INVESTIGATION OF THE EFFECTS OF HIV-1 AND SIV$_{AGM}$ TAT PROTEINS ON SLPI GENE EXPRESSION IN *CHLOROCEBUS SABAEUS* KIDNEY (VERO) CELL LINE

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF HIV-1 AND SIV$_{AGM}$ TAT PROTEINS ON SLPI GENE EXPRESSION IN *CHLOROCEBUS SABAEUS* KIDNEY (VERO) CELL LINE

Even though Human Immunodeficiency Virus (HIV) can infect Old World Monkey (i.e African Green Monkey) cells, replication of the virus is hampered with different kinds of restriction mechanisms before the integration of viral genome to host genome. After viral integration to host genome, HIV-1 TAT protein is expressed, which plays an important role for the generation of other proteins for viral production. Previous studies which were performed in our laboratory indicate that Secretory Leukocytes Protease Inhibitor (SLPI) overexpressed in existence of HIV-1 TAT protein. This result was confirmed with 2D-PAGE, qRT-PCR and Western Blot Analysis. The aim of this study is to understand how Vero Stable Cell Lines which produces HIV-1 TAT protein can affect expression levels of SLPI. At mRNA level of SLPI expression was confirmed with qRT-PCR. In order to measure SLPI expression at the protein level in the presence of HIV-1 TAT, Western Blot and Sandwich Elisa methods were used. Luciferase Assay process was performed in order to measure the effects of SLPI on HIV-LTR. Consequently, expression of SLPI in Vero Stable Cell Line increased in the existence of HIV-1 TAT protein. Additionally, SLPI inhibits HIV-LTR function through suppressing NF- κB promoter which exists in HIV-LTR.

ÖZET

CHLOROCEBUS SABAEUS BÖBREK (VERO) HÜCRE HATTINDA HIV-1 VE SIV_{AGM} TAT PROTEİNLERİNİN SLPI GEN EKSPRESYONU ÜZERİNDEKİ ETKİSİNİN ARAŞTIRILMASI

İnsan Bağışıklık Yetmezliği Virüsü (HIV) Eski Dünya Maymunu (örneğin Afrika Yeşil Maymunu) hücrelerine girse bile, virüsün çoğalması viral genom konakçı hücre genomuna entegre olmadan çeşitli engelleyici mekanizmalarla engellenir. Konakçı genomuna viral genomun entegre olmasından sonra, virüs çoğalmasında diğer proteinlerin ortaya çıkmasında önemli rol oynayan HIV-1 TAT protein eksprese edilir.. Laboratuvarımızda gerçekleşen önceki çalışmalar SLPI geninin HIV-1 TAT protein varlığında çok fazla eksprese olduğu göstermiştir. Bu sonuç, 2D-PAGE, qRT-PCR ve Western Blot yöntemleriyle doğrulanmıştır. Bu çalışmanın amacı, HIV-1 TAT protein üreten, transfekte edilmiş Vero stabil Hücre Hatlarında SLPI ekspresyon seviyesinin nasıl etkilendiğini anlamaktır. SLPI ekspresyonu mRNA seviyesinde qRT-PCR ile doğrulanmıştr. HIV-1 TAT varlığında SLPI ekspresyon seviyesinin protein seyiyesinde ölçmek için Western Blot, Sandwich Elisa yöntemleri kullanılmıştır. SLPI HIV-LTR üzerindeki etkisini ölçmek için Lüsiferaz Analizi yapılmıştır. Sonuç olarak, transfekte edilmiş Vero Stabil Hücre Hatları'nda SLPI ekspresyonu HIV-1 TAT proteini varlığında artar. Buna ek olarak, SLPI HIV-LTR işlevini HIV-LTR içinde bulunan NF-κB promotorunu baskılayarak engeller.

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CHAPTER 1

INTRODUCTION

1.1. Human Immunodeficiency Virus (HIV)

HIV-1 and HIV-2 are epidemiological agents which cause Acquired Immunodeficiency Syndrome (AIDS) in humans, belonging to Lentivirus genus of Retroviridae family. First clinical observation of AIDS emerged in 1881. Isolation of HIV which occurred in 1883 was an important development to examine what the causative agent for AIDS was. In 1996, Identification of two co-receptors, CCR5 and CXCR4, played an important role in the beginning of viral infection. Up to today, Different types of restriction mechanisms have been found such as APOBEC3G, TRIM5α, and SAMHD1. According to the phylogenetical aspect, there are three sub-groups of HIV which have different origins, e.g. M, N and O sub-groups. M group is one of the most common causes of AIDS around the world; whereas N and O groups are limited to West Africa. Beyond all of this information, the origin of HIV plays the most vital part in the understanding of its epidemiology. According to some investigations the cause of emerging viruses can be traced to the Zoonotic transmission between human and nonhuman primates. The analysis of genetic relationship shows that SIV_{SM} is very homologous to HIV-2 strain, whereas SIV_{CPZ} is mostly related to HIV-1 strain in terms of homology. Simian immunodeficiency virus (SIV) is special for infecting nonhuman primate species which was found in Sooty Mangabeys (SMs), African Green Monkeys (AGMs), Mandrills and Chimpanzees as natural hosts during the discovery of SIV. Contrary to HIV infection, the Anti-inflammatory surrounding prevents activation and proliferation of T lymphocytes during SIV infection which means that there are lower $CD4^+$ T lymphocytes and CCR5 co-receptors in SIV infection compared to HIV infection.

1.2. Structure and Genome of HIV

HIV genome has positive single stranded RNA which can be converted to double stranded RNA through the process of reverse transcription. HIV genome consists of different types and featured-genes such as tat, rev, etc. In addition to that, gag, pol and env genes can exist almost in all retroviruses. The gag, pol and open reading frame (ORF) encode inner structural proteins and replication enzymes at 5'region. In depth, gag is crucial for forming virus particles and pol serves for encoding enzymes such as integrase, reverse transcriptase and protease. Transcription of retroviral particles consists of 5'splice site (5'ss) upstream of the gag ORF in the 5'untranslated region (UTR) and 3'splice site at the end of the pol ORF. Env is also required to encode envelope proteins such as gp120 which is a surface protein and gp41 which serves for receptors to bind co-receptors in order to begin the process of infection. It is important that tat and rev genes in HIV genome are called regulators for the processing of transcription and translation. With the exception of tat and rev, there are accessory proteins which are not important for viral replication such as nef, vif, vpr and vpu. Efficient viral infection is possible only all of genes can be expressed with balance (Wodrich and Kräusslich, 2001). RNAs of HIV-1 are split into Rev-dependent which has at least one functional intron and Rev-independent which has no intron (Malim et al., 1989). Aggregation of unspliced RNA in the cytoplasm shows HIV-1 transcription does not occur at all (Staffa and Cochrane, 1994).

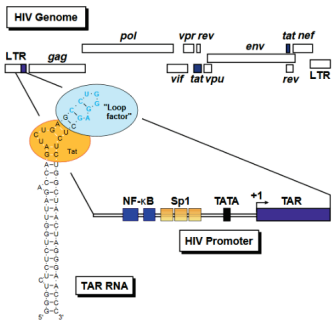


Figure 1.1. Genome Structure of HIV
(Source: Karn, 2000)

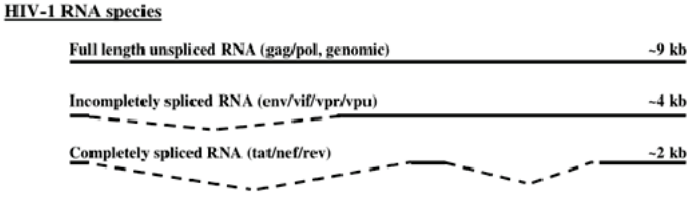


Figure 1.2. Splice Variants of HIV Genome
(Source: Morten et al., 2005)

HIV proviral genome also consists of Long Terminal Repeat Regions which are called 5' LTR (serving as promoters) & 3' LTR (signal carrier for 3' function) (Jern and Coffin, 2008). Moreover, Each LTR region contains a unique region in it, such as U3 & U5. Furthermore, The U3 region has several transcription binding sites that are important for viral gene expression. The unspliced primary transcript has approximately 9 kb. A class of singly spliced RNAs (4 kb) lacks the gag-pol coding region and a class of completely spliced RNAs (2 kb) lacks the env coding region (Bohne et al., 2005). Although, there are several splicing products, completely spliced mRNA can be transported to cytoplasm for expressing Tat, Rev and Nef proteins.

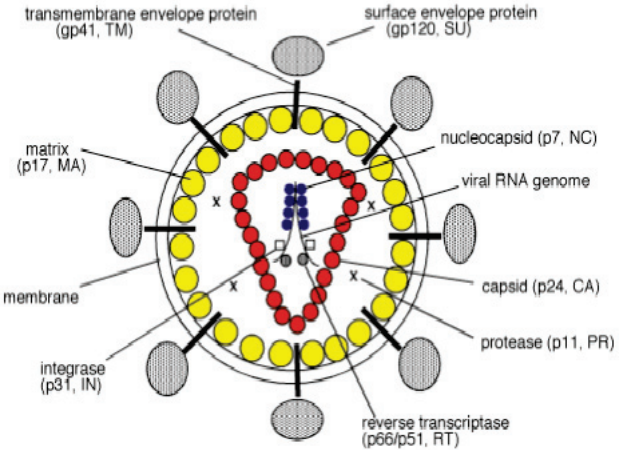


Figure 1.3. General appearance of mature virion
(Source: Freed, 1998)

1.2.1. Regulatory Genes

1.2.1.1. Trans Activator of Transcription (TAT) Protein

Firstly in order to understand the functions of Tat, it must be examined structurally. It has various types of amino acid sequences between 84 and 104, encoding from two exons with divided-six regions (Nekhai and Jeang, 2006; Shojania and O'Neil, 2006). A small RNA-binding protein Tat, regulating many processes about several cellular functions and signaling pathways, binds precisely to TAR region in the genome which has hairpin-looped structure for activating transcription and playing a significant role in replication and expression of HIV-1 (Kuppuswamy et al., 1989). For activation of HIV-LTR, Tat-TAR interaction plays an important role. On the other hand, there is another TAR-independent way for activation of the functions of Tat (Taylor et al., 1992). One of the features of Tat is the interaction with several proteins. Tat may act as a crucial role for viral gene expression with cognate DNA sequences which exists on HIV-LTR (Madore and Cullen, 1995). Binding of TAT on enhancer region of HIV-LTR which contains NF- ?B and SP1 elements is important for TAR-independent activation of Tat (Yang et al., 1997). According to Markov Model, HIV-TAT has many similarities about homology with p50 protein which exists in human and mouse and is also a member of NF- κB family (Gough et al., 2001). In addition to that, Tat proteins may start to initiate several cellular processes by interaction with different types of cellular components. There is no need to be relative with viral infection initiator. Tat may be affected not only with infected cells but also non-infected cells (Musinova et al., 2016). Tat can bind to neural membrane for depolarization through increasing ratio of Ca+ (Sabatier et al., 1991). Tat also reacts with phosphatidylinositol 4,5-bisphosphate for helping exocytosis (Tryoen-Tóth et al., 2013). As mentioned above, Tat is functional not only in cytoplasm but also in nucleus. There are two nuclear effects about Tat protein; interaction with chromatin in transduced cells expressed Tat and nucleolar Tat which exists in HIV-infected cells (Musinova et al., 2016).

1.2.1.2. Rev Protein

Rev has an important role for exporting viral messages; as there is an export signal within Rev protein which plays a crucial role in mediating post-transcriptional regulation HIV-1 (Shojania and O'Neil, 2006). Rev is small phosphoprotein which has sequence-specific and is 13 kDa. Existence of Importin and exportin for transportation between nucleus and cytoplasm emerges Rev to change place from nucleus to cytoplasm. In the structure of Rev there is an arginine-rich domain which exists between 38 and 49 amino acids for action of Rev protein (14). Arginine-Rich domain of Rev is directly binds to Rev Response Element (RRE) which is located in one of HIV introns (Malim et al., 1989). Rev has two domains which have different purposes to each other. N-terminal domain of rev supplies mutimerization of rev and binding to RRE and Near C-terminal there are sequences which contains approximately leucine-rich 10 amino acids for recognition to export (Nuclear Export Signal) (Malim et al., 1989). Rev binds to one of karyopherin family member called Crm1 to transport it to the cytoplasm (Fornerod et al., 1997; Neville et al., 1997). Crm1 interacts with its cargo in the nucleus by means of Ran GTPase. Conversion of GTP to GDP makes it possible for the cargo to be released into the export way. In addition to that, Crm1 induces to bind one of Nuclear Pore Complex components to transport from nucleus to cytoplasm (Nakielny and Dreyfuss, 1999).

1.2.2. Accessory Genes

1.2.2.1. Nef

It is one of completely spliced product with Tat and Rev. Name of nef originated from the 'negative factor' of virus replication. Absence of nef gene can cause undetermined infection in macaques which has resistance for superinfection with SIV (Daniel et al., 1992). Although, infection is delayed without nef gene, mechanism of deletion of nef gene is not understandable completely. However, nef may provide downregulation

of $CD4^+$ and MHC I. Both nef and gag have immunological dominancy regions in their movement against Cytotoxic T Lymphocytes ($CD8^+$) (Laguette et al., 2010).

1.2.2.2. Vif

Its name originated from the 'virion infectivity factor' which has an adverse effect to viral infection. Vif gene-deleted viruses also do not have APOBEC3G special protein which is important to prevent viral DNA degradation by means of hypermutation between A and G (Goila-Gaur and Strebel, 2008).

1.2.2.3. Vpu

Importance of this gene has not been fully understood for years. According to recent findings, vpu supports about $CD4^+$ downregulation as well as nef and gp120 Env (Dubé et al., 2010). Additionally, the interaction of vpu with tetherin or BST-2 host cell protein may result in causing adverse effect (Andrew and Strebel, 2010).

1.2.2.4. Vpr

Vpr is one of the early identified proteins; as a transcription activator for HIV-1 in nondividing cells (Wang et al., 1995). Moreover, vpr is crucial for different kinds of processes in virus life cycles (Subbramanian et al., 1998). Furthermore, vpr is a component of pre-integration complex which provides integration of viral genome to host genome (Popov et al., 1998).

1.2.2.5. Vpx

It only exists in HIV-2 to mediate in virus replication with either T lymphocytes or macrophages (Yu et al., 1991). Vpx is also related to SAMHD1 which is one of the

restriction mechanisms for inhibiting HIV-1 replication (Laguette et al., 2011).

1.3. Viral Life Cycle of HIV-1

The gp120 Env protein of virion provides binding to $CD4^+$ which is expressed on the surfaces of macrophages, monocytes, dendritic cells and T lymphocytes and chemokine co-receptor CCR5 and CXCR4 which are expressed differently in some T subtypes on the host cell surface. After fusion with the host cell membrane owning to gp41 Env protein of virion, uncoating process of virion occurs. Viral content goes through the Nuclear Pore complex before integration.

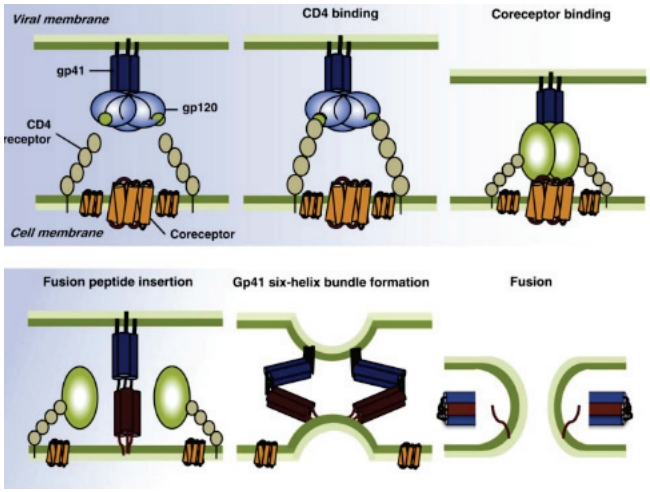


Figure 1.4. Binding Mechanism of HIV to the host cell
(Source: Marc et al., 2010)

Reverse transcription of plus-stranded RNA occurs for forming minus-stranded DNA via the process of Reverse Transcriptase in the cytoplasm of the host cell. After that, plus-strand DNA is copied from minus-strand DNA. Therefore, double-stranded DNA is formed for integration to host genome via integrase enzyme. After integration, transcription of viral genes occurs in the nucleus. Completely spliced viral RNA is exported from nucleus to cytoplasm. However, the unspliced and singly spliced viral RNAs maintain to remain in the nucleus. Translation of viral RNAs contributes to produce Gag

polyprotein precursor (55kDa) for producing virus particle and GagPol polyprotein precursor (160 kDa) for viral enzymes such as protease, reverse transcriptase and integrase. Gag polyprotein contains Matrix (MA), Capsid (CA), Nucleocapsid (NC), P6 region and P1-P2 spacer. Each region has different type of importance to form virus particle, such as MA for membrane binding and env protein inclusion, CA for regulation of nuclear import, NC for RNA encapsidation, p6 region for Vpr addition. Gag protein also provides to recruit endosomal sorting complex required for transport (ESCRT) complex which is important for budding of mature virion. Viral env proteins are generated via secretory pathway which means Rough Endoplasmic Reticulum produces Env Proteins and those proteins are transferred to Golgi in vesicle and then finally arrive to the plasma membrane. Viral proteins emerge through the translation in the cytoplasm. Mature virion starts to form in the plasma membrane and consequently, virion can be cleaved with ESCRT complex.

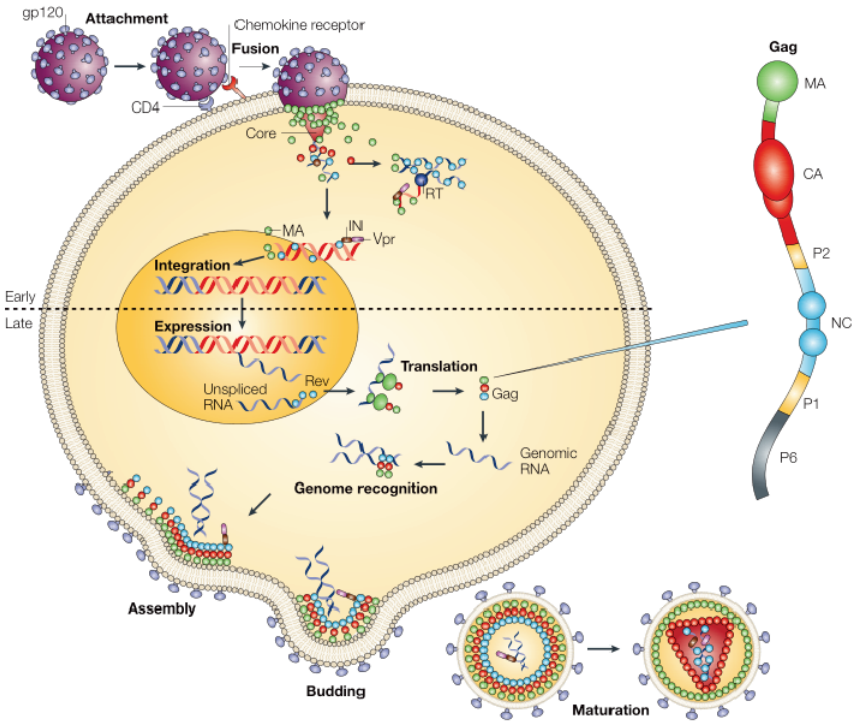


Figure 1.5. Early and Late Phases of HIV Life Cycle
(Source: D'Souza et al., 2005)

1.4. Retroviral Restriction Factors

There have been several restriction mechanisms for inhibition of HIV-1 infection during mammalian evolution. These mechanisms target different stages of viral infection. First of all, adaptive and innate immunity are important for preventing infection of HIV-1 in the beginning. Except innate and adaptive immunity for inhibition of invading of viral infection, APOBEC3, TRIM5α, SAMHD1, tetherin and Fv1 are different kind of restriction mechanisms to defend against viral infection activity (Blanco-Melo et al., 2012).

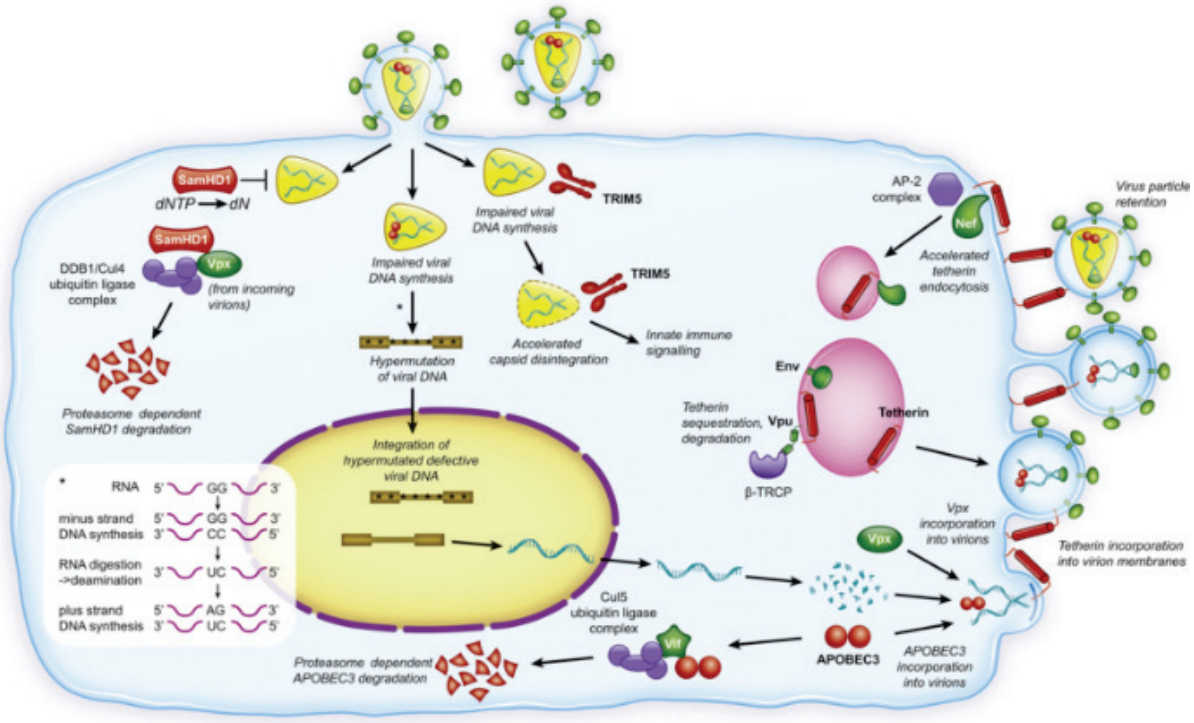


Figure 1.6. Retroviral Restriction Factors
(Source: Blanco-Melo et al., 2012)

1.4.1. Species-Specific Restriction Factor

1.4.1.1. Trim5α

Trim5 α was originated from Sodroski laboratory. They discovered that Old World Monkeys were not prone to HIV-1 infection (Meroni and Diez-Roux, 2005) (Reymond et al., 2001). In structure of TRIM5α, there is a RING construction with ubiquitin ligase activity. Modification of capsid protein can be utilized with ubiquitination in the uncoating phase. Additionally, there are B-box domains, coiled coil domains and PRYSPRY (B.30.2) domains (Reymond et al., 2001). The last domain has responsibility over specific functions of Trim5α (Nakayama and Shioda, 2010).

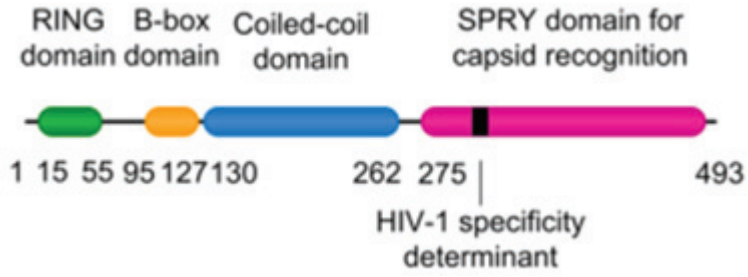


Figure 1.7. Structure of TRIM5 α
(Source: Blanco-Melo et al., 2012)

Trim5α is a natural component of intrinsic immunity; it provides inhibition of retroviral infection in primates. In rhesus macaques one of cytoplasmic proteins, Trim5 α, recognizes capsid proteins of virus in the uncoating process (Stremlau et al., 2006). Trim5α has the most diversity in primates among other restriction factors. In rhesus monkey, TFP residues (339-341) may be changed with sole glutamine in order to provide variation (Newman et al., 2006). However, Changing between TRP and glutamine provides anti-viral activity (but not against SIVmac) (Kirmaier et al., 2010). Moreover, in old World Monkeys Trim5 α prevents aggregation of newly-produced viral genomes whether in natural milieu or heterologous environments. In humans, Trim5 α is not effective for

supression of HIV-1 infection but can prevent N-tropic murine leukemia virus. (N-MLV) (Towers, 2005). Furthermore, Trim5 α can be saturated because of the pre-infected cells with viruses that depends on multimeric complexity of CA (Dodding et al., 2005). This information supports that Trim5 α is an indicator for positive selection for primates (Liu et al., 2005). B-box2 and coiled-coil domains are the highest positive selection indicators of Trim5 α; they are also crucial for the specification of Trim5 α's restrictions (Stremlau et al., 2006). In Rhesus monkey, Simian immunodeficiency virus (SIV) can be recognized with TRIM5 α after un-coating and conversion from viral RNA to cDNA. Before the identification of TRIM5 α, it is known as Ref1 for human and Lv1 for monkeys (Hrecka et al., 2011). TRIM5α-mediated provides to form either incomplete reverse transcript processes or vanishing completed reverse transcript. The most important role is that, cyclophilin protein which exists in host cells can affect the level of restriction via TRIM5 α. Cyclophilin A is also crucial for restriction of HIV-1 infection in nonhuman primates. This specific protein interacts with capsid molecules after the entry of viruses and it also affects the level of viral infection. Recent studies show that breaking interaction between capsid and cyclophilin A causes an increased potential of HIV-1 infection.

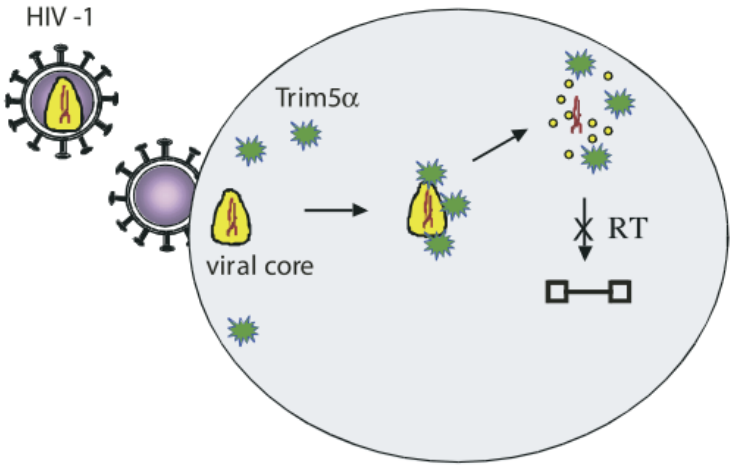


Figure 1.8. Mechanism of TRIM5 α
(Source: Emerman, 2006)

1.4.2. HIV-1 Counteracts General Restriction Factors

1.4.2.1. APOBEC3G

All of APOBEC family members have similar structure and are called cytidine deamination molecules. APOBEC can be naturally expressed by non-permissive cells for inhibition. Especially, APOBEC3G is the most powerful member for the prevention of viral effects and it also plays a role for the catalysis of hydrolysis of cytidines to uridines. APOBEC3G exclusively chooses cytidine as a target which exists in a single stranded DNA before integrating with the host cell genome. Targetting cytidine and converting to uridine are called hypermutation or DNA editing (Huthoff and Towers, 2008). In lack of HIV Vif (a small protein (23 kDa)), provirus can engulf APOBEC protein to induce editing between cytidine to uridine via deaminase-independent process. Vif is crucial not only to the inhibition of encapsidation of APOBEC protein, but also to the induction of ubiquitin-dependent APOBEC protein degradation (Goila-Gaur and Strebel, 2008). The main structural feature of APOBEC3G is having catalytic center for DNA editing. It also has a β-sheet which consists of five strands and five α-helices for forming catalytic center. Another general feature of APOBEC3G structure is that it has 384 amino acids and is a 46kDa protein. It has also conserved zinc-binding motif. According to Figure, APOBEC3G targets Vif viral protein which has wide-range between primate and non-primate viruses. One of HIV-1 proteins, Vif, has a role during HIV-1 replication. However, APOBEC3G can recognize Vif and utilize deamination of viral cDNA; this process is induced by reverse transcriptase (Stevenson, 2004).

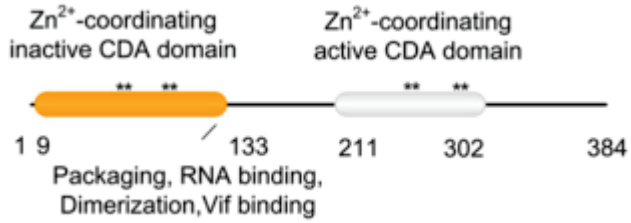


Figure 1.9. Structure of APOBEC3G
(Source: Blanco-Melo et al.,2012)

As shown in the Figure below, Viral DNA is changed by APOBEC3G in two different ways. Some of the viral DNA is known by host-uracil-DNA glycosidases enzyme for taking away dU residues which exists in viral DNA. As a result of this process, abasic region can be determined by DNA base abscission repair enzymes and provides degradation of viral DNA. Absence of vif in virion has low viral DNA, post the infection process (Mangeat et al., 2003). It is obvious that vif can bind precisely to APOBEC3G but it is not known that binding to APOBEC3G is either transient or stable. Consequently, vif may inhibit APOBEC enzyme's activity individually (Mariani et al., 2003).

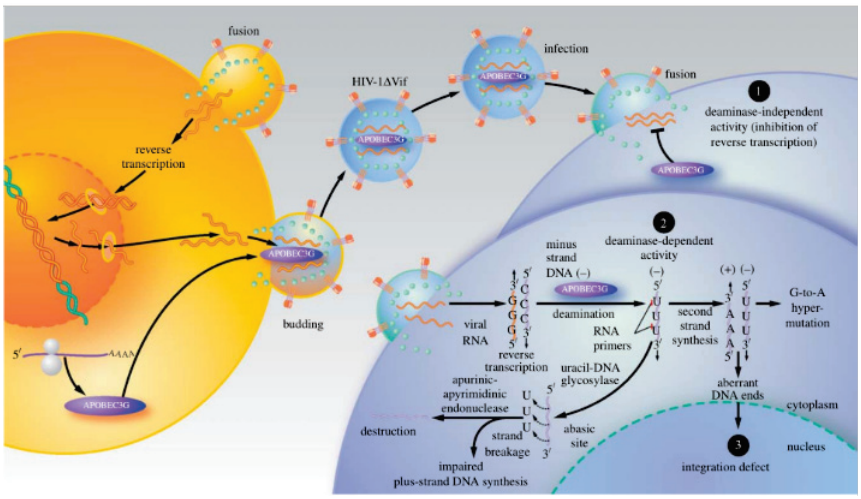


Figure 1.10. Mechanism of APOBEC3G
(Source : Ya-Lin et al., 2009)

1.4.2.2. SAMHD1

It consists of sterile alpha motif and histidine/aspartic acid domain which has protein 1. Firstly it was classified as HIV restriction factor in myeloid cells (Descours et al., 2012). SAMHD1 is also observed in resting $CD4^+$ for preventing HIV replication (Rice et al., 2009). Aicardi syndrome is an emerged mutation in SAMHD1 gene which causes overexpression of IFNα (Belshan et al., 2012). SAMHD1 is recruited by vpx for degradation of proteasomal contents by means of E3 ubiquitin ligase (Doumas et al., 2005). Thus, SAMHD1 restriction mechanism is related to hydrolysis of deoxynucleoside triphosphates for inhibiting.

Figure 1.11. Structure of SAMHD1
(Source: Blanco-Melo et al., 2012)

1.4.2.3. Tetherin

Tetherin is the 19.7 kDa transmembrane protein and also encodes glycosylphosphatidylinositol (GPI). Tetherin is important to trap mature virus particles at the cell surface, where endocytosis occurs. In other words, tetherin is activated against enveloped viruses. Tetherin exists either at cell surface or trans-Golgi Network and shuttles between them (Emerman, 2006).

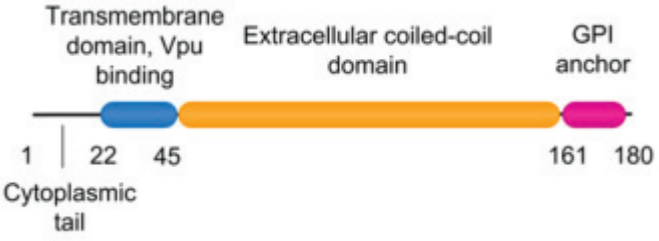


Figure 1.12. Structure of Tetherin
(Source: Blanco-Melo et al., 2012)

1.4.3. Other Restriction Factors

1.4.3.1. Fv1

Amino-terminus dimerization of Fv1 restriction mechanism provides a prevention mechanism for viral infection in the pre-integration stage. Dimeric Fv1 can interact with multiple capsid molecules in viral content. Therefore, Fv1 blocks between converting RNA to DNA with reverse transcriptase and before forming provirus phase (Bishop et al., 2006). Fv1 restriction mechanism is induced by capsid domain of gag proteins in primates and mice. In mice, Fv1 restriction mechanism can target capsid of Murine Leukemia Virus (MLV). In non-human primates, HIV-1 cannot affect because of blocking in conversion phase with reverse transcriptase, otherwise in Chimpanzees and gibbon apes, HIV-1 can infect host cells (Stevenson, 2004).

1.4.3.2. Ref1

Ref1 blocks a special residue of CA gene which is infected by MuLV like Fv1in the same manner. Ref1 is effective earlier than Fv1 while they encounter infection to block reverse transcription of viral DNA.

1.5. Preliminary Study

According to previous studies in our lab, one of the regulatory proteins, tat, was cloned to pbudCE4.1 mammalian vector. As a negative control, pBUdCE4.1 mammalian vector was used. Therefore, empty pbudCE4.1 and constructed-pBudCE4.1 HIV-TAT plasmids were transferred via transfection method, using transfection reagent to CV-1 cells (African Green Monkey-derived kidney cells). Then, protein isolation of transfected cells was done for 2D-GEL electrophoresis in order to detect the expression of several proteins. After 2D-GEL electrophoresis, different proteins were separated with their molecular weight and isoelectronic points. Divided proteins were observed and analyzed via MALDI-TOF/TOF mass spectrometry. As a result, a meaningful protein was found; namely Secretory Leucocyte Protease Inhibitor (SLPI), for leading the next experiments. It has been shown that SLPI protein inhibits HIV infection by means of preventing proliferation of T cells in saliva but there is no direct interaction with viral proteins or downregulation of $CD4^+$ cells.

1.6. Secretory Leucocyte Protease Inhibitor (SLPI)

One of the members of innate immunity, SLPI, is a highly basic, 107 amino acid and 11.7 kDa protein which is expressed by SLPI gene from trappin gene family, produced in epithelial cells (Greene et al., 2003). The main feature of SLPI is protection of tissues from harmful effects of proteolytic enzymes. Absence of SLPI causes an increase in the ratio of tissue damage (Eisenberg et al., 1990). It applies its protease activity with C-terminal domain which has active center consisted of Leu72-Met73 residues (Ying et al., 1994). N-terminus of SLPI has stabilizing features for antiprotease- protease complex (Hocini et al., 2000). In addition to this feature, SLPI can prevent a penetrate HIV-1 infection in mucosal tissues (Drenth et al., 1980). Three-dimensional structure of SLPI indicates that it is the member of a large protein family which is formed generally by neurotoxins because of four disulfide bonds which help it to hold together (Doumas et al., 2005; Grütter et al., 1988). In addition to that, Tertiary structure of SLPI contain disulfide

bridges between cysteine residues to form boomerang-shaped structure and each arm of this structure has one domain (Kikuchi et al., 1998). Human SLPI gene exists on a chromosome called 20q1213.2 (Stetler et al., 1986). SLPI gene has four exon and three introns which are about 2.6 kb on whole SLPI gene (Thompson and Ohlsson, 1986). Expression of SLPI at mRNA and protein level can occur not only in epithelial cells but also in macrophages, granulocytes and dendritic cells. It originates from parotid saliva (McNeely et al., 1995). Due to various functions SLPI provides binding especially to membrane receptor (Zhang et al., 1997). Pro-inflammatory process could trigger an increasing level of SLPI expression through binding Toll-like receptors. Moreover, cAMP-dependent signaling is important for regulation SLPI expression in cells. There are two forms for SLPI, one of them is secreted-form which is considerably important at tissue level. The other is intracellular form which can easily enter both cells and nuclear membranes. SLPI can interact with many proteases directly or indirectly through synthesis of proteases. For instance, SLPI affect serine protease directly but prevent for conversion of zymogen forms protease in monocytes indirectly (Taggart et al., 2005). SLPI plays a critical role for NF-κB activity through affecting NF-κB inhibitory components like IκBα and IκBβ. In the cytoplasm, NF-κB exists in an inactive state because of binding IκB. During cell activation, IκB is degraded, supporting transportation of NF-KB to nucleus for regulation transcription (Majchrzak-Gorecka et al., 2016). NF-κB can regulate immune response and proliferation as compensator at which body barriers. NF-κB is also important for adjusting inflammatory processes (Samsom et al., 2007). In addition to all of SLPI features, SLPI has antiviral activity which is not related to protease activity of SLPI. Scramblase 1 provides movement of phospholipids in the membrane and also blocks fusion of HIV-1 viral particle with host cell membrane. SLPI prevents interaction between main target for cell entry and scramblase (Py et al., 2009). Preventing HIV-1infection in presence of SLPI may occur with targeting host cell instead of binding to virus particle (Turpin et al., 1996). In other words, SLPI hampers HIV-1 infection with dose-dependent manner instead of binding CD4. Antiviral activity of SLPI may be led by its anti-protease activity for inhibiting viral infection (McNeely et al., 1997). In addition to that, SLPI blocks free HIV-1 in mucosal fluids such as saliva, breast milk and cervical fluid (Franken et al., 1989; Casslen et al., 1981).

1.7. Aim of the Study

It is aimed to show how the SLPI expression level in $CD4^+$ T lymphocytes and formed-stable Vero cells changed due to the existence of HIV-TAT and SIV-TAT. Some findings indicate that the prevention of viral infection with SLPI protein is observed before virus enters to host cell. However, intracellular studies about SLPI protein are limited to understand how this mechanism works. To facilitate further studies, it is important to understand intracellular SLPI effects on different types of cell lines.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Kits which was used during this work have shown in 'Comercial Kits' section, respectively. For this work, different types of plasmids were used.

2.1.1. Commercial Kits

Table 2.1. Commercial Kits which are used during experiment.

KITS	SUPPLIER
GeneJET Genomic DNA Purification Kit	Thermo Fisher
ReverAid First Strand cDNA Synthesis Kit	Thermo Fisher
CloneJET PCR Cloning Kit	Thermo Fisher
GeneJET RNA Purification Kit	Thermo Fisher
NucleoBond Xtra Midi Plus	Macherey-Nagel
High Pure Plasmid Isolation Kit	Roche
MinElute Reaction Clean-up Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
Human SLPI ELISA Kit	Fine Test

2.1.2. Plasmids

Three plasmids are required for virus production. Lentiviral plasmid was constructed with isolation Ef1ProTAT region from pBudCE4.1 HIV-TAT plasmid and ligation Ef1ProTAT region with pJET1.2/blunt plasmid. Last stage of construction of lentiviral plasmid is ligation with Ef1ProTAT region and LacZ plasmid.

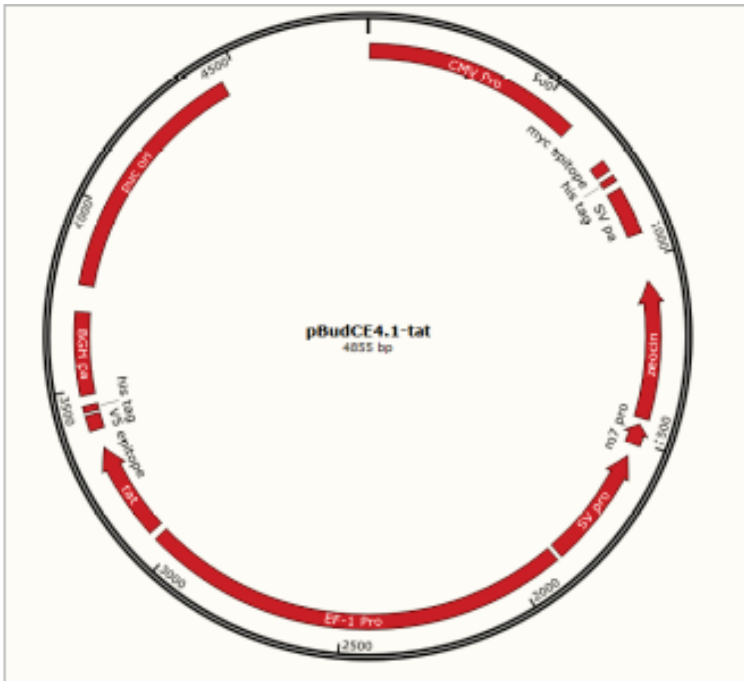


Figure 2.1. pBUDCE4.1- Tat plasmid

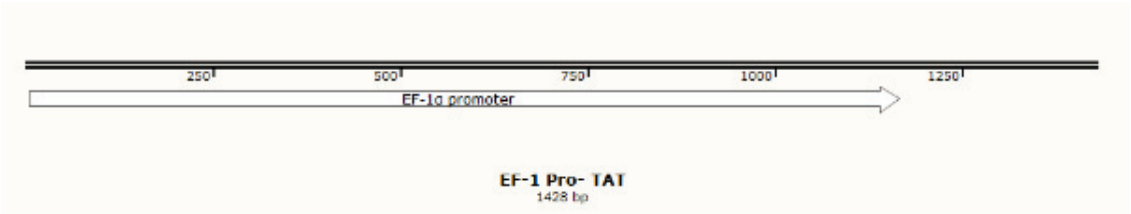


Figure 2.2. Ef1ProTAT region (separated from pBUDCE4.1- Tat plasmid)

2.1.3. Primers

From 5' to 3' Sequences of primers has shown in Table 2.2.

Table 2.2. Primer sequences which are used during experiments.

PRIMER NAME	SEQUENCES
GAPDH	Forward 5'TGCACCACCAACTGCTTAGC 3' Reverse 5' GGCATGGACTGTGGTCATGAG 3'
HIV- TAT	Forward 5' AGATCCTAGACTAGAGCCCTGGAA 3' Reverse 5' CAAACTTGGCAATGAAAGCAACAC 3'
SLPI	Forward 5'-CTAGGGAAGAAGAGATGTTG-3' Reverse 5'-CCTAAATCACAGGAATC-3'
ZEOCIN	Forward 5' TGATGAACAGGGTCACGTCGTC 3' Reverse 5' AAGTTGACCAGTGCCGTTCCG 3'
pcDNA	Forward 5'ACGACTCACTATAGGGAGACCC3' Reverse 5'TAGAAGGCACAGTCGAGGCT3'
pcDNA-SIV-TAT	Forward 5'GCTATCATTGTCCGCTTTGCTTC3' Reverse 5'CCAGGTGTATGATCTGCTGTCG3'

2.2. Cell Culture

Molt 4, Jurkat, Jurkat Tat III, Vero, HSC-F cell lines were maintained for this study. Molt 4 and Jurkat are T lymphocyte cell lines which are derived from humans. Molt 4 has hypertetraploid number of chromosomes which is also called pseudodiploid human cell line. Jurkat Cell Line was procured from a 14-years-old boy's peripheral blood. Jurkat Tat III is a human T cell leukemia cell line which expresses HIV-1 tat protein. Jurkat Tat III is an important cell line for use in the study instead of low replicating HIV strainHSC-F was isolated from a foetal Cynomolgus monkey which was immortalized with H. Saimiri. It contains either $CD4^+$ or $CD4^+CD8^+$ sub-divided colonies. It is important that these cells be infected with both SIVmac and SIVagm. Vero was isolated from the kidney of *Chlorocebus sabaeus* (African Green Monkey)in 2014. Vero has a hypodiploid chromosome count.

Figure 2.3. Construction of Ef1ProTAT-pJET1.2/blunt plasmid

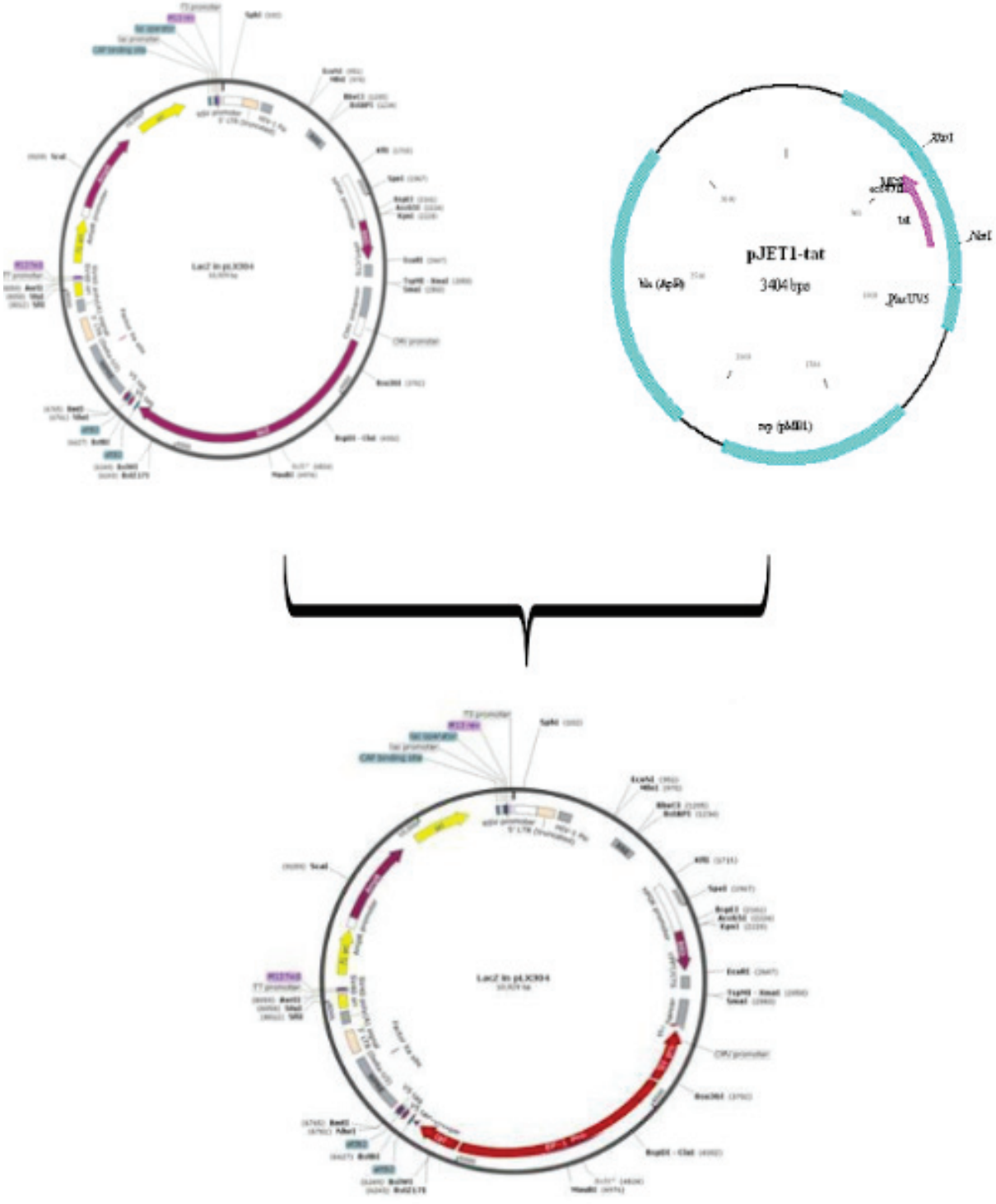
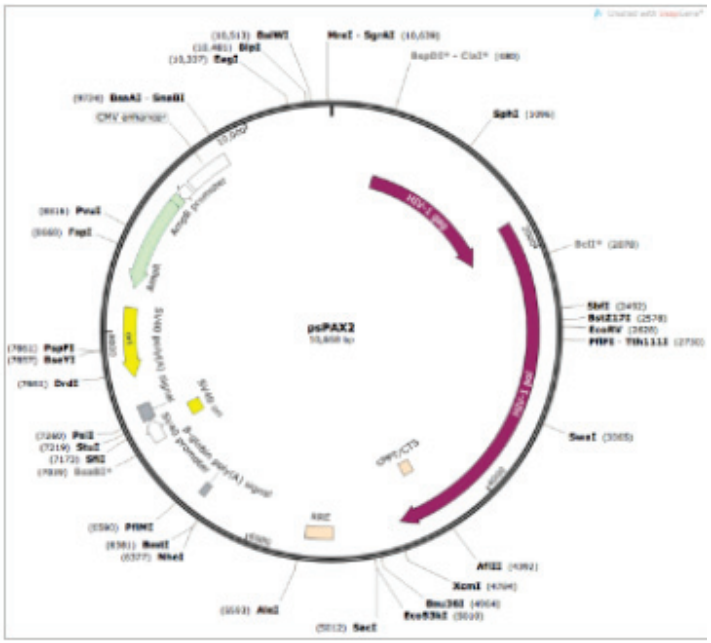


Figure 2.4. Construction of Ef1ProTAT-LacZ plasmid



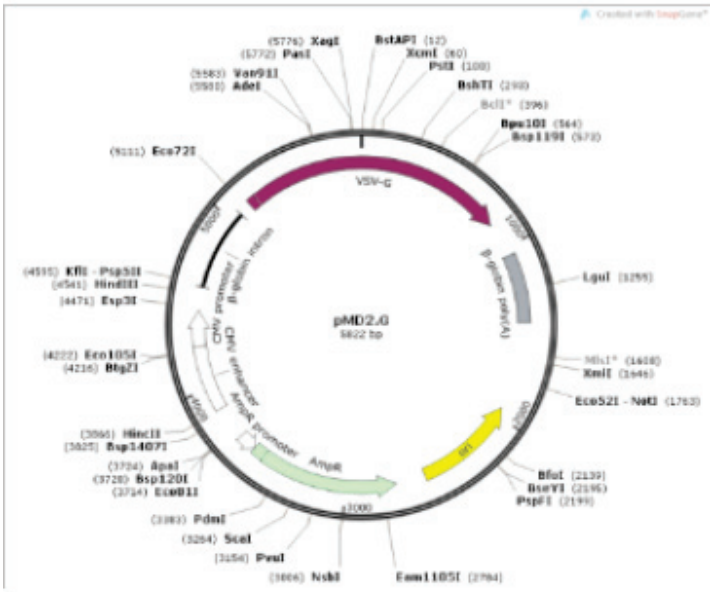


Figure 2.5. psPAX2 and pMD2.G plasmids

2.3. Freezing & Thawing of Cells

First and foremost, all of those cell lines were removed from - 80 °C freezers or from -196°C liquid nitrogen and stood in 37°C water bath (NB-9 Nuve) once those cells had thawed out completely. After that, those cells were taken to a 60 mm plate with appropriate medium according to cell types (DMEM, RPMI 1640), containing 10% FBS, 2% L-glutamine and 1% penicillin-streptomycin. Later those cells were settled in an incubator at 37 °C with 5% CO2. After one day, Medium was aspirated in order for it to take DMSO from cells for decreasing DMSO's harmful effects on the cells. Therefore, sub-culturing of those cells occurred for maintaining healthy cells 2-3 times per week. In time, new stocks were collected from healthy cell lines and they must not have too high a number of passage. When those cells were stocked, they have to have had been cultivated in 100 mm plates because the number of cells is always important for freezing them. In 100 mm plates, cells should reach 80% density for starting stocking process. After observing cells, Medium in plate was removed and washed with 1X PBS. Then 0.25% Trypsin was used for disassociating cells from surface of plate with enzymatic reaction at 37 °C. As a result of the addition of medium with FBS, Effects of Trypsin was inhibited. Trypsin/Medium mixture with cells was spun at 1250 rpm for 2 minutes. The final stages of stocking processes were dissolving cell pellet in 1 ml medium with 10% DMSO and 20% FBS and transfer to cryogenic storage vials. For a short term, Cells in cryogenic storage vials were put in -80 °C. But for long term, they must be placed into liquid nitrogen tank.

2.4. Maintenance of Cells

Molt 4, Jurkat, Jurkat Tat III, HSC-F and Vero cells were maintained with different kinds of methods and media. Molt 4, Jurkat, Jurkat Tat III, HSC-F need RPMI 1640 which contains 10% FBS and 1% penicillin-streptomycin. Vero cell needs DMEM with 10% FBS and 1% penicillin-streptomycin. Suspension cells: Molt 4, Jurkat, Jurkat Tat III and HSC-F cells were counted with the use of a Thoma chamber. In order to achieve

this goal, those cell lines were diluted with 0.5% trypan blue and put into a Thoma chamber for counting cells. According to the results, Suspension cells were split into a 60 mm plate with an appropriate rate and were completed with RPMI 1640 to total volume. Adherent cells: For Vero cells, medium was discarded from 60 mm plate. 1 X PBS was used for cleaning waste of cell debris and medium. Then, 0.25% Trypsin was added onto the cells that adhered to the bottom of the plate in order to disassociate Vero cells. Due to the inhibited harmful effects of Trypsin onto cells, medium was added to stop the activation of trypsin. Centrifugation was performed at 1250 rpm for 2 minutes to remove trypsin/medium mixture completely. After that, the cells were divided into proper ratio of the cells and cultivated onto the new 60 mm plate. For maintaining Molt 4, Jurkat and Jurkat Tat III cells, these cells reached $2X10^6$cells/ml, sub-culturing was performed every 3-4 days. Cell density is also important for splitting cells during this process; they must not exceed $3X10^6$cells/ml for each plate. Maintaining HSC-F cells is similar to maintaining Peripheral Blood Mononuclear Cells (PBMCs). They exhibit the same characteristic features in regards of culturing method. HSC-F cells cannot grow at a low concentration. Therefore, High concentration of these cells is the best way to provide cell-cell interaction and growth of these cells. It is also important that the flasks are more useful for these cells than the plates. Stand-up position of flasks inhibits loss of cells and makes them tight to grow. Every 2-3 days, half of the medium was removed and the same amount of medium was inserted into the flasks. Moreover, these cells are easily contaminated. That's why flasks are a better choice to prevent contamination with bacteria or yeasts. This property of the cells indicates that they are willing to aggregate when they have good conditions. For Vero cells, Recommendation for sub-cultivation ratio is 1:3 or 1:6. These cells should be split every 3-4 days.

2.5. Transfection

2.5.1. Virus Production

First of all, 293 T cells were plated with in a range of $5X10^6$- $6X10^6$ cells for duration of 24 hours prior to transfection in a 10 cm plate. After one day, Lentiviral plasmids were spun before use for transfection. In addition to that, transfection reagent was mixed with flicking and was made to reach room temperature. In 500 μl serum-free medium; 2 μg Lacz-Ef1ProTAT plasmid, 1.3 μg PsPAX2 (packaging vector), 0.7 μg pM2D.G (envelope vector) and 12 μl Turbofect Transfection Reagent were added per plate. For 30 minutes, DNA/Reagent mixture was kept at room temperature. Finally, the mixture was added drop by drop onto cells and a homogenous distribution was assured by mixing gently. The plate was put into an incubator with a temperature of 37 °C. Following a time lapse of 24 hours after transfection, medium was exchanged with 8 ml fresh medium in each plate. 48 hours after transfection, virus containing medium was collected into falcon tubes and stored at 4 °C. It was important that the cap of falcon tubes was covered by parafilm two times for storage. Onto the cells, 8 ml fresh medium was added and incubated at 37 °C. The following day, virus containing medium was collected onto previous collected-medium and stored at -80 °C until the Virus Infection experiment was performed.

2.5.2. Transfection Reagents

2.5.2.1. Lipofectamine Reagent

2.5 X 10^4 Jurkat cells for 24 well-plate & 1.7 X 10^4 HSC-F cells for 96 well-plate were cultivated in 500 μl growth medium with fetal bovine serum. For each well,

1.2 μg of DNA was put into 50 μl medium without serum. Moreover, for each well, 2.5 μl Lipofectamine 2000 Reagent (Invitrogen) was diluted in 50 μl medium without serum and incubated for 5 minutes at room temperature. Furthermore, DNA and Reagent were combined and kept for 30 minutes at room temperature. Exceeding 30 minutes for incubation is not good for transfection efficiency. Total DNA/Reagent mixture was added onto Jurkat cells. Plate was mixed gently by rocking back and forth and incubated at 37 °C. After 48 hours, transfection efficiency was controlled with peGFP3-C plasmid.

2.5.2.2. Turbofect Reagent

Vero cells in 6 cm plate were split with a ratio of 1:6 and approximately $5X10^4$ Jurkat cells for 24 well-plate & 1.7 X 10^4 HSC-F cells for 96 well-plate wer cells were cultivated into 24 well plates with 1ml containing serum medium one day before transfection. For efficient transfection, Cell density can reach 70-90% for adherent cells. For each well, 1 μg DNA was diluted into 100 μl medium without serum. TurboFect Transfection Reagent (Thermo) was spun immediately and added onto diluted DNA. DNA/Reagent mixture was kept for 15-20 minutes at room temperature. Then, 100 μl mixture was added onto cells drop by drop with homogenous distribution. After that, Plate was mixed by rocking back and forth. Incubation at 37 °C took place for 48 hours. Analysis of transfection efficiency was found by looking at peGFP3-C plasmid.

2.5.2.3. Stable Vero Cell Lines

pBudCE4.1 and pBudCE4.1 HIV-TAT plasmids contain Zeocin resistance gene in order to form a stable cell line. pcDNA3.1 and pcDNA3.1-SIV-TAT contain Neo resistance gene for generating a stable cell line. Zeocin and geneticin are quite different to each other in terms of affecting cells. When Zeocin was added onto cells, their shape was not becoming round and they were detaching easily from the plate; such as geneticin. Zeocin provides abnormal cell shape, breakdown of endoplasmic reticulum, Golgi apparatus, plasma and nuclear membranes in eukaryotic cells. Geneticin binds to 70S and 80S

ribosomes to inhibit polypeptide synthesis and protein elongation in eukaryotic cells. First thing to generate a stable cell line is that pBudCE4.1, pBudCE4.1 HIV-TAT, pcDNA3.1 and pcDNA3.1-SIV-TAT were cut overnight with appropriate restriction enzymes for linearization of those plasmids. On the same day $5X10^4$ cells were cultivated for each 24 well-plate.

Table 2.3. Linearization of Plasmids

	pBudCE4.1	pBudCE4.1TAT	pcDNA3.1	pcDNA3.1SIVTAT
Buffer	5 μl	5 μl	5 μl	5 μl
Plasmid	2 μg	2 μg	2 μg	2 μg
Enzyme	1 μl NheI	1 μl NheI	1 μl BglII	1 μl BglII
dH2O	to 50 μl	to 50 μl	to 50 μl	to 50 μl

Vero cell line was transfected with linearized-pBudCE4.1, pBudCE4.1 HIV-TAT, pcDNA, pcDNA-SIV-TAT respectively for each 24 well-plate. After 48 hours, according to transfected cells with peGFP3-C plasmid as indicator, transfected cell density was determined. Then all transfected cells were taken into 6 well plate for 1:4 dilution. In addition to that, ranging within 50 μg/ml - 1000 μg/ml, Zeocin was added onto Vero; cells were transfected with pBudCE4.1, pBudCE4.1 HIV-TAT and 100 μg/mL - 1 mg/ml range of Geneticin was added onto Vero cells and then transfected with pcDNA, pcDNA-SIV-TAT. After treatment with antibiotics, cells started to die immediately. And live cells were willing to aggregate and form a single colony on different locations in 6 well-plates. Then, a single colony of those transfected cells with different plasmids was taken with Scienceware ®cloning discs (3.2mm) to 24 well-plate and medium without antibiotics and added onto a single colony.

2.6. Total RNA Isolation and cDNA Synthesis

Transfection with either transfection reagents or virus particles was done before total RNA isolation with GeneJet RNA Purification Kit (Thermo #K0731). According to procedure, Pellet either suspension or adherent cells were re-suspended with lysis buffer comprised of 14.3 M β-mercaptoethanol. After Homogenization of these mixtures, 100%

ethanol was added and mixed with lysis buffer/ β-mercaptoethanol. After loading mixture to the column centrifugation and then serial washing processes were performed and RNA purification was completed by elution with nuclease-free water into the center of the column. Consequently, RNA concentration of whole samples was measured via nanodrop. Thenceforth, DnaseI treatment was applied for inhibiting DNA contamination to RNA samples. DnaseI is an important endonuclease for recognizing single and double stranded DNA and for providing breakdown of phosphodiester bonds. $Ca2^{+}$ is important for enzyme which in turn can be activated using $Mg2^{+}$ and $Mn2^{+}$ which are crucial for activation of DnaseI. In presence of $Mg2^{+}$ ion, DnaseI break double-stranded DNA (dsDNA) at each strand freely. In presence of $Mn2^{+}$ ion, Dnase I cleaves dsDNA roughly at the same site to produce blunt-end or two overhangs.

Table 2.4. Dnase Treatment Solutions and Amounts

Components	Amount
10X Reaction Buffer with MgCl2	1 μl
RNA Samples	1 μg
DNase 1	1 μl
dH2O	to 10 μl
50 mM EDTA	1 μl

After Dnase I treatment of every RNA sample, they were converted into cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo # K1621).

Basically, template RNA, Oligo (dT) and nuclease-free water were put in Rnase-free micro centrifuge tubes on ice.

Table 2.5. Synthesis of cDNA Step 1

	Amount
Template RNA	0.1 ng - 5 μg
Primer (Oligo(dT))	1 μl
Water (nuclease-free)	to 12 μl

Then, Micro centrifuge tubes were sustained at 65 °C for 5 minutes. After that, Reaction Buffer, Rnase Inhibitor, dNTP Mix and Reverse Transcriptase were put into micro centrifuge tubes, as well and incubated at 42 °C for one hour.

Table 2.6. Synthesis of cDNA Step 2

	Amount
5X Reaction Buffer	4 μl
RiboLock Rnase Inhibitor (20 U/μl)	1 μl
10 mM dNTP Mix	2 μl
RevertAid M-MuLV RT (200 U/μl)	1 μl

After all of those processes, converted RNA to cDNA samples were used for following experiments or stored at -20 °C or -80 °C temperatures.

2.7. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Transfected Molt 4, Jurkat, Jurkat Tat III, HSC-F and Vero cells with pbudCE4.1 (as a control); pbudCE4.1 HIV-TAT were observed with qRT-PCR for measuring SLPI expression in those cells. In addition to that, Quantitative expression level of SLPI was normalized with an expression of housekeeping gene such as GAPDH. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes a step of carbonhydrate metabolism; converting Glyceraldehyde-3-phosphate into inorganic phosphate and nicotinamide adenine dinucleotide (NAD). GAPDH is one of the most useful housekeeping genes generally for observing expression level with qRT-PCR as an internal control.

Table 2.7. qRT-PCR Solutions and Amounts

Component	Volume	Final Concentration
RealQ Plus 2X Master Mix	12.5 μl	1X
Primer Forward (10 mM)	0.5 μl	0.1 μM
Primer Reverse (10 mM)	0.5 μl	0.1 μM
Template DNA	-	Genomic DNA: 20 ng Plasmid DNA: 0.5 ng Bacterial DNA: 5 ng
PCR-grade Water	to 25 μl	

Table 2.8. Conditions of qRT-PCR

Cycles	Duration of Cycles	Temperature
1	15 minutes	95 °C
40	15-30 seconds	95 °C
	30 seconds	55-60 °C
	30 seconds	72 °C

2.8. Analysis of Proteins

2.8.1. Protein Isolation

First important thing for protein isolation from transfected Molt 4, Jurkat, Jurkat Tat III, HSC-F and Vero cells with pbudCE4.1 and pbudCE4.1-TAT is that Cell density should be reached to an acceptable confluency in 10cm plates. Pellets of all cells were spun with centrifugation at 1250 rpm for 2 minutes and supernatant of these cells were discarded. Then, ProteoJET Mammalian Cell Lysis Reagent was added 20:1 ratio according to pellet volume. For resuspending cells, Mixture was spun by vortex and incubated on a shaker (900-1200 rpm) for 10 minutes at room temperature. Lysate clarification was provided with centrifugation at 16.000-20.000 g for 15 minutes. Supernatant was transferred to a new micro centrifuge tube. Lysate can be stored at -80 °C until use.

2.8.2. Bradford Assay

Bradford Assay is based on attaching Coomasie Blue-G250 (CB-G250) to protein in order to measure total protein concentration of samples. At different pKa values, CB-G250 can appear in four various ionic forms such as 1.15,1.82 and 12.4. More anionic form of the dye which is blue, can bind to proteins. After binding absorbency can be measured at 595 nm. 100 mg/ml BSA was used as a protein standard, concentrated protein standard should be 10, 20, 40,60,80 and 100 μg/ml. Dilution liquid was used as a negative control for forming standard curve. Samples of unkonwn protein concentration

were diluted with ratios such as 1, 1:10, 1:100 and 1:1000. ProteoJET Mammalian Cell Lysis Reagent was used as a negative control for diluted samples. Bradford Reagent was prepared with 100 mg of Coomasie Blue G250 in 50 ml 95% ethanol and then the solution was mixed with 100 ml 85% phosphoric acid and filled with distilled water up to 1L. Thereafter, 1ml Bradford Reagent was put onto each tube containing standards, samples and negatives. Consequently, 200 ml of each tube was transferred to the 96 well-plate, respectively to measure at 595 nm via spectrophotometer. Standard curve was formed with BSA dilutions and unknown protein samples can be measured by using this standard curve.

2.8.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Western Blot is also called immunoblotting which is highly preferable in order to detect and analyze proteins. This method relies on antibody-protein complexes whereby binding specifically to each other immobilized on a membrane. Work flow of Western blot is important to understand stages from sample preparation to detection.

Figure 2.6. Workflow of Western Blot

Different types of samples can be prepared with several methods such as detergent lysis, mechanical homogenization, freeze/thaw lysis, osmotic shock lysis, etc. In our lab, Protein samples were isolated with ProteoJET Mammalian Cell Lysis Reagent which has protease inhibitors to avoid degradation of proteins during cell lysis process. Polyacrylamide gel allows proteins to move with electric field and separate with molecular weight, larger molecules are decelerated compared to smaller molecules in gels. Acrylamide is a monomer of gel matrix, Bisacrylamid is a cross-linking agent for forming polyacrylamide. APS is an initiator for polimerization and TEMED which catalyzes to provide

polimeraization. Tris-glycine (Laemmli) buffering systems are consisted of a stacking gel (pH 6.8) and resolving gel (pH 8-9) provides for a favorable separation of proteins.

SDS is an agent which provides the individual separation of proteins based on their size, not their charge or three-dimensional structure for SDS-PAGE; because detergent binds to proteins to mask with coating of negative charged micelles. SDS-PAGE was prepared with 15% seperating gel and 5% stacking gel, respectively for separating proteins according to their size. Amount of solutions for separating and stacking gels are shown in figure.

Table 2.9. 15% Resolving Gel Preparation

40% Polyacrylamide (Sigma)	3.75 ml
1M Tris (pH 6.8)	2.5 ml
10% APS (AppliChem)	100 μl
10% SDS (AppliChem)	100 μl
TEMED (Sigma)	4 μl
UltraPure Water	3.55 ml

Table 2.10. 5% Stacking Gel Preparation

40% Polyacrylamide (Sigma)	1.25 ml
1M Tris (pH 8.8)	1.25 ml
10% APS (AppliChem)	100 μl
10% SDS (AppliChem)	100 μl
TEMED (Sigma)	10 μl
UltraPure Water	7.3 ml

20-30 μg protein samples were mixed gently with protein loading dye. Protein Samples/loading dye mixture were incubated at 90 °C over 10 minutes for breakdown of three-dimensional structure of proteins to move easily in the gel. After that, Prepared-Protein samples with loading dye (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8) were loaded into vertical gel for 2 hours at 120 V in 1X SDS Running Buffer. (3.0 g of Tris base (0.25 M), 14.4 g glycine (1.92 M) and 1 g SDS in 100 ml of ultrapure water, pH 8.3) For semi-dry transfer with stacking, gel was cut away from resolving gel after running process was completed. Four Thermo Scientific Pierce Western Blotting Filter Papers were cut the

same size as gel and absorbed in transfer buffer. For PVDF membrane, it is required to be made wet in advance with 100% methanol for 30 minutes and nitrocellulose membranes should be made wet in distilled water before equilibration in transfer buffer. (25 mM Tris, 190 mM Glycine, 20% methanol) Nitrocellulose membrane is used for detection of proteins which have low molecular weight and PVDF is suggested for detection of higher weighted molecular proteins. Nitrocellulose membrane is commonly used because of low background and provides a combination of non-covalent and hydrophobic forces for interaction between biomolecules and membrane. Nitrocellulose membrane is not recommended when stripping or re-probing is performed. PVDF membrane is more powerful where binding capacity of proteins is concerned, which causes a higher background. It is also better for the stripping or re-probing process. PVDF membrane has a combination of hyrophobic interaction and dipole. After equilibration in transfer buffer, Thermo Scientific G2 Fast Blotter was used for the transfer stage of western blot. It is provided that for semi-dry transfer of proteins, ranging 10-300 kDa in 5 to 10 minutes. In Thermo Scientific Power Stain Cassette, firstly, two equilibrated-filter paper were put, then PVDF membrane which was cut the same size as gel and filter papers, than settled onto two filter papers, afterward gel was put onto membrane, and then two equilibrated-filter paper were settled on the gel. The above mentioned process was lined up from anode (+) to cathode (-). In our lab, Western Blot method was used for detection of GAPDH (37 kDa), SLPI (11.7 kDa) and TAT(10 kDa) proteins from different types of cell lines. Therefore, PVDF membrane was preferred in this study. In addition to that, 'Mixed Range MW (25-150 kDa)' program was required to observe proteins of interest. After transferring from gel to membrane, Blocking was done with blocking buffer which contained 5 g dry/non-fat milk in 100 ml TBS-T incubated for 1 hour at room temperature for immobilization of biomolecules on a membrane. The next stage after blocking is washing membrane with TBS-T (Tris-buffered saline, 0.1%Tween 20) for 30 seconds per wash and 5 times for the removal of blocking buffer excess. Following the washing step, proteins of interest can be detected by using primary and secondary antibodies. Monoclonal and polyclonal antibodies can be used for the purpose of detecting proteins. Monoclonal antibodies are more specific than polyclonal antibodies. Polyclonal antibodies are more sensitive than monoclonal antibodies. Both of them have different features compared to each other.

Polyclonal antibodies are commonly used because of lower price and because they are less-time consuming in the production process; whereas monoclonal antibodies can be bound by their specific proteins to bring about low backgrounds and obtain pure proteins. Primary antibody incubation time and temperature are important. Higher temperature causes higher binding either specific or non-specific. General suggestion for incubation time and temperature is 1 hour at room temperature or at 4 °C overnight. After incubation with primary antibody, it is crucial to wash the membrane with TBS-T for 5 minutes per wash and 5 times in order to remove remaining antibodies to prevent higher background. Thereafter, Incubation with secondary antibody was done at room temperature for 1-2 hours. Before incubation with substrate, washing processes are 5 minutes per wash and 5 times. Then, Stable Peroxide Solution was mixed with the same amount of Luminol/Enhancer Solution. (per membrane cm^2, 0.1 ml mixture of those solution) After incubation for 5 minutes with substrate, membrane was placed into imaging system.

2.9. Sandwich Elisa

96 well-plate were pre-coated with SLPI antibody as a detector for analyzing enzyme-linked assay. There are several methods for sample collection from serum, plasma, tissue homogenates, etc. Cell culture supernatant was used for this assay. Supernatant of cell culture was removed into a micro centrifuge tube. Supernatant was centrifuged for 20 minutes at 2-8 °C with 1000 x g for removal of excess cell debris. After collecting pure supernatant, assay should be started immediately. Concentration of protein which is a target for assay should be determined with an appropriate dilution factor using a dilution buffer. According to procedure of assay, high concentration samples should be diluted with a ratio of 1:100. Medium concentration of samples should be diluted according to a ratio of 1:10. Low concentration of samples should be diluted as 1:2. Very low concentration samples generally were not diluted. Several concentrations are shown in Figure

Preparation of standard is important for detection of the amount of interest of proteins. Firstly, 4000 pg/ml of standard solution was diluted with 1 ml of standard dilution buffer into tube. After that, standard solution was kept at room temperature for 10 min-

Table 2.11. 5% Stacking Gel Preparation

High Concentration	40000-400000 pg/ml
Medium Concentration	4000-40000 pg/ml
Low Concentration	62.5-4000 pg/ml
Very Low Concentration	<62.5 pg/ml

utes. Then, serial dilution was used for making standard curve for analyzing samples. Serial dilution of standard solution is shown in Figure 2.7.

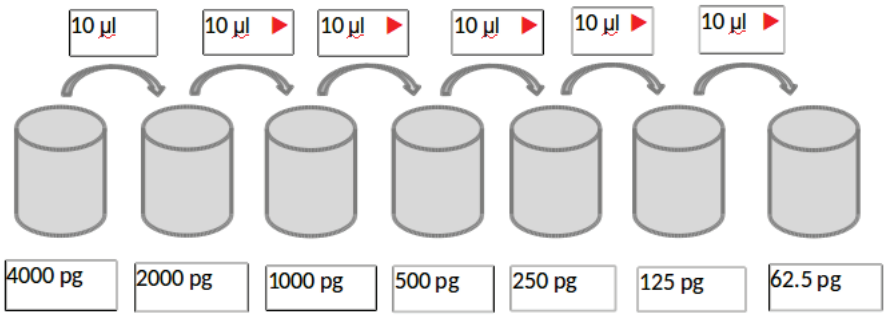


Figure 2.7. Serial Dilutions for Elisa standard

Biotin-labeled Antibody Working solution was prepared with antibody dilution buffer in ratio of 1:100. Moreover, HRP- Streptavidin Conjugate (SABC) Working Solution was diluted with SABC dilution buffer in ratio of 1:100. After preparation of standard solution, serial solutions, Biotin-labeled Antibody working solution in 1 hour before experiment and SABC working solution within 30 minutes before experiment, Assay procedure was applied. First step of procedure is washing wells 2 times with wash buffer (30 ml concentrated wash buffer, fill to distilled water to 750 ml). Then, serial dilution solutions into standard wells, supernatant into sample wells and standard solution into control well were added. Incubation at 37 °C for 90 minutes was applied and also plate was sealed before putting into incubator. After 90 minutes, plate was washed 2 times with wash buffer. Following step is adding 100 μl Biotin-labeled antibody working solution to each well without touching sidewall of the wells. Plate was incubated at 37 °C for 60 minutes. Then, each well was washed 3 times with wash buffer. After that, 100 μl

SABC working solution was added into wells while avoiding to touch the sidewalls. After adding SABC working solution, plate was incubated at 37 °C for 30 minutes. Later, it was washed 5 times with wash buffer for removal of excess of working solution. 90 μl TMB substrate was added to each well. Incubation at 37 °C was done in the dark for 15-30 minutes. Finally, 50 μl stop solution was added to each well above TMB substrate. The blue color started to convert into yellow after adding stop solution into wells. Directly, O.D absorbance of standard, sample and control wells were measured at 450 nm with spectrophotometer. Consequently, Analysis of results was completed using a software which is called 'Curve Expert 1.4'.

2.10. Cloning of Ef1ProTAT Region to LacZ in PXL304 Vector

Cloning is a method for producing genes of interest genetically. TAT gene (261 bp) with EF1 promoter (1167 bp) are found in the pBudCE4.1 HIV-TAT plasmid. In order to produce Ef1ProTAT region interest of, PCR was done with special-designed primers which amplifiy this region (1428 bp). Before cloning, Special-designed primers were controlled with PCR to produce proper region for cloning. PCR content of master mix and conditions as seen in table 2.12 and 2.13 respectively.

Table 2.12. PCR Solutions for Controlling EF-1 Pro-TAT Region

Solutions	Amount
Taq Reaction Buffer (10X)	2.5 μl
MgCl2	1.5 μl
dNTP (10 mM)	4 μl
Ef1proTat- Forward Primer	1.5 μl
Ef1proTat- Reverse Primer	1.5 μl
pBudCE4.1 HIV-TAT plasmid (50 ng/ μl)	2.5 μl
DMSO (5%)	0.5 μl
Taq Polymerase	0.5 μl
Total	25 μl

After controlling primers, total volume of PCR is increased two-fold for purification of region of interest and also Pfu Polymerase is used for forming blunt-end which is

Table 2.13. PCR Solutions for Controlling EF-1 Pro-TAT Region

Cycle Number	Cycle	Temperature	Duration
1	Initial Denaturation	94 °C	4 minutes
30	Denaturation Annealing Extension	94 °C 55 °C 72 °C	30 seconds 2 minutes 2 minutes
1	Final Extension	72 °C	10 minutes

compatible with pJET1.2/blunt vector (pJET1.2/blunt vector). At the end of PCR, products are loaded to 1% agarose gel and run for 45 minutes at 90 V. Then, region of interest is isolated with MiniElute Gel Extraction Kit (Qiagen). After purification Ef1ProTAT region was cloned to pJET1.2/blunt vector, using Clonejet PCR Cloning Kit (Thermo). 6 bp to 10 kb insert can be entered into pJET1.2/blunt linearized vector. Advantages of cloning is that blunt-end products of PCR are formed with proofreading activity of DNA Polymerase and ligated with pJET1.2/blunt vector in 5 minutes. After the cloning process, standard heat-shock transformation of chemically competent bacteria was done by following protocol for detection of the gene of interest. First of all, competent cells (DH5α, XL1blue, etc.) were removed from -80 °C and put the ice on for thawing for 20-30 minutes. Correspondingly, LB Agar plate with proper antibiotic was taken from +4 °C to room temperature. 1-5 μl cloning solution which contains DNA was put into 50 μl competent cell in micro centrifuge tube. Mixture was mixed gently. Then, mixture is settled at room temperature for 20-30 minutes. Micro centrifuge tube was dipped into 42 °C water for 30-60 seconds and put into ice again for 2 minutes. After that, 300 μl SOC media without antibiotics was put into micro centrifuge tube and incubated in shaker at 37 °C for 45 minutes. Consequently, 100 μl mixture was inoculated to 10 cm plate which contains proper antibiotic for selection of colonies and incubated overnight at 37 °C. Next day, the colony PCR was done from selecting generating colonies. It is a method for detection of which colony has the DNA of interest. Master Mix of PCR is prepared and selected colonies are picked up with tips for the template and to put into the master mix solution of PCR. Following step of observing colonies after PCR result, proper colony was taken and inoculated to LB broth with appropriate antibiotic. Then, pJET1.2/blunt vector-Ef1ProTAT plasmid was isolated with High Pure Plasmid Extrac-

tion Kit Mini Prep (Roche). Both of the cloned-Ef1ProTAT and LacZ were cut with XmaI (New England Biolabs) and NheI (New England Biolabs) restriction enzymes in CutSmart Buffer (New England Biolabs) at 37 °C for one hour. After the overnight incubation, restriction solutions were loaded to 1% Agarose gel to observe. Then, large band from LacZ (7026 bp) and small band from cloned-Ef1ProTAT (1428 bp) were cut from gel and isolated with MiniElute Gel Extraction Kit (Qiagen). Purified Ef1ProTATregion was ligated with 7026 bp region from LacZ, using T4 Ligase (Thermo) for overnight at 16 °C. After ligation, Transformation procedure was applied with ligation solution. Final stage of transformation is that Inoculation to LB-Agar with appropriate antibiotic was performed and was incubated overnight at 37 °C. Next day, selected-colonies were confirmed with colony PCR and restriction enzymes, respectively. Plasmid isolation was done from colonies which have LacZ plasmid with Ef1ProTAT region, using NucleoBond Xtra Midi plus Prep (Macherey-Nagel).

CHAPTER 3

RESULTS AND DISCUSSION

$CD4^+$ T lymphocytes are hard to be transfected. Hence optimization with different reagents is required.

3.1. Optimization of Transfection of T lymphocytes with Calcium-Phosphate Precipitation

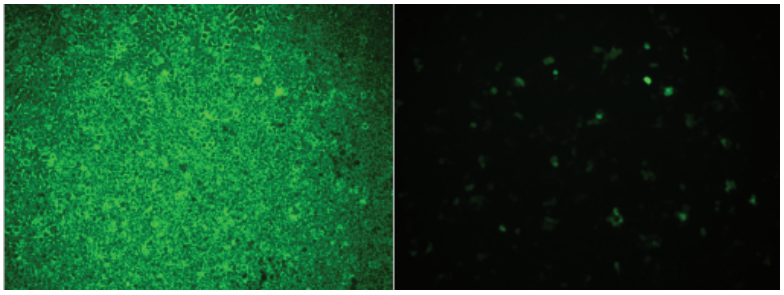


Figure 3.1. 293T (HEK) Cell Line Transfection with Calcium-Phosphate Transfection.

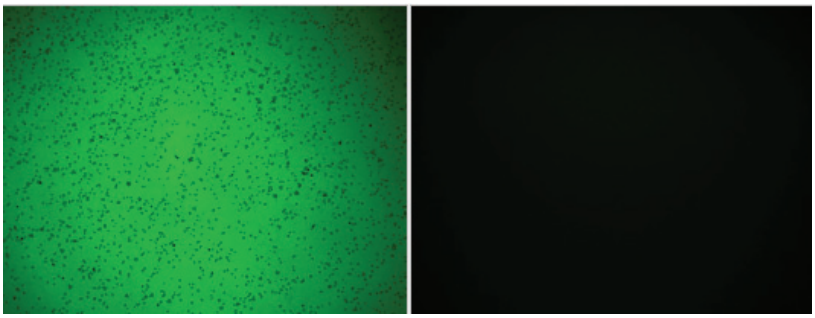


Figure 3.2. Jurkat Cell Line Transfection with Calcium-Phosphate Transfection.

Calcium- Phosphate transfection is another method for the transfection of T lymphocytes. In this method, firstly 293T (HEK) cell line was used for optimization as a positive control of transfection. Then, Jurkat cell line was used for transfection with a different plasmid concentration and 10% Glycerol durations. As seen in the table below, the results obtained after a duration of 48 hours are not effective.

Table 3.1. Tried several concentration plasmid and 10 % glycerol durations.

peGFP Plasmid Concentration	10% Glycerol with Duration
1000 ng, 2000 ng	30 sec
1000 ng, 2000 ng	1 min
1000 ng, 2000 ng	2 min
1000 ng, 2000 ng	3 min

3.2. Optimization of Transfection of T lymphocytes with Transfection Reagent

In order to understand which concentrations of plasmid and amounts of Transfection Reagent are proper for transfection; Different plasmid concentration and Lipofectamine Transfection Reagent concentration amounts were tried to obtain a higher transfection efficiency.

Table 3.2. Tried several concentration of plasmids and amounts of Reagent

peGFP Plasmid Concentration	Amount of Lipectamine 2000 Reagent
800 ng	1.5 μl, 2 μl, 2.5 μl, 3 μl
1000 ng	1.5 μl, 2 μl, 2.5 μl, 3 μl
1200 ng	1.5 μl, 2 μl, 2.5 μl, 3 μl

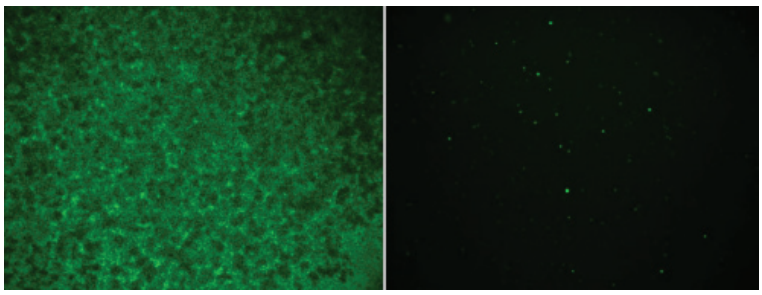


Figure 3.3. Jurkat Cell Line Transfection with Lipofectamine 2000 Reagent.

In Jurkat Cells, 1200 ng plasmids with 2.5 ul Lipofectamine 2000 Transfection Reagent after 48 hours gave the best result for transfection.

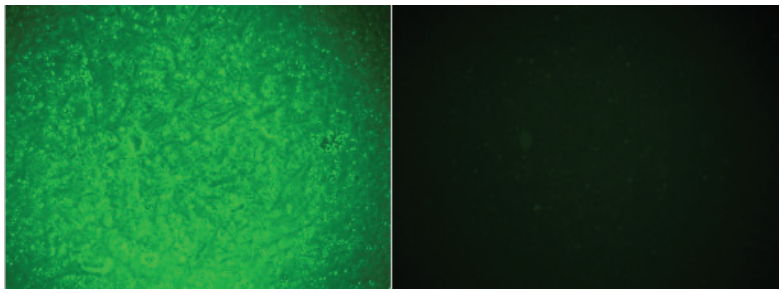


Figure 3.4. HSC-F Cell Line Transfection with Lipofectamine 2000 Reagent.

In HSC-F cells, 1200 ng plasmids with 2.5 μl Lipofectamine 2000 Transfection Reagent after 96 hours was the best result for transfection.

3.3. Obtaining Ef1ProTAT region with specific primers

Amplified-Ef1ProTAT region Primers was tested with PCR under specific conditions which mentioned before. As shown in agarose gel analysis (Figure 3.5), Ef1ProTAT region is observed in 1428 bp field.

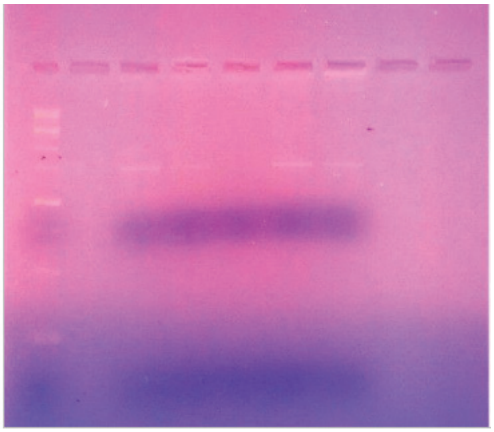


Figure 3.5. Agarose Gel Analysis of amplified-Ef1ProTAT region.

3.4. Purifying Envelope and Packaging Plasmids

psPAX2, pM2D.G and LacZ plasmids were transformed with competent cells then inoculated to LB-Agar with ampicillin for purification (Figure 3.6).

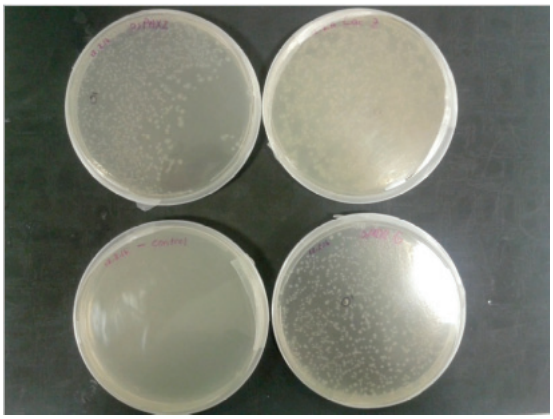


Figure 3.6. Plate image before transformation of psPAX2, pMD2.G and LacZ

Selected colonies were inoculated into LB-Broth with ampicillin. After approximately 16 hours, plasmids isolation occurred with the appropriate kit. After isolation, plasmids were controlled with restriction enzymes.

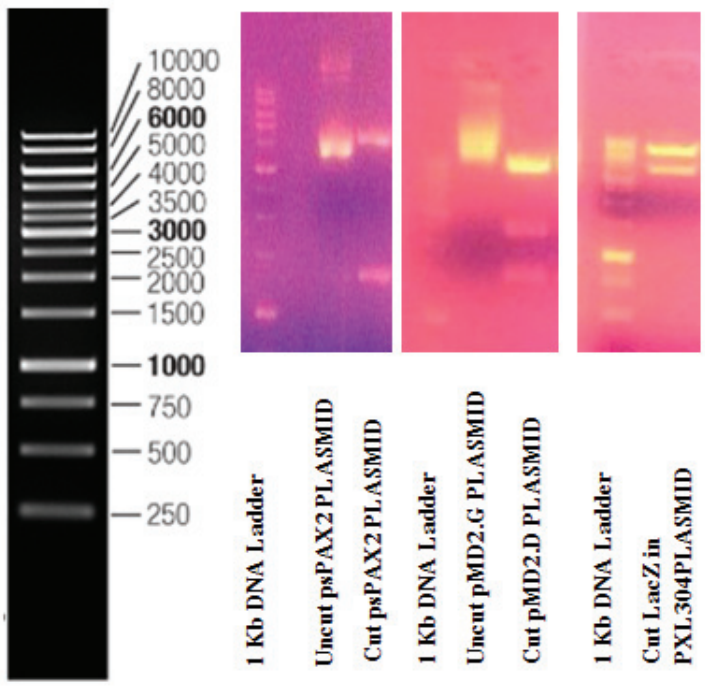


Figure 3.7. Agarose Gel Anlaysis of Plasmids for virus production.

psPAX2 Plasmid was cut with PvuI and SacI restriction enzymes. In addition to that, pMD2.G Plasmid was cut with PvuI and ApaI. Lastly, LacZ in PXL304 Plasmid was cut with SmaI and NheI.

3.5. Construction of Lentiviral Plasmid

Ef1ProTAT region was isolated from the gel and ligated with pJet1.2/blunt vector. After 16 hours, transformation was performed for the selection of colonies that carry the region of interest. Then, plasmid isolation was completed and restriction enzymes were used for controlling.

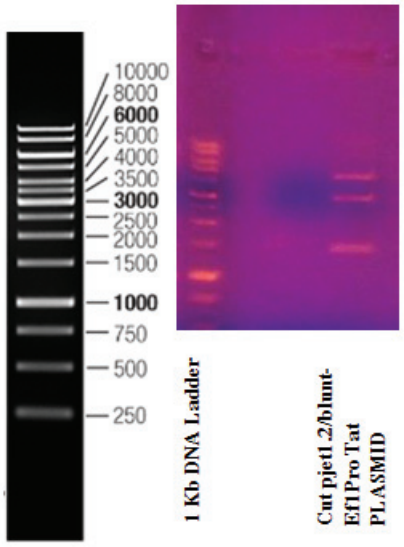


Figure 3.8. Agarose Gel analysis of pjet1.2/blunt-Ef1ProTAT plasmid.

After that, Ef1ProTAT region from pjet1.2/blunt-Ef1ProTAT plasmid and LacZ were ligated to each other and the resulting mixture was transformed with XL1blue competent cells. Colonies were observed and selected-colonies were used in colony PCR for detecting of the region of interest.

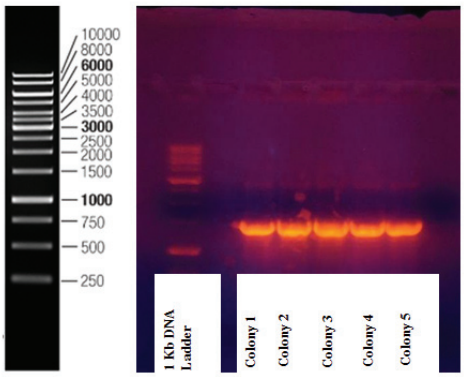


Figure 3.9. Agarose Gel Analysis after colony PCR.

After verification of the region of interest via colony PCR with specific primers, Restriction enzymes are used for another verification, as it is important that the region of interest is inserted in a right way.

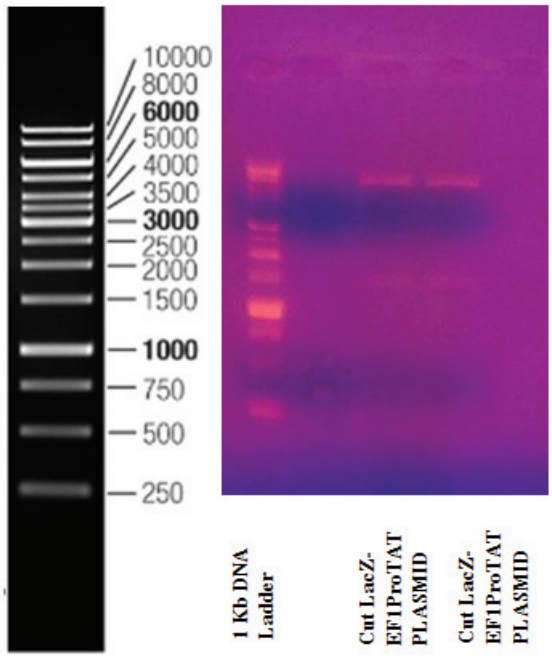


Figure 3.10. Agarose Gel Analysis of LacZ-Ef1ProTAT

After plasmid purification from Transformed colonies with Ligated LacZ-Ef1ProTAT plasmids were cut with NheI and XmaI restriction enzymes for verification of the region of interest. All of the plasmids for lentiviral production were obtained and controlled. After Virus particle collection from 293T cells; Molt 4 cell lines were infected with the obtained-virus particles. The steps to follow were, the isolation of RNA from Jurkat, Jurkat Tat III, Molt 4 and infected-Molt 4, the treatment of the RNAs of these cells with DnaseI and their conversion to cDNA.

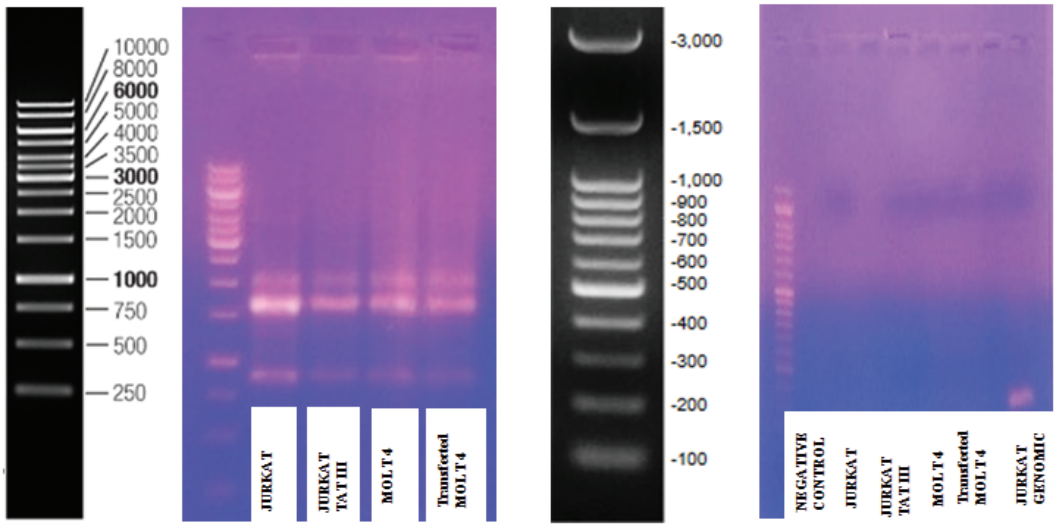


Figure 3.11. Left: Agarose Gel Analysis after RNA isolation by direct load.
Right: Agarose Gel Analysis after RNA isolation and by using PCR with GAPDH primers.

3.6. Determinaiton of Tat gene expression after Transfection in Molt 4 (Human T lymphocytes) cell line with qRT-PCR

Jurkat and Jurkat Tat III which expresses Tat protein cell lines were used as a positive control in the comparison of Molt 4 and transfected Molt 4 cell lines. According to the result, Tat expression of Jurkat Tat III and transducted- Molt 4 cell lines increased in comparison to their respective mock cell lines. This result also shows that Transfection with lentiviral particle is more beneficial for transfection of T lymphocytes than the others.

As shown in Figure 3.12, the P-values are less than 0.05 for each TAT expression in different cell types compared to their mocks. It means that expression differences between Jurkat and Jurkat Tat III - Molt 4 and Transfected Molt 4 can provide statistical significance.

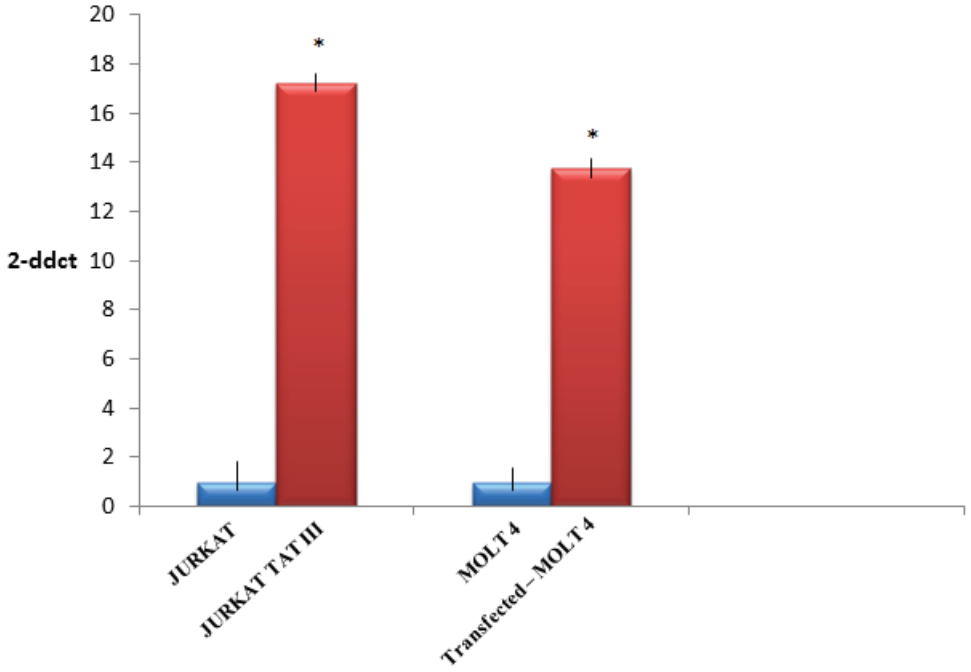


Figure 3.12. qRT-PCR Analysis for detection Tat expression for T cells.

3.7. Generating Stable Cell Lines from Vero Cell lineage

Transfection efficiency with linearized- pBudCE4.1, pBudCE4.1 HIV-TAT,pcDNA and pcDNA SIV-TAT plasmids was determined with peGFP for generating stable cell line for Vero cells (Figures 3.13 and 3.14).

After transfection, antibiotic selection was started. For pBudCE4.1 and pBudCE4.1 HIV-TAT plasmids, Zeocin was used and for pcDNA3.1 and pcDNA3.1 SIV-TAT plasmids, G418 was used For selection; Antibiotic treatment causes a decrease in the number of cells. After this rapid decrease in cells the survivours start to multipy and form a single colony (Figure 3.15).

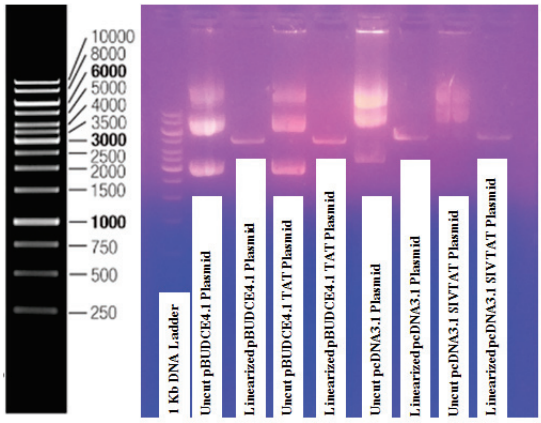


Figure 3.13. Agaose Gel Analysis for detection of linearized plasmid for generating stable cell lines.

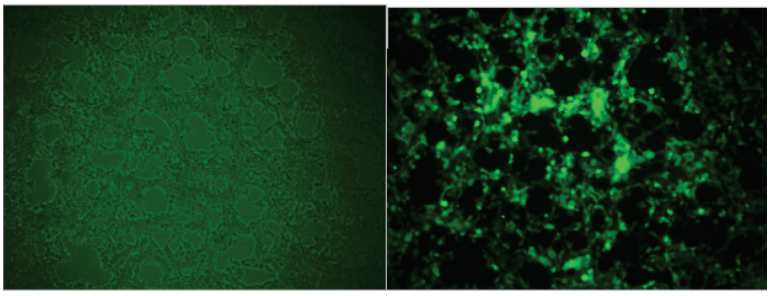


Figure 3.14. peGFP Image for detection of transfection efficiency.

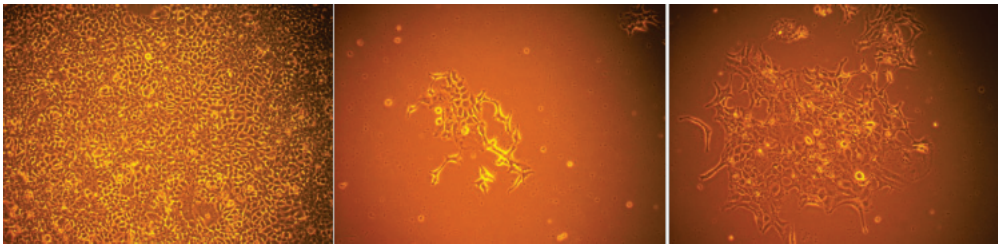


Figure 3.15. View of generating stable cell line, gradually.

3.8. Confirmation Vero Stable Cell lines for qRT-PCR

For confirmation of stable cell lines, RNAs were isolated from each stable cell lines.Then, Dnase I treatment was performed for eliminating DNA conformation from RNAs. After that, pure RNAs were checked by using a control PCR with GAPDH primers. Final step was the conversion from RNAs to cDNAs.

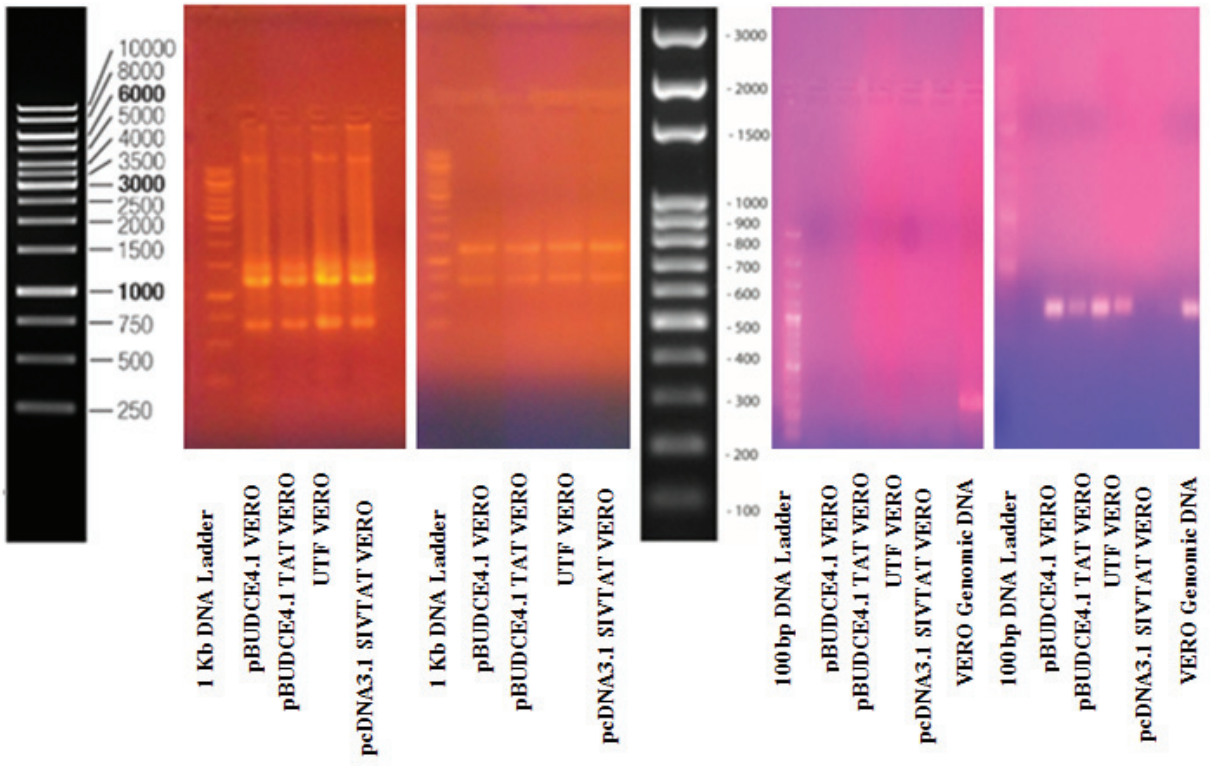


Figure 3.16. First Agarose Gel Analysis: RNAs of Vero Stable Cell Lines. Second Agarose Gel Analysis: After Dnase I Treatment RNAs of Vero Stable Cell Lines. Third Agarose Gel Analysis: After Dnase I Treatment PCR of Vero Stable Cell Lines. Fourth Agarose Gel Analysis: Confirmation of cDNA of Vero Stable Cell Lines with PCR.

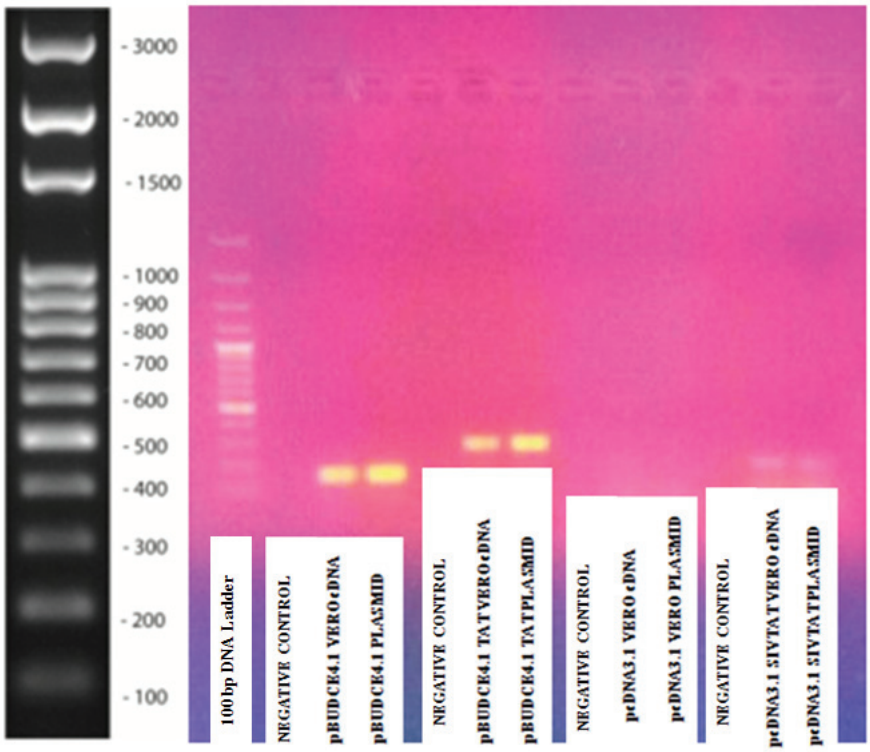


Figure 3.17. Agarose Gel Analysis after control PCR for confirmation Vero stable Cell Lines with specific primers.

After the conversion of cDNA, Stable cell lines were verified by using a control PCR with specific primers. pBUDCE4.1 Vero Cell Line was confirmed with Zeocin primers, pBUDCE4.1 HIV-TAT Vero Cell Line was confirmed with Htat primers, pcDNA3.1 Vero Cell Line was confirmed with pcDNA primers and pcDNA3.1 SIV-TAT Vero Cell Line was confirmed with Stat primers.

3.9. Determination of SLPI Gene Expression Level of Vero Stable Cell Lines with qRT-PCR

After conversion from RNAs to cDNA of Vero Stable Cell Lines, SLPI expression was measured for each stable cell lines with qRT-PCR.

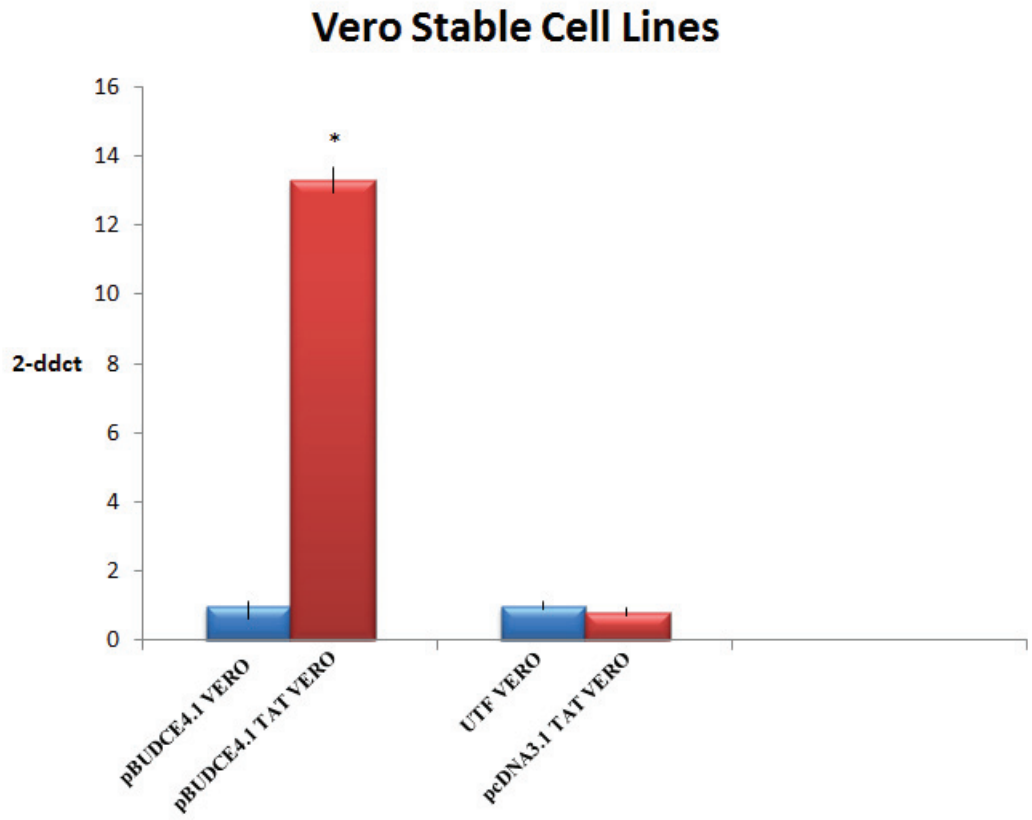


Figure 3.18. SLPI expression of generated stable Vero Cell Lines.

As seen above, SLPI expression level increased in the presence of Tat gene compared to its mock cell lines. SLPI Expression of pBUDCE4.1 HIV-TAT Vero Cell Line was approximately 13 times higher than that of pBUDCE4.1 Vero Cell Line. In addition to that, Untransfected Vero Cell Line slightly increased compared to pcDNA3.1 SIV-TAT Vero Cell Line. As a result, Expression Level of SLPI increased in the cell when Tat (one of Regulatory proteins of HIV-1) expresses. The P-value for the t-test is important as it decides whether Null hypothesis is rejected or accepted. Differences between SLPI expression level in the presence of Tat gene and in the absence of Tat gene shows that the P-value is less than 0.05 according to qRT-PCR results. (p= 0,038963). In other words, Null hypothesis could be accepted.

3.10. Bradford Assay for Detection of Amount of Unknown Protein

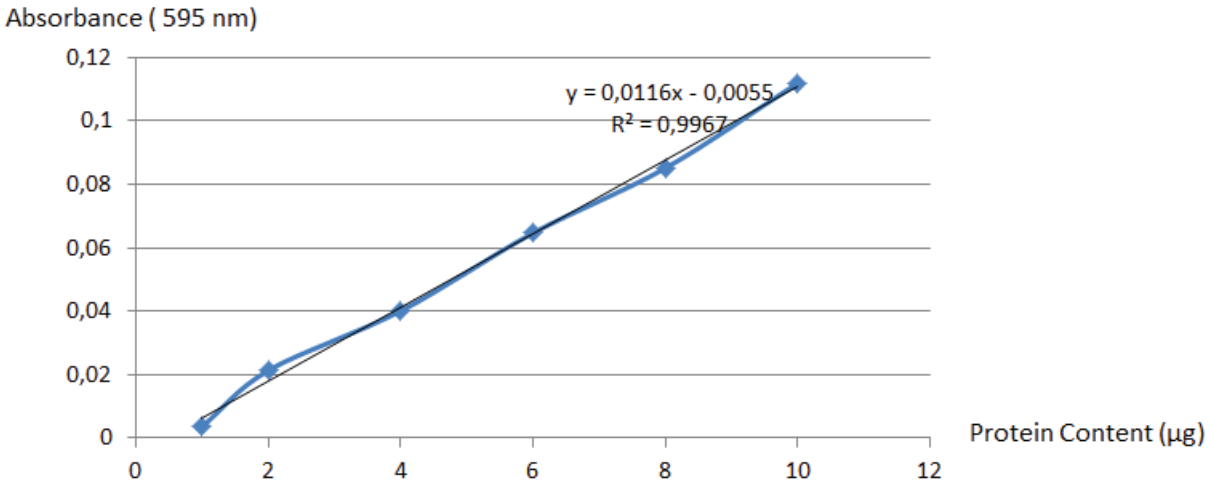


Figure 3.19. BSA standard curve for Bradford Assay.

Isolation of total protein of Vero stable cell lines with Lysis Reagent is important for detecting the amount of unknown proteins. Each of the cell lines was diluted 1:10, 1:100 and 1:1000 for Bradford Assay. Acoording to BSA standard, the graph could lead to the detection of the protein content of different diluted samples. It was important that all the protein samples were in range of the standard curve.

3.11. Detection TAT and SLPI Protein Expressions by Using Western Blot

After the detection of the amount of protein per microlitre, protein samples were used for Western Blot. All of the samples needed to be equal before loading to SDS-PAGE.

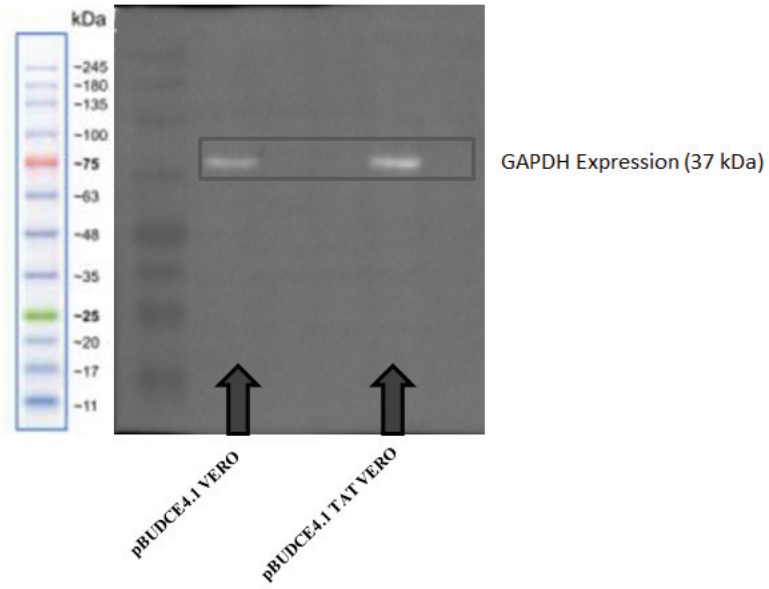


Figure 3.20. Western Blot Analysis with GAPDH primary antibody.

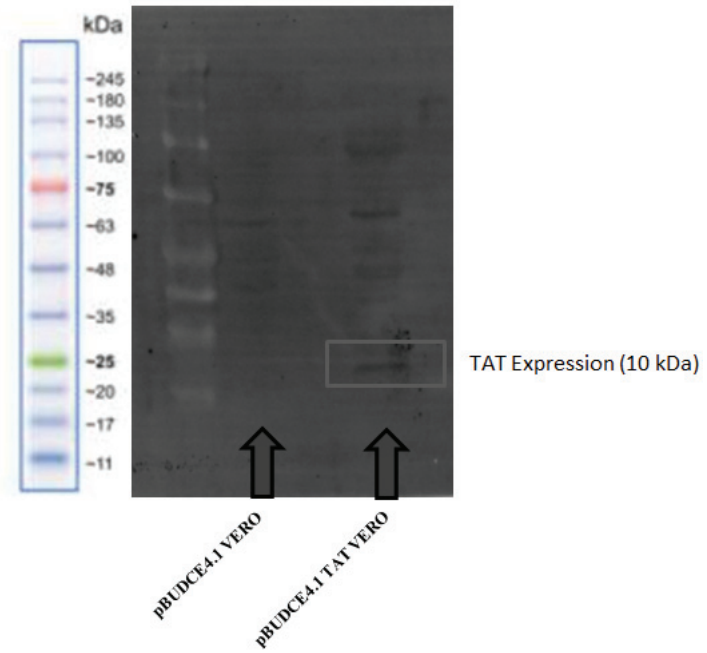


Figure 3.21. Western Blot Analysis with TAT primary antibody.

As shown in Figure 3.20 and 3.21, pBUDCE4.1 Vero Cell Line and pBUDCE4.1 HIV-TAT Vero Cell Line were used as template with GAPDH primary antibody to detect whether protein amount was equal or unequal. If protein amounts were equal, TAT expression in pBUDCE4.1 Vero Cell Line and pBUDCE4.1 HIV-TAT Vero Cell Line were detected correctly. pBUDCE4.1 HIV-TAT Vero Cell Line had higher Tat expression compared to pBUDCE4.1 Vero Cell Line.

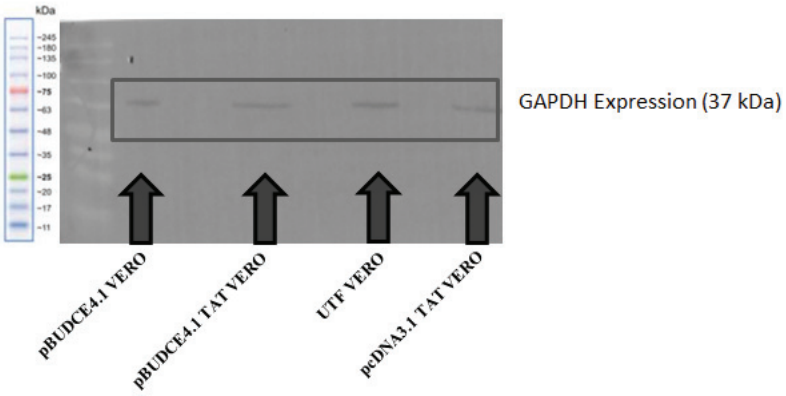


Figure 3.22. Western Blot Analysis with GAPDH primary antibody.

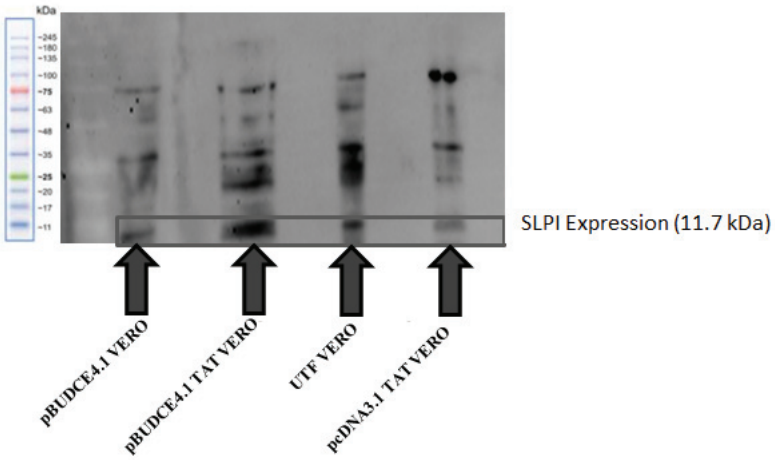


Figure 3.23. Western Blot Analysis with SLPI primary antibody.

According to Western Blot results (Figure 3.22 and 3.23), Equal loaded protein samples for each of the stable cell lines were controlled with GAPDH primary antibody. Then, SLPI expression of pBUDCE4.1 HIV-TAT Vero Cell Line increased compared to pBUDCE4.1 Vero Cell Line. Moreover, Untransfected Vero Cell Line slightly increased compared to pcDNA3.1 SIV-TAT Vero Cell Line. HIV-TAT and SIV-TAT are similar; 50% in terms of amino acid sequences. That is why SLPI Expression level differences between HIV-TAT and SIV-TAT can be understandable.

3.12. Detection Extracellular SLPI Protein Expression by Using Sandwich Elisa

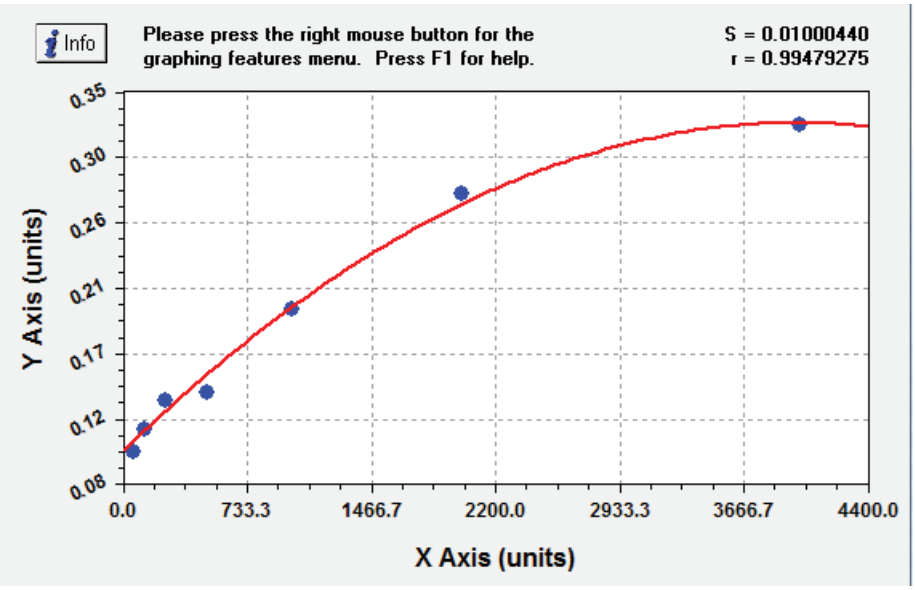


Figure 3.24. Sandwich ELISA SLPI standard Quadratic Curve from Curve Expert 1.4 program.

First off, Standard curve was formed with standard SLPI which exists in the kit. Then, unknown Extracellular SLPI concentrations were found via this standard curve.

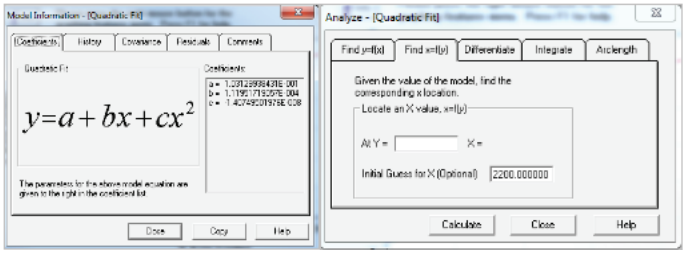


Figure 3.25. Specific equation from Curve Expert 1.4 program.

After finding Equation with the specified program, Values from spectrophotometer were calculated.

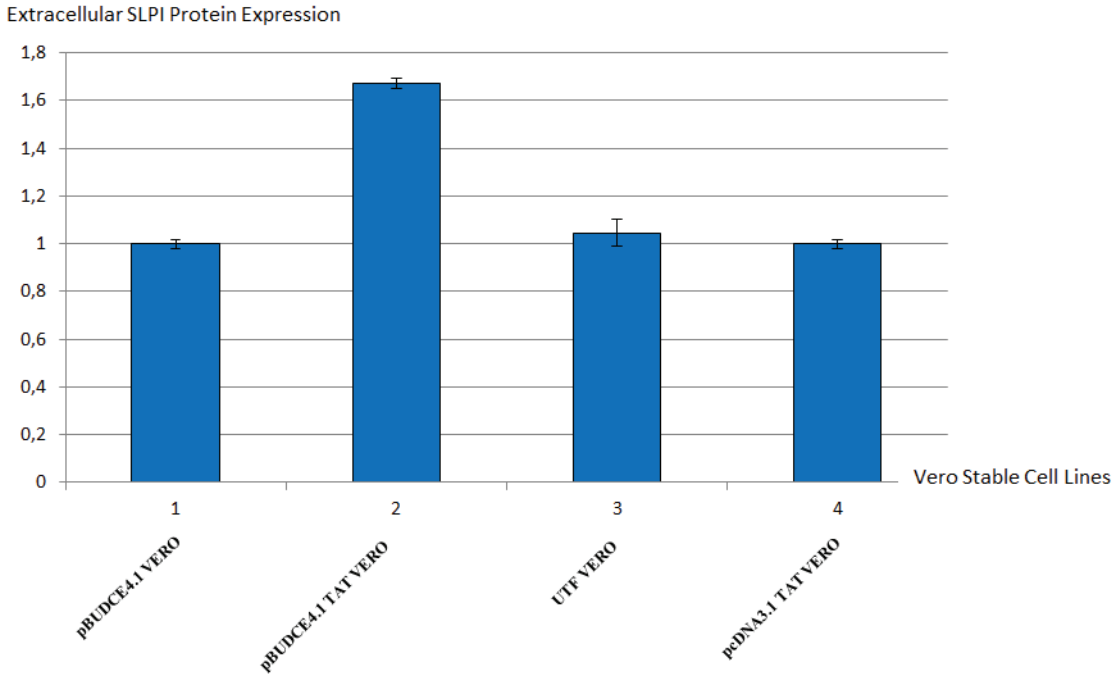


Figure 3.26. Extracellular SLPI Expression Analysis with Sandwich ELISA.

As shown in figure 3.26, Extracellular SLPI Expression of pBUDCE4.1 Vero Cell Line decreased in comparison to extracellular SLPI Expression of pBUDCE4.1 HIV-TAT Vero Cell Line. pcDNA3.1 SIV-TAT Vero Cell Line slightly decreased according to com-

parison with Untransfected Vero Cell Line. ELISA result also emphasized that Extracellular SLPI protein can give information about intracellular SLPI expression. Extracellular SLPI protein may pass between cells and may have a protector effect in uninfected cells as well.

CHAPTER 4

CONCLUSION

In this Project; pBUDCE4.1, pBUDCE4.1 HIV-TAT, untransfected and pcDNA3.1 SIV-TAT plasmids were linearized for generating stable cell lines with Vero cell lineages which were established from the female *Chlorocebus sabaeus* kidney-derived cells. Generating stable cell lines with Vero cell lineages is important to examine SLPI expression in the presence of Tat gene. Stable and long-term expression from Vero Cell Line is required for detection of accurate TAT and SLPI expression levels by using qRT-PCR, Western Blot and Sandwich ELISA. High concentration of SLPI may have a special contribution in the prevention of HIV-1 infection because it acts as an inhibitor by distrupting the process after virus binding. Generated-Vero stable cell lines were used for detection of SLPI expression through the use of qRT-PCR. According to the qRT-PCR result, SLPI expresion increased in the presence of Tat gene of HIV but SLPI expression did not change as significantly in the presence of Tat gene of SIV. Western Blot Analysis showed that SLPI expression is higher in the presence of HIV-TAT gene than in its absence. In addition to that, SLPI Expression of Untransfected Vero Cell Line is slightly higher than pcDNA3.1 SIV-TAT Vero Cell Line. Sandwich Elisa method was used for detection of Extracellular SLPI concentration of the supernatants of Vero stable cell lines. Extracellular SLPI concentration increased in HIV-TAT stable cell line in comparison to its mock cell line. On the other hand, untransfected Vero Cell Line increased inconsiderably compared to pcDNA3.1 SIV-TAT Vero Cell Line in terms of extracellular SLPI concentration. For further studies, CD4 is the most important receptor for HIV-1 infection. Hence, $CD4^+$ T lymphocytes should be used as a next step to determine the effects of HIV-1 and SIVAGM Tat proteins on SLPI gene expression. After that, a HIV-1 Infection Experiment should be done to support the data found in previous studies.

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