CHAPTER 2

Research design and methods

2.1 Research design

Genomic DNA of 638 northern Thai primigravidarum volunteers were extracted by salting out method and used for the identification of α-thalassemia genotypes by gap-PCR and PCR-RFLP. Four common genotypes of α-thalassemia including α^0-thalassemia $--^{SEA}$ type, α^+-thalassemia $-\alpha^{3.7}$ type, α^+-thalassemia $-\alpha^{4.2}$ type and HbCS were performed. For generation of a novel recombinant scFv antibody, mouse hybridoma producing monoclonal antibody highly specific to Hb Bart's (clone 2D4) was a starting material. Total ribonucelic acid (RNA) was isolated from mouse hybridoma. Complementary DNA (cDNA) was subsequently synthesized, and used as templates for the V_H and V_L gene amplification by the established antibody specific primers. The amplified products of V_H and V_L genes were individually cloned into pGEM®-T easy vector, and then, transformed into *E. coli* TOP10F. Clone of V_H and V_L genes were individually determined by the commercial direct DNA sequence of the dye termination method. The deduced amino acid sequence of the cloned V_H and V_L chains were individually identified for the complementarity determining regions (CDRs) by Kabat and Chothia numbering scheme. The selected clone of V_H and V_L genes were connected via a short linker to be the full length scFv gene by splice overlapped extension-polymerase chain reaction (SOE-PCR). The full length scFv gene was ligated into pET28a(+) expression vector, and then, transformed into *E. coli* BL21(DE3). The N-terminal histidine fusion scFv antibody expressing in BL21(DE3) was purified by Ni-NTA affinity chromatography. The specificity of the produced scFv antibody against Hb Bart's was investigated by Western blot analysis and indirect ELISA. The sensitivity of scFv antibody for capturing Hb Bart's in blood samples was determined by dot blot ELISA. Finally, the affinity constant (K_a) of the scFv

anti-Hb Bart's was calculated by mathematical equation using indirect ELISA. The overview procedures of the generation of scFv were summarized in Figure 2.1.

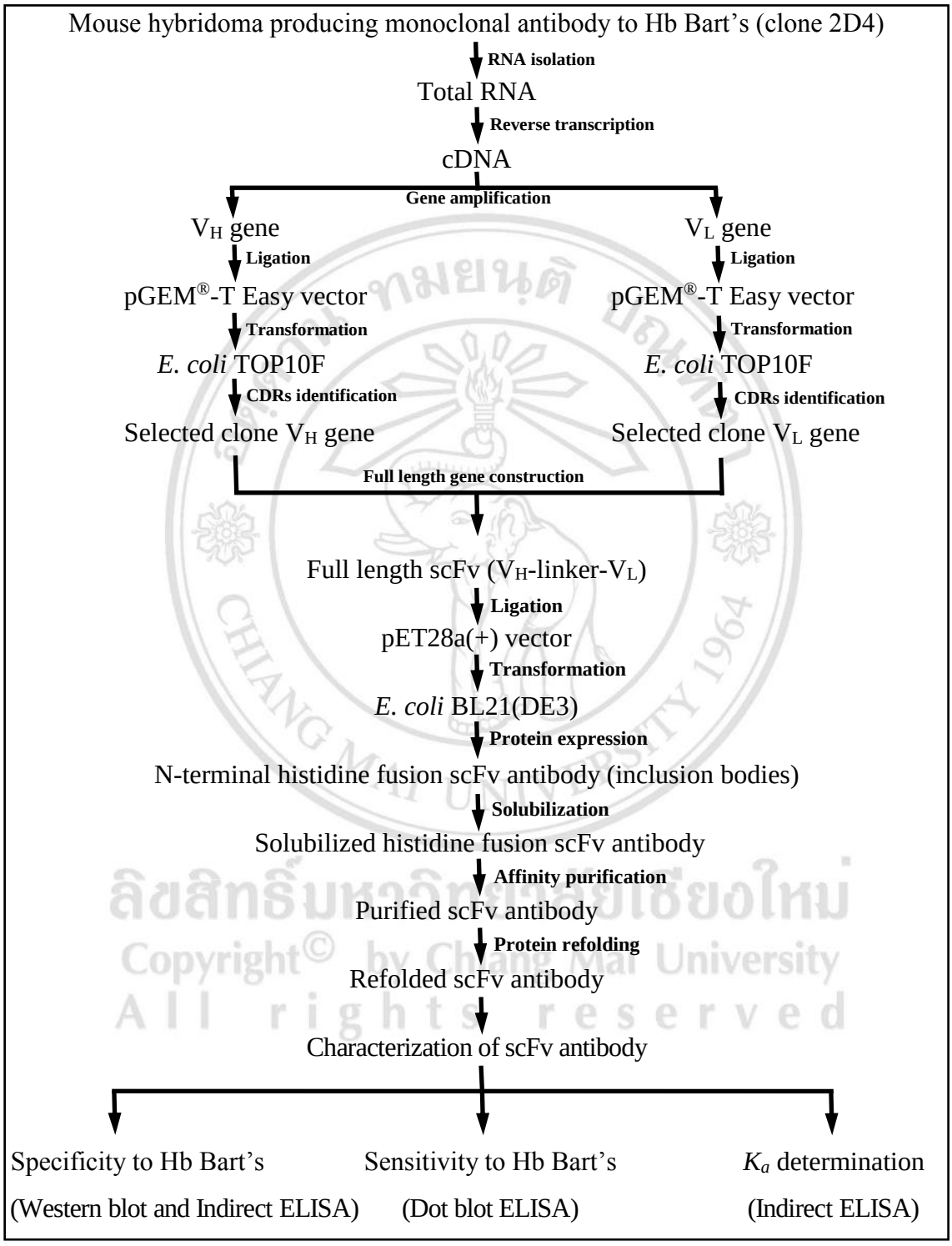

Figure 2.1 Schematic representation of the procedures of the scFv generation.

2.2 Classification of α-thalassemia genotypes by gap-PCR and PCR-RFLP

Blood samples of 638 primigravidarum (first time of pregnancy) who came to the antenatal clinic at Maharaj Nakorn Chiang Mai Hospital previously collected during July 2009 to 2010 were used in this study. All samples were approved by the Research Ethics Committee of the Faculty of Medicine of Chiang Mai University, Chiang Mai, Thailand (No. 198/2009). All genomic DNA previously extracted by the salting out method [65] were subjected for the identification of the α-thalassemia genotype by gap- PCR [4,7, 66] and PCR-RFLP [6].

2.2.1 Identification of α^0-thalassemia $--^{SEA}$ type

Detection of α^0-thalassemia $--^{SEA}$ type was modified from the previous method described by Bowden et al. [4]. Multiplex gap-PCR reaction tube containing SEA7, 8 and 9 primers (Table 2.1) was performed simultaneously. The primers amplified the $--^{SEA}$ deletion amplified between φα2 gene to 3′ hypervariable region. The product from SEA7 and 8 primers was 1,011 base pair (bp) for normal φα2 gene while product from SEA7 and 9 primers was 660 bp for $--^{SEA}$ deletion. PCR reaction mixture was performed in 50 µL of reaction tube containing 0.5 µg DNA, 1x buffer Phusion™ HF, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each primer and 1 unit of DNA polymerase (Finnzymes, Finland) . PCR was run in a Perkin Elmer Cetus model 2400 thermal cycler with the following profile: initial denaturation at 98ºC for 30 s, followed by 30 cycles of denaturation at 98ºC for 10 s; annealing at 62ºC for 30 s; extension at 72ºC for 30 s; with an additional 10 min extension at 72ºC in the final cycle. Ten microliters of PCR product were analyzed by separation on 2% agarose gel in 0.5x Tris-acetate-EDTA (TAE) buffer at 100 V for 50 min. Agarose gel was stained with 5 µg/mL of ethidium bromide solution for 5 min then destained thrice in distilled water. The DNA bands were visualized by UV light and documented by using a Bio-Rad gel doc 1000. The locations on chromosome 16 of SEA7, 8 and 9 primers for the amplification of α^0-thalassemia $--^{SEA}$ type was described in Figure 2.2.

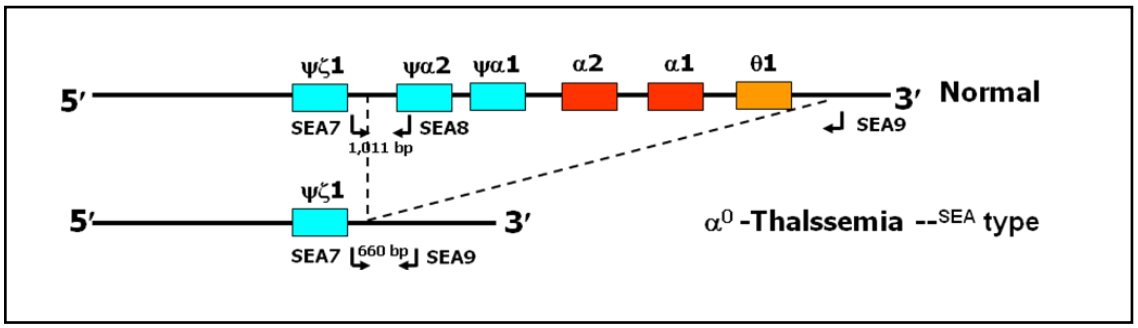

Figure 2.2 The locations on chromosome 16 of the specific primers for the amplification of α^0-thalassemia $--^{SEA}$ type.

2.2.2 Identification of α^+-thalassemia $-\alpha^{3.7}$ and $-\alpha^{4.2}$ type

Gap-PCR for detection of α^+-thalassemia $-\alpha^{3.7}$ and $-\alpha^{4.2}$ types were modified from the methods described by Smetanina and Huisman [7] and Baysal et al.[66], respectively. Detection of $-\alpha^{3.7}$ type was performed in two separate reactions for each DNA sample, the first reaction tube contained normal primers (3.7A and B) while the second tube contained deletion primers (3.7A and C) (Table 2.1). The primers amplified normal α2 gene, 3.7A and B, amplified between promoter to 3′ untranslated region behind the α2 gene. Another pair of primers, 3.7A and C, for the $-\alpha^{3.7}$ deletion amplified between promoter of α2 gene to 3′ untranslated region behind the α1 gene. Because of the products from both pair of primers were 1.76 kb for normal α2 gene and $-\alpha^{3.7}$ deletion, the two separate reactions are perform simultaneously for each DNA sample. Fifty-five microliters of reaction mixture were contained 67 mM Tris buffer pH 8.8, 16.6 mM $(NH)_2SO_4$, 10 mM β-mercaptoethanol, 2.0 mM $MgCl_2$, 0.1 mg/mL BSA, 0.16 mM dNTP, 0.45 µM of each primer, 7.5% DMSO, 2.5 units of Taq DNA polymerase (Promega, USA) and 0.5 µg of DNA. The PCR was performed in a thermal cycler with the following profile: initial denaturation at 99ºC for 6 min, followed by 25 cycles of denaturation at 95°C for 1 min; annealing at 68ºC for 1 min; extension at 72ºC for 2.5 min, with an additional 7 min extension at 72ºC in the final cycle.

Determination of $-\alpha^{4.2}$ type was performed in one tube containing 3 primers of 4.2G, E and F (Table 2.1). The reaction mixture and the PCR thermal cycler were

performed as described above with the exception of the annealing temperature at 55ºC for 1 min. PCR product from 4.2G and F primers was 228 bp while product from 4.2G and E primers was 1.76 kb. PCR product was analyzed by separation on 1.5% agarose gel electrophoresis as described in method 2.2.1. The locations on chromosome 16 of 3.7A, B, C and 4.2G, E, F primers were described in Figure 2.3A and B respectively.

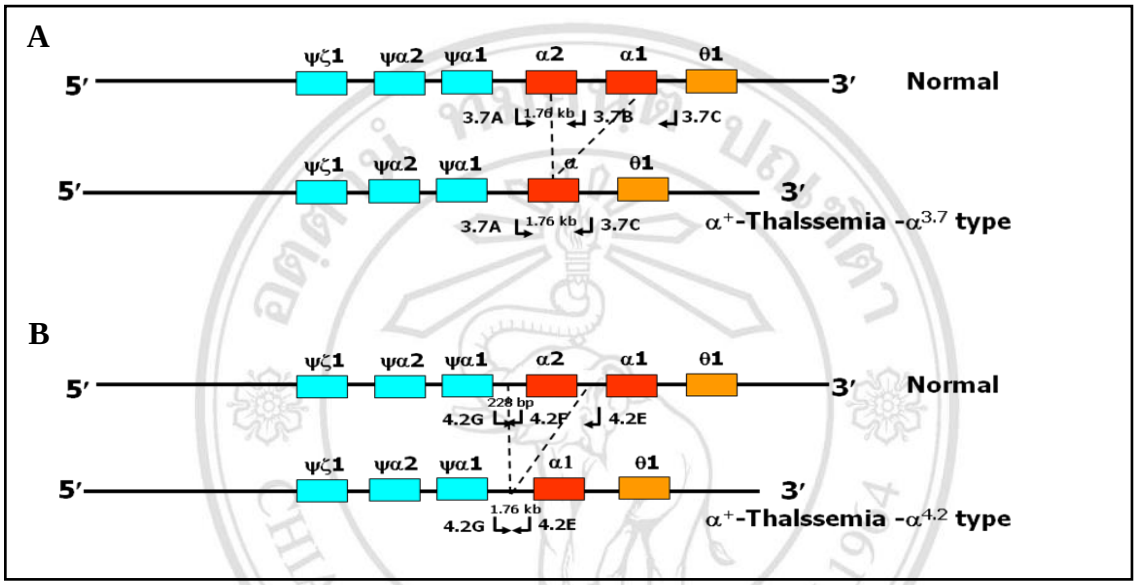

Figure 2.3 The locations on chromosome 16 of the specific primers for the amplification of α^+-thalassemia $-\alpha^{3.7}$ type (A) $-\alpha^{4.2}$ type (B).

2.2.3 Identification of nondeletional α^+-thalassemia HbCS

HbCS mutation was detected by PCR-RFLP described by Makonkawkeyoon et al. [6]. Primer CSF and R (Table 2.1) amplified between the second intron to 3′ untranslated region of the α2 gene. The reaction was carried out in a 50 μL volume contained 0.5 μg of DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer and 2 units of Taq DNA polymerase (Promega, USA). The reaction was performed using the thermal cycler with the following profile: 30 cycles of denaturation at 95ºC for 1 min; annealing at 63ºC for 1 min; extension at 72ºC for 30 s, after the initial incubation at 95ºC for 5 min. PCR product was precipitated by adding 5 μL of 5 M NaCl into 50 μL of PCR product then mixed well. One hundred and forty microliters of

cold absolute ethanol were added, mixed well then stored at -20ºC for overnight. PCR product mixture was centrifuged at 13,000 rpm for 30 min. The pellet of PCR product was found in the bottom of tube then discarded the supernatant. One hundred and forty microliters of 70% ethanol were added into a pellet of PCR product then mixed and centrifuged at 13,000 rpm for 5 min. The pellet of PCR product was washed thrice with 70% ethanol then dried at room temperature for 30 min. The pellet of PCR product was reconstituted with 10 µL of deionized water. One microliter of PCR was added into 99 µL of 10 mM Tris-EDTA (TE) buffer pH 8.0 for determination of DNA concentration by measurement the absorbance at 260 nm. One microgram of PCR product, size 276 bp, was further digested by 2 units of *MseI* (New England Biolabs, England) and incubated at 37ºC for overnight. All of digested PCR product was analyzed by separation on 3% Nusieve agarose gel electrophoresis in TAE buffer at 100 V for 50 min as described in method 2.2.1. The locations on chromosome 16 of CSF and R were described in Figure 2.4.

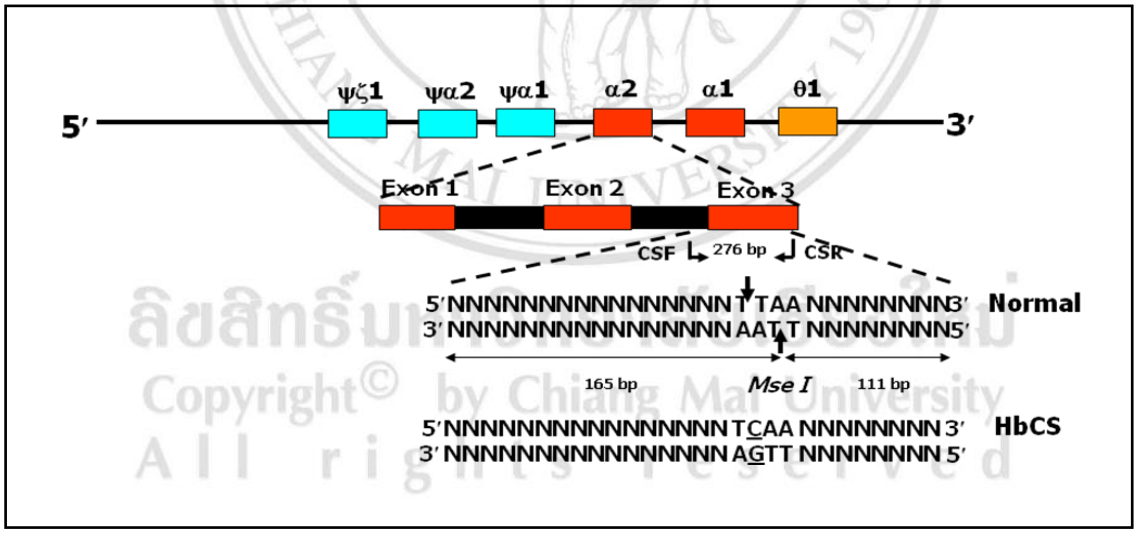

Figure 2.4 The locations on chromosome 16 of the specific primers for the amplification of HbCS and *MseI* restriction sites.

Table 2.1 Sequences of primers for PCR genotyping of α-thalassemias.

Primers	Sequences	Location on chromosome 16
SEA7	5′-CTCTGTGTTCTCAGTATTGGAG-3′	135285-135306
SEA8	5′-TGAAGAGCCTGCAGGACCAGGTCA-3′	136272-136295
SEA9	5′-ATATATGGGTCTGGAAGTGTATC-3′	155586-155608
3.7A	5′-CCCTCCCCCTCGCCAAGTCCACCCC-3′	141904-141928
3.7B	5′-GGGAGGCCCATCGGGCAGGAGGAAC-3′	143645-143669
3.7C	5′-GGGGGGAGGCCCAAGGGGCAAGAA-3′	147461-147484
4.2G	5′-CCGGTTTACCCATGTGGTGCCTC-3′	139287-139309
4.2E	5′-CCCTGGGTGTCCAGGAGCAAGCC-3′	145228-145250
4.2F	5′-GGCACATTCCGGGACAGAGAGAA-3′	139492-139514
CSF	5′-TGCGGGCCTGGGCCGCACTGA-3′	143460-143480
CSR	5′-GCCGCCCACTCAGACTTTATT-3′	143715-143735

Note: primers SEA7, 8 and 9 for the detection of $--^{SEA}$ type, primers 3.7A, B and C for the detection of the $-\alpha^{3.7}$ type, primers 4.2G, E and F for the detection of the $-\alpha^{4.2}$ type, and primers CSF and R for the detection of the HbCS mutation. Accession number of chromosome 16 is DQ431198.

2.3 Growth of hybridoma and single-cell cloning

The 2D4 mouse hybridoma producing monoclonal antibody highly specific to Hb Bart's [15] frozen in liquid nitrogen was thawed in a 37ºC water bath. Thawed cells were suspended in 10 mL of incomplete Iscove's Modified Dulbecco's Medium or IMDM (Gibco, USA), and then, centrifuged at 400 x g for 5 min. After discard the supernatant, cells were washed once with 10 mL of incomplete IMDM. After centrifugation, the supernatant was discarded and cell pellet was suspended in 2 mL of IMDM supplemented with 20% fetal bovine serum (FBS, Gibco, USA) as prepared by

the formula in appendix C. Suspended cells were plated in 24-well tissue culture plate and incubated in a 5% CO_2 incubator at 37ºC for 3-5 days. Cells were subcultured every 3 days and the culture supernatant was collected for the determination of mouse anti-Hb Bart's by indirect ELISA as mentioned in method 2.4.

Mouse hybridoma producing monoclonal antibody against Hb Bart's were subjected for the single-cell cloning by the method modified from the limiting dilution technique [67]. Briefly, spleen cells of normal BALB/c mice were prepared as feeder cells for mouse hybridoma. Normal mouse spleen cells were suspend in IMDM supplemented with 20% FBS at the concentration of 5 x 10^5 cells/mL. One hundred microliters of cells were plated in 5 plates of 96-well tissue culture plate. Feeder cells were cultured in a 5% CO_2 incubator at 37ºC for 1 day. For cells limiting dilution, the number of mouse hybridoma was counted and diluted with IMDM supplemented with 20% FBS to obtain the cell numbers of 1,000, 100, 10, 1 and 0.1 cells per milliliter, respectively. One hundred microliters of each cell dilution were plated in 96-well tissue culture plate containing feeder cells. Finally, tissue culture plates contained the number of mouse hybridoma of 100, 10, 1, 0.1 and 0.01 cells per well, respectively. All cells were cultured in a 5% CO_2 incubator at 37ºC for 7-14 days. The culture supernatant of the number of mouse hybridoma of 1, 0.1 and 0.01 cell per well were collected for determination of mouse anti-Hb Bart's by indirect ELISA as mentioned in method 2.4. The limiting dilution of mouse hybridoma was repeated twice for the selection of single cell producing monoclonal antibody specific to Hb Bart's without cross-reactivity to Hbs; A, F, S, E, A_2 and H.

2.4 Detection of mouse anti-Hb Bart's by indirect ELISA

Indirect ELISA protocol was modified from the method described previously [67]**.** Briefly, ELISA wells were coated with 50 μL of either 10 μg/mL of purified Hb Bart's or 20 μg/mL of purified Hbs; A_2, E, F, A and H (obtained from Dr. Luksana Makonkawkeyoon's laboratory) in 50 mM carbonate-bicarbonate buffer, pH 9.6. ELISA plates were incubated in a moist chamber at 4°C for overnight. After washing with phosphate buffer saline-0.05% Tween 20 (PBS-T) for 5 times, ELISA wells were blocked with 100 μL of 10% (w/v) skim milk by incubation at 37°C for 1 h. After

washing, ELISA wells were added with 50 µL of the culture supernatant (obtained from method 2.3) and incubated at 37°C for 1 h. After washing, ELISA wells were added with 50 µL of horseradish peroxidase (HRP) conjugated rabbit anti-mouse Igs (1: 1,000) and incubated at 37°C for 1 h. After washing with PBS-T, ELISA wells were added with 100 µL of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate and incubated at room temperature for 15 min. The color reaction was terminated by adding 100 µL of 2.5 N H_2SO_4 into ELISA wells and measured the optical density at 450 nm by a microplate reader (Bio-Tek).

2.5 Isolation of RNA, synthesis of cDNA and antibody gene amplification

Mouse hybridoma secreting monoclonal antibody specific to Hb Bart's (obtained from single-cell cloning, method 2.3) were grown in a 25 cm^2 tissue culture flask containing 10 mL of IMDM supplemented with 10% FBS as previously described in method 2.3 for 2 days. Total RNA was isolated from mouse hybridoma using RNeasy® mini kit (Qiagen, Germany) as described in appendix E.

Synthesis of the first-strand cDNA followed by the V_H or V_L gene amplification was performed in one-tube system using RobusT™ I RT-PCR kit (Finnzymes, Finland). Briefly, 1 µg of total RNA was added into 50 µL of reaction tube containing 1x Robust™ reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 µM of either mixed V_H or V_L forward and reverse primers as shown in Tables 2.2 and 2.3 [68], 5 units of AMV reverse transcriptase and 2 units of DyNAzyme™ EXT DNA polymerase. The profiles of the first-strand cDNA synthesis followed by the V_H or V_L gene amplification were performed as follows; denaturation of RNA secondary structure at 65°C for 5 min, reverse transcription at 48°C for 30 min, inactivation of reverse transcriptase and denaturation of cDNA-RNA at 94ºC for 2 min, DNA amplification for 30 cycles of 94°C for 30 s; 50°C for 30 s; 72°C for 1 min and final extension at 72°C for 7 min. After amplification, 10 µL of the amplified product of the V_H or V_L gene were mixed with 2 µL of 6x gel loading buffer and analyzed by separation on 2% agarose gel in 1x TAE buffer at 100 V for 30 min. Agarose gel was stained and processed as described in method 2.2.1. The amplicon of the V_H gene was 363 bp while the amplicon of the V_L gene was 321 bp.

Table 2.2 List of antibody-specific DNA primers used for the V_H gene amplification.

Primers	Sequence	d	Volume (μL)
Primer V_H-forward			
HB1	5'-GAKGTRMAGCTTCAGGAGTC-3'	8	4
HB2	5'-GAGGTBCAGCTBCAGCAGTC-3'	9	4
HB3	5'-CAGGTGCAGCTGAAGSASTC-3'	4	3
HB4	5'-GAGGTCCARCTGCAACARTC-3'	4	4
HB5	5'-CAGGTYCAGCTBCAGCARTC-3'	12	7
HB6	5'-CAGGTYCARCTGCAGCAGTC-3'	4	2
HB7	5'-CAGGTCCACGTGAAGCAGTC-3'	1	1
HB8	5'-GAGGTGAASSTGGTGGAATC-3'	4	2
HB9	5'-GAVGTGAWGYTGGTGGAGTC-3'	12	5
HB10	5'-GAGGTGCAGSKGGTGGAGTC-3'	4	2
HB11	5'-GAKGTGCAMCTGGTGGAGTC-3'	4	2
HB12	5'-GAGGTGAAGCTGATGGARTC-3'	2	2
HB13	5'-GAGGTGCARCTTGTTGAGTC-3'	2	1
HB14	5'-GARGTRAAGCTTCTCGAGTC-3'	4	2
HB15	5'-GAAGTGAARSTTGAGGAGTC-3'	4	2
HB16	5'-CAGGTTACTCTRAAAGWGTSTG-3'	8	5
HB17	5'-CAGGTCCAACTVCAGCARCC-3'	6	3.5
HB18	5'-GATGTGAACTTGGAAGTGTC-3'	1	0.7
HB19	5'-GAGGTGAAGGTCATCGAGTC-3'	1	0.7

The sequences were given using the IUPAC nomenclature of mixed bases (shown in underline capital letters, R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T), with a column listing the d-fold degeneration encode in each primer, and the volume of each primer to be used for 1/5 dilution from 100 μM stock primer to 20 μM working primer [68].

Table 2.2 List of antibody-specific DNA primers used for the V_H gene amplification (continued).

Primers	Sequence	d	Volume (µL)
Primer V_H-reverse			
HF1	5'-CGAGGAAACGGTGACCGTGGT-3'	1	1
HF2	5'-CGAGGAGACTGTGAGAGTGGT-3'	1	1
HF3	5'-CGCAGAGACAGTGACCAGAGT-3'	1	1
HF4	5'-CGAGGAGACGGTGACTGAGGT-3'	1	1

The sequences were given using the IUPAC nomenclature of mixed bases (shown in underline capital letters, R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T), with a column listing the d-fold degeneration encode in each primer and the volume of each primer to be used for 1/5 dilution from 100 µM stock primer to 20 µM working primer [68].

Table 2.3 List of antibody-specific DNA primers used for the V_L gene amplification.

Primers	Sequence	d	Volume (µL)
Primer V_L-forward			
LB1	5'-GAYATCCAGCTGACTCAGCC-3'	2	1
LB2	5'-GAYATTGTTCTCWCCCAGTC-3'	4	2
LB3	5'-GAYATTGTGMTMACTCAGTC-3'	8	5
LB4	5'-GAYATTGTGYTRACACAGTC-3'	8	3.5
LB5	5'-GAYATTGTRATGACMCAGTC-3'	8	4
LB6	5'-GAYATTMAGATRAMCCAGTC-3'	16	7
LB7	5'-GAYATTCAGATGAYDCAGTC-3'	12	6
LB8	5'-GAYATYCAGATGACACAGAC-3'	4	1.5
LB9	5'-GAYATTGTTCTCAWCCAGTC-3'	4	2
LB10	5'-GAYATTGWGCTSACCCAATC-3'	8	3.5
LB11	5'-GAYATTSTRATGACCCARTC-3'	16	8
LB12	5'-GAYRTTKTGATGACCCARAC-3'	16	8
LB13	5'-GAYATTGTGATGACBCAGKC-3'	12	6
LB14	5'-GAYATTGTGATAACYCAGGA-3'	4	2
LB15	5'-GAYATTGTGATGACCCAGWT-3'	4	2
LB16	5'-GAYATTGTGATGACACAACC-3'	2	1
LB17	5'-GAYATTTTGCTGACTCAGTC-3'	2	1
LBλ	5'-GATGCTGTTGTGACTCAGGAATC-3'	1	1

The sequences were given using the IUPAC nomenclature of mixed bases (shown in underline capital letters, R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T), with a column listing the d-fold degeneration encode in each primer and the volume of each primer to be used to for 1/5 dilution from 100 µM stock primer to 20 µM working primer [68].

Table 2.3 List of antibody-specific DNA primers used for the V_L gene amplification (continued).

Primers	Sequence	d	Volume (µL)
Primer V_L-reverse			
LF1	5'-ACGTTTGATTTCCAGCTTGG-3'	1	1
LF2	5'-ACGTTTTATTTCCAGCTTGG-3'	1	1
LF4	5'-ACGTTTTATTTCCAACTTTG-3'	1	1
LF5	5'-ACGTTTCAGCTCCAGCTTGG-3'	1	1
LFλ	5'-ACCTAGGACAGTCAGTTTGG-3'	1	0.25

The sequences were given using the IUPAC nomenclature of mixed bases (shown in underline capital letters, R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T), with a column listing the d-fold degeneration encode in each primer and the volume of each primer to be used to for 1/5 dilution from 100 µM stock primer to 20 µM working primer [68].

2.6 Cloning of the V_H and V_L gene

Both amplicons of the V_H (363 bp) and V_L (321 bp) genes obtained from method 2.5 were individually purified by PCR clean-up gel extraction kit (Macherey-Nagel, Germany) as mentioned in appendix F. After purification, either V_H or V_L purified amplicon (with 3′-dA overhang generated by DyNAzyme™ EXT DNA polymerase) were individually ligated into pGEM®-T Easy vector according to the manufacturer's instructions (Promega, USA). Briefly, 200 ng of either V_H or V_L purified amplicon were separately added into 20 µL of ligation reaction containing 50 ng of pGEM®-T Easy vector, 1x ligation buffer and 3 units of T4 DNA ligase. The ligation mixtures were incubated at 16ºC for overnight. The circle map, promoter and T overhang cloning sequence of pGEM®-T Easy vector were shown in Figure 2.5.

After incubation, the ligation mixture was transformed into *E. coli* TOP10F. Firstly, 500 µL of frozen *E. coli* TOP10F (prepared from protocol described in appendix G) were thawed, then, pipetted into 3 separate tubes in volume of 200, 200 and 100 µL, respectively. Three tubes containing *E. coli* TOP10F was added with 12, 5 and 2.5 µL of ligation mixture, respectively. For heat-shock the bacterial cells, all reaction tubes were placed on ice for 30 min and heated at exactly 42ºC for 45 s. After that, all reaction tubes were immediately placed on ice for another 2 min. The preheat SOC medium (37ºC) was added to all tubes to make the final volume of 1 mL and rotated end-to-end at 37ºC for 3 h. Bacterial cell suspension was centrifuged at 10,000 x g for 30 s, then, discarded the supernatant by remaining the volume approximately 50 µL. The remainder of bacterial cells suspension was spread on LB plate containing 100 µg/mL of ampicillin, previously spread with 40 µL of 40 mg/mL X-gal and 4 µL of 400 mg/mL isopropyl-β-D-thiogalactopyranoside (IPTG). Finally, LB plates were incubated at 37ºC for 14-16 h.

The colonies of bacteria were screened for the transformation of the pGEM®-T Easy vector containing the insertion of V_H or V_L gene (pGEM®-T-V_H or pGEM®-T-V_L) by color screening. Successful cloning of the pGEM®-T-V_H or pGEM®-T-V_L interrupted the coding sequence of β-galactosidase (*LacZ* as shown in Figure 2.5). The blue colony bacteria represented the remainder of β-galactosidase activity for digestion of X-gal, therefore, blue colony bacteria had no insertion of V_H or V_L gene into pGEM®-T Easy vector. By contrast, the white colony bacteria contained the pGEM®-T-V_H or pGEM®-T-V_L resulted in the absent of β-galactosidase activity. After color screening, white bacterial colonies were subjected for colony PCR. Briefly, the white colony bacteria was picked by a sterile toothpick into 25 µL of a PCR reaction tube containing 1 unit of Phusion® DNA polymerase (Finnzymes, Finland), 1x Phusion® HF buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each T7 promoter primer (5′-AATACGACTCACTATAGGG-3′, 19 nucleotides) and SP6 promoter primer (5′-ATTTAGGTGACACTATA-3′, 17 nucleotides). The locations of both primers on the nucleotide sequences of the pGEM®-T Easy vector were indicated in appendix D. The profiles of colony PCR were performed with following: initial denaturation at 98ºC for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s; annealing at 52°C for

10 s; extension at 72°C for 10 s, with an additional 10 min extension at 72°C in the final cycle. The amplified products were analyzed by separation in 2% agarose gel in 1x TAE buffer at 100 V for 40 min. Agarose gel was further performed as described in method 2.2.1. The amplicon of 537 bp was positive for the insertion of the V_H gene (pGEM®-T-V_H) whereas 495 bp of amplicon was positive for the insertion of the V_L gene (pGEM®-T-V_L).

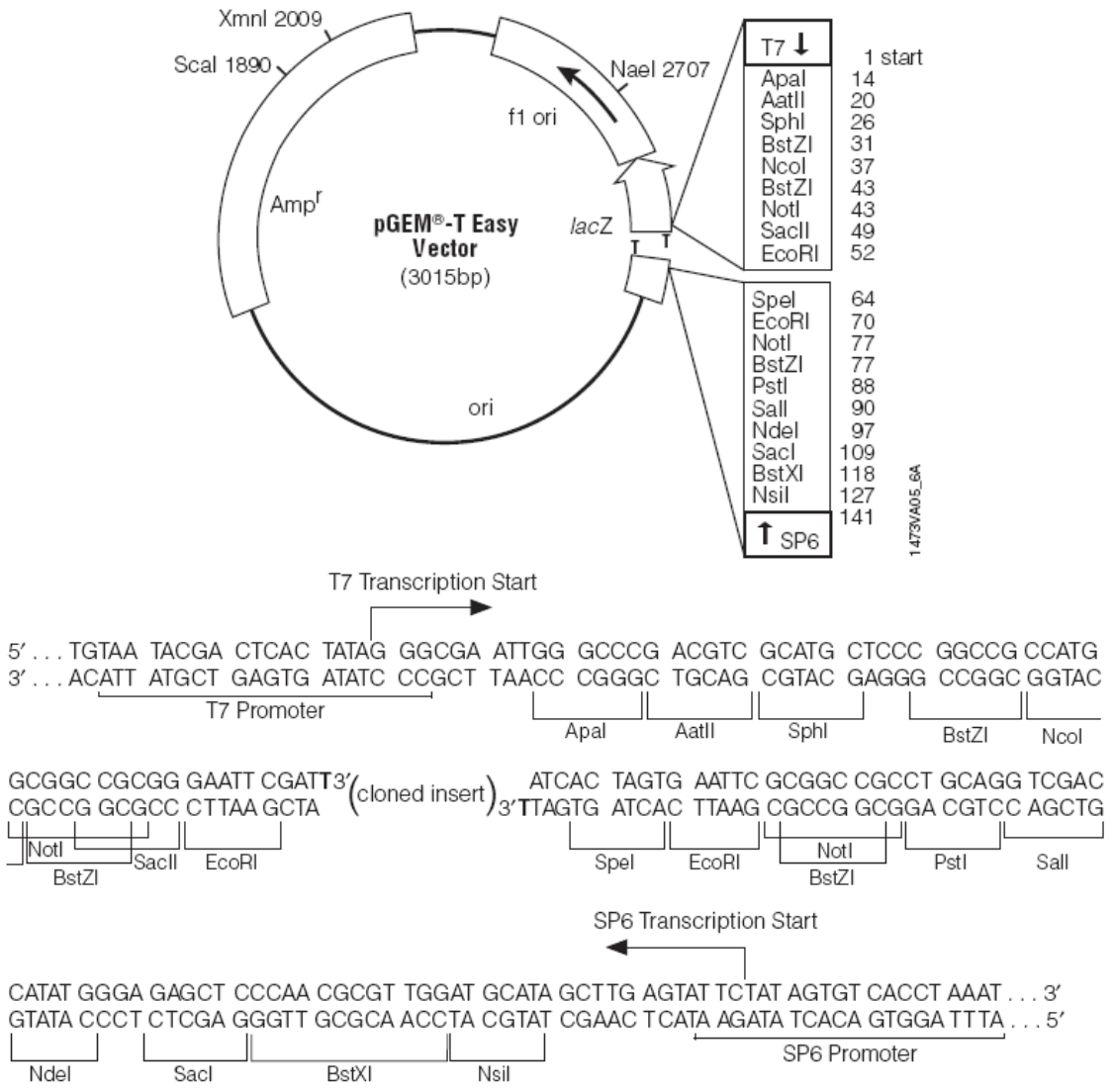

Figure 2.5 The circle map, promoter and T overhang cloning sequences of pGEM®-T Easy vector (http://www.promega.com).

2.7 Analysis of the V_H and V_L gene

Ten positive colonies of *E. coli* TOP10F carrying either the pGEM®-T-V_H or pGEM®-T-V_L (obtained from method 2.6) were randomly selected for plasmid DNA purification using kit (Macherey-Nagel, Germany) as described in appendix H. Purified plasmid DNA of either pGEM®-T-V_H or pGEM®-T-V_L were confirmed for either the V_H or V_L gene inserted by *Eco*R I restriction digestion analysis (Amersham Pharmacia Biotech, Sweden) as shown of the enzyme restriction sites in Figure 2.5 and appendix D. Briefly, 200 ng of plasmid DNA of either the pGEM®-T-V_H or pGEM®-T-V_L were added into 20 µL of reaction tube containing 1x OPA^+ buffer (Amersham Pharmacia Biotech, Sweden) and 2 units of *Eco*R I. Reaction mixtures were incubated in a 37ºC water bath with shaking for overnight. Reaction mixtures were analyzed for the insertion of either the V_H or V_L gene into the pGEM®-T Easy vector by electrophoresis in 1.5% agarose gel as described in method 2.2.1. Both amplicons of the V_H gene (363 bp) and the V_L gene (321 bp) were used as comparative base pair size to both insertion genes. The negative clone for colony PCR was used as a negative control. The cutting products of the insertion of the V_H and V_L genes in pGEM®-T Easy vector were 381 and 339 bp, respectively.

All purified plasmids DNA of either the pGEM®-T-V_H or pGEM®-T-V_L were subjected for commercial direct DNA sequence by dye termination method (Applied biosystems, USA) using T7 promoter primer. The deduced amino acid of either the V_H or V_L chain was translated from the nucleotide sequences, then, identified the CDRs-H and CDRs-L, respectively, using Kabat and Chothia numbering scheme (as shown in Figures 2.6 and 2.7).

CDR-H1

Start	approximately residue 26 (always 4 after a C) [Chothia /AbM defintion]; Kabat definition starts 5 residues later
Residues before	always C-X-X-X
Residues after	always a W. Typically W-V, but also, W-I, W-A
Length	10 to 12 residues [AbM definition]; Chothia definition excludes the last 4 residues

CDR-H2

Start	always 15 residues after the end of Kabat / AbM definition) of CDR-H1
Residues before	typically L-E-W-I-G, but a number of variations
Residues after	K/R-L/I/V/F/T/A-T/S/I/A
Length	Kabat definition 16 to 19 residues; AbM (and recent Chothia) definition ends 7 residues earlier

CDR-H3

Start	always 33 residues after end of CDR-H2 (always 2 after a C)
Residues before	always C-X-X (typically C-A-R)
Residues after	always W-G-X-G
Length	3 to 25 residues

Figure 2.6 Kabat and Chothia numbering scheme for the identification of CDRs-H (http://www.bioinf.org.uk/abs/).

CDR-L1	
Start	approximately residue 24
Residue before	always a C
Residue after	always a W. Typically W-Y-N, but also, W-L-N, W-F-N, W-Y-L
Length	10 to 17 residues
CDR-L2	
Start	always 16 residues after the end of CDR-L1
Residues before	generally I-Y, but also, V-Y, I-K, I-F
Length	always 7 residues (except NEW (7FAB) which has a deletion in this region)
CDR-L3	
Start	always 33 residues after end of CDR-L2 (except NEW (7FAB) which has the deletion at the end of CDR-L2)
Residue before	always C
Residues after	always F-G-X-G
Length	7 to 11 residues

Figure 2.7 Kabat and Chothia numbering scheme for the identification of CDRs-L (http://www.bioinf.org.uk/abs/).

2.8 Construction of the full length scFv gene

The purified plasmid DNA of either the pGEM®-T-V_H or pGEM®-T-V_L (obtained from method 2.6) were used as the template for the construction of the full length scFv gene (V_H-linker-V_L format). The V_H and V_L genes were amplified, then, linked to be the full length scFv gene by SOE-PCR using 2 pairs of designed primers. Two pairs of primers containing *Nde* I and *Bam*H I restriction sites, peptide linker $(Gly_4Ser)_3$ and a stop codon were designed for cloning of the full length scFv gene into pET28a(+) expression vector. Nucleotide sequences of all primers used were indicated in Table 2.4. Firstly, 100 ng of either the pGEM®-T-V_H or pGEM®-T-V_L were individually added into the 25 µL of PCR reaction tube containing 1 unit of Phusion® DNA polymerase, 1x Phusion® HF buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of either the V_H or V_L forward and reverse primers. The amplicon of V_H gene was 402 bp while the amplicon of V_L gene was 364 bp. Both amplicons were purified using PCR clean-up gel extraction kit as described in appendix F. Finally, both purified amplicons of the V_H and V_L genes were further amplified using V_H forward and V_L reverse primers to obtain the amplicon of the full length 751 bp. Briefly, 100 ng of purified amplicon of both V_H and V_L genes were added into the 25 µL of PCR reaction tube containing 1 unit of Phusion® DNA polymerase, 1x Phusion® HF buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each V_H forward and V_L reverse primers. All of PCR condition for amplification of V_H and V_L genes and construction of the full length scFv gene were undertaken in the following profile: initial denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s; 72°C for 25 s, with final extension at 72°C for 10 min. The amplicon was analyzed as previously described in method 2.2.1. Construction of the full length scFv gene by SOE-PCR was shown in schematic diagram of Figure 2.8.

Table 2.4 Primers designed for construction of the full length scFv gene.

Primers	Sequence	Annealing T°
V_H forward	5′GCGCATATGCAGGTTCAGCTGCAGCAGTCT-GGG-3′	72°C
V_H reverse	5′*GGAGCCGCCGCCGCCAGAACCACCACCACC*G-GAGGAGACGGTGACTGA-3′	81°C
V_L forward	5′*GGCGGCGGCGGCTCCGGTGGTGGTGGTTCA*A-TTGTGATGACCCAGTCT-3′	78°C
V_L reverse	5′CGCGGGATCC**TTA**TTTCCAGCTTGGTCCCCC-CTC-3′	73°C

V_H forward primer contained the *Nde* I restriction site (underlined). V_H reverse and V_L forward primers contained the linker sequence (italic). V_L reverse primer contained the *Bam*H I restriction site (underlined) and a stop codon (bold).

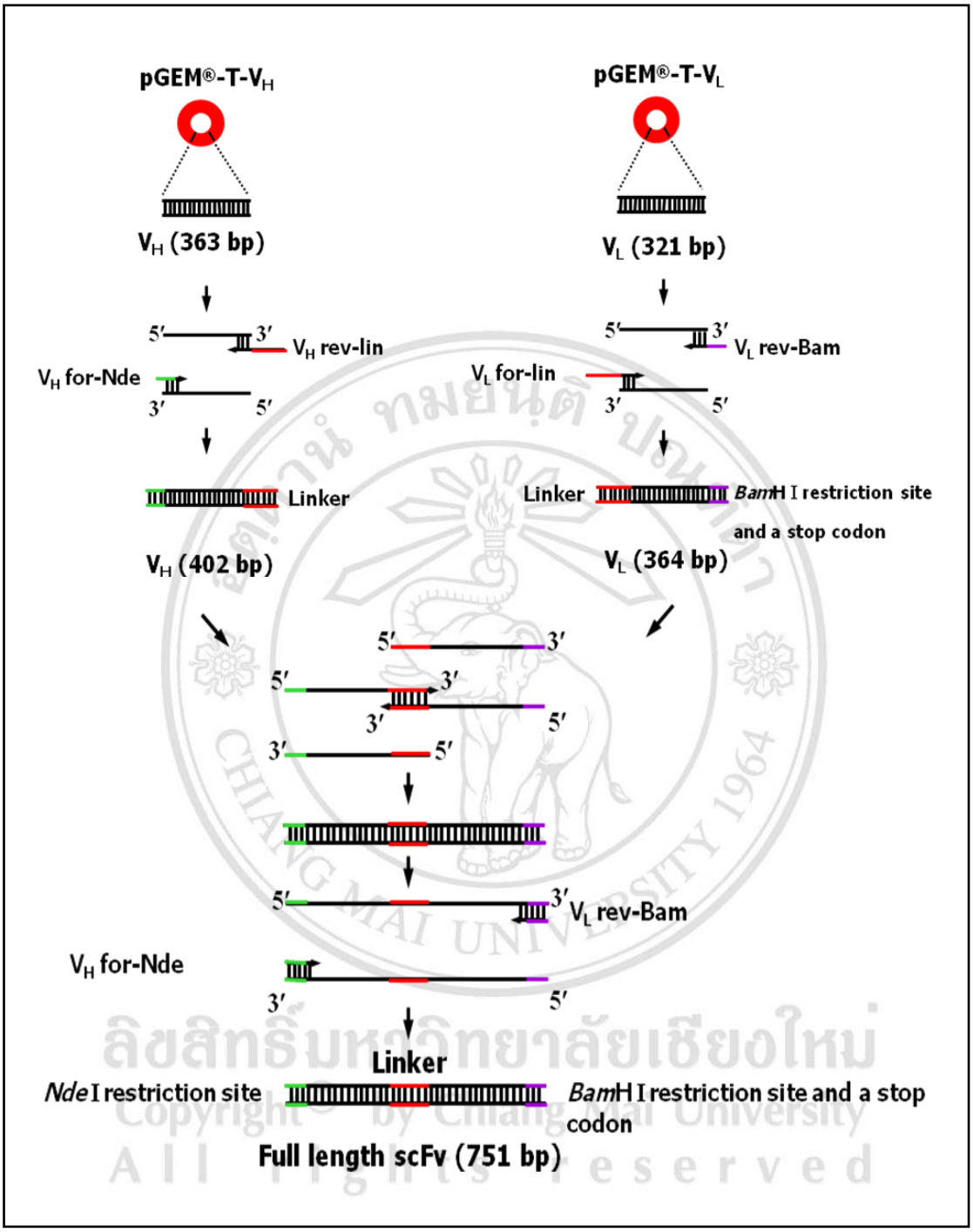

Figure 2.8 The schematic diagram of the construction of the full length scFv gene by SOE-PCR.

2.9 Cloning of the full length scFv gene [69]

The amplicon of the full length scFv gene (obtained from method 2.8) was purified by PCR clean-up gel extraction kit as described in appendix F. After purification, the full length scFv gene was subcloned into pET28a(+) expression vector (Novagen, USA). The circle map, promoter and multiple cloning sequences of the expression vector were shown in Figure 2.9. The full length scFv gene was ligated into pET28a(+) vector via *Nde* I and *Bam*H I restriction sites to obtain the pET28a(+)-scFv anti-Hb Bart's as shown in Figure 2.10 and appendix D. Before ligation reaction, both of the full length scFv gene and pET28a(+) vector were individually double cut with *Nde* I and *Bam*H I. Briefly, 10 µg of the purified amplicon of the full length scFv gene (751 bp) and 3 µg of pET28a(+) vector were individually added into 30 µL of reaction tube containing 1x buffer and each of 20 units of *Nde* I and *Bam*H I (Amersham Pharmacia Biotech, Sweden). The reaction mixture was incubated in a 37ºC water bath for overnight. The product of enzyme cut was purified by PCR clean-up gel extraction kit as described in appendix F.

After purification, 100 ng of the cutting full length scFv gene (737 bp) were added into 20 µL of ligation reaction containing 100 ng of the cut pET28a(+) vector (5,329 bp), 1x ligation buffer and 1 unit of T4 DNA ligase, then, incubated at 16ºC for overnight. After incubation, ligation product was transformed into *E. coli* BL21(DE3) as described in method 2.6. The bacterial cells suspension were spread on a LB plate containing 100 µg/mL of kanamycin and incubated at 37ºC for 16-18 h.

Bacterial colonies were subjected for screening of the insertion of the full length scFv gene by colony PCR as described in method 2.5 using 0.5 µM of each T7 promoter primer (5′-AATACGACTCACTATAGGG-3′, 19 nucleotides) and T7 terminator primer (5′-CTAGTTATTGCTCAGCGGT-3′, 19 nucleotides). The locations of both primers on the nucleotide sequences of pET28a(+) vector were shown in Figure 2.9 and appendix D. The profiles of the colony PCR were performed as follows: initial denaturation at 98ºC for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s; annealing at 54°C for 10 s; extension at 72°C for 15 s, with an additional 10 min extension at 72°C in the final cycle. The amplified products were analyzed by

separation in 2% agarose gel as described in method 2.2.1. The empty pET28a(+) vector was used as a DNA template control for the uninsertion of gene (316 bp). The amplicon of the pET28a(+)-scFv anti-Hb Bart's was 1,013 bp.

Ten positive colonies of *E. coli* BL21(DE3) carrying the pET28a(+)-scFv anti-Hb Bart's were randomly selected for the plasmid DNA purification by kit as described in appendix H. The purified plasmid DNA of the pET28a(+)-scFv anti-Hb Bart's was analyzed for the integrity of the nucleotide sequences by commercial DNA sequencing using T7 promoter primer.

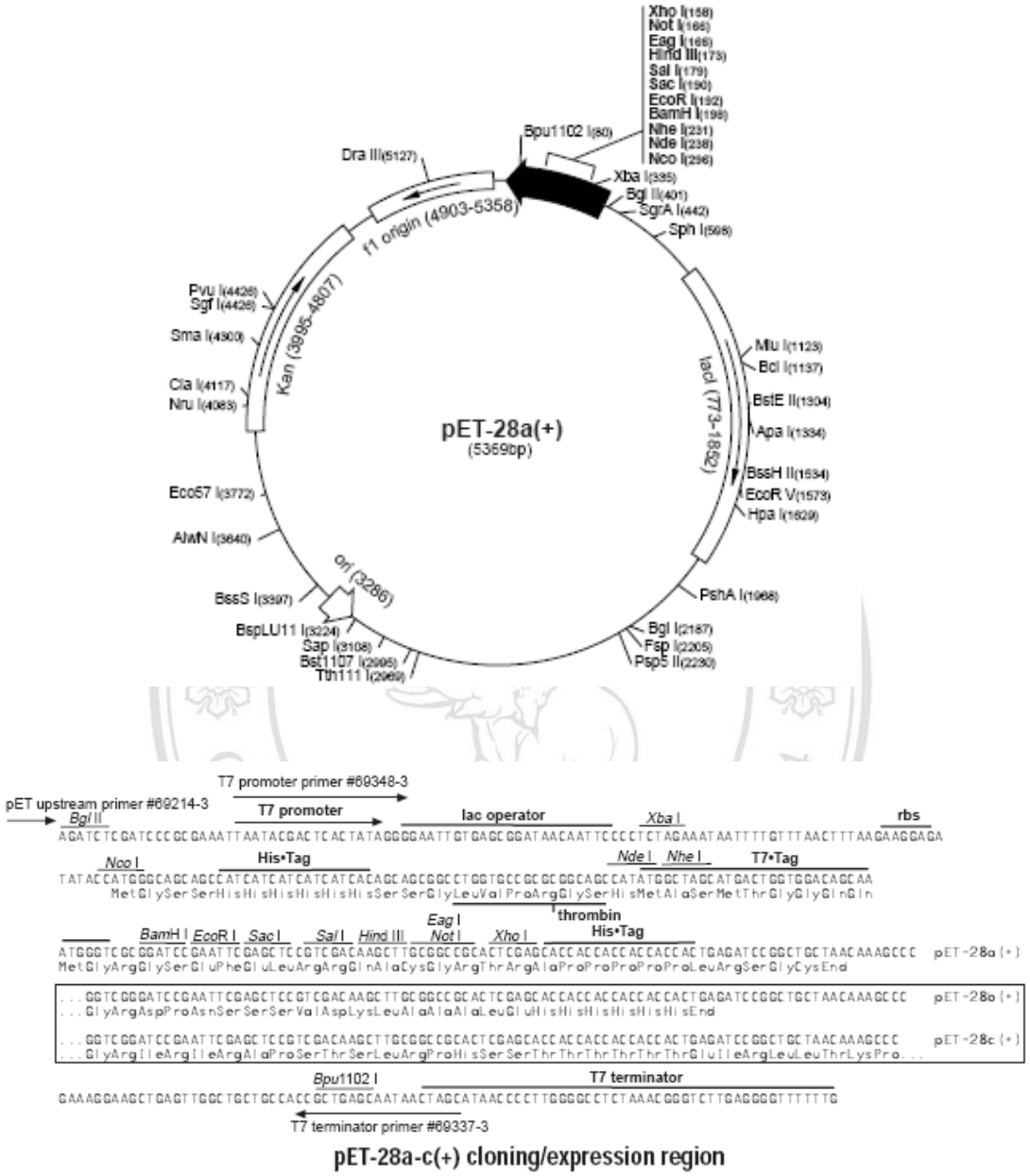

Figure 2.9 The circle map, promoter and multiple cloning sequences of pET28a(+) expression vector. The sequence was numbered by the PBR322 convention, therefore, the T7 expression region was reversed on the map circle (http://www.novagen.com).

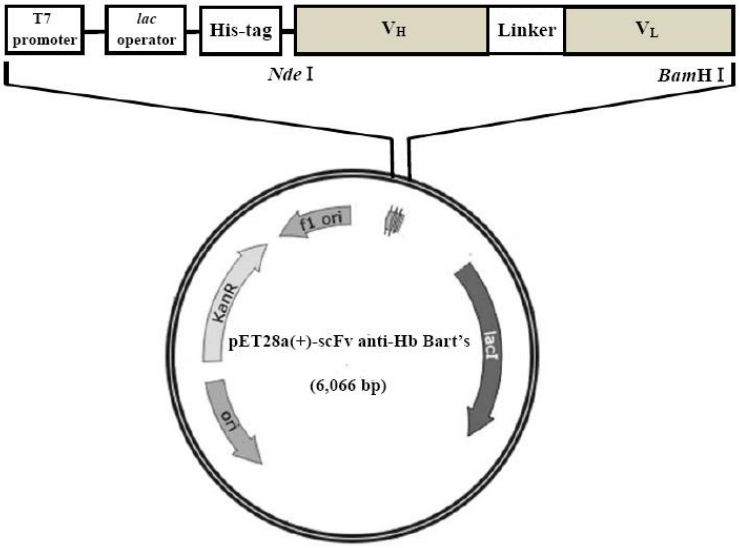

Figure 2.10 Expression vector map of the pET28a(+)-scFv anti-Hb Bart's. Expression vector consecutively contained T7 promoter, *lac* operator, hisitdine tag fusion partner, and the full length scFv gene ligation into the multiple cloning sites via *Nde* I and *Bam*H I restriction sites.

2.10 Optimization of IPTG induction

E. coli BL21(DE3) carrying the pET28a(+)-scFv anti-Hb Bart's was grown in 10 mL of LB broth containing 100 µg/mL kanamycin with shaking at 37ºC for 2-3 h until the OD_{600} of culture broth reached to 0.5-0.6. After that, 1 mL of culture broth was collected for use as the uninduced control (induction time at 0 h). The remainder culture broth was added with 5.4 µL of 400 µg/mL IPTG (to make the final concentration of 1 mM IPTG). After 1, 2, 3, 4, 6, 12 and 24 h, 1 mL of culture broth was collected. The culture broth at various times was centrifuged at 10,000 rpm for 5 min. After discard the supernatant, bacterial pellet was suspended in 100 µL of SDS gel-loading buffer and boiled at 100ºC for 5 min. The expressed proteins of the scFv antibody at various times were analyzed by 12% SDS-PAGE [70]**.** Briefly, 20 µL of bacterial cell lysate at various time mixed with SDS gel-loading buffer were apply into 12% SDS-polyacrylamide gel (prepared from the protocol described in appendix I), then, electrophoresed at 100 V for 1.2 h using Tris-glycine electrophoresis buffer. PageRuler® prestained protein ladder (Fermentas, USA) was used as a protein marker. After electrophoresis, gel was stained with 0.25% Coomassie brilliant blue

R-250 for 4 h. Gel was destained with destaining solution for overnight. The protein bands were documented by computer scanning.

2.11 Expression of the scFv antibody

E. coli BL21(DE3) carrying the pET28a(+)-scFv anti-Hb Bart's were cultured in 30 mL of LB broth as mentioned in method 2.10. After the OD_{600} of culture broth reached to 0.5-0.6, 1 mL of culture broth was collected as uninduced control. The remainder culture broth was added with 17.3 µL of 400 µg/mL IPTG and incubated at 37ºC for 4 h. One milliliter of culture broth was collected as IPTG induced. The remainder culture broth was centrifuged at 4,000 rpm, 4°C for 20 min, then, discarded the supernatant. Bacterial pellet was washed once with 50 mL of 50 mM Tris-HCl, pH 8.0. Bacterial cell pellet was resuspended in 20 mL of bacterial lysis buffer and incubated at room temperature for 30 min. Bacterial suspension was added with Triton X-100 and 5 M NaCl to make the final concentration as 1% Triton X-100 and 0.5 M NaCl, respectively, then, incubated at room temperature for another 30 min. Bacterial suspension was conducted for cell lysis on ice by the sonicator (Sonics & Materials, USA) with the 6 cycles of sonicated for 10 s and stood for 10 s. After lysis of bacteria, the inclusion bodies (IBs) expressed protein was collected by centrifugation at 14,000 rpm, 4°C for 20 min. The supernatant was collected for as the soluble fraction of bacterial extract. The IBs protein was washed with 30 mL of 50 mM Tris-HCl, pH 8.0 for 5 times, then, solubilized with 2 mL of 8 M urea, 50 mM Tris-HCl, pH 8.0 and 1 mM PMSF. For analysis of bacterial proteins, 20 µL of samples mixed with SDS gel-loading buffer including bacterial cell lysate of uninduced, bacterial cell lysate of IPTG induced, soluble fraction of bacterial extract and solubilized IBs were analyzed by 12% SDS-PAGE as described in method 2.10.

The expression of N-terminal histidine fusion scFv antibody was determined by Western blot analysis. Proteins of bacterial cell lysate of uninduced and IPTG induced were run by 12% SDS-PAGE, then, transferred onto a polyvinylidene difluoride (PVDF) membrane (NEN Life Science, USA) using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, USA) as described in appendix J. After transfer, the membranes were blocked with 10% (w/v) skim milk at room temperature for 2 h. After washing 3 times with PBS-T, HRP conjugated mouse

anti-His-tag (1:1,000) was added and incubated at room temperature for 1 h. After washing with PBS-T, 4-chloro-1-naphthol substrate was added and the color reaction was subsequently developed at room temperature for 10-15 min. The reaction was stopped by repeatedly washing with distilled water.

2.12 Purification of the scFv antibody by affinity chromatography and refolding of the scFv antibody

E. coli BL21(DE3) carrying the pET28a(+)-scFv anti-Hb Bart's was cultured in 1 L of LB broth as described in methods 2.11. Four grams of bacterial cells pellet were suspended in 80 mL of lysis buffer. Further protocol for IBs isolation was performed as previously described in method 2.11. The IBs was dissolved in 60 mL of 8 M urea, 50 mM Tris-HCl, pH 8.0, and 1 mM PMSF, then, the protein concentration was determined by the modified Bradford assay (described in appendix K). All of solubilized IBs contained the N-terminal histidine fusion scFv antibody was purified under denaturing conditions by Ni-NTA His-Bind® resin affinity chromatography according to the method modified from the manufacturer's instructions as described in appendix L. After elution of protein, 20 mL of Ni-NTA purified scFv antibody were directly refolded by drip dilution into 2 L of the redox refolding buffer and gently stirred at 4°C for overnight. After centrifugation at 12,000 rpm, 4°C for 20 min, the aggregate protein was discarded. The supernatant of refolded scFv antibody was concentrated by the Centriprep centrifugal filter (Millipore, Germany) at 3,000 x g for 1 h. Six milliliters of concentrated solution of the refolded scFv antibody were dialyzed against phosphate buffer saline (PBS) at 4°C for overnight. After centrifugation at 12,000 rpm, 4°C for 20 min, the aggregated protein was discarded. The protein concentration of the refolded scFv antibody was quantified by the modified Bradford assay. The integrity of the refolded scFv antibody was evaluated by SDS-PAGE as described in method 2.10.

2.13 Specificity of the refolded scFv antibody and the parent monoclonal antibody by Western blot and indirect ELISA

Specific binding of scFv anti-Hb Bart's was investigated by Western blot analysis. Parent monoclonal antibody specific to Hb Bart's (obtained from Dr. Luksana

Makonkawkeyoon's laboratory) was used as a positive control. Fifty micrograms of purified hemoglobins; A, F, E, A_2, standard HbS and approximately 120 µg of HbH disease and Hb Bart's hydrops fetalis hemolysate (obtained from Dr. Luksana Makonkawkeyoon's laboratory) were electrophoresed in 12% native-polyacrylamide gel (prepared in the protocol described in appendix I) at 100 V for 6 h using Tris-asparagine electrophoresis buffer. After electrophoresis, all hemoglobin proteins as indicated were transferred onto a nitrocellulose membrane as described in appendix J. The membrane was blocked with 10% (w/v) skim milk and incubated at room temperature for 2 h. After washing 3 times with PBS-T, 100 µg/mL of either the refolded scFv antibody (obtained from method 2.12) or the parent monoclonal antibody specific to Hb Bart's were separately added and incubated at room temperature for 1 h. HRP conjugated mouse anti-His-tag (1:1,000) or HRP conjugated rabbit anti-mouse Igs (1:500) were separately added and incubated at room temperature for 1 h after washing with PBS-T. Finally, 4-chloro-1-naphthol substrate was added and color reaction was developed as previously described in method 2.11.

Specific binding of the refolded scFv anti-Hb Bart's was also determined by indirect ELISA (previously described in method 2.4) using 20 µg/mL of refolded scFv antibody and HRP conjugated mouse anti-His-tag (1:2,500).

2.14 Sensitivity of the refolded scFv antibody and the parent monoclonal antibody by dot blot ELISA

Sensitivity of the refolded scFv antibody and the parent monoclonal antibody to capture Hb Bart's was analyzed by dot blot ELISA. Firstly, 1 µL of a serial 2-fold dilution of Hb Bart's hydrops fetalis hemolysate (40-0.31 µg) was individually spotted onto a nitrocellulose membrane and incubated in a moist chamber at room temperature for 1 h. The membrane was subsequently blocked with 10% (w/v) skim milk for 2 h. After washing 3 times with PBS-T, the membrane was added with 100 µg/mL of the refolded scFv antibody or parent monoclonal antibody, then, incubated at room temperature 1 h. After washing 3 times with PBS-T, HRP conjugated mouse anti-His-tag (1:500) or HRP conjugated rabbit anti-mouse Igs (1:500) were separately added and incubated at room temperature for 1 h. Finally, 4-chloro-1-naphthol substrate was added and color reaction was developed as previously described in method 2.11.

2.15 Affinity constant (K_a) determination of the refolded scFv antibody and the parent monoclonal antibody by indirect ELISA [71]

Indirect ELISA was performed simultaneously for K_a of the refolded scFv anti-Hb Bart's (obtained from method 2.12) and the parent monoclonal antibody. Briefly, ELISA wells were coated with 50 µL of four concentrations (40, 20, 10 and 5 µg/mL) of purified Hb Bart's in 50 mM carbonate-bicarbonate buffer, pH 9.6 and incubated in a moist chamber at 4°C for overnight. ELISA wells were blocked with 10% (w/v) skim milk at 37°C for 1 h. After washing 3 times with PBS-T, 50 µL of a serial 2-fold dilutions of either the refolded scFv anti-Hb Bart's or the parent monoclonal antibody (80-0.62 µg/mL) were added into each wells. The reaction was incubated at 37°C for 1 h. After washing with PBS-T, 50 µL of either HRP conjugated mouse anti-His-tag (1:2,500) or HRP conjugated rabbit anti-mouse Igs (1:1,000) were separately added into wells and incubated at 37°C for 1 h. Finally, the color reaction was developed using 100 µL of TMB substrate and the absorbance was measured at 450 nm as described in method 2.4.

The mathematical equation for calculation of K_a is $K_a = (n-1)/2(n[Ab2]-[Ab1])$ where K_a is the antigen-antibody affinity constant in M^{-1}, [Ab1] and [Ab2] are the antibody concentration required to achieve 50% of the maximum absorbance obtained at two antigen concentrations and n is the dilution factor between the two antigen concentrations.

2.16 Stability (shelf life) of the refolded scFv antibody

The stability of the refolded scFv antibody was evaluated by dot blot ELISA as described in method 2.14. The storage conditions of the refolded scFv antibody at 4°C and -20°C for 1 week, 1 and 3 months were evaluated for the stability of the refolded scFv antibody.