CHAPTER 3

Results

3.1 Genotyping of α-thalassemias in primigravidarum volunteers

PCR genotyping of α-thalassemias were performed using various specific primers. Detection and interpretation of α-thalassemia genotypes by gap-PCR, PCR-RFLP and gel separation were shown in Figure 3.1.

Determination of α^0-thalassemia $--^{SEA}$ type by multiplex gap-PCR generated a 1,011 bp amplicon (Figure 3.1A, lane 1) for normal haplotype (αα/) and a 660 bp amplicon (Figure 3.1A, lane 2) for α^0-thalassemia $--^{SEA}$ haplotype ($--^{SEA}$/).

Detection of α^+-thalassemia $-\alpha^{3.7}$ type in two separate pairs of primers generated both a 1,766 bp amplicon by the primers 3.7A and B for normal haplotype (αα/) (Figure 3.1B, lanes 1 and 3) but only a 1,766 bp amplicon by the primers 3.7A and C for α^+-thalassemia $-\alpha^{3.7}$ haplotype ($-\alpha^{3.7}$/) (Figure 3.1B, lanes 2 and 4).

Detection of α^+-thalassemia $-\alpha^{4.2}$ type by multiplex gap-PCR generated a 228 bp amplicon (Figure 3.1C, lane 2) for normal haplotype (αα/) and a 1,761 bp amplicon (Figure 3.1C, lane 1) for α^+-thalassemia $-\alpha^{4.2}$ haplotype ($-\alpha^{4.2}$/).

Detection of HbCS mutation was done in two steps. First, amplification of the α2 specific gene generated a 276 bp amplicon as shown in Figure 3.1D (lanes 1 and 3). Second, the amplicon from the first amplification was differentiated by restriction enzyme cutting. Normal haplotype (αα/) could be completely cut to yield a 165 and a 111 bp fragments (Figure 3.1D, lanes 2 and 4), while HbCS haplotype ($\alpha^{CS}\alpha$/) could not be cut by the enzyme yielding the remainder of 276 bp fragment (Figure 3.1D, lane 4). Therefore, heterozygous HbCS genotype ($\alpha^{CS}\alpha/\alpha\alpha$) showed 3 bands after

enzyme cutting (Fig 3.1D, lane 4). Fragment sizes 165 and 111 bp resulted from αα/ haplotype and half of the uncut 276 bp was belong to $\alpha^{CS}\alpha$/ haplotype.

With the detection systems mentioned above, genotypes of α-thalassemia from all DNA samples of 638 primigravidarum volunteers could be revealed and analyzed. There were 409 samples (64.11%) with normal genotypes and 229 samples (35.89%) with various genotypes of α-thalassemia as shown in Table 3.1.

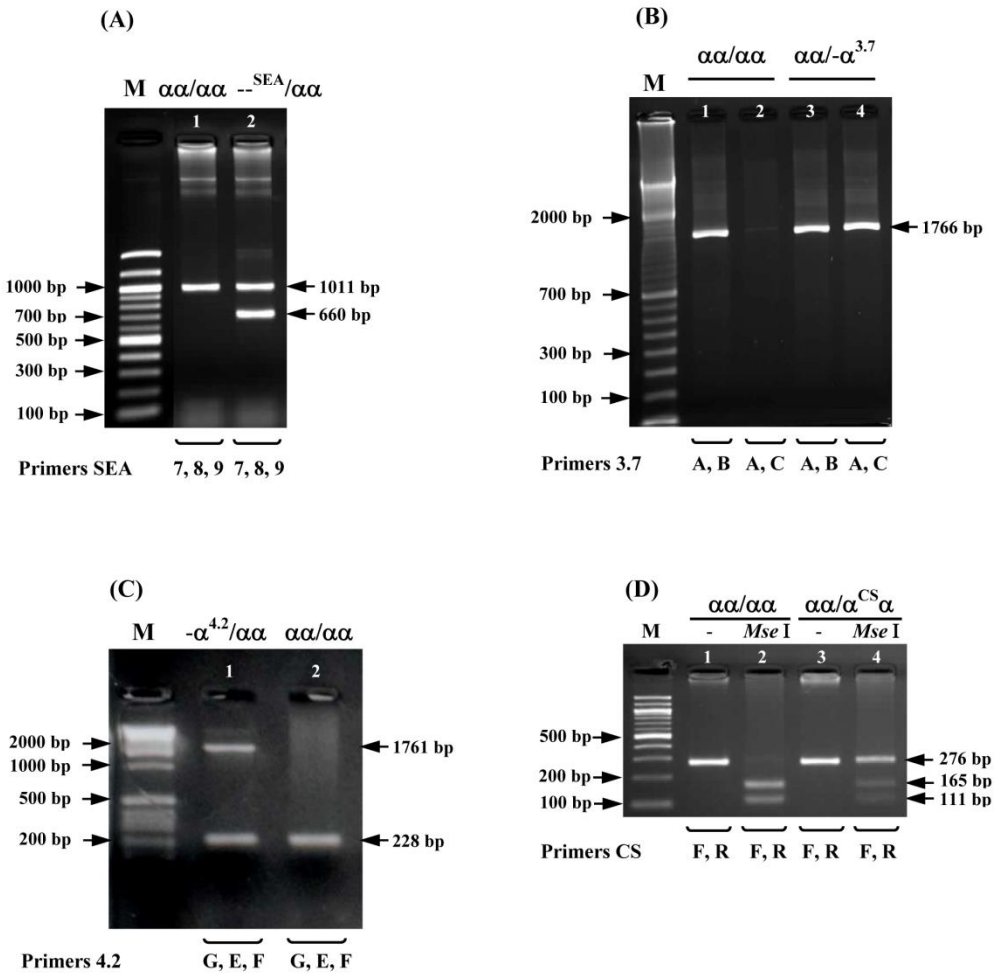

Figure 3.1 PCR detection of the common genotypes of α-thalassemias: $--^{SEA}$ type (A), $-\alpha^{3.7}$ type (B), $-\alpha^{4.2}$ type (C) and nondeletional type $\alpha^{CS}\alpha$ (Hb Constant Spring) (D).

M = standard 100 bp DNA ladders

Mse I = restriction enzyme *Mse* I

Table 3.1 Classification of α-thalassemia genotypes in 638 primigravidarum volunteers from Maharaj Nakorn Chiang Mai Hospital.

Genotype	Number of samples	Percent (%)
1. Normal (αα/αα)	409	64.11
2. Alpha-thalassemias	229	35.89
2.1 Deletional HbH disease ($--^{SEA}/-\alpha^{3.7}$)	18	2.82
2.2 Heterozygous α^0-thalassemia $--^{SEA}$ type ($--^{SEA}/\alpha\alpha$)	78	12.23
2.3 Heterozygous α^+-thalassemia $-\alpha^{3.7}$ type ($-\alpha^{3.7}/\alpha\alpha$)	99	15.52
2.4 Homozygous α^+-thalassemia $-\alpha^{3.7}$ type ($-\alpha^{3.7}/-\alpha^{3.7}$)	5	0.78
2.5 Heterozygous α^+-thalassemia $-\alpha^{4.2}$ type ($-\alpha^{4.2}/\alpha\alpha$)	2	0.31
2.6 Heterozygous HbCS ($\alpha^{CS}\alpha/\alpha\alpha$)	27	4.23
Total	638	100.00

3.2 Specificity of mouse anti-Hb Bart's monoclonal antibody

The specific binding of mouse monoclonal antibody against Hb Bart's was determined by indirect ELISA. Both culture supernatant of mouse hybridoma before and after single-cell cloning were determined for the specificity of mouse monoclonal antibody against Hb Bart's. All of culture supernatant containing mouse monoclonal antibody was added into ELISA well individually coated with Hbs; Bart's, A_2, E, F, A and H. Results revealed that mouse monoclonal antibody could bind specifically to Hb Bart's without cross reactivity to other hemoglobins. The specific binding of mouse monoclonal antibody to Hb Bart's after second round of the single-cell cloning was shown in Table 3.2 and Figure 3.2. Similar results were observed when specificity test were determined in the culture supernatant of mouse hybridoma before and after first round of the single-cell cloning.

Table 3.2 Specificity of mouse monoclonal antibody to Hbs; Bart's, A_2, E, F, A and H by indirect ELISA.

Type of Hb	OD 450 nm (1)	OD 450 nm (2)	OD 450 nm (mean)
Hb Bart's	1.202	1.464	1.333
HbA_2	0.093	0.065	0.079
HbE	0.085	0.069	0.077
HbF	0.105	0.103	0.104
HbA	0.059	0.053	0.056
HbH	0.069	0.065	0.067

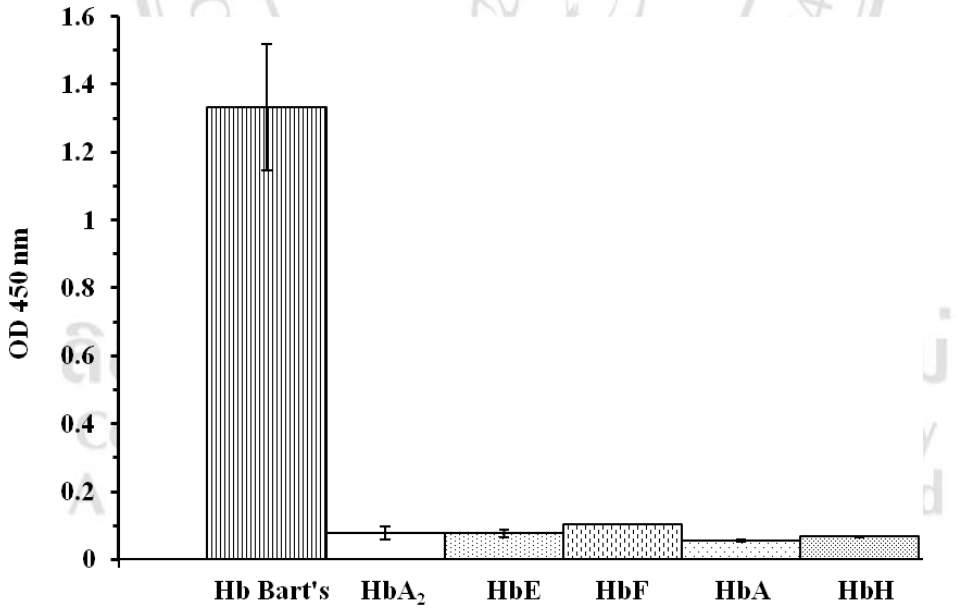

Figure 3.2 Reactivity of mouse monoclonal antibody to Hbs; Bart's, A_2, E, F, A and H by indirect ELISA.

3.3 RNA isolation, cDNA synthesis and antibody gene amplification

Thirty-three micrograms of total RNA were isolated from 2.22 x 10^6 cells of mouse hybridoma after second round of the single-cell cloning. The first-strand cDNA was subsequently synthesized from mRNA, then, the V_L and V_H genes were individually amplified using cDNA as templates. The amplicons of V_L and V_H genes electrophoresed in 2% agarose gel were 321 and 363 bp, respectively (Figure 3.3).

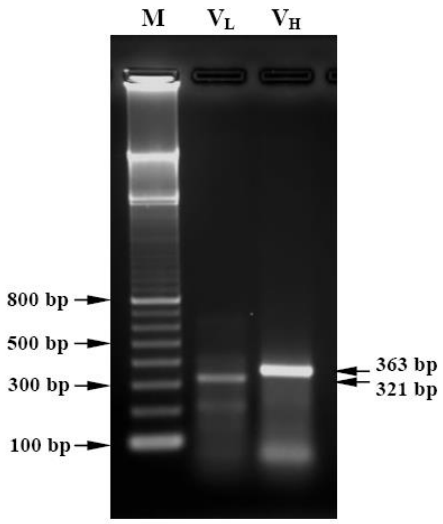

Figure 3.3 Agarose gel electrophoresis of amplicons of V_L and V_H gene from cDNA of mouse hybridoma.

Lane M = standard 100 bp DNA ladders

Lane V_L = amplicon of V_L gene

Lane V_H = amplicon of V_H gene

3.4 V_H and V_L gene cloning

Sixty-nine bacterial colonies were performed by colony PCR after V_H genes were cloned into pGEM®-T Easy vector. Results revealed 18 positive clones for the insertion of the V_H gene or pGEM®-T-V_H (537 bp) as shown in Figure 3.4.

After cloning of the V_L gene, 70 clones were subjected for colony PCR. Fourteen colonies of V_L were positive for the insertion of the V_L gene or pGEM®-T-V_L (495 bp) as shown in Figure 3.5.

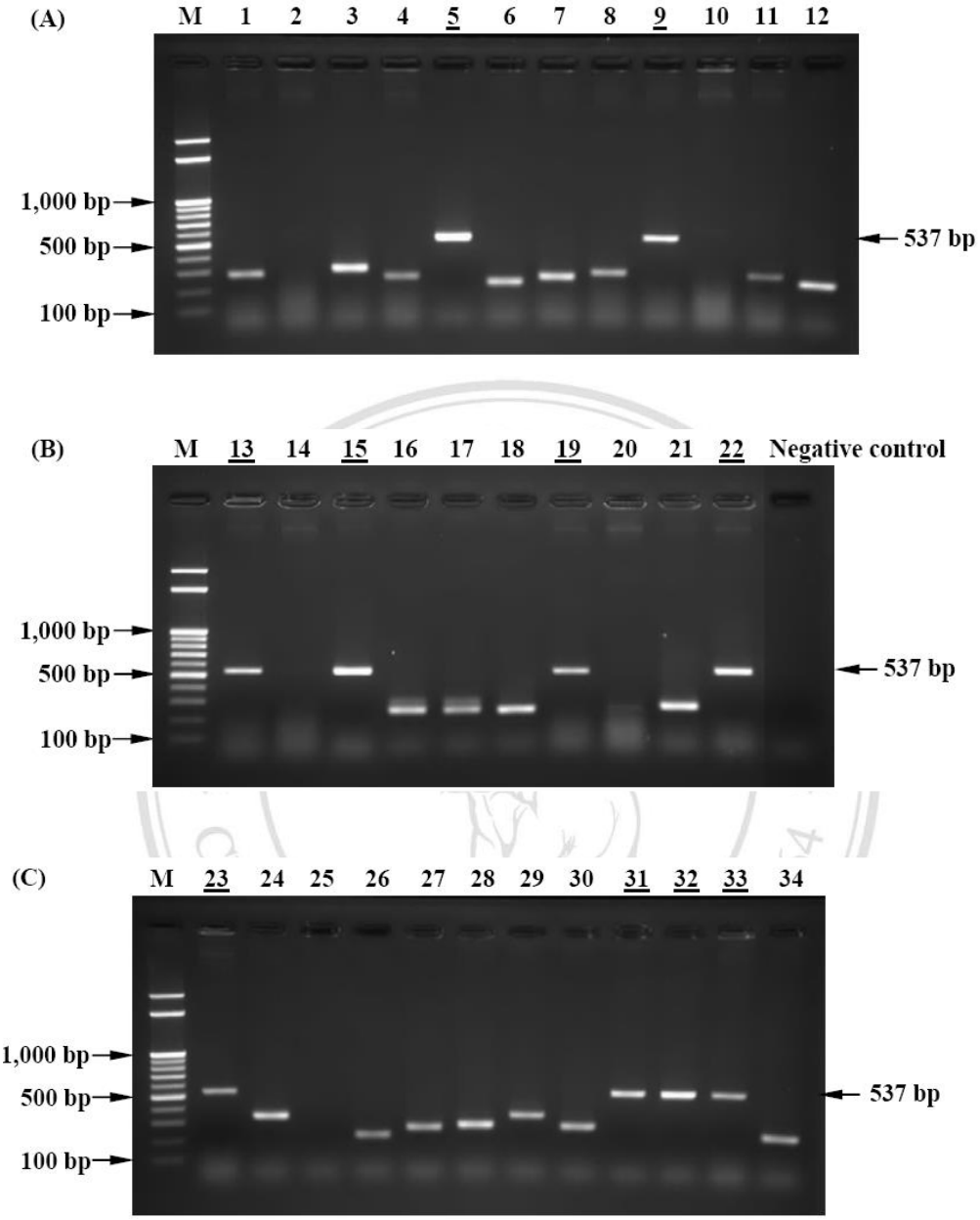

Figure 3.4 Agarose gel electrophoresis of amplicons of *E. coli* TOP10F white bacterial colonies for the presence of pGEM®-T-V_H by colony PCR.

M = standard 100 bp DNA ladders

Negative control = amplicon of PCR reaction without DNA template

Underlined number = positive bacterial clones for colony PCR

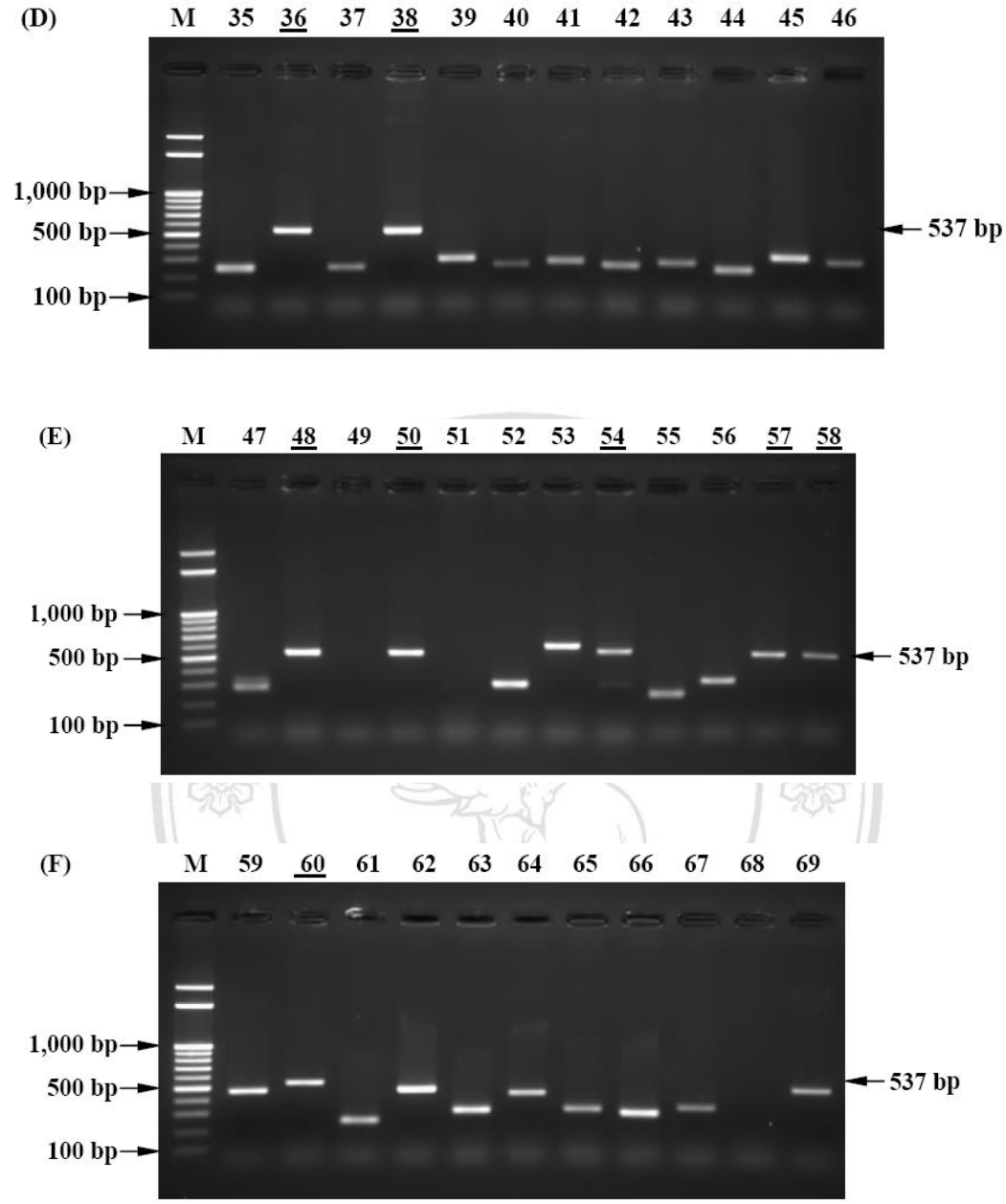

Figure 3.4 Agarose gel electrophoresis of amplicons of *E. coli* TOP10F white bacterial colonies for the presence of pGEM®-T-V_H by colony PCR (continued).

M = standard 100 bp DNA ladders

Underlined number = positive bacterial clones for colony PCR

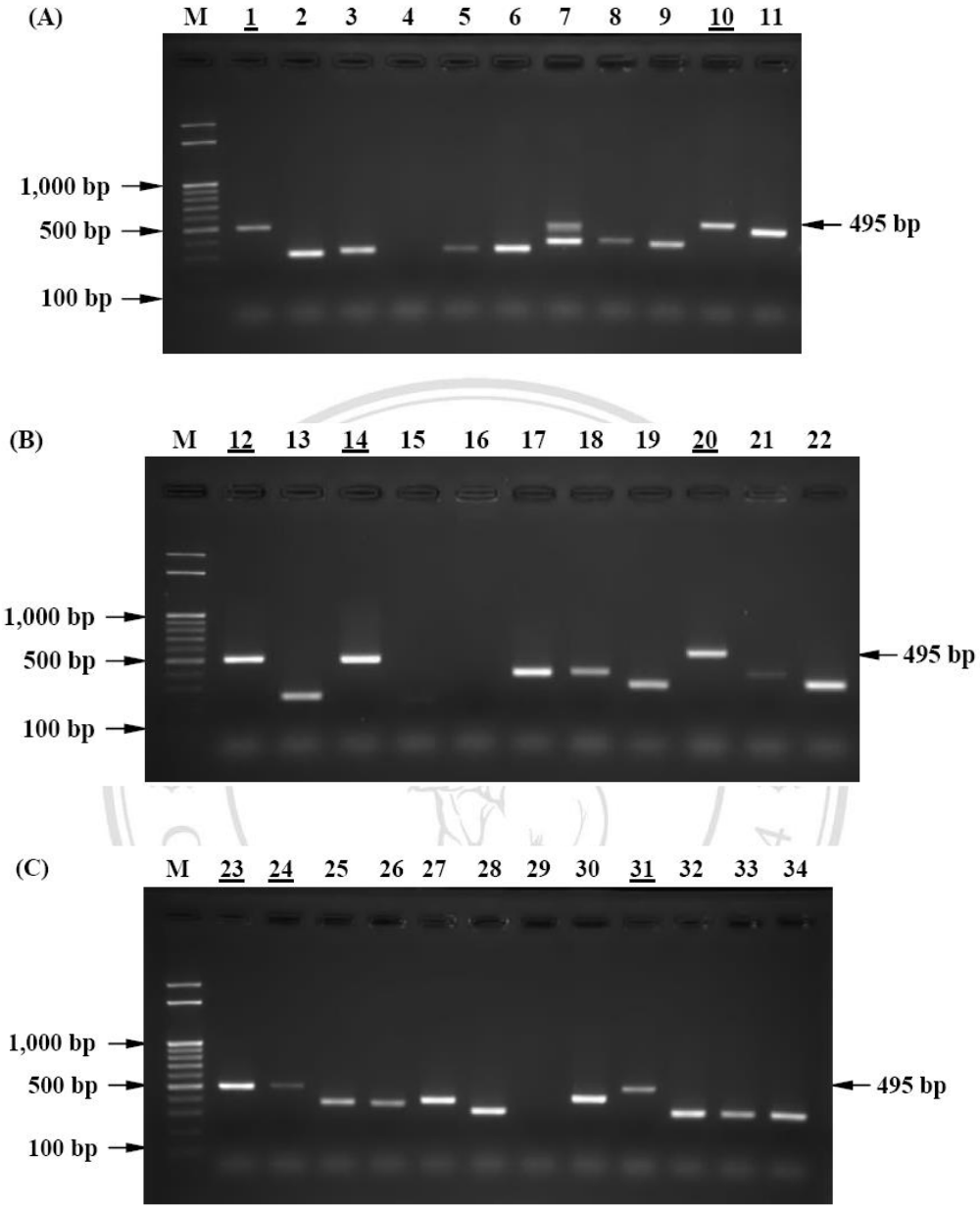

Figure 3.5 Agarose gel electrophoresis of amplicons of *E. coli* TOP10F white bacterial colonies for the presence of pGEM®-T-V_L by colony PCR.

M = Standard 100 bp DNA ladders

Underlined number = positive bacterial clones for colony PCR

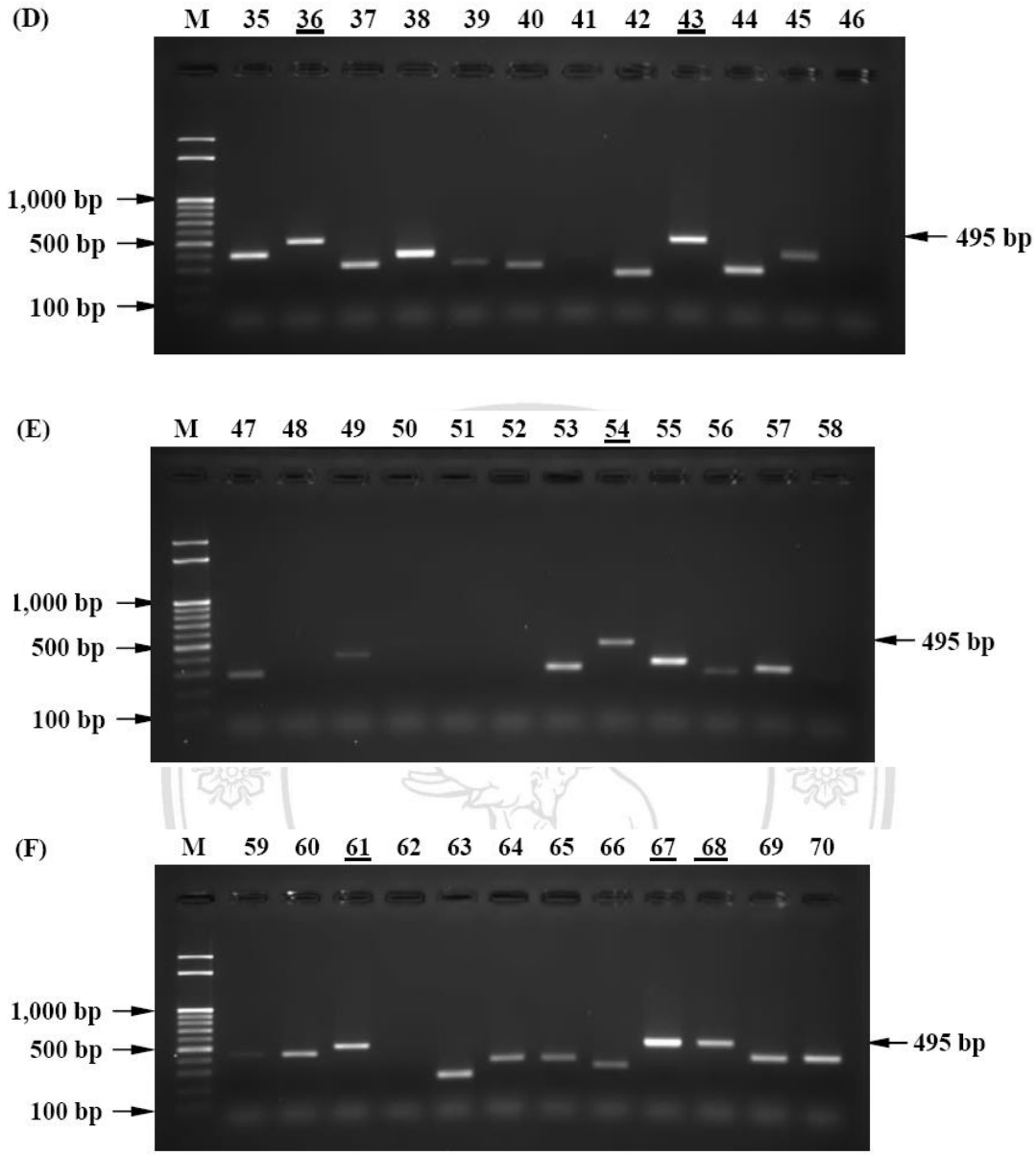

Figure 3.5 Agarose gel electrophoresis of amplicons of *E. coli* TOP10F white bacterial colonies for the presence of pGEM®-T-V_L by colony PCR (continued).

M = standard 100 bp DNA ladders

Underlined number = positive bacterial clones for colony PCR

3.5 Analysis of V_H and V_L DNA sequence

Nine of ten clones of purified plasmid DNA of the pGEM®-T-V_H were selected for *Eco*R I restriction digestion analysis. Results revealed the insertion of V_H gene in the pGEM®-T Easy vector after restriction digestion analysis as shown in Figure 3.6. After DNA sequencing and identification of CDRs-H, all of ten clones of V_H were identical and unique as shown in Figure 3.7.

All ten clones of purified plasmid DNA of the pGEM®-T-V_L revealed the insertion of V_L gene after restriction digestion analysis. After DNA sequencing and CDRs-L identification, two populations of V_L were obtained. Nucleotide sequences, deduced amino acid sequences and CDRs-L of 5/10 clones of V_L were shown in Figure 3.8. The other V_L populations (5/10), deduced amino acid sequences represented the premature termination and causing of shortened polypeptide products as shown in Figure 3.9.

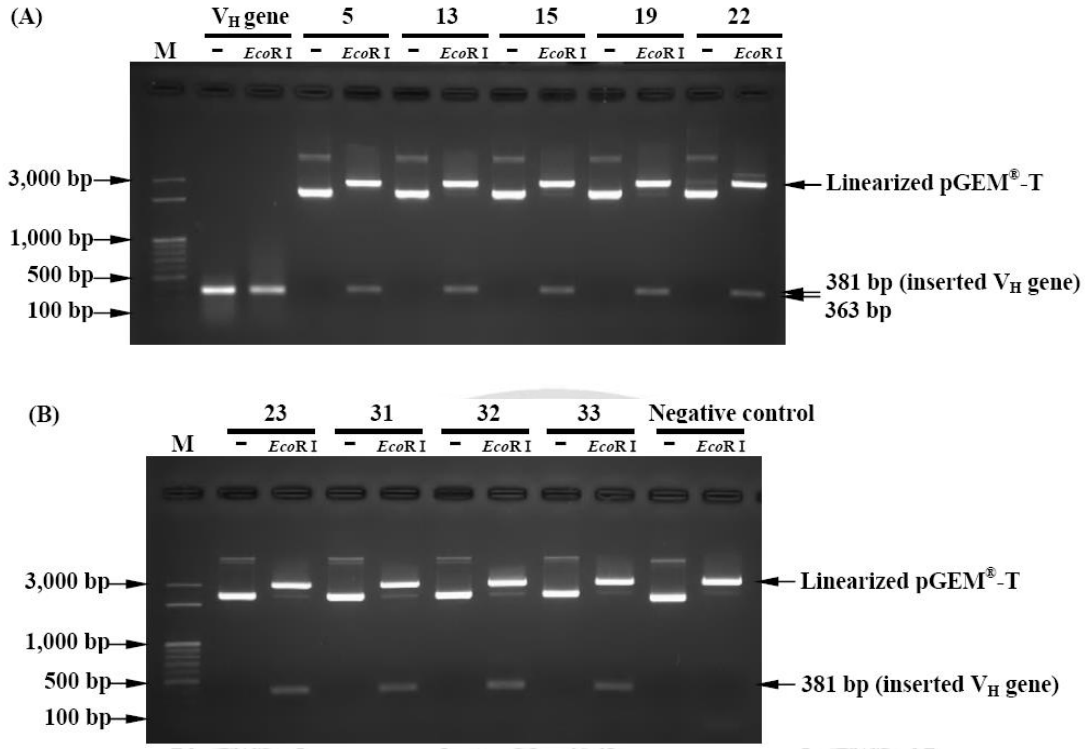

Figure 3.6 Agarose gel electrophoresis of *Eco*R I restriction digestion analysis of purified plasmid DNA from the pGEM®-T-V_H.

M = standard 100 bp DNA ladders

*Eco*R I = cutting of *Eco*R I restriction enzyme

V_H gene = amplicon of V_H gene (363 bp)

Negative control = circular pGEM®-T vector from negative colony PCR

Number = bacterial clone number

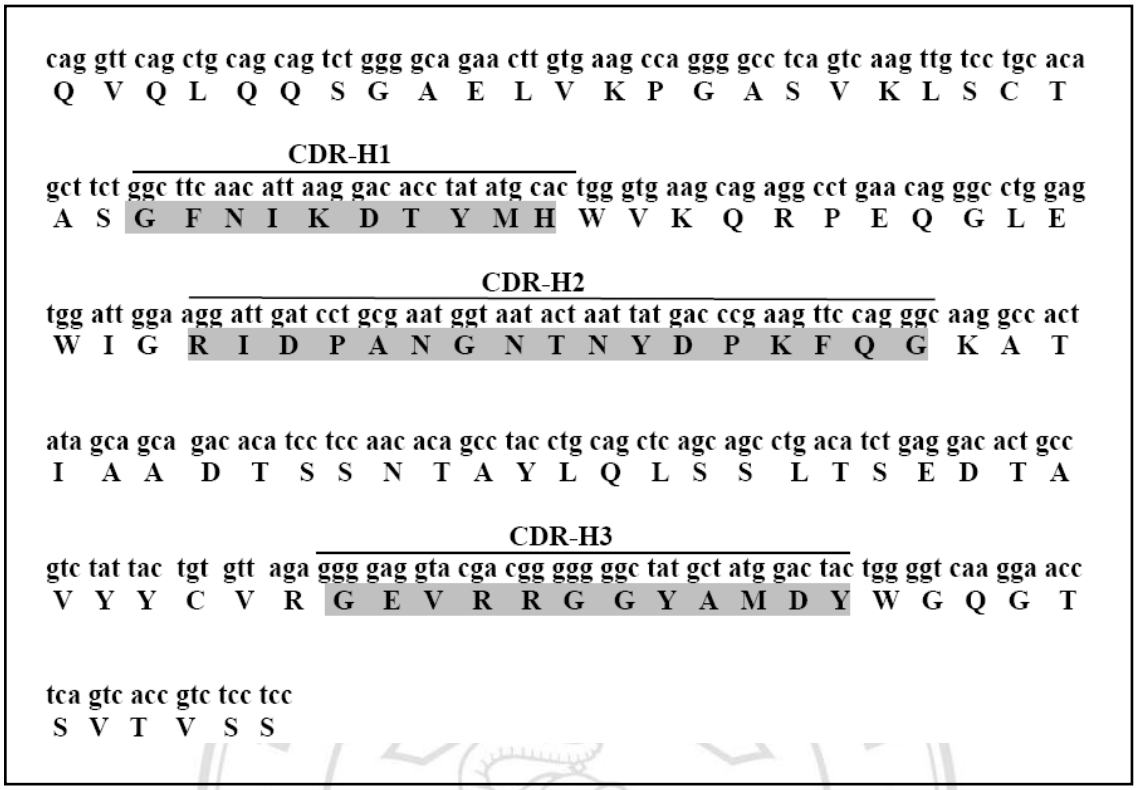

Figure 3.7 Nucleotide and amino acid sequences of V_H. CDR-H1, 2 and 3 were the complementarity determining regions (CDRs) 1, 2 and 3 of V_H.

Shaded regions = all of CDRs

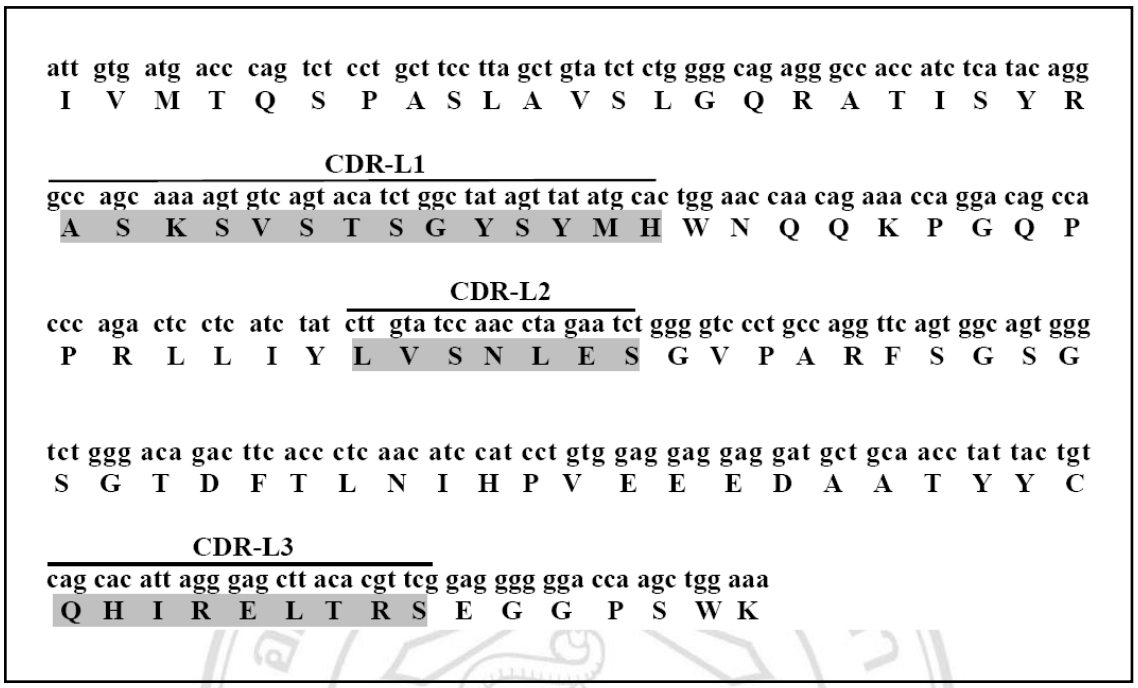

Figure 3.8 Nucleotide and amino acid sequences of V_L. CDR-L1, 2 and 3 were the complementarity determining regions (CDRs) 1, 2 and 3 of V_L.
Shaded regions = all of CDRs

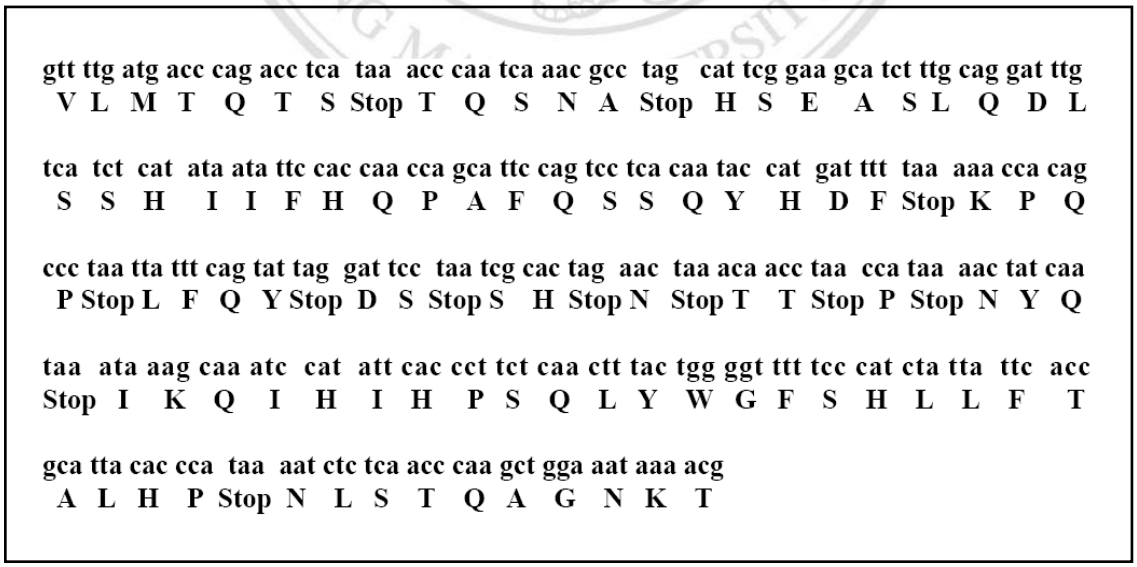

Figure 3.9 Nucleotide and amino acid sequences of the other V_L.

3.6 Full length scFv gene construction

After individual amplification of V_H and V_L genes followed by the assembly of V_H and V_L genes into full length scFv, the amplicons of the V_H, V_L and full length scFv genes were shown in Figure 3.10. The amplicon of V_H gene was 402 bp (Figure 3.10, lane 1). The amplicon of V_L gene was 364 bp (Figure 3.10, lane 2). The amplicon of full length scFv gene was 751 bp (Figure 3.10, lane 3).

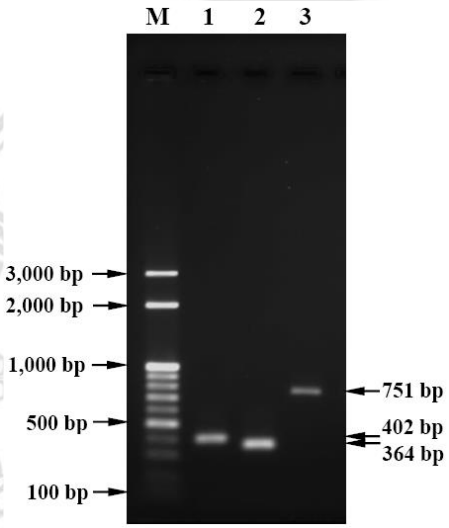

Figure 3.10 Agarose gel electrophoresis of amplicons of the V_H, V_L and full length scFv genes.

Lane M = standard 100 bp DNA ladders

Lane 1 = amplicon of V_H gene

Lane 2 = amplicon of V_L gene

Lane 3 = amplicon of full length scFv

3.7 Full length scFv gene cloning

After subcloning of full length scFv gene into pET28a(+) expression vector and transforming into *E. coli* BL21(DE3), 33 clones were subjected for colony PCR. Seventeen colonies were positive for the insertion of full length scFv gene or pET28a(+)-scFv anti-Hb Bart's (1,013 bp) as shown in Figure 3.11.

3.8 Full length scFv gene analysis

DNA sequence of full length scFv gene was identical to the designed V_H -linker-V_L gene. The nucleotides and deduced amino acid sequences of the N-terminal histidine fusion full length scFv were shown in Figure 3.12. Importantly, the nucleotides and deduced amino acid sequences of the full length scFv as V_H-linker-V_L format were submitted to GenBank public database as the accession no. KF663616.

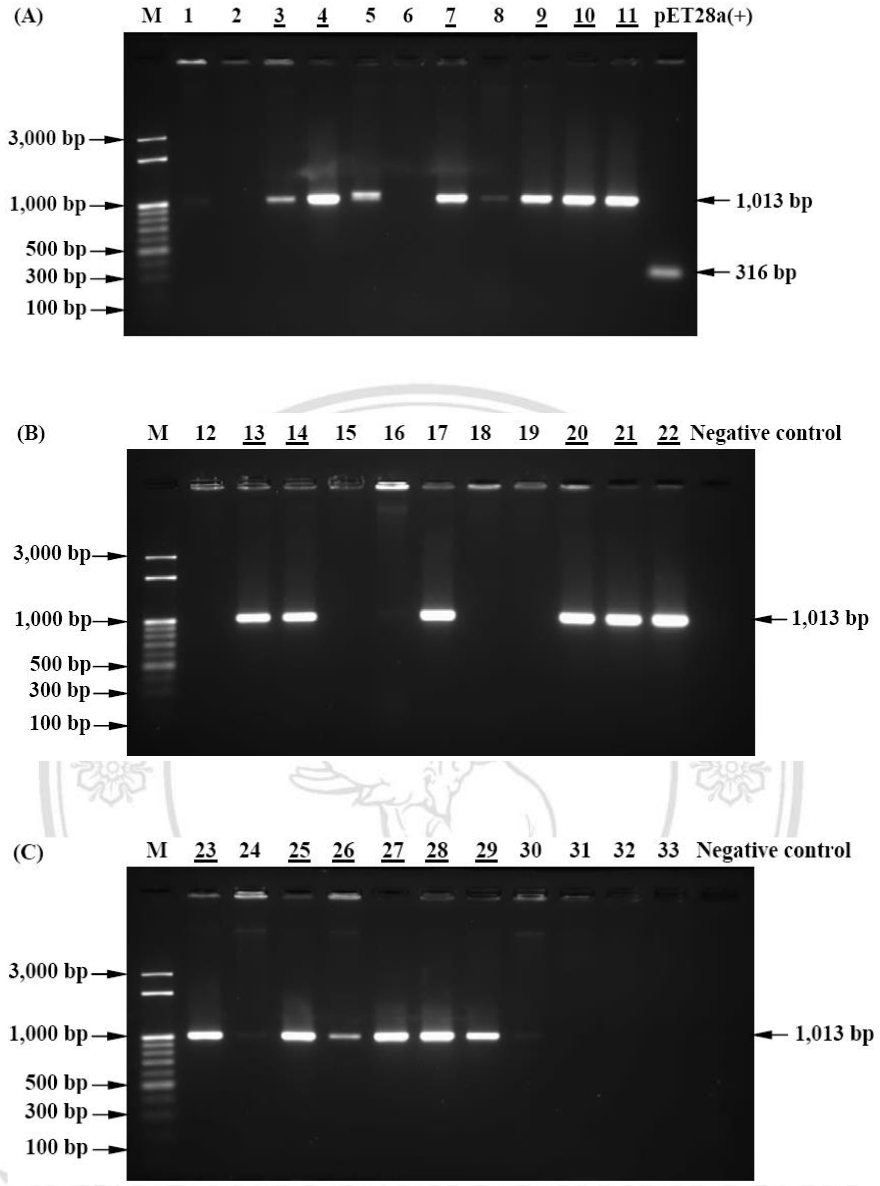

Figure 3.11 Agarose gel electrophoresis of amplicons of *E. coli* BL21(DE3) colonies for the presence of pET28a(+)-scFv anti-Hb Bart's by colony PCR.

M = standard 100 bp DNA ladders

pET28a(+) = empty pET28a(+) vector for uninserted gene control

Negative control = amplicon of PCR reaction without DNA template

Underlined number = positive bacterial clones for colony PCR

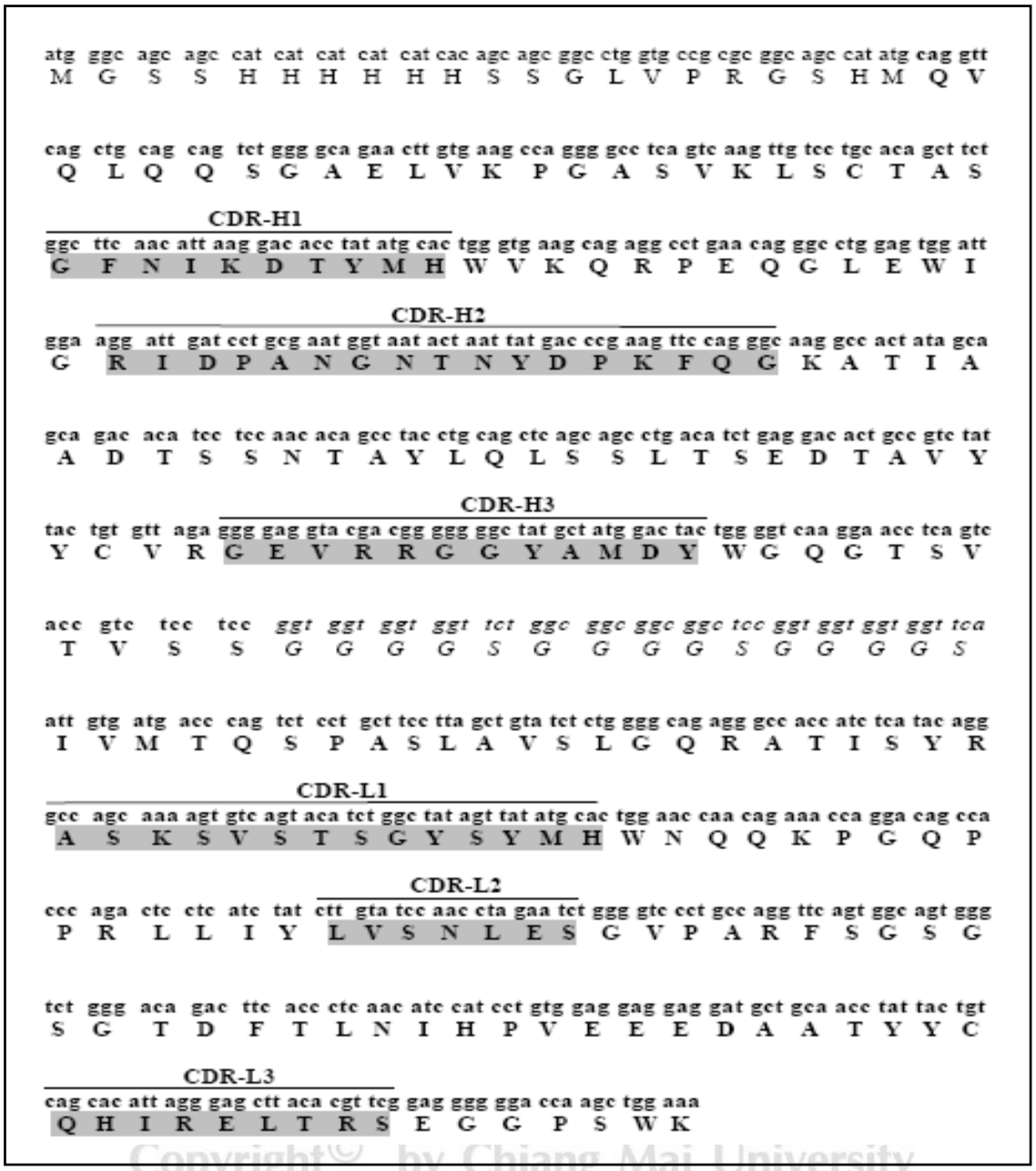

Figure 3.12 Nucleotide and amino acid sequences of the N-terminal histidine fusion full length scFv.

Bold letter = sequences of V_H and V_L

Italic letter = linker

CDR-H1, 2, and 3 = CDR 1, 2 and 3 of V_H

CDR-L1, 2, and 3 = CDR1, 2 and 3 of V_L

Shaded regions = all of CDRs

3.9 Optimization of IPTG induction and the full length scFv expression

Protein expression from various times in *E. coli* was analyzed by SDS-PAGE. The density of proteins analyzed by ImageJ program was increased by the induction time in dependent manner. The density of protein was highest at 4 h and being plateau at 6-24 h after IPTG induction. Therefore, the optimized time of IPTG induction was 4 h as shown in Figure 3.13.

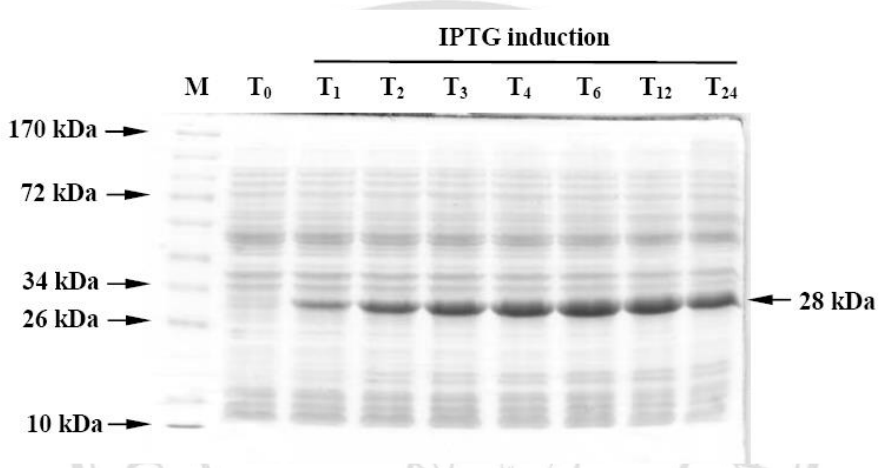

Figure 3.13 Polyacryamide gel electrophoresis of the expression of the scFv antibody in *E. coli* BL21(DE3) after IPTG induction at various time.

M = PageRuler® prestained protein molecular weight markers

T_0-T_{24} = IPTG induction time at 0-24 h

3.10 ScFv antibody expression

Bacterial cell lysate of uninduced and IPTG induced, soluble fraction of bacterial extract and solubilized inclusion bodies (IBs) were analyzed by SDS-PAGE. After IPTG induction, the scFv antibody was mostly expressed as inclusion bodies with the predicted molecular weight of 28 kDa as shown in Figure 3.14. In addition, the expression of the scFv antibody with N-terminal histidine tag was confirmed by Western blot analysis as shown in Figure 3.15.

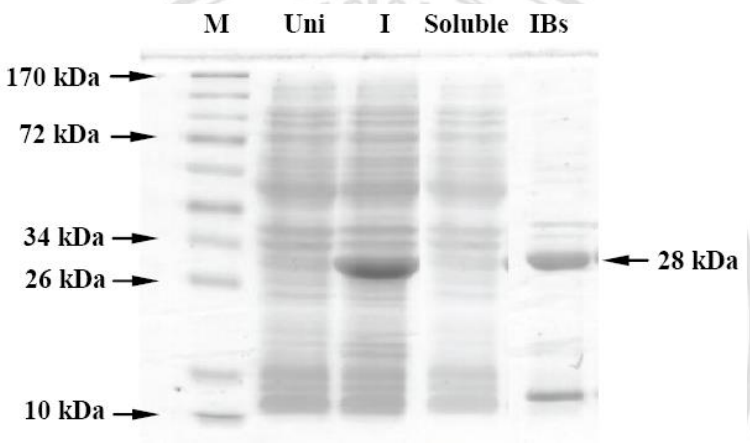

Figure 3.14 Polyacryamide gel electrophoresis of the expression of the scFv antibody in *E. coli* BL21(DE3) for analysis of induction condition.

M = PageRuler® prestained protein molecular weight markers

Uni = bacterial cell lysate of uninduced

I = bacterial cell lysate of IPTG induced

Soluble = soluble fraction of bacterial extract

IBs = solubilized inclusion bodies

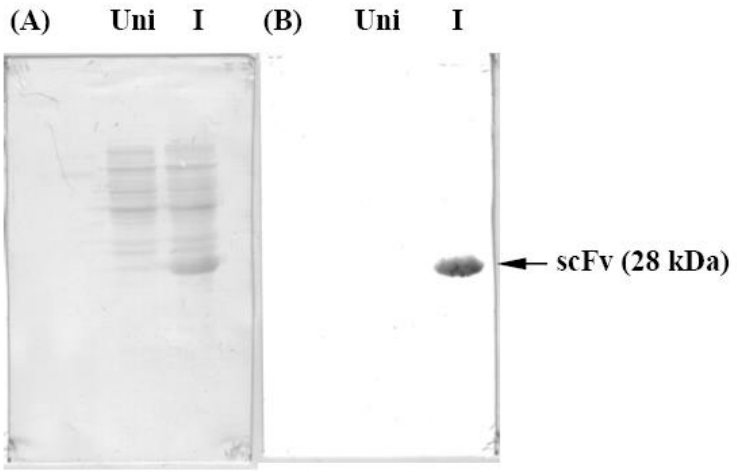

Figure 3.15 Western blot analysis of the expression of N-terminal histidine fusion scFv antibody. The protein of uninduced and IPTG induced fractions were run in 12% SDS-PAGE, then, either transferred onto a PVDF membrane for staining with Coomassie brilliant blue R-250 (A) or Western blot analysis using HRP conjugated mouse anti-His-tag (B).

Uni = bacterial cell lysate of uninduced

I = bacterial cell lysate of IPTG induced

3.11 ScFv antibody purification and refolding

The total proteins, purity and percent of recovery yield of scFv antibody in each purification step were summarized in Table 3.3. Protein concentration was determined by the modified Bradford assay. Purity was evaluated by analysis the density of protein bands from SDS-PAGE of Figure 3.14 and 3.16 using ImageJ program. Results revealed that approximately 1 mg of refolded scFv antibody was recovered from 1 L of bacterial culture (2% recovery yield). The refolded scFv antibody of 28 kDa was confirmed by SDS-PAGE as shown in Figure 3.16.

Table 3.3 Recovery yield of the scFv antibody from each step of purification.

	Protein concentration (mg/mL)	Total volume (mL)	Total protein (mg)	Purity (%)	scFv antibody (mg)	Yield (%)
Bacterial cell lysate	1.4	80	112	47	53	100
Denatured inclusion bodies	0.9	60	54	52	28	53
Ni-NTA chromatography	1.1	20	22	90	19.8	37
Ultrafiltration from refolded scFv	0.2	6	1.2	90	1	2

Approximately 4 g of cells (wet weight) was obtained from 1 L of bacterial culture; protein concentrations determined by the modified Bradford assay.

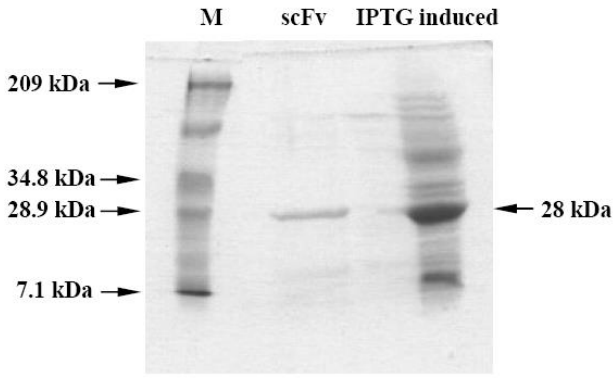

Figure 3.16 Polyacryamide gel electrophoresis of the refolded scFv antibody.
M = Kaleidoscope protein molecular weight markers
scFv = 2 µg of the refolded scFv antibody
IPTG induced = bacterial cell lysate of IPTG induced

3.12 Specificity of the refolded scFv antibody and the parent monoclonal antibody

The specific binding of the refolded scFv antibody and the parent monoclonal antibody to Hb Bart's was determined by Western blot analysis. The native-PAGE of hemoglobins was either stained with Coomassie brilliant blue (Figure 3.17A) or transferred onto a nitrocellulose membrane (Figure 3.17B). Results revealed that the scFv antibody could bind specifically to Hb Bart's (Figure 3.17B, lanes 1 and 8) without cross-reactivity to Hbs; A, F, S, E, A_2 and H (Figure 3.17B, lanes 2-7). Similar results were observed when specificity test was undertaken in the parent monoclonal antibody (Figure 3.17C).

The specific binding of the refolded scFv antibody to Hb Bart's was also determined by indirect ELISA. Results revealed that the refolded scFv antibody could bind specifically to Hb Bart's without cross reactivity to other hemoglobins as shown in Table 3.4 and Figure 3.18.

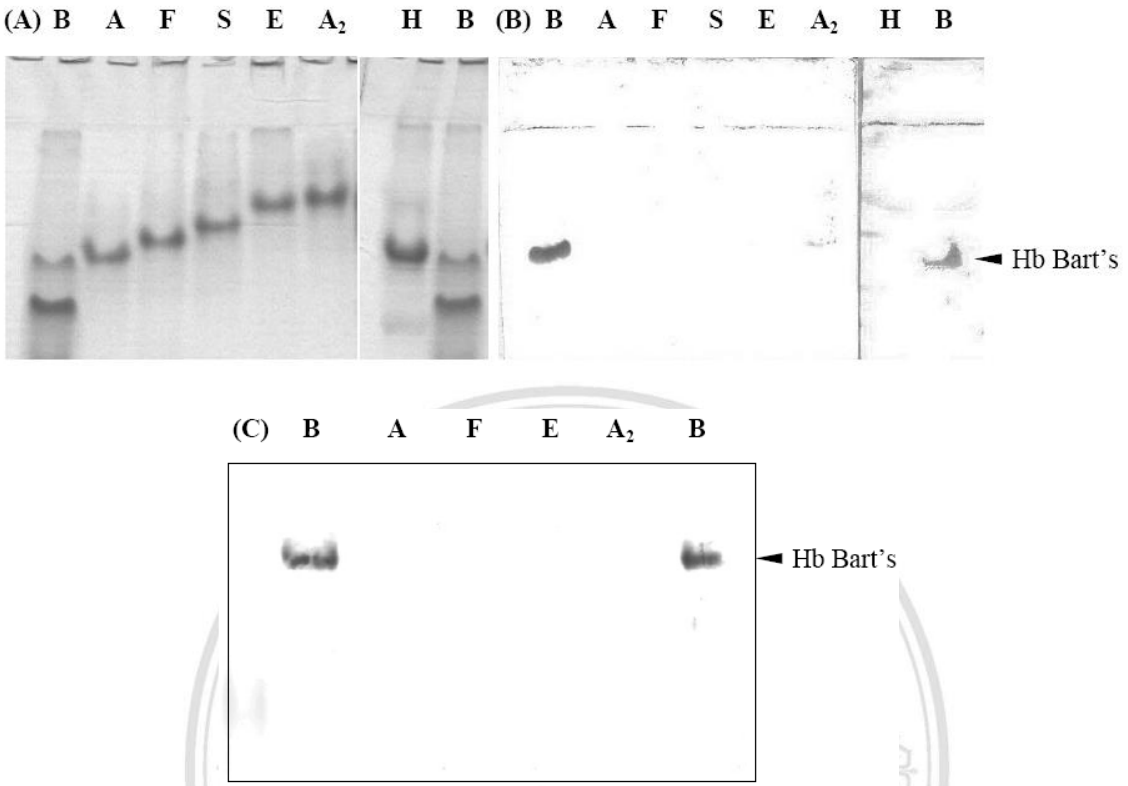

Figure 3.17 Native polyacrylamide gel electrophoresis and Western blot analysis of the specific binding of the refolded scFv antibody to Hb Bart's. Fifty micrograms of each standard hemoglobins (A, F, S, E and A_2) and 120 µg of hemolysates of Hb Bart's hydrops fetalis and HbH were run in 12% native-PAGE. Hemoglobin proteins were either stained with Coomassie brilliant blue R-250 (A) or transferred onto a nitrocellulose membrane for Western blot analysis by the refolded scFv anti-Hb Bart's (B) and the parent monoclonal antibody (C).

Lane B = hemolysate of Hb Bart's hydrops fetalis
Lane A = HbA
Lane F = HbF
Lane S = HbS
Lane E = HbE
Lane A_2= HbA_2
Lane H = hemolysate of HbH disease

Table 3.4 Specificity of the refolded scFv antibody to Hbs; Bart's, A_2, E, F, A and H by indirect ELISA.

Type of Hb	OD 450 nm (1)	OD 450 nm (2)	OD 450 nm (mean)
Hb Bart's	2.510	2.490	2.500
HbA_2	0.167	0.210	0.189
HbE	0.194	0.212	0.203
HbF	0.139	0.147	0.143
HbA	0.131	0.141	0.136
HbH	0.144	0.119	0.132

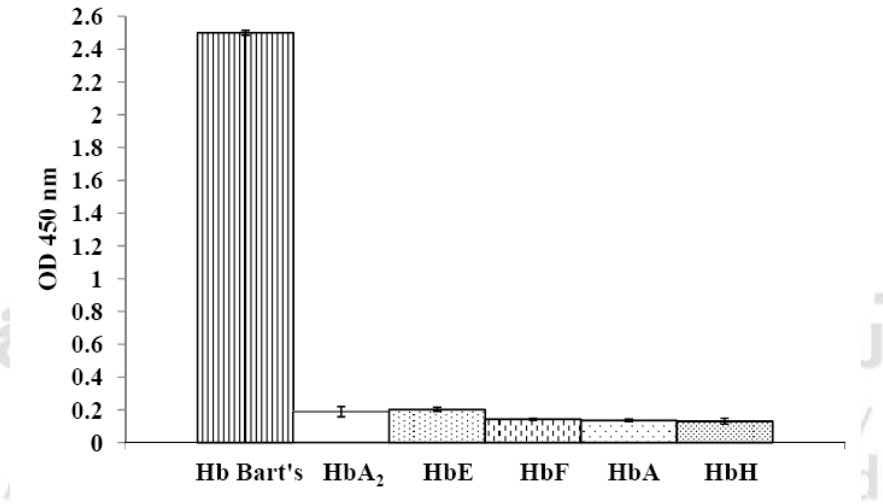

Figure 3.18 Reactivity of the refolded scFv antibody to Hb Bart's, A_2, E, F, A and H by indirect ELISA.

3.13 Sensitivity of the refolded scFv antibody and the parent monoclonal antibody

Dot blot ELISA using the refolded scFv antibody was developed for the sensitivity determination of Hb Bart's. The detection sensitivity of Hb Bart's was 5 μg/μL of Hb Bart's hydrops fetalis hemolysate as shown in Figure 3.19A, spot 4. Similar results were obtained when using the parent monoclonal antibody (Figure 3.19B).

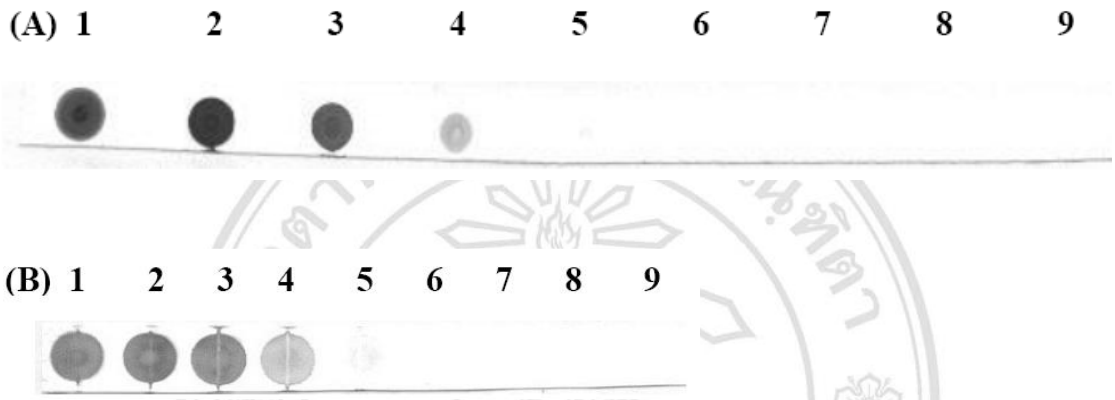

Figure 3.19 Sensitivity of the refolded scFv antibody and the parent monoclonal antibody to Hb Bart's by dot blot ELISA. Hb Bart's hydrops fetalis hemolysate at various concentrations were spotted onto a nitrocellulose membrane and detected with 100 μg/mL of the refolded scFv antibody (A) and 100 μg/mL of the parent monoclonal antibody (B).

Spot 1 = 40 μg of Hb Bart's

Spot 2 = 20 μg of Hb Bart's

Spot 3 = 10 μg of Hb Bart's

Spot 4 = 5 μg of Hb Bart's

Spot 5 = 2.5 μg of Hb Bart's

Spot 6 = 1.25 μg of Hb Bart's

Spot 7 = 0.63 μg of Hb Bart's

Spot 8 = 0.31 μg of Hb Bart's

Spot 9 = 0 μg of Hb Bart's

3.14 Affinity constant (K_a) of the refolded scFv antibody and the parent monoclonal antibody

The K_a of the refolded scFv antibody was determined by indirect ELISA. A total of four concentrations of Hb Bart's were analyzed and six K_a (three for n = 2, two for n =4 and one for n = 8) were obtained for affinity binding calculation of the refolded scFv antibody as shown in Figure 3.20A. The mean K_a of the refolded scFv to Hb Bart's was 8.31 ± 1.28 x 10^5 M^{-1}. The K_a of the parent monoclonal antibody was performed simultaneously (Figure 3.20B) as K_a = 2.11 ± 0.20 x 10^6 M^{-1}.

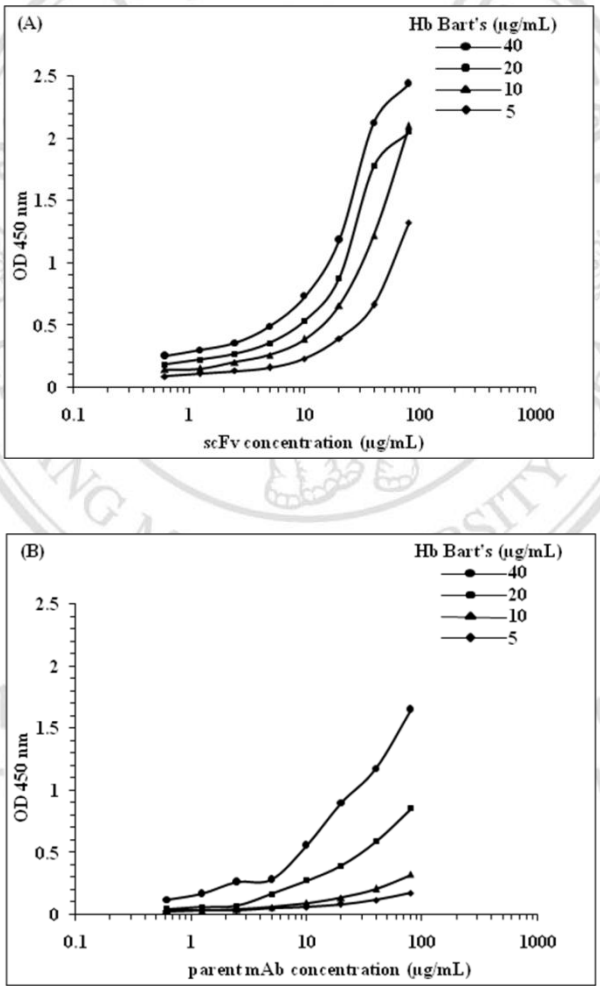

Figure 3.20 Reactivity of the refolded scFv antibody (A) and the parent monoclonal antibody (B) to various concentrations of Hb Bart's for analysis of the affinity constant (K_a) by indirect ELISA.

3.15 Stability of the refolded scFv antibody

The stability of the refolded scFv antibody storing at various temperature and time were evaluated by dot blot ELISA. Results revealed that the refolded scFv antibody was remained the activity for capturing Hb Bart's when stored at -20°C for 1 week. However, the function of scFv antibody was lost when storage at 4°C for 1 week and -20°C for 1 month or more.