CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and equipments

Chemicals and equipments used in this study were shown in Appendix A. List of restriction enzymes are detailed in Appendix D. The recipes for reagent preparations are shown in Appendix E.

2.2 *E. coli* strains and vectors

XL-1 Blue (Stratagene, La Jolla, CA) was used as a host for the library generation and the amplification of phage displaying artificial ankyrin repeat protein library. Examination of protein expression and production of recombinant HIV-1 protease were performed using *E. coli* strain BL21 (DE3) (Stratagene, La Jolla, CA) in CoFi blot experiment to evaluate the quality of constructed library. The pHDiExDsbA-Ank15 phagemid vector (Nangola et al., 2010) was used for the library construction and phage library production. The pQE-30 expression vector (Qiagen, Hilden, Germany) was used for the soluble protein production in M15 [pREP4] (Qiagen, Hilden, Germany). pET14bELP105KnewTat (kindly provided by Prof. Wilfred Chen, California, USA) was used as a template for the amplification of gene encoding HIV-1 protein. The pCEP4 based vector (Invitrogen, Carlsbad, CA) was

exploited to generate the stable cell lines expressing ankyrin repeat protein. pNL4-3 (kindly provided by Prof. Pierre Boulanger, Lyon, France) was used as the template for MA-CA domain and pBlueBac4.5 (Invitrogen, Carlsbad, CA) was used to produce the recombinant H_6MA-CA in insect cells. Genotype of all *E. coli* is detailed in Appendix B.

2.3 Cell culture

Sf9 cells (the pupal ovarian tissue of *Spodoptera frugiperda*, Invitrogen, Carlsbad, CA) was used for the generation of recombinant baculovirus carrying gene encoding H_6MA-CA, and H_6-CA protein. Cells were grown in the non-humidified environment at 27ºC in Grace's insect medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10 mM L-glutamine (Gibco-BRL, Gaithersburg, MD), 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD), penicillin (100 units/ml), and streptomycin (100 µg/ml).

Sup-T1 (human T cells lymphoblastic lymphoma) cell (kindly provided by Prof. Watchara Kasinrerk) was used to generate the stable cell line expressing the ankyrin repeat proteins in two versions: membrane-targeting proteins and cytoplasmic proteins. Cells were grown in a humidified atmosphere of 5% CO_2 at 37 ^{o}C in the RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10 mM L-glutamine, 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml).

2.4 Construction of artificial ankyrin repeat protein library

2.4.1 Sequence analysis and design of oligonucleotide fragments for library construction

The sequence of artificial ankyrin repeat proteins library is based on the sequence design designated as designed ankyrin repeat proteins (DARPins) library previously described by Plücktun A (Binz et al., 2003) with two minor modifications:

- Limited changes in the Ankyrin repeat concensus sequence has been introduced in order to create a non symetric restriction site (*Bsm* BI). The presence of this site between consecutive ankyrin repeat coding fragments simplify library construction and module shuffling processes. The 3D structure of modified consensus was subjected to Discovery 2.5 program using DARPin E3_5 (1JM0) as the template to analyze the optimized structure compared to the template structure.

- The randomization scheme used in the variable position of the ankyrin repeats was targeted to mimic the natural residue frequency at each variable position. The frequency of amino acids in each variable position was computed from Ankyrin domain families as defined in Pfam (Coggill et al., 2008; Finn et al., 2008) and Prosite (Ribeiro Ede et al., 2005; Sigrist et al., 2005; Hulo et al., 2006). The conservation at each position in multi alignment of ankyrin modules can be visualized as a sequence logo generated using the WebLogo3 server (http://weblogo.berkeley.edu/).

The amino acid distribution at each variable position was approximated by encoding each of these positions with a set of partially degenerated codons. The repeat sequences were made by a set of oligonucleotides. The sequence coding a

single repeat was divided into four fragments: Va (variable fragment a), Vb (variable fragment b), Vc (variable fragment c) and C1 (constant fragment) as shown in **Figure 2.1**. The C1 fragment was designed to have the site for *Bsm* BI restriction enzyme. The sequences of each fragment are detailed in **Table 2.1**.

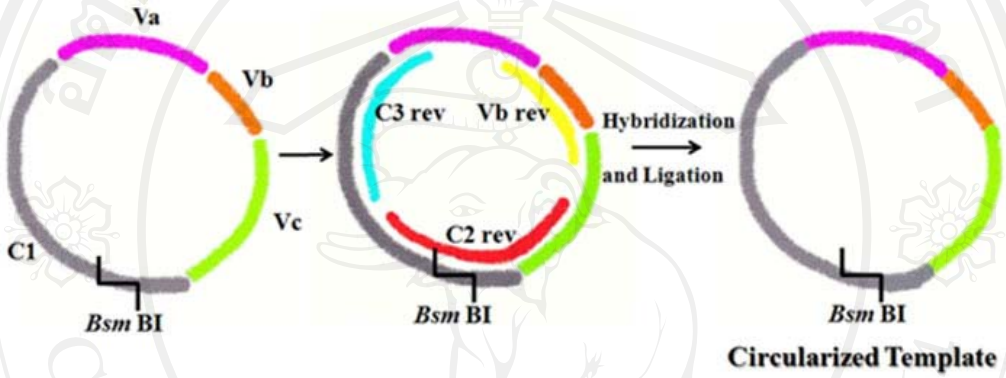

Figure 2.1 The generation of circularized template. All fragments of Va, Vb, Vc, C1 and short bridging fragments (Vb rev, C2 rev and C3 rev) are mixed together with an equal molar ratio. Hybridization and ligation resulted in the circularized template.

Table 2.1 Oligonucleotide for the artificial ankyrin repeat protein library construction.

Oligonucleotide	Sequence (5'->3')
Variable fragment a (Va)	amino acid position: 33, 1, X, X, 4, X, 6, 7
Va1	cgt gac vdk vdk ggt vdk acc ccg
Va2	cgt gac vdk vdk ggt dmy acc ccg
Va3	cgt gac vdk dmy ggt vdk acc ccg
Va4	cgt gac vdk dmy ggt dmy acc ccg
Va5	cgt gac dmy vdk ggt vdk acc ccg
Va6	cgt gac dmy vdk ggt dmy acc ccg
Va7	cgt gac dmy dmy ggt vdk acc ccg
Va8	cgt gac dmy dmy ggt dmy acc ccg
Va9	cgt gac raa vdk ggt dmy acc ccg
Va10	cgt gac raa dmy ggt vdk acc ccg
Va11	cgt gac raa vdk ggt vdk acc ccg
Va12	cgt gac raa dmy ggt dmy acc ccg
Va13	cgt gac vdk van ggt dmy acc ccg
Va14	cgt gac dmy van ggt vdk acc ccg
Va15	cgt gac dmy van ggt dmy acc ccg
Va16	cgt gac vdk van ggt vdk acc ccg
Va17	cgt gac vdk dmy ggt van acc ccg
Va18	cgt gac dmy vdk ggt van acc ccg
Va19	cgt gac dmy dmy ggt van acc ccg
Va20	cgt gac vdk vdk ggt van acc ccg
Va21	cgt gac vdk dmy ggt tgg acc ccg
Va22	cgt gac dmy vdk ggt tgg acc ccg
Va23	cgt gac vdk vdk ggt tgg acc ccg
Va24	cgt gac dmy dmy ggt tgg acc ccg
Variable fragment b (Vb)	amino acid position: 8, 9, X
Vb1	ctg cac ctg
Vb2	ctg cac tgg
Vb3	ctg cac tac
Vb4	ctg cac rtc

Table 2.1 Oligonucleotide for the artificial ankyrin repeat protein library construction (con't).

Oligonucleotide	Sequence (5'->3')
Variable fragment c (Vc)	amino acid position: 11, 12, X, X, 15, 16, 17
Vc1	gct gcg kck kck ggt cat ctg
Vc2	gct gcg kck var ggt cat ctg
Vc3	gct gcg kck aac ggt cat ctg
Vc4	gct gcg kck sgy ggt cat ctg
Vc5	gct gcg kck yay ggt cat ctg
Vc6	gct gcg kck ntg ggt cat ctg
Vc7	gct gcg tac var ggt cat ctg
Vc8	gct gcg tac kck ggt cat ctg
Vc9	gct gcg tac yay ggt cat ctg
Vc10	gct gcg cgy kck ggt cat ctg
Vc11	gct gcg cgy var ggt cat ctg
Vc12	gct gcg cgy aac ggt cat ctg
Vc13	gct gcg cgy sgy ggt cat ctg
Vc14	gct gcg cgy yay ggt cat ctg
Vc15	gct gcg cgy ntg ggt cat ctg
Vc16	gct gcg var kck ggt cat ctg
Vc17	gct gcg var var ggt cat ctg
Vc18	gct gcg var aac ggt cat ctg
Vc19	gct gcg var sgy ggt cat ctg
Vc20	gct gcg var yay ggt cat ctg
Vc21	gct gcg ktk kck ggt cat ctg
Vc22	gct gcg ktk var ggt cat ctg
Vc23	gct gcg ktk aac ggt cat ctg
Vc24	gct gcg ktk sgy ggt cat ctg
Vc25	gct gcg ktk yay ggt cat ctg
Constant fragment	amino acid position: 18-32*
C1	gaa atc gtt cgt ctc ctg ctg gaa cac ggc gca gac gta aac gcg

Table 2.1 Oligonucleotide for the artificial ankyrin repeat protein library construction (con't).

Oligonucleotide	Sequence (5'->3')
Bridging fragments	
Vb1rev	cgc agc cag gtg cag cgg ggt
Vb2Rev	cgc agc cca gtg cag cgg ggt
Vb3rev	cgc agc gta gtg cag cgg ggt
Vb4rev	cgc agc gay gtg cag cgg ggt
C2rev	cag gag acg aac gat ttc cag atg acc
C3rev	gtc acg cgc gtt tac gtc tgc gcc gtg ttc cag

Footnote: * Recognition site for *Bsm* BI is represented as underlined letters.

Neucleotides at randomized positions are higlighted in red color.

Code for mixed bases nomenclature:

d = g, a or t

k = g or t

m = a or c

v = g, a or c

r = a or g

s = g or c

y = c or t

2.4.2 Preparation of DNA cassettes encoding a single internal repeat for library construction.

All synthetic fragments (Va, Vb, Vc, and C1) were hybridized with reverse oligonucleotides linkers (bridging fragments; Vb rev, C2 rev, and C3 rev) at equal molar by heating at 95ºC for 5 min followed by cooling down to 25ºC at the rate 0.1 ºC/minute. To generate the circularized template, the hybridized product was ligated by T4 DNA ligase enzyme (NEB, Pickering, Ontario) resulting in the circular templates. The ligation product was purified using NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany). The circularized templates were hybridized with random hexamer primers provided in the illustra TempliPhi 100 amplification kit and amplified by Phi29 polymerase (GE healthcare Bio-Sciences Co. Piscataway, NJ) at 30ºC for 15 hr. The amplification process using Phi29 polymerase is based on the rolling circle amplification (RCA) processes (Dean et al., 2001) as shown in **Figure 2.2.**

The polymerized product was incubated at 65ºC for 15 min and subsequently treated with *Bsm* BI (NEB, Pickering, Ontario) at 55ºC for 4 hr. The *Bsm* BI-treated product represented mixture of a monomeric fragment of DNA cassette was purified using NucleoSpin® Extract II. The complete digestion was analyzed by agarose gel electrophoresis.

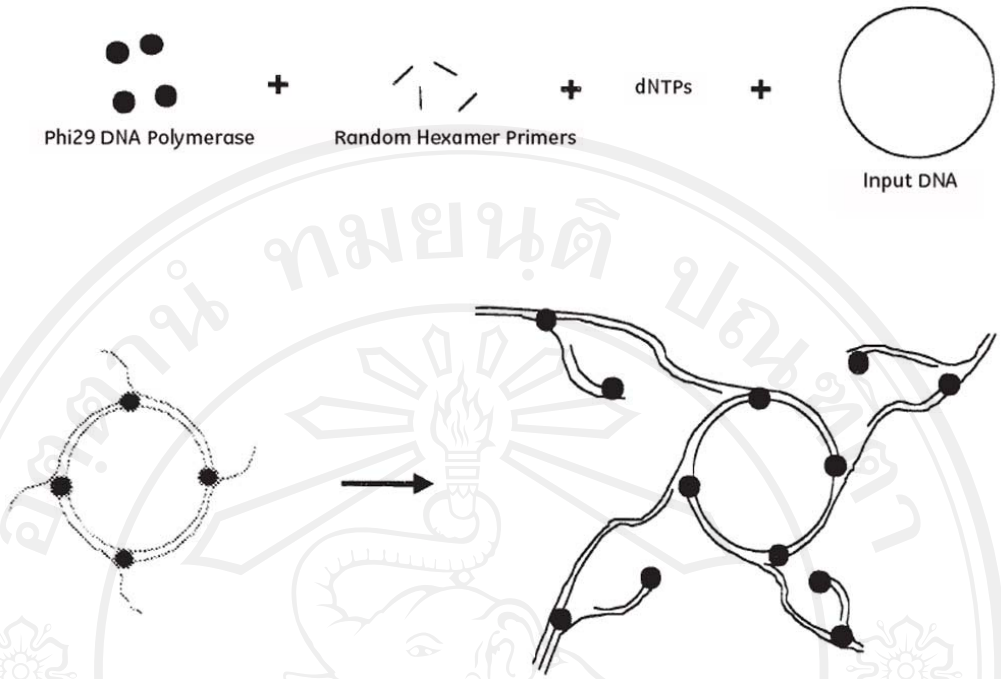

Figure 2.2 Schematic of the TempliPhi amplification process. Random hexamer primers anneal to the circular template DNA at multiple sites. Phi29 DNA polymerase extends each of these primers. When the DNA polymerase reaches a downstream extended primer, strand displacement synthesis occurs. The displaced strand is rendered single-stranded and available to be primed by more hexamer primers. The isothermal amplification was continued at 30ºC for 4-6 hr without the need for thermal cycling (Dean et al., 2001).

2.4.3 Generation of intermediate vector

The phagemid pHDiExDsbA-Ank15 vector (Nangola et al., 2010) was used for the construction of intermediate vector termed acceptor vector (pHDiExDsbA-AccV). The synthetic adaptor cassette containing restriction sites required for the library construction process;

Ank ModAcc Fw (5'-TGCTGGAGGAGGGTACCGAGGAGACCTGCTA ATTGCTGAAGCACGGTGCTGACGTTAACGCTAAT-3') and Ank ModAcc Rev (5'-GATCATTAGCGTTAACGTCAGCACCGTGCTTCAGCAATTAGCAGGTCT CCTCGGTACCCC CTCC-3') were mixed at the equal molar ratio (5 μM) and hybridized by heating at 95ºC for 5 min followed by cooling down to 25ºC with the constant rate at 0.1ºC/second. The hybridized cassettes were cloned into *Not* I and *Bcl* I site of pHDiExDsbA-Ank15 and transformed into competent XL-1 Blue resulting in pHDiExDsbA-AccV vector.

2.4.4 Library construction

The pHDiExDsbA-AccV vector (30 μg) was treated with *Kpn* I at 37ºC for 4 hr and then purified using NucleoSpin® Extract II. The purified product was further digested with *Bsm* BI at 55ºC for 16-18 hr and purified using NucleoSpin® Extract II. The mixture of single-stranded oligonucleotides, Biotin link *Kpn* I (biotin-5'-ACGACAGGGTAC-3') and Link *Kpn* I Rev (5'-CTGTCTGT-3') was pre-hybridized prior to the ligation step with treated acceptor vector to seal the *Kpn* I site. The ligation product was further purified by NucleoSpin® Extract II. The DNA cassette which was prepared in the previous step and the intermediate vector (pHDiExDsbA-

AccV vector linked with biotinylated-*Kpn* I linker) were mixed at the molar ratio 1:5. DNA fragments corresponding to single ankyrin modules were hetero polymerized in the acceptor vector with T4 DNA ligase at 20 ºC for 4 hr. Due to the non palyndromic *Bsm* B1 cohesive ends, the modules can be ligated in only one orientation. The excess cassettes were eliminated by capturing the biotinylated acceptor vector on pre-washed streptavidin coated magnetic beads (Sigma, St. Louis, MO). Beads were agitated at 25ºC for 1 hr and washed three times with NEB buffer containing 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 (NEB, Pickering, Ontario). The vectors which are captured on beads were released out by incubating with *Bsp* MI enzyme at 50ºC for 3 hr resulting in the cohesive ends designed to compatible with *Bsm* BI site at 3' end of the polymerized DNA cassette. The mixture of released vector was glued to generate the circularized vector by T4 DNA ligase at 20ºC for 4 hr, as shown in **Figure 2.3**. The ligation product was purified and then electroporated into XL-1 Blue electrocompetent cells. The transformed cells were spread on the 20 cm x 20 cm plates of 2YT agar containing ampicillin (100 µg/ml) and 1% (w/v) glucose. Some of the transformed cells were taken to perform the serial dilution and plated on 2YT agar containing 100 µg/ml ampicillin and 1% (w/v) glucose to evaluate the library size. The number of colonies of each dilution was use for library size calculation. Colonies on the solid medium were collected after overnight growth, pooled and stored at -80ºC in the 2YT medium containing 20% (v/v) glycerol. The DNA was extracted from a sample of pooled cells for restriction analysis and collective transformation in an expression host.

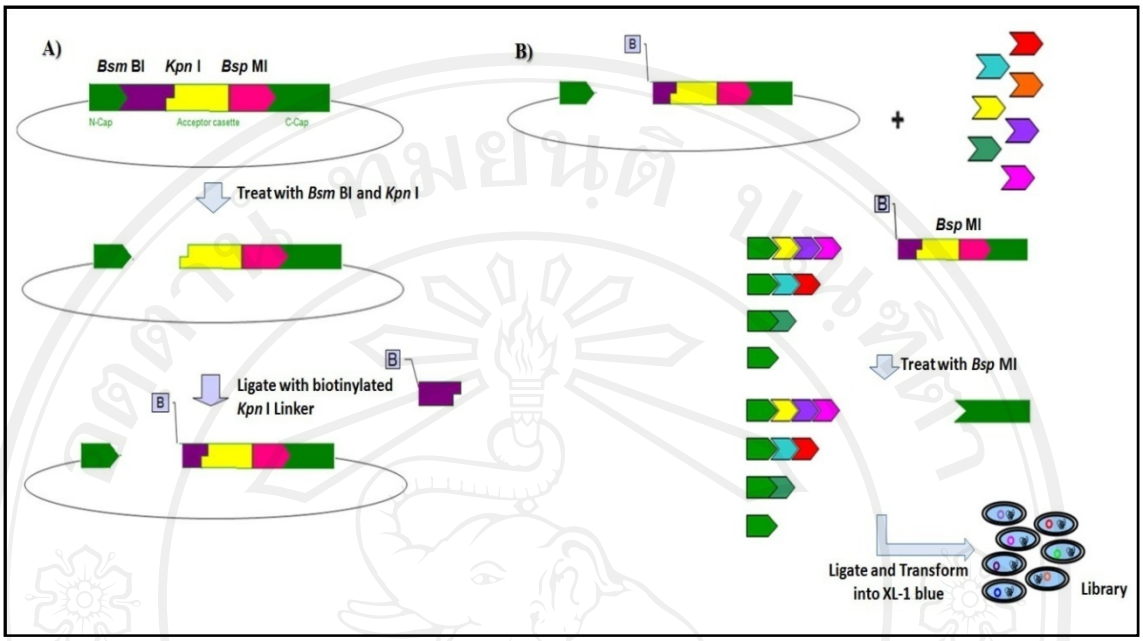

Figure 2.3 Process for library generation. **(A)** The intermediate vector was treated with *Bsm* BI and *Kpn* I restriction enzymes to generate the cohesive ends for polymerization with DNA cassettes at C-terminus of N-cap and for ligation with biotinylated linker. **(B)** The polymerization process was undergone randomly during the ligation process with the mixture of DNA cassettes. Free-DNA cassettes were removed by trapping the vector on streptavidin beads and washed. Then vectors were released from the beads by digestion with *Bsp* MI to generate the compatible end with *Bsm* BI and closed by the ligation process. Finally, released vectors were closed by the ligation process and then transformed into *E. coli*.

2.4.5 Characterization of the library

Thirty randomized colonies from the library were picked and their plasmid was extracted to analyze the sequence by the standard sequencing method. To validate the distribution of the repeat number, plasmid from DNA pooled clones was treated with *Nde* I and *Hind* III at 37ºC for 3 hr and separated using gel electrophoresis. Moreover, the soluble protein expression was evaluated by colony filtration (CoFi) blot analysis as described elsewhere (Dahlroth et al., 2006). Briefly, the pooled plasmid from the library was transformed into *E. coli* BL21 (DE3). 72 randomized single colonies were picked and cultured in 200 µl of LB broth containing ampicillin (100 µg/ml), and 1% (w/v) glucose in 96-well plate with shaking at 550 rpm, 37ºC for 5 hr. Pre-cultured cells were transferred to culture on Durapore® membrane filter (Millipore, Billerica, MA) which pre-covered on LB agar containing ampicillin (100 µg/ml), and 1% (w/v) glucose using 96-well replicator at 37ºC for 16-18 hr. The membrane containing colonies was transferred to another LB agar induction plate containing ampicillin (100 µg/ml), and 1mM IPTG for protein induction and incubated at 37ºC for 4 hr. The membrane was taken out from agar and placed on the pre-wet filter sandwich containing layers of nitrocellulose membrane on the top of three pieces of Whatman 3MM paper (GE healthcare Bio-Sciences Co. Piscataway, NJ). The filter was then subjected to freeze-thawing process at -80ºC for 10 min following by incubation at 37ºC for 10 min. Three consecutive repeats were performed. Consequently, the Durapore membrane was discarded. Soluble proteins were recovered on the nitrocellulose membrane and probed with a mouse anti-His tag antibody (GenScript, Piscataway, NJ) at dilution 1:5,000 in blocking solution (5% BSA in TBST) after the blocking step. The membrane was washed three times with

TBST (0.05% Tween-20 in TBS) and then revealed with goat anti-mouse immunoglobulins conjugated Alexa Fluor® 680 at dilution 1:10,000 in blocking solution. After washing step, the membrane fluorescent signal was observed using Odyssey® infrared scanner (LI-COR Biosciences, Lincoln, NE) with excitation at 680 nm and emission at 700 nm. Positive clones were detected by quantitative fluorescence signal analysis comparing to signal from background and negative control.

2.5 Preparation of phage-displayed artificial ankyrin repeat protein library

Five hundred microliters of stock library were cultured in 500 ml 2X YT broth (1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 0.5% (w/v) sodium chloride) containing ampicillin (100 µg/ml), 1% (w/v) glucose and tetracycline (10 µg/ml) at 37ºC with shaking at 200 rpm until the $OD_{600\ nm}$ was as reached 0.5. The bacterial culture was infected further with 20 MOI of K07 helper phages and then incubated at 37ºC for 30 minmin without shaking and followed by 37ºC for 30 min with shaking at 150 rpm. Phage-infected cells were centrifuged at 1,200 g for 10 min at 25 °C. Pellets were resuspended and cultured in 500 ml of 2X YT broth containing ampicillin (100 µg/ml), and kanamycin (70 µg/ml) with shaking at 200 rpm at 30 °C for 16-18 hr. Bacterial culture was clarified by centrifugation 1,200 g for 10 min at 4°C. The culture supernatant was collected and phage particles were harvested by PEG/NaCl (20% (w/v) PEG 8000, 2.5 M NaCl) precipitation. Finally, the pellets were resuspended with 10 mM Tris-buffer saline (TBS) pH 7.4.

2.6 Phage titration

The concentration of phage was titrated using two methods, which are measuring by UV spectrophotometer as described elsewhere (Barbas, 2001) and re-infecting into *E. coli* strain XL-1 Blue resulting in the concentration in particles per volume (particles/ml) and colony forming units per volumn (CFU/ml), respectively. The first method, precipitated phages were diluted at dilution 1: 50 in TBS and scanned from 240 to 320 nm with UV-2450/2550 spectrophotometer (Shimadzu, Columbia, MD). The absorbance at 269 nm (A_{269}) which is a maximum peak was used for calculation as follow:

$$\text{Phage particles per ml} = \frac{(\text{Adjusted } A_{269}) \times (6 \times 10^6) \times (\text{dilution factor})}{(\text{The number of nucleotides in the phage genome})}$$

The second method, fifty microliters of serial dilutions of phages were used to infect 450 µl of cultured *E. coli* (OD_{600nm} at 0.5) and incubated at 37ºC for 30 min. Fifty microliters of viral-infected bacteria were spread on LB agar containing ampicillin (100 µg/ml). Plates were incubated at 37ºC overnight and the ampicillin resistant colonies were counted and calculated for phage concentration using the formula below.

$$\mathbf{A = B \times C \times (1000/v)}$$

A = The concentration of phages (CFU/ml)

B = The number of ampicillin resistant colonies.

C = Dilution factor of viral infected bacteria.

V = Volume (µl) of viral infected bacteria.

2.7 Phage filtration using *Strep*-tactin® coated magnetic beads

To enrich the population of coding phage in the library, 1 x 10^{13} particles of phage library were diluted in filter-sterile 2% BSA in TBS and incubated with pre-washed *Strep*-tactin® coated magnetic beads (IBA GmbH, Göttingen, Germany) with agitation at 25ºC for 1 hr. Unbound phage particles were eliminated by washing nine times with washing buffer (sterile-filtered 0.1% Tween 20 in TBS). Bound phage particles were specifically eluted with 500 µl of 2.5 mM desthiobiotin and collected by centrifugation. The eluted phage particles in the supernatant were rescued by infecting *E. coli*. The infected cells were spread on LB agar containing ampicillin (100 µg/ml) and incubated at 37ºC overnight. The antibiotic resistant colonies were collected, pooled and stored at -80ºC in the 2YT medium containing 20% (v/v) glycerol. The phage particles were prepared from the glycerol stock for the next round filtration. This step was performed for three rounds. DNA was extracted from pooled clones of each round and transformed into *E. coli* BL21 (DE3) to evaluate the soluble expression by CoFi blot analysis as described in the previous step.

2.8 Production of H_6MA-CA and H_6-CA recombinant protein by baculovirus expression system

2.8.1 Vector construction

A DNA fragment coding for H_6MA-CA was generated by standard PCR method using the following pairs of primers: the first pair consisted of primers 5'-CTAGCATGGGTGCGAGAG-3' and 5'-CATGGGTGCGAGAGCG-3' and the

second pair consisted of primers 5'-CTTACTACAAAACTCTTGCTTTATG-3' and 5'-GTACCTTACTACAAAACTCTTGC-3'. Two PCR reactions were performed using the HIV-1 plasmid pNL4-3 as the template. The PCR products from both reactions were then mixed, denatured, and hybridized to obtain DNA fragments with *Nhe* I and *Kpn* I cohesive ends, resulting in the H_6MA-CA-encoding fragment. This fragment was competent for ligation to *pBlueBac4.5-His intermediate vector* linearized with *Nhe* I and *Kpn* I. Plasmid *pBlueBac4.5-His was derived from pBlueBac4.5 (Invitrogen*, San Diego, CA) by *insertion of a* sequence coding for the 6Histidine (H_6) tag and a GSGSAS linker upstream to the *Nhe* I site. The H_6MA-CA-encoding fragment was cloned into the *Nhe I and Kpn I sites of pBlueBac4.5 intermediate vector* using the standard ligation method as described in previous step to generate *pBlueBac*-H_6MA-CA vector.

2.8.2 Production of recombinant H_6MA-CA and H_6-CA in Sf9 cells

Sf9 cells were cotransfected with pBlueBac4.5-H_6MA-CA (10 μg) and Bac-N-Blue™ DNA using Cellfectin® II reagent following the Bac-N-Blue™ transfection and expression manual (Invitrogen, San Diego, CA). These two vectors recombined inside the cells resulting in the recombinant AcMNPV DNA (**Figure 2.4**) containing genes encoding for proteins for viral replication, H_6MA-CA and LacZ for blue plaque selection as shown in **Figure 2.5**. The recombinant virus (BV-H_6MA-CA) in the supernatant was isolated using the blue plaque selection method as described in the instruction manual. Briefly, the culture supernatant of transfected cells was harvested after 48 hr of incubation. Serial dilutions of the supernatant containing the

recombinant virus were used for infection with Sf9 cells seeded in 6-well plates. Plate was rotated gently and incubated at 27ºC for 1 hr. Medium was discarded after incubation prior to carefully adding the TNM-FH medium containing 2% of low melting agar and 150 µg/ml of 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal). The blue plaques were picked up and resuspended in Grace's Insect medium and used for infection in Sf9 cells. After 48 hr of infection, infected cells were harvested and lysed cells by the freeze-thawing method. The clarified lysate was yielded by centrifugation at 15,000 g, 4ºC for 30 min. The presence of H_6MA-CA protein was detected by Western immunoblotting. The cell lysate was separated in 12% SDS-PAGE and then transferred to nitrocellulose membrane. The blotted membrane was blocked with 5% skim milk in TBS at RT for 1 hr and then detected using monoclonal anti-His tag antibody diluted 1:5,000 in the blocking solution at RT for 1 hr with slow agitation. After an incubation time, the membrane was washed with TBST (0.05% Tween 20 in TBS) and revealed using goat anti-mouse immunoglobulins conjugated HRP at dilution 1:8,000 in 5% skim milk in TBS. Unbound conjugate was washed five times. The specific bands were visualized using TMB membrane peroxidase substrate (KPL, Gaithersburg, MD). H_6MA-CA protein was recovered from a clarified Sf9 cell lysate by affinity chromatography on HisTrap column using ÄKTA prime™ plus (GE healthcare Bio-Sciences Co. Piscataway, NJ). Protein concentration of purified H_6MA-CA was determined by Bradford protein assay (Thermo Fisher Scientific Inc., Rockford, IL). Purified H_6MA-CA protein was separated in 15% SDS-PAGE under reducing condition and then the gel was stained using Coomassie's blue G to determine the purity.

Recombinant H_6-CA was produced by baculovirus expression system. A gene fragment coding CA was amplified from parental vector (pNL4-3) by standard PCR protocol using pair of primers: FWD_p24 *Nhe* I, 5'-GAGGAGGAGGTGCTATA GTGCAGAACCTCCAG-3' and REV_p24 *Kpn* I, 5'-GAGGAGGAGCTGGT ACCTTACAAAACTCTTGCTTTATGGCC-3'. PCR fragment was treated with *Nhe* I and *Kpn* I and subsequently cloned into *pBlueBac*-H_6MA-CA plasmid resulting pBlueBac-H_6-CA. This vector was co-transfected with Bac-N-Blue^{TM} DNA using the standard protocol as described. The subsequent steps for recombinant viral production, protein expression and detection were followed the same protocol as described in H_6MA-CA production.

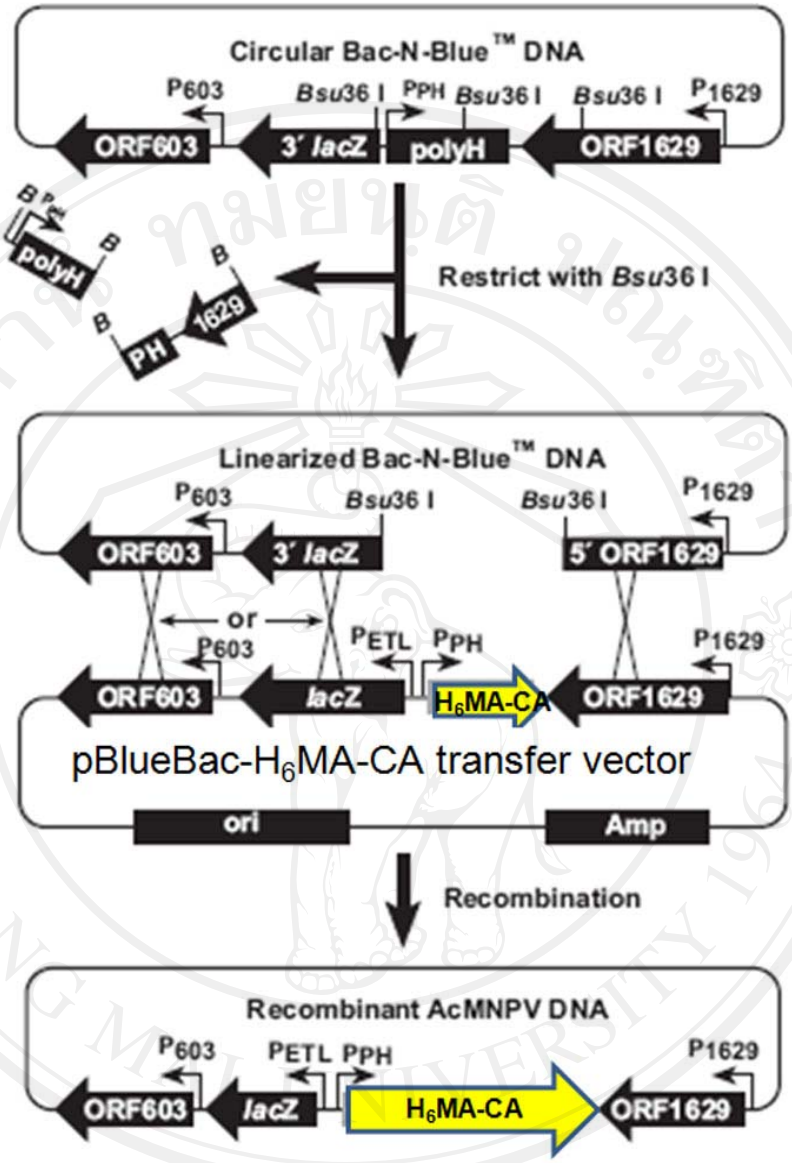

Figure 2.4 Recombination between Bac-N-Blue™ DNA and pBlueBac-H_6MA-CA transfer vector (modified from the instruction of Bac-N-Blue™ transfection kit, Invitrogen).

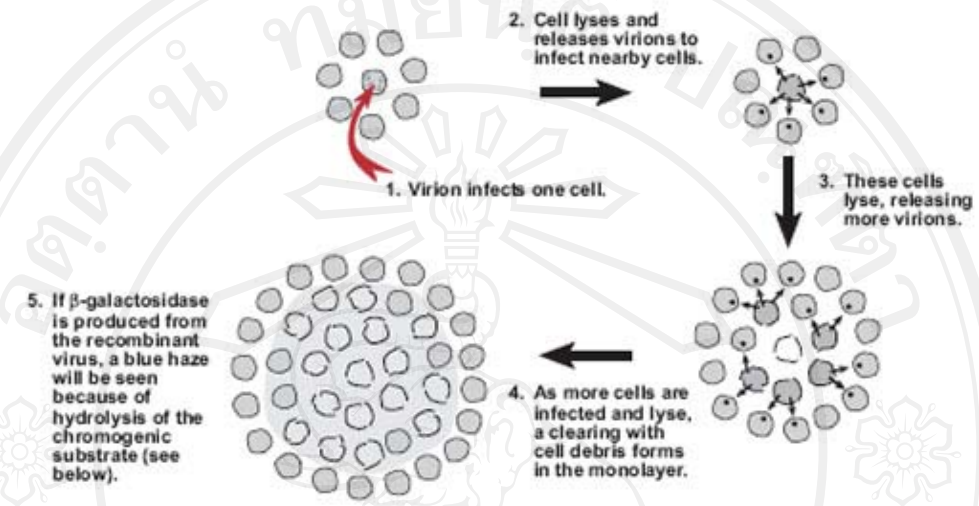

Figure 2.5 Formation of blue plaques. The infected cells are lysed and released virions to infect nearby cells. The plaque with cell debris as observed a clearing area indicated more cells are infected and lysed. In the presence of X-gal, a blue haze was seen because of hydrolysis of the substrate (modified from the instruction of Bac-N-Blue™ transfection kit, Invitrogen).

2.9 Selection of specific binders from the constructed artificial ankyrin repeat protein library

2.9.1 Selection procedure

Microtiter plate (NUNC, Roskilde, Denmark) was coated with 100 µl of purified H_6MA-CA protein (20 µg/ml) or A3 protein diluted in sterile PBS overnight at 4ºC. A3 protein was used as the folded target protein for evaluating the quality of constructed artificial ankyrin library. Purified A3 protein was produced as described (Urvoas et al., 2010). Coated wells were washed four times with sterile-filtered TBST. The wells were blocked the non-specific binding with 200 µl of sterile-filtered blocking buffer (2% (w/v) BSA in TBST) at RT for 1 hr with shaking at 150 rpm in Eppendorf Thermomixer® (Eppendorf, Hauppauge, NY). After the washing step, 100 µl (1×10^{11} particles) of diluted phage were added and incubated at RT for 1 hr with shaking. After an incubation time, wells were washed 20 times with TBST and 10 times with TBS to eliminate unbound phage. 100 µl of 0.1M Glycine pH2.5 was added and incubated at RT for 10 min with shaking. Then 12.5 µl of 1M TrisHCl pH8 was added to neutralize the acid condition. The eluted phage was collected and subsequently infected with 5 ml of XL-1 Blue ($O.D_{600nm}$ = 0.6-0.8) at 37ºC for 30 min. The infected cells were centrifuged at 1,200 g, 25ºC for 10 min and re-suspended with 1 ml of 2X YT broth. The cells were plate on LB agar containing ampicillin (100 µg/ml). Some of the cells were taken to evaluate the number of phage output by performing serial dilution in 2X YT broth and 50 µl of each dilution was spread on LB agar containing ampicillin (100 µg/ml). The colonies on agar were collected and used for phage preparation as described in previous step to perform the next round of

selection. In addition for the third round of selection, free antigen was used as specific elutor in the elution step instead of 0.1M Glycine pH 2.5 to obtain the specific binders.

2.9.2 Screening of specific binding clones

Phage particles were prepared in small scale using the 96-wells format. Briefly, random colonies from each round of selection were picked and culture in 150 µl of 2X YT broth containing ampicillin (100 µg/ml) and 1% (w/v) glucose in 96-well plate (master plate) with shaking at 600 rpm in Eppendorf Thermomixer®, 37ºC overnight. 10 µl of overnight culture was transferred to new plate containing 150 µl of 2X YT broth containing ampicillin (100 µg/ml), tetracycline (10 µg/ml), and 1% (w/v) glucose and cultured at 37ºC with shaking for 4 hr. Bacterial cells were infected with 20 MOI of KO7 helper phage and incubated at 37ºC without shaking for 30 min and at 37ºC with shaking for 30 min. 200 µl of infected cells was transferred to the 96-well storage plate containing 1.5 ml of 2X YT broth adding ampicillin (100 µg/ml) and kanamycin (70 µg/ml). The plate was covered with gas permeable adhesive seals (Thermo scientific, Surrey, UK) and cultured at 30ºC with shaking for 16-18 hr. Phages in the culture supernatant were collected after the centrifugation and used for phage ELISA to determine the binding activity.

Hundred microliters of 20 µg/ml of purified H_6MA-CA or A3 diluted in PBS was added into a microtiter plate and left for overnight at 4ºC in the moisture chamber. The coated wells were washed four times with TBST. The wells were then blocked the non-specific binding with 200 µl of blocking solution at RT for 1 hr with

shaking. After the washing step, 100 µl of the culture supernatant of each clone containing phage particles was added and incubated at RT for 1 hr. 100 µl of mouse anti-M13 conjugated HRP (GE healthcare Bio-Sciences Co. Piscataway, NJ) at dilution 1:5,000 in blocking buffer was added after washing step. The wells were then washed again prior to adding 100 µl SureBlue™ TMB Microwell Substrate (KPL, Gaithersburg, MD) and measured the optical density at 450 nm (OD_{450nm}) after adding 1 N HCl by a MTP-120 ELISA plate reader (Corona Electric, Japan).

2.10 Production of soluble ankyrin binders

2.10.1 Construction of pQE-30 expressing soluble ankyrin binders

pQE-30 ankyrin acceptor vector was constructed by inserted the hybridization product of two synthetic oligonucleotides, pQE-Ank-Adapt-Fw (5'-GATCCGCGGC CGCAAACGCGTAAA-3') and pQE-Ank-Adapt-Re (5'-AGCTTTTACGCGTTTGC GGCCGCG-3'), into *Bam* HI and *Hind* III sites on pQE-30 vector resulting in additional *Not* I restriction site in pQE-30. The DNA fragment encoding ankyrin binders was isolated from pHDiExDsbA phagemid vector by treated with *Not* I and *Hind* III and then subcloned into the same sites on acceptor vector resulting in pQE-30 containing DNA fragment encoding ankyrin binders. These vectors were transformed into chemical competent M15 [pREP4] (Qiagen, Hilden, Germany).

2.10.2 Expression and purification of soluble ankyrin binders

M15 [pREP4] containing certain vectors were cultured in 500 ml of LB broth containing ampicillin (100 µg/ml), kanamycin (25 µg/ml), and 1% (w/v) glucose at 37ºC with shaking until OD_{600nm} reached 0.8. Bacterial cells were induced for protein production by adding 1mM IPTG and cultured at 30ºC for 4 hr with shaking. The induced cells were collected by centrifuged at 3000 rpm 4ºC for 30 min. The cell pellets were resuspended with lysis buffer (TBS pH7.4, complete EDTA free protease inhibitor, 1 µg/ml lysozyme) and then subjected to three cycles of freeze-thawing. The lyzed cells were centrifuged at 15,000 g 4ºC for 30 min. The soluble form of ankyrin binders was purified by injected the clarified lysate into HisTrap column (GE healthcare Bio-Sciences Co. Piscataway, NJ) followed by Sephadex G-75. The purified proteins were analyzed the purity by separated in 15% SDS-PAGE and performed Coomassie's blue staining.

2.10.3 Biotinylation of soluble ankyrin binders and monoclonal antibodies

The purified ankyrin binders, and monoclonal antibodies were chemically linked to biotin molecule following the instruction of the EZ-Link Sulfo-NHS-LC-Biotin kit (ThermoScientific, Rockford, IL). Briefly, 100 µM of purified protein was mixed with 500 µM of Sulfo-NHS-Biotin solution at the final volume 2 ml and then incubated at 25ºC for 1 hrs. Free biotin molecules were removed by applying the mixture into pre-equilibrated Zeba™ Desalt Spin Column (ThermoScientific, Rockford, IL) and allowed sample to absorb into resin. The column was centrifuged at 1,000 g for 2 min. The biotinylated protein solution in flow-through was collected and

measured the concentration using NanoDrop 2000 (ThermoScientific, Rockford, IL). The purified proteins were evaluated the biotinylation efficiency by dot blot analysis. 10 μmole of biotinylated proteins was spotted on nitrocellulose membrane. The membrane was occupied with blocking solution and revealed by Extravidin-HRP (Sigma, St Louis, MO) at dilution 1:5,000 in blocking solution for 1 hr with shaking. The BM Blue POD Substrate, precipitating (Roche, Mannheim, Germany) was added to visualize the reaction.

2.11 Production of monoclonal antibodies

2.11.1 Mouse immunization

Two female Balb/c mice at six weeks of ages were used in this study. Blood was collected by tail-bleeding to prepare the pre-immunized serum. Mice were immunized intraperitoneal route (IP) with 100 μg purified H_6MA-CA in 500 μl sterile PBS. The immunizations were repeated every two weeks. Blood was collected after the third immunization. Sera were isolated from the collected blood and determined for the antibody responses by indirect ELISA described in 2.11.4.1.

2.11.2 Hybridoma technique

An immunized Balb/c mouse, that appearing high antibody titer was selected and used for hybridoma generation. The selected mouse was boosted IP with 100 μg of purified H_6MA-CA. Five days after boosting, mouse was sacrificed and spleen was taken. Spleen cells were isolated by homogenizing carefully. The obtained

splenocytes were counted with Turk's solution using hematocytometer. Myeloma cells were collected from culture flasks and counted by 0.2% trypan blue using hematocytometer. Then, the splenocytes were fused with mouse myeloma cells using 50% polyethylene glycol (PEG) by standard hybridoma techniques. The fused cells were subsequently resuspended in the HAT selection medium and transferred to 96 well culture plates at 10 plates/fusion. The hybridoma cells were incubated at 37^{o}C in 5% CO_2 incubator for five days. After that the culture plates were added with HT supplement medium. The hybridoma clones were monitored by an inverted light microscope.

The culture supernatant from the wells containing hybridoma cells were collected and screened for antibody reactivity with the corresponding antigens by ELISA as was described in 2.11.4.1.

2.11.3 Single cell cloning by limiting dilution

Hybridoma cells from the antibody screening positive wells clones were counted by hematocytometer. The cells were diluted in IMDM medium containing 10% FCS and 10% BM condimed at the final concentration of 400 cells/15 ml. After that the hybridoma cells were diluted in serial two-fold dilution into three concentrations: 400 cells/7.5 ml, 200 cells/7.5 ml and 100 cell/15 ml respectively. Then the cell suspension was added into 96 well culture plates at 150 µl/well, the final concentration of each well was 4 cells/well, 2 cells/well and 1 cell/well respectively. The culture plates were incubated at 37^{o}C in 5% CO_2 incubator for two weeks. The hybridoma clones in the cultured wells were monitored by an inverted light

microscope. Cell culture supernatants were collected from wells containing a single clone determined their activity by ELISA as was described in 2.11.4.1.

2.11.4 Characterization of monoclonal antibodies by Indirect ELISA and Western immunoblotting

2.11.4.1 Indirect ELISA

Indirect ELISA was set up for analyzed the presence of polyclonal antibodies in serum and for characterization of monoclonal antibodies. H_6MA-CA at concentration 10 µg/ml diluted in the coating buffer was coated on microtiter plate 50 µl/well at for overnight at 4ºC in a moisture chamber. Wells were washed three times with washing buffer (0.05%Tween 20 in PBS) and then blocked with 2% skim milk in PBS 200 µl/well at RT for 1 hr. Fifty microliters of the diluted serum or culture supernatant of each clone was added into the wells. After 1 hr of incubation time, wells were washed three times with washing buffer and goat anti-mouse immunoglobulins conjugated HRP at dilution 1:3,000 in blocking buffer was added into the wells. The wells were washed and added 100 µl of TMB substrate and measured the optical density (OD) at 450 nm after adding 1 N HCl by a MTP-120 ELISA plate reader.

2.11.4.2 Western immunobloting

Cell lysate form BV-H_6MA-CA infected Sf9 cells were separated in 12% SDS-PAGE under denaturing condition prior to PVDF membrane transfer. The

blotted was blocked with 5%BSA in TBS and then cut to small strip (5 mm). The culture supernatants of each clone were separately reacted with strips at RT for 1 hr with shaking. Goat anti-mouse immunoglobulins conjugated HRP at dilution 1:5,000 in blocking buffer was added after washing step and incubated for 1 hr at RT with shaking. The reactive bands were visualized by adding TMB membrane substrate after washing step.

2.11.5 Purification of monoclonal antibodies

Hybridoma clones were adapted to grow in serum-free medium before purification using protein G column. Briefly, Cells were cultured by using four or more consecutive dilutions of the culture medium (10%FBS/DMEM) with the protein-free hybridoma serum-free medium (PFHM-II: Gibco BRL, Caithersburg, MD) during a period of 8 to 14 days. Each dilution with PFHM-II reduced the FBS concentration by half. Rate of dilution of the 10%FBS/DMEM was decreased when the percentage of viable cells as less than 80%. Finally, cells were maintained in serum-free medium for 10 days. The culture supernatant was collected by centrifugation 1,200 g, 4ºC for 30 min and filtered through a 0.2 μm syringe filter. The clarified supernatant was subjected into HiTrap protein G HP column (GE health care). Column was washed by binding buffer (20 mM sodium phosphate buffer, pH 7) and elute by elution buffer (0.1M glycine-HCl, pH 2.7). The elute fractions were immediately neutralized with neutralizing buffer (1M Tris-HCl pH 9) and dialyzed against PBS. Purified monoclonal antibodies were quantified the concentration by measuring the absorbent at 280 nm.

2.12 Evaluation of binding activity of ankyrin binders

2.12.1 Assessment the reactivity of ankyrin binders by Indirect ELISA techniques

Microtiter plates were coated with 100 µl of 1 µg/ml of purified H_6MA-CA or A3 diluted in PBS and left for overnight at 4ºC in the moisture chamber. The coated wells were washed four times with TBST. The wells were blocked the non-specific binding with 200 µl of blocking solution (2% BSA in TBS) at RT for 1 hr. After the washing step, 100 µl of 10 µM biotinylated-ankyrin binders or the mixture of biotinylated-ankyrin binders and inhibitors (non-biotinylated ankyrin binders, and irrelevant ankyrin binders), or biotinylated-monoclonal antibodies were applied and incubated at RT for 1 hr. After an incubation time, wells were washed prior to adding Extravidin-HRP at dilution 1:5000 in blocking solution. The wells were washed and added 100 µl of TMB substrate and measured the optical density (OD) at 450 nm after adding 1 N HCl by a MTP-120 ELISA plate reader.

2.12.2 Epitope identification of ankyrin binders by Western immunoblotting and Indirect ELISA

The cell lysate of BV-H_6MA-CA infected Sf9 cells was separated in 12% SDS-PAGE under reducing condition. The separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membrane. The blotted membranes were blocked at 4°C overnight in blocking buffer (5% BSA in TBS pH 7.2) and then incubated with 1 µM biotinylated-ankyrin binders for 1 h at room temperature on a shaking platform. After washing step, Extravidin-HRP at dilution 1:10,000 in blocking solution was

added to detect the bound binders. An excess conjugate was eliminated by washing and then the reactive bands were visualized by TMB membrane peroxidase substrate.

Furthermore, the recognition site of these binders was confirmed by indirect ELISA. BV-H_6-CA-infected cell lysate was added into Nickel pre-coated wells as described in 2.13.2.1. Biotinylated-ankyrin binders were individually reacted with CA-captured on well after the washing step as described in 2.13.2.2, and incubated at 37ºC for 1 hr. The binding reaction was monitored by adding Extravidin-HRP at dilution 1:5,000. Wells were washed after an incubation time and subsequently developed signal by adding TMB substrate. Signal was measured at 540 nm after stopping reaction with 1N HCl.

2.12.3 Epitope mapping of ankyrin binders by sandwich ELISA

Monoclonal antibodies against CA domain, clone G18 and M88, were coated on microtiter plates at the concentration 1 µg/ml diluted in PBS, 100 µl/well and left at 4ºC for overnight in the moisture chamber. The coated wells were washed four times with TBST. H_6MA-CA at 10 µg/ml diluted in blocking solution was added after blocking step and incubated at RT for 1 hr. Excess antigen was eliminated by washing four times with washing buffer. The biotinylated-ankyrin binders or biotinylated-monoclonal antibodies were then added into wells at concentration 10 µg/ml diluted in blocking buffer. After an incubation time, wells were washed four times and monitored the binding reaction by adding Extravidin-HRP at dilution 1:5,000 in blocking solution. The wells were washed and added 100 µl of TMB substrate and measured OD at 450 nm after adding 1 N HCl.

2.12.4 Evaluation of binding activity using isothermal titration calorimetry (ITC)

The H_6MA-CA, A3, and ankyrin binders were subjected to the MicroCal iTC_{200} isothermal titration calorimeter (Microcal) to evaluate the binding activity. The experiment was performed with the same condition as described previously (Nicaise et al., 2004). All proteins were diluted in 20 mM phosphate buffer pH7.5, 150 mM NaCl. For each injection, 2 µl of binders was added from a computer-controlled 300 µl microsyringe at intervals of 1 minute into H_6MA-CA solution. A theoretical titration curve was fitted to the experimental data using the ORIGIN software (Microcal). This software uses the relationship between the heat generated by each injection to calculate several parameters such as ΔH^o (enthalpy change in kcal $mole^{-1}$), K_a (the association binding constant in M^{-1}), n (number of binding sites per monomer).

2.13 Interference of HIV protease activity by ELISA-based method

2.13.1 Production of recombinant HIV-1 protease in *E. coli*

The gene encoding HIV-1 protease was amplified from vector pET14bELP105KnewTat using a pair of primers: Fw HIV-PR *Nhe* I (5'-GACGACG CTAGCATGCCTCAGATCACTCTTTGG-3') and HIV-PR H6 *Hind* III rev (5'-GTC GTCAAGCTTTTAATGGTGATGGTGATGGTGTGCGCCAAAATTTAAAGTGA G CCAAT-3'). The reverse primer was designed to insert 6Histidine tag at C-terminus of protein. PCR reaction was amplified using Accuprime™ Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The PCR product was purified using

GeneJet™ PCR purification kit (Fermentas). The purified PCR product and pET21a vector (kindly provided by Dr. Matthew DeLisa, Cornell University, Ithaca, NY) were treated with *Nhe* I and *Hind* III at 37ºC for 16 hr. The treated insert fragment and vector were ligated using T4 DNA ligase after purification step at 4ºC for overnight. The ligation product was transformed into chemical competent XL-1 blue. The clones were analyzed by standard sequencing method. The corrected clone was further transformed into the BL21 (DE3) expression strain. Hundred microliters of pre-culture cells were transferred into 100 ml of terrific broth containing ampicillin (100 µg/ml) and cultured at 37ºC with shaking until OD_{600nm} reached 0.8. IPTG was added to the cultured to final concentration 0.1 mM and continued shaking at 16ºC for 18 hr. The induced cells were collected by centrifuged at 1,200 g 4ºC for 30 min. The cell pellets were resuspended with TBS pH7.4 and centrifuged again. Pellet was resuspended by TBS to yield the OD_{600nm} at 75 and subjected to lyse by ultrasonication. The lyzed cells were centrifuged at 15,000 g 4ºC for 30 min. Cell lysate was quantified the concentration by BCA Protein Assay (Pierce). The presence of HIV-1 protease in the cell lysate was analyzed by Western Immunoblotting using the same protocol as described in 2.11.4.2.

2.13.2 Evaluation of ankyrin binders on HIV-1 protease interfering effect by ELISA-based HIV protease assay.

2.13.2.1 Preparation of the nickel pre-treated plate

Microtiter plate was pre-treated using described procedures (Paborsky et al., 1996; Cressey et al., 2008). Briefly, wells were coated with 100 µl of 10mM

BCML (N,N-bis(carboxymethyl)lysine hydrate) diluted in 0.1 M $NaPO_4$ and incubated at 4ºC for overnight. Wells were washed three times with 200 µl of 0.05%Tween 20 in ddH_20 (washing buffer) by shaking at 25ºC for 5 min and then added 200 µl of 0.05%BSA in 0.05% Tween 20 in TBS. After 1 hr of incubation, wells were washed with a series of buffer as follows: first, 50 mM Tris HCl pH7.5 containing 500 mM imidazole and 0.05% Tween20; second, washing buffer; third, 100 mM EDTA, pH 8.0. The plate was then incubated with 10 mM $NiSO_4$ for 1 hr at room temperature. The plate was sequential washed with washing buffer, then 50 mM Tris HCl (pH7.5), and finally 500 mM NaCl (pH 7.5) and ready for detection step.

2.13.2.2 Detection process

Purified H_6MA-CA proteins were added 50 µl at concentration 5 µg/ml in pre-treated plate and incubated at 4ºC for overnight. The excess antigen was removed by washing four times with serial buffer as follows; first, 0.5 M NaCl; second, 20 mM Tris HCl; third, 6M urea; and consecutively washed with buffer containing the serial concentration of imidazole ranging from 20, 40, 60, and 80 mM, respectively. Subsequently, wells were extensively washed once with 0.1%BSA in washing buffer. Biotinylated ankyrin binders at concentration 10 µM were added into the target coated wells and incubated for 1 hr. Wells were washed after incubation time with high-stringency washing buffer (50 mM Tris HCl, pH7.5, 50 mM NaCl, 0.1% BSA. 0.05% Tween20) followed by 0.1%BSA in washing buffer. The bacterial lysate containing HIV-1 protease at concentration 100 µg/ml was added into the wells and incubated for 1 hr at 37ºC with shaking. The presence of C-terminus of MA and the decreasing of CA were separately detected using monoclonal anti-MA specifically

binds to the C-terminus, MH-SVM33, (Lee et al., 2010), after washing steps. Finally, goat anti-mouse immunoglobulins conjugated HRP (KPL, Gaithersburg, MD) was subsequently added and incubated for 1 hr at RT. Wells were then washed again prior to adding 100 µl 3, 3', 5, 5'-tetramethyl-benzidine (TMB) substrate. The OD at 450 nm was measured by an ELISA plate reader after adding 1 N HCl to stop the reaction.

2.14 Generation of stable cell line expressing selected ankyrin binders

2.14.1 Construction of pCEP4 vector harboring ankyrin binders

Two versions of vectors which are pCEP4Myr-Ank-GFP and pCEP4Cyt-Ank-GFP vector were constructed. These two vectors were used for expressing and directing ankyrin protein to inner membrane of cells (Myr) and cytoplasm (Cyt), respectively. The DNA encoding ankyrin repeat proteins, which bind to H_6MA-CA were fused with or without myristoylation signal and green fluorescent protein (GFP) at N- and C- terminus respectively, by overlapping PCR using two sets of primers, as shown in **Table 2.2**. Genes encoding ankyrin binders were amplified from pHDiExDsbA-encoding ankyrin binders vector and DNA encoding GFP fragment was amplified from pTriEx-GFP (Sakkhachornphop et al., 2009) using Accuprime™ *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). PCR products encoding ankyrin binders and GFP were used as a template for the second round of PCR resulting in the recombined fragment of these two genes. The PCR products of the second round PCR were treated with *Kpn* I and *Xho* I restriction enzymes and cloned into corresponding sites of pCEP4 based vector. The sequences of these vectors were analyzed by standard sequencing method.

Table 2.2. The sequences of primers for generation of pCEP4 expressing ankyrin binders

Primers	Sequences (5'-->3')
Primers for ankyrin amplification	
Fw KpnIAnkMyr-pCep4	gag gag gag ctg gta cca tgg gga gta gca aga gca agg cgg ccg cag acc t
Fw KpnIAnk-pCep4	gag gag ctg gta cca tgg cgg ccg cag acc tgg gta ag
Rev AnkHindIII-pCep4	acc aga gaa gct ttg cag gat ttc agc cag gtc ctc gt
Primers for GFP amplification	
Fw HindIIIGFP-pCep4	ctg caa agc ttc tct ggt acg att gat gac gac ga
Rev GFPHisXhoI-pCep4	gac ctc ctc gag cta tta gtg atg gtg gtg atg gtg act agt ttt gt

2.14.2 Establishment of stable cell line expressing MA-CA binders

1 x 10^6 cells of SupT1 cell line were electroporated with pCEP4Myr-Ank-GFP and pCEP4Cyt-Ank-GFP vector using the Nucleofector™ (Amaxa, Koeln, Germany) with the Nucleofector™ transfection reagent V following protocol T-16. pCEP4 based vector contains the hygromycin resistance gene for selection of stable lines. The transfected cells were selected by limiting dilution and maintained in complete RPMI containing hygromycin B (Hyclone) ranging from 10-400 µg/ml. The transfected cells were observed the expression and the localization of protein under the fluorescence microscopy and flow cytometry.

2.14.3 Assessment of intracellular distribution of ankyrin binders by fluorescence microscopy

The SupT1 stable lines expressing GFP-fused ankyrin binders were harvested by centrifuged at 500 g at 25ºC for 5 min and washed with PBS. Cells were fixed with 4% paraformaldehyde at 25ºC for 10 min and washed two times with PBS. Fixed cells were permeabilized with 0.2% Triton X-100 at 25ºC for 12 min then washed three times. Finally, DAPI solution at dilution 1:1,000 was mixed with cells and left in the dark at least 5 min before capturing pictures by fluorescence microscopy (OLYMPUS AX70, Tokyo, Japan).

2.14.4 Flow cytometric analysis for expression of GFP-fused ankyrin binder

The stable lines were collected and centrifuged at 8,000 g for 15 seconds at 4ºC. Cell pellets were washed twice with 1% BSA-PBS-NaN_3 and resuspended with 350 µl 1% BSA-PBS-NaN_3. Percentage of GFP-positive cells was determined by flow cytometry (BD FACSort™, San Diego, CA). Non-transfected SupT1 cells were used as the negative control.

2.15 Intracellular function of ankyrin protein on viral assembly

2.15.1 Challenging Sup-T1 stable lines by HIV-1_{NL4-3} viral particles

The SupT1 stable lines (1×10^6 cells) were seeded in 6 well-plate containing 1.4 ml complete-RPMI containing Hygromycine 400 µg/ml and challenged with 1MOI of HIV-1_{NL4-3} virus at 37ºC in 5% CO_2 incubator for 16 hours. Cells were washed 3 times with incomplete-RPMI and seeded in 6-well plate containing 3 ml of complete-RPMI concluding Hygromycine 400 µg/ml and continued cultured at 37ºC in 5% CO_2 incubator. Culture supernatants were collected every 3 days.

2.15.2 Monitoring the viral production by measuring level of p24

The concentration of p24 in culture supernatant was measured using the GENETIC SYSTEM™ HIV-1 Ag EIA kit (BioRad, Hercules, CA, USA). Apply culture supernatant directly wells without prior washing of the plate, in succession covered the microplate with adhesive film and incubate the plate for 60 min at 37 ˚C

dry-heat incubator. Reaction wells were washed 4 times with washing solution provided in the kit and subsequently added working conjugate solution 1 (biotinylated sheep anti-p24 antibody, incubated plate for 30 min at 37 ˚C dry-heat incubator. Working Conjugate Solution 2 (avidin coupled to peroxidase of Raifort) was added into wells after washing step and incubated for 30 min at 37 ˚C. Substrate development was done by adding development solution (TMB solution) and allowed reaction to develop in the dark for 30 min at room temperature followed by adding stopp solution before detecting the signal at 450 nm.