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Cloning BIV Tat sequences into the pFliTrx vector for use in peptide display

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CLONING BIV TAT SEQUENCES INTO THE PFLITRX VECTOR FOR USE IN PEPTIDE DISPLAY

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ABSTRACT

CLONING BIV TAT SEQUENCES INTO THE PFLITRX VECTOR FOR USE IN PEPTIDE DISPLAY

by Chun-Jung Chu

Bovine immunodeficiency virus (BIV) TAR-Tat peptide is a useful RNA-protein model which can be used to understand the interactions between RNA and proteins. Previous studies suggested that substitution of more flexible residues in the Tat peptide can increase binding by increasing amino acid access for contact with nucleotides in TAR. Calculations have predicted which peptide sequences have the best binding with TAR, but the calculations have not been confirmed by experiments. In this project, cDNA coding for the 14-mer and 17-mer peptide sequences in the BIV Tat binding site were designed and extended using mutually primed synthesis. The gene coding for 14-mer and 17-mer residue Tat sequences was first inserted into the pGEM®-T Easy vector, double digested with ApaI and XhoI, and cloned into the pFliTrx vector. This vector can be transformed into *E. coli*. The resulting peptide display of the Tat sequences will allow the binding strength between wild-type BIV TAR-Tat to be measured.

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There have been so many people who provided guidance, advice, education, training, and support as I worked on this research. My professors, Dr. Melanie McNeil, Dr. Elaine Collins, and Dr. Brooke Lustig, have taught me a lot and shaped me professionally. I deeply appreciate all of you. My teammates at Dr. Collins' laboratory have helped me much, and I thank each of you.

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CHAPTER ONE
INTRODUCTION

Acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV), was first recognized in the early 1980s and has become a major worldwide epidemic. HIV is a lentivirus, an infectious retrovirus that inserts virus RNA into the genetic structure of the body cells, propagating itself to weaken host immune responses and to overpower the immune system [1]. The immune system loses its function along with certain immune cells-CD4 T cells [1,2]. The HIV virus binds to CD4 T cells and becomes internalized as it systemically destroys the immune system. HIV latency in humans, defined as the time it takes to get a response from the HIV, can arise during normal CD4 T cell differentiation in the thymus which was indicated by Brooks *et al.* in 2001 [3]. Gene expression was examined in latently infected cells. HIV infects the cells of the immune system within a short period of time, and allows the spread of the HIV infection to others. During the latency, the virus RNA is reverse transcribed, and viral DNA integrates into the host DNA, enabling further replication. Once the viral DNA is incorporated into the host genome, the cell's own machinery then replicates the virus, eventually destroying the cell. The viral regulatory protein, the trans-activator protein (Tat), regulates transcription of the HIV genome at the level of elongation when the virus is no longer latent. Therefore, Tat is an important element of the progression of HIV disease.

The life cycle of the HIV begins when the retrovirus enters the host cell, integrates into the host genome, and reproduces its own genome and assembles into retrovirus particles, shown in Figure 1.

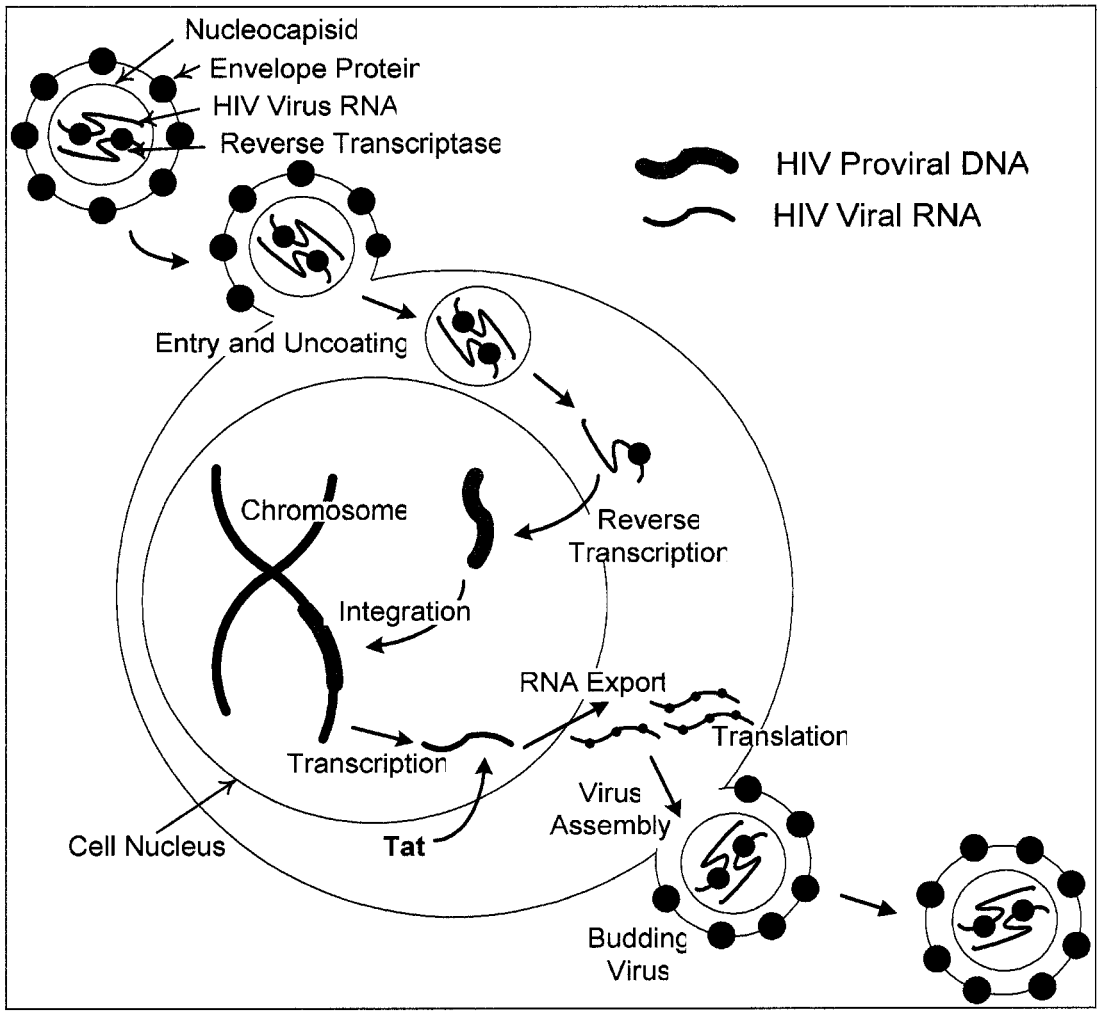

Figure 1. A typical wild-type HIV life cycle. Retroviral particle attaches to the host cell through envelop proteins. Once the retrovirus inserts virus RNA into the cell, the virus RNA is reverse transcribed into DNA through reverse transcriptase, and the DNA integrates into the host DNA to become proviral DNA. After the latently infected cell is activated, transcription occurs followed by viral protein synthesis, and the new retrovirus particles are formed.

Most retroviruses consist of two single-stranded RNA molecules and reverse transcriptase enzymes that are contained within a protein shell surrounded by a lipid membrane with envelope proteins [4]. The envelope proteins play an important role in viral infection because they are responsible for the attachment to and fusion with target cell membranes. After the retrovirus inserts virus RNA into the target cell, the virus RNA generates a double-stranded DNA through reverse transcription. The double-stranded viral DNA then passes into the nucleus of the infected cell, integrates with the host cell genome, and lives temporarily within the chromosomes of the infected cell. The proviral DNA is replicated along with the cell's own DNA for the expression of viral genes to produce the HIV proteins [4]. Once integration has occurred, the integrated provirus can remain transcriptionally silent until appropriate signals activate gene expression. The integrated provirus must wait for more materials it needs to complete the reproductive process [5]. Significant transcription of the proviral genome requires the viral genes of the HIV regulatory protein Tat in the nucleus. The Tat gene encodes a protein that accelerates transcription, and there is an increase in the levels of long transcripts [5]. Activation of the latently infected cell results in the transcription of viral DNA into mRNA that can be read by the host cell's protein-making machinery. After HIV mRNA is processed in the nucleus, viral proteins are synthesized, and the next generation of retrovirus particles is formed [4].

HIV gene transcription, which is regulated by the activity of the HIV promoter contained within a region for stimulation of gene expression directed by the long-terminal-repeat (LTR), was first described by Sodroski *et al.* [6]. They discovered the

LTR region is located at the 5' end of the DNA, and the transactivation of the transcription is caused by the presence of the trans-activating factors, the key HIV regulatory proteins Tat and Rev [6].

Figure 2 illustrates the genome organization of the human immunodeficiency virus (HIV) [7]. The HIV genome is encoded on the single-stranded RNA when it is a free retrovirus particle. After the integration, the information is encoded in the host's DNA as a provirus DNA [8]. Figure 2 shows how the genes of HIV are located in the central region of the proviral DNA. It has nine open reading frames but encodes more than nine proteins [8]. These proteins fall into different categories including: *gag*, *pol,* and *env* as the major structural proteins, meaning they are incorporated into virus particles; *tat* and *rev* as the regulatory proteins are required for a growth process such as gene expression; and *vpu*, *vpr*, *vif*, and *nef* as the accessory proteins can be removed from the virus genome without affect on growth in laboratory conditions [7]. Both ends of the HIV genome are flanked by LTR which is not a gene but a sequence for regulatory purposes [9]. HIV gene expression requires virally encoded trans-activating factors, and Tat plays an important role during the HIV infection by binding directly to the transactivation response region (TAR) of the viral mRNAs in order to stimulate transcription from viral LTRs thereby enhancing the elongation [7,10]. The HIV promoter is transcribed by RNA polymerase II [10]. The HIV LTR promoter contains several binding sites for different cellular transcription factors, such as nuclear factor κB (NFκB) and stimulating protein 1 (SP1), which bind within the first 100 bp of the transcriptional initiation site [11,12]. A promoter that only contains the TATA box and the TAR element is insufficient to allow

efficient transcription of HIV; therefore, the addition of the sites for NFκB and Sp1 are required for maximal transactivation by Tat [13]. They stimulate the activity of the HIV LTR and associate with RNA polymerase II, as well as the Tat protein [10,11].

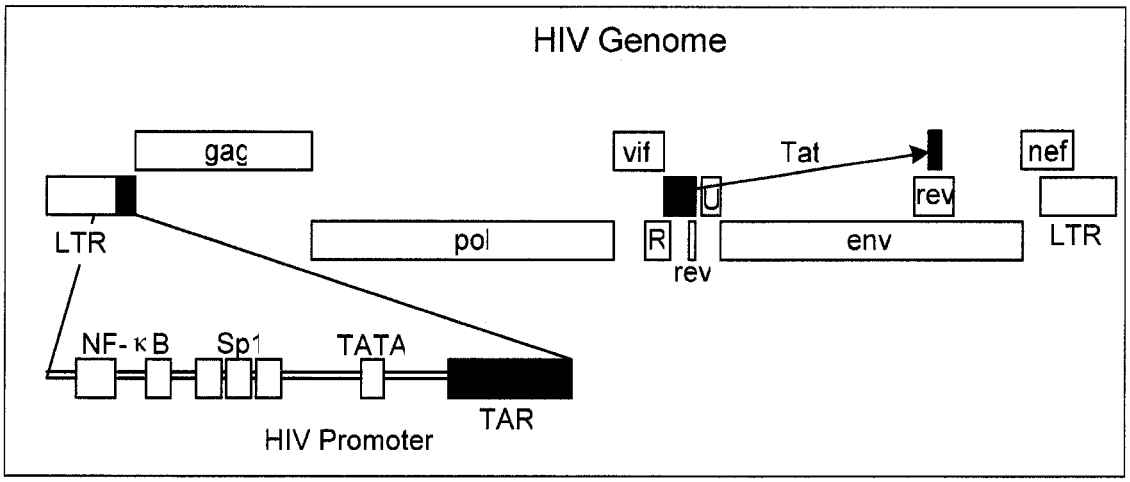

Figure 2. The genome organization of HIV and the HIV viral promoter. Tat protein is encoded by the two exons, which are highlighted in red, that partially overlap exons for the regulator of virus expression (rev) and envelop (env) genes. The HIV promoter is transcribed by RNA polymerse II. The HIV promoter has two tandem NF-κB binding sites and three tandem SP-1 binding sites. Structurally, these two are positioned before the TATA binding site and the transcriptional initiation site. Source: [7,8]

Trans-activator protein, Tat, is one of the key elements of lentiviral transcription. Tat is a small peptide and its structure contains a transcriptional activator and includes an activation domain and a nucleic acid binding domain [14,15]. Tat function is dependent on a stem-bulge-loop trans-activation-response (TAR) RNA element [7,13,16,17], which is located downstream of the transcriptional initiation site. TAR is only functional when it is placed downstream of the start of the transcription and in the correct orientation [7].

HIV uses specific viral RNA structures to control viral replication. Tat is the transactivator that acts through an RNA Target, the TAR RNA, which is located at the 5' end of the LTR [13]. TAR plays a key role for transmission of the genetic information. Each viral mRNA carries a copy of TAR at its 5' end [13]. HIV TAR consists of 59 nucleotides forming a stable stem-bulge-loop structure, consisting of a loop of six nucleotides, a bulge of three nucleotides, and two strands of complementary sequence [7,13,16,17]. The three-nucleotide bulge is separated from the six-nucleotide-loop by an upper stem [7,13,16,17], and the bulge induces a bend in the RNA helix that widens the major groove to allow for Tat binding [13,17,18,19]. Some studies showed that the hexa-nucleotide loop is also important for the Tat binding because mutation of this region inhibits transactivation [16,18,19].

HIV gene expression depends upon the interaction between viral regulatory proteins (Tat) and specific RNA target sequences (TAR). A major function for TAR is to recruit Tat to the retrovirus LTR where it can transactivate transcription by interacting with the RNA polymerase II transcription complex [17,20]. RNA polymerase II, a large complex of 12 subunits, transcribes genetic information into a message that can be read by the ribosome to produce protein [21]. The Tat-TAR binding interaction is through electrostatic interactions [17,20] between the basic domain of Tat and the negatively charged phosphates of the RNA bulge and also through a hydrogen bond in the groove of the RNA [17,20]. Tat acts by a complex mechanism. Several groups demonstrated experimentally that Tat binds specifically to the bulge of the TAR and recruits the cellular protein cyclin T (CycT) and its associated cyclin-dependent kinase (CDK9)

[22,23,24]. CycT increases the affinity of Tat for TAR [25,26]; however, Tat binds to cycT, cycT binds to CDK9 but Tat does not bind to CDK9. CDK9, part of a transcription elongation complex, was found to bind to the activation domain of Tat, and the phosphorylate carboxyl-terminal domain (CTD) of RNA polymerase II [22,23,24,25,26]. This multi-protein-RNA complex (Tat/CycT/CDK9/TAR RNA) is the functional complex for Tar-TAR interaction [20,21,22]. Phosphorylation of the CTD of the RNA polymerase II is essential during transcription and mRNA metabolism [22,23,24,25,26].

The model for Tat stimulation of transcriptional elongation and the requirement for TAR are shown in Figure 3. If Tat is present and assembles on TAR, CDK9 is able to phosphorylate the CTD of the RNA polymerase II. Tat then stimulates transcriptional elongation of mRNA and produces a significant increase in the amount of longer transcripts [14,15,26]. However, without Tat binding to TAR, elongation is inefficient because of hypo-phosphorylation of the CTD of RNA polymerase II. RNA polymerase II transcriptional complexes assembled on the DNA pause, disengage, and release short mRNAs that contain TAR [14,15,26].

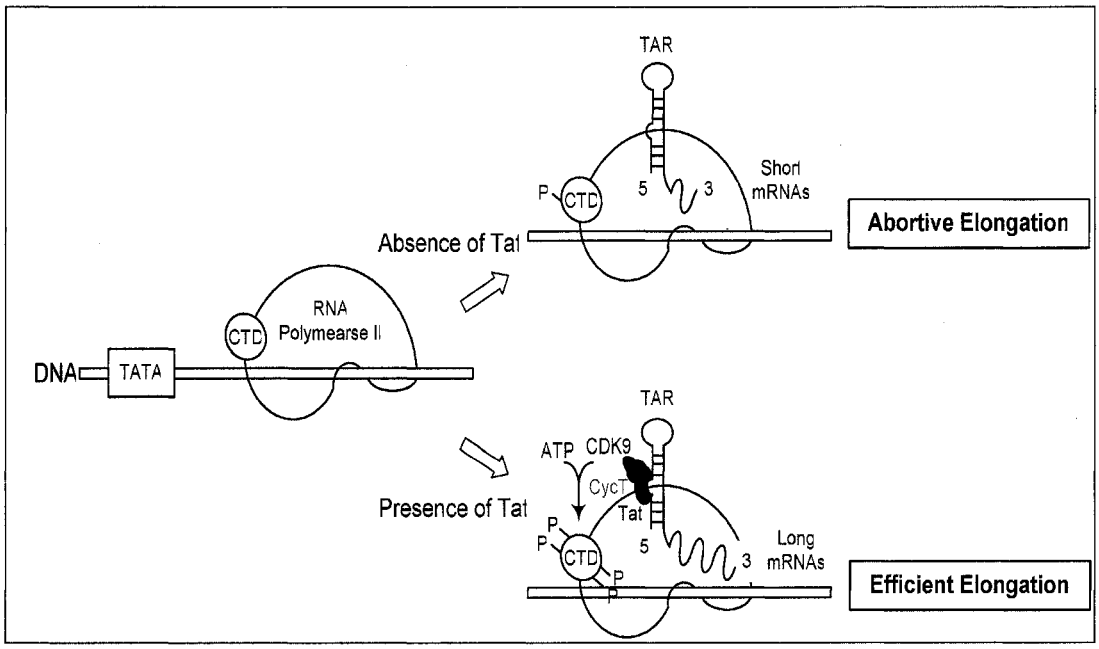

Figure 3. The control of transcription elongation by Tat. (Top) In the absence of Tat, short transcripts terminate near the end of the viral LTR. (Bottom) In the presence of Tat, there is a significant increase in the levels of the viral mRNAs found downstream of the promoter.

Bovine immunodeficiency virus (BIV), a member of the same lentivirus subfamily of retroviruses as HIV, was first isolated by Van Der Maaten *et al.*, from infected cows with persistent lymphocytosis, lymphadenopathy, and central nervous system lesions [27]. Like other retroviruses, once the proviral DNA integrates into the host cell genome, BIV establishes a permanent infection. BIV is related to human immunodeficiency virus types 1 and 2, as well as the simian immunodeficiency virus [24,28]. Studies have used BIV as a model for HIV since it is a simpler system, presents fewer human risks and can be studied more easily in the laboratory [28,29]. Figure 4 compares the genome organization of the human immunodeficiency virus (HIV) with bovine immunodeficiency virus (BIV) [28]. The major required retroviral structural genes identified in the HIV and

BIV genomes are *gag*, *pol*, and *env* genes. The non-structural accessory genes identified in the HIV genome are the viral infectivity factor (*vif*), trans-activator of transcription (*tat*), regulator of virus expression (*rev*), another potential trans-activator (R), the virus release factor (U), and the negative factor (*nef*). As for BIV, *vif*, *tat*, *rev*, *vpy*, and *vpw* genes are the non-structural accessory genes that reside between the *rev* and *env* genes. The *tat* gene is encoded by two exons that are highlighted in red on both the HIV and BIV genome maps. Previous studies have demonstrated that the structural *gag*-encoded capsid protein (p26), *env*-encoded surface (SU) and transmembrane (TM) proteins are highly immunogenic because they have been shown to induce the production of specific serum antibodies to BIV [30]. In addition, the bovine immunodeficiency virus TAR-Tat is an RNA-protein model system for understanding the interactions between RNA and proteins because it is a simple and well-characterized system.

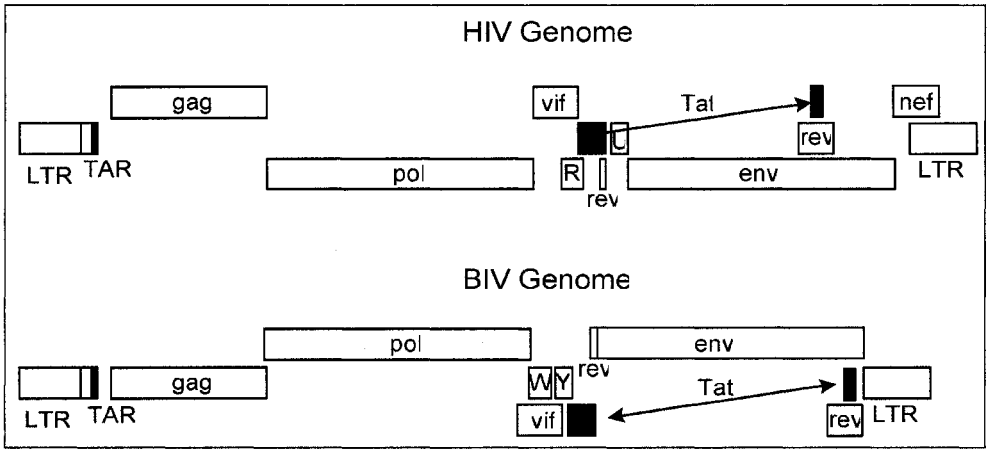

Figure 4. The genome organization of HIV and BIV. The major required retroviral structure genes identified in the HIV and BIV genomes are gag, pol, and env genes. The non-structural accessory genes identified in the HIV genome are vif, tat, rev, R, U, and nef. The non-structural accessory genes identified in the BIV genome are vif, tat, rev, vpy, and vpw. Source: [7,8,28]

Figure 5 illustrates the fragment of the BIV TAR and Tat sequence. NMR studies of the un-bond BIV TAR RNA demonstrate that it folds into a stem-bulge-loop [31,32], as shown in Figure 5A. The BIV Tat protein can be divided into several segments: N-terminal, cysteine-rich, core, basic (where it binds TAR), glutamine-rich, and C terminal [32]. However, the high affinity and specificity of TAR binding can be narrowed down to a minimal arginine-rich domain [31,32,33], as shown in Figure 5B. Nucleotides that are important for protein binding are boxed. Amino acid residues of the BIV peptide sequence that are critical for binding to the TAR RNA are in bold. Therefore, the 3 major RNA base-amino acid contacts are Arg70-G14, Arg73-G11, and Ile79-U10. More detail about the BIV TAR RNA-Tat peptide residue binding will be discussed in greater detail in the next chapter.

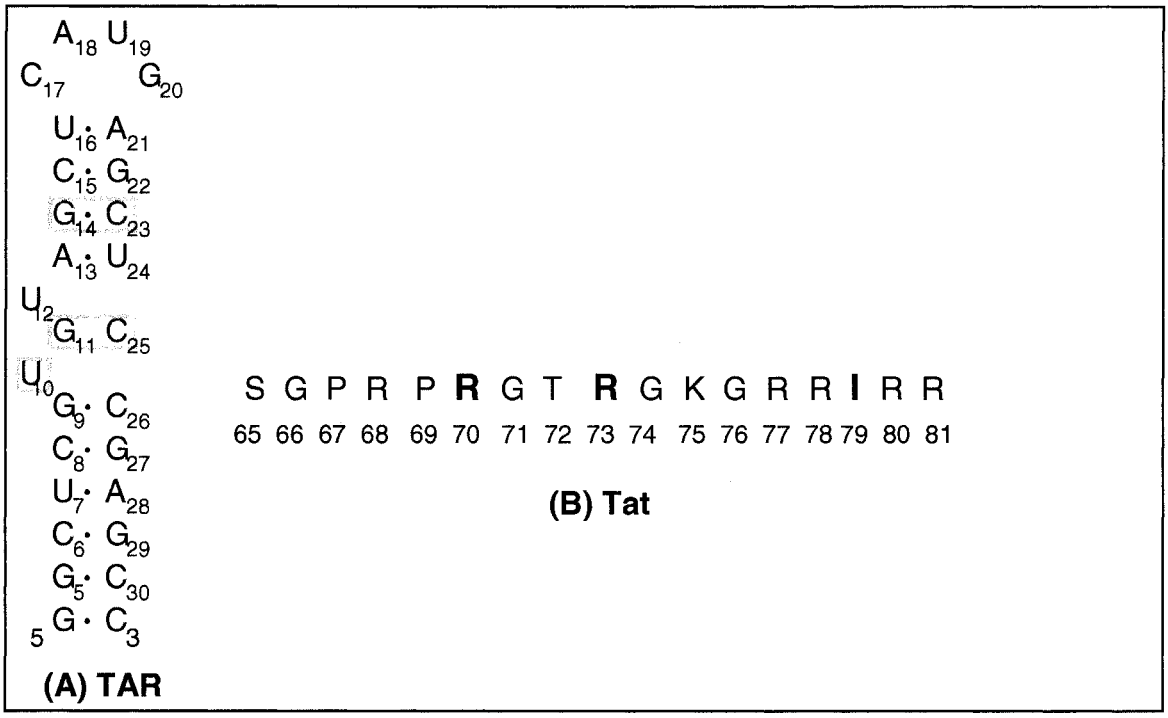

Figure 5. Fragments of the BIV TAR and Tat sequence. (A) is a fragment of the BIV TAR RNA sequence; numbering corresponds to nucleotide positions in BIV mRNA. (B) is the sequence of the BIV Tat peptide; numbering corresponds to positions in the intact protein. Nucleotides that are important for protein binding are boxed. Amino acid residues of the BIV peptide sequence that are critical for binding to the TAR RNA are in bold.

CHAPTER TWO
LITERATURE REVIEW

The binding of Tat to TAR is important for Tat to function as a transcriptional activator. By interacting with the TAR RNA of the transcribed mRNA, Tat stimulates transcription of the integrated provirus. In 1992, Puglisi *et al.* performed NMR studies on wild-type and mutant TAR RNAs in the presence or absence of arginine analog and determined that the bulge of HIV TAR RNA (U23 and A27·U38) stabilizes arginine hydrogen bonding to G26 and phosphates [31]. It was found that the BIV encodes a TAR similar to that of HIV-1 and is an analogous Tat protein that contains an arginine-rich RNA-binding domain (residues 68 to 81) that is closely related to the activation domain [34]. However, unlike HIV, the BIV RNA-binding domain recognizes BIV TAR with high affinity and specificity in a loop-independent manner and in the absence of cyclin T1 or other cellular proteins [31].

Some studies have shown that specific base pairs G11·G25 and G14·C23 of the BIV RNA, as well as a bulged U10, are involved in the binding of the BIV Tat peptide, both *in vitro* and *in vivo* [35,36,37]. Additionally, the BIV Tat peptide residues Arg70, Arg73, and Ile79 are also important [35,36,37]. Two independent groups [34,38] concluded that specific contacts exist between TAR and Tat using NMR solution studies of BIV TAR-Tat binding. Global features of both structures are similar, and the TAR fragment had the same characteristics in both studies. The Puglisi group described the structure of the BIV Tat-TAR peptide-RNA complex where the peptide not only adopts a β-turn conformation, but resides in the major groove of the RNA [34]. Moreover,

specific contacts are apparent between critical amino acids in the peptide and bases and phosphates in the RNA [34]. However, the differences in both the Puglisi and Ye structures are the formation of the U-A-U base triple, the dynamic flexibility of the Tat peptide, the interactions at the binding interface, and the calculation of the relative binding of different Tat peptide mutants to TAR RNA [32,39]. These differences exist in the RNA protein contacts between the two structures. The Puglisi group's 14- residue Tat peptide is three amino acids shorter in size at the N-terminus than the peptide used in the Ye group's. These additional residues do not contribute to RNA affinity, although they do contact the hairpin loop of the TAR RNA [37].

In 1997, Lustig *et al.* used frequencies of contacts between RNA bases and amino acids to derive relative interaction energies from the RNA-protein complexes [40]. They suggested the strongest RNA interaction is between arginine and guanine; in addition, less bulky residues in Tat increase flexibility [40]. They computationally investigated the flexibility of bulges of various sizes and determined that the original higher flexibility of the TAR may optimize intermolecular interaction for the bound state, and the resulting relatively high probability of the bound conformation may possibly contribute to the observed correlation [40].

Utilizing the previous study of interaction potentials [40,41], Lustig *et al.* determined the relative amino acid flexibility of BIV TAR-Tat from RNA base-amino acid contact maps of twelve million lattice chains [42]. They calculated the energies for the wild-type sequence of the Tat binding peptide as well as a set of eleven BIV Tat peptides and TAR fragments. They successfully demonstrated the results of contact

frequencies for wild-type BIV-RNA bases with Tat peptide amino acids using an approach which identified potential sites that are amenable to substitution of more flexible residues which enhance peptide binding to RNA targets [42]. They suggested the possible substitutions to enhance flexibility are Lys75 to Gly75 and Arg78 to Gly75.

Collins *et al.* used gel shift assays, using BIV TAR RNA fragment (nucleotides 4-31) and BIV Tat peptide (residues 65-81) or a variant peptide (Lysine at 75 position was substituted with Glycine), to demonstrate the theoretical calculations. The relative binding affinity can be determined by comparing the intensity from the gel shift assays. The result indicates that the binding characteristic of the variant peptide including Glycine substitutions at non-contact positions for the purpose of increasing is better than the BIV wild-type Tat. Tat peptide flexibility enhances binding to the TAR fragment.

It has become an increasingly popular strategy in various applications to display foreign proteins on the surface of microorganisms that can also be enabled by means of recombinant DNA technology. Millions of short peptides can be easily examined by binding an antibody, receptor, or protein using a combinatorial library. Bacterial surface display is one of many new combinatorial gene expression technologies. Through using this technology, bacteria have been employed as a tool to display short peptides that are capable of specific binding [43,44]. The foreign molecules can be displayed through genetic in-frame fusion with the bacterial flagellum, and could then be utilized as a carrier for transportation of DNA into a cell [43,44]. In 1995, Lu *et al.* developed a system for exploring protein-protein interactions which display random peptides in the exposed domain of thioredoxin (trxA) which is fused to the flagellin gene (fliC) that

targets the fusion protein to the bacterial membrane [45]. The FliTrx peptide display library can be utilized in this study to identify BIV TAR RNA-Tat peptide binding specificity.

CHAPTER THREE
RESEARCH OBJECTIVES

The research objectives of this project are to design the primers coding for 14-mer and 17-mer residue Tat sequences, extend the primers using mutually primed synthesis, and insert the resulting cDNA into the pFliTrx vector. For the rest of the thesis, the cDNA sequences will be referred to as 14-mer Tat DNA and 17-mer Tat DNA, respectively, for the purpose of identification.

CHAPTER FOUR
EXPERIMENT METHODS AND MATERIALS

4.1 Overview of Experimental Methods

Figure 6 illustrates the overall flowchart of the experiment techniques used in this research.

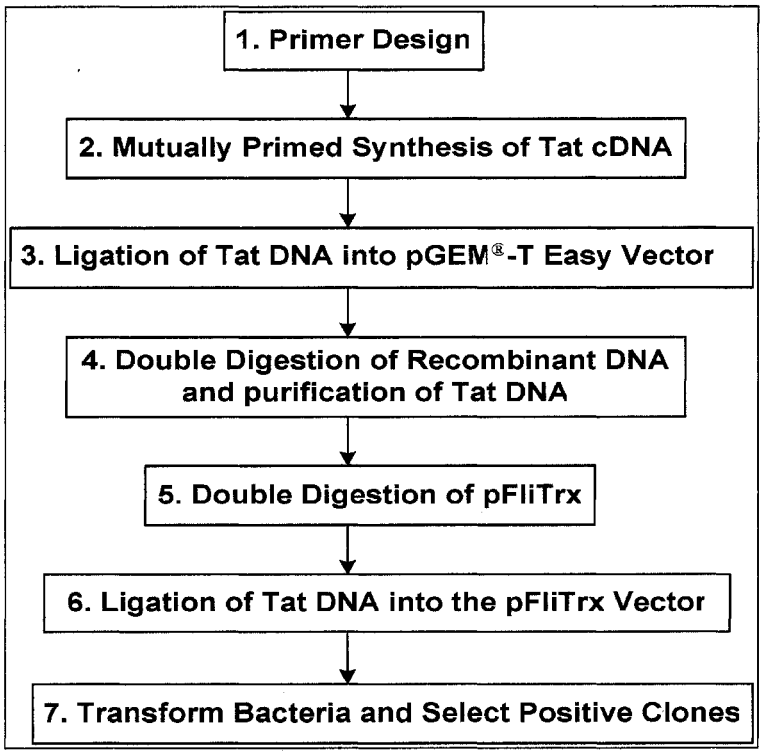

Figure 6. An overview flow chart of the experimental techniques used in this research.

In step one, 14-mer and 17-mer residue Tat primers were designed based on previous reports of the BIV TAR-Tat active binding region [34,38]. In step two, each primer was extended using mutually primed synthesis. In step three, the prepared 14-mer and 17-mer Tat DNAs were individually cloned into the pGEM®-T Easy vector in order

to improve the subsequent restriction enzyme digestion. In step four, a double digestion was preformed on the pGEM®-T Easy containing the Tat DNA insert. The restriction enzymes, ApaI and XhoI, were used, and the resulting products were run on an agarose gel and purified. In step five, a double digestion was performed on the pFliTrx with ApaI and XhoI to open the multiple cloning site. In step six, an insertion of the Tat DNAs into the pFliTrx vector was performed to obtain recombinant DNA. In step seven, the recombinant pFliTrx vector was transformed into *E. coli* followed by selection of positive clones.

4.2 Materials

Table 1. Buffer and Solutions.

Buffers and Solutions	Ingredients and Concentration
Ampicillin Solution	100 mg/mL (Ampicillin Sodium Salt-Mediatech). Filter sterilize using 0.22 μM filter.
Chloroform	Chlorofor/Isoamylalcohol: 24/1
LB Broth	10 g Tryptone (Bacto), 5 g Yeast Extract (Bacto), and 10 g NaCl (Sigma) per 1000 mL DI water. Autoclave sterilize 20 to 30 minutes.
NZY+ Broth	11 g NZCYM powder (which is for use of maintenance and propagation of bacteriophage lambda) (Difco) per 500 mL DI water, 12 mM $MgSO_4$, 0.02 M glucose. Filter sterilize using 0.22 μM filter.
Phenol	Buffered in Tris buffer to pH8.
Sodium Chloride	5M
TAE 1x Buffer	40 mM Tris, 40 mM Glacial acetic acid, 1 mM EDTA (Trisodium Ethylenediaminetetraacetate Trihtdrate)
TBE 1x Buffer	89 mM Tris, 89 mM Boric acid, 2 mM EDTA
TE 1x Buffer	10 mM Tris-HCl, 2 mM EDTA

Table 2. Plates.

Plates	Ingredients
LB/Ampicillin Plate (The formula is prepared with 1L distilled water)	10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g Sodium chloride, 15 g Bacto-agar. After autocleaving and cooling to 50°C, add ampicillin to final concentration of 100 μg/mL.
LB/Ampicillin/IPTG/XGal Plate (The formula is prepared with 1L distilled water)	10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g Sodium chloride, 15 g Bacto-agar. After autocleaving and cooling to 50°C, add ampicillin to final concentration of 100 μg/mL, IPTG to final concentration of 0.5 mM, and Xgal to final concentration of 80 μg/mL.

4.3 Primer Design

The 14-mer and 17-mer Tat DNA molecules constructed in this study code for the Puglisi group [34] 14-mer residue Tat peptide and the Ye group [38] 17-mer residue Tat peptide, respectively. Figure 7 shows the 14-mer and 17-mer Tat DNA segments in blue. ApaI and XhoI restriction endonuclease cleavage sites were positioned on either side of the Tat DNA as shown in the red and pink sections, respectively, in Figure 7. These two restriction sites are also contained in the multiple cloning site of the pFliTrx vector (Invitrogen). The pFliTrx vector map is shown in Appendix D. The A, G, and C nucleotides, shown in green in Figure 7, were added in order to match the inserts to the coding frame of the pFliTrx vector. The forward and reverse of 14-mer and 17-mer Tat primers were ordered from a commercial vendor (Stratagene).

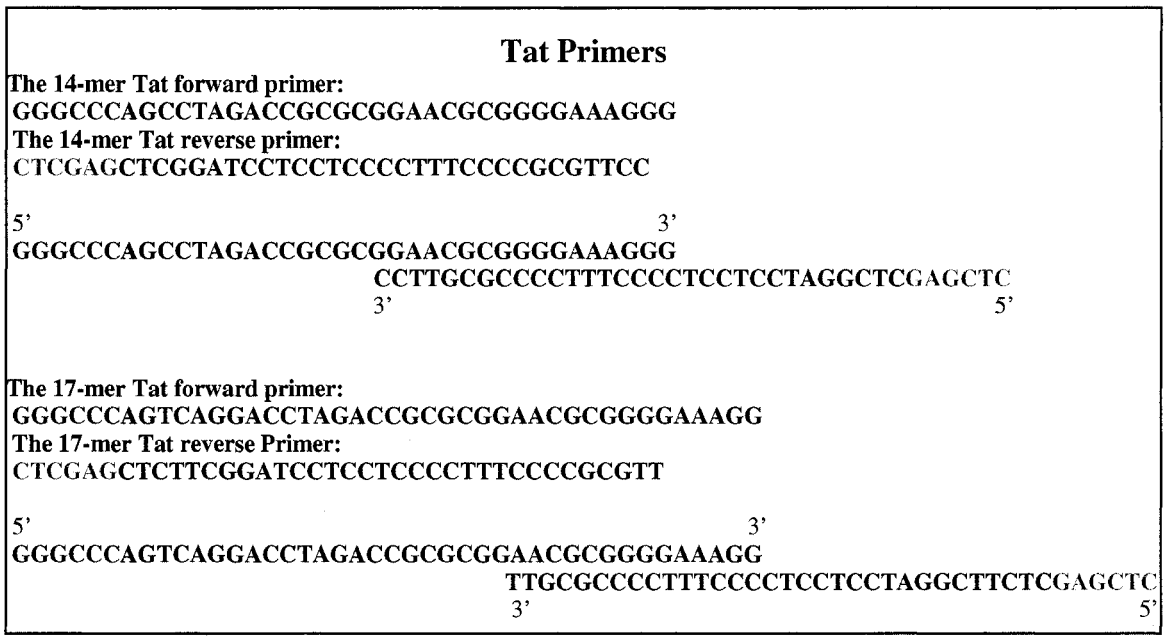

Figure 7. Tat forward and reverse primers. The14-mer and the 17-mer Tat sequences are highlighted in blue. The ApaI and the XhoI restriction sites are designed to occur on either side of the primers. The ApaI restriction site is highlighted in red and the XhoI restriction site is highlighted in pink. A, G, and C nucleotides were added to match the coding frame of the vector and are highlighted in green.

4.4 Mutually Primed Synthesis

The designed primers were subject to mutually primed synthesis to convert single-stranded DNA into double-stranded DNA for subsequent cloning into the pGEM®T-Easy vector (Promega). The primers were annealed and extended using the following mutually primed synthesis protocol. 2 μL of 1 μM forward primer and 2 μL of 1 μM reverse primer were transferred to a 500 μL micro-centrifuge tube. A 5 μL of 2x PCR master mix (containing 50 units/ml of Taq DNA polymerase, 400 μM dNTPs, 3 mM $MgCl_2$, and reaction buffers pH8.5, Promega) was added in the mixture and the final volume was

adjusted with nuclease-free water to 10 μL before the heating process. A total of 10 μL mixture was heated up using the MJ Research Minicycler for 2 minutes at 95 °C, 1.5 minutes at the melting temperature, 2 minutes at 72 °C, followed by a hold step at 4 °C. The mutually primed synthesis products were immediately subjected to gel electrophoresis after reaching 4 °C. The presence of the 14-mer and 17-mer Tat DNA was confirmed using gel electrophoresis.

4.5 DNA Ligation

The ligation of the mutually primed synthesis product into the pGEM®-T Easy vector was performed using the following standard ligation protocol. A mixture containing 4 ng of mutually primed synthesis product and 50 ng vector along with 2 μL of nuclease-free water, 5 μL of 2x rapid ligation buffer, and 3 U of T4 DNA ligase (Promega) was incubated for 20 minutes at room temperature. A background control of 50 ng pGEM®-T Easy vector (Promega), 3 μL of nuclease-free water, 5 μL of 2x rapid ligation buffer, and 3 U of T4 DNA ligase (Promega) was also prepared and incubated for 20 minutes at room temperature.

4.6 Bacterial Cell Transformation

The ligation product was transformed in to Epicurian *E. coli* XL-1 Blue competent cells (Stratagene) using the following standard transformation protocol. Falcon 2059 tubes (Fisher) were prechilled on ice for 15 minutes before adding 50 μL XL-1 blue cells. Either 1 μL or 4 μL of the ligation reaction were individually added to separate tubes

containing the competent cells. The cell mixtures were then mixed by gently flicking and incubated on ice for 30 minutes. The cells were heat-shocked for 45 seconds in a Precision 183 water bath at exactly 42 °C and then incubated on ice for 2 minutes. NZY+ broth (450 mL), preheated to 42 °C, was added to each tube, and the mixture incubated for 1 hour at 37 °C in a Lab-Line Orbit Environ Shaker incubator at 220 rpm. Following incubation, either 100 μL or 400 μL of each transformation were plated onto a LB/ampicillin/IPTG/XGal plate. The plates were incubated at 37 °C for sixteen-fourty hours in a Thermodyne Type 37900 Culture Incubator. White colonies were selected at the end of the incubation period, and up to twelve positive colonies were re-grown on a LB/ampicillin/IPTG/XGal master plate.

4.7 Plasmid Purification

Positive colonies (white colonies) from the master plates were selected for overnight cultures. NZY+ broth (5 mL) with 100 μg/mL of ampicillin was placed into a Falcon 2059 tube for each overnight culture. The culture tubes were incubated overnight at 37 °C in a Lab-Line Orbit Environ Shaker incubator operating at 280 rpm. The plasmids were purified from 3 mL of the overnight culture using a FastPlasmid Mini kit (Eppendorf), following the kit directions [46]. In the final step, the plasmid DNA was re-suspended in 10 μL of nuclease-free water.

Large scale plasmid purification was performed as follows. Two to five mL of culture was grown using antibiotic selection by incubating for 8 hours at 37 °C in a Lab-Line Orbit Environ Shaker incubator operating at 280 rpm. The starter culture was

transferred to a 500 mL culture flask and was diluted with LB broth (250 mL) containing 100 μg/mL of ampicillin before incubating overnight at 37 °C in a Lab-Line Orbit Environ Shaker incubator operating at 280 rpm. Plasmid DNA was purified using an EndoFree Plasmid Maxi kit (Qiagen), following the kit directions [47]. In the final step, DNA was re-suspended in TE 1x Buffer, to inhibit the degradation of DNA, to a final concentration of 250 μg/μl approximately [47]. Some plasmids were purified with the Qiagen HiSpeed Plasmid Maxi kit, following the kit directions [48].

4.8 Restriction Digestion of Plasmid DNA

The restriction digestion of plasmid DNA was performed using the following standard digestion protocol. A 2 μg DNA sample of the plasmid preparation was digested with a 10 U selected restriction enzyme (Promega), 1 μL of the 10x restriction enzyme buffer (Promega), and nuclease-free water in a total volume of 10 μL. The digestion was mixed gently by pipeting, centrifuged for a few seconds in a microcentrifuge, incubated for 1 hour at 37 °C, followed by a hold step at 4 °C. The resulting products were subjected to gel electrophoresis after reaching 4 °C. The presence of the Tat DNA was confirmed using gel electrophoresis.

4.9 Gel Electrophoresis

The DNA (primer, plasmid, and insert) was visualized using agarose gel electrophoresis, which separates DNA fragments based on their size.

The gel was made with an agarose concentration appropriate for the DNA size being analyzed, 0.75% to 4%. An aliquot of 5 mg/mL ethidium bromide solution was added to the gel solution before it was poured into a Fisher Scientific FB-SB-710 gel apparatus and allowed to set about 20 minutes. The gel was then covered with 1x TBE buffer as the conducting buffer. The DNA fragments were loaded into wells formed in the gel. Blue-orange loading dye (6x, Promega) was added into the DNA samples. DNA size standards of known molecular weight were run along with the DNA samples to determine the size of the DNA fragments. The completed gel was electrophoresed with appropriate voltage and time, and visualized using the AlphaInnotech FluorChem System with FluorChem 8900 software.

4.10 DNA Sequencing

Dideoxy sequencing of positive colonies was performed using a fmole DNA cycle sequencing system (Promega) and DNA sequencing gel apparatus (Life Technologies, Inc. Model S2). T7 promoter primer (Promega) was used for dideoxy sequencing for inserts cloned into the pGEM®-T Easy vector. The T7 primer was end-labeled with γ-[^{33}P]- ATP by mixing 1 µL of 10 pmole/µL T7 primer solution, 2.5 µL of γ-[^{33}P]- labeled ATP (Amersham Pharmaceuticals- 3000 Ci/mmol specific activity), 5 µL of nuclease-free water, 1 µL of 10x T4 Polynucleotide kinase buffer (Promega), and 0.5 µL of 10 U/µL T4 Polynucleotide kinase enzyme, and incubating the mixture for 30 minutes at 37 °C, and then 2 minutes at 90 °C.

Dideoxy sequencing was performed by mixing 0.1 μg of the DNA, 5 μL of fmol DNA sequencing 5x buffer (Promega), 1.5 pmol of T7 γ-[^{33}P]- labeled primer, 5 U of Taq polymerse (Promega), and nuclease-free water in a total volume of 17 μL. An aliquot (4 μL) of the sequencing reaction containing a different didoxynucleotide mix (G, A, T, or C) was prepared. The samples were then run through 30 replication cycles after heating at 95 °C for 2 minutes using a MJ Research Minicycler, which had been preheated to 95 °C. The cycles were as follows: 30 seconds at 95 °C, 30 seconds at 42 °C, and 1 minute at 70 °C. The tubes were then stored at -20 °C following a 4 °C hold.

The sequencing reactions were resolved on the DNA sequencing system, using a 6% urea-polyacrylamide gel. 3 μL of DNA sequencing stop solution (Promega) was added in the reaction tubes, followed by a heating process for 2 minutes at 70 °C, and incubation on ice until ready to load onto the gel. The gel was made with Sequa-Gel-6 solution (National Diagnostics), Sequagel Complete Buffer (National Diagnostics), and 10 % ammonium persulfate, and was dried overnight. The gel was prerun for 40 minutes before the regular performance. 3 μL of each dideoxy reaction sample was loaded on the gel and electrophoresed for 90 minutes at 70 Watts constant power. The gel was dried in a Savant gel dryer, and exposed to a Kodak Biomax film in a lightproof cassette for 3-7 days. The film was developed using the Kodak RP-X-Omat Model M-7B Processor and read manually.

4.11 Gel Purification of DNA Fragments

The DNA was digested with ApaI restriction enzymes (10 U/μL, Promega) and XhoI restriction enzymes (10 U/μL, Promega), and run on 0.75% low melting gel as described in Section 4.10. The desired DNA fragment, visualized by ethidium bromide fluorescence was cut out of the gel using a razor blade. One volume of phenol was then added to the cut out sample in order to elute the DNA fragment from the gel. The mixture was disrupted by vortexing and then placed in a -70 °C freezer for at least 30 minutes. It was then defrosted for 15 minutes, vortexed, and refrozen. After three freezing and defrosting cycles, the mixture was centrifuged at 4 °C for 15 minutes, and the upper aqueous phase was transferred to a fresh tube. The organic phase was back extracted with a 100 μL 1x TE buffer and the aqueous phases were combined. An equal volume of chloroform was added to the aqueous phase, vortexed for 2 minutes, and centrifuged for 15 minutes. The upper aqueous phase was transferred to another new tube. A one to ten volume of 5 M sodium chloride and two volumes of 100% ethanol were added to the aqueous phase, vortexed and incubated at -70 °C for 30 minutes. The mixture was centrifuged for 30 minutes and the ethanol was discarded. A two and a half to three volume of 70% ethanol was then added to wash the DNA. The DNA was air dried for 10-15 minutes, and dissolved in 10μL of nuclease-free water. The DNA was run on a 4% agarose E-Gel (Invitrogen) as described in Section 4.9.

CHAPTER FIVE
RESULTS

5.1 Mutually Primed Synthesis of Tat DNA

Both 14-mer and 17-mer Tat primers were extended to double-stranded DNA using mutually primed synthesis. Mutually primed synthesis products were run on a 4% agarose E-Gel, since the DNA fragments were less than 100 base pair in size. In order to identify the mutually primed synthesis products' sizes and to compare the length of mutually primed synthesis products with the single-stranded DNA, a 10 bp DNA step ladder, the 14-mer forward free primer, and the 17-mer forward free primer were also run on the same gel.

The results are shown in Figure 8. Each sample lane is marked on the figure. In addition, the DNA ladder bands corresponding to 50 bp and 60 bp are marked with arrows since the 14-mer and 17-mer Tat DNAs are shown to be 56 bp and 65 bp, respectively.

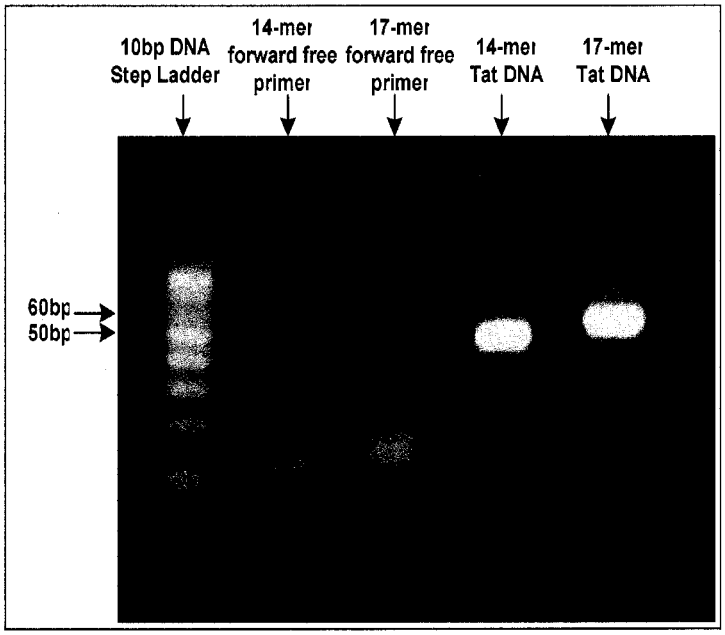

Figure 8. Mutually primed synthesis of Tat DNA. The mutually primed synthesis products were resolved electrophoretically on 4% agarose E-Gel (Invitrogen), using a 10 bp DNA Step Ladder (0.65 μg, Promega) for identification. The DNA samples consisted of 1 μL of the mutually primed synthesis product (1 μM), 16 μL of 1x TAE buffer, and 3 μL of blue-orange loading dye (6x, Promega). Two free primers, ran as controls, consisted of 1 μL of the 14-mer forward primer or the 17-mer forward primer, 16 μL of 1x TAE buffer, and 3 μL of blue-orange loading dye (6x, Promega).

5.2 Cloning Tat DNA into pGEM®-T Easy Vector

The cDNA fragments corresponding to the Tat 14-mer and 17-mer were then both cloned by ligation into pGEM®-T Easy vectors (Promega). The resulting plasmids were transformed into competent *E. coli* cells, as described in Section 4.7. Positive colonies (white) were selected from those growing on the LB/Ampicillin/IPTG/XGal plates. Thirty-seven blue colonies and eleven white colonies were obtained on the plate transformed with pGEM®-T Easy with the 14-mer Tat (pGEM-Tat14) insert. Forty-three blue colonies and fourteen white colonies were obtained on the plate transformed with

pGEM®-T Easy with the the 17-mer Tat (pGEM-Tat17) insert. The background control was *E.coli* transformed with ligated pGEM®-T Easy with no insert grown on a LB/Ampicillin/IPTG/XGal plate. Fifty blue colonies were present on the background control plate. White colonies indicate bacteria containing potential successfully ligated plasmids, since the *LacZ* gene is interrupted by the insert.

Two white colonies were selected from the pGEM-Tat14 plate and six white colonies were selected from the pGEM-Tat17 plate. The plasmids from each selection were purified using a FastPlasmid Mini kit (Eppendorf) [46]. The purified plasmids from each colony were separated into three samples. One sample was undigested, one sample was digested with BamHI (Promega), and one sample was digested with EcoRI (Promega). The digestion products were run on a 1% agarose gel, and the results are shown in Figures 9 and 10.

As shown in Figure 9, lanes 1 and 4 are the possible pGEM-Tat14 plasmids digested with BamHI, lanes 2 and 5 are the plasmids digested with EcoRI, and lanes 3 and 6 are the undigested plasmids. The plasmids from the two colonies are color-coded for identification (Plasmid 1 and Plasmid 2 in Figure 9). The undigested plasmids were used as controls to show whether digestion was successful because linearized plasmids will appear at a different location than circular plasmids. In addition, linear plasmids often appear as sharper bands than circular plasmids. Therefore, as shown in Figure 9, the plasmids from each colony were digested by both BamHI and EcoRI and they migrated in the gel as sharp bands in a different lane location than the bands of the undigested plasmid. By comparing to the HindIII/EcoRI cut Lambda Markers shown in

Figure 9, it was calculated that approximately 100 ng of Plasmid 1 and 98 ng of Plasmid 2 appeared on the gel.

Similarly, Figure 10 shows the results for the digestions of the possible pGEM-Tat17 plasmids. As shown, BamHI and EcoRI only digested the plasmids obtained from colonies 1 and 3 (Plasmid 1 and 3 in Figure 10). The plasmids from colonies 2, 4, 5, and 6 were undigested by either restriction enzyme. By comparing to the HindIII/EcoRI cut Lambda Markers shown in Figure 10, approximately 1400 ng of Plasmid 1 and 900 ng of Plasmid 3 appeared on the gel.

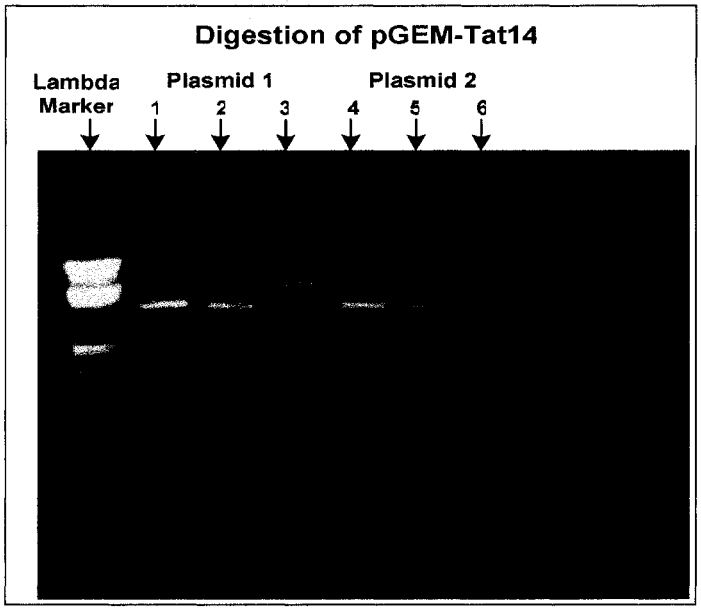

Figure 9. Restriction digestion of pGEM-Tat14 with BamHI and EcoRI. A 5 μL sample of the plasmid prep was single digested with BamH1 (10 U, Promega) (lanes 1 and 4) or EcoR1 (10 U, Promega) (lanes 2 and 5) for 1 hour at 37 °C. The undigested controls (lanes 3 and 6) were prepared to run with the digested samples in order to verify a cut DNA or uncut DNA. 1% agarose gel electrophoresis was applied in 1x TBE buffer, using HindIII/EcoRI cut Lambda Markers for identification (0.5 μg, Promega). An aliquot of 5 mg/mL ethidium bromide (3 μL) solution was added to the gel solution before it was poured into a Fisher Scientific FB-SB-710 gel apparatus and allowed to set about 20 minutes until the gel dried.

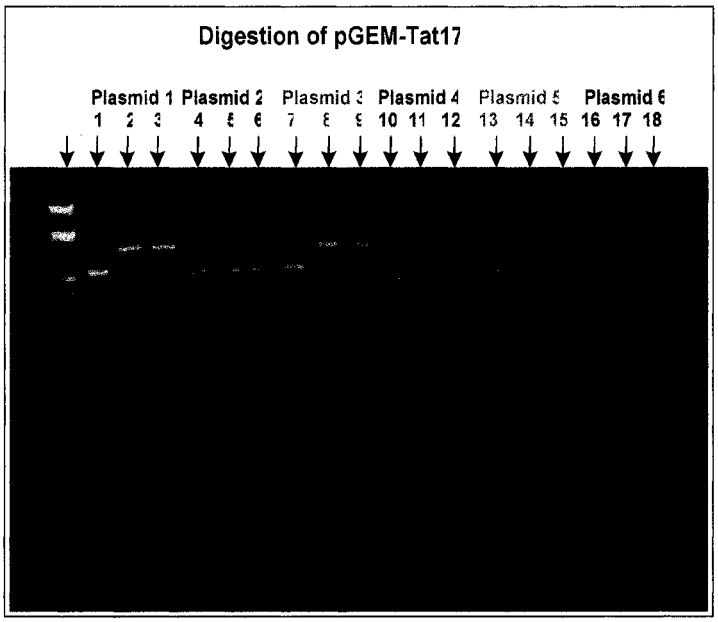

Figure 10. Restriction digestion of pGEM-Tat17 with BamHI and EcoRI. A 5 μL sample of the plasmid prep was single digested with BamH1 (10 U, Promega) (lanes 2, 5, 8, 11, 14, and 17) or EcoR1 (10 U, Promega) (lanes 3, 6, 9, 12, 15, and 18) for 1 hour at 37°C. The undigested controls (lanes 1, 4, 7, 10, 13, and 16) were prepared to run with the digested samples in order to verify a cut DNA or uncut DNA. 1% agarose gel electrophoresis was applied in 1x TBE buffer, using HindIII/EcoRI cut Lambda Markers for identification (0.5 μg, Promega). An aliquot of 5mg/mL ethidium bromide (3 μL) solution was added to the gel solution before it was poured into a Fisher Scientific FB-SB-710 gel apparatus and allowed to set about 20 minutes until the gel dried.

5.3 DNA Sequencing

Based on the results shown in Figures 9 and 10, DNA sequencing was performed on the putative positive plasmids to determine the sequence of the insert. A sample sequencing gel is shown in Figure 11 where the 14-mer and 17-mer Tat DNA are boxed in red. Comparing the nucleotide order shown with the known sequence it was determined that clones were obtained with the correct insert for both the 14-mer and 17-mer Tat DNA. See Appendix B for the sequencing data. Colonies containing these clones were used for the rest of the experiments.

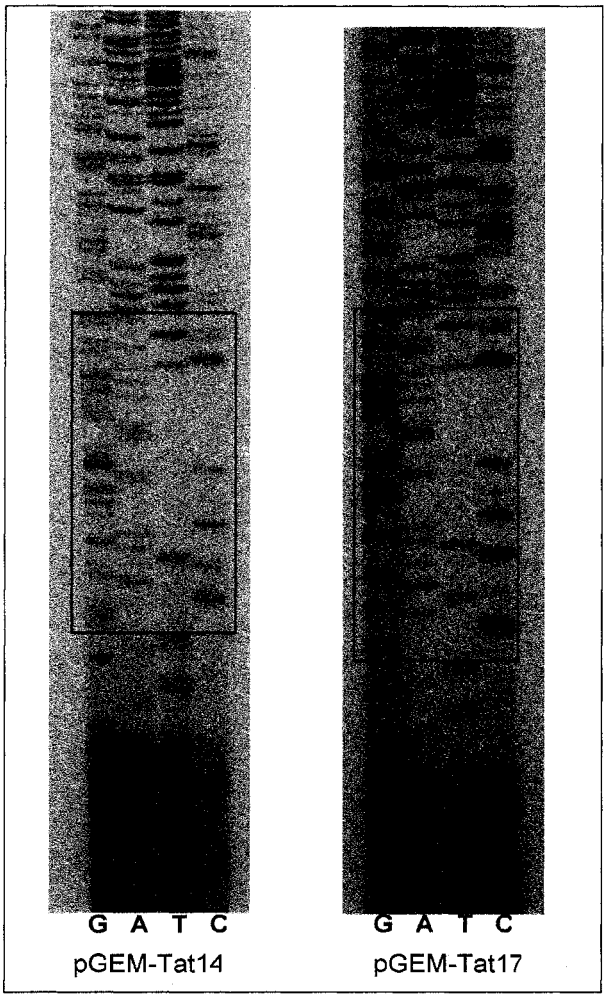

Figure 11. DNA sequencing results for the pGEM-Tat14 and pGEM-Tat17 recombinant plasmids. Dideoxy sequencing of positive colonies was performed using a fmole DNA cycle sequencing system (Promega) and DNA sequencing gel apparatus (Life Technologies, Inc. Model S2). T7 promoter primer (Promega) was used for dideoxy sequencing for inserts cloned into the pGEM®-T Easy vector. The T7 primer was end-labeled with γ-[^{33}P]- ATP (Amersham Pharmaceuticals- 3000 Ci/mmol specific activity). Each dideoxynucleotide, G, A, T, or C, is labeled at the bottom of the DNA sequencing results, and the sequences for the 14-mer Tat DNA and the 17-mer Tat DNA are outlined in red.

5.4 Tat DNA Isolation

The pGEM®-T Easy vector containing either the cDNA fragments corresponding to the Tat 14-mer or 17-mer cDNA, was digested with ApaI (Promega) and XhoI (Promega)

and run on a 0.75% low-melting-point agarose gel as shown in Figure 12. Digestion resulted in two different sizes of the linear DNA fragments. By comparing to the DNA size standards shown in Figure 10, the bands at approximately 3000 bp corresponding to the pGEM®-T Easy linear vector are indicated with blue arrows and the bands at approximately 60 bp corresponding to the 14-mer cDNA and 17-mer cDNA are indicated with red arrows. See Appendix C for details.

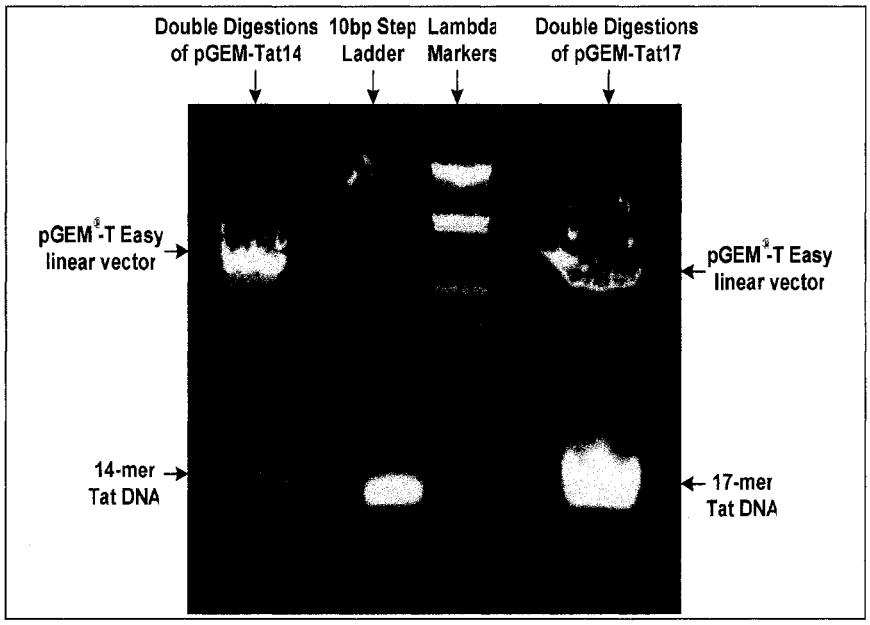

Figure 12. Double restriction digestions of pGEM-Tat14 and pGEM-Tat17 with ApaI and XhoI. Each DNA sample of pGEM-Tat14 and pGEM-Tat17 was digested with ApaI (10 U, Promega) and XhoI (10 U, Promega). A 10 bp DNA step ladder (0.65 μg, Promega) and a HindIII/EcoRI Lambda Markers (0.5 μg, Promega) were prepared to run with the DNA sample. Two different sizes of the linear DNA were presented on a 0.75% agarose gel. The bands at approximately 60 bp correspond to Tat DNA; the bands at approximately 3000 bp correspond to the pGEM®-T Easy linear vector.

The desired DNA fragment containing either the cDNA for the 14-mer or 17-mer Tat DNA was visualized and removed by cutting the bands out of the gel. The gel slices

containing the 14-mer Tat DNA (56 bp) and the 17-mer Tat DNA (65 bp) were purified as described in Section 4.9. The presence of the purified Tat DNA of the correct base pair sizes were confirmed using a 4% agarose E-Gel as shown in Figure 13. By comparing to the 10 bp DNA step ladder shown in Figure 13, the bands at approximately 56 bp and 65 bp corresponding to the 14-mer and the 17-mer Tat DNA, respectively, are indicated with arrows. The products were assumed to have a 50% yield after the phenol extraction.

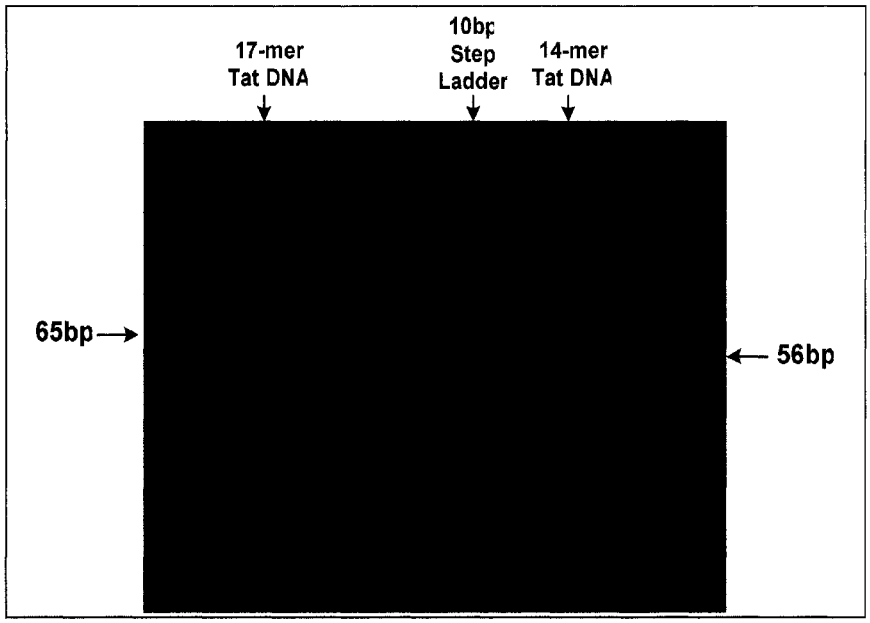

Figure 13. Gel purified 14-mer Tat DNA and 17-mer Tat DNA. The presence of the 14-mer and 17-mer Tat DNA fragments of the correct base pair size were confirmed using a 4% agarose E-Gel (Invitrogen). The DNA samples consisting of 1 μL of the phenol/chloroform extraction products (Tat DNAs), 16 μL of 1x TAE buffer, and 3 μL of blue-orange loading dye (6x, Promega) were prepared. A 10 bp DNA step ladder (0.65 μg, Promrga) was prepared to run with the DNA samples. A 20 μL aliquot of each DNA sample and the 10 bp DNA step ladder were then loaded into the 4% agarose E-Gel, and electrophoresed for 30 minutes. The gel was visualized using the AlphaInnotech FluorChem System with FluorChem 8900 software.

5.5 Cloning Tat DNA into pFliTrx Vector

In order to obtain large quantities of the pFliTrx cloning plasmids, a large-scale plasmid purification of pFliTrx was performed. To create the correct single-stranded overhangs for cloning the inserts, a double digestion was performed with ApaI (Promega) and XhoI (Promega) as shown in Figure 14. Digestion resulted in two different sizes of the linear DNA fragments. However, the larger DNA fragment was present on the gel while the smaller DNA fragment was not visible due to its smaller size and the lower mass amount. By comparing to the HindIII/EcoRI cut Lambda Markers shown in Figure 14, the band at approximately 4940 bp corresponding to the pFliTrx linear vector is indicated with arrow. See Appendix D for the pFliTrx vector map.

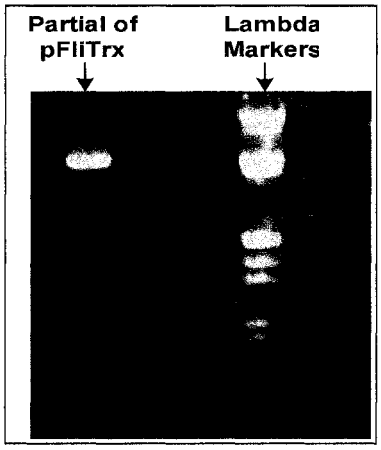

Figure 14. Double restriction digestion of pFliTrx plasmid with ApaI and XhoI. A 31.6 µL aliquot of pFliTrx plasmid (378 ng) was double digested with 2 µL of ApaI (10 U, Promega) and 2 µL of XhoI (10 U, Promega), 4 µL 10x buffer B (Proemga), and 0.4 µL 100x BSA (New England Biolabs), for 3 hours at 37 °C, followed by a 4 °C hold. A 0.75% agarose low-melting gel was applied in 1x TBE buffer, using 4 µL of HindIII/EcoRI Lambda Markers (0.5 µg, Promega) for identification. Two different sizes of the DNA fragments were expected to cleave. One DNA fragment was about 4940 bp in size and the other DNA fragment was about 100 bp in size. The partial pFliTrx shown in the figure corresponds to 4940 bp in size.

After a double digestion, the pFliTrx vector was run on a 0.75% low-melting-point agarose gel. The desired DNA fragment containing the linear pFliTrx vector was visualized and removed by cutting the correct band out of the gel. The gel slice containing the pFliTrx vector was first purified, and run on a 1% agarose gel as shown in Figure 15. By comparing to the HindIII/EcoRI cut Lambda Markers shown in Figure 15, approximately 7.2 ng of the pFliTrx vector was obtained.

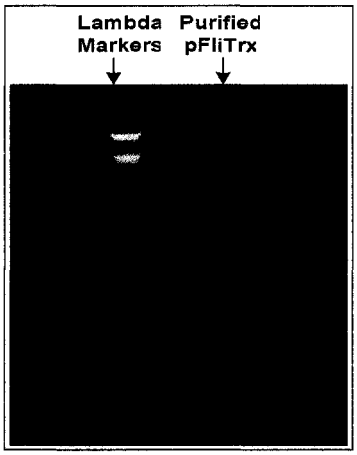

Figure 15. Purification of pFliTrx. 1% agarose gel electrophoresis was performed on the purified pFliTrx in order to determine the concentration. A HindIII/EcroRI Lambda Markers (0.5 μg, Promega) was prepared to run with the sample. Approximately 7.2 ng of the pFliTrx vector was obtained.

After the purification, the cDNA fragments corresponding to the Tat 14-mer and 17-mer were then both cloned into pFliTrx (Invitrogen), using the restriction sites, ApaI and XhoI. The resulting plasmids were transformed into competent *E. coli* cells, as described in Section 4.7. Colonies were selected from those growing on the LB/Ampicillin plates. Three colonies were obtained of the pFliTrx containing the 14-mer

Tat (pFliTrx-Tat14). Nine colonies were obtained of the pFliTrx containing the 17-mer Tat (pFliTrx-Tat17). The background control was *E.coli* transformed with pFliTrx with no insert grown on a LB/Ampicillin plate. One colony was present on the background control plate.

All the colonies that were present on either the pFliTrx-Tat14 or the pFliTrx-Tat17 plate were selected and purified. The plasmids from each selection were purified using a FastPlasmid Mini kit (Eppendorf) [47]. The purified plasmids from each colony were separated into three samples. One sample was undigested, one sample was digested with BamHI (Promega), and one sample was digested with EcoRV (Promega). The digestion products were run on a 1% agarose gel, and the results are shown in Figures 16.

As shown in Figure 16, lanes 3, 6, and 9 are the possible pFliTrx-Tat14 plasmids digested with BamHI, lanes 2, 5, and 8 are the plasmids digested with EcoRV, and lanes 1, 4, and 7 are the undigested plasmids. Similarly, lanes 10 to 32 are the results for the digestions or the undigested samples of the possible pFliTrx-Tat17 plasmids. The plasmids from each colony are color-coded for identification. The undigested plasmids were used as controls to show whether digestion was successful because linearized plasmids will appear at a different location than circular plasmids. In addition, linear plasmids often appear as sharper bands than circular plasmids. Therefore, as shown in Figure 16, the plasmids from each colony were digested by BamHI appearing as sharp bands at a different migration than the undigested plasmid and the digested plasmid by EcoRV. In addition, the undigested plasmid migrated the same distance as the plasmid digested by EcoRV.

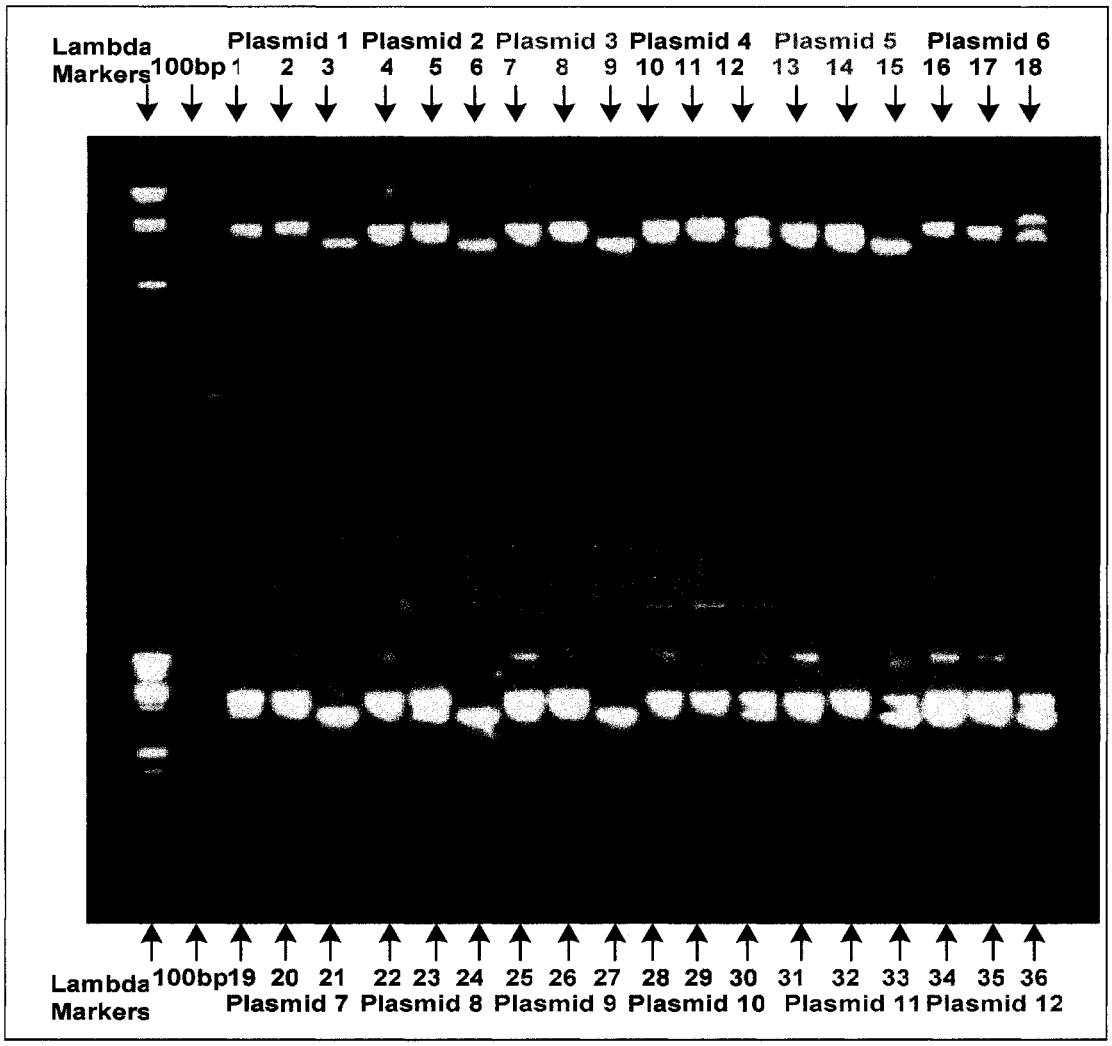

Figure 16. Single restriction digestion on the purified plasmids with BamHI and EcoRV. A 5 μL sample of the plasmid prep was single digested with BamH1 (10 U, Promega) (lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, and 36) or EcoRV (10 U, Promega) (lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, and 35) for 1 hour at 37 °C. The undigested controls (lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, and 34) were prepared to run with the digested samples in order to verify a cut DNA or uncut DNA. 1% agarose gel electrophoresis was applied in 1x TBE buffer. An aliquot of 5 mg/mL ethidium bromide (about 3 μL) solution was added to the gel solution, before it was poured into a Fisher Scientific FB-SB-710 gel apparatus and allowed to set about 20 minutes until the gel dried. A 2 μL of blue-orange loading dye (6x, Promega) was added into each 10 μL DNA digestion sample. A HindIII/EcoRI Lambda Markers (0.5 μg, Promega) and a 100 bp DNA step ladder (0.65 μg, Promrga) were prepared to run with the DNA samples.

CHAPTER SIX
DISCUSSION

6.1 Overview

In this research, cloning experiments were performed in order to obtain the 14-mer or 17-mer Tat DNA inserted in either pGEM®-T Easy or pFliTrx vector. Cloning the inserts into pGEM®-T Easy was successful. However, cloning into pFliTrx was not successful. Although the pFliTrx cloning was not successful, it is important to continue this type of research.

It has been reported in the literature that flexibility can affect binding between protein and RNA, for instances Tat-TAR binding [40,41,42]. In order to evaluate that claim, it is necessary to determine the binding strength of wild-type TAR Tat and the combinatorial library of mutated sequences. The purpose of this study was to generate peptide of the important sequence of the Tat-TAR binding on the surface of E.coli where the binding strength between the peptide and TAR could be measured e.g. by gel shift assay. Later, the binding strength between the library of mutated sequences and TAR could be measured in order to determine if flexibility affects binding. This research is just the start of that process. If flexibility affects binding, reasonable drug design approaches might be aimed at blocking the Tat-TAR interaction [42]. The present knowledge about the inhibition of TAR-Tat interaction is an approach to develop new anti-HIV compounds. RNA molecules can fold into a wide variety of structures, although RNA plays a key role in many biological processes from the control of gene expression to the translation processes [42]. Therefore, targeting proteins is still

dominant in anti-HIV drug design. Understanding how flexibility affects Tat-TAR binding may be useful to develop drugs to treat or stop AIDS [42].

6.2 Cloning Tat DNA into pGEM®-T Easy Vector

As shown in Figure 8, after mutually primed synthesis, both the 14-mer and 17-mer primers had extended into two double-stranded DNA. The 14-mer and 17-mer forward free primers were used as controls to show that elongation step in mutually primed synthesis were successful. The comparison between DNA band location of the free primers and the mutually primed synthesis products show that elongation occurred. As expected due to their single-stranded nature, the 14-mer and 17-mer forward free primers migrated further down the gel than their respective mutually primed synthesis products. The 17-mer free primer is slightly behind the 14-mer free primer due to extra base pairs. Furthermore, the bands for the 14-mer and 17-mer mutually primed synthesis products are located at positions corresponding to 56 bp and 65 bp, respectively. The size of each sample indicates that the mutually primed synthesis process was successful.

Using the mutually primed synthesis products, ligation into pGEM®-T Easy was attempted. The pGEM®-T Easy was chosen for this step since the XhoI restriction enzyme can not cut efficiently if the restriction site is at the end of the DNA. That limitation could be overcome by cloning the 14-mer and the 17-mer Tat DNA into the pGEM®-T Easy vector to make a circular plasmid so that XhoI could cut efficiently.

Initially, many ligation reactions failed as shown by the fact that transformed bacteria either did not grow at all or only grew blue (negative) colonies. There were several possible reasons for the failed ligation reaction, such as: 1) the Taq DNA polymerase was still active during the ligation reaction, 2) pGEM®-T Easy might lost the T overhangs, 3) the ligation molar ratio was not acceptable. The following experiments were performed in order to determine the most probable causes.

Since the Taq DNA polymerase is thermostable [49], it might still be active during the ligation reaction. If Taq DNA polymerase is still active, the T overhangs on pGEM®-T Easy will fill. In order to check if the Taq DNA polymerase was still active in the ligation mixture, the mutually primed synthesis products were run on a low-melting-point agarose gel. The purified bands were cut out and purified using phenol extraction and ethanol precipitation in order to remove the extra enzymes and agarose. However, the subsequent ligation reactions still failed, as shown by the fact that the transformed bacteria grew high colony number, but none of them were white colonies.

In order to check if the pGEM®-T Easy vector was contaminated, e.g., if the vector had religated or generated a dimer, new pGEM®-T Easy vector was purchased. It was possible that the pGEM®-T Easy vector was contaminated since it was old. Since pGEM®-T Easy has unstable 3'-T overhangs at the insertion sites, there may have been instances where the plasmids had lost their T overhangs. In addition, since the T overhang is linked by a covalent phosphodiester bond to the vector, the plasmids that lost the T overhangs could religate to form a circular plasmid [50]. However, the subsequent ligation reaction failed again as shown by the fact that the transformed bacteria grew high

colony number, but none of them were white colonies. Religated plasmids could generate blue colonies after transformation since the *lacZ* gene would be intact [50].

In order to investigate if the failed ligation reactions were due to an incorrect ligation molar ratio, some experiments were run with various molar ratios. For these experiments, 100 ng of the new pGEM®-T Easy vector was used. The successful molar ratio was found to be at one vector to four inserts. This ligation molar ratio gave successful cloning results since white colonies were formed after transformation as shown in Section 5.2.

After the successful transformation, plasmids from selected white colonies were purified and then single digested with EcoRI or BamHI. BamHI restriction site is in the inserts. The drawing in Appendix E shows that EcoRI cuts the vector twice while BamHI cuts it once. However, the 14-mer and 17-mer inserts are only 56 bp or 65 bp, respectively. Thus, the relative migration between the EcoRI and BamHI successful digestions are essentially the same, at approximately 3000 bp, as shown in Figures 9 and 10. As shown in Appendix E, the EcoRI digestion would result in two DNA fragments. However, as shown in Figure 9 and 10, all the successful digestions only show one band. The reason for this is that the insert DNA fragments were so small that they had already run out of the gel or too low in mass to be detected. In Figure 9, both plasmid 1 and plasmid 2 are clearly cut and linearized by either EcorRI or BamHI. By comparing the undigested lanes to the digested lanes, the undigested lanes are more indistinct than the digested lanes. Since the successful digestions appeared a different location than the undigested controls, and appeared as sharper bands than the undigested controls, it was

concluded that the plasmids were successfully linearized. Therefore, both Plasmid 1 and Plasmid 2 are predicted as putative positive plasmids in Figure 9. Similarly, in Figure 10, Plasmid 1 and Plasmid 3 are predicted as putative positive plasmids.

6.3 Tat DNA Isolation

The DNA sequencing results shown in Section 5.3 confirmed the inserts were 14-mer and 17-mer Tat DNA. Some experiments were run to isolate the Tat DNA so that it could be isolated in preparation for insertion into pFliTrx. A double digestion with ApaI and XhoI was performed on the successful recombinant pGEM-T Easy plasmids and the products were run on a low-melting-point gel as shown in Figure 12. The gel slices containing each desired fragment (14-mer and 17-mer Tat DNA) were removed and both of the Tat DNA were purified as described in Section 5.4. The purified Tat DNA fragments were run on an agarose gel as shown in Figure 13.

Figure 13 shows two close bands in the 17-mer Tat DNA lane, indicating that it was contaminated. By comparing the two DNA bands to the 10 bp step ladder, approximately 56 bp and 65 bp were read corresponding to sizes for the cDNA for 14-mer and 17-mer Tat DNA, respectively. This indicates the 17-mer Tat DNA was somehow contaminated with the 14-mer Tat DNA due to improper handling. Most likely this occurred during transformation when both the pGEM-Tat14 and pGEM-Tat17 grew on the same plate and the colonies intermingled. Therefore, other positive colonies on the same master plate and they were purified and digested. Unfortunately, in all cases, the agarose gel results showed that the 17-mer Tat DNA was contaminated with the 14-

mer Tat DNA. Due to time restrictions, the 14-mer Tat DNA and the contaminated 17-mer Tat DNA were used in subsequent ligation into the pFliTrx vector. Since the 17-mer samples were contaminated, it must be noted that either 14-mer or 17-mer Tat DNA fragments could be cloned into the pFliTrx vector during the ligation step.

6.4 Cloning Tat DNA into pFliTrx Vector

An attempt was made to transform competent *E. coli* cells with the recombinant pFliTrx plasmids. After transformation, colonies grew on LB/Ampicillin plates. All colonies were white but this was expected since the pFiTrix plasmid does not have the *lacZ* gene so IPTG/Xgal could not be used for selection. Thus, several colonies were purified and digested with EcoRV and BamHI in order to identify if the cloning step was successful.

As shown in Appendix D, the original pFliTrix plasmid is approximately 5000 bp in size. Appendix G shows that EcoRV will cut once on the plasmid at the 3585 position at the multiple cloning site only if the insert (14-mer Tat DNA or 17-mer Tat DNA) is not successfully cloned into the vector. EcoRV would not cut plasmids containing the desired inserts since the EcoRV site would have been removed in the ApaI/XhoI (Appendix F) double digestion step. Appendix G also shows that BamHI will cut the plasmid three times at the 3583, 3998 and 4597 positions only if the insert is cloned into the pFliTrx vector, resulting in three different DNA fragments. One is 4000 bp in size, one is 600 bp in size, and another is 400 bp in size. Without the insert, BamHI would only cut twice on the plasmid at the 3583 and 4597 positions, resulting in two different

sizes of the DNA fragments. Furthermore, as shown in Appendix G, plasmids containing the desired inserts would be cut three times by BamHI, removing a 1000 bp section. Plasmids that do not contain an insert would only be cut twice removing a 600 bp section.

In Figure 16, the lanes for BamHI restriction digestion showed one band corresponding to approximately 3000 bp in size (lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, and 36). The size is unexpected since the large fragment should correspond to approximately 4000 bp. Also, the lanes for EcoRV restriction digestion (lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, and 35) are in identical position with the lanes containing undigested plasmid (lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, and 34) indicating EcoRV did not cut the plasmid. This result, taken individually, might indicate that the digestion step was successful, removing the multiple cloning site containing the EcoRV restriction site, but that the insert ligation step had failed. However, the EcoRV results in combination with the BamHI size results indicated that pGEM®-T Easy is the plasmid instead of pFliTrx. Both the BamHI results and EcoRV results indicate that Tat DNA was not successfully cloned into the pFliTrx vector. None of the plasmids could be predicted as putative positive plasmids from Figure 16. DNA sequencing was performed to verifity that the plasmid was pGEM®-T Easy instead of pFliTrx.

6.5 DNA Sequencing

In order to confirm the plasmid identity, DNA sequencing was performed on the plasmids to verify their sequence. Purified plasmids from the attempts to transform the colonies with pFliTrix were used as the template. Appendix H showed the DNA

sequencing if the Tat DNA is successfully cloned into pFliTrx. Unfortunately, no sequences showed on the sequencing film at all. The failure might have been due to insufficient or incorrect templates.

For each set of four sequencing reactions, approximately 0.1 μg of template was recommended. In the experiment, 65 ng of each template was added to each set of four sequencing reactions with the appropriate deoxy/didexoy nucleotide solution. Therefore, the concentration for each template was a little bit low but still should have given visible bands.

Another possible cause for no sequencing data is that the primers used were chosen assuming that the template was recombinant pFliTrx. If the plasmids were not pFliTrx then the DNA sequence will not show on the film because there are no corresponding sequences on the pGEM®-T Easy plasmid. If this is the reason that the DNA sequencing gave no results after several attempts were made to sequence the DNA, then this is consistent with the results shown in Figure 16 that indicate the plasmid size is closer to pGEM®-T Easy than to pFliTrx. The digestion results also correspond to what would be expected for pGEM®-T Easy instead of pFliTrx. The position of the BamHI restriction digest bands to the Lambda Markers in Figure 16 has the same relative position as the BamHI restriction digest bands to the Lambda Marker in Figures 9 and 10. Assuming those templates are either 14-mer or 17-mer Tat DNA with the pGEM®-T Easy vector, the BamHI restriction enzyme would cut once at the multiple cloning site to linearize the plasmid to approximately 3000 bp in size, corresponding to the results shown on the agarose gel, and the EcoRV restriction enzyme would not cut the plasmid since there are

no EcoRV sites on pGEM®-T Easy or the Tat DNA inserts. A likely cause is that the gel-purified Tat fragments were contaminated with pGEM®-T Easy linear plasmid, and the fragments relegated to pGEM®-T Easy rather than to pFliTrx. The most likely reason that this occurred was that the pFliTrx plasmid was not completely digested by both of the restriction enzymes and so the ends were incompatible for insertion of the Tat fragments.

6.6 Future Work

Before the ligation with the Tat DNA and the pFliTrx vector, the pFlixTrx vector was prepared by double digestion with ApaI and XhoI in the Promega buffer B. It is likely that the pFliTrx plasmid was not fully digested because buffer B is not the optimal buffer for either ApaI or XhoI; ApaI is only 50-75% active in buffer B and XhoI is only 75-100% active in buffer B [51]. If the efficiency can be improved then the cloning might have a better chance of being successful. The pFliTrx digestion can be improved by performing two single digestions sequentially on the pFliTrx plasmid instead of running one double digestion. A single restriction digestion with the restriction enzyme ApaI, which has a 100 percent activity, in the Promega buffer A should be performed on the pFliTrx plasmid. And this can be followed by another single-restriction digestion with the restriction enzyme XhoI, which has 100 percent activity, in the Promega buffer D on the pFliTrx plasmid. The problem is that the difference in size of the single or double digestied pFliTrx is only about 160 bp which cannot really be distinguished by gel electropheresis. The pFliTrx vector will then be fully digested and both the 14-mer and

17-mer Tat DNA should be able to clone into the pFliTrx vector with a higher probability of success.

CHAPTER SEVEN
CONCLUSIONS

In this research, primers coding for the 14-mer and 17-mer residue Tat sequences were successfully designed. The primers were also successfully used to produce cDNA coding for 14-mer and 17-mer residue Tat sequences. The results of DNA sequencing showed the 14-mer and 17-mer Tat DNA were the correct sequences. These sequences were each successfully inserted into the pGEM®-T Easy vector, completely digested by the restriction enzymes, ApaI and XhoI, and purified, resulting in the availability of 14-mer and 17-mer Tat DNA for cloning into the pFliTrx vector. However, the 14-mer and 17-mer Tat DNA did not insert into the pFliTrx vector successfully.

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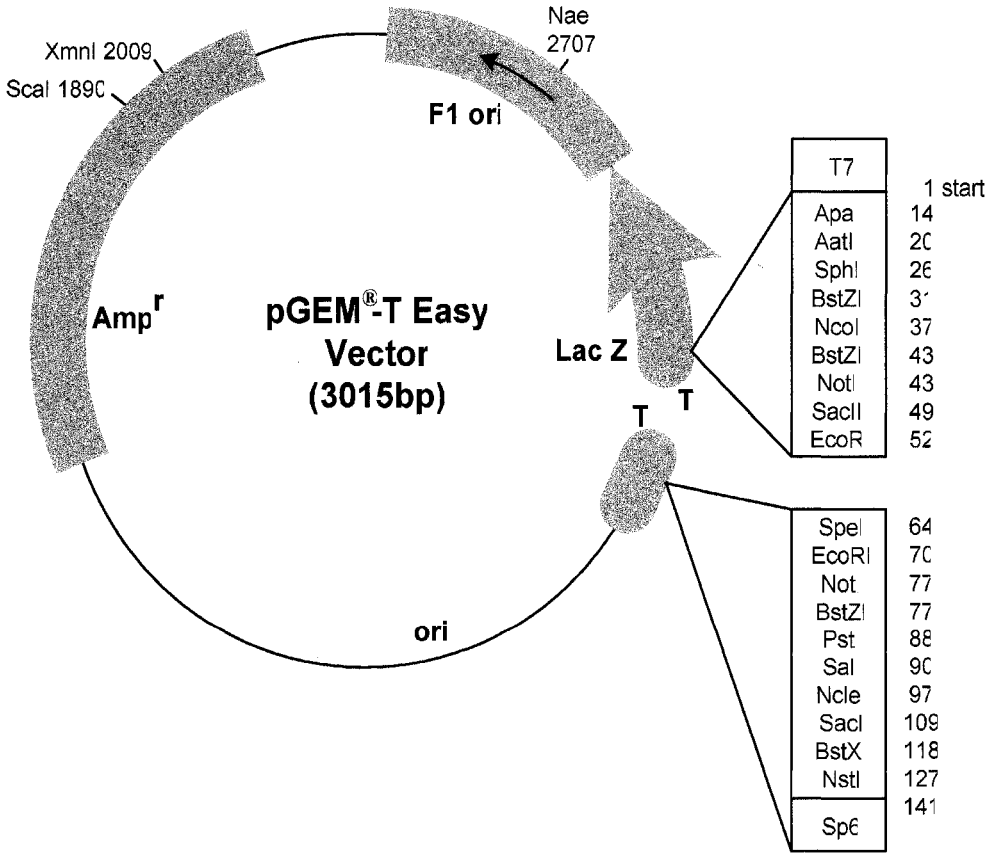
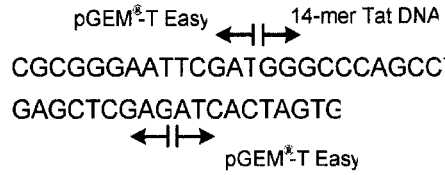
Appendix A. pGEM®-T Easy Vector Map and Sequence Reference Points

Promega pGEM®-T Easy Vector/Amp-3015 nucleotides	
T7 RNA polymerase transcription initiation site	1
multiple cloning region	10-113
SP6 RNA polymerase promoter (-17 to +3)	124-143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161-177
lacZ start codon	165
lacZ operator	185-201
β-lactamase coding region	1322-2182
phage f1 region	2365-2820
lac operon sequences	2821-2981, 151-380
pUC/M13 Forward Sequencing Primer binding site	2941-2957
T7 RNA polymerase promoter (-17 to +3)	2984-3



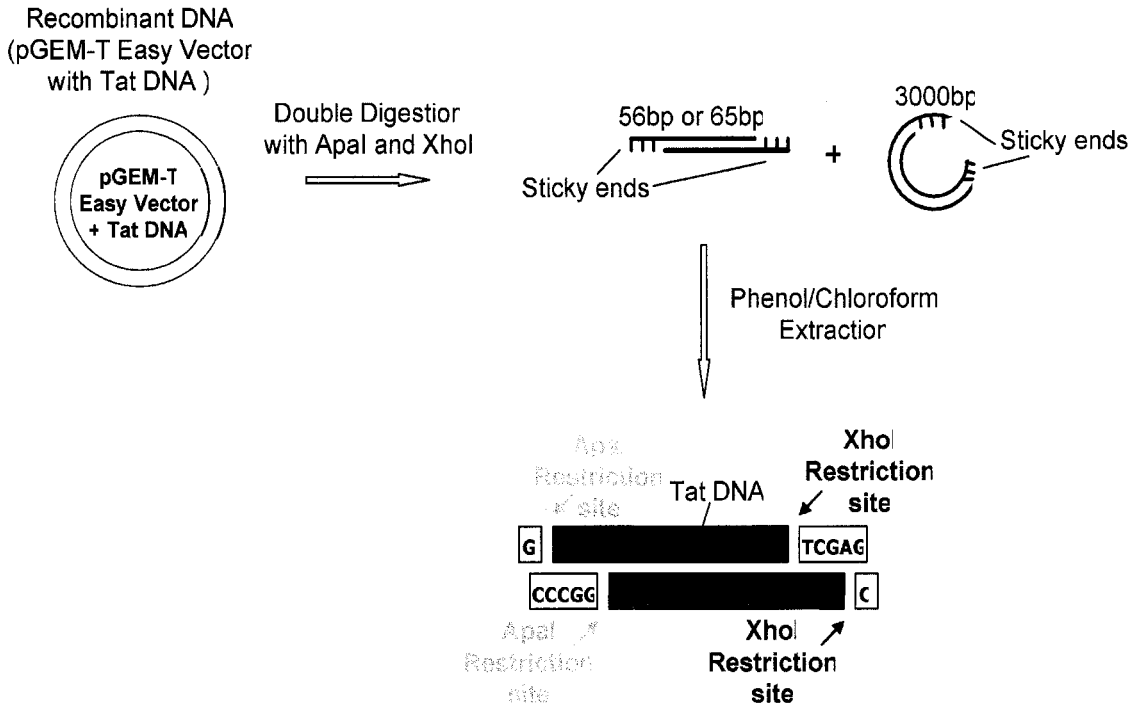
Appendix B. Sequencing Data of the pGEM®-T Easy vector with 14-mer Tat DNA or the pGEM®-T Easy vector with 17-mer Tat DNA

Theoretical DNA Sequencing of the pGEM®-T Easy Vector with 14-mer Tat DNA :



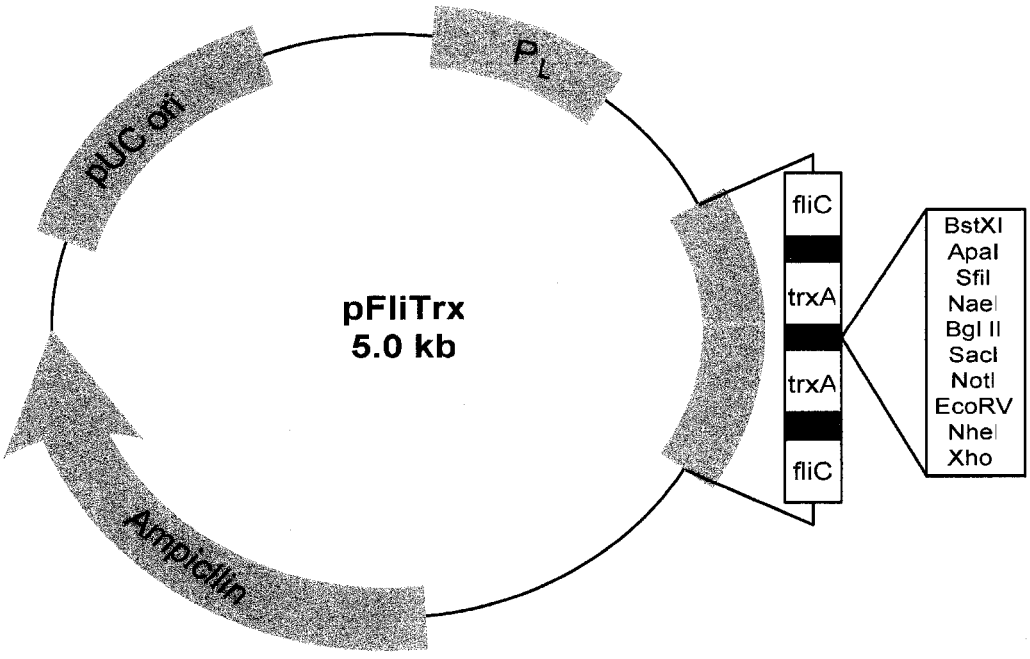
Theoretical DNA Sequencing of the pGEM®-T Easy Vector with 17-mer Tat DNA :

Appendix C. Double Restriction Digestion of pGEM-Tat14 or pGEM-Tat17 with ApaI and XhoI

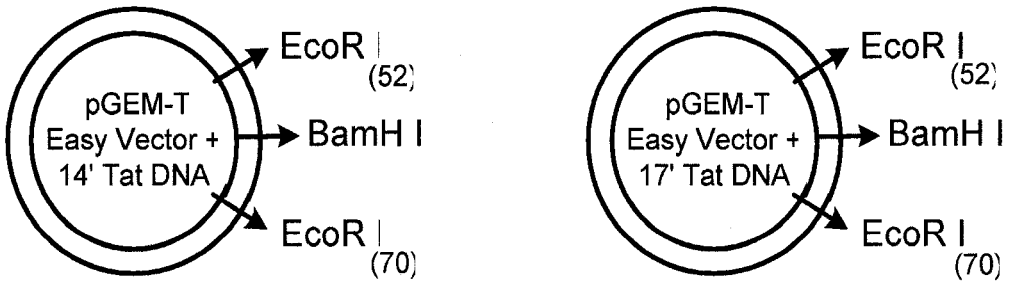


Appendix D. pFliTrx/Amp Map

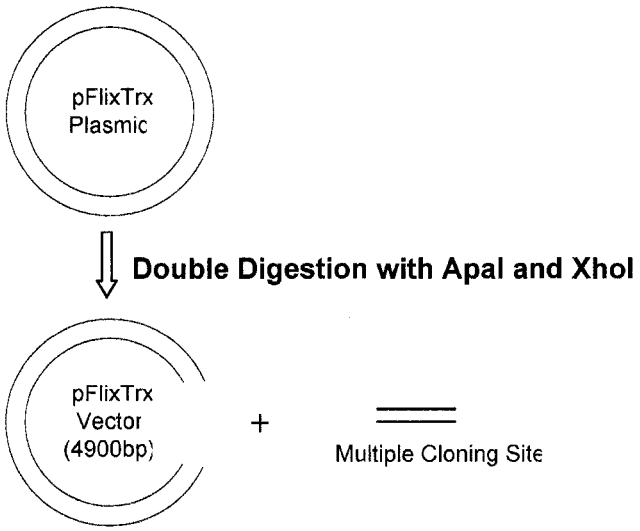
Invitrogen pFliTrx/Amp-5032 nucleotides	
Ampicillin Resistance Gene	200-1061
pUC origin	1206-1879
P_L Promoter	2061-2510
fliC 5' ORF	2706-3434
trxA 5' ORF	3435-3536
multiple cloning site	3537-3597
trxA 3' ORF	3598-3821
fliC 3' ORF	3825-4271



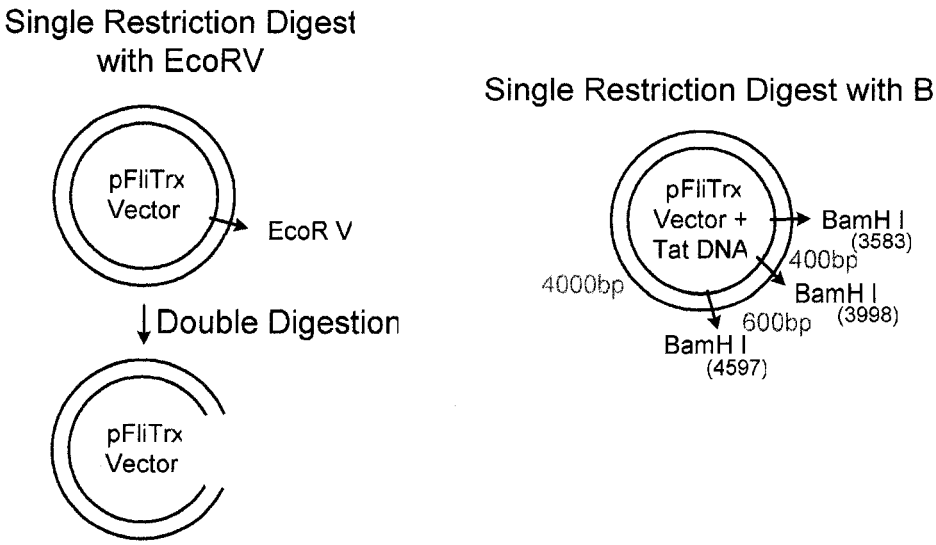
Appendix E. Single Restriction Digestion of the pGEM-Tat14 and pGEM-Tat17 with EcoRI and BamHI



Appendix F. Double Digestion with ApaI and XhoI on pFliTrx Plasmid



Appendix G. Single Digestion with BamHI and EcoRV on the pFliTrx vector with 14-mer Tat DNA or 17-mer Tat DNA



Appendix H. Sequencing Data of the pFliTrx vector with 14-mer Tat DNA or the pFliTrx Vector with 17-mer Tat DNA

DNA Sequencing of the pFliTrx Vector with 14-mer Tat DNA :

pFliTrx Vector ←|→ 14-mer Tat DNA

CAGTGTGCTGGGCCGGGCCCAGCCTAGACCGCGCGCAACGCGGGAAAGGGGAGGAGGATCC

GAGCTCGAGATCACTAGTG

←|→ pFliTrx Vector

DNA Sequencing of the pFliTrx Vector with 17-mer Tat DNA :

pFliTrx Vector ←|→ 17-mer Tat DNA

CAGTGTGCTGGGCCGGGCCCAGTCAGGACCTAGACCGCGCGGAACGCGGGGAAAGGGGAGG

AGGATCCGAAGAGCTCGACCTCGAGGTCCG

17-mer Tat DNA ←|→ pFliTrx Vector