APPENDIX

APPENDIX A

CHEMICALS AND EQUIPMENTS

All chemicals used in this study were analytical grade reagents

Table A-1. Lists of chemicals used in this study

Chemicals/Substances	Source
For hemolysate, Hb and globin chains preparation	
Carbon tetrachloride	May & Baker Dagenham, Ikeja Lagos, Nigeria.
Ethylenediamine tetra acetic acid (EDTA) disodium salt	BDH Laboratory Supplies, TD, England
Ponceau S	Sigma, St. Louis, MO, USA
Sodium chloride	Merck, Darmstadt, Germany
Tris base	Amersham Biosciences, Uppsala, Sweden
Boric acid	Sigma, St. Louis, MO, USA
Hydrochloric acid	Merck, Darmstadt, Germany
Glacial acetic acid	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany

Chemicals/Substances	Source
Potassium cyanide	Reidel-DE Haen AG Sellze-Handnover, Seelze, Germany
Trichloroacetic acid	Fluka, Buchs, Switzerland
Tetramethylene ethylenediamine (TEMED)	Sigma, St. Louis, MO, USA
Urea	Sigma, St. Louis, MO, USA
Cellulose acetate membrane	Helena Laboratories, Beaumont, TX, USA
Potassium hexacyano ferrate (II)-3-hydrate	Reidel-DE Haen AG Sellze-Handnover, Seelze, Germany
DEAE Sepharose	Amersham Biosciences, Uppsala, Sweden
Sodium hydrogen carbonate	Merck, Darmstadt, Germany
Naphthol blue black (Amido black 10B)	Sigma, St. Louis, MO, USA
Ammonium persulfate	Merck, Darmstadt, Germany
Pyronin Y	Sigma, St. Louis, MO, USA
Octyl phenoxy polyathoxyethan (Triton X-100)	Sigma, St. Louis, MO, USA
Sodium hydroxide	EKA Nobel, Göteborg, Sweden

Chemicals/Substances	Source
Reagents for Hybridoma technique and cell culture	
Ethanol	Merck, Darmstadt, Germany
Diethyl ether	Merck, Darmstadt, Germany
Fungizone (Amphotericin B)	Bristol-Myer Squibb, Cincinnati, OH, USA
Isocove's Modified Dulbecco's Medium (IMDM)	Gibco, Gran Island, N.Y., USA
Gentamycin	Atlantic Labs, Selangor, Malaysia
10X BM condimed HI	Roche, Mannheim, Germany
100X Hypoxanthine Aminopterin Thymidine (HAT)	Gibco, Gran Island, N.Y., USA
100X Hypoxanthine Thymidine (HT)	Gibco, Gran Island, N.Y., USA
Gential violet/ Crytal violet	Sigma, St. Louis, MO, USA
Trypan blue powder	Sigma, St. Louis, MO, USA
Fetal bovine serum	Gibco, Gran Island, N.Y., USA
Dimethyl sulfoxide (DMSO)	Sigma, St. Louis, MO, USA
Ammonium chloride	Sigma, St. Louis, MO, USA
Potassium hydrogen carbonate	Fluka, Buchs, Switzerland
Ethylenediamine tetra acetic acid (EDTA)	BDH Laboratory Supplies, TD, England

Chemicals/Substances	Source
Reagents for ELISA	
Potassium chloride	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Sodium carbonate anhydrous	Merck, Darmstadt, Germany
Di-sodium hydrogen orthophosphate anhydrous	Fisher Scientific, Cough borough, UK
Polyoxyethylenes orbitan monolaurate (Tween 20)	Sigma, St. Louis, MO, USA
Bovine serum albumin	Sigma, St. Louis, MO, USA
Sodium azide	Reidel-DE Haen AG Sellze-Handnover, Seelze, Germany
3,3',5,5;-Tetramethylbenzidine (TMB)	Zymed, South san Francisco, CA, USA
Reagents for purification of mAbs and SDS-PAGE	
Isopropanol	Merck, Darmstadt, Germany
Ammonium sulfate	Fluka, Buchs, Switzerland
2-mercaptoehanol (2-ME)	Sigma, St. Louis, MO, USA
Sodium dodecyl sulfate (SDS)	Fisher Scientific, Cough borough, UK
Acrylamide	Sigma, St. Louis, MO, USA
Bis-acrylamide	Sigma, St. Louis, MO, USA

Chemicals/Substances	Source
Bromphenol blue	Matheson Coleman and Bell, Ohio, USA
Glycerol	Merck, Darmstadt, Germany
Coomassie blue R250	Bio-rad, Hercules, CA, USA
Reagents for Western blotting	
Enhanced Chemiluminescence (ECL)	Pierce Biotechnology, Rockford, IL, USA
Nitrocellulose membrane	Pall life sciences, Pensacola, FL, USA

Lists of instruments and equipments used in this study

- Biological safety cabinet class II, NUAIRE, USA
- Inverted light microscope, Olympus, Japan
- Light microscope, Olympus, Japan
- Water bath**,** Memmert, Germany
- CO_2 incubator, Thermo electron corporation, USA
- 2-20 µl Autopipette, Bio-rad, USA
- 20-200 µl Autopipette, Bio-rad, USA
- 100-1000 µl Autopipette, Bio-rad, USA
- 40-350 µl multichanel autopipette, Socorex, Switzerland
- Hemacytometer, Boeco, Germany
- Refrigerated centrifuge, Sorvall® Legend RT, Kendo Laboratory, Germany
- Microcentrifuge, Sorvall® Biofuge pico, Kendo Laboratory, Germany
- Vivaspin 2 ml, Vivascience sartorius group, Germany
- Fraction collector, AKTA prime, Amershame Biosciences, Sweden
- 37^{o}C incubator, Lenton thermal designs, England
- Analytical balance, Mettler Toledo, Canada
- Hotplate stirrer, Daihan labtech, Korea
- pH/conductivity meter, Accument® Fisher scientific, USA
- Power supply, Bio-rad, USA
- Centrifuge, Kokusan, Japan
- Microcentrifuge, Sorvall® Instroments, Kendo Laboratory, Germany

- Vortex, Labnet VX100, Mo Bio Laboratories, USA
- Microplate reader, Sunrise tecan, Austria
- CO_2 calibrator, Fyrite® Bacharach, new Kensington, PA
- Autoclave, Huxey, Taiwan
- Ultrasonic cleaner, Elma® Transonic digital, Germany
- Pipet-aid, Drummond, USA
- Rotator, Technomara, Switzerland
- Semi-dry blotting, Amershame Biosciences, Sweden
- Power supply, Amershame Biosciences, Sweden
- UV1601 Spectrophotometer, Shimadzu, Japan
- Refrigerated microcentrifuge MRX-150, Tomy, Japan

APPENDIX B

REAGENTS PREPARATION

1. Reagents for cellulose acetate electrophoresis

1.1. 10X Tri-Borate-EDTA (TBE) buffer pH 8.6 (0.85 M Tris 0.0015 M EDTA 0.055 M Boric acid)

Tris base	121	gm
EDTA (disodium salt)	11	gm
Boric acid	15	gm
ddH_2O	800	ml

Adjusted the pH to 8.6 with saturated Boric acid.

Adjusted the volume to 1000 ml. with ddH_2O and store at room temperature.

1.2. Working TBE buffer pH 8.6

10X TBE buffer pH 8.6	100	ml
ddH_2O	900	ml

Mix thoroughly and kept at room temperature

1.3. Drabkin's solution

$K_3Fe(CN)_6.3H_2O$	0.23	gm
KCN	0.05	gm
$NaHCO_3$	1	gm
dH_2O	1000	ml

Mix thoroughly and kept at room temperature

1.4. Ponceau S staining (0.5% W/V)

Ponceau S	0.5	gm
Trichloroacetic acid	5	gm

Adjusted the volume to 100 ml with dH_2O and store at room temperature

1.5. Destaining solution (5% Acetic acid)

Acetic acid	50	ml
dH_20	950	ml

1.6. Clearing solution

Methanal : Gracial acetic acid = 4:1

2. Reagents for purification of hemoglobins

2.1. 10X Tris-HCl buffer pH 9.0

Tris base	60.57	gm
dH_2O	800	ml

Adjusted the pH to 9.0 with 4 N HCl.

Adjusted the volume to 1000 ml. with ddH_2O and stored at room temperature.

2.2. Working Tris-HCl-KCN (THK) buffer pH 9.0

10X Tris-HCl buffer pH 9.0	100	ml
ddH_2O	900	ml
KCN	0.1	gm

Mix thoroughly and filtrated by 0.2 μm Millipore membrane filter

kept at room temperature

2.3. Working THK buffer pH 6.5

10X Tris-HCl buffer pH 9.0	100	ml
ddH_2O	800	ml
KCN	0.1	gm

Adjusted the pH to 6.5 with 4 N HCl.

Adjusted the volume to 1000 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter

kept at room temperature

3. Reagents for Hybridoma technique and cell culture

3.1. Incomplete Isocove's Modified Dulbecco's Medium (IMDM)

IMDM powder	1	pack
$NaHCO_3$	3.024	gm
Gentamycin (40 mg/ml)	1	ml

Dissolve in ddH_20 and adjust volume to 1000 ml

Filtrated through 0.2 μm Millipore membrane filter

Add Fungizone (5 mg/ml)	500	μl

Mix thoroughly and stored at 4^oC

3.2. Inactivated bovine serum (FCS)

Fetal bovine serum was inactivated the complement by heated at 56^{o}C in a water bath for 30 min. Inactivated FCS was aliquot to 10 ml/tube and stored at -20^{o}C until used.

3.3. Complete IMDM (10% FCS IMDM)

FCS	10	ml
Complete IMDM	90	ml

Mix well, stored at 4^{o}C

3.4. 10% BM condimed in complete IMDM

BM-condimed	10	ml
Complete IMDM	90	ml

Mix well, Stored at 4^{o}C

3.5. 0.6% 2-mercaptoethanol (2-ME)

Complete IMDM	5	ml
2-mercaptoethanol	30	μl

Filtrated through 0.2 μm Millipore membrane filter

Aliquot 30 μl/tube, stored at -20^{o}C

3.6. 1xHAT medium

Complete IMDM	79	ml
FCS	10	ml
BM condimed HI	10	ml
0.6% 2-ME	30	μl
100X HAT	1	ml

Mix well, stored at $4^{o}C$

3.7. 1xHT medium

IMDM	119	ml
FCS	15	ml
BM condimed HI	15	ml
0.6% 2-ME	30	μl
100X HT	1	ml

Mix well, stored at $4^{o}C$

3.8. 50% PEG

3.9. Turk's solution

Glacial acetic acid	3	ml
1% gential violet	1	ml

Adjust volume to 100 ml with dH_2O

Filtrated by Whatman filter paper no. 1

Stored at room temperature

3.10. Trypan blue (0.2%)

Trypan blue powder	0.2	gm
PBS pH 7.2	100	ml

Filtrated by Whatman filter paper No. 1

Stored at room temperature

3.11. Freezing medium (10%DMSO in 25%FCS-IMDM)

Incomplete IMDM	65	ml
FCS	25	ml
DMSO (Hybrimax)	10	ml

Mix well, stored at $4^{o}C$

3.12. Hypotonic solution (0.083% NH4Cl) for RBC lysing

NH_4Cl	0.829	gm
$KHCO_3$	0.1	gm
EDTA	0.0037	gm
ddH_2O	90	ml

Adjust pH to 7.2 with 1N HCl

Adjust volume to 100 ml

Filtrated through 0.4 μm Millipore membrane filter

Mix well, store at $4^{o}C$

4. Reagents for ELISA

4.1. Coating buffer (0.1 M carbonate/bicarbonate buffer, pH 9.6)

Na_2CO_3	2.12	gm
$NaHCO_3$	2.52	gm
ddH_2O	400	ml

Adjusted the pH to 9.6 with conc. HCl

Adjusted volume to 500 ml and stored at 4^oC

4.2. 10X Phosphate buffer saline (PBS) pH 7.2

NaCl	80	gm
Na_2HPO_4	11.5	gm
KCl	2	gm
KH_2PO_4	2	gm
dH_2O	800	ml

Adjusted the pH to 7.2 with 5 N NaOH.

Adjusted the volume to 1000 ml. with dH_2O and store at room temperature.

4.3. Working PBS pH 7.2

10X PBS pH 7.2	100	ml
dH_2O	900	ml

Mix thoroughly and kept at room temperature

4.4. Blocking reagent (2% BSA, 0.02% NaN_3 in PBS)

Bovine serum albumin (BSA)	2	gm
PBS pH 7.2	100	ml
10% NaN_3-PBS	200	µl

Mix well, Stored at 4°C

4.5. 0.05% Tween PBS pH 7.2 (Fresh prepared before used)

PBS pH 7.2	1000	ml
Tween 20	500	µl

4.6. Stop reaction solution (1N HCl)

dH_2O	91.7	ml
Conc. HCl	8.3	ml

Stored at room temperature

5. Reagent for globin chain analysis

5.1. Running buffer (5% acetic acid)

Acetic acid	200	ml
dH_20	3,800	ml

5.2. 60% Monomer (60% acrylamide, 0.4% bis-acrylamide)

Acrylamide	60	gm
Bis-acrylamide	0.4	gm
ddH_2O	100	ml

Filtrated through 0.2 µm Millipore membrane filter

Stored in dark at 4°C

5.3. 8 M urea (Fresh prepared before used)

Urea	5.76	gm
dH_2O	12	ml

5.4. Slab gel (12% TAU-polyacrylamide gel)

60% monomer	2.92	gm
Glacial acetic acid	731	µl
8 M urea	10.97	ml
Triton X-100	292	µl
Ammonium persulfate	0.03	gm

Mix thoroughly and degas for 5 min

Add NNN'N'-tetra-methylene ethylenediamine (TEMED) 198 µl

for starting the polymerization

6. Reagents for Western blot

6.1. Blotting buffer

Tris base	1.515	gm
Glycine	7.205	gm
Sodium dodecyl sulfate (SDS)	0.5	gm
ddH_2O	350	ml

Mix thoroughly, added methanol 100 ml

Adjust volume to 1000 by ddH_20

Filtrated through 0.2 µm Millipore membrane filter

Stored at room temperature

6.2. Amido black 10B

Amido black 10B (Naphthal blue)	0.1	gm
Methanol	45	ml
Acetic acid	10	ml

Adjust volume to 100 ml by dH_20

Store at room temperature

6.3. 0.1% Tween-PBS (Fresh prepared before used)

PBS pH 7.2	1000	ml
Tween 20	1	ml

7. Reagent for purification of mAbs

7.1. 1 M Na_2HPO_4

Na_2HPO_4	14.2	gm
ddH_2O	100	ml

Mix well, store at room temperature

7.2. 1 M NaH_2PO_4

$NaH_2PO_4.H_2O$	13.8	gm
ddH_2O	100	ml

Mix well, store at room temperature

7.3. Reagents for IgM purification

7.3.1. Binding buffer (20 mM sodium phosphate, 0.8 M $(NH_4)_2SO_4$, pH 7.5)

1 M Na_2HPO_4	5.8	ml
1 M NaH_2PO_4	4.2	ml
$(NH_4)_2SO_4$	52.856	gm
ddH_2O	400	ml

Adjusted the pH to 7.5 with 5 N NaOH.

Adjusted the volume to 500 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter kept at 4°C**,** degas for 30 min before used

7.3.2. 4X Binding buffer (100 ml)

1 M Na_2HPO_4	4.6	ml
1 M NaH_2PO_4	3.36	ml
$(NH_4)_2SO_4$	42.284	gm
ddH_2O	70	ml

Adjusted the pH to 7.5 with 5 N NaOH.

Adjusted the volume to 100 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter kept at 4°C**,** degas for 30 min before used

7.3.3. Eulting buffer (20 mM sodium phosphate pH 7.5)

1 M Na_2HPO_4	11.6	ml
1 M NaH_2PO_4	8.4	ml
ddH_2O	800	ml

Adjusted the pH to 7.5 with 5 N NaOH.

Adjusted the volume to 1000 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter kept at 4^{o}C**,** degas for 30 min before used

7.3.4. Regeneration buffer

1 M Na_2HPO_4	5.8	ml
1 M NaH_2PO_4	4.2	ml
Isopropanol	150	ml
ddH_2O	200	ml

Adjusted the pH to 7.5 with 5 N NaOH.

Adjusted the volume to 500 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter kept at 4^{o}C**,** degas for 30 min before used

7.4. Reagents for IgG purification

7.4.1. Binding buffer (20 mM sodium phosphate buffer pH 7.0)

1 M Na_2HPO_4	11.6	ml
1 M NaH_2PO_4	8.4	ml
ddH_2O	800	ml

Adjusted the pH to 7.0 with 5 N NaOH.

Adjusted the volume to 1000 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter kept at 4°C**,** degas for 30 min before used

7.4.2. Eluting buffer (0.1 M glycine-HCl, pH 2.7)

Glycine	3.753	gm
ddH_2O	350	ml

Adjusted the pH to 2.7 with conc. HCl.

Adjusted the volume to 1000 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter kept at 4°C**,** degas for 30 min before used

7.4.3. Neutralizing buffer (1 M Tris-HCl, pH 9.0)

Tris base	12.114	gm
ddH_2O	60	ml

Adjusted the pH to 9.0 with conc. HCl.

Adjusted the volume to 100 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter kept at 4°C**,** degas for 30 min before used

7.5. Reagents for SDS-PAGE

7.5.1. 1 M Tris-HCl pH 6.8

Tris base	12.11	gm
ddH_2O	80	ml

Adjusted the pH to 9.0 with conc. HCl.

Adjusted the volume to 100 ml. with ddH_2O and stored at room temperature

7.5.2. 1% Bromphenol blue

Bromphenol blue	0.1	gm
dH2O	1	ml

7.5.3. 10X non-reducing buffer (NRB)

ddH2O	1.25	ml
1 M Tris-HCl pH 6.8	0.625	ml
Glycerol	1	ml
10% SDS	2	ml
1% Bromphenol blue	125	µl

Aliquot 300 µl/tube, kept at -20^{o}C

7.5.4. 5X reducing buffer (RB)

10X NRB	250	µl
2-ME	25	µl
ddH_2O	225	µl

Aliquot 100 µl/tube, kept at -20^{o}C

7.5.5. Running buffer

Glycine	14.413	gm
Tris base	3.028	gm
SDS	1	gm
ddH_2O	1000	ml

7.5.6. Slab gel

7.5.6.1. 30% Monomer (30.8% acrylamide, 2.7% bis-acrylamide)

Acrylamide	60	gm
Bis-acrylamide	1.6	gm
ddH_2O	200	ml

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter, kept in dark at 4^oC

7.5.6.2. 4X Stacking buffer (0.5 M Tris-HCl pH 6.8)

Tris base	6.0	gm
ddH2O	80	ml

Adjusted the pH to 6.8 with conc. HCl.

Adjusted the volume to 100 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter, kept at 4^oC

7.5.6.3. 4X Separating buffer (1.5 M Tris-HCl pH 8.8)

Tris base	18.15	gm
ddH_2O	80	ml

Adjusted the pH to 8.8 with conc. HCl.

Adjusted the volume to 100 ml. with ddH_2O

Mix thoroughly and filtrated through 0.2 μm Millipore membrane filter, kept at 4°C

7.5.6.4. 10% Ammonium persulfate (APS)

APS	0.5	gm
ddH_2O	5	ml

Mix thoroughly and aliquot 100 μl/tube, stored at -20°C

7.5.6.5. 10% Sodium dodecyl sulfate (SDS)

SDS	0.3	gm
ddH_2O	3	ml

Mix thoroughly and aliquot 150 μl/tube, stored at -20°C

7.5.6.6. Separating gel (12.5% SDS-polyacrylamide gel)

ddH_2O	3.2	ml
30% Monomer	4.2	ml
4X separating buffer	2.5	ml
10% SDS	100	μl
10% APS	50	μl
TEMED	20	μl

7.5.6.7. Stacking gel (4% SDS-polyacrylamide gel)

ddH_2O	1.5	ml
30% Monomer	332.5	µl
4X stacking buffer	625	µl
10% SDS	25	µl
10% APS	12.5	µl
TEMED	10	µl

7.5.7. 0.025% Coomassie brilliant blue R250

Coomassie brilliant blue R250	0.125	gm
Methanol	200	ml
Acetic acid	35	ml

Adjusted volume to 500 ml by dH_2O

Stored at room temperature

7.5.8. Destaining gel solution I (40% methanol, 7% acetic acid)

Methanol	400	ml
Acetic acid	70	ml

Adjusted volume to 1000 ml by dH_2O

Stored at room temperature

7.5.9. Destaining gel solution II (5% methanol, 7% acetic acid)

Methanol	50	ml
Acetic acid	70	ml

Adjusted volume to 1000 ml by dH_2O

Stored at room temperature

CURRICULUM VITAE

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Poster Presentation :

1. **Khamrin** S, Tatu T, Chiampanichayakul S, Khamnoi P, Kasinrerk W. Production and characterization of monoclonal antibodies against hemoglobins in Bart's hydrops fetalis syndrome. The sixth national symposium on graduate research. October 13-14 2006, Chulalongkorn University, Bankok, Thailand.
2. **Khamrin** S, Tatu T, Chiampanichayakul S, Khamnoi P, Kasinrerk W. Production of monoclonal antibody to Zeta globin chain. วันวิชาการ มหาวิทยาลัยเชียงใหม่ ครั้งที่ 2 วิถีวิจัย “ตามรอยพระยุคลบาท”. 8-10 ธันวาคม 2549, ณ หอประชุมใหญ่ มหาวิทยาลัยเชียงใหม่, เชียงใหม่, ประเทศไทย.