APPENDIX A

List of chemicals and materials used in study

Chemicals/Materials	Companies
Absolute ethanol	E. Merck, Germany
Acetic acid	E. Merck, Germany
Acrylamide	Plusone, Sweden
Agarose	Gibco, USA
Ammonium persulfate	Sigma-Aldrich, USA
Amphotericin B	Bristol-Myers Squibb, USA
Ampicillin	Biobasic, Canada
Arginine	Biobasic, Canada
Asparagine	E. Merck, Germany
Bacto®-tryptone	Becton Dikinson, USA
Bacto®-yeast extract	Becton Dikinson, USA
*Bam*H I	Amersham Pharmacia Biotech, Sweden
Blot absorbent filter paper	Bio-Rad, USA
Bromophenol blue	Sigma-Aldrich, USA
Calcium chloride	E. Merck, Germany
Centrifuge tube 15, 50 mL	Nunc, Denmark
Centriprep centrifugal filter	Millipore, USA
4-Chloro-1-naphthol	Sigma-Aldrich, USA
Coomassie brilliant blue G-250	Sigma-Aldrich, USA
Coomassie brilliant blue R-250	Sigma-Aldrich, USA
Deoxynucleotide triphosphates	Promega, USA
Dimethyl formamide	Sigma-Aldrich, USA
Dimethyl sulfoxide	Sigma-Aldrich, USA
Disodium hydrogen phosphate	E. Merck, Germany

Chemicals/Materials	Companies
Dithiothreitol	Sigma-Aldrich, USA
DNA polymerase	Finnzymes, Finland
E. coli BL21(DE3)	Novagen, USA
E. coli TOP10F	Novagen, USA
EcoR I	Amersham Pharmacia Biotech, Sweden
ELISA plate	Nunc, USA
Eppendorf tube 1.5 mL	Corning, USA
Ethidium bromide	American research product, USA
Ethylenediaminetetraacetic acid	E. Merck, Germany
Fetal bovine serum	Gibco, USA
Filter paper	Whatman, England
Gentamycin	Atlantic Laboratories, Thailand
Glucose	E. Merck, Germany
Glutathione oxidized	Biobasic, Canada
Glutathione reduced	Biobasic, Canada
Glycerol	E. Merck, Germany
Glycine	E. Merck, Germany
Hemoglobin S standard	Sigma-Aldrich, USA
HRP conjugated mouse anti-His-tag	US Biological, USA
HRP conjugated rabbit anti-mouse Igs	Dako, Denmark
Hydrochloric acid	E. Merck, Germany
Hydrogen peroxide	Vittayasom, Thailand
Iscove's Modified Dulbecco's Medium	Gibco, USA
Isopropanol	E. Merck, Germany
Isopropyl-β-D-thiogalactopyranoside	Biobasic, Canada
Kaleidoscope protein standard marker	Bio-Rad, USA
Kanamycin	Biobasic, Canada
LB agar	Biobasic, Canada
LB broth	Biobasic, Canada
Ligation buffer	Promega, USA
Lysozyme	Amresco, USA

Chemicals/Materials	Companies
Magnesium chloride	E. Merck, Germany
Magnesium chloride 25 mM	Promega, USA
β-Mercaptoethanol	Sigma-Aldrich, USA
Methanol	E. Merck, Germany
N, N′-Methylene-bis-acrylamide	Sigma-Aldrich, USA
Nde I	Amersham Pharmacia Biotech, Sweden
Ni-NTA resin	Novagen, USA
Nitrocellulose membrane	Schleicher & Schuell, Germany
Oligonucleotide primer	Pacific Science, Thailand
PageRuler® prestained protein ladder	Fermentas, USA
Pepstatin	Sigma-Aldrich, USA
pET-28a(+) expression vector	Novagen, USA
pGEM®-T Easy vector	Promega, USA
Phosphoric acid	E. Merck, Germany
Phenylmethyl sulfonyl fluoride	Biobasic, Canada
Phusion® HF buffer 5x	Finnzymes, Finland
Phusion® DNA polymerase	Finnzymes, Finland
Pipette tip	Corning, USA
Polyvinylidene difluoride membrane	NEN Life Science, USA
Potassium chloride	E. Merck, Germany
Potassium dihydrogen phosphate	E. Merck, Germany
Skim milk	Difco, USA
Sodium acetate	E. Merck, Germany
Sodium carbonate anhydrous	E. Merck, Germany
Sodium chloride	E. Merck, Germany
Sodium citrate	E. Merck, Germany
Sodium dodecyl sulphate	Sigma-Aldrich, USA
Sodium hydrogen carbonate	E. Merck, Germany
Sodium hydroxide	E. Merck, Germany
Sulfuric acid	E. Merck, Germany
Supor® membrane filter	Pall Life Science, USA

Chemicals/Materials	**Companies**
T4 DNA ligase	Promega, USA
Taq DNA polymerase	Promega, USA
3, 3′, 5, 5′-Tetramethylbenzidine	Sigma-Aldrich, USA
N, N, N′, N′-Tetramethylethylenediamine	Promega, USA
Thin wall 0.2 mL tube	Corning, USA
Tissue culture flask 25 cm^2	Nunc, USA
Tissue culture plate 24, and 96-well	Nunc, USA
Tris (hydroxymethyl aminomethane)	E. Merck, Germany
Triton X-100	Sigma-Aldrich, USA
Tween 20 (polyethylenesorbitan)	Sigma-Aldrich, USA
Urea	E. Merck, Germany
X-gal	Biobasic, Canad

APPENDIX B

List of instruments used in study

Instruments	Models	Sources
Balance	500C	Precisa, UK
Centrifuge	Universal 16R	Hettich Zentrifugen, Deuthchland
Centrifuge hi-speed	5C	Sorval, USA
CO_2 incubator	TC2323	Shel Lab, USA
Freezer -20°C	FC-27	Sharp, Japan
Gel Doc	1000	Bio-Rad, USA
Hot air oven	UM600	Memmert, Germany
Incubator 37°C	U25	Memmert, Germany
Invert microscope	IX50-S8F2	Olympus, Japan
Laminar flow	NU-440-300E	Nuaire, USA
Light microscope	CH30	Olympus, USA
Magnetic stirrer	MS 3000	Biosan, USA
Microcentrifuge	Minispin plus	Eppendorf, Germany
Microplate reader	EL311	Bio-Tek, USA
Microplate washer	GLW 1000	Genelabs Diagnostic, Singapore
Microwave	NN-6653	National, Japan
Orbital incubator shaker	Gyromax™ 737R	Amerex Instruments, USA
PCR thermal cycler	2400	Perkin Elmer, USA
pH meter	ΦPHI 34	Beckman, USA
Refrigerator	The elegance	Whirpool, Japan
Semi-Dry Electrophoretic Transfer Cell (Trans-Blot® SD)	170-3940	Bio-Rad, USA
Shaker	VS50 rocker	Shelton scientific, USA
Sonicator	MTO-SSPC1111670	Sonics & Materials, USA

Instruments	Models	Sources
Spectrophotometer	GeneQuan pro	Amersham Bioscience, USA
Submarine electrophoresis	Mupid-exu	Advance, Japan
Vertex mixer	VX 100	Labnet, USA
Vertical electrophoresis	Mini-protean	Bio-Rad, USA
Water bath	1086	GFL, UK

APPENDIX C

List of buffers and media used in study

1. Buffers and media for growth of mouse hybridoma and single-cell cloning

1.1 Incomplete Iscove's Modified Dulbecco's Medium (IMDM)

IMDM	17.2 g
$NaHCO_3$	3.024 g
40 mg/mL Gentamycin	1 mL
5 mg/mL Amphotericin B	0.5 mL
Deionized water to	1 L

Sterilized by filter through 0.2 µM Supor® membrane filter and kept at 4ºC.

1.2 IMDM supplemented with 10% or 20% fetal bovine serum (FBS)

Incomplete IMDM	90 mL or 80 mL
FBS	10 mL or 20 mL
0.6% β-Mercaptoethanol	60 µL

Kept at 4ºC.

1.3 0.6% β-Mercaptoethanol

β-Mercaptoethanol	30 µL
Incomplete IMDM	5 mL

Dispensed into aliquots of 60 µL per vial and kept at-20ºC in a brown bottle.

1.4 0.2% Trypan blue

Trypan blue	0.2 g
Phosphate buffer saline, pH 7.4 to	100 mL

Kept at 4ºC.

1.5 Turk's solution

1% Aqueous gentian violet	1 mL
Glacial acetic acid	3 mL
Distilled water to	100 mL

Kept at 4ºC.

2. Buffers and reagents for indirect ELISA, Western blot analysis and dot blot ELISA

2.1 50 mM Carbonate-bicarbonate buffer, pH 9.6 (coating buffer)

Na_2CO_3	1.59 g
$NaHCO_3$	2.94 g
Distilled water to	1 L

2.2 10x Phosphate buffer saline (PBS)

NaCl	80.0 g
Na_2HPO_4	11.5 g
KCl	2.0 g
KH_2PO_4	2.0 g
Distilled water to	1 L

2.3 1x PBS, pH 7.4

10 x PBS	100 mL
Distilled water	900 mL

2.4 PBS-Tween 20 (0.05% Tween 20)

Tween 20 (polyethylenesorbitan)	0.5 mL
1x PBS, pH 7.4 to	1 L

2.5 10% (w/v) Skim milk

Skim milk	10 g
1x PBS, pH 7.4 to	100 mL

Kept at -20ºC.

2.6 TMB substrate

Dissolved TMB	1.6 mg in 0.5 mL DMSO
0.1 M Acetate buffer, pH 5.5	9.5 mL
6% H_2O_2	29.2 µL

Freshly prepared before used.

2.7 0.1 M Acetate buffer, pH 5.5

Sodium acetate.$3H_2O$	3.4 g
Distilled water	200 mL

Adjusted pH to 5.5 with glacial acetic acid before adjusting final volume to 250 mL with distilled water. Kept at 4ºC.

2.8 Stock 4-chloro-1-naphthol substrate

4-chloro-1-naphthol	0.06 g
Absolute methanol to	20 mL

Kept at -20ºC in a brown bottle.

2.9 Working 4-chloro-1-naphthol substrate

Stock 4-chloro-1-naphthol	2 mL
10 mM Tris-HCl, pH 7.5	10 mL
5 M NaCl	0.3 mL
6% H_2O_2	30 µL

Freshly prepared before used.

2.10 5 M NaCl

NaCl	292.2 g
Distilled water to	1 L

Sterilized by autoclaving.

2.11 1 M Tris-HCl, pH 8.0

Tris (base)	121.14 g
Distilled water	800 mL

Adjusted pH to 8.0 by adding concentrate HCl. Allowed to cool to room temperature before adjusting final volume to 1 L with distilled water and sterilized by autoclaving.

2.12 10 mM Tris-HCl, pH 7.5

1 M Tris-HCl, pH 8.0	10 mL
Distilled water to	800 mL

Adjusted pH to 7.5 with HCl before adjusting final volume to 1 L with distilled water. Sterilized by autoclaving.

2.13 2.5 N H_2SO_4

Concentrate H_2SO_4	7.0 mL
Distilled water to	100 mL

Cautiously added acid to distilled water.

3. Buffers and reagents for agarose gel, and polyacrylamide gel electrophoresis

3.1 50x Tris-acetate-EDTA (TAE) buffer

Tris (base)	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA, pH 8.0	100 mL
Distilled water to	1 L

3.2 1x TAE

50 x TAE	20 mL
Distilled water to	1 L

3.3 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0

EDTA disodium salt.$2H_2O$	186.1 g
Distilled water to	800 mL

Adjusted pH to 8.0 with NaOH (~20 g of NaOH pellets) before adjusting final volume to 1 L with distilled water. Sterilized by autoclaving.

3.4 6x Gel loading buffer

Bromophenol blue	25 mg
Sucrose	4 g
Distilled water to	10 mL

Kept at 4°C.

3.5 10 mg/mL Ethidium bromide

Ethidium bromide	0.1 g
Distilled water to	10 mL

Kept at room temperature in a brown bottle.

3.6 30% Acrylamide

Acrylamide	29 g
N, N′-Methylene-bis-acrylamide	1 g
Distilled water to	100 mL

Heated at 37ºC to dissolve the chemicals. Sterilized by filter through 0.45 µM Supor® membrane filter and kept at room temperature in a brown bottle.

3.7 10% Ammonium persulfate

Ammonium persulfate	0.1 g
Distilled water to	1 mL

Freshly prepared before used.

3.8 1.5 M Tris-HCl, pH 8.8

Tris (base)	181.71 g
Distilled water to	800 mL

Adjusted pH to 8.8 with HCl. Allowed to cool to room temperature before adjusting final volume to 1 L and sterilized by autoclaving.

3.9 1 M Tris-HCl, pH 6.8

Tris base	121.14 g
Distilled water to	800 mL

Adjusted pH to 6.8 with HCl. Allowed to cool to room temperature before adjusting final volume to 1 L and sterilized by autoclaving.

3.10 5x Tris-glycine electrophoresis buffer

Tris base	15.1 g
Glycine	94 g
10% SDS	50 mL
Distilled water to	1 L

3.11 10% Sodium dodecyl sulfate (SDS)

SDS	100 g
Distilled water to	1 L

Heated at 68ºC to assist dissolution. Adjusted pH to 7.2 with a few drop of concentrated HCl.

3.12 1x Tris-glycine electrophoresis buffer

5x Tris-glycine	200 mL
Distilled water to	1 L

3.13 5x Tris-asparagine electrophoresis buffer

Tris (base)	15.1 g
Asparagine	96.1 g
Distilled water to	1 L

3.14 1x Tris-asparagine electrophoresis buffer

5x Tris-asparagine	200 mL
Distilled water to	1 L

3.15 1x SDS gel-loading buffer

1 M Tris-HCl, pH 6.8	0.5 mL
1 M Dithiothreitol	1 mL
10% SDS	2 mL
Bromophenol blue	10 mg
87% Glycerol	1.15 mL
Distilled water to	10 mL

1 x SDS gel-loading buffer without dithiothreitol can be kept at room temperature. 1 M Dithiothreitol should be added before used.

3.16 1 M Dithiothreitol

Dithiothreitol	3.09 g
0.01 M Sodium acetate, pH 5.2	20 mL

Sterilized by filter through 0.2 µM Supor® membrane filter and kept at -20ºC.

3.17 6x SDS gel-loading buffer

1 M Tris-HCl, pH 6.8	3 mL
SDS	1.2 g
Bromophenol blue	60 mg
87% Glycerol	6.9 mL
Distilled water to	10 mL

After mixing 4 µL of 6x gel-loading buffer with 20 µL of protein samples (making final dilution to 1x gel-loading buffer), 2.4 µL of 1 M Dithiothreitol were added.

3.18 6x Native gel loading buffer

1 M Tris-HCl, pH 6.8	3 mL
Bromophenol blue	60 mg
87% Glycerol	6.9 mL
Distilled water to	10 mL

3.19 0.25% Coomassie brilliant blue R-250

Coomassie brilliant blue R-250	0.25 g
Absolute methanol	45 mL
Distilled water	45 mL
Glacial acetic acid	10 mL

Filtered through a Whatman® No.1 to remove the fine particular matter.

3.20 Destaining solution

Absolute methanol	450 mL
Distilled water	450 mL
Glacial acetic acid	100 mL

4. Buffers and medias for bacterial culture

4.1 Luria Bertani (LB) agar for 4 plates

LB agar	4 g
Deionized water to	100 mL

Sterilized by autoclaving then allowed to cool to 50ºC before adding 100 µL of antibiotics. Poured 25 mL of media to the 10 cm perti dish and kept at 4ºC.

4.2 LB broth

LB broth	25 g
Deionized water to	1 L

Sterilized by autoclaving then allowed to cool to 50ºC before adding 100 µL of antibiotics. Kept at 4ºC.

4.3 100 mg/mL Amplicilin

Amplicilin	1 g
Deionized water to	10 mL

Sterilized by filter through 0.2 µM Supor® membrane filter and kept at -20ºC in a brown bottle.

4.4 30 mg/mL Kanamycin

Kanamycin	0.3 g
Deionized water to	10 mL

Sterilized by filter through 0.2 µM Supor® membrane filter and kept at -20ºC in a brown bottle.

4.5 40 mg/mL X-gal

X-gal	40 mg
Dimethyl formamide	1 mL

Kept at -20ºC in a brown bottle.

4.6 400 mg/mL Isopropyl-β-D-thiogalactopyranoside (IPTG)

IPTG	0.4 g
Deionized water to	1 mL

Sterilized by filter through 0.2 µM Supor® membrane filter and kept at -20ºC in a brown bottle.

4.7 0.1 M $CaCl_2$

$CaCl_2.2H_2O$	1.47 g
Deionized water to	100 mL

Sterilized by filter through 0.2 µM Supor® membrane filter and kept at room temperature.

4.8 SOC medium

Bacto®-tryptone	20 g
Bacto®-yeast extract	5 g
NaCl	0.5 g
Deionized water	800 mL

Mixed until the solutes have dissolved, then added 10 mL of 250 mM KCl. Adjusted pH to 7.0 with NaOH, and adjusted final volume to 1 L with deionized water. Sterilized by autoclaving. Before used, added 5 mL of a sterile solution of 2 M $MgCl_2$ and 20 mL of 1 M glucose.

4.9 250 mM KCl

KCl	1.86 g
Deionized water to	100 mL

4.10 2 M $MgCl_2$

$MgCl_2$	19 g
Deionized water to	100 mL

Sterilized by autoclaving.

4.11 1 M Glucose

Glucose	18 g
Deionized water to	100 mL

Sterilized by filter through 0.2 µM Supor® membrane filter.

5. Buffers for scFv antibody expression and purification

5.1 Bacterial lysis buffer (1 mg/mL lysozyme, 50 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol)

Lysozyme	80 mg
1 M Tris-HCl, pH 8.0	80 mL
14.3 M β-Mercaptoethanol	5.6 µL

Freshly prepared before used.

5.2 Solubilization buffer (8 M urea, 50 mM Tris-HCl, pH 8.0, 1 mM PMSF)

Urea	240.24 g
1 M Tris-HCl, pH 8.0	25 mL
100 mM PMSF	5 mL
Deionized water to	500 mL

5.3 100 mM Phenylmethyl sulfonyl fluoride (PMSF)

PMSF	0.35 g
Isopropanol to	20 mL

Kept at -20ºC.

5.4 Buffer for washing and eluting Ni-NTA affinity column chromatography, pH 6.3, 5.9, 4.9 and 2.5

Solubilization buffer	100 mL

Adjusted pH to the desired pH with HCl.

5.5 Redox refolding buffer (0.1 M Tris-HCl, pH 7.4, 0.4 M L-arginine, 0.2 mM PMSF, 1 µg/mL pepstatin, 0.5 mM oxidized glutathione, 5 mM reduced glutathione)

L-Arginine	84.26 g
1 M Tris-HCl, pH 7.6	100 mL
100 mM PMSF	2 mL
1 mg/mL Pepstatin	1 mL
Oxidized glutathione	0.31 g
Reduced glutathione	1.54 g
Deionized water to	1 L

5.6 1 mg/mL Pepstatin

Pepstatin	10 mg
Dimethyl sulfoxide (DMSO) to	10 mL

Kept at -20ºC.

5.7 50 mM Tris-HCl, pH 8.0

1 M Tris-HCl, pH 8.0	50 mL
Distilled water to	1 L

6. Reagents for determination of protein concentration

6.1 Coomassie brilliant blue G-250

Coomassie brilliant blue G-250	10 mg
85% H_3PO_4	10 mL
Absolute ethanol	5 mL
Distilled water to	100 mL

6.2 40 mg/mL BSA

BSA	40 mg
Distilled water to	1 mL

6.3 1 N NaOH

NaOH	4 g
Distilled water to	100 mL

APPENDIX D

Bacterial strains and their vectors

1. Bacterial strains

Table 1. Bacterial strains.

Strain	Genotype	Product
TOP10F	*E. coli* F^- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Novagen, USA
BL21(DE3)	*E. coli* F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen, USA

2. Nucleotide sequence of the pGEM®-T Easy Vector (3,015 bp)

The pGEM®-T Easy Vector has been linearized by E*coR* V restriction enzyme at base 60 of the sequence (indicated by an asterisk) and a T added to both 3′-ends. The added T is not included in this sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. Underlined sequence indicated binding sites of T7 promoter and SP6 promoter primer for colony PCR (174 bp); underlined and italic sequence indicated restriction sites of *Eco*R I for digestion analysis (http://www.promega.com).

T7 promoter primer 19 nt (position 3000-3)

1 gggcgaattg ggcccgacgt cgcatgctcc cggccgccat ggcggccgcg

EcoR I restriction site (52) and (70)

51 g*g▼aattc*gat* atcactagt*g▼aatt*cgcggc cgcctgcagg tcgaccatat

SP6 promoter primer 17 nt (position 142-158)

101 gggagagctc ccaacgcgtt ggatgcatag cttgagtatt ctatagtgtc

151 acctaaatag cttggcgtaa tcatggtcat agctgtttcc tgtgtgaaat

201 tgttatccgc tcacaattcc acacaacata cgagccggaa gcataaagtg

251 taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc

301 gctcactgcc cgctttccag tcgggaaacc tgtcgtgcca gctgcattaa

351 tgaatcggcc aacgcgcggg gagaggcggt ttgcgtattg ggcgctcttc

401 cgcttcctcg ctcactgact cgctgcgctc ggtcgttcgg ctgcggcgag

451 cggtatcagc tcactcaaag gcggtaatac ggttatccac agaatcaggg

501 gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa

551 ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc cgcccccctg

601 acgagcatca caaaaatcga cgctcaagtc agaggtggcg aaacccgaca

651 ggactataaa gataccaggc gtttcccccc ggaagctccc tcgtgcgctc

701 tcctgttccg accctgccgc ttaccggata cctgtccgcc tttctccctt

751 cgggaagcgt ggcgctttct catagctcac gctgtaggta tctcagttcg

801 gtgtaggtcg ttcgctccaa gctgggctgt gtgcacgaac cccccgttca

851 gcccgaccgc tgcgccttat ccggtaacta tcgtcttgag tccaacccgg

901 taagacacga cttatcgcca ctggcagcag ccactggtaa caggattagc

951 agagcgaggt atgtaggcgg tgctacagag ttcttgaagt ggtggcctaa

1001 ctacggctac actagaagaa cagtatttgg tatctgcgct ctgctgaagc
1051 cagttacctt cggaaaaaga gttggtagct cttgatccgg caaacaaacc
1101 accgctggta gcggtggttt ttttgtttgc aagcagcaga ttacgcgcag
1151 aaaaaaagga tctcaagaag atcctttgat cttttctacg gggtctgacg
1201 ctcagtggaa cgaaaactca cgttaaggga ttttggtcat gagattatca
1251 aaaaggatct tcacctagat ccttttaaat taaaaatgaa gttttaaatc
1301 aatctaaagt atatatgagt aaacttggtc tgacagttac caatgcttaa
1351 tcagtgaggc acctatctca gcgatctgtc tatttcgttc atccatagtt
1401 gcctgactcc ccgtcgtgta gataactacg atacgggagg gcttaccatc
1451 tggccccagt gctgcaatga taccgcgaga cccacgctca ccggctccag
1501 atttatcagc aataaaccag ccagccggaa gggccgagcg cagaagtggt
1551 cctgcaactt tatccgcctc catccagtct attaattgtt gccgggaagc
1601 tagagtaagt agttcgccag ttaatagttt gcgcaacgtt gttgccattg
1651 ctacaggcat cgtggtgtca cgctcgtcgt ttggtatggc ttcattcagc
1701 tccggttccc aacgatcaag gcgagttaca tgatccccca tgttgtgcaa
1751 aaaagcggtt agctccttcg gtcctccgat cgttgtcaga agtaagttgg
1801 ccgcagtgtt atcactcatg gttatggcag cactgcataa ttctcttact
1851 gtcatgccat ccgtaagatg cttttctgtg actggtgagt actcaaccaa
1901 gtcattctga gaatagtgta tgcggcgacc gagttgctct tgcccggcgt
1951 caatacggga taataccgcg ccacatagca gaactttaaa agtgctcatc
2001 attggaaaac gttcttcggg gcgaaaactc tcaaggatct taccgctgtt
2051 gagatccagt tcgatgtaac ccactcgtgc acccaactga tcttcagcat
2101 cttttacttt caccagcgtt tctgggtgag caaaaacagg aaggcaaaat
2151 gccgcaaaaa agggaataag ggcgacacgg aaatgttgaa tactcatact
2201 cttccttttt caatattatt gaagcattta tcagggttat tgtctcatga
2251 gcggatacat atttgaatgt atttagaaaa ataaacaaat aggggttccg
2301 cgcacatttc cccgaaaagt gccacctgat gcggtgtgaa ataccgcaca
2351 gatgcgtaag gagaaaatac cgcatcagga aattgtaagc gttaatattt
2401 tgttaaaatt cgcgttaaat ttttgttaaa tcagctcatt ttttaaccaa
2451 taggccgaaa tcggcaaaat cccttataaa tcaaaagaat agaccgagat
2501 agggttgagt gttgttccag tttggaacaa gagtccacta ttaaagaacg
2551 tggactccaa cgtcaaaggg cgaaaaaccg tctatcaggg cgatggccca

2601 ctacgtgaac catcacccta atcaagtttt ttggggtcga ggtgccgtaa
2651 agcactaaat cggaacccta aagggagccc ccgatttaga gcttgacggg
2701 gaaagccggc gaacgtggcg agaaaggaag ggaagaaagc gaaaggagcg
2751 ggcgctaggg cgctggcaag tgtagcggtc acgctgcgcg taaccaccac
2801 acccgccgcg cttaatgcgc cgctacaggg cgcgtccatt cgccattcag
2851 gctgcgcaac tgttgggaag ggcgatcggt gcgggcctct tcgctattac
2901 gccagctggc gaaaggggga tgtgctgcaa ggcgattaag ttgggtaacg
2951 ccagggtttt cccagtcacg acgttgtaaa acgacggcca gtgaattgta
3001 atacgactca ctata

3. Nucleotide sequence of the pET-28a(+) expression vector (5,369 bp)

The sequence is numbered by the PBR322 convention, therefore, the T7 promoter primer binding site is reversed in this sequence. Underlined sequence indicated binding sites of T7 terminator and T7 promoter primer for colony PCR (316 bp); underlined and italic sequence indicated restriction sites of *Bam*H I and *Nde* I for cloning the full length scFv (http://www.informaxinc.com).

1 atccggatat agttcctcct ttcagcaaaa aacccctcaa gacccgttta gaggccccaa

T7 terminator primer 19 nt (position 70-88)

61 ggggttatgc tagttattgc tcagcggtgg cagcagccaa ctcagcttcc tttcgggctt
121 tgttagcagc cggatctcag tggtggtggt ggtggtgctc gagtgcggcc gcaagcttgt

*Bam*H I restriction site (198) *Nde* I restriction site (238)

181 cgacggagct cgaattcgga tccgcgaccc atttgctgtc caccagtcat gctagccata
241 tggctgccgc gcggcaccag gccgctgctg tgatgatgat gatgatggct gctgcccatg
301 gtatatctcc ttcttaaagt taaacaaaat tatttctaga ggggaattgt tatccgctca

T7 promoter primer 19 nt (position 367-385)

361 caattcccct atagtgagtc gtattaattt cgcgggatcg agatctcgat cctctacgcc
421 ggacgcatcg tggccggcat caccggcgcc acaggtgcgg ttgctggcgc ctatatcgcc
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601 ccattccttg cggcggcggt gctcaacggc ctcaacctac tactgggctg cttcctaatg
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901 ggagctgaat tacattccca accgcgtggc acaacaactg gcgggcaaac agtcgttgct
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5341 cgccgctaca gggcgcgtcc cattcgcca

APPENDIX E

Protocol of RNA isolation

Mouse hybridoma cells secreting monoclonal antibody specific to Hb Bart's cultured in 25 cm^2 tissue culture flask for 2 days were a starting material for RNA isolation using RNeasy® mini kit according to the manufacturer's instructions (Qiagen, Germany). The overview of protocol was shown in Figure 1.

Procedure

1. Harvested mouse hybridoma cells by flush using Pasteur pipette and transferred to a 15 mL centrifuge tube.
2. Centrifuged for 5 min at 400 x g. Carefully removed all supernatant.
3. Added 10 mL of serum free IMDM and centrifuged for 5 min at 400 x g. Washed cells with serum free IMDM for 2 times. Suspended cells in 5 mL of serum free IMDM.
4. Determined the number of cells by using Improved Neubauer Hemacytometer. By recommendation of starting cells, no more than 3-4 x 10^6 cells were used.
5. Completely aspirated the supernatant after centrifuged for 5 min at 400 x g.
6. Added 350 µL of buffer RLT. Loosen the cell pellet thoroughly by flicking the tube.
7. Homogenized the cell lysate by pass through a 20-gauge needle attached to a sterile plastic syringe for 5-10 times or until a homogeneous lysate was achieved.
8. Added 350 µL of 70% ethanol to the homogenized lysate and mixed well by pipetting up and down.

9. Placed an RNeasy spin column into a 2 mL collection tube and loaded all sample into the column. Closed the lid gently and centrifuged for 15 s at 8,000 x g. Discarded the filtrate.

10. Added 700 µL of buffer RW1 to the RNeasy spin column and incubated at room temperature for 5 min. Closed the lid gently and centrifuged for 15 s at 8,000 x g. Discarded the filtrate.

11. Added 500 µL of buffer RPE to the RNeasy spin column. Closed the lid gently and centrifuged for 15 s at 8,000 x g. Discarded the filtrate.

12. Added another 500 µL of buffer RPE to the RNeasy spin column. Closed the lid gently and centrifuged for 2 min at 8,000 x g. Discarded the filtrate.

13. To avoid the remainder of buffer RPE, placed the RNeasy spin column in a new 2 mL collection tube. Closed the lid gently and centrifuged at full speed for 1 min.

14. Placed the RNeasy spin column in a clean 1.5 mL eppendorf tube. Added 30 µL of deionized water directly to the spin column membrane and incubated at room temperature for 10 min. Closed the lid gently and centrifuged for 1 min at 8,000 x g.

15. Collected the eluent and determined the concentration of RNA by measuring the optical density at 260 nm.

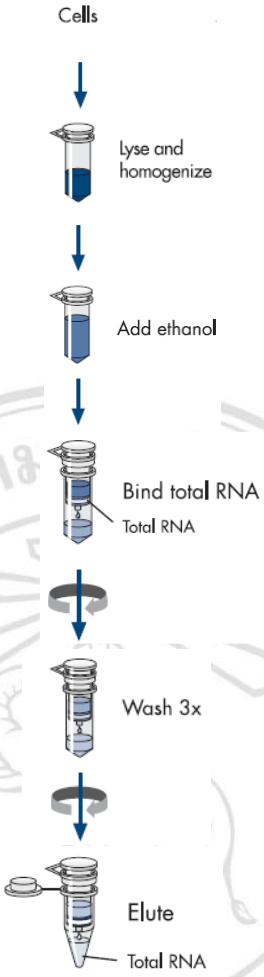


Figure 1 Schematic diagram of the protocol for RNA isolation.

APPENDIX F

Protocol of PCR product purification from agrose gel

First of all, 300 µL of the amplified product of either V_H or V_L were separated in 1.5% low melting point agarose gel in 1x TAE buffer at 50 V for 1 h. Agarose gel was then stained with 5 µg/mL ethidium bromide and visualized by UV light. After electrophoresis and staining of agarose gel, the amplified products of V_H and V_L were purified by PCR clean-up gel extraction kit according to the manufacturer's instructions (Macherey-Nagel, Germany). The overview of protocol was shown in Figure 1.

Procedure

1. Take a clean scalpel to excise the DNA fragment from an agarose gel. Determined the weight of the gel slice and transferred it to a clean 1.5 mL eppendorf tube.

2. For recommendation of 100 mg of agarose gel per 200 µL of buffer NT, added 400 µL of NT buffer to 200 mg of the gel slice and incubated at 50ºC for 5-10 min. Vortexed the DNA sample briefly every 2-3 min until the gel slice was completely dissolved.

3. Placed a NucleoSpin® Extract II column into a 2 mL collection tube and loaded the DNA sample into the column.

4. Centrifuged for 1 min at 11,000 x g. Discarded the filtrate and placed the NucleoSpin® Extract II column back into the collection tube.

5. Added 700 µL of buffer NT3 to the NucleoSpin® Extract II column. Centrifuged for 1 min at 11,000 x g. Discarded the filtrate and placed the NucleoSpin® Extract II column back into the collection tube.

6. Added another 500 µL of buffer NT3 to the NucleoSpin® Extract II column. Centrifuged for 1 min at 11,000 x g. Discarded the filtrate and placed the NucleoSpin® Extract II column back into the collection tube.

7. Centrifuge for 2 min at 11,000 x g to remove buffer NT3 quantitatively.

8. Placed the NucleoSpin® Extract II column into a clean 1.5 mL eppendorf tube. Added 30 µL of prewarmed deionized water (70ºC) and incubated at room temperature for 5 min. Centrifuged for 1 min at 11,000 x g.

9. Collected the eluent and determined the concentration of DNA by measuring the optical density at 260 nm.

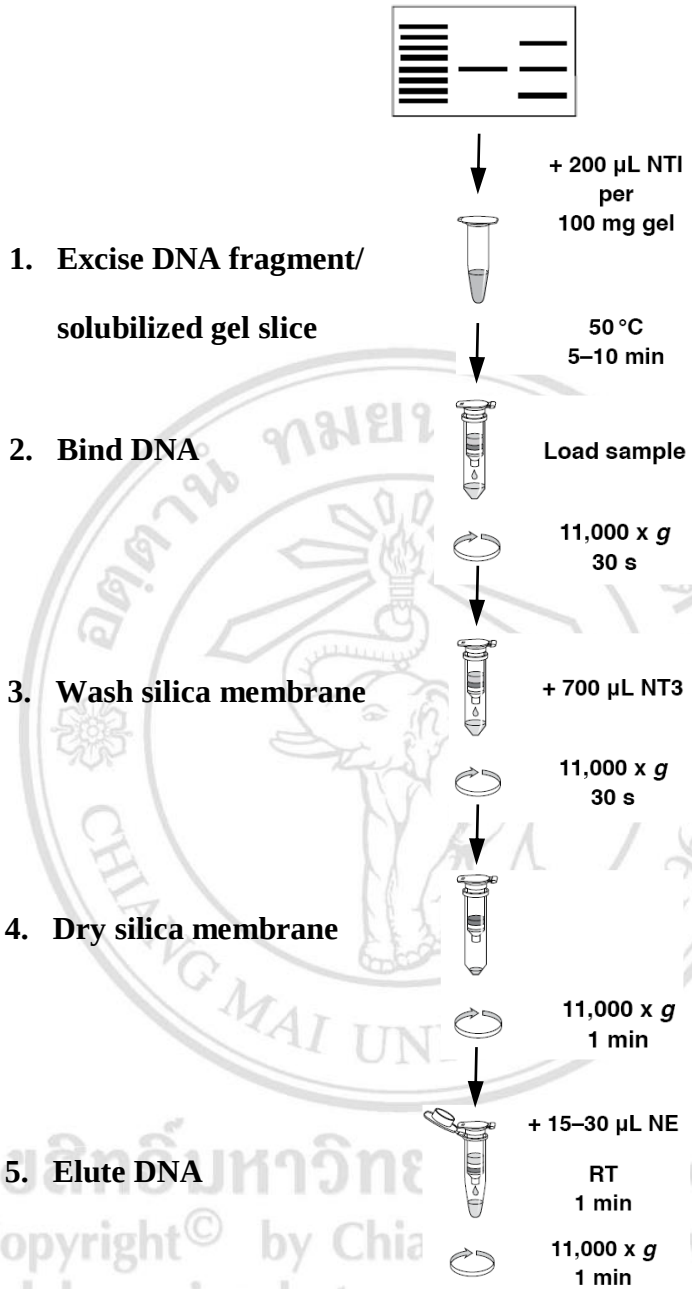


Figure 1 Schematic diagram of the protocol for PCR product purification from agarose gel.

APPENDIX G

Protocol of bacterial competent cells preparation

Both competent *E. coli* strains TOP10F and BL21(DE3) were individually prepared for transformation of plasmid DNA (the pGEM®-T-V_H and pGEM®-T-V_L, and the pET28a(+)-scFv anti-Hb Bart's, respectively). Glycerol frozen (-70ºC) of both *E. coli* strains were a starting material for the bacterial competent cells preparation. The protocol was modified from the method described by Sambrook and Russell [69].

Procedure

1. Individually streaked *E. coli* strains TOP10F and BL21(DE3) on LB agar without any antibiotics and incubate at 37ºC for 16-18 h.

2. Picked the isolated colony, then cultured in 2 mL of LB broth without any antibiotics and incubated at 37ºC for 16-18 h.

3. Expanded the 2 mL bacterial culture in 100 mL of LB broth without any antibiotics and incubated at 37ºC until the OD_{600} reached 0.15-0.3 (2-3 h).

4. Transferred all bacterial culture to a 50 mL centrifuge tube. Centrifuged at 4,000 rpm, 4ºC for 10 min. Discarded the supernatant.

5. Suspended the bacterial pellet in 30 mL of 0.1 M $CaCl_2$ and immediately placed on ice for 30 min. Centrifuged at 4,000 rpm, 4ºC for 10 min. Discarded the supernatant.

6. Suspended the bacterial pellet in 3 mL of 0.1 M $CaCl_2$ and 15% glycerol. Pipetted the bacterial suspension into aliquots of 500 µL in a 1.5 mL eppendorf tube.

7. Stored the bacterial competent cells at -70ºC until used.

APPENDIX H

Protocol of plasmid DNA purification

The plasmid DNA of pGEM®-T-V_H, pGEM®-T-V_L and pET28a(+)-scFv anti-Hb Bart's were purified by using plasmid DNA purification kit according to the manufacturer's instructions (Macherey-Nagel, Germany). The overview of protocol was shown in Figure 1.

Procedure

1. Picked the isolated colony of the *E. coli* TOP10F carrying either the pGEM®-T-V_H or pGEM®-T-V_L and *E. coli* BL21(DE3) carrying the pET28a(+)-scFv anti-Hb Bart's, and cultured in 5 mL of LB broth with appropriate antibiotics, then, incubated at 37ºC for 16-18 h.

2. Centrifuged the bacterial culture for 30 s at 11,000 x g. Discarded the supernatant and removed as much of the liquid as possible.

3. Suspended the bacterial cell pellet in 250 µL of buffer A1. Pipetted up and down until no cell clumps.

4. Added 250 µL of buffer A2. Mixed gently by inverting the tube for 6-8 times and incubated at room temperature for up to 5 min or until lysate appeared clear.

5. Added 300 µL of buffer A3. Mixed thoroughly by inverting the tube for 6-8 times. Centrifuged for 5 min at 11,000 x g.

6. Placed a Nucleospin® plasmid column in a 2 mL collection tube, then, decanted the supernatant from step 5 or pipette a maximum of 750 µL of the supernatant into the column.

7. Centrifuged for 1 min at 11,000 x g. Discarded the filtrate, then, placed the Nucleospin® plasmid column back into the collection tube.

8. Added 500 µL of preheated buffer AW (50ºC) and incubated at room temperature for 2 min. Centrifuged for 1 min at 11,000 x g. Discarded the filtrate, then, placed the Nucleospin® plasmid column back into the collection tube.

9. Added 600 µL of buffer A4. Centrifuged for 1 min at 11,000 x g. Discarded the filtrate, then, placed the Nucleospin® plasmid column back into the collection tube.

10. Centrifuged for 2 min at 11,000 x g and discarded the collection tube.

11. Placed the Nucleospin® plasmid column in a clean 1.5 mL eppendorf tube, then, added 50 µL of preheated deionized water (70ºC). Incubated at room temperature for 1 min. Centrifuge for 1 min at 11,000 x g.

12. Collected the eluent and determined the concentration of DNA by measuring the optical density at 260 nm.

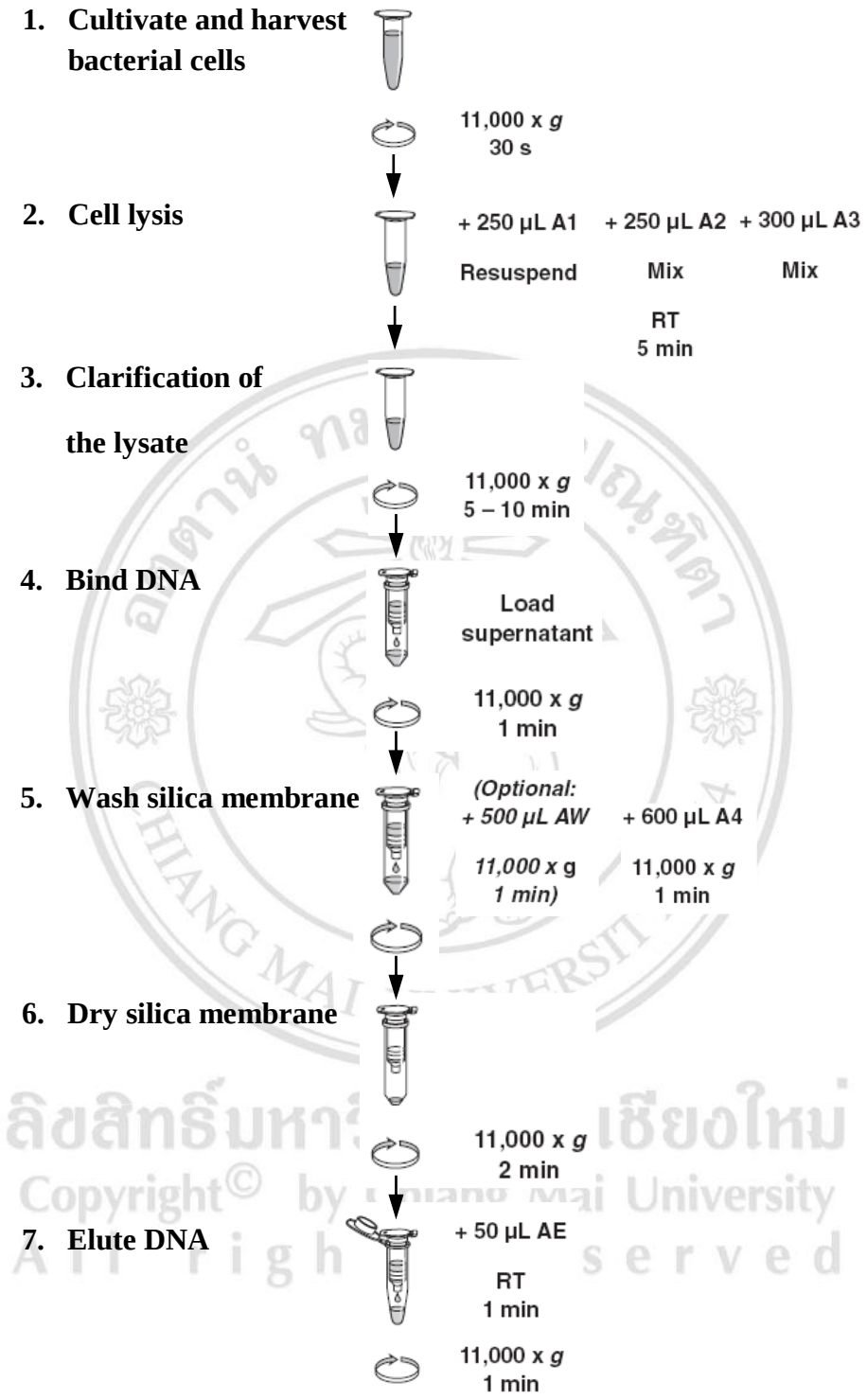


Figure 1 Schematic diagram of the protocol for plasmid DNA purification.

APPENDIX I

Protocol of SDS-polyacrylamide and native polyacrylamide gel preparation

Preparation of 12% SDS-polyacrylamide and 12% native polyacrylamide gel were done by the protocol described by Sambrook and Russell [69]. The solution for preparation of resolving gel and stacking gel were mentioned in Tables 1 and 2. Preparation of 12% native polyacrylamide gel was modified from Tables 1 and 2 by lacking SDS.

Table 1 Composition of the 12% resolving gels.

Composition	Volumes (mL) per gel mold	
	5 mL	10 mL
Distilled water	1.6	3.3
30% Acrylamide	2.0	4.0
1.5 M Tris-HCl, pH 8.8	1.3	2.5
10% SDS	0.05	0.1
10% Ammonium persulfate	0.05	0.1
TEMED	0.005	0.01

Table 2 Composition of the 5% stacking gels.

Composition	Volumes (mL) per gel mold	
	3 mL	5 mL
Distilled water	2.1	3.4
30% Acrylamide	0.5	0.83
1 M Tris-HCl, pH 6.8	0.38	0.63
10% SDS	0.03	0.05
10% Ammonium persulfate	0.03	0.05
TEMED	0.003	0.005

APPENDIX J

Protocol of proteins transfer from polyacrylamide gels to membranes

Bacterial proteins of uninduced and IPTG-induced or hemoglobin proteins were transferred to PVDF membrane or nitrocellulose membrane, respectively by Semi-Dry Electrophoretic Transfer Cell (Trans-Blot® SD) according to the manufacturer's instructions (Bio-Rad, USA).

Procedure

1. After electrophoresis, soaked the SDS-polyacrylamide gel in Bjerrum and Schafer-Nielsen transfer buffer for 15 min.

2. Cut a PVDF membrane (NEN Life Science, USA) as the same size of the gel. Soaked the PVDF membrane in absolute ethanol for at least 1 min or until it changed from opaque white to a uniform translucent gray. Rinsed the membrane in distilled water for 2-3 min. Soaked the PVDF membrane in Bjerrum and Schafer-Nielsen transfer buffer for 15-30 min.

3. Cut a filter paper (Bio-Rad, USA) as the same size of the gel. Soaked the filter paper in Bjerrum and Schafer-Nielsen transfer buffer for 15-30 min.

4. For assembly of Semi-Dry Electrophoretic Transfer Cell unit, firstly placed the pre-soaked filter paper onto the platinum anode. Removed air bubbles by roll a pipette or test tube over the surface of the filter paper.

5. Placed the pre-wetted PVDF membrane on top of the filter paper.

6. Carefully placed the equilibrated gel on top of the PVDF membrane.

7. Placed the other sheet of pre-soaked filter paper on top of the gel. Carefully removed air bubbles from between the gel and filter paper.

8. Carefully placed the stainless cathode onto the stack. Pressed to engage the latches without disturbing the stack.

9. Placed the safety cover on the unit. Plug the unit into the power supply.

10. Turned on the power supply. Run the electrophoretic step at 15 V for 30 min.

11. After the transfer step, turned the power supply off and disconnected the unit from the power supply. Removed the safety cover and the stainless cathode assembly.

12. Discarded the filter paper. Briefly washed the PVDF membrane in PBS, then, performed the next step for Western blot analysis as described in method 2.10.

The protocol for transfer of hemoglobin proteins from native polyacrylamide gel to nitrocellulose membrane (Schleicher & Schuell, Germany) was modified from the protocol described above. Instead of using Bjerrum and Schafer-Nielsen transfer buffer, Dunn carbonate was used as the transfer buffer and the electrophoretic step was performed at 25 V for 1 h.

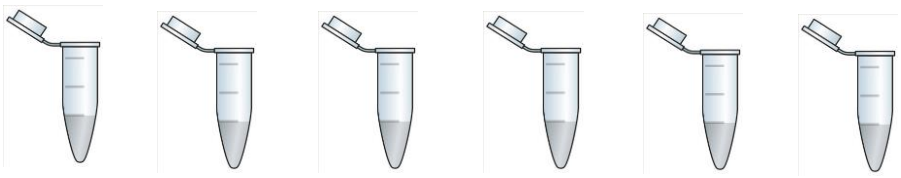
APPENDIX K

Protocol of protein concentration determination

Protein concentration of the purified scFv antibody was determined by the method modified from Bradford assay [67].

Procedure

1. Pipetted 20 μL of either scFv antibody or standard proteins (40, 20, 10, 5 and 2.5 mg/mL of bovine serum albumin, BSA) into a 1.5 mL eppendorf tube. The volumes of BSA used in 2-fold dilution were described in Figure 1.
2. Added 50 μL of 1 N NaOH. Mixed by vortex.
3. Added 1 mL of Coomassie brilliant blue G-250. Mixed by vortex and stood for at least 5 min at room temperature.
4. Measured the absorbance at 595 nm.
5. Plotted the standard curve of protein (BSA) concentration as presented in Table 1 and Figure 2.
6. Calculated the protein concentration of scFv antibody by using standard curve.



						discard
40 mg/mL BSA (μL)	2	20	20	20	20	-
Distilled water (μL)	38	20	20	20	20	20
Protein concentration (mg/mL)	40	20	10	5	2.5	0

Figure 1 Preparation of standard protein concentrations for performing the standard curve. The standard proteins (BSA) were diluted as 2-fold dilution to obtain the final protein concentrations of 40, 20, 10, 5, 2.5 and 0 mg/mL, respectively.

Table 1 The absorbance of various standard protein (BSA) concentrations at wavelength 595 nm.

Protein concentration (mg/mL)	OD_1 595 nm	OD_2 595 nm	ΔOD 595 nm
2.5	0.096	0.101	0.098
5	0.194	0.168	0.185
10	0.296	0.297	0.296
20	0.613	0.540	0.589
40	1.198	0.896	1.097

Δ = average

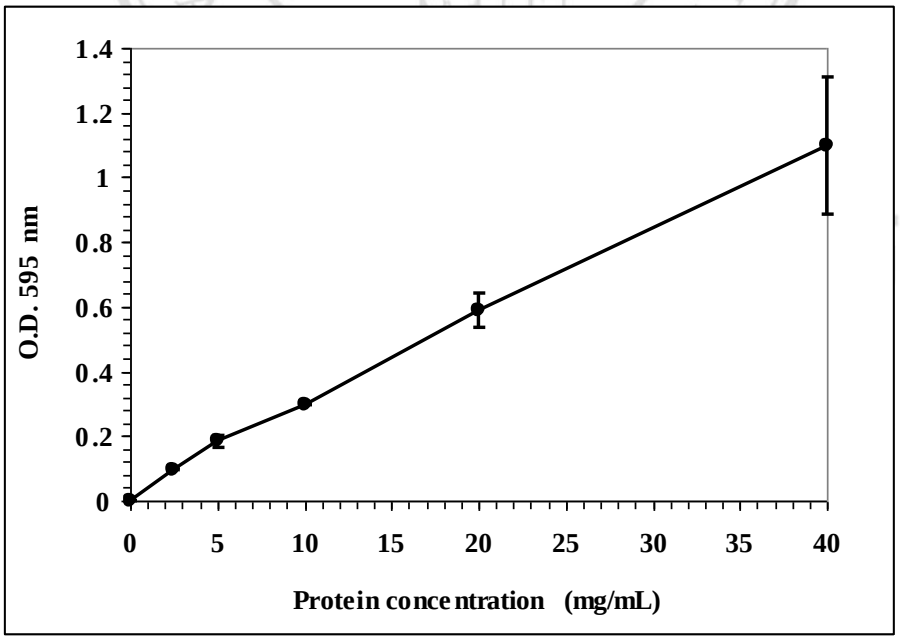


Figure 2 Standard curve of standard protein (BSA) by the modified Bradford assay.

APPENDIX L

Protocol of scFv antibody purification by Ni-NTA affinity chromatography

The N-terminal histidine fusion scFv antibody was purified by Ni-NTA His-Bind® Resins affinity chromatography according to the manufacturer's instructions (Novagen, USA) with some modification. The purification protocol was conducted under denaturing condition by urea for dissolving the IBs containing scFv antibody.

Procedure

1. Mixed 54 mg of solubilized IBs (60 mL) with 10 mL of Ni-NTA His-Bind® Resins. Gently inverted the mixture at room temperature for 1 h.

2. Applied all mixture into a chromatography column and allowed the free drop of filtrate. Discarded the filtrate.

3. Added 8 volumes (80 mL) of 8 M urea, 50 mM Tris-HCl pH 6.3 for 2 times. Discarded the filtrate.

4. Added 5 volumes (50 mL) of 8 M urea, 50 mM Tris-HCl pH 5.9, followed by 10 volumes (100 mL) of 8 M urea, 50 mM Tris-HCl pH 4.9. Discarded all of the filtrate.

5. Added 10 volumes (100 mL) of 8 M urea, 50 mM Tris-HCl pH 2.5. Collected the filtrate for 10 mL per fraction.

6. Determined the protein concentration of the filtrate by measuring the optical density at 280 nm.

7. Pooled all fractions with high protein concentration for refolding process as described in method 2.12.

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Publications	1) Pharephan S, Intorasoot S, Tuntiwechapikul W, Makonkawkeyoon S, Makonkawkeyoon L. Cloning and expression of a recombinant single-chain variable fragment antibody specific to hemoglobin Bart's. Asian Pac J Health Sci 2015;x: pp.
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