CHAPTER IV
MATERIALS AND METHODS

1. *Leptospira* cultivation

Pathogenic and non-pathogenic *Leptospira* spp. used throughout this study were kept under liquid nitrogen at the Molecular Immunology Laboratory, Faculty of Allied Health Sciences, Thammasat University, Rangsit Center, Pathum-thani. The bacteria used in this study are listed in **Table 9**.

The leptospires were cultivated and maintained in the liquid and semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, USA) supplemented with BSA, Tween-80, pyruvate, and other minor supplements (Johnson and Harris, 1967; Johnson *et al*., 1973b).

Approximately 5-10 ml of the sterile culture medium (basal medium:supplement; 10:1) **(Appendix A)** was aliquoted in screw-capped glass tubes. After inoculating leptospires into the medium, the tubes were incubated aerobically at 29-30°C in a low-temperature incubator (Memmert, Germany). Subculture was performed every 2-3 weeks. The cultures were monitored weekly for growth and any contamination under dark-field microscopy (DFM) (Olympus, USA). In case of contamination, 5-fluorouracil (5-FU) was added to a final concentration of 200 μg/ml in order to inhibit the contaminants. Alternatively, the contaminated culture was filtered through a sterile 0.22 μm Millipore membrane (Millipore, USA). The leptospires are actively motile and could penetrate through the membrane while the others are retained on the membrane. For large-scale preparation, leptospires were cultured in 100 ml liquid EMJH medium in a 250 ml bottle and incubated under the condition mentioned above.

2. Preparation of *Leptospira* whole cells homogenate

Log phase leptospires in liquid EMJH medium were harvested by centrifuging the culture at 12,000 × *g* at 4°C for 30 minutes. After the supernatant was discarded, the pellet was washed three times with sterile phosphate buffered saline, pH 7.4 (PBS) by centrifugation at the same speed and temperature for 5 minutes. The bacterial

pellet was resuspended in a small volume of sterile distilled water (DW) and the bacterial suspension was sonicated by Ultrasonic-homogenizer LABSONIC® P (Sartorius AG, Goettingen, Germany) for 5 minutes under the ice bath. The protein contents of the homogenate were determined by Bradford assay. Aliquots (50 µl) of the samples were kept at -70°C until use.

3. Protein content determination

The Bio-Rad protein assay, based on the method of Bradford, is a simple and accurate procedure for determining a concentration of solubilized proteins. It is a dye-binding assay in which a differential color of the dye occurs in response to various concentrations of proteins (Bradford, 1976).

Ten microliters of individual *Leptospira* homogenates was placed into the separate wells of a microtiter plate. One hundred and ninety microliters of the diluted dye reagent (one part of Coomassie brilliant blue G 250 dye reagent concentrate (Bio-Rad, USA) and four part of ultra-pure distilled water [UDW]) was added to each well. The protein sample and the dye reagent were mixed thoroughly by gently aspiration and mixing, using a microtiter plate mixer (Multiscan *EX*, Labsystems, Helsinki, Finland). The plate was incubated at 25°C for 5 minutes, and the absorbance (OD) of the content in each well was determined at 595 nm with a microtiter plate reader (Multiscan *EX*). The protein concentration was calculated from the standard curve obtained against the OD of the standard solutions containing bovine serum albumin (BSA) at various concentrations (0-0.5 mg/ml). The buffer used for dissolving the sample was used as a blank.

Table 9 List of *Leptospira* spp. used in this study

Species	Serogroup	Serovar (strain)
L. interrogans	Icterohaemorrhagia	Copenhageni*
L. interrogans	Icterohaemorrhagia	Icterohaemorrhagia*
L. interrogans	Canicola	Canicola*
L. interrogans	Bataviae	Bataviae*
L. interrogans	Australis	Bangkok*
L. interrogans	Pomona	Pomona*
L. interrogans	Pomona	Pomona**
L. borgpetersenii	Tarassovi	Tarassovi*
L. borgpetersenii	Javanica	Javanica*
L. borgpetersenii	Sejore	Sejore*
L. biflexa	Andamana	Andamana*
L. biflexa	Semaranga	Patoc*

* High number of passages in EMJH medium

** Virulent strain, primary culture from leptospirosis patient

4. Cultivation of murine hybridoma secreting specific monoclonal antibodies (MAb) against pathogenic *Leptospira* spp.

Murine hybridoma clone LPF1 secreting monoclonal antibodies specific to pathogenic *Leptospira* has been produced and maintained at the Molecular Immunology Laboratory, Faculty of Allied Health Sciences, Thammasat University, Rangsit Center, Pathum-thani. The clone produces IgG1/kappa isotype. At the maximum phase of growth in serum-free medium (Gibco, USA), the clone produces monoclonal antibodies with indirect ELISA titer about 1:1,024 against *Leptospira* whole cells homogenate.

LPF1 hybridoma and P3x-63-Ag8.653 myeloma cells freshly thawed out from the liquid N_2 tank at the Molecular Immunology Laboratory, Faculty of Allied Health Sciences, Thammasat University, Rangsit Center, Pathum-thani were cultured initially in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum **(Appendix B)** in tissue culture flasks (Costar, USA) at 37°C in a 5% CO_2 incubator (SL Shellab, USA). At log phase of growth, they were transferred to grow in serum-free medium containing 4% L-glutamine by centrifuging the cultures at 3,500 × *g*, 25°C for 10 minutes and resuspending hybridoma pellet with the prewarmed serum free medium. The culture was allowed to grow in the condition as described above. When 75% confluent cell growth was obtained, the culture was harvested by centrifugation at 3,500 × *g*, 25°C for 10 minutes. The quantity and viability were determined under light microscope. The supernatants containing monoclonal antibodies were collected and characterized for its antigenic specificity by Western blotting (WB), titrated by indirect ELISA and tested for the protective efficacy against pathogenic leptospires both *in vitro* and *in vivo* assays.

5. Indirect enzyme linked-immunosorbent assay (indirect ELISA)

The wells of the microtiter plate (Costar) were coated with 100 µl coating buffer containing 10 µg/ml of pathogenic leptospires whole cells homogenate. The plate was incubated at 37°C for 16 hours until wells were completely dried. The unbound antigens were removed by excessive washing with washing buffer (PBS-T). Unoccupied sites of the wells were blocked with blocking buffer (1% BSA) and incubated at 37°C for 1 hour in a humid chamber. The excess BSA was removed by

washing with PBS-T for three times. Serial dilutions of antibody preparation (100 µl) were added to individual wells. Undiluted P3x-63-Ag8.653 spent culture supernatant and PBS, pH 7.4 served as the negative and blank control, respectively. The antigen-antibody reaction was allowed to take place for 1 hour at 37°C in a humid chamber. After washing with PBS-T as described previously, 100 µl of a 1:1,000 dilution of rabbit anti-mouse immunoglobulin (Ig)-horseradish peroxidase conjugate (Southern Biotech Associates, Inc, USA) in the diluent (0.2% BSA, 0.2% gelatin) was added to each well and incubated as above for 1 hour. The unbound antibody-enzyme conjugate was removed by washing as above, and then the enzyme substrate solution **(Appendix C)** was added to all wells (50 µl/well). The reaction was allowed to take place in the dark for 30 minutes and the reaction was stopped by adding 50 µl of stop solution per well. The optical density of the content of each well was measured at 492 nm against negative control and blank with an ELISA reader. The ELISA titer of the antibody was the highest dilution of the antibody that gave an OD ≥ 0.05. One indirect ELISA unit was the smallest amount of the MAb that give positive ELISA.

6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful technique for separating molecules with different molecular sizes in a complex mixture. This technique was described by Laemmli in 1970. In this study, the SDS-PAGE was carried out in a 10.0 × 7.5 cm vertical slab gel by using the casting apparatus (Mini-PROTEAN® 3 Cell, Bio-Rad) and electrophoresis was done in an electrophoretic chamber with a power supply (Model 3,000/300, Bio-Rad).

A 4% acrylamide stacking gel and 10-12% acrylamide separating gel were used **(Appendix D)**. Approximately 5.0 ml of the separating gel was poured per a slab gel, overlaid with UDW and allowed to polymerize for 30 minutes at 25°C. Thereafter, the overlaid UDW was removed. The stacking gel was then poured on top of the polymerized separating gel and the comb was inserted. The stacking gel was allowed to polymerize for at least 45 minutes at 25°C. The comb was then carefully removed and all the wells so-formed were washed thoroughly with UDW and electrode buffer, respectively. The polymerized casted gel was removed from the casting apparatus and

placed vertically in an electrophoresis chamber. The protein sample mixed with an equal volume of the sample buffer was heated for 4 minutes before loading into the well. Various protein standard markers were included in at least one well of the same gel. The sample was electrophoresed using an initial constant current of 10 mA per gel for 15 minutes followed by 20 mA per gel. At the end of the electrophoresis, the gel was carefully removed and either subjected to WB or staining for the direct visualization of the separated components.

6.1 Coomassie Brilliant Blue staining

The acrylamide gel containing the separated components/proteins was stained for proteins with working Coomassie Brilliant Blue dye (CBB) either CBB G250 or CBB R250 **(Appendix D)**. For CBB R250 staining, the gel was stained in staining solution for 1-2 hours at 25°C on a rocking platform (Bioline model GT25, Thailand). The stained gel was destained with several changes of the destaining solution until the blue colored bands become visible above the background. The gel was submerged in 2% glycerol and air-dried on a cellophane paper (Bio-Rad). For colloidal CBB G250 staining, the gel was placed in fixing solution for at least 1 hour at 25°C on the rocking platform. The fixed gel was then stained with the staining solution for 1 hour up to 16 hours. Destaining of the gel was made by washing with 25% ethanol until the background was cleared. The stained gel was pH neutralized with neutralizing buffer for 5 minutes and preserved in the stabilizing buffer.

6.2 Silver staining

Silver staining allows detection of protein bands at a concentration as low as nanograms level. It is approximately a hundred times more sensitive than CBB staining. In this study, the Silver Staining Kit (Amersham BioSciences, USA) was used **(Appendix D)**. First, the gel containing the separated proteins was fixed in the fixing solution for 30 minutes. After removal of the fixing solution, the sensitizing solution was added and placed on a horizontal rocker for 30 minutes. The sensitized gel was washed three times (5 minutes per cycle) with UDW, and then silver reaction was developed for 20 minutes. The gel was washed thoroughly on the rocker for cycle 1 minute each. The stained bands were visualized by incubating in a developing solution for 2-5 minutes. Stop solution was added to the container containing the gel

and incubated for 10 minutes. The gel was washed with UDW and preserved in a 2% glycerol solution.

7. Western blot analysis

Western blotting was performed by transferring the separated proteins on the acrylamide gel onto a nitrocellulose membrane (NC) (Amersham BioSciences) by the method described by Towbin *et al*. (1979). The gel and nitrocellulose membrane were separately equilibrated in the transfer buffer **(Appendix E)** for 15 minutes. The separated proteins were electro-transferred to the NC by blotting apparatus (Bio-Rad) with constant voltage at 100 V for 1.5 hours on an ice bath. The transblotted NC was air dried and the unoccupied sites on the blotted membrane were blocked with blocking buffer **(Appendix E)** with gentle agitation for 1 hour at 25°C. The excess blocking reagent was removed by washing the membrane with PBS-T three times. The NC was reacted with the antibody preparations for 1 hour at 25°C on the rocking platform. The NC was washed three times with washing buffer. Thereafter, 1:3,000 dilution of anti-mouse IgG-horserdish peroxidase conjugate in the diluent was added to the NC. The reaction was allowed to take place at 25°C for 1 hour and the NC was thoroughly washed as described above. NC was submerged in 1/15 M phosphate (PB) buffer, pH 7.6 for 15 minutes before the bands of the antigens antibody complex were developed with the substrate solution. The NC was washed with UDW until the background was cleared. NC was air dried at 25°C.

8. Bioinformatics

The BLAST program (Altschul, 1990) was used for searching public DNA and protein databases (GeneBank, EMBL, Swissport) of related sequences. Multiple sequence alignments were done using CLUSTAL W (Thompson, 1994) program through EMBOSS emma interface program. Murine CDRs and human canonical frameworks were identified and selected by using NCBI Ig BLAST (Altschul, 1990) search and IMGT program (Giudicelli *et al*., 2004). An automated homology modeling program, ESyPred3D was used to build three dimensional structures of scFv antibodies (Lambert *et al*., 2002). Visualization was performed *via* PyMOL software (http://pymol.sourceforge.net/).

9. Hemolysis dose fifty (HD50) determination and hemolysis test

In this study, hemolysis assay was performed with modification from the method of Hathaway and Marshall in 1980. Log phase primary isolate *Leptospira interrogans*, serogroup Pomona serovar Pomona, from a leptospirosis patient cultured in liquid EMJH medium was harvested by centrifuging at 12,000 × *g* at 4ºC for 5 minutes and the culture medium was discarded. Bacterial pellet was resuspended with sterile PBS, pH 7.4. The bacterial suspension was adjusted to 1×10^9 cells/ml by determining OD at 420 nm against diluent and calculating the cell density from leptospires concentration standard curve.

Leptospires were two-fold serially diluted in 100 µl of PBS. Each concentration of the leptospires (1×10^9, 5×10^8, 25×10^7, 12.5×10^7, 6.25×10^7 and 3.125×10^7) was aliqouted in a U-bottom microtiter plate (Costar). Washed human red blood cells (RBC) (group O, Rh positive) was prepared by washing EDTA treated whole blood with sterile PBS three times and the cell suspension was adjusted to 1%. One hundred microliters of the 1% RBC suspension was added into each well containing each leptospire concentration. The equal volume of UDW or PBS was mixed with the 1% RBC suspension and served as the 100% hemolysis control or the autohemolysis control, respectively. The reaction was allowed to take place in humid chamber at 37ºC for 16 hours. The optical density of the hemolysate of each well was read at the wavelength of 540 nm using PBS as the blank. The percentage of the hemolysis was calculated as follow: **percent hemolysis is equal to (OD of sample/OD of 100% hemolysis) × 100**. The amount of leptospires that caused 50% RBC hemolysis (1 HD50) was calculated from Table Curve 2D program version 5.0.

For hemolysis inhibition assay, 1% RBC suspension was prepared in PBS containing the antibody preparations (original murine MAb LPF1, murine single chain variable fragment [muscFv] and humanized-murine single chain variable fragment [huscFv]). An equal volume (100 µl) of the erythrocytes suspension containing each antibody preparations was mixed with either 1.4 or 2.8 HD50 of *Leptospira interrogans,* serogroup Pomona serovar Pomona. Appropriate hemolysis controls, *i.e.*, positive (1% RBC in UDW) and autohemolysis (1% RBC in PBS), were included in the same plate. After incubation under the same condition for the hemolysis test, the percentage of hemolysis in each well was similarly measured. The percent hemolysis

inhibition by each antibody preparation was calculated from OD of hemolysate of each sample. **Percent hemolysis inhibition is equal to [(OD of 1.4/2.8 HD50 – OD of sample)/ OD of 1.4/2.8 HD50] × 100**.

10. Experimental animals

Female outbred strain of Syrian hamsters (*Mesocricetus auratus*), 3-4 weeks of age, 40-50 g of weight, and New Zealand white rabbits (1.5-1.7 kg) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakhon Pathom province, Thailand. They were allowed to adapt to the domestication condition at our laboratory for one week before commencing the experiment under the animal ethics of the Faculty of Allied Health Sciences, Thammasat University.

11. Lethal dose fifty (LD50) determination of *Leptospira interrogans* serogroup Pomona serovar Pomona

Six groups of hamsters (6/group) were intraperitoneally injected (i.p.) with 100 µl of various amounts of leptospires (5×10^4, 5×10^3, 500, 250, 100, and 50). PBS injected group served as the negative control (group seven). The numbers of dead hamsters were recorded daily for 14 days post-infection. The dose of the leptospire that killed 50% of the animals (1 LD50) was calculated according to Reed and Muench (Reed and Muench, 1938).

12. *In vivo Leptospira* MAb LPF1neutralization test

MAb LPF1 was tested for its neutralizing activity in experimental leptospirosis model. On day zero, 12 female Golden Syrian hamsters, aged ~ 4 weeks old, were individually i.p. injected with 5 LD50 (500 cells) of *L. interrogans*, serogroup Pomona serovar Pomona. The infected animals were divided into two groups (groups 1 and 2). Six other hamsters were injected with PBS instead of the *Leptospira* and served as negative leptospirosis controls (group 3). Individual animals in group 1 were injected i.p. with purified LPF1 MAb (250 µg in 200 µl PBS per dose) on days 1 to 7 post-*Leptospira* infection. The animals in group 2 (positive leptospirosis controls) and group 3 were injected with PBS (antibody diluent) instead of the MAb. Morbidity (*i.e.*, rectal temperature, body weight, and consumption of food pellets and water) and

mortality of experimental hamsters in all groups were recorded daily. After 21 days post-infection, all survived hamsters were sacrificed and their internal organs (*i.e.*, lung, heart, liver, spleen and kidney), blood and urine were collected and subjected to culture in liquid EMJH medium for detection of live leptospires as described in **section 43**. Gross and microanatomy lesions of the individual organs were studied.

13. Hybridoma cells collection and total RNA extraction

Hybridoma cells and P3x-63-Ag8.663 myeloma cells were cultured in serum free medium. At the log phase of growth at about 75% cells confluent, they were harvested by centrifuging at 3,500 × *g*, 25°C for 30 minutes and washing with 1x PBS, pH 7.4, three times by centrifugation as above. The cell viability was examined by Trypan blue staining **(Appendix B)**. The number of cells was counted by a hemocytometer under a light microscope.

To prepare the total RNA, approximately 10^7 hybridoma cells were homogeneously resuspended in 1 ml of TRIZOL reagent (Invitrogen, USA). The preparation was kept at 25°C for 5 minutes. Two hundred microliters of chloroform was added and the mixture was shaken vigorously, incubated 2-3 minutes at 25°C and subsequently centrifuged at 12,000 × *g* at 4°C for 15 minutes. The upper phase of the mixture was transferred to a new tube. Total RNA was precipitated by adding 500 µl of cold isopropranol. The preparation was gently mixed, kept at 25°C for 10 minutes and centrifuged at 4°C for 10 minutes. The pellet containing the total RNA was washed with 75% ethanol-diethylpyrocarbonate (DEPC)-treated water. After centrifugation at 7,500 × *g*, for 5 minutes at 4°C, total RNA pellet was air-dried at 25°C, then dissolved by adding 50 µl of DEPC-treated water and kept at least 1 hour at 25°C to completely dissolve. The preparation was kept at -70°C until use. Quantity and purity of the total RNA preparation were determined by measuring its OD at the wavelengths 260 and 280 nm. The integrity of total RNA was examined by 1% agarose electrophoresis, ethidium bromide staining and visualizing using UV illuminator **(Appendix H)**.

14. Messenger RNA purification

Messenger RNA (mRNA) was isolated from the total RNA using Oligotex™ column (Qiagen, Germany). The poly-A-tail mRNA was hybridized to Oligotex [d(T) oligomer coupled to a solid phase matrix] in high-salt condition. The specific mRNA was eluted after washing out the other RNA. Purified mRNA was subsequently used as the template for synthesizing a first strand cDNA.

Approximately 750 µg of total RNA from the previous step was heated at 60°C for 3 minutes, subsequently, 250 µl of OEB binding buffer and 15 µl of oligo(dT) were added. The preparation was incubated at 70ºC for 3 minutes followed at 25°C for 10 minutes. The preparation was transferred to the d(T) oligomer column. After the column was centrifuged at 12,000 × *g*, for 2 minutes at 25°C, the first eluate which passed through the matrix in the column was discarded. The matrix in the column was washed twice with 400 µl of OW2 buffer. The mRNA was eluted from the matrix by adding 20 µl of pre-heated (70°C) OEB buffer. The column was subsequently centrifuged at 12,000 × *g*, for 1 minute at 25°C. The eluate containing mRNA was kept at -70°C or immediately subjected to cDNA synthesis.

15. First strand complementary DNA (cDNA) synthesis

The Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, USA) was used to synthesize first strand cDNA from the purified mRNA. The Oligo d(T) was specifically hybridized with mRNA and catalyzed along the chain using SuperScript™ II *RNase* H reverse transcriptase. The *RNase* H was added after the synthesis of the first-strand cDNA was completed in order to eliminate the mRNA.

The mixture of cDNA synthesis consisted of 7 µl of mRNA, 1 µl of 10 mM dNTPs, 1 µl of oligo d(T) and 1 µl of DEPC-treated water. The preparation was incubated at 65°C for 5 minutes and placed on ice bath for 1 minute. These reagents were then added to the preparation, 2 µl of 10x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT and 1 µl of recombinant *RNase* inhibitor. The preparation was incubated at 42°C for 50 minutes and followed by 70°C for 15 minutes and at 0°C on ice bath for 1 minute. One microliter of *RNase* H was added and the preparation was further incubated at 37°C for 20 minutes.

The preparation (cDNA) was kept at -20ºC until use. The cDNA was used as a template for amplification of the *VH* and *VL* DNA sequences using degenerate primers by PCR as described in the next step.

16. Amplification of heavy and light antibody variable region genes (*VH/VL*)

Genes encoding variable regions of heavy (*VH*) and light (*VL*) chains were separately amplified by polymerase chain reaction (PCR) using the respective first strand cDNA of hybridoma clone LPF1 as a template. The *VH/VL* gene segments were amplified using the degenerate primers according to Cheng *et al*. (2003). The nucleotide sequences are shown in **Table 10**.

The PCR mixture consisted of 16.3 µl of UDW, 2.5 µl of 10x *Tag* buffer with KCl, 2.0 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 1.0 µl of forward primer (10 pg/µl), 1.0 µl of reverse primer (10 pg/µl), 1.0 µl of cDNA and 0.2 µl of *Taq* DNA polymerase (5 U/µl). The preparation was thoroughly mixed and placed into PCR machine. The PCR condition for amplifying *VH* and *VL* are shown in **Table 11**. PCR amplicons were examined by 1% agarose electrophoresis, ethidium bromide staining and visualizing using a UV illuminator.

Table 10 Nucleotide sequences of degenerate primers used for murine scFv amplification

Region of coding sequence to amplified	Name of primers	Nucleotide sequence (5'→ 3') of primer
VH	**HL For**	ATG GRA TGS AGC TGK GTM ATS CTC TT
	Cγ Back	GGG GCC AGT GGA TAG AC
VL	**VL For**	GAY ATT GTG MTS ACC CAA ACT CCA
	Cκ Back	GTT GGT GCA GCA TCA GC
EVH	**VH Back**	GGC GGC GGC GGC TCC GGT GGT GGT GGA TCC GAG GT CCA GCT GCA GCA GCA GTC
	VH For	AGG ATT CGC GGC CGC TGA CGA GAC GGT GAC TGA GGT
EVL	**VL Back**	AGC CGG CCG AYA TTG TGM TSA CCC AAA CTC CA
	VL For	GGA GCC GCC GCC GCC AGA ACC ACC ACC ACC AGA ACC ACC ACC ACC CCG TTT G ATT TCC AGC GTG G
scFv	**sc Back**	AAG GAA GGC CCA GCC GGC CGA YAT TGT G
	sc For	AGG ATT CGC GGC CGC TG

(In degenerate primers, R = A or G, S = C or G, K = G or T, M = A or C, Y = C or T, W = A or T); For, forward; Back, backward

Table 11 PCR condition for amplifying *VH/VL*, *EVH/EVL* and *scFv* genes

Amplification	***VH***	***VL***	***EVH/EVL***	***scFv***
Pre-denaturation	94°C, 5 min	94°C, 5 min	94°C, 5 min	94°C, 5 min
Denaturing	94°C, 1 min	94°C, 1 min	94°C, 1 min	94°C, 1min
Annealing	55°C, 1 min	60°C, 1 min	55°C, 1 min	60°C, 1 min
Extension	72°C, 1 min	72°C, 1 min	72°C, 2 min	72°C, 2 min
Cycles	30 cycles	30 cycles	30 cycles	30 cycles
Final extension	72°C, 10 min	72°C, 10 min	72°C, 10 min	72°C, 10 min

17. Cloning of *VH* and *VL* amplicons into pGEM®-T vector

The *VH*/*VL* amplicons amplified by *Taq* DNA polymerase were designed to contain d(A) at both ends of the products such that they could be hybridized and thus ligated with d(T) overhanged pGEM®-T vector (Promega, USA).

VH and *VL* amplicons were purified to eliminate other contaminants using UltraClean™ PCR clean-up kit (SLG, Germany). The amplicons from PCR reaction was purified by mixing 5 volumes of SpinBind (buffer salt solution) to the amplicons and the mixture was applied to a spin filter unit which was spun down at 10,000 × *g*. The column was washed with 300 µl of Spinclean buffer, and then the amplicons were eluted out with UDW.

Individually purified *VH* or *VL* PCR product was ligated into pGEM®-T Easy vector according to pGEM®-T Easy vector protocol. The ligation mixture which consisted of 3 µl of purified PCR amplicons, 5 µl of 2x T4 buffer, 1 µl of linearlized pGEM-T vector and 1 µl of T4 ligase (3 U/µl) was prepared. The preparation was allowed to take place by incubating the mixtures at 4°C, overnight to get highest efficiency. The product was collected by a brief centrifugation before being introduced into *E. coli* host cells.

18. *E. coli* transformation of recombinant pGEM®-T vector

Escherichia coli strain JM109 was used as a host of either pGEM-T-*VH* or pGEM®-T-*VL* recombinant vector. The competent cells and procedure were according to the standard molecular cloning protocol (Sambrook *et al*., 1989). The ligation mixture (10 µl) from the previous step was added into prechilled 2 ml ependorf tube which contained 100 µl of the competent *E. coli* cells **(Appendix H)**. The mixture was placed on ice bath for 20 minutes to allow attachment of the vector to the competent *E. coli* cells. Subsequently, the mixture was heat shocked at 42°C for 50-60 seconds and immediately placed on ice bath for at least 2 minutes. Warmed SOC medium (950 µl) was then added to the mixture and incubated at 37°C for 1 hour with shaking at 250 rpm. The transformant cells were plated on LB/ampicillin (100 µg/ml) /40 µl of 100 mM IPTG/ 40 µl of 5% X-Gal plates and allowed to growth at 37°C for 18 hours.

19. Screening of the recombinant *E. coli* clones carrying the recombinant plasmid

White and blue *E. coli* colonies obtained from each sample were individually picked for culture in 1.5 ml of LB-ampicillin (100 μg/ml) at 37°C for 18 hours with shaking at 250 rpm. The *E. coli* cultures were harvested by centrifugation at 7,000 × *g* for 5 minutes. The plasmids were prepared by the alkali lysis protocol **(Appendix H)**.

E. coli pellet was resuspended with 200 μl of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). *RNase* A (8 mg/ml) (1 μl) was added before vortexing for 30 seconds. Solution II (0.2 N NaOH, 1% SDS) (300 μl) was then added and the preparation was placed on ice for 3-4 minutes. Solution III (3 M potassium acetate, pH 5.2, 300 μl) was added and the mixture was centrifuged at 12,000 × *g*, 25°C for 5 minutes. The supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The plasmid was precipitated from the supernatant of the mixture after centrifuged by adding 0.6 volume of cold-isopropranol. The plasmid pellet was washed with 500 μl of cold 70% ethanol and the plasmid pellet was allowed to dry at 25°C before dissolving in a small amount of UDW.

The integrity and sizes of the plasmids were analyzed under a UV-illuminator after ethidium bromide staining of the 1% agarose gel electrophoresed products. The positive plasmids which showed increased sizes of the plasmids when compared to the empty plasmid in the stained gel were subjected to amplification of their inserted *VH* or *VL* genes by PCR. Positive plasmids were sent for nucleotide sequencing and the nucleotide sequences were subjected to align by BLASTing against the database (Altschul, 1990).

20. Construction of recombinant murine *scFv* antibody (*muscFv*)

VH and *VL* DNA sequences were extended with the linkers $(Gly_4Ser)_4$ at the C- and N-termini, respectively. The respective products were called extended VH (*EVH*) and extended VL (*EVL*). The primer sequences used to amplify the respective DNA are listed in **Table 10** (according to Cheng *et al*., 2003). The PCR mixture consisted of 15.3 μl of UDW, 2.5 μl of 10x *Taq* buffer, 2.0 μl of 25 mM $MgCl_2$, 1.0 μl of 10

mM dNTPs, 1.0 µl of forward primer (10 pg/µl), 1.0 µl of reverse primer (10 pg/µl), 1.0 of either *VH* amplicon or 1.0 µl of *VL* amplicon and 0.2 µl of *Taq* DNA polymerase (5 U/µl). The condition for amplifying *EVH/EVL* are summarized in **Table 11**.

Extended *VH/VL* (*EVH/EVL*) were purified as described previously and subjected to overlapped extension PCR (SOE-PCR) for constructing murine *scFv* (*muscFv*) DNA sequences which consisted of *VH* and *VL* linked together *via* $(Gly_4Ser)_4$ linker. *Sfi* I-*Not* I restriction sites were incorporated into the sequence by using outer primers set. The primer sequences used to amplify the respective DNA are listed in **Table 10**. The PCR mixture consisted of 15.3 µl of UDW, 2.5 µl of 10x *Taq* buffer, 2.0 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 1.0 µl of forward primer (10 pg/µl), 1.0 µl of reverse primer (10 pg/µl), 1.0 of *EVH* amplicon, 1.0 µl of *EVL* amplicon and 0.2 µl of *Taq* DNA polymerase (5 U/µl). PCR amplicons were examined after 1% agarose electrophoresis, ethidium bromide staining and visualizing using UV illuminator.

21. Ligation of *Sfi* I-*Not* I digested *muscFv* into pCANTAB5E phagemid vector

SuperClean™ column was used to clean the so-prepared *muscFv* amplicons. The DNA purity was determined by calculating the readout OD at 260 and 280 nm. Purified *muscFv* DNA sequence was subsequently digested with *Sfi* I and *Not* I at N- and C- termini, respectively, to generate cohesive ends for ligating to linearlized *Sfi* I-*Not* I pCANTAB5E cloning vector. The amount and the purity of the purified *Sfi* I-*Not* I-digested *muscFv* were determined by UV-spectrophotometry (Amersham BioSciences).

To digest *muscFv* segment with *Sfi* I restriction enzyme, the preparation consisted of 44 µl purified *muscFv* DNA solution, 5 µl of 10x buffer, 1.0 µl of *Sfi* I and the mixture was then incubated at 50°C for 6 hours. *Sfi* I digested *muscFv* DNA was precipitated by adding 5 µl of 3 M sodium acetate, pH 6.5, followed by adding 100 µl of ice cold absolute ethanol. The preparation was kept at -20°C for 10 min before centrifuging at 12,000 × *g*, 25°C for 10 minutes. The supernatant was discarded and the pellet was washed twice with 70% ethanol. The pellet (*Sfi* I digested *muscFv*) was then subjected to digestion with *Not* I restriction endonuclease. The

mixture consisted of 44 µl of *Sfi* I digested *muscFv*, 5 µl of 10x buffer and 1.0 µl of *Not* I. The preparation was incubated at 37°C for 6 hours.

The *Sfi* I-*Not* I digested *muscFv* was precipitated as described above and the pellet was resuspended with 10 µl of UDW. The digested DNA was ligated into corresponding *Sfi* I- *Not* I digested pCANTAB5E phagemid vector according to the manufacture's instruction. The ligation mixture consisted of 2 µl of *Sfi* I-*Not* I *muscFv* (35 ng/µl), 1 µl of *Sfi* I-*Not* I linearlized phagemid (50 ng/µl), 1 µl of 10x OPA buffer, 5 µl of 10 mM ATP, and 1 µl of T4 DNA ligase(10 U/µl). The preparation was incubated 18 hours at 16°C and kept at -20°C until use.

22. *E. coli* transformation of murine *scFv* recombinant phagemid

The electroporation technique was used to transform recombinant phagemid into competent *E. coli* strain TG1.

E. coli strain TG1 competent cells were prepared according to the manufacture's instruction. Four microliters of ligation product was added into a pre-chilled cuvette (1 mm gap) which contained 50 µl of competent *E. coli* TG1 cells. The preparation was placed on ice bath for 1 minute. After *E. coli* cells were electrophoresed at 2,500 V, 950 µl of pre-warmed 2x-YT-G was then added. The preparation was placed in a 15 ml tube and shaked at 250 rpm, 37°C for 1 hour. All transformants were either subjected to rescue phages or kept frozen in glycerol at -70°C.

23. Rescue of phages carrying muscFv antibody

Nine hundred microliters aliquot of *E. coli* transformants was added to 9.1 ml of 2x-YT medium, then shaked at 250 rpm, 37°C for 1 hour. Subsequently, 50 µl of ampicillin (20 mg/ml) and 4 $\times 10^{10}$ pfu of M13KO7 helpher phages were added to select ampicillin-resistant recombinant *E. coli* cells for rescuing antibody phage clones. The recombinant *E. coli* were grown at 37°C, at 250 rpm for 1 hour. The *E. coli* culture was centrifuged at 3,500 × *g* at 25°C for 15 minutes and the culture supernatant was discarded. Ten milliliters glucose-deficient medium containing ampicillin and kanamycin (2x-YT-AK) was added and the *E. coli* pellet was resuspended. *E. coli* culture was allowed to grow at 37°C at 25°C for 18 hours. The

culture medium containing phages were collected after centrifuging at 7,000 × *g* at 25°C for 30 minutes and filtration through the 0.45 µm sterile Millipore membrane.

24. Preparation of phages expressing muscFv antibody

Phages displaying recombinant muscFv in the spent culture supernatant were precipitated by adding 2 ml of PEG/NaCl solution to the culture supernatant. The preparation was allowed to precipitate on ice bath for 1 hour. Precipitated phages were collected by centrifuging at 12,000 × *g* at 4°C for 20 minutes. The pellet was resuspended in 16 ml of 2x-YT medium and 14 ml of a blocking buffer with 0.1% Triton X-100, 0.01% NaN_3 and the preparation was incubated for 15 minutes at 25°C before subjecting to the biopanning process.

25. Immobilization of *Leptospira* antigen onto solid surface

Pathogenic *Leptospira interrogans* serogroup Icterohaemorrhagia serovar Copenhageni whole cell homogenate (10 µg/ml) in 3 ml of coating buffer was coated onto the surface of a 25 cm^2 tissue culture flask. The flask was incubated at 37°C for 24 hours and the uncoated antigens were removed by washing with 1x PBS, pH 7.4, three times. The unoccupied sites were blocked with blocking solution **(Appendix G)** at 37°C for 1 hour. The *Leptospira* antigen coated surface is ready for biopanning process.

26. Biopanning

Phages suspension (20 ml) was allowed to bind to pre-blocked immobilized *Leptospira* antigen at 37°C for 2 hours. Thereafter, the unbound phages were removed by excessive washing 20 times with 1x PBS, pH 7.4, followed 30 times with PBS-Tween-20 (PBS-T). Phages displaying muscFv attached to the immobilized antigen. Log phase *E. coli* TG1 (10 ml) was then added to the flask and subsequently incubated with shaking at 250 rpm at 37°C for 1 hour. An aliquot (100 µl) of the culture was spread onto the surface of SOBAG agar plate and incubated for 18 hours at 37°C. *E. coli* colonies appeared on the plate indicated successful phage transfection. *E. coli* cells were collected from the remaining culture in the flask by centrifugation at 3,500

× *g*, 25°C for 30 minutes. Bacterial cells were resuspended in 2x-YT medium containing 2x-YT-AK previously added with helper phages. The preparation was incubated 18 hours at 37°C with shaking (250 rpm). Supernatant containing the phages were collected and the phages were precipitated by adding PEG/NaCl solution **(Appendix G)**. The phages were resuspended in 2x-YT medium and used in the next round of biopanning process as described above. Thereafter, biopanning was repeated for 5 cycles. Phages collected after the fifth round of the biopanning were used to transfect *E. coli* TG1 (without helper phage). Several aliquots of the *E. coli* culture were kept as phage stocks at -70°C. One aliquot was spreaded onto SOBAG agar plate and incubated overnight at 37°C. Individual *E. coli* colonies were randomly selected and grown in 2x-YT-A medium at 37°C until OD at 600 nm was 0.5. Bacterial cells derived from individual *E. coli* colonies were collected after centrifugation and resuspended in 2x-YT-AK containing the helper phages (2.5×10^{10} pfu) and incubated with shaking for 18 hours at 37°C. Complete phages collected from individual *E. coli* culture supernatants were subjected to phage ELISA to select only for *E. coli* harboring the phages that yielded high optical densities against reagent blank.

27. Phage ELISA

Leptospira spp. whole cells homogenate or BSA (10 μg/ml) in coating buffer **(Appendix C)** which serve as a negative control were coated onto 96 wells microtiter plate (100 μl/well), incubated at 37°C for 24 hours. Unbound antigens were removed by excessive washing with 1x PBS, pH 7.4, three times and unoccupied sites were blocked with blocking solution (2% BSA) for 1 hour at 37°C in a humid chamber. After washing away of the blocking solution as described above, phages prepared from individual *E. coli* colonies were then added appropriately into antigen/BSA coated wells. PBS serves as reagent blank in the same plate. The antigen-scFv reaction was allowed to occur for 2 hour at 37°C in a humid chamber. Unbound phages were removed and all wells were washed five times with PBS-T. Horseradish peroxidase conjugated anti-M13 antibody (100 μl of 1:5,000 dilution in diluent) was added to individual wells and incubated as above for 1 hour. Unbound enzyme-

antibody conjugate were removed by washing as described above. Enzyme substrate solution [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] (ABTS) (Zymed, USA) were then added to all wells (50 µl/well). The reaction was allowed to take place in the dark for 30 minutes and stopped by adding 50 µl of stop solution in each well. The optical density at 405 nm of the content in each well was measured against negative control and blank using an ELISA reader. Positive result was the content which had optical density equal to or more than two times against the negative control.

28. Amplification of *muscFv* gene from selected phage clones

Recombinant *E. coli* clones containing *muscFv* phagemid with high optical densities against reagent blank as detected by the phage ELISA were confirmed for the presence of phagemid containing *scFv* DNA fragment by PCR. The pCANTAB5E specific primers, pCANTAB-R1 primers: (5' CCATTACGCCAAGCTTTGGAGCC-3') and R2 (5'-CGATCTAAAGTTTTGTCGTCTTTCC-3'), specific to upstream and downstream of the *scFv* DNA fragment inserted in pCANTAB5E phagemid, respectively were used. PCR mixture consisted of 16.3 µl of UDW, 2.5 µl of 10x *Tag* buffer with KCl, 2.0 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 1.0 µl of forward primer (10 pg/µl), 1.0 µl of reverse primer (10 pg/µl), 1.0 µl of 1:10 diluted plasmid and 0.2 µl of *Taq* DNA polymerase (5 U/µl). PCR amplicons were examined under 1% agarose electrophoresis, ethidium bromide staining and visualizing using UV illuminator.

29. *Mva* I restriction endonuclease analysis

A number of unique *scFv* antibody clones could be analyzed by *Mva* I DNA fragment endonuclease restriction analysis (Hyland *et al.*, 2003). The *scFv* PCR amplicons were subjected to digestion by *Mva* I restriction enzyme. The reaction consisted of 34 µl of UDW, 10 µl of *scFv* amplicon, 5 µl of 10x buffer and 1 µlof *Mva* I. The mixture was incubated at 37°C for 4 hours and 10 µl of reaction was subjected to 12% polyacrylamide gel electrophoresis, ethidium bromide staining and visualizing using UV illuminator for the digested DNA patterns. Different DNA patterns implied the different nucleotides within *scFv* nucleotide sequences.

30. Expression of recombinant scFv antibody

Selected phage clones carrying high-optical density antibodies from phage ELISA were used to transfect log-phase *E. coli* strain HB2151 for production of soluble scFv proteins. Three microliters of selected phages was added to 1 ml of log-phase *E. coli* HB2151 and the phages were allowed to transfect for 1 hour, 37°C with shaking at 250 rpm. Thereafter, a loopful of the infected *E. coli* was streaked onto the selective medium (SOBAG-N) containing ampicillin, nalidixic acid and glucose. Transfected *E. coli* were allowed to grow at 37°C for 18 hours. Isolated colonies of *E. coli* HB2151 on the SOBAG-N plate were individually and randomly picked and confirmed for phagemid carrying *muscFv* gene by PCR as described in **section 28**. Positive *E. coli* clones were kept frozen in a glycerol at -70°C. A loopful of aliquot from the stock culture was streaked on SOBAG-N plate. Isolated *E. coli* colonies were individually inoculated into 10 ml of 2x-YT-A medium and cultured at 37°C with shaking at 250 rpm for 18 hours. Five milliliters of *E. coli* culture was used as a starter for adding into the fresh 500 ml of 2x-YT-A medium. The preparation was incubated with shaking at 250 rpm until the OD at 600 nm reaches 0.5. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for induction of transcription of *scFv via lac*Z promotor. The culture was further incubated with continuous shaking as described above for 4 hours. Bacterial cells were harvested by centrifugation at 4,200 × *g* at 25°C for 30 minutes. The pellet was washed three times with 1x PBS. Washed pellet was kept at -20°C until used as a material for extracting recombinant scFv protein.

31. Extraction of periplasmic contents of the recombinant *E. coli*

The procedure used for extraction of protein in periplasmic space was performed using the method from Skerra and Pluckthun (1988) with modification. *E. coli* pellet prepared as described in section 30 was resuspended in 10 ml of ice-cold 1x Tris-EDTA-Sucrose pH 8.0 (1x TES) and the suspension was mixed thoroughly, followed by adding 15 ml of 1/5x TES, pH 8.0. Osmotic shock was performed by placing the preparation on an ice bath with occasional stirring for 1-2 hours. The spheroplasts were collected by centrifugation at 10,000 × *g* for 10 minutes at 4°C. The supernatant

containing recombinant scFv was collected and kept at 4°C. Protein contents of the preparation were determined by measuring the optical densities at 280 nm and the recombinant antibody were subsequently purified.

32. Purification of recombinant scFv antibody by anion-exchange chromatography column

In this study, recombinant scFv was purified by the conventional anion-exchange chromatography column (Adapted from Laroche-Traineau *et al*., 2000). Crude scFv periplasmic protein in TES buffer was dialyzed against 50 mM Tris-HCl, pH 7.5 buffer for 48 hours with several changes of buffer and subsequently concentrated by 10 kDa cut-off amicon (Millipore) to final volume of 50 ml. The concentrated preparation was filtered through 0.45 µm sterile Millipore membrane. The amount of crude proteins was measured by UV spectrophotometer at wavelength 280 nm. The crude preparation was then loaded to the 50 mM Tris-HCl, pH 7.5 pre-equilibrated DEAE sepharose flast flow (Amersham BioSciences) packed into a 2.0 × 30 cm column. The proteins were allowed to bind to the matrix for 1.5 hours. The unbound proteins were thoroughly washed out from the column with 50 mM Tris-HCl, pH 7.5 until the optical density (280 nm) of the eluate was zero. The bound proteins were eluted from the column by a linear gradient 0-0.5 M NaCl. Two milliliter fractions were collected and optical density at 280 nm was measured. Three microliters of each fraction was individually dotted onto NC and probed with anti-E-tag-HRP conjugate. The signal was developed with the substrate 2',6' dichlorophenol indophenol (DCIP). Positive fractions were monitored for their purity by 12% SDS-PAGE under reducing condition and stained with either CBB or silver stain. Purified scFv protein were pooled together, dialyzed against 1x PBS, pH 7.4 and concentrated by Amicon. The amount of the purified scFv protein was determined by Bradford assay. Purified scFv recombinant protein preparation was aliquoted and stored at -20ºC until use.

33. Purification of recombinant scFv antibody by anti-E-tag affinity chromatography

The constructed scFv carried the 13 amino acids peptide tag (E-tag) at the C-terminal which can be recognized by anti-E-tag monoclonal antibodies. E-tag scFv

bound specifically to anti-E-tag column at neutral pH and could be eluted out from the column using the buffer with decreasing pH.

The crude scFv protein in TES buffer was adjusted the pH to 8.0 before loading to binding buffer pre-equilibrated anti-E tag column. The recombinant scFv was allowed to bind specifically to the matrix for 2 hours at 25°C. Unbound proteins were washed out with binding buffer until the OD at 280 nm of the eluate was zero. The bound scFv was eluted from the matrix with eluting buffer. One milliliter fractions were collected and immediately neutralized with 150 µl of the neutralizing buffer. The eluted fractions were collected until the optical density at 280 nm of the eluate was equal to baseline of the eluting buffer. The positive fractions which had optical density at 280 nm higher than the baseline were pooled and dialyzed against PBS, pH 7.4. The purity of purified scFv was assessed by 12% SDS-PAGE and either Coomassie Brilliant Blue or silver staining.

34. Purification of recombinant scFv antibody by *Leptospira* coupled CNBr-activated Sepharose-4B affinity chromatography

34.1 Coupling of leptospire antigen to CNBr-activated sepharose-4B

CNBr activated-sepharose-4B powder (Pharmacia, Sweden) was swollen in 1 mM HCl (200 ml of the HCl solution per 1 g of resin) at 25°C for one day to gave a volume of gel about 3.5 ml. The gel was then washed in coupling buffer for at least 2 times. The buffer was discarded by centrifuging the resin at 700 × *g* for 5 minute at 25°C and the *Leptospira* homogenate (serovar Copenhageni) (80 mg in 10 ml) was immediately transferred into the gel preparation. The mixture was mixed by an end-over-end mixer for 18 hours at 4°C. The unbound *Leptospira* antigen was extensively washed away by coupling buffer. The remaining active groups of the gel were blocked by blocking solution for 1 hour at 25°C. The excess blocking buffer was washed two times of a series buffers, *i.e.* a coupling buffer, sodium acetate buffer and coupling buffer. Thereafter, the *Leptospira* antigen bound-gel was subsequently equilibrated with starting buffer. This gel was stored at 4°C until use.

34.2 Purification of *Leptospira* specific scFv

Crude murine or humanized scFv in PBS was applied to the column containing CNBr-activated sepharose-4B coupled with *Leptospira* antigen. The gel preparation was mixed by an end-over-end mixer for 18 hours at 4°C. The gel was then washed with starting buffer in order to wash the unbound proteins. The bound protein (scFv specific to *Leptospira* antigen) was eluted out from the gel with eluting buffer. One milliliter fractions were collected and immediately neutralized with 150 µl of the neutralizing buffer. The used gel was re-equilibrated with strating buffer. The eluted fractions were collected until the optical density at 280 nm of the eluate was equal to baseline of the eluting buffer. The positive fractions were similarly determined as **section 33**.

35. Identification of murine immunoglobulin frameworks (FRs) and complementarity determining regions

Selected *E. coli* harboring murine anti-*Leptospira* scFv amino acid sequences were analyzed for the regions of their FRs and CDRs of both VH and VL. Phagemids carrying *muscFv* were isolated from the selected *E. coli* colonies and the *muscFv* nucleotides were sequenced by an automated DNA sequencer (ABI PRISM model 377) using pCANTAB5E specific primers; R1 and R2 as described in **section 28**. Murine *VH* and *VL* DNA sequences were deduced for amino acid sequences by EMBOSS program. Their FRs and CDRs were identified by using NCBI Ig BLAST and V-Quest software provided by the International ImMunoGeneTics database.

36. Selection and amplification of most matched-human VH and VL sequences to the murine VH and VL sequences

Murine VH and VL amino acid sequences obtained from selected phagemids were individually aligned to the human VH and VL, respectively, using the immunoglobulin sequence database. The human VH and VL amino acid sequences with high homology against murine VH and VL were selected and used as an acceptor for transferring murine CDRs onto the human frameworks.

Specific primers were designed from the selected human *VH* and *VL* nucleotide sequences from the database for amplification of the genes using pooled human naïve immunoglobulin cDNA as the template by PCR.

Human naïve immunoglobulin cDNA was synthesized from total RNA of pool peripheral blood mononuclear cells (PBMCs) obtained from healthy Thai blood donors. The PCR mixture consisted of 16.3 µl of UDW, 2.5 µl of 10x *Tag* buffer with KCl, 2.0 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 1.0 µl of forward primer (10 pg/µl), 1.0 µl of reverse primer (10 pg/µl), 1.0 µl of pooled human immunoglobulin cDNA and 0.2 µl of *Taq* DNA polymerase (5 U/µl). DNA amplicons were analyzed by 1% agarose gel electrophoresis, ethidium bromide staining and visualizing using a UV illuminator. Selected human *VH* and *VL* amplicons were purified and used as templates for grafting murine *CDRs* onto human *FRs*.

37. Construction of humanized-murine *scFv* (*huscFv*) against pathogenic *Leptospira*

To construct humanized-murine *scFv*, the murine DNA sequences encoding the three *CDRs* specific against *Leptospira* were grafted onto the respective human immunoglobulin frameworks (*FRs*) by using primers induced mutagenesis and SOE-PCR techniques.

For humanization of the murine *VH*, four DNA segments were constructed sequentially: (1) N-terminus containing the *Sfi* I restriction site, human *VH FR1* sequence, and murine *CDR1* sequences; (2) murine *CDR1*, human *FR2*, and murine *CDR2* sequences; (3) murine *CDR2,* human *FR3,* and murine *CDR3* sequences; and (4) murine *CDR3*, human *FR4,* and $(Gly_4Ser)_4$ nucleotide sequences. The first and the second segments were linked together by SOE-PCR and the third and the fourth segments were similarly linked. Finally, the two segments were brought into contiguity, *i.e.,* the humanized-murine *VH* sequence, also by the SOE-PCR. **Figure 15** depicts the sequential steps for murine *VH* humanization. The humanized-murine *VL* sequence was similarly constructed: (1) N-terminus with $(Gly_4Ser)_4$ nucleotide sequences, human *VL FR1* sequence, and murine *CDR1* sequences; (2) murine *CDR1*, human *FR2*, and murine *CDR2* sequences; (3) murine *CDR2,* human *FR3,* and murine *CDR3* sequences; and (4) murine *CDR3*, human *FR4,* and *NotI* restriction site. The humanized *VL* was similarly linked. **Figure 15** also shows the sequential steps for murine *VL* humanization. The humanized *VH* and *VL* segments were linked together

via the $(Gly_4Ser)_4$ nucleotide sequence at 3'-end of the humanized *VH* and 5'-end of the humanized *VL* to make a complete *huscFv* DNA sequence.

The *huscFv* DNA sequence was purified and subjected to *Sfi* I and *Not* I restriction enzyme endonuclease digestions, respectively as described the reaction mixture in **section 21**. *Sfi* I-*Not* I digested *huscFv* fragment was ligated into the corresponded restriction sites of the linearlized pCANTAB5E phagemid vector and the recombinant phagemids were introduced into competent *E. coli* strain TG1. The recombinant *E. coli* harboring phagemid containing *huscFv* were selected on SOBAG plate. Individual *E. coli* colonies were screened for high reactivity against various pathogenic *Leptospira* using the phage ELISA. Phages from the selected clones were used to transfect *E. coli* strain HB2151 for the protein expression. The selected recombinant *E. coli* clones were kept frozen in glycerol stock at -70°C.

Recombinant huscFv antibodies were overexpressed in *E. coli* and subsequently purified by the same method described in **section 32**.

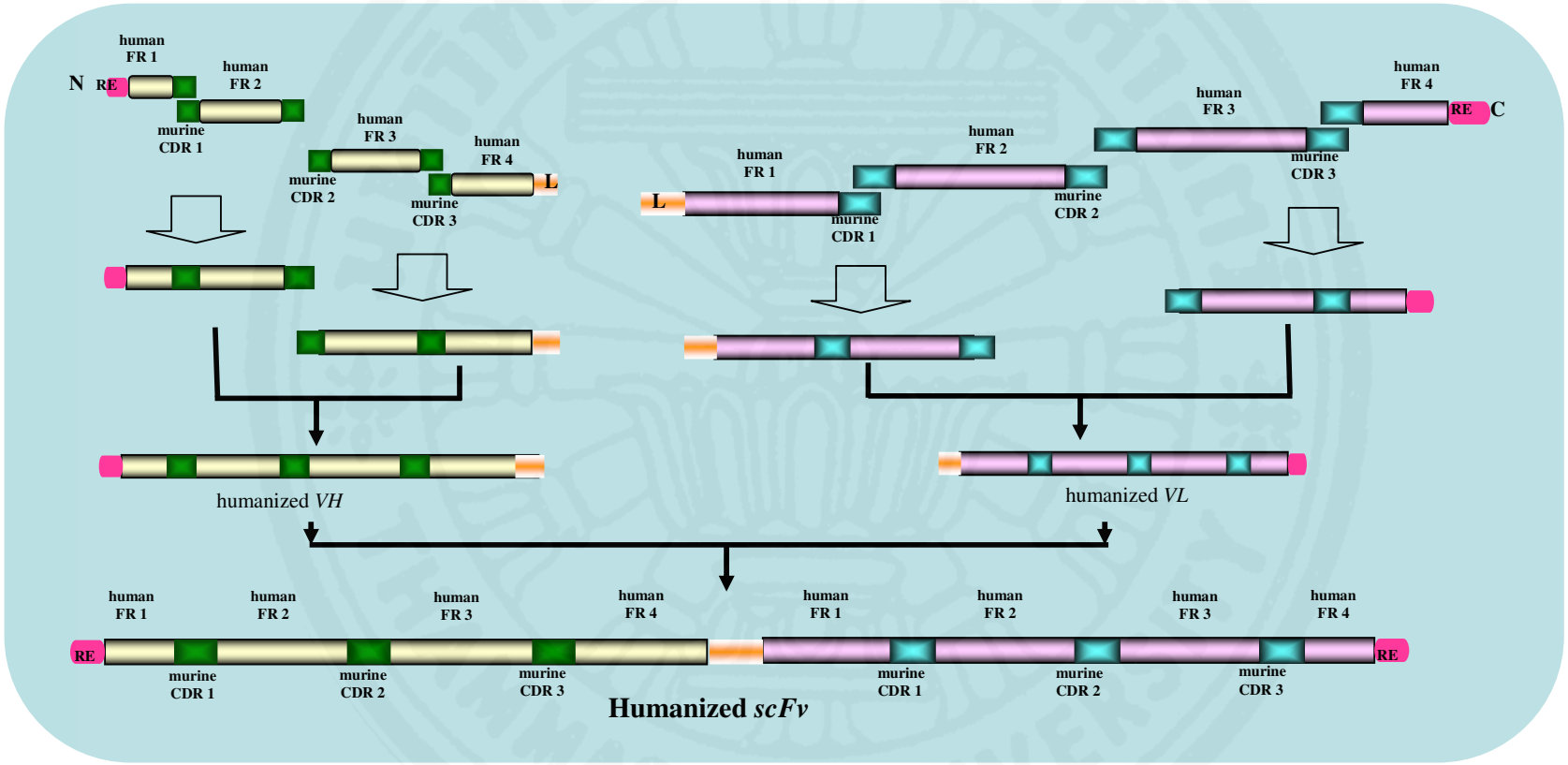

Figure 15 Sequential steps for construction of humanized *scFv*.

38. Preparation of human immunoglobulins

Human immunoglobulins (Igs) were prepared from the pooled human sera by slowly and dropwise adding of an equal volume of saturated ammonium sulfate solution to the sera while gently stirring in an ice bath. The solution was kept at 4°C for 18 hours to ensure complete precipitation. Thereafter, the solution was centrifuged at 12,000 × *g* for 15 minutes at 4°C. The pellet was dissolved in small volume of PBS, pH 7.4. The preparation was placed in a dialysis tube and extensively dialyzed against PBS at 4°C for 48 hours with several changes of buffer. The protein content was measured by Bradford protein assay. The Igs was analyzed by 10% SDS-PAGE under either non or reducing condition and Coomassie Brilliant Blue staining. The Igs solution was aliquoted and kept at -70°C.

39. Purification of human immunoglobulin by protein A-sepharose CL-4B

In this study, human Igs were purified by the affinity chromatography using protein A-sepharose CL-4B column chromatography method. One milliliter of protein A-sepharose CL-4B was equilibrated with PBS, pH 8.0 in a screw capped tube. Human IgG were allowed to bind specifically to the gel by adding approximately 20 mg of the precipitated Igs to the tube and the tube was mixed on an end to end mixer at 4°C for 18 hours. The unbound antibodies and other contaminants were extensively washed away with PBS, pH 8.0, until the OD at 280 nm of the washing buffer was zero. The protein bounded resin was loaded in a column and the bound Igs were then eluted from the column with 0.1 M citric acid, pH 4.5 in 1 ml-fractions in microtubes containing 50 µl of 1 M Tris-HCl, pH 8.5. The fractions were collected until the optical density of the eluate was dropped to the baseline. The individual eluted fractions containing detectable optical density were pooled and analyzed for the purity of the Igs by 10% SDS-PAGE. The remaining gel was equilibrated in PBS, pH 8.0 and stored at 4°C.

40. Preparation of polyclonal rabbit anti-human light chain antibody

Purified human Igs was separated by 10% SDS-PAGE under reducing condition. Under the reducing condition, Igs were separated into Fab and light chain fragments

with relative molecular mass ~50 and 27 kDa, respectively. The gel containing the separated protein was stained with Coomasssie Brilliant Blue R250, destained and preserved in 2% glycerol. The lower protein band (expected human light chain) was cut from the gel and minced in PBS, pH 7.4 buffer and used as immunogen for rabbit immunization.

To produce rabbit polyclonal antibody, one milliliter of blood was collected from the ear of the rabbit before immunization and the serum was used as pre-immunized serum. Thereafter, separated human light chain in stained polyacrylamide gel in PBS buffer was injected intramascularly (i.m.). The animal received three booster doses at 2 week intervals of the same immunogen and the same route. Before each immunization, the blood sample was collected and the antibody titer was determined by an indirect ELISA against purified human Igs. The antigenic specificity of the antibody was determined by Western blot analysis.

When the maximum serum antibody titer of the immunized animal was reached, the blood sample was collected from the heart puncture. The rabbit anti-human light chain serum was aliqouted in small volumes and kept at -70°C. Rabbit Igs were prepared by precipitation from the immune serum by the method as described in **section 38**. They were dialyzed against PBS, pH 7.4 buffer. The preparation was used as the secondary antibody in the antigenic specificity determination of scFv derivatives.

41. Characterization of antigenic specificity of recombinant muscFv and huscFv

In this study, SDS-PAGE and Western blotting were used for determining the antigenic specificities of the antibody preparations. First of all, whole cell homogenates of various *Leptospira* serovars were prepared. Approximately 30 μg of each homogenate was separated by 12% polyacrylamide gel electrophoresis. Separated *Leptospira* components were electro-transblotted onto NC for Western blot analysis. The unoccuplied sites of transblotted NC were blocked with the blocking solution (3% BSA) at 25°C for 1 hour. After washing the excess buffer with PBS-T, blocked NC was incubated with the purified antibody derivatives, *i.e.*, murine single chain variable antibody fragments (muscFv) or huscFv, at 25°C for 1-2 hours. After the unbound antibody was washed away, the NC were incubated with a 1:1,000

dilution of rabbit anti-human light chain. The reaction was allowed to take place at 25°C for 1 hour. The excess unbound antibody was washed as described above. The NC was subsequently incubated with alkaline phosphatase goat-anti-rabbit IgG isotype. NC was equilibrated in 0.15 M Tris, pH 9.6 for 15 minutes before adding the BCIP/NBT chromogenic substrate. The reaction was stopped by washing NC with UDW until the background was cleared.

42. *In vivo Leptospira* neutralization test by recombinant huscFv

The huscFv recombinant antibody was tested for its neutralizing activity in an experimental leptospirosis model. In trial 1, female Golden Syrian hamsters, aged ~ 4 weeks old, were divided into 3 groups. Group 1: 6 hamsters were intraperitoneally (i.p.) injected with 1,000 LD50 of *L. interrogans*, serogroup Pomona, serovar Pomona in PBS containing 100 µg of purified huscFv. Group 2: 6 hamsters were i.p. injected with 1,000 LD50 in PBS without antibody. This group served as positive leptospirosis control. Group 3: 6 hamsters were i.p. injected with PBS (antibody diluent) instead of leptospires (negative leptospirosis control). The mortality of experimental hamsters in all groups were recorded daily until 14 days post-infection.

In trial 2, 20 hamsters were individually i.p. injected with 10 LD50 of *L. pomona.* The infected animals were divided into two groups, *i.e*. groups 1 and 2. Ten other hamsters were injected with PBS instead of the *Leptospira* and served as negative leptospirosis control (group 3). Individual animals in group 1 were i.p. injected with purified huscFv (100 µg in 100 µl PBS per dose), 5 doses at 12 hours interval post-*Leptospira* infection. The animals in group 2 (positive leptospirosis controls) and group 3 were injected with PBS (antibody diluent) instead of the huscFv. Percent survival of the animals was monitored along 14 days after treatment.

In trial 3, on days zero, 12 female Golden Syrian hamsters, aged ~ 4 weeks old, were i.p individually injected. with 5 LD50 of *L. pomona.* The infected animals were divided into two groups (groups 1 and 2). Six other hamsters were injected with PBS instead of the *Leptospira* and served as negative leptospirosis controls (group 3). Individual animals in group 1 were i.p. injected with purified huscFv (250 µg in 200 µl PBS per dose) on days 1 to 7 post-*Leptospira* infection. The animals in group 2 (positive leptospirosis controls) and group 3 were injected with PBS (antibody diluent)

instead of the scFv. Morbidity (*i.e.*, rectal temperature, body weight, and consumption of food pellets and water) and mortality of the experimental hamsters in all groups were recorded daily for 21 days post-treatment. The survived hamster in each group were terminated. The internal organs including lung, heart, liver, spleen and kidney were studied for gross lesions and the persistence of the leptospires in the particular organs. Moreover, leptospires in blood and urine were cultured and all the culture was monitored for the growth of leptospires under the dark field microscopy (DFM).

43. *Leptospira* culture from clinical samples

After 21 days post-infection, survived hamsters in all groups were sacrificed. One to two drops of whole blood from the heart puncture were immediately inoculated into 5 ml of liquid EMJH medium containing 200 μg/ml of 5-FU and the mixture was incubated at 29-30°C in a low-temperature incubator for 6 weeks. The growth of the leptospires was weekly monitored under DFM. Internal organs of the survived hamsters were individually washed with sterile NSS and a piece of individual tissues was minced in sterile basal EMJH medium. The supernatant was collected by centrifugation at 3,000 × *g*, at 25°C for 5 minutes and 100 μl of each sample was inoculated in 5 ml of 5-FU supplemented liquid EMJH medium. The growth of leptospires was monitored as described above.

44. Hematoxylin and eosin (H&E) staining of tissue sections

The internal organs of each hamster including lung, heart, liver, spleen and kidney were fixed with buffered neutral formalin buffer for at least 1 day, dehydrated with ethanol and embedded in paraffin. The paraffin sections (4-6 μm thick) of individual tissues were placed individually on the microscopic slides and deparaffined by placing the slides in a xyline solution for 2-4 minutes. The slides were washed thoroughly by dipping into an absolute ethanol solution and rehydrated with 95% and 70% ethanol, respectively. The preparations were placed in Lugol's iodine solution for 15 minutes, rinsed by a running tap water and placed in a 5% aqueous sodium thiosulfate solution for 3 minutes. The tissue sections were stained by placing them into a Mayer's hematoxylin solution for 15 minutes, rinsed under the running tap water for 20 minutes, then counter-stained by placing them in an eosin solution for 1

minute. Thereafter, the slides were dehydrated with gradients of 70%, 95% and absolute alcohol ethanol. The slides were dipped in xylene solution, mounted with a mounting medium and covered with coverslips. They were examined under a light microscope.