CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and equipments

Chemicals and equipments used in this study are shown in Appendix A. The lists of restriction enzymes are detailed in Appendix D. The recipe for reagent preparations is shown in Appendix E.

2.2 *E. coli* strains and vectors

E. coli strain, XL-1 Blue (Stratagene, La Jolla, CA) was used as a host for molecular cloning and the production of phage-displayed scFv-MA HB-8975 whereas *E. coli* strain, HB2151 (provided by Dr. Andrew D Griffiths, MRC, Cambridge, UK) was applied as a host for production of soluble scFv anti-MA HB-8975. The expression of recombinant HIV-1 protease (HIV-PRH_6) was performed using *E. coli* strain, BL21(DE3) (Stratagene, La Jolla, CA). The pComb3X-SS phagemid, kindly provided by Dr. Carlos F Barbas, Scripps Research Institute, USA, was used for producing phage-displayed- and soluble scFv-MA HB-8975. The pBlueBac4.5 (Invitrogen, Carlsbad, CA) was used to produce baculovirus displayed scFv-MA HB-8975 and the recombinant H_6MA-CA in insect cells. The pNL4-3 (kindly provided by Prof. Pierre Boulanger, Lyon, France) was used as the template for constructing HIV-PR substrate, H_6MA-CA domain. The gene encoding HIV-PRH_6 was amplified from pET14bELP105KnewTat vector (kindly provided by Prof. Wilfred Chen, California, USA) and cloned into the pET21 vector (kindly provided by Dr. Matthew DeLisa,

Cornell University, Ithaca, NY) for HIV-PRH_6 production. Genotype of entire *E. coli* was detailed in Appendix B.

2.3 Cell culture

Hybridoma cells producing anti-MA mAb, MH-SVM33C9/ATCC HB-8975, was obtained from the American Type Culture Collection (ATCC, Manassas, VA) (Tewari et al., 1998). Hybridoma cells producing anti-CA mAb, G18, was kindly provided by Dr. Watchara Kasinrerk, Chiang Mai University, Chiang Mai, Thailand (unpublished data). Cells were cultured in Iscove's Modified Dulbecco's Media (IMDM) containing 10% fetal bovine serum, penicillin (100 Units/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37ºC.

Sf9 (the pupal ovarian tissue of *Spodoptera frugiperda*) cells (Invitrogen, Carlsbad, CA) was used for the generation of recombinant baculovirus carrying gene encoding scFv-MA HB-8975 and H_6MA-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).

2.4 Computational assisted modeling

In this study, we have collaborated with Dr. Vannajan Sanghiran Lee (Computational Simulation and Modeling Laboratory (CSML), Department of

Chemistry and Center for Innovation in Chemistry, Chiang Mai University) to study the relevant information and functional role of scFv-MA HB-8975 using computational approaches e.g. homology modeling, molecular docking and molecular dynamics (MD) simulations and binding free energy calculation.

2.4.1 Homology modeling

The primary sequence of the scFv-MA HB-8975 protein has previously been obtained by Tewari et al. (1998). The sequence of the heavy (V_H) and light (V_L) chain variable domains of scFv-MA HB-8975 were aligned and compared with the primary sequences of all immunoglobulins deposited in the Protein Data Bank using the BLAST program (Altschul et al., 1997). The Complementary Determinant Region (CDR) definition of scFv-MA HB-8975 variable domains was investigated using the Kabat method (Kabat, National Institutes of, and Columbia, 1991). Regarding the best match V_H and V_L template, the three-dimension (3D) structure of scFv-MA HB-8975 was constructed using the MODELLER program and the structural orientations were generated by superimposition strategy. Next, the 3D structure was performed based on energy minimization using AMBER03 force field (Case DA, 2006) and validated using PROCHECK. The Ramachandran plot and G-factor were used for determining the most favored regions. Finally, the proper 3D structure of scFv-MA HB-8975 was obtained.

2.4.2 Molecular docking

The 3D structure of scFv-MA HB-8975 was docked with nine peptides, an original HIV-1 epitope at the C-terminal (^{121}DTGHSSQVSQNY^{132}) on MA and eight natural mutants **(Table 2.1)**. Nine peptides were built partly based on a crystal structure from the Protein Databank. The initial structures of the nine modeled peptides had energy minimized using CHARMm force field in Discovery Studio 1.7. Structures of scFv-MA HB-8975 (a homology model) complexed with the peptides were constructed using the docking procedure in the BioMedCaChe 2.0 (Fujitsu, Inc.) program, in which the CDR loops (H1–H3 and L1–L3) were defined as the potential binding sites. Both the peptides and the binding sites were set to be flexible during the docking simulation. Each of the docking complexes were energetically evaluated based on the potential of mean force (PMF) that describes the potential energies involving bond stretching, angle bending, torsional, and non-bonded interactions such as Amber van der Waals and hydrogen bond interactions of molecules (Morris et al., 1998). The PMF scores of each peptide were evaluated by a genetic algorithm. Finally, the complex structures were analyzed and the interaction energy between the peptides and antibody was calculated.

Table 2.1 The amino acid sequences of nine synthetic C-terminal MA epitope peptides.

Peptide names	Epitope	Origin or HIV-1 isolate
p17.1	^{121}DTGHSSQVSQNY^{132}	LAI (Peden, Emerman, and Montagnier, 1991)
p17.2	^{121}DTGHS<u>N</u>QVSQNY^{132}	HXB2R(Buseyne et al., 1998)
p17.3	^{121}DTGHSSQ<u>I</u>SQN Y^{132}	1M-1005 (Sanchez-Merino, Nie, and Luzuriaga, 2005)
p17.4	^{121}DTGH<u>N</u>SQVSQNY^{132}	(Brown et al., 1997)
p17.5	^{121}<u>N</u>TGHSSQVSQNY^{132}	(Cloyd and Moore, 1990)
p17.6	^{121}DTG<u>N</u>SSQVSQNY^{132}	(Shibata et al., 1995)
p17.7	^{121}DTGHSSQ<u>A</u>SQNY^{132}	g22s2 (Saurya S, 2002)
p17.8	^{121}DTGHS<u>K</u>QVSQNY^{132}	^{121}DY^{132}4 (Nagai et al., 2005)
p17.9	^{121}DTG<u>NN</u>SQVSQNY^{132}	pNL4-3 (Adachi et al., 1986)

2.4.3 Molecular dynamics (MD) simulations and binding free energy calculation

MD simulations were carried out at the molecular mechanics level using the AMBER03 force field as implemented in the AMBER9 suite of programs (Case et al., 2005). Structures of antibody-peptide were solvated in a cubic box of TIP3P water extending at least 10 Å in each direction from the solute, and the cut-off distance was kept to 12 Å to compute the non bonded interactions. Based on the selected MD snapshots, the binding free energy for each antibody-peptide system could be

estimated using MM-PBSA (Molecular Mechanics Poisson–Boltzmann Surface Area) (Kollman et al., 2000) and MM-GBSA (Molecular Mechanics Generalized Born Solvent Area) (Chong et al., 1999). The binding free energies ($\Delta G_{binding}$) were determined from the free energies of the complex, protein and peptide according to the equation:

$$\Delta G_{binding} = \Delta G_{water}(complex) - [\Delta G_{water}(protein) + \Delta G_{water}(peptide)]$$

In addition, the scFv-MA/peptide interaction energy profiles were generated by decomposing the total binding free energies into residue-residue interaction pairs by the MM-GBSA decomposition process in the mm pbsa program of AMBER9 (Gohlke, Kiel, and Case, 2003; Hou et al., 2008).

2.5 Construction of the gene encoding scFv-MA HB-8975

2.5.1 Extraction and quantification of total RNA from hybridoma cells producing anti-MA mAb HB-8975

Hybridoma cells producing anti-MA mAb, HB-8975 (isotype IgG1) were cultured in Iscove's Modified Dulbecco's Media (IMDM) containing 10% fetal bovine serum, penicillin (100 Units/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37ºC. Total RNA was extracted from 5×10^6 anti-MA mAb HB-8975-producing hybridoma cells using an RNeasy Mini kit (Qiagen, Hilden, Germany). Initially, the hybridoma cells were lysed by adding Buffer RLT and subsequently homogenized by vortex. The supernatant containing RNA was collected

by centrifugation at 12,000 rpm for 10 minutes at 4°C. Then the supernatant was transferred to RNeasy spin column which was placed on a 2-ml collecting tube and centrifuged at 12,000 rpm for 60 seconds. The flow through was discarded and the RNeasy spin column was washed using buffer RPE and centrifugation for twice. Eventually, the RNA binding column was eluted with RNase-free water for 20 µl to obtain total RNA. The amount of the total RNA was determined by UV spectrophotometer (1 OD at 260 nm is equal to 40 µg/ml).

2.5.2 The generation of scFv-MA HB-8975 fragment

First stranded cDNA was synthesized from 1 µg of total RNA using a Transcriptor High Fidelity cDNA synthesis kit (Roche, Mannheim, Germany). Briefly, the total RNA was hybridized with Anchored-oligo$(dT)_{18}$ primer at 65ºC for 10 minutes and immediately cooled down on ice. Subsequently, the total RNA was reversed transcribed to cDNA by adding Transcriptor High Fidelity Reverse Transcriptase enzyme and other components (Transcriptor High Fidelity buffer, RNase inhibitor, dNTPs, and DTT) and incubating reaction at 55ºC for 30 minutes. The resulting cDNA was further amplified for generating the variable regions of heavy (V_H) and light chains (V_L) using specific primers **(Table 2.2).** Fw-VHP17 and Rev VHP17 were used for the V_H fragment and, Fw VLP17 and Rev VLP17 for V_L fragment. Next, the fragment encoding the scFv-MA HB-8975 was constructed by overlapping PCR using Fw-VHP17 and Rev VLP17 primer.

Table 2.2 Primers for construction of scFv-MA HB-8975 fragment

Primers	Sequences
Heavy chain primers	
FW VHP17	5´-ATAT**GCTAGC**GGCCCAGGCGGCCCAGATCCAGTTGGTGCAGT-3´
Rev VHP17	5´-CGACCCTCCACCGCCGGACCCGCCACCTCCAGACCCTCCGCCACCTGCAGAGACAGTGACCAGAGTCCC- 3´
Light chain primers	
FW VLP17	5´-GGGTCCGGCGGTGGAGGGTCGGATGTTGTGATGACCCAGACTCCA-3´
Rev VLP17	5´-ATAT**AAGCTT**TCATTAAGCGTAGTCCGGAACGTCGTACGGGTACTGGCCGCCCTGGCCTTTGATTTCCAGCTTGGTACCTCC-3´

Footnote: Restriction site for *Sfi* I is represented as underlined letters, with CGG CCC AGG CGG CC and GGC CGC CCT GGC C. Restriction site for *Nhe* I and *Hind* III are indicated in bold letters; GCTAG for *Nhe* I, and AAGCTT for *Hind* III.

2.6 Construction of phagemid vector encoding scFv-MA HB-8975

2.6.1 Construction of pComb3X-SS processing scFv-MA HB-8975 gene

The scFv-MA HB-8975 was treated with *Sfi* I restriction enzyme and cloned into *Sfi*I-treated pComb3X phagemid vector (a gift from Dr. Carlos F Barbas, Scripps Research Institute, USA) using standard ligation method. Briefly, hundred nanogram of *Sfi*I-treated pComb3X phagemid vector was mixed with 66 ng of *Sfi*I-treated scFv-MA HB-8975 and 5 units of T4 DNA ligase enzyme in total volume 20 µl. Then the reaction mixture was incubated at 4ºC for 16 hr. Following ligation process, five microlitres of ligation product was transformed into *E.coli* strain, XL-1 Blue (Stratagene, La Jolla,CA). The ligated DNA was co-incubated with 200 µl of cold-thawed $CaCl_2$ competent cells on ice for 1 hr. The mixture was transferred into cooled screw cap tube and subsequently shocked at 42°C for 1.5 minutes, then abruptly chilled on ice for 1 minute. Three milliliter Luria-Bertani (LB) broth without antibiotic was added and bacteria were further cultured with shaking at 120 rpm, 37°C for 3 hr. The transformed bacteria were centrifuged at 2,500 rpm at RT for 10 minutes and plated on LB agar containing 100 µg/ml of ampicillin. The plates were incubated at 37°C for 14-16 hr.

2.6.2 Phagemid purification by using alkaline lysis method

Ampicillin resistant colonies were picked and grown in 3 ml of LB broth containing 100 µg/ml of ampicillin with vigorous shaking (180 rpm) at 37°C for 16 hr. A half of culture volume was centrifuged at 10,000 rpm at 4°C for 5 minutes.

The supernatant was discarded and the bacterial cell pellet was lysed by adding 100 µl of 1×glucomix-lysozyme and vortexed vigorously. Two hundred microlitres of freshly prepared NaOH/SDS was added and mixed by inverting. Then, 150 µl of potassium acetate was added and gently mixed by vortexing. The solution was centrifuged at 10,000 rpm at 4°C for 5 minutes for collecting the clear supernatant. Nine hundred microlitres of absolute ethanol was added and kept on ice for 2 minutes. The DNA was spun down at 10,000 rpm at 4 °C for 10 minutes and discarded the supernatant. The DNA pellet was reconstituted by 100 µl of sterile DW and followed by adding 50 µl of 7.5 M ammonium acetate and incubated at -70°C for 10 minutes. The supernatant was collected by centrifugation at 10,000 rpm at 4 °C for 5 minutes. Three hundred microliters of absolute ethanol was added to the solution and incubated at -70°C for 10 minutes. The solution was spun down to harvest the pellet. The pellet was cleaned up with 1 ml 70% ethanol by centrifugation at 10,000 rpm at 4°C for 10 minutes. The DNA pellet was dried at 37°C for 30 minutes, reconstituted with 30 µl of sterile DW and stored at -20°C.

2.6.3 Characterization of recombinant clones

The purified phagemids were firstly checked by fractionating on 1% agarose gel electrophoresis. In order to verify the corrected *E. coli* clones, the purified phagemid from individual clone was characterized by digesting with *Sfi* I restriction enzyme and amplifying by PCR to identify the corrected band of insertion fragment. Furthermore, the sequence of scFv-MA HB-8975 was analyzed by standard

sequencing methods (1st Base, Singapore). Finally, the constructed phagemid namely pComb3X-scFv-MA was obtained.

2.7 Production of phage-displayed scFv-MA HB-8975

2.7.1 Phage production

The phagemid vector, pComb3X-scFv-MA, was transformed into the amber-suppressing (supE) *E. coli* strain, XL-1 Blue and selected on LB agar plate containing ampicillin. The transformed bacteria were grown in 10 ml of 2×TY broth containing ampicillin (100 μg/ml) at 37°C with shaking at 200 rpm. The precultured bacteria were subsequently transferred to the same medium containing 1% (w/v) glucose, 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) and cultivated at 25°C until the optical density at 600 nm (OD_{600}) reach 0.8. After induction, the bacterial culture was further infected with 10^{12} colony forming units per volume (CFU/ml) of VCSM13 helper phages and left at 37°C for 30 minutes without shaking. Phage infected XL-1 Blue was spun down at 3,000 rpm for 10 minutes at 4°C. The pellet was resuspended in 30 ml 2×TY broth containing ampicillin (100 μg/ml) and kanamycin (70 μg/ml) and then transferred to 220 ml of the same broth and shaken at 180 rpm at 25°C for 16 hr.

2.7.2 Harvesting phage by polyethylene glycol (PEG) precipitation

Bacteriophage harboring scFv-MA HB-8975 *via* gpIII was pelleted at 3,000 rpm, 4°C for 30 minutes. The culture supernatant was further collected. The recombinant phages were harvested by 4% w/v of PEG 8,000 and 3% w/v of NaCl

precipitation with shaking at 180 rpm at RT for 15 minutes or until PEG/NaCl completely dissolved. The supernatant was kept on ice for 1 hr and centrifuged 10,800 rpm at 4°C for 30 minutes. The pellet was air dried for 30 minutes and reconstituted with 2.5 ml of PBS pH 7.2. The suspension was subsequently centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was preserved in 10% glycerol and stored at -70°C.

2.7.3 Phage-displayed scFv-MA HB-8975 titration by *E. coli* infection

A single colony of *E. coli* strain, XL-1 Blue was inoculated into 10 ml 2XTY broth and grown at 37°C until the OD_{600} reaches 0.6. Ten microlitres of phage-displayed scFv-MA HB-8975 was added into 990 µl of cultured bacteria (dilution $1:10^2$) and then 10 µl of mixture was added into 990 µl of 2XTY broth (dilution $1:10^4$) and incubated for 15 min. The infected *E. coli* strain, XL-1 Blue was further diluted into 10^8 and 10^{10} 2XTY broth. Fifty microlitres of each dilution was plated onto LB agar containing 100 µg/ml ampicillin and incubated at 37°C overnight. The ampicillin resistant colonies were counted and calculated for the phage concentration using the formula at below.

$$A = B \times C \times (1000/V)$$

A = The original amount of phage obtained from preparation process (CFU/ml)

B = The number of ampicillin resistant colonies

C = Titer of viral infected bacteria

V = Volume (µl) of viral infected bacteria

In addition, *E. coli* strain, XL-1 Blue harboring pCom3X-scFv-MA M100G and M100E which used for phage-displayed scFv-MA M100G and M100E *via* gpIII preparation was performed with the same method as described in section 2.7.1- 2.7.3.

2.7.4 Determining of phage expressing scFv-MA HB-8975 by SDS-PAGE and Western immunobolting

10^{13} CFU/ml of recombinant phage-displayed scFv-MA HB-8975 were separated by SDS-PAGE under reducing conditions on a 12% polyacrylamide gel. The sample were boiled for 5 minutes and loaded onto the well of SDS-PAGE. Electrophoresis was carried out by applying constant voltage at 100 Volts using 0.025 M Tris/glycine, pH 8.5 containing 0.1% SDS as an electrophoresis buffer. After electrophoresis, the separated proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane. Blocking was performed for 1 hr at RT with 5% skimmed milk in PBS pH 7.2 and further incubated with mouse anti-gpIII mAb (Exalpha Biologicals, Water town, MD) (dilution 1:2,500) for 1 hr. The membrane was washed five times with 0.05% Tween 20 in PBS pH 7.2 and then incubated with HRP-conjugated goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD) diluted 1:3,000 in 5% skimmed milk in PBS pH 7.2 for 1 hr. Unbound conjugate was washed out five times with 0.05% Tween 20 in PBS pH 7.2; the specific bands were visualized using a SuperSignal West Pico Substrate (Pierce, Rockford, USA).

2.8 Preparation of soluble scFv-MA HB-8975 protein

2.8.1 Production of soluble scFv-MA HB-8975 protein

The phagemid, pComb3X-scFv-MA was transformed into the competent non-suppressor *E. coli* strain, HB2151. The bacterial cells harboring the vector were grown in 10 ml of terrific broth (1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% [w/v] glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) containing ampicillin (100 µg/ml) at 37ºC for 18 hr with shaking. One hundred microlitres of precultured bacteria were inoculated in 100 ml of the same medium containing 1% (w/v) glucose and ampicillin (100µg/ml), with shaking continued at 37ºC until an OD_{600} was reached to 1.5. To induce the protein expression, IPTG was added to the culture at a final concentration of 1 mM. After induction, the bacteria were grown at 25ºC for 20 hr. The culture supernatant containing extracellular soluble scFv anti-MA HB-8975 was collected by centrifugation at 5,000 rpm for 30 minutes at 4ºC. Proteins were precipitated with saturated $(NH_4)_2SO_4$ in an ice bath and concentrated with Amicon Ultra centrifugal filter units (Millipore, Cork, Ireland). Finally, the concentrated protein was reconstituted with 1.5 ml of 0.15 M PBS, pH7.2.

2.8.2 Western blotting

The concentrated soluble scFv-MA HB-8975 protein was separated in 12% SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The blotted membrane was blocked with 5% skimmed-milk in PBS for 1 hr at RT with shaking and then treated with anti-HA tag

mAb (Sigma-Aldrich, St. Louis, MO). After incubation, the membrane was washed 5 times with washing buffer (0.05% Tween-20 in PBS) and HRP-conjugated goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD) were added to the membranes. The peroxidase reaction was visualized using a SuperSignal West Pico Substrate (Pierce, Rockford, USA).

2.9 Evaluation of the binding activity of phage-displayed scFv-MA HB-8975 and soluble scFv-MA HB-8975 with HIV-1 MA epitope variants by ELISA

2.9.1 Indirect ELISA

Nine HIV-1 MA epitope peptides were synthesized (GenScript, Piscataway, New Jersey, USA) and tested with phage-displayed scFv-MA HB-8975 and soluble scFv-MA HB-8975 protein using a standard ELISA procedure. Peptide p17.1 represented the wild-type epitope peptide while other peptides represented the mutant peptides. The amino acid sequences of all synthetic peptides are shown in **Table 2.1.** Briefly, one hundred microlitres of 50 µg/ml of each peptide in coating buffer (0.1 M $NaHCO_3$, pH 8.6) was added to microtiter plates (NUNC, Roskilde, Denmark) and incubated overnight at 4ºC. The coated wells were then blocked with 200 µl of blocking buffer (2% BSA in TBS) for 1 hr at RT. The wells were washed five times with washing buffer (0.05% Tween-20 in TBS). One hundred microlitres of phage-displayed scFv-MA HB-8975 or 200 µg/ml of soluble scFv anti-MA HB-8975 protein in blocking buffer were added to each well and incubated for 1 hr at RT. After incubation, the excess antibody was eliminated by washing. Subsequently, the wells

were incubated for 1 hr at RT with 100 µl of HRP-conjugated anti-M13 phage mAb (Amersham Pharmacia Biotech, Buckinghamshire, UK) or HRP-conjugated mouse anti-HA tag mAb (Roche, Indiapolis, IN) diluted 1:3,000 in blocking solution, respectively. Wells were then washed again prior to adding 100 µl of 3,3´,5,5´-tetramethyl-benzidine (TMB) substrate. The optical densities OD_{450} was measured by an ELISA plate reader (TECAN, Austria) after adding 100 µl of 1 N HCl.

2.9.2 Competitive ELISA

A peptide competitive ELISA was performed following the same procedure as described for the indirect ELISA; the phage-displayed scFv-MA HB-8975 or soluble scFv-MA HB-8975 protein were mixed with 100 ng/ml of each variant peptide and incubated for 1 hr at RT. One hundred microlitres of the mixture were applied into individual peptide p17.1 pre-coated wells and incubated for 1 hr at RT. After washing the wells, the bound phage-displayed scFv-MA HB-8975 or soluble scFv-MA HB-8975 protein were monitored by adding 100 µl of HRP-conjugated anti-M13 phage mAb (Amersham Pharmacia Biotech, Buckinghamshire, UK) or HRP-conjugated mouse anti-HA tag mAb (Roche, Indiapolis, IN) diluted 1:3,000 in blocking solution, respectively. Wells were then washed again and 100 µl of TMB substrate was subsequently added. The enzymatic reaction was stopped by adding 100 µl of 1 N HCl. The OD_{450} was measured by an ELISA plate reader. The OD values were converted to percentage inhibition values (PI) by using the following formula:

$$PI = 100 - ((B/B_0) \times 100)$$

B and B_0 are the OD values of scFv anti-MA with peptide inhibitor and without peptide inhibitor respectively.

2.10 Construction and evaluation of scFv-MA HB-8975 mutants

2.10.1 Site specific mutagenesis of scFv-MA HB-8975

Amino acid residue 100, methionine (M), of scFv-MA HB-8975 was mutated to glycine (G) and glutamate (E) using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the primers; M100G Fw and M100G Rev for generating scFv-MA M100G, and M100E Fw and M100E Rev for constructing scFv-MA M100E **(Table 2.3).** The pComb3X-scFv-MA was hybridized with each pair of primer and the plasmid was replicated by PfuTurbo DNA polymerase. Subsequently, the parental template was treated with *Dpn* I endonuclease before performing transformation into *E. coli* strain, XL-1 Blue. The transformed colony of scFv mutants were analyzed by standard sequencing method. Finally, the plasmid pComb3X-scFv-MA M100G and pComb3X-scFv-MA M100E were obtained.

Table 2.3 Primers for generation of scFv-MA mutants

Primers	Sequences
scFv-MA M100G	
scFv-MA M100G Fw	5´-ACATATTTCTGTTCAAGATCTGGCAAAGGTCT TACTGGGGCCAA-3´
scFv-MA M100G Rev	5´-TTGGCCCCAGTAAGAACCTTTGCCAGATCT TGAACAGAAATATGT-3´
scFv-MA M100E	
scFv-MA M100E Fw	5´-TTTCTGTTCAAGATCTGAAAAAGGTTCTTA CTGGG- 3´
scFv-MA M100E Rev	5´-CCCSGTAAGAACCTTTTTCAGATCTTGAACA GAAA-3´

Footnote: The mutagenesis residue was indicated in underlined letters; GGC and GCC for scFv-MA M100G, and GAA and CTT for scFv-MA M100E

2.10.2 Production of phage-displayed scFv-MA mutants and soluble scFv-MA mutants

The phage-displayed scFv-MA mutants (scFv-MA M100G, and scFv-MA M100E) were produced as describe in previous step (2.7) and the soluble scFv-MA mutant proteins were expressed as describe in previous step (2.8).

2.10.3 Evaluation of the binding activity of phage-displayed scFv-MA mutants and soluble scFv-MA mutant proteins by ELISA

The binding interaction of phage-displayed scFv-MA mutants and soluble scFv-MA mutant proteins with MA epitope peptides were determined by indirect and competitive ELISA as describe in previous step (2.9)

2.11 Engineering of gene encoding scFv-MA HB-8975 in baculovirus expression system.

2.11.1 Construction of two versions of scFv-MA HB-8975

The DNA fragment encoding scFv-MA HB-8975, obtained as described in 2.5, was cut with the *Nhe* I and *Hind* III restriction enzymes and cloned into *Nhe* I and *Hind* III treated pBlueBac4.5 plasmid to generate pBlueBac4.5-scFv-MA plasmid. Subsequently, the 5´ and 3´ ends of scFv-MA HB-8975 fragment in the pBlueBac-scFv-MA vector were modified by oligonucleotide insertion at both *Nhe* I and *Hind* III sites, to obtain two versions of the scFv-MA HB-8975 **(Table 2.4).** One encoded the dipeptide Met-Glu using oligonucleotide E2Fw and oligonucleotide E2Rev, generating the scFvE2/MA clone, the other encoded the dipeptide Met-Gly at the N-terminus of scFv-MA using oligonucleotide G2Fw and oligonucleotide G2Rev, generating the scFvG2/MA clone. The oligonucleotides for each version were hybridized and inserted into *Nhe* I treated-pBluebac-scFv-MA vector to obtain pBluebac-scFvE2/MA and pBluebac-scFvG2/MA plasmid. At the 3´ end of both clones, an oligonucleotide encoding the Influenza A virus hemagglutinin epitope

YPYDVPDYA (HA tag) was inserted. The vectors were separately transformed into *E. coli* strain, XL-1 Blue and selected on LB agar containing 100 µg/ml of ampicillin. The pBlueBac-scFvE2/MA and pBlueBac-scFvG2/MA plasmid were extracted and analyzed by standard sequencing method.

Table 2.4. Oligonucleotides for construction of two versions of scFv-MA HB-8975 fragment in baculovirus expression system

Primers	Sequences
scFvE2/MA	
oligonucleotide E2Fw	5´-CTAGAGCCATGGAAGCTTCG-3´
oligonucleotide E2Rev	5´-CGAAGCTTCCATGGCTCTAG-3´
scFvG2/MA	
oligonucleotide G2Fw	5´-GCTAGCGAAGCTTCCATGGGA-3´
oligonucleotide G2Rev	5´-TCCCATGGAAGCTTCGCTAGC-3´

2.11.2 Production of recombinant scFvE2/MA and scFvG2/MA in Sf9 cells

Sf9 cells were cotransfected with pBlueBac-scFvE2/MA or pBluebac-scFvG2/MA (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 2.1)** containing genes encoding for proteins for viral replication,

scFvE2/MA or scFvG2/MA, and LacZ for blue plaque selection as shown in **Figure 2.2**. The recombinant virus (BV-scFvE2/MA or BV-scFvG2/MA) 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 at 4ºC for 30 minutes. The presence of scFvE2/MA and scFvG2/MA 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-HA tag mAb (Sigma, St Louis, MO, USA) diluted 1:3,000 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 HRP conjugated goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD) at dilution 1:3,000 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).

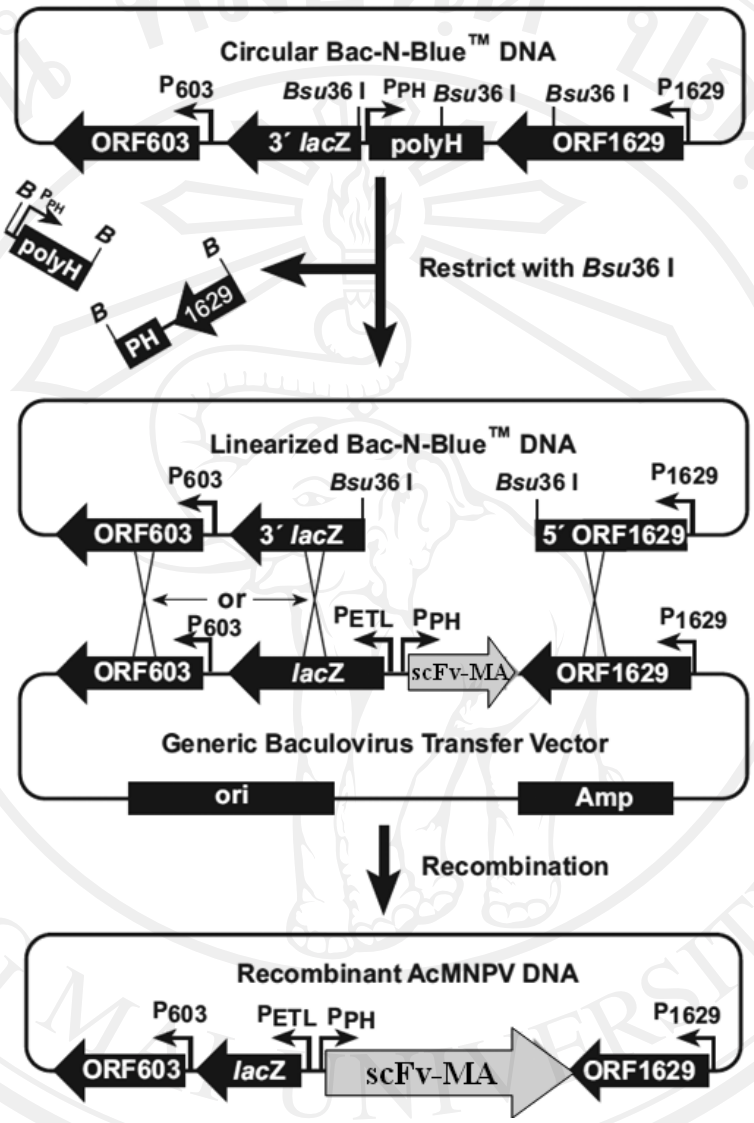

Figure 2.1 Recombination between Bac-N-Blue™ DNA and pBlueBac-scFv-MA transfer vector (modified from the instruction of Bac-N-Blue™ transfection kit, Invitrogen).

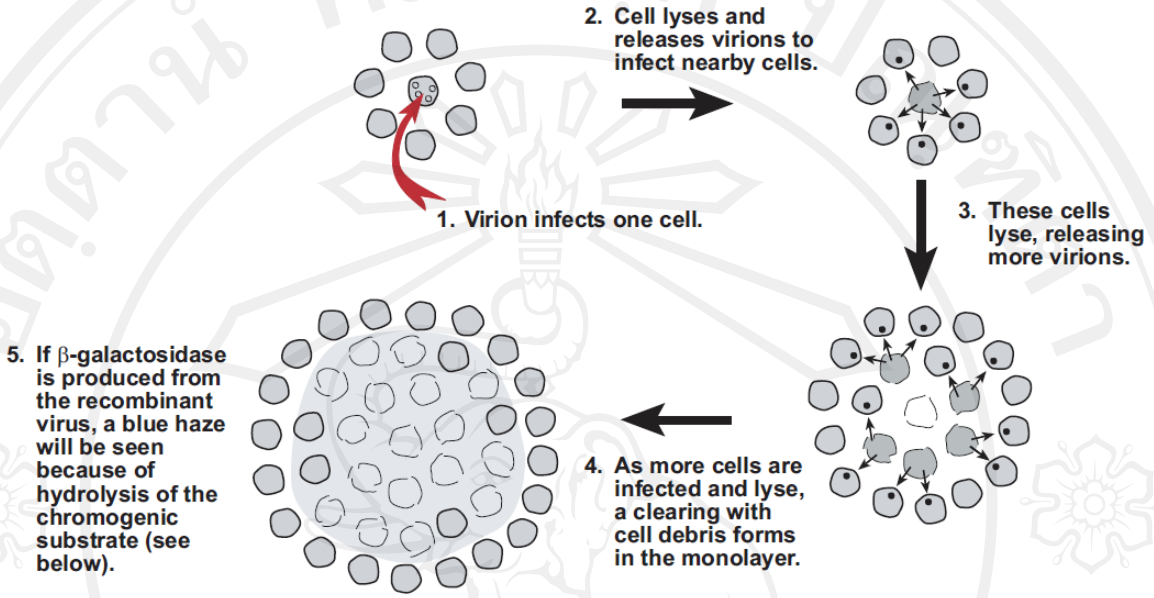

Figure 2.2 Formation of blue plaques. The infected cells are lysed and released virions to infect nearby cells. The plaque with cell debris as observed a clearing area indicated more cells are infected and lysed. In the presence of X-gal, a blue haze was seen because of hydrolysis of the substrate (modified from the instruction of Bac-N-Blue™ transfection kit, Invitrogen).

2.11.3 Purification of scFv-MA HB-8975 protein from infected Sf9 cells

The scFvE2/MA protein was purified by affinity selection on anti HA tag antibody Affimatrix gel (Roche Applied Science, IN, USA), following the manufacturer's instructions. Each fraction eluted from the affinity gel, using elution buffer containing HA peptide (Roche Applied Science) as binding competitor to displace the bound proteins, was analyzed by SDS-PAGE and transferred to a

nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The blotted membrane was blocked with 5% skimmed-milk in PBS for 1 hr at RT with shaking and then treated with anti-HA tag mAb (Sigma–Aldrich, St. Louis, MO). After incubation, the membrane was washed 5 times with washing buffer (0.05% Tween-20 in PBS) and HRP-conjugated goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD) were added to the membranes. Unbound conjugate was washed five times. The scFvE2/MA protein was visualized using TMB membrane peroxidase substrate (KPL, Gaithersburg, MD).

2.12 Localization of scFv-MA on infected Sf9 cells

2.12.1 Cell fractionation

The Sf9 cells were infected with BV-scFvE2/MA and BV-scFvG2/MA. After 48 and 72 hr post infection, BV-infected Sf9 cells were harvested and cellular fractionation was performed using the FractionPREP™ Cell Fractionation System (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). Four subcellular protein fractions were thus obtained, cytosol, nucleus, membrane/particulate and cytoskeletal fractions. The proteins were separated in 12% SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The blotted membrane was blocked with 5% skimmed-milk in PBS for 1 hr at RT with shaking and then treated with anti-HA tag mAb (Sigma–Aldrich, St. Louis, MO). After incubation, the membrane was washed 5 times with washing buffer (0.05% Tween-20 in PBS) and HRP-conjugated goat anti-mouse

immunoglobulins (KPL, Gaithersburg, MD) were added to the membranes. Unbound conjugate was washed five times. The specific bands were visualized using TMB membrane peroxidase substrate (KPL, Gaithersburg, MD).

2.12.2 Flow cytometry

The Sf9 cells were infected with BV-scFvE2/MA and BV-scFvG2/MA. The infected Sf9 cells were resuspended in 200 µl PBS and incubated with monoclonal anti-HA tag mAb (Sigma–Aldrich, St. Louis, MO) for 1 hr at RT diluted 1:3,000 in PBS. After washing, the cell pellet was resuspended in 200 µl PBS and reacted with Alexa Fluor® 488-labeled goat anti-mouse immunoglobulins (Molecular Probes, Invitrogen). The cell suspension was then diluted with 10 volumes of PBS, and analyzed by flow cytometry using a BD FACS Canto™ II cytometer (Becton Dickinson Biosciences). At least 10,000 events were acquired for each experiment using the DIVA 6 software (Becton Dickinson).

2.13 Baculovirus-displayed scFv-MA particle

2.13.1 Isolation of BV

Concentrated stocks of recombinant BV expressing scFv-MA molecules, BV-scFvE2/MA and BV-scFvG2/MA, respectively, were prepared (Granio et al., 2009). Infected Sf9 cell culture supernatants were harvested at 50 to 60 hr post infection (pi), and clarified by centrifugation at 2,400 rpm and 4°C for 10 minutes. Aliquots (11-ml) of clarified culture supernatant were subjected to ultracentrifugation at 28,000 rpm for

1 hr at 4°C through a 1-ml sucrose cushion (20% sucrose, w/v in PBS) in Beckman SW41 rotor. Each baculoviral pellet was resuspended by gentle shaking in sterile phosphate-buffered saline (PBS) overnight at 4°C (100 µl per centrifuge tube). The titers of BV suspensions ranged usually between 5×10^9 and 1×10^{10} pfu/ml, as determined by plaque titration in Sf9 cells (pfu/ml), which corresponded to 1×10^{12} to 5×10^{12} BV physical particles per ml (Granio et al., 2009; Slack and Arif, 2006).

2.13.2 Western blotting of BV particles

BV particles were further purified by isopycnic ultracentrifugation in linear sucrose-D2O gradient (Bardy et al., 2001). Gradients (10-ml total volume, 30-50%, w/v) were generated from a 50% sucrose solution made in D2O buffered to pH 7.2 with NaOH, and a 30% sucrose solution made in 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5.7 mM Na2EDTA. The gradients were centrifuged for 18 hr at 28,000 rpm in a Beckman SW41 rotor. 0.5 ml-fractions were collected from the top. The proteins from each fraction were separated in 12% SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The blotted membrane was blocked with 5% skimmed-milk in PBS for 1 hr at RT with shaking and then treated with anti-HA tag mAb (Sigma–Aldrich, St. Louis, MO) and anti-baculoviral envelope glycoprotein GP64. After incubation, the membrane was washed 5 times with washing buffer (0.05% Tween-20 in PBS) and HRP-conjugated goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD) were added to the membranes. Unbound conjugate was washed five times. The specific bands were visualized using TMB membrane peroxidase substrate (KPL, Gaithersburg, MD). BV

particles were recovered in fractions with an apparent density ranging from 1.08 to 1.15.

2.13.3 Electron microscopy

The BV particles were processed for EM and observed as previously described (Granio et al., 2009). In brief, pelleted virions of BV were resuspended in 20 μlaliquots of 0.14 M NaCl, 0.05 M Tris-HCl buffer, pH 8.2, and adsorbed onto carbon-coated formvar membrane on nickel grids. The grids were incubated with anti-HA tag mAb (Sigma–Aldrich, St. Louis, MO) at a dilution of 1: 50 in TBS for 1 hr at RT. After rinsing with TBS, the grids were post-incubated with 20-nm colloidal gold-tagged goat anti-mouse immunoglobulins (British Biocell International Ltd, Cardiff, UK; diluted to 1: 50 in TBS) for 30 minutes at RT. After rinsing with TBS, the specimens were negatively stained with 1% uranyl acetate in H20 for 1 minute at RT, rinsed again with TBS, and examined under a JEM 1400 Jeol electron microscope equiped with an Orius-Gatan digitalized camera (Gatan France, 78113 Grandchamp).

2.14 Evaluation of purified- and baculovirus-displayed scFvE2/MA and scFvG2/MA by ELISA

2.14.1 Indirect ELISA

The functionality of recombinant scFvE2/MA was evaluated by their binding activity to the synthetic MA epitope peptide, ^{121}DTGHSSQVSQNY^{132} (GenScript; Piscataway, USA) in standard indirect ELISA procedure. In brief, hundred microlitres of 50 μg/ml of MA epitope peptide in coating buffer (0.1 M $NaHCO_3$, pH 9.6) were

incubated overnight at 4°C in 96-well microtiter plates (NUNC, Roskilde, Denmark). The coated wells were blocked with 200 µl of blocking buffer (2% bovine serum albumin in TBS; TBS-BSA) for 1 hr at RT, then washed five times with washing buffer (0.05% Tween-20 in TBS; TBS-T). Hundred microlitres of purified scFv proteins or BV particles were added to each well and incubation proceeded for 1 hr at RT. After five cycles of washing with TBS-T, anti-HA tag mAb (Sigma–Aldrich, St. Louis, MO) and anti-baculoviral envelope glycoprotein GP64 diluted 1:3,000 in blocking solution were added, respectively. After incubation, wells were washed 5 times with washing buffee TBS-T and HRP-conjugated goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD) were added. Wells were then washed again prior to adding 100 µl of 3,3´,5,5´- tetramethyl-benzidine (TMB) substrate. The optical densities (OD) at 450 nm will be measured by an ELISA plate reader (TECAN, Austria) after adding 100 µl of 1 N HCl.

2.14.2 Competitive ELISA

Microtiter plates (NUNC) were coated with 50 µl of 10 µg/ml of avidin in coating buffer (0.1 M NaHCO3, pH 6.8) and incubated overnight at 4°C in moist chamber. The coated wells were then blocked with 200 µl of blocking buffer (2% BSA in TBS) for 1 hr at RT, then washed four times with washing buffer (0.05% Tween-20 in TBS). Fifty microlitres of 50 µg/ml of biotinylated MA epitope peptides in blocking buffer were added to each well and incubated for 1 hr at RT. In parallel, purified scFvE2/MA was mixed with MA epitope peptides **(Table 2.5)** at the final peptide concentration of 1 µg/ml, and incubated for 1 hr at RT. After washing the

wells, the mixture was added to the wells and incubated for 1 hr at RT. Bound scFvE2/MA was monitored by adding 50 µl of HRP-conjugated monoclonal anti-HA tag mAb (Sigma) at dilution 1:1,000 in blocking buffer. The wells were washed four times prior to the addition of 50 µl of 3,3',5,5'-tetramethyl- benzidine (TMB) substrate. Reaction was stopped by addition of 50 µl of 1 N HCl, and OD_{450} were measured using an ELISA plate reader. The percentage of inhibition (PI) was given by the following formula: PI = 100-[(B:Bo) × 100], where B and Bo were the OD values for scFvE2/MA with and without inhibitor, respectively.

Table 2.5 The HIV-1 MA epitope peptides of natural varaints.

Peptide names	**Epitope**	**Origin or HIV-1 isolate**
p17.1	^{121}DTGHSSQVSQNY^{132}	LAI (Peden, Emerman, and Montagnier, 1991)
p17.3	^{121}DTGHSSQ<u>I</u>SQNY^{132}	1M-1005 (Sanchez-Merino, Nie, and Luzuriaga, 2005)
p17.7	^{121}DTGHSSQ<u>A</u>SQNY^{132}	g22s2 (Saurya S, 2002)
p17.8	^{121}DTGHS<u>K</u>QVSQNY^{132}	4 (Nagai et al., 2005)
p17.9	^{121}DTG<u>NN</u>SQVSQNY^{132}	pNL4-3 (Adachi et al., 1986)
Inverted p17.1	^{121}YNQSVQSSHGTD^{132}	-

2.15 Production and evaluation of baculovirus-displayed scFv-M61B9

2.15.1 Vector construction and baculovirus display production

The construction and characterization of scFv-M61B9 directed against CD147 have been described in previous study (Intasai et al., 2009; Tragoolpua et al., 2008). The DNA fragment for the HA-tagged scFv-M61B9 was amplified using plasmid pComb3X-scFv-M61B9 as the template, with the M6-1B9 Fw primer (5´-GAGGAGGAGCTGGCCCAGGCGGCCCAGATCCAGTTGGTGCAGTCTGGAG AGCTAGTGATGACCCAGACTCCAGC-3´) encoding the N-terminal 18 amino acids of scFvE2/MA (^{1}MEASLAAQAAQIQLV QSG^{18}), and the M61B9 Rev primer (5´-CTCCTCCTCGGCCGCCCTGGCCACTA GTGACAGATGGGGCTG-3´). The scFv-M61B9 was then cloned into the *Sfi* I site of *Sfi* I-restricted pBlueBac-scFvE2/MA. The recombinant BV was isolated as described above, and abbreviated BV-N18E2/M61B9.

2.15.2 Indirect ELISA

The antigen-binding activity of chimeric scFvE2/M61B9 was assessed in ELISA using immobilized recombinant CD147-biotin carboxyl carrier protein (BCCP) fusion protein as the antigen, produced as previously described.

2.16 Construction and expression of HIV-PRH_6

2.16.1 Vector construction

The HIV-PR gene was amplified from the pET14bELP105Knew/Tat vector (kindly provided by Prof. Wilfred Chen, University of California, Riverside, CA) with the primers HIV-PR *Nhe*I For (5´-GACGACGCTAGCATGCCTCAGATCACTCTT TGG-3´) and HIV-PR H6 *Hind*III Rev (5´-GTCGTCAAGCTTTTAATGG TGATGGTGATGGTGTGCGCCAAAATTTAAAGTGAGCCAAT-3´). The reverse primer was designed to add a C-terminal six histidine (His_6) tag for detection purposes. PCR was performed using Accuprime Pfx DNA polymerase (Invitrogen, Carlsbad, CA), and the products were purified using a GeneJet PCR Purification Kit (Fermentas, St. Leon-Rot, Germany). The purified PCR product and the pET21a vector (kindly provided by Dr. Matthew DeLisa, Cornell University, Ithaca, NY) were digested with *Nhe*I and *Hind*III at 37°C for 16 hr. The digested fragment and vector were ligated using T4 DNA ligase at 4°C overnight. The ligated product (pET21-HIV-PRH_6) was purified and transformed into chemically competent XL1-Blue cells. For the construction of the previously described mutant HIV-PR I54V (Jiménez et al., 2005; Šašková et al., 2008), amino acid residue 54 of PR in pET21-HIV-PRH_6 was mutated using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the primers PR-I54Vfw (5´-GAATTGGAGGTTTTGTG AAAGTAAGACAGTATG-3´) and PR-I54Vrev (5´-CATACTGTCTTACTTTCACA AAACCTCCAATTC-3´). Transformation of the plasmid was carried out as described

above. The clones containing HIV-PRH_6 or the variant were analyzed using standard sequencing methods.

2.16.2 Expression of HIV-PRH_6 in *E. coli*

The pET21-HIV-PRH_6 or pET21-HIV-PRH_6 I54V was transformed into the BL21(DE3) expression strain. Pre-cultured cells (100 µl) were transferred into 100 ml of terrific broth (1.2% [w/v] tryptone, 2.4% [w/v] yeast extract, 0.4% [w/v] glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) containing ampicillin (100 µg/ml), and the culture was incubated at 37°C with shaking until OD_{600} reached to 0.8. To induce protein expression, IPTG was added to the culture at a final concentration of 0.1 mM, and the culture was shaken continuously at 16°C or 30°C for 18 hr. After induction, the cells were harvested via centrifugation at 5,000 rpm at 4°C for 30 minutes, and the pellets were washed with TBS (pH 7.4) and centrifuged. The resulting pellets were resuspended in TBS to OD_{600} = 75 and lysed via ultrasonication. The cell lysate was clarified via centrifugation at 10,000 rpm at 4°C for 30 minutes, and the soluble fraction was collected. The protein concentration was quantified using a BCA Protein Assay (Pierce, Rockford, USA). The presence of soluble HIV-PRH_6 or the mutant was further analyzed by western blotting. The same total amount of proteins were separated in 15% gels using SDS-PAGE under denaturing conditions and transferred to a polyvinylidene fluoride (PVDF) membrane (Pierce). The membrane was blocked with 5% skim milk in TBS (blocking solution) for 1 hr and then incubated with a monoclonal anti-His_6 tag antibody (1:3,000 dilution in blocking solution; GenScript, Piscataway, NJ, USA) at 4°C overnight with shaking. After incubation, the excess

antibody was removed using washing buffer (0.05% Tween 20 in TBS), and the blots were incubated with an HRP-conjugated goat anti-mouse immunoglobulin antibody (1:5,000 dilution in blocking buffer; KPL, Gaithersburg, MD) at RT for 1 hr with shaking. After washing, the bands were visualized using TMB Membrane Peroxidase Substrate (KPL).

2.17 Production of H_6MA-CA substrate by baculovirus expression system

2.17.1 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-CAencoding 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.

2.17.2 Preparation of recombinant H_6MA-CA protein in Sf9 cells

The production of the H_6MA-CA substrate has been described previously (Kitidee et al.). Briefly, Sf9 cells were infected with a baculovirus expressing recombinant H_6MA-CA (BV-H_6MA-CA) for 48 hr. After infection, H_6MA-CA protein was recovered from a clarified Sf9 cell lysate by affinity chromatography on a HisTrap column using the ÄKTAprime plus system (GE Healthcare Bio-Sciences, Piscataway, NJ). The concentration of the purified H_6MA-CA was determined using a Bradford protein assay (Thermo Fisher Scientific, Rockford, IL). The purified H_6MA-CA protein was separated in 15% gels using SDS-PAGE under reducing conditions, and the gels were stained using Coomassie blue to verify the purity of the preparation.

2.18 Production of monoclonal antibodies

2.18.1 Mouse immunization

To generate a monoclonal antibody against CA, two female six-week-old Balb/c mice were intraperitoneally immunized with 100 μg purified H_6MA-CA in complete Freund's adjuvant. The immunizations were repeated every two weeks with the same dose in incomplete Freund's adjuvant. Blood samples were collected via tail-bleeding prior to immunization and after the third immunization. Serum was isolated from the collected blood and screened for an antibody response using an indirect ELISA.

2.18.2 Hybridoma technique

One immunized mouse that appeared to have a high antibody titre was selected and used for hybridoma generation. The selected mouse was intraperitoneally boosted with 100 μg of purified H_6MA-CA. Five days after boosting, the mouse was euthanized, and splenocytes were fused with myeloma cells using 50% polyethylene glycol and standard hybridoma-generation techniques. Supernatants were collected from the plated hybridoma cells and screened for antigen recognition via ELISA. Hybridoma cells from the antigen-specific wells were selected for limiting dilution, single-cell cloning. Cell culture supernatants were collected and examined for their antigen specificity using indirect ELISAs and Western immunoblotting.

For the anti-MA antibody, the hybridoma clone, MH-SVM33C9/ATCC HB-8975, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). This antibody is known to bind to an epitope (DTGHSSQVSQNY) located in the C terminus of the Gag polyprotein (Tewari et al., 1998). The hybridoma cells were expanded in Iscove's Modified Dulbecco's Media (IMDM) containing 10% FCS, and the culture supernatant was collected to determine the binding activity using indirect ELISA and Western immunoblotting.

2.19 Purification of monoclonal antibodies

Hybridoma clones were grown in serum-free hybridoma medium (PFHM-II: Gibco BRL, Gaithersburg, MD) at a concentration of 1×10^6 cells/ml for 7 days. The culture supernatant was collected via centrifugation (1,200 × *g* at 4° C for 30 min) and filtered through a 0.2 µm filter. Antibodies were purified from the clarified supernatant using Protein G affinity chromatography (HiTrap protein G HP column; GE Healthcare Bio-Sciences). The concentration of the purified monoclonal antibodies was quantified by measuring the absorbance at 280 nm.

2.20 Characterization of monoclonal antibodies by Indirect ELISA and Western blotting

2.20.1 Indirect ELISA

An indirect ELISA was used to detect the presence of polyclonal antibodies in the serum and for characterization of the monoclonal antibodies. H_6MA-CA (10 µg/ml diluted in coating buffer; 50 µl/well) was coated on a microtiter plate, which was incubated at 4°C overnight in a humidified chamber. The wells were washed three times with washing buffer (0.05% Tween 20 in PBS) and then blocked with 2% BSA in PBS (200 µl/well) at RT for 1 hr. The diluted serum or culture supernatant from each hybridoma clone (50 µl) was added to the wells. After 1 hr incubation, the wells were washed three times with washing buffer, and an HRP-conjugated goat anti-mouse immunoglobulin antibody (diluted 1:3,000 in blocking buffer) was added.

The wells were washed, and 100 µl of TMB substrate was added. The reaction was stopped via the addition of 1 N HCl, and OD_{450} using an MTP-120 microplate reader (Biodirect, MA, USA).

2.20.2 Western blotting

Western blots were used to evaluate the recognition of recombinant H_6MA-CA by the monoclonal antibodies. Purified H_6MA-CA (50 µg/ml) was incubated with 1.5 mg/ml of the soluble fraction of HIV-PRH_6 in 0.1% BSA in TBS at 37°C for 18 hr. The digested proteins were separated in 15% gels using SDS-PAGE under denaturing conditions and transferred to PVDF membranes. The membranes were blocked with 5% BSA in TBS and cut into small strips (5 mm). The monoclonal antibody–containing culture supernatants were incubated with the strips at RT for 1 hr with shaking. After washing, the strips were incubated with an HRP-conjugated goat anti-mouse immunoglobulin antibody (diluted 1:3,000 in blocking buffer) for 1 hr at RT with shaking. The membranes were washed, and bands were visualized using TMB Membrane Peroxidase Substrate (KPL).

2.21 Development of the ELIS-based HIV-PR activity assay (ELIB-PA)

2.21.1 HIV-PR activity assay by ELIB-PA

Microtiter plates (Greiner Bio-one, Frickenhausen, Germany) were pre-treated according to a previously described procedure (Cressey et al., 2008; Paborsky et al., 1996). Briefly, 100 µl of 10 mM BCML (N, N-bis(carboxymethyl) lysine hydrate) diluted in 0.1 M $NaPO_4$ was added to the plate, which was then incubated at 4 °C overnight. The wells were washed three times with 200 µl washing buffer (0.05% Tween 20 in water) at 25 °C for 5 minutes with shaking and then blocked with 200 µl of 0.05% BSA in TBS containing 0.05% Tween 20. After 1 he of blocking, the wells were washed with the following buffer series: 50 mM Tris-HCl (pH 7.5) containing 500 mM imidazole and 0.05% Tween 20, washing buffer, and 100 mM EDTA (pH 8.0). The plate was then incubated with 10 mM $NiSO_4$ for 1 hr at RT. The plate was sequentially washed with washing buffer and 50 mM Tris HCl (pH 7.5) containing 500 mM NaCl. Then, 100 µl of purified H_6MA-CA (5 µg/ml) in 0.5% BSA in TBS was added to the pre-treated plate and incubated at 4°C overnight. The unbound protein was removed by washing four times with a buffer containing 0.5 M NaCl, 20 mM Tris-HCl, and 6M urea with increasing concentrations of imidazole (20, 40, 60, and 80 mM). The wells were then washed once with 0.1% BSA in TBS containing 0.05% Tween 20. The soluble HIV-PRH_6 and HIV-PRH_6 I54V (2 mg/ml) were mixed with or without 1.6 µM of lopinavir, a protease inhibitor (PI) (a gift from Dr. Timothy Cressey, RD174-PHPT, Chiang Mai University, Chiang Mai, Thailand). Subsequently, the reaction mixture was added into the coated wells and the plate was

incubated at 37°C for 1 hr. The wells were then washed with high-stringency washing buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1% BSA, and 0.05% Tween 20) followed by 0.1% BSA/0.05% Tween 20 in TBS. The presence of the free C terminus of MA and the decreased CA level were separately detected using 0.5 µg/ml of an anti-MA mAb HB-8975 that specifically binds to the free C terminus MA and 0.5 µg/ml of an anti-CA mAb G18 (generated in our laboratory). Finally, HRP-conjugated goat anti-mouse immunoglobulins (diluted 1:3,000 in 0.5% BSA in TBS) were added, and the plates were incubated for 1 hr at RT. The wells were then washed, and 100 µl of TMB substrate was added. The reaction was stopped using 1 N HCl, and the OD_{450} was measured using a microplate reader (Biodirect).

2.21.2 The efficacy of ELIB-PA with HIV protease inhibitors

The efficacy of ELIB-PA was validated by determining the median 50% inhibitory concentrations (IC_{50}). The reaction was performed as describe in previous step. The soluble HIV-PRH_6 (2 mg/ml) was mixed with or without various concentrations (0–2 µM) of HIV protease inhibitors (PIs), lopinavir (LPV), ritonavir (RTV), and nelfinavir (NFV), while efavirenz (EFV), reverse transcriptase inhibitor was used as a control inhibitor (a gift from Dr. Timothy Cressey, RD174-PHPT, Chiang Mai University, Chiang Mai, Thailand). Subsequently, the free C-terminus MA was detected using 0.5 µg/ml of anti-MA mAb HB-8975 followed with HRP-conjugated anti-mouse immunoglobulins. After washing step, TMB substrate was added and the reaction was stopped using 1 N HCl. The OD_{450} was measured using a microplate reader (Biodirect). The percentage of inhibition was given by the

following formula: Percentage of inhibition = 100 – ((B/B_0) x 100), where B and B_0 are the OD values of anti-MA mAb HB-8975 with protease inhibitor and without protease inhibitor respectively.

The dose-response curve was generated using percentage of inhibition and concentration of each inhibitor. The IC_{50} of each protease inhibitor using ELIB-PA was calculated by dose-response curve. Moreover, a straight line of each inhibitor was plotted between the percentage of inhibition and the concentrations of inhibitor by calculating from the equation of each inhibitor from the dose-response curve. The steep line that represented the accelerated inhibition of protease inhibitor with HIV-PRH_6 could be observed.