Currently, new technologies and sciences have been developed that should help to limit the complications of human disease. Many diseases such as heart attack, urinary tract infection, and others can be cured, whereas with HIV infection HAART can control the disease, but can’t cure the infection. HAART, a combination therapy containing nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PRIs), and is the therapeutic mainstay for HIV treatment (De Clercq, 2001; Flexner, 2007). Recently a new class of inhibitors, the integrase (IN) inhibitors, are now using in clinical trials (Evering and Markowitz, 2008; Grant and Zolopa, 2008). However the resistance and toxicities have been reported (Charpentier et al., 2008; Delelis et al., 2009; Hicks et al., 2009). Although, HAART is effective to for dramatic reduction of viral load and does improves the health of patients (Cavert et al., 1997; Palella et al., 1998), HIV infection still persists due to the a reservoir of cellular infection, which are memory CD4+ T cells (Han et al., 2004). Mostly likely other cellular reservoirs exist such as the hematopoietic stem cells, macrophages, and microglia within the central nervous system. When the antiviral therapy is discontinued, the return of viral replication occurs within weeks. This is a serious disadvantage of drug therapy which is the inability to affect an HIV cure. HAART has favorably altered the clinical course of HIV-1 infection in individuals from an acute to a long-term, managed, chronic viral
infection. However, individuals on long-term HAART are reported to have cardiac changes, loss of bone density, as well as other complications significant to consider alternative therapies (Sabin et al., 2008; Ofotokun and Weitzmann, 2010; Deeks, 2011). Therefore, it is a need for a life-long antiviral therapy, which protects cells from viral infection, thereby reducing viral load without additional medical complications. Gene therapy is one potential treatment. In this study, a gene therapy based antiviral strategy using to inhibit HIV-1 integration relied on a designed zinc finger protein, is investigated.

Gene therapy for HIV-1 infection has been continuously developing. Several strategies of gene therapy are based on inhibiting HIV-1 replication by interfering with the functions of HIV-1 RNAs or proteins. More examples of RNA approach have been shown to inhibit HIV replication. One of them as antisense RNA against the HIV-1 env gene has demonstrated of its activity (Cohli et al., 1994; Lu et al., 2004). Rz1-9 was a multimeric hammerhead ribozyme targeting a highly conserved region in the env coding of HIV-1 RNA, which also shown to inhibit HIV-1 replication (Ramezani et al., 2002). Tat-activated HIV-1 transcription have been inhibited by a small circular TAR RNA decoy (Bohjanen et al., 1996), while small interfering RNAs (siRNAs) was used to target HIV-1 rev transcripts in human cells (Lee et al., 2002). However, RNA approaches may also be limited by the escape mutations in the site targeting regions, even without the changing the encoded protein.

In the direction to interfere protein strategies, the inhibition of HIV-1 replication was observed in CD4+ cells expressing trans-dominant negative mutants of Tat and Rev (Liem et al., 1993). Even many single-chain antibodies (intrabodies) have been demonstrated to inhibit the function of HIV-1 proteins such as IN (Levy-
Mintz et al., 1996), reverse transcriptase (RT) (Shaheen et al., 1996), Rev (Vercruysse et al., 2010), gp120 (Marasco et al., 1993), Gag p17 (Tewari et al., 2003). However, proper single-chain antibodies folding need disulfide bond formation, which is an obstacle for the use of these intrabodies in gene therapy.

Mutations in IN enzyme encoded by pol gene have been reported (Charpentier et al., 2008; Delelis et al., 2009; Hicks et al., 2009). On the other hand, for IN substrate, numerous studies have indicated that the important 2-7 base pairs upstream and the well conserved CA dinucleotide at the 3’ end of viral cDNA play key roles in processing and strand transfer by HIV-1 IN in the early phase of viral replication (LaFemina et al., 1991; Vink et al., 1991; van den Ent et al., 1994; Yoshinaga and Fujiwara, 1995; Katzman and Sudol, 1996; Balakrishnan and Jonsson, 1997). Therefore, IN substrate is a qualified target for antiviral therapy development.

Many Cys2His2 ZFPs have been demonstrated to be specific binding proteins to the recognition sequences (Dreier et al., 2000; Dreier et al., 2001; Blancafort et al., 2004; Dreier et al., 2005; Zimmerman et al., 2008). Promising alternatives to intrabody strategies are designing ZFPs targeting the viral genome, and many investigations have been shown to block viral replication at the level of transcription. The engineered ZFPs targeting the Sp1 binding site in promoter region of HIV-1 have been reported, and one of them was found to inhibit HIV-1 replication by 75% (Reynolds et al., 2003). In 2004, Segal et al. demonstrated that a transcriptional repressor protein, namely KRAB-HLTR3, was able to achieve 100-fold repression of transcription from the HIV-1 promoter. This transcription factor also repressed the replication of several HIV-1 strains 10-to 100-fold in T-cell line and primary blood mononuclear cells (PBMCs) with no significant cytotoxicity (Segal et al., 2004).
Recently, ZFPs were designed to bind DNA sequences in the duck hepatitis B virus (DHBV) to inhibit viral transcription in tissue culture. Two candidate ZFPs decreased production of viral products and progeny viral genomes (Zimmerman et al., 2008). These studies supported the use of ZFPs to inhibit viral replication, opening new avenues in gene therapy. Therefore, the advantage of targeting the 3’-end terminal part of HIV-1 LTR to interfere with the IN enzyme is an attractive strategy for a new approach to gene therapy.

There are various methods available to deliver a protein for gene therapy. The \textit{in vivo} adenoviral gene transfer of ZFPs has been used in a mouse model (Rebar et al., 2002), whereas retroviral and lentiviral gene transfer has been delivered the artificial zinc finger transcription factors to bind sites in the HIV-1 promoter to repress the replication of several HIV-1 strains (Segal et al., 2004). Recently, various non-biological and biological carrier systems have been developed for HIV-1 gene therapy, such as nanoparticles, liposomes, or synthetic polymers, which can be taken up by many cell types (Lanao et al., 2007).

Herein, 2LTRZFP-GFP can be designed to target integrase recognition sequences at 2-LTR circle junctions of HIV-1 DNA, (Sakkhachornphop et al., 2009). Although 2-LTR circles have been detected as a small amount in total HIV DNA (Pauza et al., 1994; Hazuda et al., 2000; Butler et al., 2001) and are thought to be a dead end product, this protein was designed to provide a steric barrier to the processing of partially 2-LTR circle junctions of HIV-1 DNA before integration. A six-finger protein was constructed specifically to the 18 bp of IN recognition sequence at 2-LTR-circle junctions. The 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} fingers can target the terminal sequence of 5’ LTR, whereas the terminal sequence of 3’ LTR can be targeted by the other
three fingers. The 2LTRZFP-GFP was expressed and purified from *E. coli* in order to investigate its binding properties. The binding activity was on the nanomolar scale, which is similar to the affinity level of HIV-1 IN (Bugreev et al., 2003; Deprez et al., 2004). This finding suggested that the HIV-1 IN might be interfered with by the 2LTRZFP as well. The specificity of this binding was obtained by competitive SPR, which indicated that there was specific binding of 2LTRZFP-GFP and its target DNA. However, this binding affinity was decreased when the concentration of EDTA in binding buffer was increased. This data demonstrated the influence of zinc ion on correct folding of 2LTRZFP domains in recognizing a specific DNA sequence. Moreover, 2LTRZFP can specifically bind to its target ds-DNA, whereas binding of GFP in the C-terminal part was not facilitated.

Compared to cells expressing control ZFP, a dramatic suppression of RFP expression was observed in 293T cells expressing 2LTRZFP-GFP that were challenged with VSV-G pseudotyped RFP lentivirus at both 1 and 10 MOI. To determine whether 2LTRZFP-GFP inhibited HIV-1 replication, HIV-1\_NL4-3 was used for the challenge. A third-generation lentiviral vector (Dull et al., 1998; Zufferey et al., 1998) was used for delivery of the 2LTRZFP-GFP gene into the human T-lymphocytic cell line (SupT1). HIV-1 integration was inhibited in cells expressing 2LTRZFP-GFP in contrast to those expressing the control ZFP, and inhibition depended on the amount of transgene expression represented by mean fluorescence intensity (MFI). Therefore, we demonstrated that the expression of 2LTRZFP-GFP, in the nucleus of human T-lymphocytic cells markedly inhibited viral replication and integration as measured by the p24 antigen assay and *Alu-gag* qPCR, respectively.
To improve safety and gene transfer efficiency, a third-generation lentiviral vector has been widely used for gene delivery into the host cell genome (Dull et al., 1998; Zufferey et al., 1998). Integration into certain sites can cause tumours by directly activating proto-oncogenes (Marchetti et al., 1995; Li et al., 2002). Therefore, gene delivery vehicles that direct therapeutic gene integration into “safe sites” within the human genome or that use technology based on a Sleeping Beauty transposon system should be considered for clinical gene therapy (Tan et al., 2004; Tamhane and Akkina, 2008; Staunstrup et al., 2009; Su et al., 2009).

In the present study, the pCEP4 vector was also used as a non-viral gene transfer system to deliver 2LTRZFP-GFP into human T-lymphocytic cell lines. A complete inhibition of viral integration, as evidenced by undetectable levels of p24 antigen, was observed in the supernatants of cells expressing 2LTRZFP-GFP. This implied that the vanishing of p24-production is due to a blockade of viral integration since the replicable HIV was used. In comparison with the inhibition of viral integration by 2LTRZFP-GFP into stably transduced SupT1 (clone ZFP-5E3 and ZFP-4F6), it was likely that stably transfected with pCEP4-2LTRZFP-GFP (clone ZFP-9B1 and ZFP-9B2) showed a higher efficiency. These evidences may be contributed with the higher copy number of episomal and transcriptional level under CMV promoter by pCEP4-based vector than the lentiviral vectors controlled by mPGK promoter. This observation was designated by the level of MFI.

In this study, the efficiency of HIV integration was monitored by the modified first-round Alu-gag qPCR (O’Doherty et al., 2002; Agosto et al., 2007) and second-round RU5 kinetic PCR (Liszewski et al., 2009). We observed integration impairment upon 2LTRZFP-GFP expression. The modified Alu-gag qPCR method used in the
present study may be used to screen compounds for their ability to inhibit HIV integration in cell culture. Moreover, this assay was competent to differentiate the integration forms of lentiviral vector and HIV-1 into the host genomes. Therefore, this modified method was also useful for detecting the efficiency of transduction by lentiviral vector as well.

The ultimate goal of HIV gene therapy is to improve the health and survival of HIV-infected individuals. Since, 2LTRZFP-GFP has been shown to be a highly effective inhibitor of HIV-1 replication in human T-lymphocytes that interferes with the integration step of the virus with no obvious toxicity to the host cells. Thus, 2LTRZFP-GFP could be delivered to hematopoietic stem cells (HSCs) to yield a long-term transgene expression for inhibiting HIV-1 integration into host cells (Tesio et al., 2008).

Of note, the continuing development of ZFP technology by gene therapy should be a significant issue for the future of HIV-1 clinical application. Since the cost of antiretroviral therapy was costly to expand the survival of an infected individual, it is conceivable to combine antiretroviral therapy with gene therapy. However the combating with HIV using gene therapy is still required the optimization of vector types, effective doses and routes of manipulation. In order to remedy the T-lymphocyte quality, stem cell gene transfer should be considered as a probable clue for ZFP gene therapy of HIV in the future.