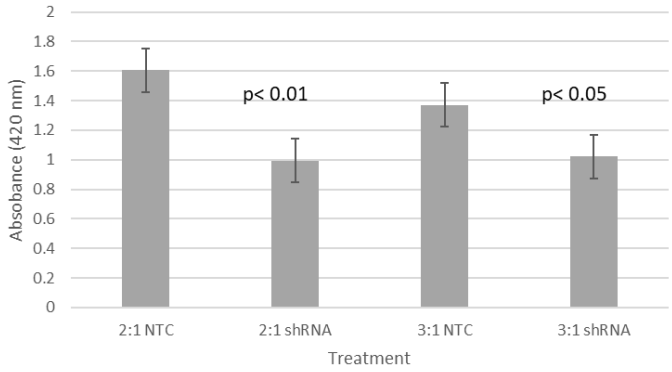

Figure 5. Revsh8526 decreases β-galactosidase activity. The level of ß-galactosidase activity as measured by ONPG assay indicates a significant decrease in X-gal expression in cells transfected with pH1Revsh8526 as compared to the non-template control at both a 2:1 and 3:1 shRNA to reporter ratio ($p<0.01$). NTC – non-targeted control, shRNA – Revsh8526.

Overall, these results demonstrate the utility of a chimeric β-galactosidase reporter assay as a simple and rapid initial screen to determine the efficacy of siRNAs targeted to HIV-1 genes, such as Rev. This reporter assay is an effective method for screening any number of siRNAs targeted to Rev exon 2. Moreover, the utility of the chimeric design allows substitution of various HIV-1 gene sequences, which in turn will allow for screening of appropriately targeted shRNAs. Although this chimeric system is useful in analyses of siRNA-mediated activity, the effects on reporter gene expression results cannot be extrapolated to an effect on viral replication. For this, more complex testing will be necessary. However, this chimeric reporter plasmid and assay does provide an important first step in the overall analysis of siRNA function.

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Notes and References

*Corresponding author email: billj@usca.edu

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