TABLE OF CONTENTS

| | Page |
|--|--------------------------|
| | |
| ACKNOWNLEDGMENTS | iii |
| | |
| THAI ABSTRACT | V |
| ENGLISH ABSTRACT | vi |
| | |
| LIST OF TABLES | xiv |
| | |
| LIST OF FIGURES | xvi |
| | Y // |
| ABBREVIATIONS AND SYMBOLS | xxvii |
| | |
| CHAPTER 1 INTRODUCTION | 1 |
| 1 Statement and significance of the problem | - 19 |
| 1. Statement and significance of the problem | Reializi |
| 2. Literature review | UOUINU |
| | 5 |
| 2.1 Human hemoglobin | Uni ₃ /ersity |
| | |
| | erved |
| | |

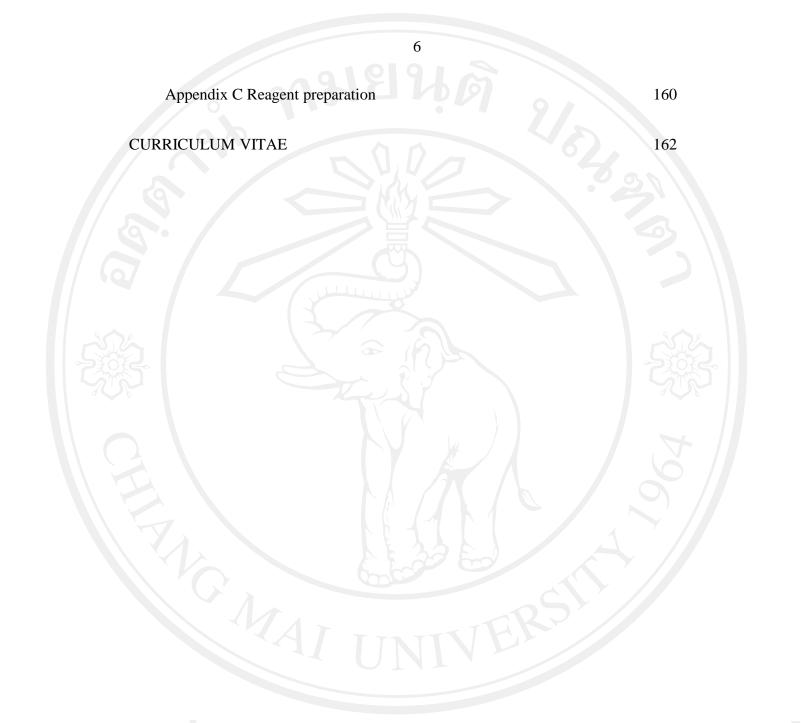
| 2 | |
|--|------------|
| 2.2 Globin gene clusters, globin genes and hemoglobin | 5 |
| switching | |
| 2.3 Thalassemia | 2 10 |
| 2.4 Alpha (α)-thalassemia | -11 |
| 2.5 Beta (β)-thalassemia | 14 |
| 2.6 Hemoglobinopathies | 15 |
| 2.7 Incidence of thalassemia and hemoglobinopathies in | 17 |
| Thailand | |
| 2.8 Clinical classification of thalassemia and | 18 |
| hemoglobinopathies | |
| 2.9 Laborarory diagnosis of thalassemia and | 19 |
| hemoglobinopathies | |
| 2.10 DNA analysis for diagnosis of thalassemia and | 21 |
| hemoglobinopathies | |
| 2.11 PCR from whole blood without DNA preparation | 24 |
| Objectives | 26 |
| TER 2 MATERIALS AND METHODS | nizversity |
| | |

| 2.1 Materials | 27 |
|---|----|
| 2.1.1Chemicals and instruments used in this thesis are | 27 |
| indicated in the Appendix. | |
| 2.1.2 Blood samples | 27 |
| 2.2 Methods | 27 |
| 2.2.1 Development of the in-house multiplex allele-specific | 28 |
| PCR | |
| 2.2.1.1 Extraction of genomic DNA | 28 |
| 2.2.1.1 Extraction of genomic DIVA | |
| 2.2.1.2 Determination of locations of allele-specific primers | 29 |
| in the corresponding globin genes | |
| 2.2.1.3 Optimization of the singleplex allele-specific PCR | 33 |
| 2.2.1.4 Development and optimization of developed in- | 38 |
| house multiplex allele-specific PCR | |
| 2.2.1.5 Evaluation of efficiency of the developed in-house | 43 |
| multiplex allele-specific PCR | |
| 2.2.2 Development of whole-blooded PCR protocols for | 48 |
| identifying globin gene mutations | |
| | |

2.2.2.1 Determining optimal thermal cycling pattern and 48 suitable form of blood samples to be used in the PCR reaction

| | 2.2.2.2 Search for the best PCR facilitators | 49 |
|------|--|----|
| | 2.2.2.3 Determining appropriate volume of blood samples | 50 |
| | used in the whole-blooded PCR | |
| | 2.2.2.4 Applying the developed technique in detecting the | 50 |
| | carriers of α - and β -thalassemia and hemoglobinopathies | |
| | common in Thailand | |
| СНАР | TER 3 RESULTS | 54 |
| 3.1 | Development of the in-house multiplex allele-specific PCR | 54 |
| | 3.1.1 Determination of locations of allele-specific primers in | 54 |
| | the corresponding globin genes | |
| | 3.1.2 Optimization of the singleplex allele-specific PCR | 58 |
| | 3.1.3 Development and optimization of in-house multiplex | 73 |
| | allele-specific PCR | |
| | 3.1.4 Evaluation of efficiency of the developed in-house | 99 |
| | multiplex allele-specific PCR | |
| | | |

| | 5 | |
|-------|--|-------------------------|
| 3.2 | Development of whole blood PCR protocols for identifying | 105 |
| globi | in gene mutations | |
| | 3.2.1 Determining optimal thermal cycling pattern and suitable form of blood samples to be used in the PCR | 105 |
| | reaction | |
| | 3.2.2 Search for the best PCR facilitators | 109 |
| | | |
| | 3.2.3 Determining appropriate volume of blood samples used in the whole blood PCR | 113 |
| | 3.2.4 Applying the developed technique in detecting the | 115 |
| | carriers of α - and β -thalassemia and | |
| | hemoglobinopathies common in Thailand | |
| СНА | APTER 4 DISCUSSION | 127 |
| СНА | APTER 5 CONCLUSIONS | 136 |
| REFI | ERENCES | 140 |
| APPI | ENDICES | 154 |
| | Appendix A List of chemicals | 155 |
| | Appendix B List of instruments | 158 niversity |
| | | |



<mark>ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่</mark> Copyright[©] by Chiang Mai University All rights reserved

LIST OF TABLES

| Table | Page |
|--|------|
| 1.1 Differences of length of UTR and IVS of the human globin genes | 8 |
| 1.2 Hemoglobin constituents in each developmental stage in human | 9 |
| 1.3 Mutations of β -globin genes commonly found in Thailand | 16 |
| 2.1 Names and sequences of oligonucleotide primers utilized in the | 32 |
| developed PCR protocols | |
| 2.2 "Checker board" pattern used in optimization of oligonucleotide | 38 |
| primers for α-thalassemia/hemoglobinopathies | |
| 3.1 Numerical positions of α - and β -globin gene specific primers in | 57 |
| the GenBank database | |
| 3.2 Sizes of amplified products deduced from position of each primer | 58 |
| in the AE006462 and HUMHBB GenBank databases | |
| 3.3 The optimized condition of the in-house multiplex allele-specific | 84 |
| PCR for α-thalassemia and hemoglobinopathy | |
| | |

| 3.4 The optimized condition of the in-house multiplex allele-specific | 98 |
|---|-----|
| PCR for β -thalassemia and hemoglobinopathy | |
| 3.5 Frequencies of globin gene mutations in 70 thalassemia carriers | 100 |
| as detected by the in-house multiplex allele-specific PCR | |
| 3.6 The optimized condition of the whole blood Gap PCR for | 119 |
| detecting SEA- α thalassemia 1 | |
| 3.7 The optimized condition of the whole blood PCR for detecting | 123 |
| HbCS | |
| 3.8 The optimized condition of the whole blood multiplex allele- | 126 |
| specific PCR for detecting the common β- | |
| thalassemia/hemoglobinopathy | |
| | |
| | |

ลิ<mark>ปสิทธิ์มหาวิทยาลัยเชียงใหม่</mark> Copyright[©] by Chiang Mai University All rights reserved

LIST OF FIGURES

| Figure | Page |
|--|------|
| 1.1 Structure of functional human hemoglobin molecule | 4 |
| 1.2 Structure of the human α -like globin gene clusters and β -like | 6 |
| globin gene clusters | |
| 1.3 Developmental stage specific production of globin genes in | 7 |
| human | |
| 1.4 Structure of the human α -globin gene and β -globin gene | 8 |
| 1.5 Deletions of α -globin gene cluster in α -thalassemia 1 | 13 |
| 1.6 Reciprocal crossovers on α -globin cluster causing 3.7-kb (Z-Z | |
| boxes) and 4.2-kb (X-X boxes) deletions causing α -thalassemia 2 | |
| or α ⁺ -thalassemia Chiang Mai | |
| | |

| 10 | |
|---|----|
| 2.1 Schematic locations of α -globin and β -globin genes common | 30 |
| mutations in Thailand | |
| 2.2 Locations of oligonucleotide primers used in the multiplex allele- | 31 |
| specific PCR | |
| 2.3 Locations of PCR primers and sequencing primer used for β - | 45 |
| globin gene sequencing | |
| 3.1 Part of the "AE006462" GenBank database as source of α -globin | 55 |
| gene sequences used in this thesis | |
| 3.2. Part of the "HUMHBB" GenBank database as source of β -globin | 56 |
| gene sequences used in this thesis | |
| 3.3 PCR products (391-bp) of internal control primers; αG-17 + C3 | 59 |
| 3.4 The optimization of annealing temperature for the singleplex | 60 |
| Gap-PCR for detection of the SEA- α thalassemia 1. | |
| 3.5 The optimization of concentration of MgCl ₂ for the singleplex | 61 |
| Gap-PCR for detection of the SEA- α thalassemia 1. | |
| 3.6 Titration of amount of SEA primers for the singleplex Gap-PCR | 62 |
| for detection of the SEA- α thalassemia 1. | |
| | |
| | |

| 3.7 The titration of amount of SEA primers for the singleplex Gap- | 63 |
|--|----------|
| PCR for detection of the SEA- α thalassemia 1. | |
| 3.8 The titration of amount of SEA primers for the singleplex Gap- | 64 |
| PCR for detection of the SEA- α thalassemia 1. | |
| 3.9 The titration of amount of SEA primers for the sin66gleplex Gap- | 65 |
| PCR for detection of the SEA- α thalassemia 1. | |
| | |
| 3.10 The titration of amount of SEA primers for the singleplex Gap- | 66 |
| PCR for detection of the SEA- α thalassemia 1. | |
| 3.11 Agarose electrophoretic pattern of the amplified fragments of | 67 |
| the single plex Gap-PCR for detection of the SEA- α | |
| thalassemia 1. | |
| 3.12 Titration of amount of α G-17 primer in the single plex allele- | 68 |
| specific PCR for HbCS. | |
| specific relevies. | |
| 3.13 Agarose electrophoresis pattern of the amplified fragment of the | 69 |
| singleplex allele-specific PCR for detection of the HbCS | |
| employing the optimized conditions. | |
| 3.14 Titration of concentration of MgCl ₂ in the singleplex allele- | 000 JUNU |
| specific PCR for α -thalasseia 2 (3.7-kb deletion). | |
| | |
| | |
| | |

3.15 Titration of concentration of betaine in the singleplex allele-71 specific PCR for α -thalasseia 2 (3.7-kb deletion). 3.16 Agarose electrophoresis pattern of the amplified fragment of the 72 singleplex allele-specific PCR for detection of the α -73thalassemia 2 (3.7 kb deletion) after addition of aG-17 and C3 primers and 22-round thermal cycle performed. 3.17 Agarose electrophoresis pattern of the amplified fragment of the 73 singleplex allele-specific PCR for detection of the α thalassemia 2 (3.7 kb deletion). 3.18 Agarose gel electrophoresis of amplified fragments for the inhouse multiplex allele-specific PCR for αthalassemia/hemoglobinopathy. 3.19 Agarose gel electrophoresis of amplified fragments for the in-75 house multiplex allele-specific PCR for αthalassemia/hemoglobinopathy. 3.20 Agarose gel electrophoresis of amplified fragments for the in-76 house multiplex allele-specific PCR for αthalassemia/hemoglobinopathy.

3.21 Agarose gel electrophoresis of amplified fragments for the in-77 house multiplex allele-specific PCR for athalassemia/hemoglobinopathy for the titration of MgCl₂ concentration. 3.22 Agarose gel electrophoresis of amplified fragments for the inhouse multiplex allele-specific PCR for αthalassemia/hemoglobinopathy. 3.23 Agarose gel electrophoresis of amplified fragments for the inhouse multiplex allele-specific PCR for αthalassemia/hemoglobinopathy. 3.24 Agarose gel electrophoresis of amplified fragments for the in-80 house multiplex allele-specific PCR for αthalassemia/hemoglobinopathy. 3.25 Agarose gel electrophoresis of amplified fragments for the in-81 house multiplex allele-specific PCR for αthalassemia/hemoglobinopathy. 3.26 Agarose gel electrophoresis of amplified fragments for the inhouse multiplex allele-specific PCR for αthalassemia/hemoglobinopathy.

3.27 Agarose gel pattern of amplified products generated by the 83 optimized in-house multiplex allele-specific PCR for αthalassemia and hemoglobinopathy. 3.28 Agarose gel electrophoresis of amplified fragments for the in-86 house multiplex allele-specific PCR for β thalassemia/hemoglobinopathy. 3.29 Agarose gel electrophoresis of amplified fragments for the in-87 house multiplex allele-specific PCR for βthalassemia/hemoglobinopathy. 3.30 Agarose gel electrophoresis of amplified fragments for the in-88 house multiplex allele-specific PCR for βthalassemia/hemoglobinopathy. 89 3.31 Titration of concentration of MgCl₂ to be used in the in-house multiplex allele-specific PCR for βthalassemia/hemoglobinopathy. 3.32 Agarose gel electrophoresis of amplified fragments for the in-90 house multiplex allele-specific PCR for βthalassemia/hemoglobinopathy.

| 15 | |
|---|----|
| 3.33 Agarose gel electrophoresis of amplified fragments for the in- | 91 |
| house multiplex allele-specific PCR for β- | |
| thalassemia/hemoglobinopathy. | |
| 3.34 Agarose gel electrophoresis of amplified fragments for the in- | 92 |
| house multiplex allele-specific PCR for β- | |
| thalassemia/hemoglobinopathy. | |
| 3.35 Agarose gel electrophoresis of amplified fragments for the in- | 93 |
| house multiplex allele-specific PCR for β- | |
| thalassemia/hemoglobinopathy. | |
| 3.36 Agarose gel electrophoresis of amplified fragments for the in- | 94 |
| house multiplex allele-specific PCR for β- | |
| thalassemia/hemoglobinopathy. | |
| 3.37 Agarose gel electrophoresis of amplified fragments for the in- | 95 |
| house multiplex allele-specific PCR for β- | |
| thalassemia/hemoglobinopathy. | |
| 3.38 Agarose gel electrophoresis of amplified fragments for the in- | 96 |
| house multiplex allele-specific PCR for β- | |
| thalassemia/hemoglobinopathy. | |
| | |
| | |

| 16 | |
|--|-----|
| 3.39 Agarose gel electrophoresis of amplified fragments genetared | 97 |
| from the optimized in-house multiplex allele-specific PCR for | |
| β-thalassemia/hemoglobinopathy. | |
| 3.40 Nucleotide sequences of SEA- α thalassemia 1 breakpoint in | 101 |
| blood sample. | |
| 3.41 Nucleotide sequences of HbCS in blood sample | 102 |
| 3.42 Nucleotide sequences of $\beta^{17(A-T)}$ in blood sample | 102 |
| 3.43 Nucleotide sequences of $\beta^{41/42(-TTCT)}$ in blood sample | 103 |
| 3.44 Nucleotide sequences of $\beta^{26(G-A)}$ or HbE in blood sample | 104 |
| 3.45 Nucleotide sequences of $\beta^{-28(A-G)}$ in known blood sample | 104 |
| 3.46 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments using | 106 |
| blood lysate | |
| 3.47 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments using | 107 |
| fresh blood with three repeating extra heat-cool steps | |
| 3.48 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments using | 108 |
| fresh blood with five repeating extra heat-cool steps | |
| | |
| | |
| | |

| 3.49 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments using | 109 |
|--|------|
| fresh blood with ten repeating extra heat-cool steps | |
| 3.50 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments in | 110 |
| presence of varying amount of BSA. | |
| 3.51 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments in | 111 |
| presence of varying amount of BSA. | |
| 3.52 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments in | -111 |
| presence of varying amount of betaine. | |
| 3.53 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments in | 112 |
| presence of varying amount of betaine. | |
| 3.54 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments in | 113 |
| presence of fixed amount of betaine at 9% and varying | |
| concentration of BSA. | |
| 3.55 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments using | 114 |
| varying volume of blood lysate. | |
| 3.56 Amplification of 665-bp $^{G}\gamma$ -globin specific fragments using | |
| varying volume of fresh blood. | |
| | |
| | |

| 18 | |
|--|-------|
| 3.57 Titration of SEA1 primer concentration used in the whole blood | 115 |
| Gap-PCR of SEA-α thalassemia 1. | |
| 3.58 Titration of SEA2 primer concentration used in the whole blood | 9 116 |
| Gap-PCR of SEA-α thalassemia 1. | |
| 3.59 Titration of SEA3 primer concentration used in the whole blood | 117 |
| Gap-PCR of SEA-α thalassemia 1. | |
| 3.60 Detection of the SEA- α thalassemia 1 by the whole blood Gap | 118 |
| PCR | |
| 3.61 Titration of CS-2 primer concentration used in the whole blood | 120 |
| allele-specific PCR for HbCS. | |
| 3.62 Titration of α G-17 primer concentration used in the whole blood | 121 |
| allele-specific PCR for HbCS. | |
| 363 Detection of carrier of HbCS by the whole blood allele-specific | 122 |
| PCR | |
| 3.64 Titration of concentration of "beta-E multiplex" primer used in | 124 |
| whole blood multiplex allele-specific PCR of common β - | |
| thalassemia in Thailand. | |
| | |
| | |

3.65 Whole blood multiplex allele-specific PCR for detecting the

common β -thalassemia/hemoglobinopathy in Thailand.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่ Copyright[©] by Chiang Mai University All rights reserved

ABBREVIATIONS AND SYMBOLS

| bp | base pair |
|---------------|----------------------------------|
| conc. | concentration |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxynucleotide triphosphates |
| DMSO | dimethyl sulfoxide |
| EDTA | ethylenediamine tetraacetic acid |
| g/dl | gram per deciliter |
| Hb Bart's | hemoglobin Bart's |
| HbCS | hemoglobin Constant Spring |
| HbE | hemoglobin E |
| нын by Chiang | hemoglobin H |
| | eserved |

21 intervening sequence IVS kb kilobase pairs potassium chloride KCl Μ molar MgCl₂ magnesium dichloride milliliter ml millimolar mМ nucleotide nt SEA Southeast Asia weight/volume w/v microliter μl micromolar μM

ลิ<mark>ปสิทธิ์มหาวิทยาลัยเชียงใหม่</mark> Copyright[©] by Chiang Mai University All rights reserved