CHAPTER III

RESULTS

3.1 Construction of pTat8-CD147 phagemid

3.1.1 Site-directed mutagenesis of pComb8-CD147Ex phagemid

In order to generate phage expressing CD147 molecule *via* Tat pathway, pComb8-CD147Ex phagemid (Intasai *et al*., 2003) was modified by substituting the leader sequence from PelB with that from TorA. *Nsi* I restriction site at upstream of PelB signal sequence in pComb8-CD147Ex phagemid was created by Site-directed mutagenesis using MutPelBa and MutPelBb primers. After the PCR process, the mixture was treated with *Dpn* I restriction enzyme and the resulting phagemid was named MpComb8-CD147Ex. The MpComb8-CD147Ex was subsequently cleaved with *Nsi* I and *Xba* I to prepare the cloning site. The *Nsi* I- and *Xba* I-digested MpComb8-CD147Ex fragment with the molecular weight of 2,942 and 74 bp was obtained (Figure 3.1).

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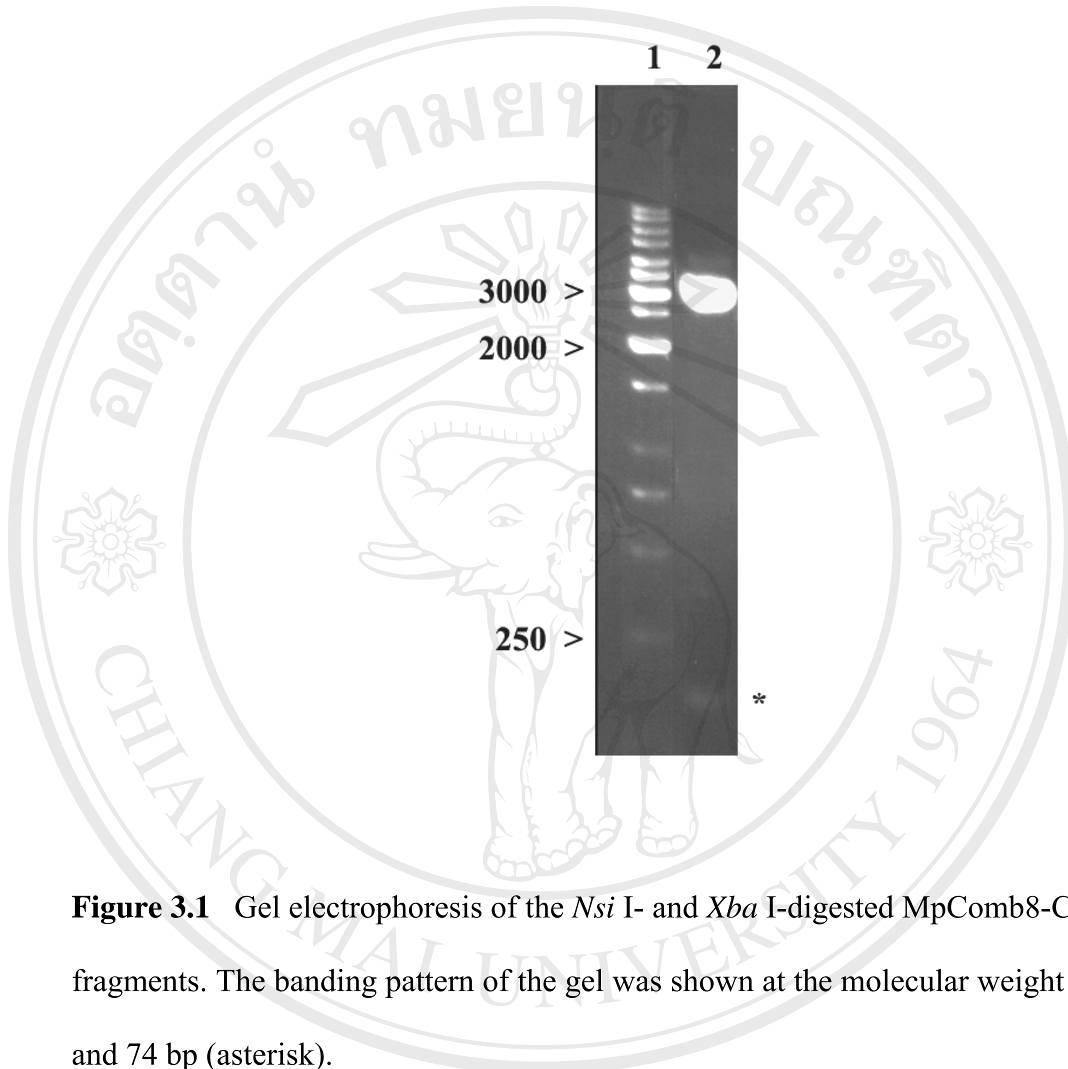

Figure 3.1 Gel electrophoresis of the *Nsi* I- and *Xba* I-digested MpComb8-CD147Ex fragments. The banding pattern of the gel was shown at the molecular weight of 2,942 and 74 bp (asterisk).

Lane 1. 1 kb DNA markers

Lane 2. *Nsi* I- and *Xba* I-digested MpComb8-CD147Ex

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3.1.2 TorA signal sequence amplification by PCR

Tat signal sequence (TorA) was amplified from pSPL04 by PCR using primers TatNsiIFw and TatXhoIRev. The PCR product of 161 bp was obtained (Figure 3.2). The amplified TorA was purified using PCR purification kit and followed by digesting with *Nsi* I and *Xho* I and the digested TorA with the molecular weight of 125 bp was used in the next ligation step.

3.1.3 Construction of phagemid containing Tat-CD147Ex gene

The *Nsi* I- and *Xho* I-digested TorA with the molecular weight of 125 bp was ligated to pComb8-CD147Ex of 844 bp which generated by digesting pComb8-CD147Ex with *Xho* I and *Xba* I (Figure 3.3). The 969 bp ligation product was obtained and named Tat-CD147 fragment and used as template for PCR which performed to increase the Tat-CD147 fragment for using in the next ligation step. The PCR product with the molecular weight of 1005 bp (Figure 3.4) was digested with *Nsi* I and *Xba* I and then inserted into dephosphorylated *Nsi* I -and *Xba* I-digested MpComb8-CD147, thus, the new phagemid vector, pTat8-CD147, was generated.

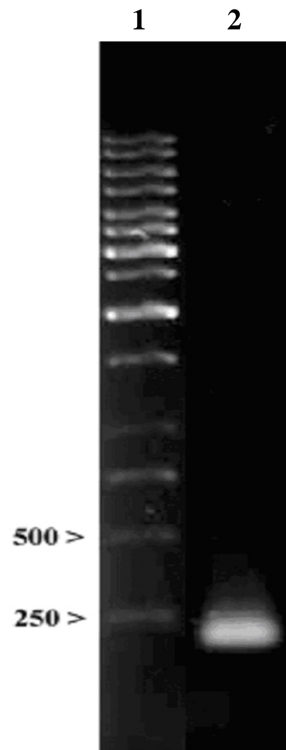


Figure 3.2 Analysis of PCR product of the TorA signal sequence, 161 bp, amplified from pSPL04 vector using TatNsiIFw and TatXhoIRev primers. The samples were electrophoresed in 1% agarose gel.

Lane 1. 1 kb DNA markers

Lane 2. Amplified product of TorA signal sequence

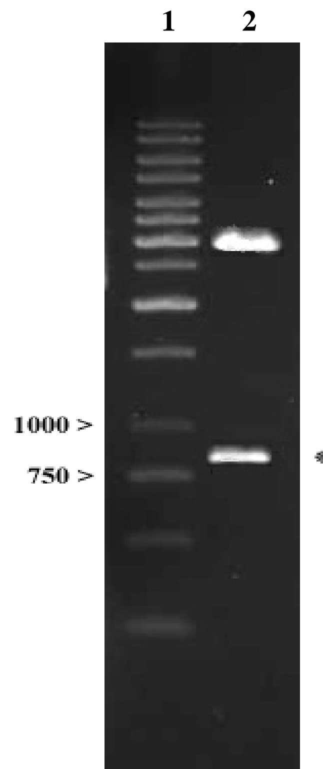


Figure 3.3 The fragment of 844 bp (asterisk) of *Xho* I- and *Xba* I-digested pCom8-CD147Ex which used for ligating with TorA signal sequence.

Lane 1. 1 kb DNA markers

Lane 2. *Xho* I- and *Xba* I-digested pCom8-CD147Ex

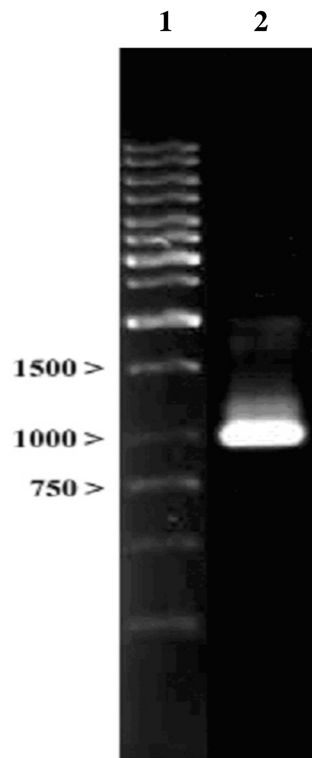


Figure 3.4 Gel electrophoresis of the Tat-CD147 fragment which was amplified by using TatNsiIFw and Tat-CD147Rv primers. The inserted fragment of Tat-CD147 with the molecular weight of approximately 1005 bp is shown.

Lane 1. 1 kb DNA markers

Lane 2. Amplified product of Tat-CD147 fragment

3.1.4 Characterization of recombinant clones

pTat8-CD147 was successfully transformed into *E. coli*. The ampicillin resistant colonies were selected for purification of the phagemid. The purified phagemid was treated with *Nsi* I and fractionated in 1% agarose gel electrophoresis to determine the size of pTat8-CD147. The linear form of *Nsi* I-digested pTat8-CD147 at the molecular weight of 3911 bp was shown (Figure 3.5, lane 2). The correct insertion of Tat-CD147 fragment, CD147Ex and TorA signal sequence were verified by restriction fragment analysis. pTat8-CD147 was digested with the combination of enzymes; *Nsi* I and *Xba* I, *Xho* I and *Spe* I, and *Nsi* I and *Xho* I. (Figure 3.5, lane 3, 4 and 5, respectively). The verification in the correct insertion of Tat-CD147 fragment was further performed by PCR reamplification (Figure 3.6). The correct nucleotide sequence of TorA-CD147Ex-gpVIII was determined according to dideoxychain terminator procedure using BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems, CA, USA). The resulting map of a novel constructed phagemid, pTat8-CD147, is shown in Figure 3.7.

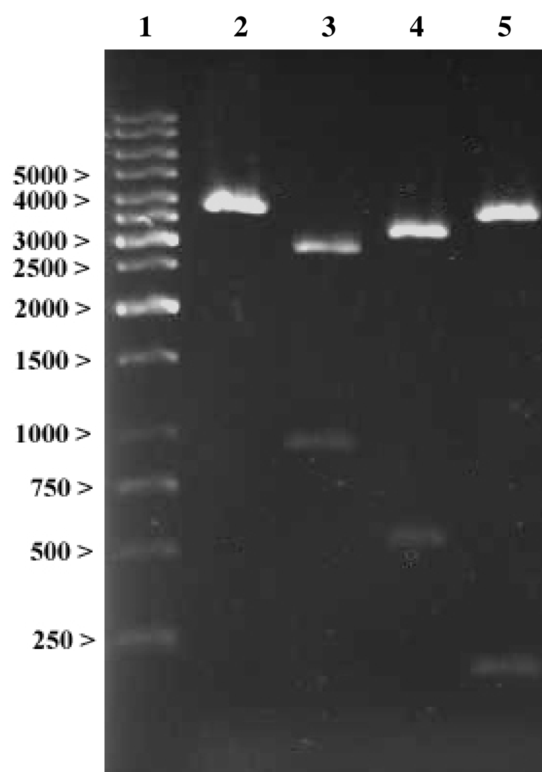


Figure 3.5 Characterization of recombinant clone containing the inserted Tat-CD147 by restriction fragment analysis. Purified pTat8-CD147 was digested with *Nsi* I and the combination of *Nsi* I and *Xba* I, *Xho* I and *Spe* I, and *Nsi* I and *Xho* I. The inserted fragments of Tat-CD147 fragment, CD147Ex and TorA signal sequence, were retrieved from pTat8-CD147 (Lane 3, 4 and 5, respectively).

Lane 1. 1 kb DNA markers

Lane 2. *Nsi* I-digested pTat8-CD147

Lane 3. *Nsi* I- and *Xba* I-digested pTat8-CD147

Lane 4. *Xho* I- and *Spe* I-digested pTat8-CD147

Lane 5. *Nsi* I- and *Xho* I-digested pTat8-CD147

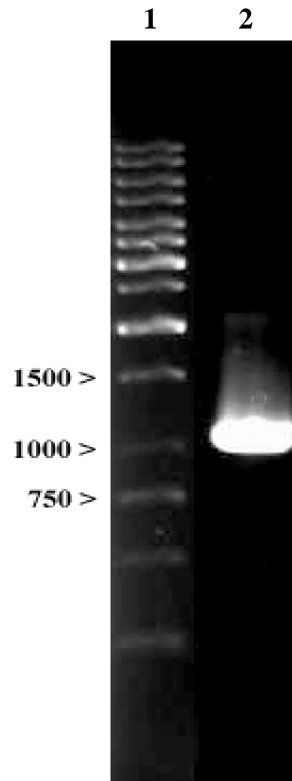
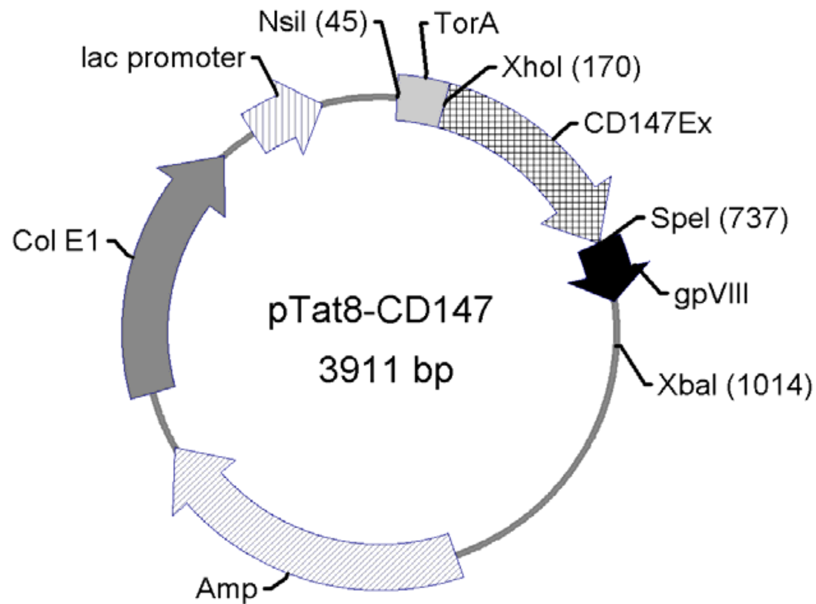


Figure 3.6 Reamplified product of Tat-CD147 fragment from pTat8-CD147 using TatNsiIFw and Tat-CD147Rv primers. The 1005 bp reamplified product from pTat8-CD147 is shown.

Lane 1. 1 kb DNA markers

Lane 2. Reamplified product of Tat-CD147 fragment.



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101 **taaccgtcgc cgggatgctg gggccgtcat tgttaacgcc gcgacgtgcg**
151 **actgcggcgc aagcggcg**ct cgaggctgcc ggcacagtct tcactaccgt
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3851  accatgatta cgccaagctc gaaattaacc ctcactaaag ggaacaaaag
3901  ctggccaccg c
```

Figure 3.7 Map of pTat8-CD147 phagemid. The *Nsi* I- and *Xba* I-cloning sites are where the Tat-CD147 fragment was inserted; the origin of replication (ColE1), lac promoter, Tat signal sequence (TorA) and gpVIII are shown. The complete nucleotide sequences were demonstrated (below). TorA signal sequence (Gene ID: 946267) was designated in bold letters and CD147Ex coding sequence (Gene ID: 682) was underlined. gpVIII gene (Gene ID: 927333) was indicated in box.

3.2 Comparison of phage-displayed CD147Ex expression by Sandwich ELISA

Phage displaying CD147Ex *via* gpVIII by Tat pathway (ΦTat-CD147gpVIII) was produced by infecting the pTat8-CD147 transformed TG-1 with VCSM13 helper phage. In addition, phage display of CD147 fusion protein from TG-1 Δ*tatABC* mutant was performed to validate the TorA signal peptide in directing the CD147 fusion protein *via* the Tat pathway. The expression of recombinant CD147Ex-gpVIII through the Tat pathway was determined in comparison with Sec pathway by Sandwich ELISA. A panel of anti-CD147 mAbs (M6-1D4, M6-1E9, M6-1F3, M6-2B1 and M6-2F9) was used as the capture antibody. By using HRP-conjugated anti-gpVIII, ΦTat-CD147gpVIII showed higher absorbance unit than did ΦSec-CD147gpVIII with all anti-CD147 mAbs (Figure 3.8). However, the binding activity of phage which was produced from TG-1 Δ*tatABC* mutant (ΦTatmut-CD147gpVIII) to the mAb-coated wells was unexpected comparably to ΦTat-CD147gpVIII. None of CD147 mAbs captured VCSM13, which assured the specificity of assay. In addition, the nonspecific binding of phages was ruled out since the signal was undetectable in any uncoated wells.

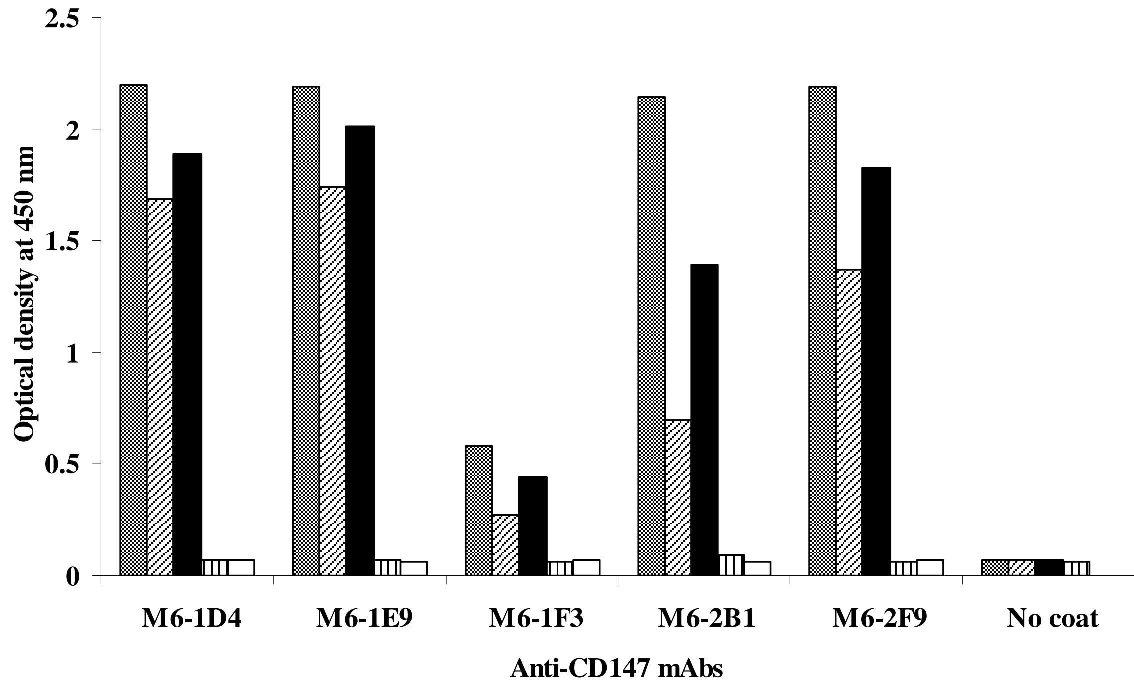

Figure 3.8 Comparison of the expression of phage-displayed CD147Ex *via* Tat and Sec pathway by Sandwich ELISA. Anti-CD147 mAbs (M6-1D4, M6-1E9, M6-1F3, M6-2B1, and M6-2F9) were used to capture recombinant phages. The binding activity of each tested phage was compared using an arbitrary optical density unit at 450 nm. This experiment was done in duplicate. Phage displayed CD147Ex on gpVIII *via* Tat pathway, ΦTat-CD147gpVIII (first bar); phage displayed CD147Ex on gpVIII *via* Sec pathway, ΦSec-CD147gpVIII (second bar); phage displayed CD147Ex on gpVIII produced from tat mutant, ΦTatmut-CD147gpVIII (third bar); VCSM13 phage (forth bar) and no phage (last bar).

3.3 Demonstration of anchored CD147Ex on phage particles by Sandwich ELISA

To confirm that CD147 molecules were linked to the phage particles, the biotinylated anti-gpIII mAb/HRP-conjugated anti-biotin antibody detection system was applied. As shown in Figure 3.9, ΦTat-CD147gpVIII showed higher binding activity than ΦSec-CD147gpVIII against most of anti-CD147 mAbs excepted for M6-1F3 in which the similar signal was observed. ΦTatmut-CD147gpVIII and VCSM13 were not recognized by each anti-CD147 mAbs and this result was also obtained in uncoated wells.

3.4 Western immunoblotting

ΦTat-CD147gpVIII and ΦTatmut-CD147gpVIII (10^{10} cfu each) were fractionated by SDS-PAGE under reducing conditions, electroblotted and probed with pooled CD147 mAbs (M6-1D4 and M6-1E9). An immuno-reactive band with molecular weight of 28 kDa was visualized (Figure 3.10). This band appeared at the same size of ΦSec-CD147gpVIII in previous study (Intasai *et al*, 2003). The band was considered a fusion protein of CD147Ex (20 kDa) and gpVIII (6 kDa). No reactive band was detected in the control lane in which VCSM13 phages were used.

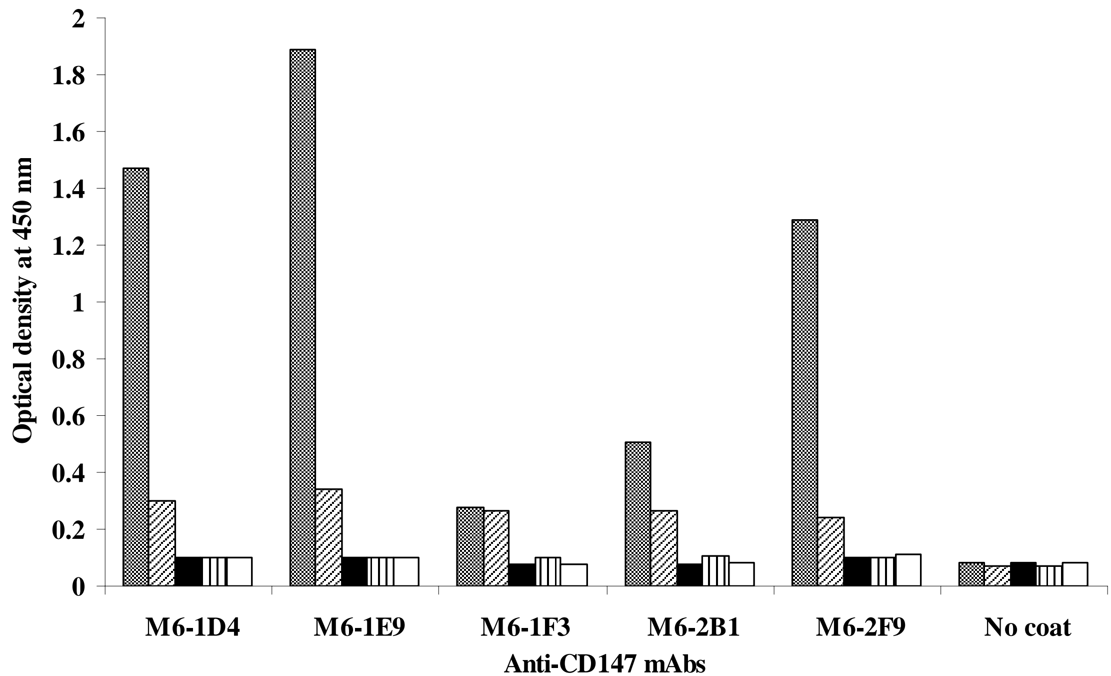

Figure 3.9 Detection of CD147Ex presenting on phage particles *via* gpVIII by Sandwich ELISA. The recombinant phages were captured with five of anti-CD147 mAbs (M6-1D4, M6-1E9, M6-1F3, M6-2B1, and M6-2F9). The biotinylated anti-gpIII mAb/streptavidin conjugated HRP detection system was used for tracing the antibody-bound phages. The experiment was done in duplicate and the symbols of phage in each bars were used the same as described in Figure 3.8.

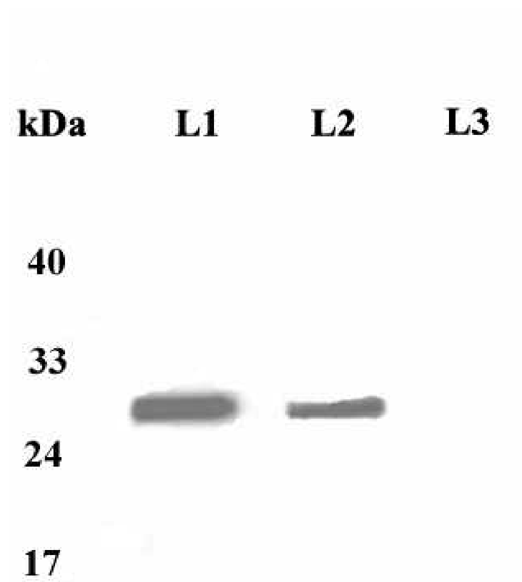

Figure 3.10 Western immunoblotting of phage-displayed CD147ExgpVIII. Immunological assay was performed by probing with a combination of anti-CD147 mAbs (M6-1D4 and M6-1E9). The immuno-reactive bands were visualized by chemiluminescence substrate detection system. Molecular weight markers in kDa were indicated.

Lane 1. ΦTat-CD147gpVIII

Lane 2. ΦTatmut-CD147gpVIII

Lane 3. VCSM13 phage