CHAPTER 3

RESULTS

3.1 Construction of phagemid carrying CD147Ex gene

In order to amplify the extracellular domain of CD147 (CD147Ex) gene from the mammalian expression vector pCDM8-CD147, a set of primer containing a short restriction sequence for *Sfi* I at 5' terminal. In the first round of amplification, the over-hang of *Sfi* I restriction site of the primers was not hybridized to the amplified gene. After the first amplified product appeared, the over-hang of *Sfi* I restriction sites were complementarily bound. From this technique, the PCR products carrying the *Sfi* I-restricted site at the terminus of CD147Ex gene were generated. The PCR products were further used for clone into the phagemid vector, pComb3HSS. To confirm the PCR amplification, the PCR product was fractionated in 1% agarose gel electrophoresis. Successful amplification of the CD147Ex gene was clearly indicated by the presence of a strong band of approximately 552 bp. (Figure 3.1.).

The amplified CD147Ex from PCR was purified using PCR Purification Kit and followed by digesting with *Sfi* I. The *Sfi* I-digested CD147Ex was inserted and ligated into the *Sfi* I-treated pComb3HSS (pComb3HSS /*Sfi* I) (Figure 3.2.) at the upstream of gene III and downstream of *Omp* A signal sequence. The ligated product was successfully transformed into the hosts, *E. coli* XL-1 Blue or TG-1 strains. The ampicillin resistant colonies were selected for

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purification of the plasmid vector. The molecular weight of the purified plasmid vector was determined by fractionating in 1% agarose gel electrophoresis. The DNA patterns in gel showed the characteristic of open circular (upper band) and supercoil (lower band) plasmid (Figure 3.3.). The purified recombinant plasmid vectors were treated with *Sfi* I and fractionated in 1% agarose gel electrophoresis. The banding pattern of the gel was shown at the molecular weight of 3,300 bp of the pComb3HSS/*Sfi* I and 552 bp of CD147Ex inserted gene whose sizes were estimated from DNA ladders running on the same gel (Figure 3.4.). Moreover the PCR reamplification of the purified vector was performed for checking the CD147Ex was precisely inserted in pComb3HSS phagemid vector. The gel electrophoresis demonstrated a single band at the molecular weight about 550 bp (Figure 3.5.) which was approximately the size of CD147Ex (552 bp). The newly synthesized recombinant phagemid vector was named as pComb3H-CD147Ex (Figure 3.6.).

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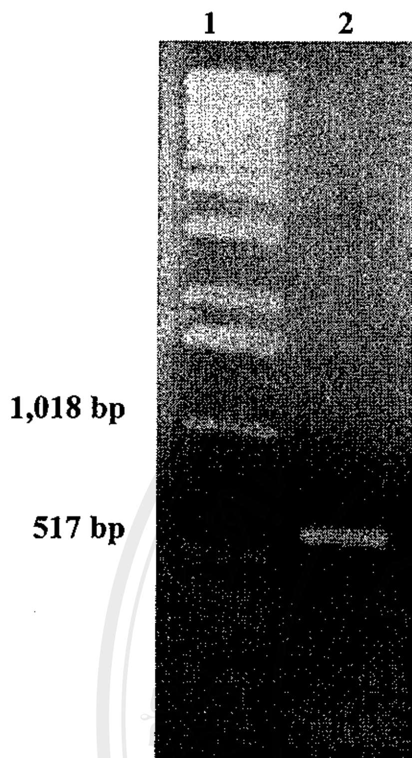

Figure 3.1. Analysis of the amplified CD147Ex gene fragment which are amplified from pCDM-8-CD147 using CD147ExFw and CD147ExRev primers. Lane 1 demonstrates 1 kb DNA marker. Lane 2 was loaded with 5 μl of amplified product. A single band at about 550 bp was depicted.

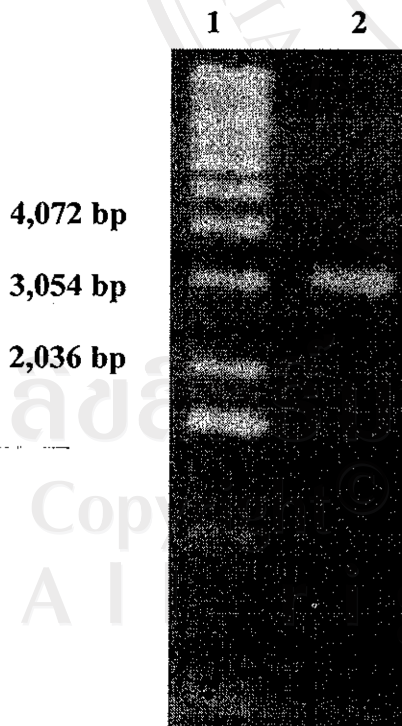

Figure 3.2. Ethidium bromide-stained DNA gel demonstrates the purified *Sfi* I-treated pComb3HSS. Lane 1: 2μl of 1 kb DNA marker. Lane 2: 2 μl of the purified *Sfi* I-treated pComb3HSS.

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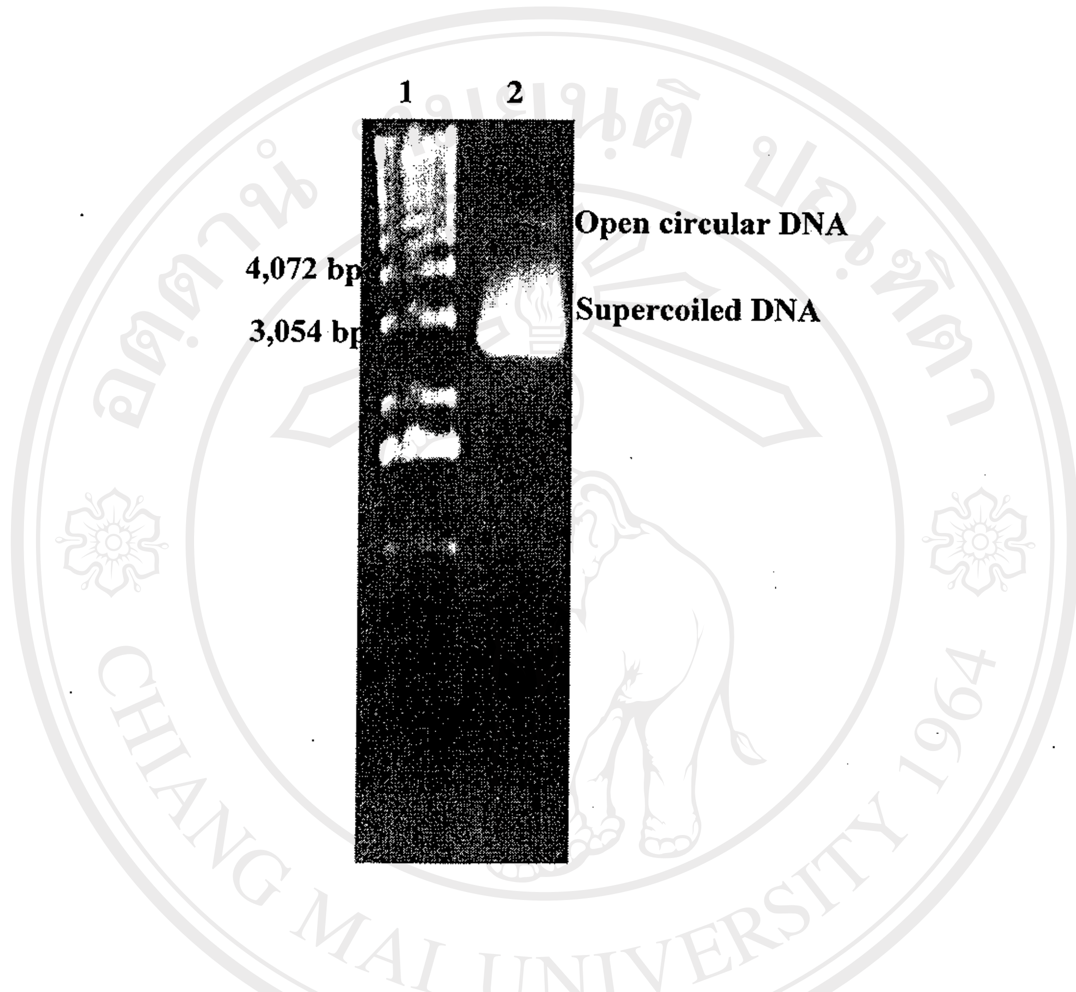

Figure 3.3. Gel electrophoresis of the purified pComb3H-CD147Ex by plasmid mini-prep. Lane 1: 2 μl of 1 kb DNA molecular weight marker. Lane 2: 2 μl of plasmid DNA purified from plasmid mini-prep.

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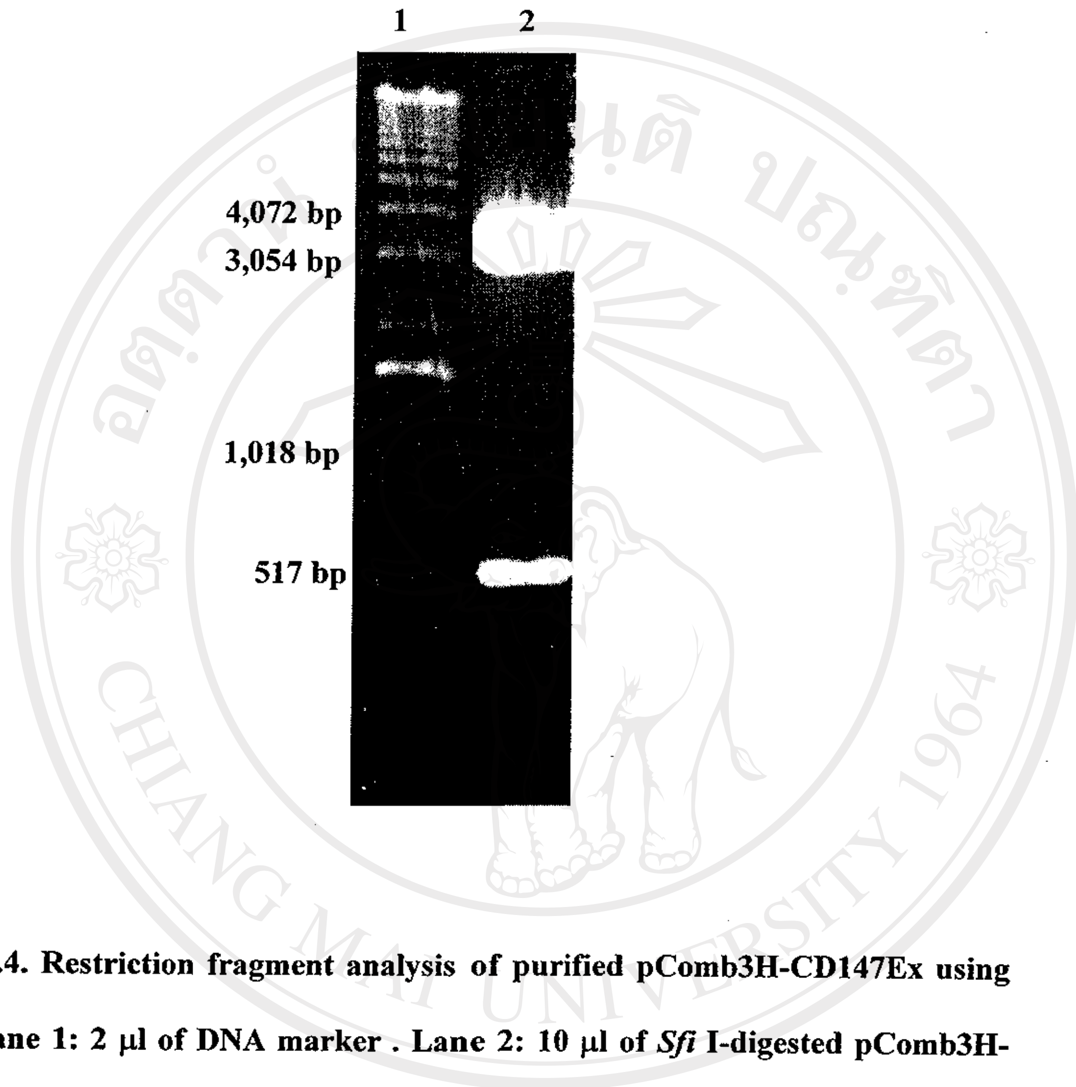

Figure 3.4. Restriction fragment analysis of purified pCombЗH-CD147Ex using *Sfi* I. Lane 1: 2 μl of DNA marker . Lane 2: 10 μl of *Sfi* I-digested pComb3H-CD147Ex.

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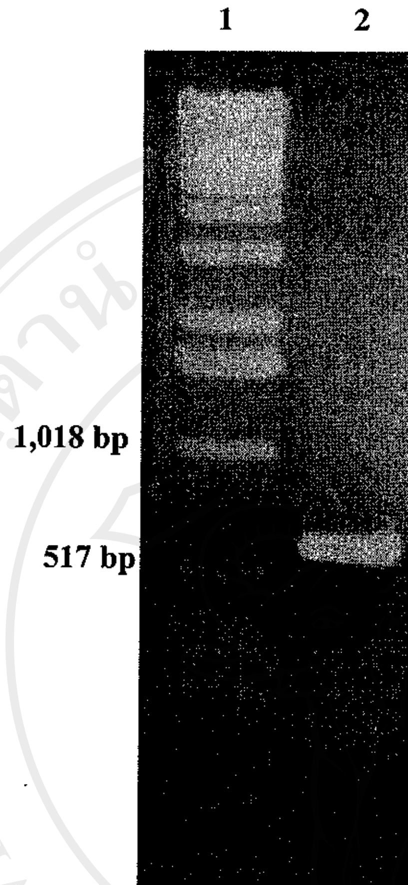

Figure 3.5. PCR reamplification of CD147Ex gene from the purified pComb3H-CD147Ex using CD147ExFw and CD147ExRev primers. Lane 1: 1 μl of 1 kb molecular weight marker. Lane 2: 5 μl of the product of PCR reamplification.

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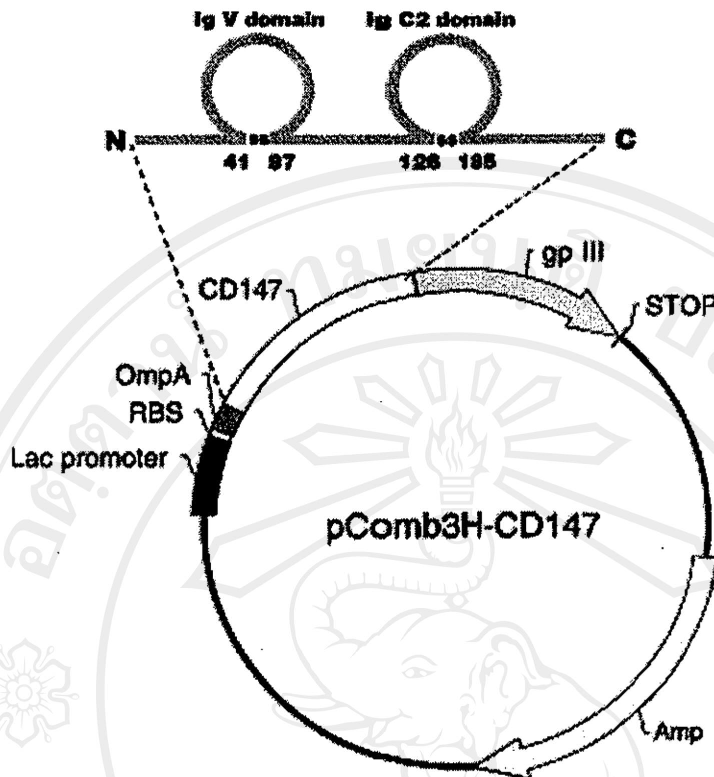

Figure 3.6. Construction of CD147Ex expression vector pComb3H-CD147. The pComb3H-CD147 was constructed by inserting the 552 bp *Sfi* I-*Sfi* I fragment containing the CD147 gene to the double *Sfi* I-cloning sites of pComb3HSS. The recombinant vector contains *lac* promoter followed by ribosome binding site (RBS), the signal sequence (*Omp*A), gpIII gene and ampicillin resistance gene (Amp). The gpIII gene is followed by stop codon for CD147-gpII translation. The derived primary structure of CD147 ectodomain containing two immunoglobulin-like domains is shown. The cysteine residues which form disulfide bridges are labeled.

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3.2 Production of phage-displayed recombinant CD147Ex (CD147Ex-Φ)

3.2.1 Production of CD147Ex-Φ using *E. coli* TG-1 host

After transformation of the pCombH-CD147 into *E. coli* strain TG-1, production of the progeny viruses was performed by growing the selected transformed *E. coli* and rescued by VCSM13 helper phage. The generated phages were harvested by precipitating with PEG 8000. The concentration of precipitated phages was determined by reinfecting the prepared phages into *E. coli* TG-1 strain at various dilutions. The 1,300 colonies of ampicillin resistance colonies were obtained from the culture plate spreaded with 50 μl of 1:10^6 viral-infected bacteria (Figure 3.7.). By calculation, the 2.6x10^4 colonies were received from 1 ml of reinfection. According to the formula in section 2.2.2.6, the concentration of precipitated phage was 2.6x10^{10} t.u./ml.

3.2.2 Production of CD147Ex-Φ using *E. coli* XL-1 Blue host

The transformed *E. coli* XL-1 Blue were cultured and infected with VCSM13 helper phages to rescue the phagemid with its CD147 gene insert. The phage particles were precipitated using PEG 8000. The concentration of the precipitated phage generated from *E. coli* strain XL-1 Blue was compared against the precipitated phage generated from *E. coli* strain TG-1 by direct ELISA. The multi-well plate was separately coated with various dilutions of the precipitated phages harvested from *E. coli* strain XL-1 Blue and TG-1 for

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finding the optimum dilution and concentration. Peroxidase-conjugated sheep anti-M13 Ab was used to determine the phage particles on each ELISA well. The suitable dilution of precipitated phage obtained from XL-1 Blue was 1: 10 which gave the equal OD of TG-1 at 1: 80 dilution. The concentration of phages obtained from *E. coli* XL-1 Blue by calculation was approximate as 3.5×10^9 t.u./ml using the correlation of OD and concentration of phage produced in TG-1 determined by phage titration as standard.

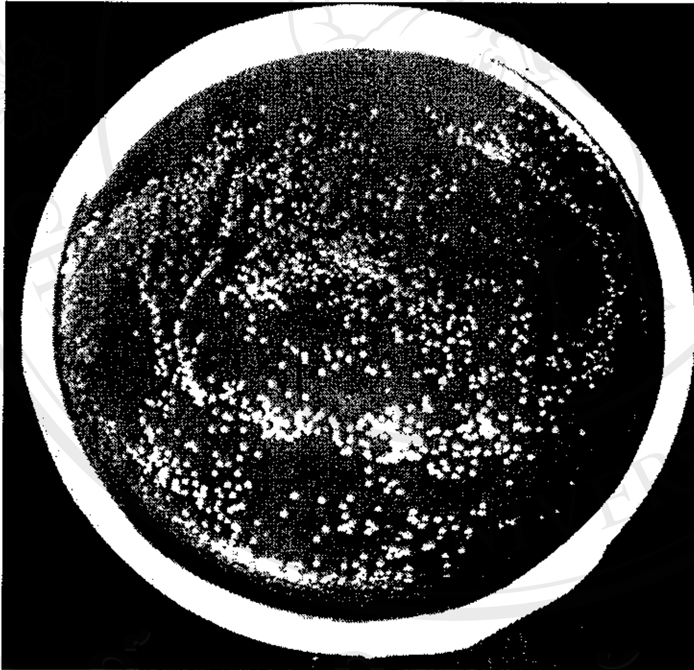

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Figure 3.7. The *E. coli* strain TG-1 after reinfected with CD147Ex-Φ at the dilution of $1{:}10^6$ and plated on LB-agar containing ampicillin. The colonies were counted and the original concentration of CD147Ex-Φ phage was calculated following the formula as described in section 2.2.2.6.

3.3 Comparison of the expression of CD147Ex in *E. coli* XL-1 Blue and TG-1 Strains

The pComb3H-CD147-transformed *E. coli* was cultured in it standard growth condition and rescued by VCSM13 helper phage. During the process of assembly, the CD147-gpIII fusion proteins were randomly incorporate into the new virus. To verify if the CD147 linked to the gpIII molecule on filamentous phages, the sandwich ELISA was performed. As described in section 2.2.3.1, by coating ELISA wells with anti-CD147 mAbs for capturing the CD147-Φ and detected by peroxidase-conjugated sheep anti-M13 Ab. The ELISA result of CD147-Φ derived from *E. coli* strain XL-1 Blue was demonstrated in Figure 3.8. Only four of the six mAbs (M6-1B9, M6-1D4, M6-1E9, and M6-2F9) reacted against CD147Ex-Φ derived from *E. coli* XL-1 Blue host. In contrast, the CD147Ex-Φ derived from TG-1 host were recognized by all CD147 mAbs tested. The CD147Ex-Φ proliferated from both strains were not recognized by the irrelevant antibody-coated well (CD54 mAb, MT54). As control, all CD147 mAbs were not bound to CD99-Φ. These controls confirmed the specificity of this experiment.

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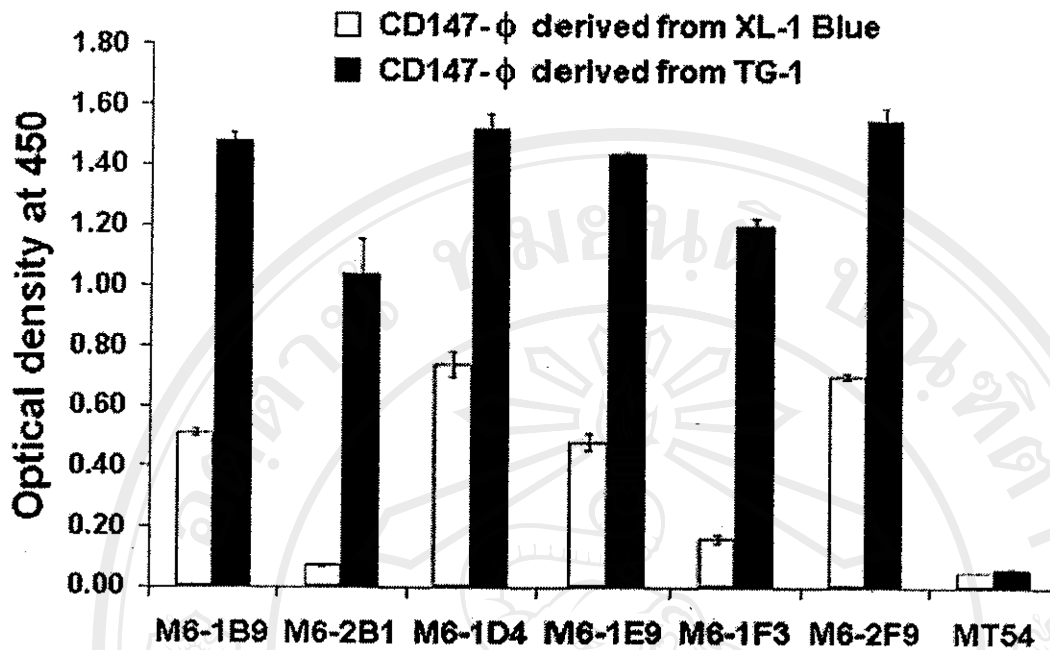

Figure 3.8. Comparison of the binding efficiency of CD147-Φ derived from *E. coli* XL-1 Blue or TG-1 host to the indicated CD147 mAbs by sandwich ELISA. ELISA wells were separately coated with six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9) and an irrelevant CD54 mAb (MT54) for capturing the CD147-Φ. The specific binding of CD147 mAbs to CD147-Φ were detected with peroxidase-conjugated sheep anti-M13. The experiment was performed twice with two preparations of CD147–Φ from both bacterial strains. The histograms demonstrated the mean value and standard deviation.

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3.4 Western immunoblotting

The protein components of CD147Ex-Φ derived from *E. coli* TG-1 were fractionated in 12% SDS-PAGE under the non-reducing condition and electrotransferred to the NC paper. The protein-blotted paper was probed with six CD147 mAbs. Peroxidase-conjugated rabbit anti-mouse Ig Ab was added and the enzymatic reaction was performed on the antibody-probed NC paper by adding the chemiluminescent substrate. The specific reactive bands were visualized by exposing the substrate-treated NC paper to the X-ray film. Four of six CD147 mAbs (M6-1B9, M6-1D4, M6-1E9, and M6-1F3) were reacted against protein-blotted NC paper at the molecular weight about 38 kDa (Figure 3.9.). The reactive band of 38 kDa represented CD147Ex (20 kDa) fused to truncated gpIII (18 kDa). The fractionated proteins did not interact with the irrelevant mAb (MT54). The specific band with molecular weight of ~40 kDa was observed when probing with anti-gpIII mAb, demonstrating the presence of the VCSM13 component in the loaded sample.

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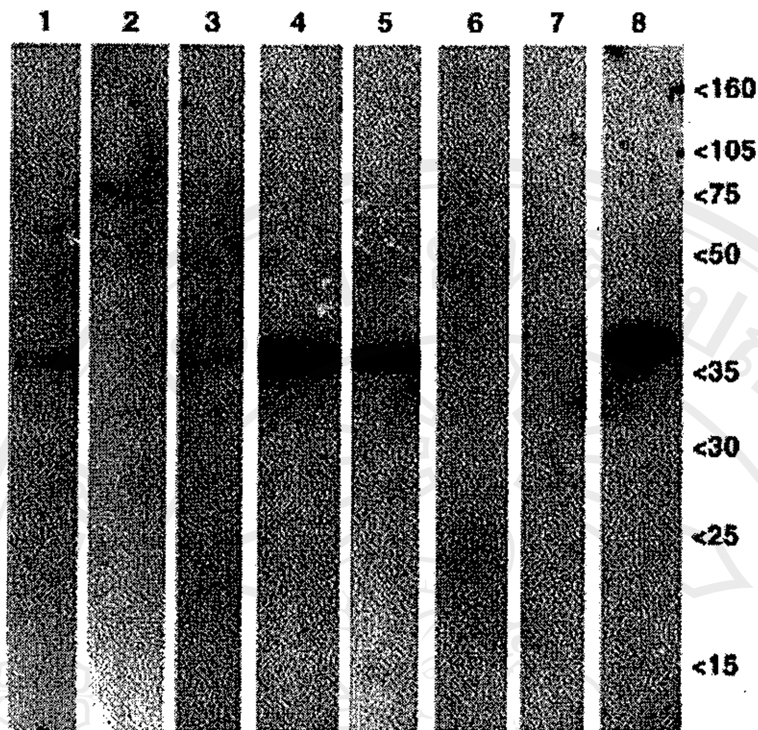

Figure 3.9. Analysis of CD147-Φ by using the western immunoblotting technique. The CD147-Φ proteins were electrophoretically fractionated under the non-reducing condition SDS-PAGE and subsequently blotted onto nitrocellulose paper. The fractionated proteins were probed with CD147 mAbs; M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9 (lanes 1–6, respectively), CD54 mAb (MT54) (lane 7) or anti-gpIII of VCSM13 mAb (lane 8). The immunoreactive bands were visualized by chemiluminescent substrate detection system. Molecular weight markers in kDa were indicated by arrows.

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3.5 Epitope mapping of CD147 mAbs

Competitive inhibition ELISA was used for epitope mapping analysis of CD147 ectodomain presented on phage particles derived from *E. coli* TG-1. Four CD147 mAbs (M6-1B9, M6-1E9, M6-1F3, and M6-2F9) were used for the epitope mapping. In this experiment, each CD147 mAb, which was used as the inhibitor, was incubated with CD147-Φ in the soluble phase. The same set of CD147 mAb was separately immobilized on ELISA wells and used as the catcher. The peroxidase-conjugated sheep anti-M13 Ab were used to determine whether CD147 mAb pre-incubated CD147-Φ was captured on the solid phase by the catcher. If the competitor and catcher bound to the same region on CD147 molecule, CD147- Φ would not be caught on the solid phase. Self-inhibition was used to indicate maximal inhibition control. The data of competitive inhibition ELISA is shown as absorbance units in Table 3.1. Each reaction pair of inhibitor and catcher, which gave more than 35% reduction of absorbance unit in comparison with the non-competitor well, was taken as indicating an overlapping epitope. In this experiment, mAbs M6-1B9 and M6-1E9 inhibited each other. MAb M6-2F9 did not hamper the binding of either mAb M6-1B9 or M6-1E9, and vice versa. Binding of mAb M6-1F3 was interfered with by all tested mAbs. In contrast, mAb M6-1F3 did not block the occupation of other mAbs. As a result, the epitopes of extracellular domain CD147 were proposed to fall into four groups (Figure 3.10.).

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Table 3.1. The optical density of competitive inhibition ELISA for epitope mapping of CD147 mAbs.

Inhibitors	Catchers			
	M6-1B9	M6-1E9	M6-1F3	M6-2F9
M6-1B9	**1.06**[a]	0.78[b]	0.07	1.69
M6-1E9	1.07	**0.96**	0.06	1.32
M6-1F3	1.63	1.59	**0.22**	1.50
M6-2F9	1.50	1.57	0.07	**1.04**
No inhibitor	1.69	1.64	1.56	1.61

[a] **The absorbance units of self-inhibition were shown in bold letters.**

[b] **The underlined values represent the absorbance units which showed more than 35% reduction in comparison with no inhibition.**

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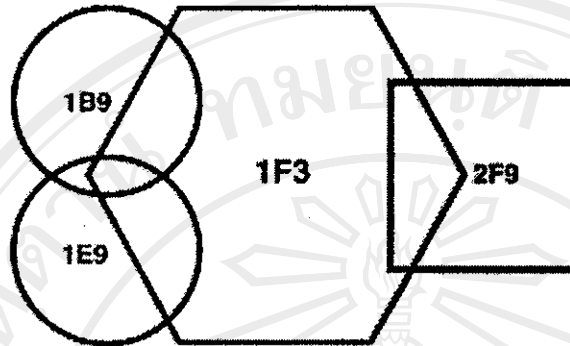

Figure 3.10. Relation of the bioactive epitope recognized by M6-1B9, M6-1E9, M6-1F3 and M6-2F9 on CD147 molecule. Each geometric form represented individual epitope. The intersections of the polygons indicate the overlapping regions of the different epitopes.

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