# CHAPTER 2

# EXPERIMENTAL

## 2.1 Materials and equipments

### 2.1.1 Chemicals

**All chemicals used in the experiment were as the following:**

- **Acrylamide (BDH Laboratory Supplies, UK)**
- **Agarose (electrophoresis grade) (Sigma-Aldrich Co.,USA)**
- **Ammonium persulphate (Amersham Biotech, Sweden)**
- **Ampicillin (Sigma-Aldrich Co., USA)**
- **Bis-acrylamide (BDH Laboratory Supplies, UK)**
- **ECL™ Western Blotting Detection Reagents (Amersham Biosciences, UK)**
- **EDTA (Sigma-Aldrich Co.,USA)**
- **Ethidium bromide (Sigma-Aldrich Co.,USA)**
- **Glacial acetic (BDH Laboratory Supplies, UK)**
- **Glycerol (Sigma-Aldrich Co.,USA)**
- **MOPS (Amresco, USA)**
- **NaCl (BDH Laboratory Supplies, UK)**
- **NaOH (BDH Laboratory Supplies, UK)**
- **PEG MW 8000 (Sigma-Aldrich Co.,USA)**
- **SDS (Sigma-Aldrich Co.,USA)**

- **Sheep anti-M13 conjugated HRP antibody (Amersham Biotech, Sweden)**
- **TEMED (Sigma-Aldrich Co.,USA)**
- **Tetracycline (Sigma-Aldrich Co.,USA)**
- **Tris Base (Sigma-Aldrich Co.,USA)**
- **Tryptone (Life Technologies, Scotland)**
- **Yeast extract (Life Technologies, Scotland)**

**2.1.2 Molecular reagents and materials**

- **1 kb DNA marker (Roche Molecular Biochemicals, Germany)**
- **dNTPs (Roche Molecular Biochemicals, Germany)**
- **Ligase (Roche Molecular Biochemicals, Germany)**
- **Primers (Life Technology, USA)**
- **QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany)**
- **QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany)**
- **QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany)**
- **Restriction enzymes (Amersham Pharmacia Biotech, Sweden)**
- **T4 ligase (Roche Molecular Biochemicals, Germany)**
- **Taq DNA polymerase and 10X reaction buffer (Roche Molecular Biochemicals, Germany)**

**2.1.3 Microorganisms**

- ***Escherichia coli* strain TG-1 {*supE hsdΔ5 thiΔ(lac-proAB)* F' [*traD36proAB+*, *lacI*$^q$ *lacZ*ΔM15]} (Stratagene, USA)**
- ***Escherichia coli* strain XL-1 Blue {*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*F' [*proAB*$^+$, *lacI*$^q$ *lacZ*ΔM15 Tn10 (tet$^r$)]} (Stratagene, USA)**
- **VCSM13 filamentous phage (Stratagene, USA)**

**2.1.4 Equipments**

- **Electrophoresis power supply, ECPS 3000/150 (Amersham Pharmacia Biotech, Sweden)**
- **FOTODYNE Incoporated Mitsubishi video copy Processor (Mitsubishi, Japan)**
- **Microplate Reader EL340 (BIO-TEK Instruments, USA)**
- **MRX-150 refrigerated microcentrifuge (TOMY, Japan)**
- **PCR Mastercycler personal (Eppendorf, USA)**
- **RT6000 D refrigerated centrifuge (Sorvall, USA)**
- **UV160 Spectrophotometer (Shimadzu, Japan)**
- **UV Transilluminator (Hoefer Scientific Instruments, USA)**

## 2.2 Methods

**2.2.1 Construction of the recombinant phagemid vector containing the extracellular domain gene of human CD147**

**2.2.1.1 Primer design**

**In order to amplify the extracellular domain of human CD147 gene (CD147Ex), the specific primers were synthesized as follow: CD147ExFw [5'-GAG GAG GAG GTG GCC CAG GCG GCC GCT GCC GCC GGC ACA GTC TTC-3'] and CD147ExRev [5'-GAG GAG GAG GTG GCC GGC CTG GCC GTG GCT GCG CAC GCG GAG-3']. This pair of specific primers was designed based on the human CD147 gene from the NCBI databases (accession number g34448). The synthesized primers contain the 5'-overhang containing the *Sfi* I restriction sites (underlined portions). The primers were suitable for annealing the extracellular domain of CD147 gene which encoding for $Ala_{22}$ to $His_{205}$ (Figure 1.1.) in pCDM-8-CD147 (Kasinrerk *et al.* 1992).**

**2.2.1.2 CD147 gene amplification by PCR**

**The 100 µl PCR reaction mixture contained 50 ng of DNA template, pCDM8-CD147 (Kasinrerk *et al.* 1992) and 250 ng of each of CD147ExFw and CD147ExRev primers, 2.5 mM dNTPs, 1X PCR buffer and 2.5 U Taq polymerase were included in this mixture. The amplification condition was started with jump-start at 85 °C for 4 min**

followed by 35 rounds of 3 steps of amplification: denaturation at 95 °C for 50 sec, annealing at 42 °C for 50 sec and extension at 72 °C for 1.5 min. Finally, the mixture was incubated at 72 °C for 10 min. The amplified product was checked for the correct molecular size (552 bp) by 1% agarose gel electrophoresis and purified by a QIAquick PCR Purification Kit.

2.2.1.3 Purification of PCR product by QIAquick PCR Purification Kit

Five volume of PB buffer was mixed together with 1 volume of PCR product and transferred to a QIAquick spin column, which was placed on a 2-ml collection tube. To bind the DNA, the QIAquick column was subsequently centrifuged at 10,000 g for 60 sec and discarded the flow through. Then, the QIAauick spin column was washed by 0.75 ml of PE buffer and centrifuged 2 times for eliminating the flow through solution at 10,000 g for 60 sec. Finally, the DNA-binding column was replaced in a clean 1.5 ml microcentrifuge tube and eluted by loading 30 µl of distilled water and centrifuge at 10,000 g for 1 min. The size of DNA was checked by fractionating in 1% gel electrophoresis.



2.2.1.4 Construction of pComb3H containing CD147Ex gene

Four micrograms of purified PCR product, which were estimated by comparing to the known concentration band from DNA marker, were treated with 60 U of *Sfi* I at 50 °C for 18 hr, whereas 20 µg

of phagemid vector, pComb3HSS (kindly provided by Dr. Carlos F. Barbas, Scripps Institute, USA) was treated with 100 U of the same enzyme. Both *Sfi* I-treated DNA fragments were further purified by the PCR Purification Kit (QIAGEN). Five units of T4 ligase was introduced to the ligation mixture containing 50 ng of purified *Sfi* I-digested CD147Ex and 100 ng of purified *Sfi* I-digested pComb3HSS (M.W. 3,300 bp). The total volume of the ligation reaction was 15 µl. The ligation mixture was subsequently incubated at 4 °C for 18 h.

2.2.2 Phage display technique for the expression of recombinant CD147Ex

2.2.2.1 Bacterial cell transformation

The similar protocol was employed for transformation of *E. coli* XL-1 Blue and TG-1 strains. Fifteen microliters of the ligated DNA were co-incubated with 200 µl of cold-thawed $CaCl_2$ competent cells on ice for 1 h. The transforming mixture was transferred into cooled screw cap tube and subsequently shocked at 42 °C for 1.5 min, then abruptly chilled on ice for 1 min. Three milliliter of non-antibiotic LB broth were added and further cultured with shaking (120 rpm) at 37 °C for 3 h. The transformed cell was centrifuged (1,900 g) at RT for 10 min and plated on ampicillin-containing LB agar (100 µg/ml). Several ampicillin resistance colonies were selected and grown for plasmid miniprep using the alkaline lysis protocol. The purified phagemids were firstly checked by fractionating in 1% agarose gel electrophoresis. In

order to verify the correct *E. coli* clones, the purified phagemid from the individual clone was further digested with *Sfi* I to identify the band of correct insert (approximately 552 bp). To confirm that the CD147Ex was inserted in pComb3HSS phagemid vector, the PCR reamplification was used to determine the correct size of the PCR product as described in section 2.2.1.2. The newly synthesized phagemid was named pComb3H-CD147.

2.2.2.2 Purification of phagemid by using alkaline lysis method

An ampicillin resistant colony was picked and grown in 3 ml of ampicillin containing LB broth (100 μg/ml) with vigorous shaking (180 rpm) at 37 °C for 8 h. The 1.5 ml of cultured were centrifuged 10,000 g at 4 °C for 5 min. The supernatant was discarded and the cell wall of bacterial pellet was lysed by 100 μl of 1X glucomix-lysozmyme and vortex vigorously. Two hundred microliters of freshly prepared NaOH/SDS were added and mixed by inverted. Then, 150 μl of potassium acetate were added and gently mixed by vortex. The solution was centrifuged 10,000 g at 4 °C for 5 min for collecting the clear supernatant. The nine hundred microliters of analytical grade absolute ethanol were added and kept on ice for 2 min. The DNA was spun down at 10,000 g at 4 °C for 5 min and the supernatant was discarded. 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 min. The supernatant was collected

by centrifugation at 10,000 g at 4 °C for 5 min. Three hundred microliters of absolute ethanol were added to the supernatant and incubated at -70 °C for 10 min. The solution was spun down to harvest the pellet. The pellet was washed with 1 ml of 70% ethanol by centrifugation (10,000 g) at 4 °C for 5 min. The DNA pellet was dried at 37 °C about 30 min and reconstituted with 30 μl of sterile DW and stored at -20 °C until use.

2.2.2.3 Preparation of phage-displayed CD147 (CD147-Φ) using the *E. coli* XL-1 Blue

The selected clone of transformed *E. coli* XL-1Blue harboring pComb3H-CD147 was cultured in antibiotic-containing superbroth (100 μg/ml of ampicillin and 10 μg/ml of tetracycline) with shaking (180 rpm) at 37 °C until OD at wavelength 600 nm was 1.5. The preculture was diluted in 100 ml of the same media and further cultivated for 2 h at 37 °C. The $10^{12}$ t.u. of VCSM13 helper phage were added into the shaking flask and culture for 3 h at 37 °C. Finally, kanamycin was added into the culture at a final concentration of 70 μg/ml and the culture phage was grown at 37 °C until 18 h.



2.2.2.4 Preparation of phage-displayed CD147Ex (CD147-Φ) using the *E. coli* TG-1

The E. coli containing pComb3H-CD147 was grown in 10 ml of 2xTY broth containing ampicillin (100 μg/ml). The bacteria was

culture with shaking (180 rpm) at 37 °C until the OD at wavelength 600 nm was 0.8. The preculture was subsequently diluted in 100 ml of the same media, which contained 2 ml of 50% glucose and cultured in the same condition until 2 h or the OD at wavelength 600 nm was reached 0.5. Thirty milliliters of the culture were divided to infect with 3 ml of $10^{11}$ t.u./ml of VCSM13 helper phage and incubated without shaking in waterbath at 37 °C for 30 min. Virus-infected bacterial cells were centrifuged 2,500 g at 4 °C for 10 min. The supernatant was discarded and the pellet was resuspended in 2xTY broth containing antibiotics (100 μg/ml of ampicillin and 70 μg/ml of kanamycin). Fifteen milliliters of resuspended cells were transferred into 250 ml of the same media and further cultured with shaking (180 rpm) at 37 °C for 18 h.

2.2.2.5 Harvesting of phages by PEG precipitation

The same protocol was used for harvesting the phage progeny from pComb3H-CD147 transformed XL-1 Blue and TG-1 cultures was used. The VCSM13-infected *E. coli* from section 2.2.2.3 or 2.2.2.4 were centrifuged 2,500 g at 4 °C for 30 min. The culture supernatant was collected and phages in the culture supernatant were precipitated by adding 4% w/v of PEG 8,000 and 3% w/v of NaCl with shaking (180 rpm) for 15 min at RT or until PEG and NaCl were completely dissolved. The supernatant was kept on ice for 30 min and centrifuged (8,000 g) at 4 °C for 30 min. The pellet was air dried for 30 min and reconstituted in 2.5 ml of 1 mM PBS pH 7.2. The solution was

centrifuged (9,000 g) at 4 °C for 10 min and the supernatant was preserved in 30% glycerol. The precipitated phages were stored at -70 °C.

2.2.2.6 Phage titration by reinfecting the *E. coli*

CD147Ex-Φ obtained from *E. coli* strain TG-1 was further titration by reinfecting into *E. coli* strain TG-1. One microliter of precipitated phages was transferred to 999 µl of 1 mM PBS to make a dilution of $1:10^3$, then 1 µl of the mixture was used to infect 1 ml of cultured *E. coli* TG-1 having OD at wavelength 600 nm about 0.4-0.6 (final dilution of phage is 1: $10^6$). Fifty microliters of viral-infected bacteria were plated on LB agar containing 100 µg/ml of ampicillin. The final dilution of phage infected TG-1 at 1: $10^8$ and 1: $10^{10}$ were performed by the same procedure. The culture plates were incubated at 37 °C for 8 h and the ampicillin resistant colonies were counted and calculated using the formula below:

$$X = Y \times (V/1000) \quad (\text{t.u./ml})$$

X: The original amount of phages obtained from preparation process.

Y: The ampicillin resistant colonies

V: Volume (µl) of viral infected bacteria

**2.2.3 Detection of the phage-displayed CD147 (CD147-Φ)**

**2.2.3.1 Direct ELISA for detection the precipitated phage**

**The aims of this experiment was to compare the quantity of precipitated phages generated from either *E. coli* strain XL-1 Blue and TG-1. To find the optimum dilution and concentration, the multi-well plate was separately coated with various dilutions of the precipitated phages from both strains in carbonate/bicarbonate buffer pH 9.6 at room temperature (RT) for 2 h. The phage-coated wells were blocked with 2% skimmed milk diluted in 1 mM PBS at RT for 1 h. The wells were washed 4 times with washing solution (0.05% Tween 20 diluted in 1 mM PBS). The phage in each well was detected by peroxidase-conjugated sheep anti-M13 Ab and incubated at RT for 1 h. After washing, the substrate, TMB (3', 3', 5', 5'-Tetramethylbenzidine), was applied to each well. The enzymatic reaction was stopped using 1 N HCl and measured the absorption at OD at wavelength 450 nm.**

**2.2.3.2 Detection of CD147-Φ by sandwich ELISA**

**Multi-well plate was separately coated with 50 µl/well of 10 µg/ml anti-CD147 monoclonal antibodies (mAbs) (including M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3 and M6-2F9), which diluted in carbonate/bicarbonate buffer pH 9.6, at 4 °C for 18 h. The antibody-coated wells were blocked with 2% skimmed milk diluted in 1 mM PBS pH 7.2 at RT for 1 h. The wells were washed four times with 0.05% tween-20 diluted in 1 mM PBS. Then, precipitated phages were added**

into each well and incubated at RT for 1 h. After washing, the peroxidase-conjugated sheep anti-M13 Ab was added and incubated at RT for 1 h. The reaction wells were washed and TMB (3', 3', 5', 5'-Tetramethylbenzidine) substrate was applied to each well. The enzymatic reaction was stopped using 1 N HCl and measured the absorption at OD at wavelength 450 nm.

2.2.3.3 SDS-PAGE and western immunoblotting

The $1.25\times10^8$ t.u. of precipitated phages, obtained from TG-1, were mix with the non-reducing buffer and heated for 5 min in boiled water. Then, the samples were loaded in each lane and fractionated in 12% polyacrylamide gel electrophoresis (PAGE). The separated proteins were electrophoretically transferred onto the nitrocellulose (NC) membrane. Protein-blotted NC membrane was blocked overnight at 4 °C in 5% skimmed milk diluted in 1 mM PBS pH 7.2. CD147 mAbs (10 μg/ml) were separately applied to capture the CD147Ex epitopes on the NC membrane and incubated for 1 h at RT. The NC membrane was washed 5 times, 5 min each, with 0.05% Tween-20 diluted in 1 mM PBS pH 7.2. Peroxidase-conjugated rabbit anti-mouse immunoglobulins was added and incubated for 1 h at RT. After washing, the enzymatic reaction on the antibody-probed NC paper was developed by chemiluminescent substrate system. The specific reactive bands were visualized by exposing the substrate-treated NC paper to the X-ray film.

**2.2.4 Epitope mapping of CD147-Φ by competitive inhibition ELISA**

**The epitope mapping of CD147Ex linking to gpIII was performed by competitive inhibition ELISA (Figure 2.1.). The core purpose of this study is to characterize the specific epitopes of CD147Ex recognized by a panel of CD147 mAbs. Six relevant CD147 mAbs, including M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3 and M6-2F9, were employed in this study. For tests, the ELISA wells were separately coated with 50 µl/well (final concentration: 10 µg/ml) of CD147 mAbs, which diluted in carbonate/bicarbonate buffer pH 9.6, at 4 °C for 8 h. The wells were blocked with 200 µl of 2% skimmed milk diluted in 1 mM PBS pH 7.2 at RT for 2 h. While blocking, the CD147Ex-Φ was separately incubated at 37 °C with the same panel of CD147 mAbs which used for coating. The final concentration of each antibody was 10 µg/ml while the final concentration of CD147Ex-Φ was $1.3x10^9$ t.u./ml. The antibody-coated wells were washed four times with 0.05% Tween-20 diluted in 1 mM PBS, followed by adding 50 µl of the antibody-incubated phages and incubated at RT for 1 h. The plate was incubated at RT for 1 h and washed four times with 0.05% Tween-20 diluted in 1 mM PBS. Fifty microliters of peroxidase-conjugated sheep anti-M13 Ab was added into each reaction wells and incubated at RT for 1 h. After washing, 100 µl of TMB (3', 3', 5', 5'-Tetramethylbenzidine) substrate was added and incubated for 10 min. The reaction was stopped by adding 100 µl of 1N HCl and detected the OD at wavelength 450 nm. The OD of inhibitions**

were compared the OD of sandwich ELISA with no inhibitor. The cut off of inhibitions was remarked when the OD of competitive inhibition wells was reduced from the OD of sandwich ELISA ≥ 35%.

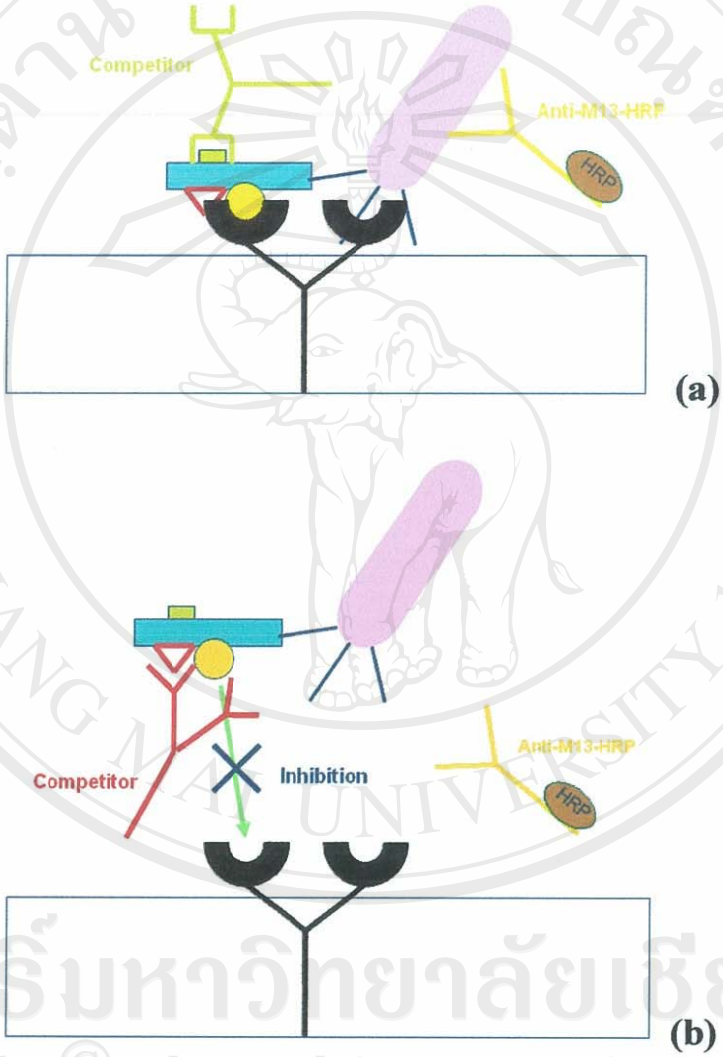




**Figure 2.1. Schematic diagram of competitive inhibition ELISA: (a) non-interfering of competitor which gives positive signal and (b) interfering of competitor which gives negative signal.**