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DIAGNOSIS OF BETA - GLOBIN GENE MUTATIONS IN SINGLE BLASTOMERE BY REVERSE DOT BLOT HYBRIDIZATION

Miss Jiraporn Pansatha

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for the Degree of Master of Science in Medical Science
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เหตุผลของการศึกษา เทคนิครีเวอร์สดอทบล็อทไฮบริไดเซชั่นเป็นวิธีที่ใช้ในการวินิจฉัยทารกก่อนคลอดของโรคเบต้าธาลัสซีเมีย เนื่องจากยีนเบต้ากลอบบินมีชนิดการกลายพันธุ์ที่หลากหลาย ซึ่งส่วนใหญ่เกิดจากเบสเปลี่ยนแปลงไปเฉพาะจุด การศึกษาวิจัยครั้งนี้คือการนำเทคนิคนี้ มาใช้ในการตรวจตัวอ่อนก่อนการย้ายฝากในโพรงมดลูก ซึ่งเป็นการวินิจฉัยความผิดปกติของยีนเบต้าธาลัสซีเมียในระดับดีเอ็นเอของบลาสโตเมียร์เดี่ยว คุณภาพและปริมาณของผลิตผลจากพีซีอาร์ เป็นปัจจัยสำคัญของการทำไฮบริไดเซชั่น ของเทคนิครีเวอร์สดอทบล็อทไฮบริไดเซชั่น ปฏิกริยาพีซีอาร์จะถูกปรับเปลี่ยนให้เหมาะสม เพื่อเพิ่มปริมาณยีนเบต้ากลอบบินให้เพียงพอ สำหรับการตรวจกรองความผิดปกติของยีนเบต้ากลอบบิน โดยวิธีรีเวอร์สดอทบล็อทไฮบริไดเซชั่นต่อไป **วิธีการ** การวิจัยมุ่งประเด็นที่จะแก้ปัญหาการหาสภาวะที่เหมาะสมในการเพิ่มปริมาณยีนเบต้ากลอบบินของเซลล์เดี่ยวตัวอ่อนโดยใช้การทำพีซีอาร์ 2 ขั้นตอน เริ่มต้นจากการหาความเข้มข้นของดีเอ็นเอจากเลือดที่ต่ำที่สุดที่สามารถเพิ่มปริมาณดีเอ็นได้ จากนั้นทำการเพิ่มปริมาณยีนเบต้ากลอบบินของบลาสโตเมียร์เดี่ยวจำนวน 106 เซลล์ การหาอุณหภูมิแอลนีลลิ่งที่เหมาะสม การศึกษาผลของไพรเมอร์คู่ในต่อการทำพีซีอาร์ และศึกษาเปรียบเทียบวิธีไลสีสเซลล์ ใช้บลาสโตเมียร์เดี่ยว 25 50 และ 256 เซลล์ ตามลำดับ ขั้นตอนสุดท้าย วินิจฉัยการกลายพันธุ์ของยีนเบต้ากลอบบิน ของดีเอ็นเอจากเลือดและและบลาสโตเมียร์เดี่ยวที่เพิ่มปริมาณเอ็นเอแล้ว **ผลการศึกษา** เมื่อเพิ่มปริมาณดีเอ็นเอจากเลือดที่ลดความเข้มข้นเป็นลำดับ สามารถเพิ่มปริมาณดีเอ็นเอของเลือดลดความเข้มข้นจนถึงระดับต่ำสุดที่ 12.5 พิโครกรัม เมื่อเพิ่มปริมาณยีนเบต้ากลอบบินของบลาสโตเมียร์เดี่ยว มีอัตราความสำเร็จเท่ากับร้อยละ 26.4 ซึ่งค่อนข้างต่ำ จึงทำการปรับองค์ประกอบของการทำพีซีอาร์ เริ่มจากการปรับอุณหภูมิแอลนีลลิ่ง พบว่าที่อุณหภูมิ 58 องศาเซนเซียส สามารถเพิ่มปริมาณดีเอ็นเอได้สำเร็จ ศึกษาผลของไพรเมอร์ต่อการทำพีซีอาร์ จึงทำการเปลี่ยนเนสเต็ดไพรเมอร์ อัตราความสำเร็จในการเพิ่มปริมาณดีเอ็นเอเพียงร้อยละ 22 เมื่อเปลี่ยนไพรเมอร์คู่นอกและปรับสภาวะในการทำพีซีอาร์อีกครั้ง พร้อมกับการไตเตรดความเข้มข้นของไพรเมอร์ พบว่าความเข้มข้นของไพรเมอร์ที่เหมาะสมคือ 0.1 ไมโครโมลาร์ จึงใช้สภาวะที่เหมาะสมนี้ในการศึกษาเปรียบเทียบวิธีไลสีสเซลล์ พบว่าไม่มีความแตกต่างระหว่างวิธีที่ใช้น้ำ (ต้มที่ 94 องศาเซลเซียส เป็นเวลา 15 นาที) กับวิธีที่ใช้ทีอีบัฟเฟอร์ (ต้มที่ 95 องศาเซลเซียส เป็นเวลา 10 นาที) ซึ่งให้อัตราความสำเร็จในการเพิ่มปริมาณยีน เบต้ากลอบบินของเซลล์เดี่ยวตัวอ่อนเท่ากับ ร้อยละ 58 และ 55.4 ตามลำดับ ต่างจากการใช้อัลคาไลน์บัฟเฟอร์ ซึ่งให้อัตราความสำเร็จในการเพิ่มปริมาณยีนเบต้ากลอบบินของเซลล์เดี่ยวตัวอ่อนร้อยละ 0 ในการทดลองสุดท้าย นำดีเอ็นเอที่เพิ่มปริมาณแล้วจากดีเอ็นเอ 10 พิโครกรัม ที่ทราบตำแหน่งการกลายพันธุ์ของยีนเบต้ากลอบบินแล้วมาตรวจสอบตำแหน่งการกลายพันธุ์อีกครั้ง เพื่อตรวจสอบความถูกต้องและแม่นยำของวิธีเรีเวอร์สดอทบล็อทไฮบริไดเซชั่น พบว่าตำแหน่งการกลายพันธุ์ทุกตำแหน่งถูกต้องตรงกับผลการตรวจเดิม บลาสโตเมียร์เดี่ยวที่เพิ่มปริมาณดีเอ็นเอแล้วประสบผลสำเร็จในการทำไฮบริไดเซชั่นเช่นกัน **สรุป** การศึกษาครั้งนี้ ประสิทธิภาพของการเพิ่มขยายดีเอ็นเอจำเพาะในเซลล์เดี่ยวตัวอ่อนด้วยเทคนิคพีซีอาร์ 2 ขั้นตอน ยังมีประสิทธิภาพค่อนข้างต่ำ จึงยังไม่สมควรที่จะนำวิธีรีเวอร์สดอทบล็อทไฮบริไดเซชั่น มาใช้ในการวินิจฉัยความผิดปกติของยีนเบต้ากลอบบิน ในตัวอ่อนของคน ก่อนที่จะได้นำไปฝังตัวในมดลูกได้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....
ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

437 52097 30 : MAJOR MEDICAL SCIENCE

KEY WORD : BETA GLOBIN GENE, PREIMPLANTATION DIAGNOSIS, REVERSE DOT BLOT HYBRIDIZATION

JIRAPORN PANSATHA : DIAGNOSIS OF BETA - GLOBIN GENE MUTATIONS IN SINGLE BLASTOMERE BY REVERSE DOT BLOT HYBRIDIZATION. THESIS ADVISOR : ASSOC.PROF. KAMTHORN PRUKSANANONDA, M.D., THESIS CO-ADVISOR : ASST.PROF. PRANEE SUTCHARITCHAN, M.D.

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BACKGROUND: Reverse dot blot hybridization (RDB) has been employed for prenatal diagnosis (PND) of beta thalassemia due to the variety of point mutation of beta globin gene. This study try to apply this technique performed preimplantation genetic diagnosis (PGD) for beta thalassemia. The quality and quantity of PCR product is the key factor for successful hybridization of RDB. PCR reactions were modified for single blastomere to provide adequate PCR product for hybridization. **METHODS:** Two-step (nested) PCR was performed to amplify beta globin gene. The genomic DNA was serial diluted for determining the minimum DNA concentration could be amplified. One hundred and six single blastomeres were subjected to genotype. The optimization of annealing temperature, changing nested primer and comparing lysis method was evaluated by genotyping of 25, 50 and 256 single blastomeres respectively. Amplified product was diagnosed beta globin mutation for RDB. **RESULTS:** The minimum DNA concentration successful amplified was 12.5 pg. Twenty-eight of 106 (26.4%) single blastomeres were successful amplified. The optimized annealing temperature was 58°C. Amplification efficiency of changing location of nested PCR primer was 22.0%. The optimized primer concentration was 0.1 μM. Comparing five lysis methods (i) boiling in water at 94°C for 15 (ii) 30 min, (III) incubation in an alkali lysis buffer for 30 min at 94°C or (iv) at 65°C for 10 min, and (v) boiling in TE buffer at 95°C for 10 min. the amplification percentages were formed to be 58, 10.8, 0, 0 and 55.4% respectively. Applications of reverse dot blot hybridization to detect beta globin gene mutation of amplified DNA from 10 pg DNA of which known mutation, it was found that the detection of beta globin gene mutations were all correct. Amplified DNA from single blastomere successful hybridized. **CONCLUSION :** At the present study, amplification efficiency of beta globin gene in single blastomere is quite low, so that it is not appropriate for clinical applications.

Field of study: Medical Science Student's signature…….

Academic year: 2003 Advisor's signature…….

Co-advisor's signature……..

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LIST OF ABBREVIATION

°C	Degree Celsius
bp	Base pair
BSA	Bovine serum albumin
ddH_2O	Deionized distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethyl diamine tetraacetic acid (disodium salt)
g	Gram
HCl	Hydrochloric acid
IVF	In vitro fertilization
M	Molar
µl	Microlitre (10^{-6} litre)
min	Minute
ml	Millitre (10^{-3} litre)
ng	Nanogram (10^{-9} gram)
PCR	Polymerase chain reaction
RDB	Reverse dot blot
SDS	Sodium dodesyl sulfate
‘sec	Second
SDW	Sterilized deionized water
T_A	Annealing temperature
TdT	Terminal deoxynucleotidyl transferase
TE	Tris EDTA
TMB	Tetrametylbenzidine
Tris	Tris (hydroxy methyl) aminomethane
V	Volt

CHAPTER I

INTRODUCTION

The beta thalassemia is one of the most common autosomal recessive single gene disorders, having particularly high incidences in affected endemic population (Weatherall,1995). In Thailand the gene frequencies of beta thalassemia are 3-9%. Hemoglobin (Hb) E is one of beta hemoglobinopathies which is the hall mark of southeast Asia, attaining a gene frequency of 50-60% at the junction of Thailand, Laos and Cambodia. Therefore, beta thalassemia is a significant public health problem in Thailand and over 5,000 of the annual one million newborn are thalassemia major which include homozygous beta thalassemia and beta thalassemia / Hb E (Winichagoon,1999).

A couple of thalassemia carrier has a 25% probability of having an affected baby with each pregnancy. Current therapies for thalassemic patients are blood transfusion, iron chelation and allogenic bone marrow transplantation (Rodgers and Rachmilewitz,1995), while chorionic villus sampling (CVS) (Brambati et al,1987), amniocentesis and genetic analysis by the polymerase chain reaction (PCR) (Saiki et al.,1985) have made first- and second- trimester prenatal diagnosis possible by DNA analysis.

Although the number of beta thalassemia births has been reduced by the treatments and the method as above, pregnancy termination is still unacceptable for many families and couple at risk are looking for an alternative way of producing non-affected offspring.

Recent progress in in-vitro fertilization (IVF), gamete and embryo micromanipulations and molecular biology as well have now made preimplantation genetic diagnosis possible of genetic diseases (Handyside et al.,1990; Harper,1996)

Hemoglobin production

Hemoglobin (Hb) is produced in the bone marrow and resides in the red blood cell. It carries the oxygen that is breathed into the lungs to different parts of the body. Hemoglobin consists of four polypeptide chains and four heme

group; it is a dimmer of dimmers with two chains from the alpha family and the other from the beta family. The four chains are held together by non-covalent attractions. Each chain contains a heme group that binds a molecule of oxygen.

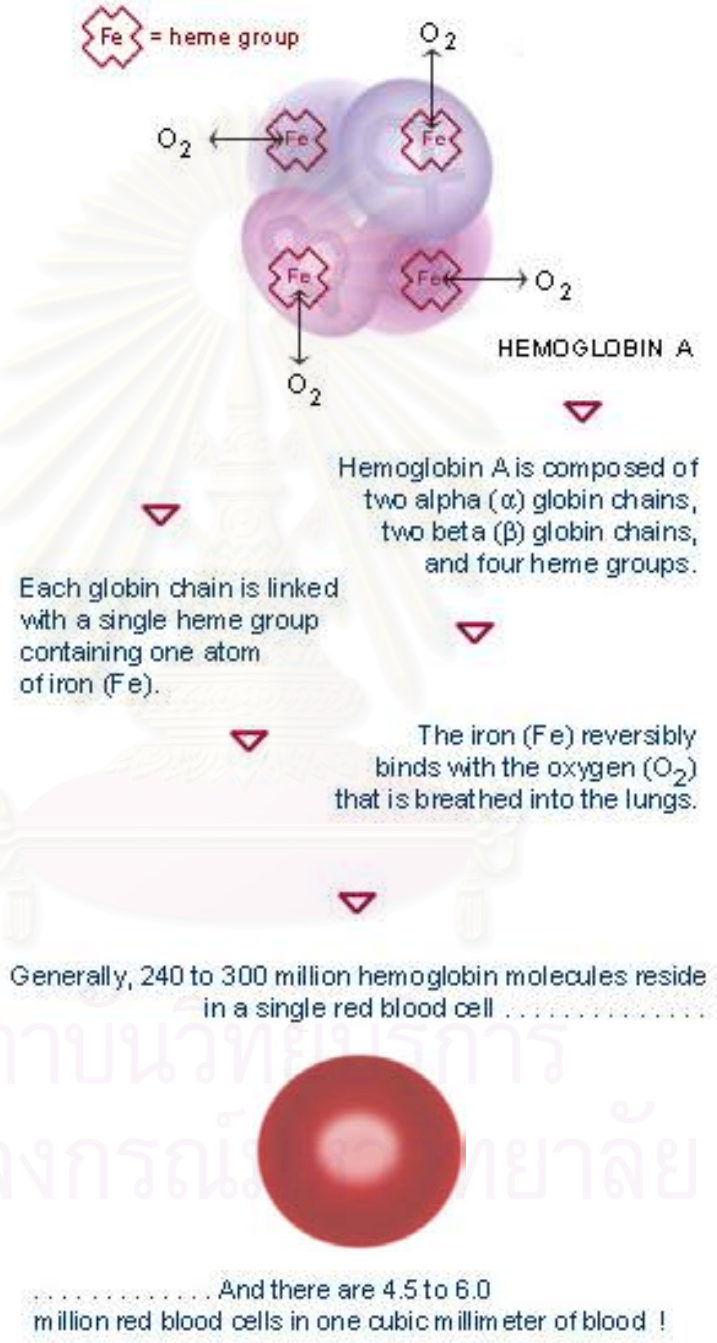

Figure 1.1 Hemoglobin production

(Image from URL: http://www.thalassemia.com)

The genes controlling globin production are on chromosome 16 (alpha α globin genes) and chromosome 11 (beta β, gamma γ, and delta δ genes).

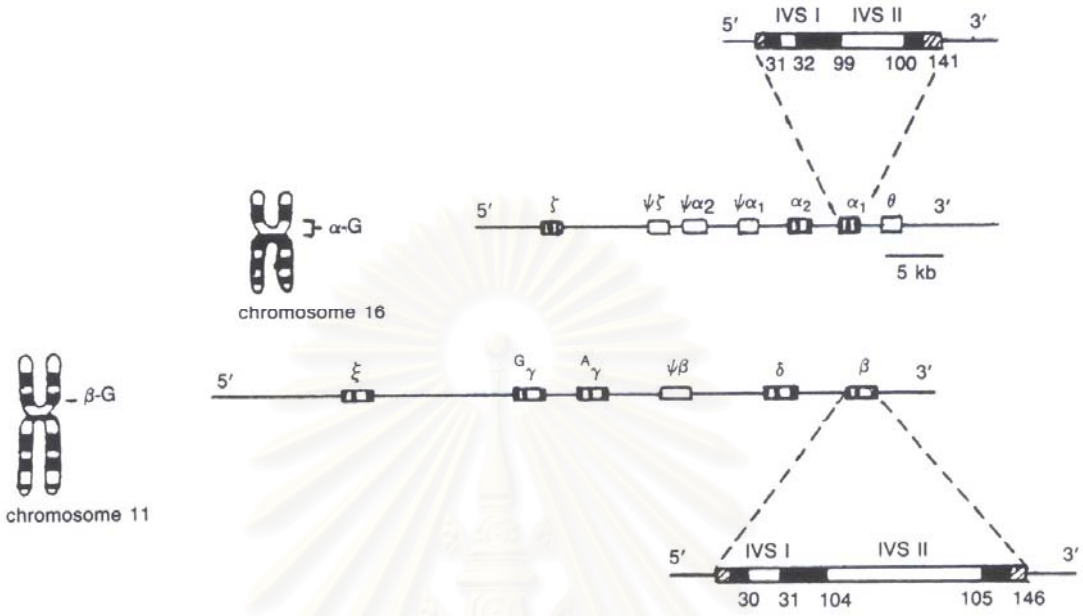

Figure 1.2 Location of the alpha family and beta family of globin chains (Image from Winichagoon P et al.,1998)

In the normal adult, hemoglobin A which is composed of two alpha and two beta globins ($\alpha^2\beta^2$) is the most prevalent, and comprised about 95% of all hemoglobin. Two minor hemoglobins also occur : hemoglobin A_2, composed of two alpha and two delta globins ($\alpha^2\delta^2$) comprises 2-3.5% of hemoglobin, while hemoglobin F, composed of two alpha and two gamma globins ($a^2\gamma^2$), comprises less than 2% of hemoglobin.

Hemoglobin F, or fetal hemoglobin, is produced by the fetus in utero and until about 48 weeks after birth. Hemoglobin F has a high oxygen-affinity in order to attract oxygen from maternal blood and deliver it to the fetus. After birth, the production of adult hemoglobin rapidly increases and fetal hemoglobin production drops off.

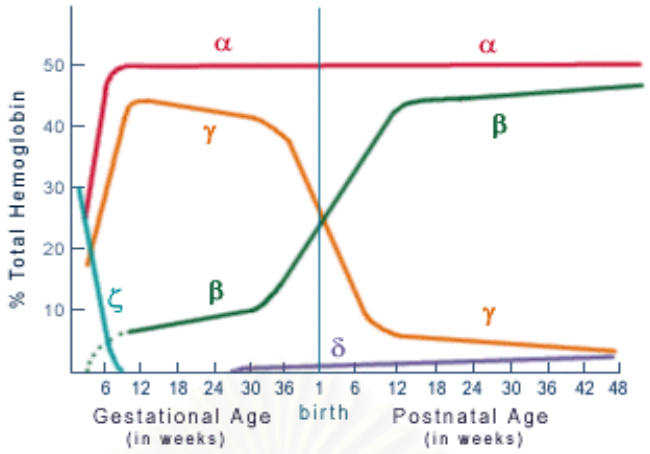

Figure 1.3 The progression of human globin chains synthesis
(Image from URL: http://www.thalassemia.com)

As seen in the Figure 1.3, the alpha globin molecule concentration is rather stable in fetal and adult life, because it is needed for both fetal and adult hemoglobin production. The beta globin appears early in fetal life at low levels and begins to rapidly increase after 30 weeks of gestational age, reaching a maximum about 30 weeks posnatally. The gamma globin molecule reaches a high level early in fetal life at about 6 weeks and begins to decline about 30 weeks of gestational age, reaching a low level about 48 weeks postgestational age. The delta globin appears at a low level at about 30 weeks of gestational age and maintains a low profile throughout life.

Beta thalassemia

There are hundreds of mutations within the beta globin gene, but approximately 20 different alleles comprise 80% of the mutations found world wide. Within each geographic population there are unique mutations. Individuals who have beta thalassemia major are usually homozygous for one of the common mutations, or heterozygous for one of the common mutations and one of the geographically-unique mutations. Both lead to absence of beta globin chain production.

The beta thalassemia syndromes are much more diverse than the alpha thalassemia syndromes due to the diversity of the mutations that produce the defects in the beta globin gene. Unlike the deletions that constitute most of

the alpha thalassemia syndromes, beta thalassemias are caused by mutations on chromosome11 that affect all aspects of beta globin production: transcription, translation, and the stability of the beta globin product. Most hematologists feel there are three general categories of beta thalassemia: beta thalassemia trait, beta thalassemia intermediate and beta thalassemia major.

Splice site mutations also occur and are of clinical consequence, when combined with a thalassemia mutation. Three splice site mutations occur in exon 1 of the beta globin gene. These mutations result in three different abnormal hemoglobins: Malay, E, and Knossos. **Hemoglobin E** is a very common abnormal hemoglobin in the Southeast Asian population, and when paired with a β^o thalassemia mutation, can produce severe transfusion-dependent ($E\beta^o$) thalassemia. Hemoglobin E is described in the next section on variant hemoglobins.

Individuals who have beta thalassemia trait have microcytosis and hypochromia; there may be targetting, elliptocytosis, though some individuals have an almost normal smear. Hemoglobins A_2 and F will be elevated on hemogram results. These hematologic features can be accentuated in women with trait who are pregnant and in individuals who are folate or iron deficient. If iron deficiency is concurrent with beta thalassemia trait there may be a normal Hb A_2. Iron deficiency causes decreased hemoglobin production, and folate or vitamin B_{12} deficiency can lead to megaloblastic anemia with increased Hb A_2. Both of these deficiencies need to betreated prior to evaluation for thalassemia trait. In iron, B_{12}, and folate replete individuals, the Hb A_2 can be as high as 3.5 to 8% and the Hb F as high as 1 to 5%. Generally, beta thalassemia trait is milder in African-Americans (who frequently have a promoter gene mutation) but has a similar presentation in individuals of Chinese, Southeast Asian, Greek, Italian, and Middle Eastern heritage.

Beta thalassemia major was first described by a Detroit pediatrician, Thomas Cooley, in 1925. The clinical picture he described is prevalent today in countries without the necessary resources to provide patients with chronic transfusions and desferoxamine therapy. Children who have untreated thalassemia major have ineffective erythropoiesis, decreased red cell deformability, and enhanced clearance of defective red cells by macrophages

(immune system cells). The result is a very hypermetabolic bone marrow with thrombocytosis,leukocytosis and microcytic anemia in the young child prior to the enlargement of their spleens. At presentation they have almost 100% percent Hb F (these cells have a longer life span due to a balanced globin ratio, as γ rather than β, globin is present Hb F). These children have little or no Hb A_2 and a low reticulocyte count. The diagnosis can be confirmed by demonstrating thalassemia trait in both parents, by globin biosynthetic ratios, or by beta gene screening. Beta gene screening identifies the most common and some uncommon mutations, but not all mutations. An electrophoresis showing only Hb F, a complete blood count and a smear will generally be diagnostic.

Variant hemoglobins

Any hemoglobin that contains a mutation is a variant hemoglobin. A mutation can involve a deletion of all or a portion of the gene leading to a reduced or absent production of globin proteins, or small changes in the DNA that affect the genes ability to function normally leading to the production of different types of hemoglobin. A mutation on chromosome 16 in the alpha globin gene can produce a different hemoglobin such as Constant Spring. However, mutations in this gene usually lead to the inactivation of the alpha globin protein production coded by the gene. A mutation can occur on chromosome 11, affecting one of the beta globins, leading to an abnormal hemoglobin. Beta globin gene mutations can lead to decreased production as well as inactivation of the gene. Not all of these mutations have been defined with DNA analysis, though many of them have and many are constantly being defined.

There are over 150 known mutations in the beta globin gene, producing beta thalassemia trait in the heterzygotic individual, and causing a variable degree of anemia in the homozygote. In addition, there are mutations such as that leading to hemoglobin E, which do not produce disease even in the homozygous state. However, hemoglobin E combined with other variant beta globins can produce mild to severe anemia. Many variant hemoglobins exist worldwide. Hemoglobins E and S are significant because they can be co-inherited with thalassemia trait to produce a disease that requires treatment. Hemoglobin E is very common among Southeast Asians; California Newborn Screening Program found that 1 in 12 Southeast Asians, and 1 in 4 Cambodian

newborns had Hb E trait. Hemoglobin EE (homozygous) has not been shown to have serious medical implications. Hemoglobin E - Beta thalassemia has a wide range of clinical manifestations, from mild anemia to significant anemia requiring chronic transfusions.

Hemoglobin S (sickle cell) is prevalent among Africans and African Americans. Hemoglobin S and beta thalassemia trait can be co-inherited to produce sickle beta zero (SB^o) thalassemia. The clinical manifestations of this disease resemble Hb SS disease.

Hemoglobin E is the most common abnormal hemoglobin identified in Laos, Cambodia and Thailand. Hemoglobin E results from a mutation in an exon (exon 1, codon 26: GAG to AAG) that creates an alternate splice site competing with the normal splice site. This results in abnormal hemoglobin production and mild thalassemia in the homozygous state. In the homozygous state it produces a mild microcytic anemia with a hemoglobin usually above 10 gm/dl. Electrophoresis reveals approximately 90% Hb E with varying amounts of Hb F. The heterozygote has a hemoglobin of about 12 gm/dl with microcytosis and an electrophoretic pattern consistent with Hb E plus Hb A_2 of 20 to 30%. When Hb E trait combines with other more severe beta thalassemias, Hb E-beta thalassemia can produce an anemia that is profound requiring chronic transfusion therapy. All children who have Hb E and Hb F on their state screen require scrutiny for emergence of a severe thalassemia syndrome. Individuals who have Hb EE do not have significant anemia and do not require special care except that they should not be treated with iron for anemia.

Preimplantation Genetic Diagnosis

Before the clinical application of preimplantation genetic diagnosis (PGD) the only way of preventing the birth of an affected child for couples that risk transmitting an inherited genetic disease, was to undergo prenatal diagnosis (PND), followed by pregnancy termination if the fetus was affected or having a disease child. However, selective termination of pregnancy in the first or second trimesters is a difficult and painful decision and might be

unacceptable for moral and some religious reasons. Preimplantation genetic diagnosis is a good technique for the diagnosis of genetic disease in the earliest stage of development of a human embryo will enable the carrier of mutant gene to avoid a pregnancy with an affected fetus (Whittingham and Penketh,1987)

At first, this technique has been successful with the rabbit blastocyst in which sex chromatin can be identified histologically (Gardner and Edwards,1968). There have been a number of mouse model system for preimplantation diagnosis. Monk et al. (1987) have diagnosed a deficiency of the X-linked enzyme hypoxanthine phosporibosyl transferase (HPRT) in mouse preimplantation embryos as a model for Lesch-Nyhan syndrome. Morsey et al. (1992) has demonstrated that in mice, preimplantation diagnosis of X-linked recessive mutation could be achieved by blastomere biopsy and followed by DNA-specific sequence amplification.

In 1989, the first successful preimplantation diagnosis in humans was been undertake for X-linked diseases by Handyside and his colleagues (Handyside et al, 1989) and in 1990, the first PGD case was reported by blastomere biopsy on cleavage stage embryos and sexing by Y-specific DNA amplification for couples that risk for having children with X-linked disease. (Handyside et al, 1990). Since then , there have been babies born free from genetic disease by using preimplantation diagnosis of some X-linked condition, such as cystic fibrosis (Handyside et al.,1992), haemophlilia A (Grifo et al., 1992a) and Tay-Sachs disease (Gibbons et al., 1995). Theorytically, preimplantation diagnosis could be applied to any genetic disease in which the responsible genes are known. This would provide an alternative for couple wishing to prevent the birth of an affected child, but not willing to consider pregnancy termination.

Methods for PGD

Embryo biopsy

In the future, it may be possible to diagnose some inherited diseases in early human embryos by non-invasive procedure (Edwards&Hollands,1998), but currently PGD requires the removal of one or more cells from each embryo. Embryo biopsy is a two-step process involving the puncture and removal of a

cell or cells. Theoretically, this can be accomplished at any developmental stage between the mature oocyte and blastocyst, but three stages have been suggested : polar body, cleavage stage and blastocyst stages. Clearly, each of these stages is biologically different and therefore each biopsy requires a different technical approach and strategy giving varying prospects of success. The majority of centers use cleavage stage biopsy to obtain genetic material for PGD. However, polar body biopsy has only been applied by two groups in the USA (Verlinsky et al.,1990; Munn′e et al.,1998) and blastocyst biopsy has not yet been reported to be clinically applied to PGD.

A. Polar body biopsy

The removal of the polar body (a by-product of the first meiotic division) is an incident approach allowing the genetic status of the oocyte to be inferred from that of polar body. The first polar body is not require for successful fertilization or normal embryonic development. The second polar body, although a product of fertilization, is similarly not required for subsequent embryo development (Kaplan et al., 1995). Thus removal of either the first or second polar body or both for the purposes of genetic diagnosis should have no deleterious effect on the developing embryo.

Polar body biopsy has been used by two PGD groups to investigate chromosome abnormalities (Munn′e et al.,1995), translocation (Munn′e et al.,1998) and single gene defects (Strom et al.,1997; Kuliev et al.,1998) in a large number of cycles. However, the use of polar body biopsy for PGD is still not widespread because the procedure only allows the detection of maternal genetic defects and crossing over of homologous chromosomes leads to a reduction in the number of embryos available for transfer (Dreensen et al.,1995)

B. Cleavage stage biopsy

The human zygote undergoes one mitotic division every 24 hours before compacting to form a morular on day 4. Each cleavage division subdivides the cytoplasm of the zygote into successively smaller cell and there appears to be a lower limit of embryo mass compatible with implantation and development. Reduction of 50% or more of the cell mass frequently result in cell proliferation in the absence of the normal differentiation. However, cell reduction

within this limit is compatible with normal fetal growth. It is this premise that makes cleavage-stage biopsy for PGD a viable option.

Studies examining the effect of embryo biopsy have shown that at the eight-cell stage, removal of up to two cells is not detrimental to embryo metabolism or development (Hardy et al.,1990) and is an efficient process with more than 90% of the embryos surviving (Ao and Handyside, 1995) and a successful biopsy achieved in 97% of cases (ESHRE PGD Consortium,1990; 2000).

A variety of different method have been developed for the removal of blastomere from early cleavage stage embryos prior to the establishment of tight junctions at the late eight-cell stage. Many of the biopsy techniques currently in use for human embryo were pioneered in animal model such as the mouse (Monk et al.,1998 ; Witton et al.,1989) and marmoset (Summers et al., 1998). Of these methods, only a few are in clinical use today. So far the most common (ESHRE PGD consortium,1999; 2000) is zona drilling using acid tyrode's solution and aspiration of the blastomere.

One limitation of cleavage stage biopsy is the small number of cells available for genetic analysis. However, it is possible to increase the number of cells by allowing the biopsied blastomere to proliferate in culture before genetic analysis. (Geber et al., 1995)

C. Blastocyst biopsy

The human blastocyst consists of an outer layer of trophectoderm cells (TE) which goes on to make the placenta and an inner cell mass (ICM) from which the embryos proper is derived.

Blastocyst biopsy was first attempted in murine blastocyst in which a small slit was made in the zona pellucida and as the blastomere herniated through the slit, the cells were excised. Successful blastocyst biopsies have been performed in mice (Monk et al.,1988) and primates (Summers et al.,1988) with the delivery of healthy individual. In human, blastocyst biopsy can be performed on day 5 or day 6 post insemination (Dolras et al.,1990,1991 ; Muggleton-tharris et al 1995).

However, there are some limitations of this technique. For example, there are a lot of cell to cell interactions in the developing blastocyst,

the cells become very sticky, making their manipulation technically difficult so that there are a low number of embryos that reach the blastocyst stage in vitro, even with improved culture condition. There were reports of a high implantation rate from blastocyst transfer and lower multiple pregnancies (Gardner et al., 1998 ; Jone et al., 1998), but these reports selected groups of patient with a high follicular response. Since it took several hours to perform blastocyst biopsy, there would be a limited time to perform the diagnosis.

Genetic diagnostic technique

There are two basic diagnostic techniques currently used for PGD. The first involves amplification of known DNA sequence by the polymerase chain reaction (PCR) (Saiki et al., 1985; Saiki et al., 1988). The second involves detection of chromosome in an intact nucleus by Fluorescent in-situ hybridization (FISH).

a. Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an invitro method for enzymatically synthesizing defined sequence of DNA; the reaction uses two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified. The elongation of the primers is catalyzed by Taq polymerase, a heat-stable DNA polymerase that is isolated from the thermophilic eubacterium Thermus aquaticus. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealing primers by Taq DNA polymerase results in exponential accumulation of a specific DNA fragment. The ends of the fragment are defined by the 5’ ends of the primer (Soharf et al.,1986). Because the primer extension products synthesized in a given cycles can serve as a template in the next cycle, the number of target DNA copies approximately double every cycle : thus, 20 cycles of PCR yield about a million copies (2^{20}) of the target DNA. (Boehringer Muannheim,1995)

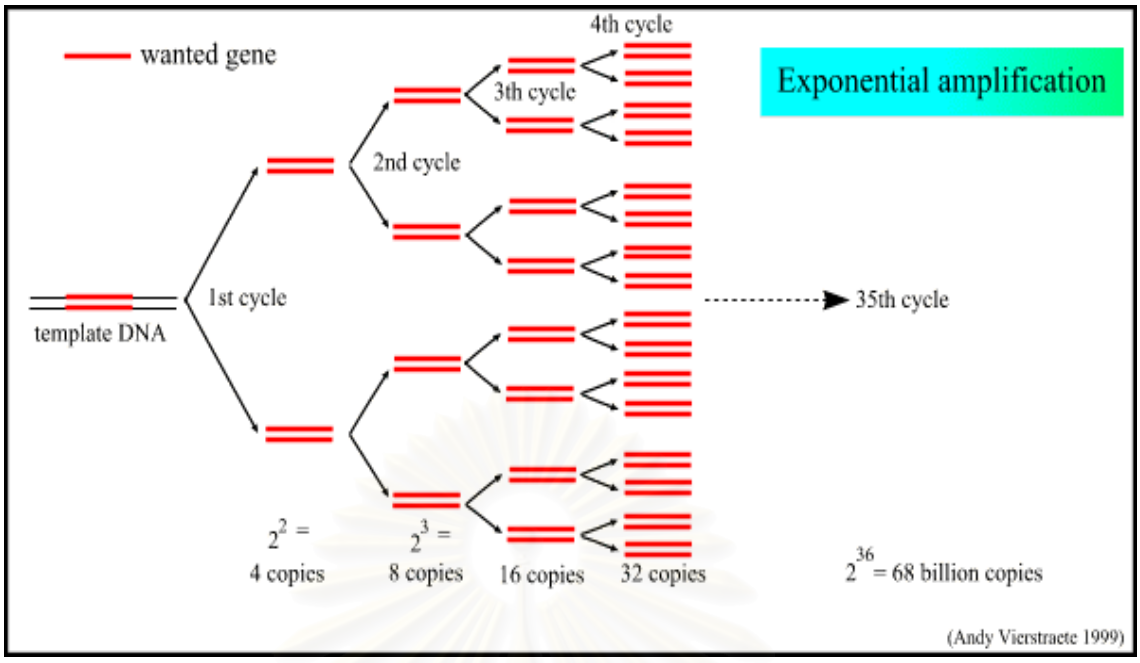

Figure 1.4 Schematic diagram of the Polymerase Chain Reaction
(Image from URL: http://allserv.rug.ac.be/~avierstr/principles/pcr.html)

One strategy which enhances the specificity of PCR, as well as reducing the risk of “carry over” contamination, is termed **Nested PCR.** This method which has been widely applied to mutation detection in single cell, uses two sequential amplification reaction. The first amplification use a pair of locus-specific primers to generate a DNA fragment encompassing the mutation site. The number of PCR cycles employed in this initial reaction is insufficient for visualization of the amplified fragment. PCR product from the first reaction are transferred to a new PCR tube and are further amplified, this time to detectable levels.

This is accomplished utilizing a different set of primers, situated within the first amplicon, such that a smaller DNA fragment is produced. This second amplicon, produced in extremely high number to enable visualization poses no threat of contamination to subsequent primary amplifications, as it cannot be amplified by the outer set of primer. Only product from the first round of PCR, a mush smaller number of which have been generated, are a contamination risk.

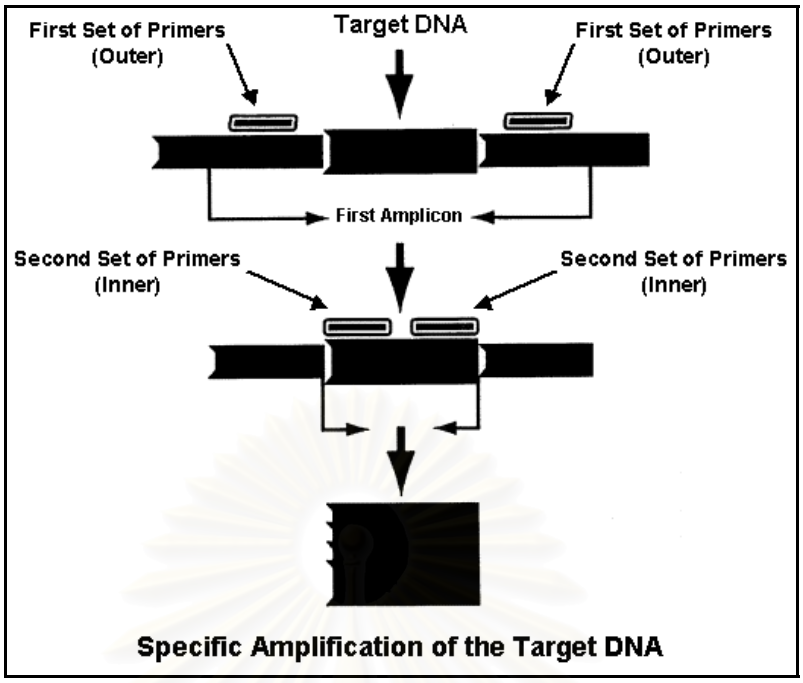

Figure 1.5 Schematic diagram of the Nested PCR
(Image from URL: http://www.ivpresearch.org/nested_pcr.htm)

b. Fluorescent In Situ Hybridization

Fluorescent In Situ Hybridization (FISH) is a relatively new technology utilizing fluorescence - labeled DNA probes to detect or confirm gene or chromosome abnormalities that are generally beyond the resolution of routine cytogenetics. The sample DNA (metaphase chromosomes or interphase nuclei) is first *denatured*, a process that separates the complimentary strands within the DNA double helix structure. The fluorescence labeled probe of interest is then added to the denatured sample mixture and hybridizes with the sample DNA at the target site as it *reanneals* (or reforms itself) back into a double helix. The probe signal can then be seen through a fluorescent microscope and the sample DNA scored for the presence or absence of the signal.

FISH has been used to examine chromosome in embryo because it is difficult to obtain a karyotype from those cell. Since 1991, FISH has been used to sex embryos from patient at risk of X-link disease (Griffin et al., 1994) and the same technique is used today, but with the addition of an autosomal probe. (Staessen et al.,1999).

Diagnostic technique for beta globin gene

Detection method of beta globin gene mutation from amplified DNA can divide into 2 major types :-

A) Unknown mutation

1. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis has been shown to detect differences in the melting behavior of small DNA fragments (200-700 bp) that differ by as little as a single base substitution. The small (200-700 bp) genomic restriction fragments are run on a low to high denaturant gradient acrylamide gel; initially the fragments move according to molecular weight, but as they progress into higher denaturing conditions, each (depending on its sequence composition) reaches a point where the DNA begins to melt. The partial melting severely retards the progress of the molecule in the gel, and a mobility shift is observed. It is the mobility shift which can differ for slightly different sequences (depending on the sequence, as little as a single base pair change can cause a mobility shift). Alleles are detected by differences in mobility. (Helms C., 1990)

2. Single Strand Conformation Polymorphism (SSCP)

The principle of this method is based on the fact that the electrophoretic mobility of nucleic acids in a non-denaturing gel is sensitive to both size and shape. Unlike double-stranded DNA, single-stranded DNA is flexible and will adopt a conformation determined by intra molecular interactions and base stacking that is uniquely dependent on sequence composition. This conformation can be affected when even a single base is changed. Conformational changes can be detected as alterations in the electrophoretic mobility of the single-stranded DNA in non-denaturing polyacrylamide gels. (Baylor college of medicine, 2003).

B) Known mutation

1. Gel eletrophoresis

Electrophoresis is one of the most convenient method to separate molecule which differ in any combination of size or charge. Using this technique in case of known mutation, that mutation is deletion or insertion more than 2 base pair (bp). Separate this mutant from normal control by polyacrylamine gel electrophoresis. An example is codon 41/42, 4 bp deletion (Sutcharitchan P, 1998)

2. Restriction Enzyme digestion

Cutting DNA by using restriction enzyme digestion is one of the most common molecular biology technique. This enzyme works by cutting the DNA at a specific nucleotide sequence, the recognition sequence of the enzyme. Any time this sequence appears in DNA it will be cleaved by the enzyme. For beta globin gene, this technique used for detecting mutant gene that mutate at the restriction site of restriction enzyme. This mutation makes the site of amplified DNA differ from normal DNA after digested with restriction enzyme. Diagnosis of this mutant gene by electrophoresis and then compare the size of DNA (Virtual lab book, n.d.)

3. Amplification Refractory Mutation System (ARMS)

Amplification Refractory Mutation System (ARMS) is also known as Allele Specific PCR (ASPCR) or PCR Amplification of Specific Alleles (PASA). This technique uses for detection of known single-base substitutions or micro deletion / insertions. The principle of this technique is two complementary reactions- one contains a primer specific for the normal allele and the other contains one for the mutant allele (both have a common 2^{nd} primer): one PCR primer perfectly matches one allelic variant of the target but is mismatched to the other. Genotyping is based on whether there is amplification in one or both reactions; band in normal reaction only indicates normal allele, band in mutant reaction only indicates mutant allele and bands in both reactions indicate a heterozygote (CMGS MRCPath Course, 1998).

4. Dot blot hybridization

The general procedure of dot blotting involves taking an aqueous target DNA and simply spotting on to a nitrocellulose or nylon membrane then allowing it to dry. The variant technique of slot-blotting involves pipetting the DNA through an individual slot in a suitable template. In both method the target DNA sequences are denatured either by previously exposing to heat, or by exposure of thefilter containing them to alkali. The denatured target DNA sequence now immobilized on the membrane are exposed to a solution containing single-stranded labeled probe sequence. After allowing sufficient time for probe-target heteroduplex formation, the probe solution is decanted, and the membrane is washed to remove excess probe that may have become nonspecifically bound to the filter. it is then dried and exposed to an autoradiographic film.

4.1 Allele Specific Oligonucleotide hybridization

Allele Specific Oligonucleotide hybridization (ASO) is an application of dot-blotting involves distinguishing between alleles that differ by even a single nucleotide substitution. To do this, ASO probe are constructed from sequence spanning the variant nucleotide site. ASO probe are typically 15-2o nucleotide long and are normally employed under hybridization conditions at which the DNA duplex between them: a single mismatch between probe and target sequence is sufficient to make the short heteroduplex unstable. Typically, this involves designing the oligonucleotide so that the single nucleotide difference between alleles occurs in a central segment of the oligonucleotide sequence, thereby maximizing the thermodynamic instability of a mismatch duplex (Strachan and Trad, 1999).

4.2 Reverse dot blot hybridization

Reverse dot blot (RDB) assays use unlabeled probe immobilized on a membrane. The membranes are hybridized with sample DNA, which is labeled during PCR amplification. The presence of DNA hybrid on the membrane is usually detected with an enzyme and color forming substrate. This is in contrast to a standard dot blot, in which the sample DNA is spotted on the membrane and then hybridized with labeled probe. A key feature of the

reverse dot blot technique is that a sample can be screened against any number of different probes in one assay:

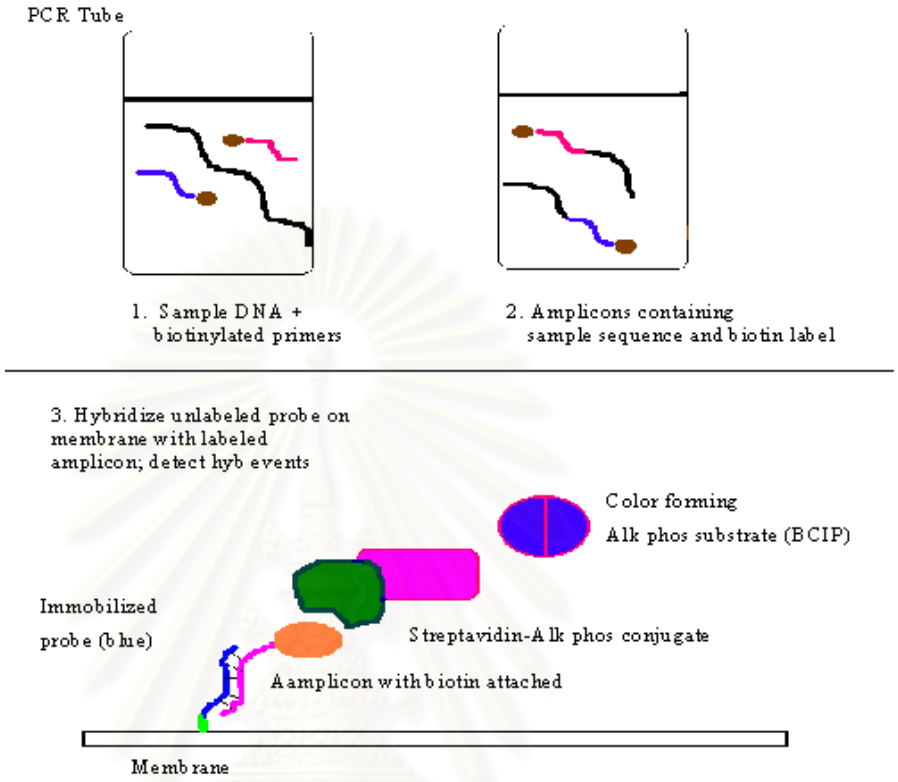

Figure 1.6 Reverse dot blot hybridization
(Image from URL: http://domino.pall.com)

In this study, I have used RDB hybridization which is a routine method using in prenatal diagnosis to detect beta globin gene mutation in human single blastomere. The problem of using this method is how to amplify DNA from single blastomere. To solve this problem, I have to optimize the PCR condition for beta globin gene amplification from single blastomere. Reverse dot blot hybridization technique would be further used to detect mutant gene from those of amplified single blastomere DNA.

Aim of the study

1. To optimize the PCR condition for amplification of beta globin from single blastomere and compare the lysis method and assess their respective effectiveness as to the developmental potential of amplified beta globin sequence in single blastomere

2. To diagnose of common beta thalassemia mutation in human single blastomere by Reverse dot blot hybridization.

Experimental design

This study consists of two sections according to its aim.

1. Section I

Optimization of PCR protocol which used for amplify beta globin gene from single blastomere using two-step (Nested) PCR in term of Annealing temperature, primer design and primer concentration for greater result of amplification efficiency and compared the PCR success rate from different lysis methods that it could ascertain which methods would be the most applicable to use for the diagnosis of beta globin mutation.

2. Section II

The amplified product from optimal PCR condition was assessed by Reverse dot blot hybridization

CHAPTER II

LITERATURE REVIEW

The first clinical application of preimplantation genetic diagnosis (PGD) using in vitro fertilization (IVF), cleavage stage embryo biopsy and single cell genetic analysis to identify unaffected female embryo in a series of couple at risk of x-linked disease, was report almost a decade ago (Handyside et al.,1990). Since then the number of centers offering PGD has increased slowly, but steadily mainly because of the difficulty of single cell analysis and the need to combine expertise in assisted reproduction and molecular genetics. In contrast, the range of genetic defects causing disease that can be detected at the single cell level has increased dramatically and now includes most common autosomal dominant and recessive single gene defects as well as structural chromosome abnormalities and aneuploidy screening using various polymerase chain reaction (PCR) or fluorescent in situ hybridization (FISH) method (Lissens and Sermon,1997).

Since 1989, diagnosis of beta globin gene mutation have been reported by Holding and Monk, they used mouse model for study. Mouse preimplantation embryos were accurately diagnosed as normal or mutant at the beta-major haemoglobin locus by amplification of specific DNA sequence in a single cell. A DNA sequence containing the whole of exon 3 and some 3′ untranslated sequence within the beta-major haemoglobin gene was amplified in single blastomere by mean of the PCR and nested PCR. Blastomeres were removed from embryo of 4-8 cells from normal BALB/c mice and from mutant (Thalassemic) BALB/c mice homologous for detection of the whole beta-major haemoglobin gene (Holding and Monk, 1989).

In 1990, Monk and Holding amplified beta-haemoglobin sequence in individual human oocytes and polar bodies. The amplified region includes some 5′ untranslated sequence, exon 1, intervening sequence 1, exon 2 and a small part of intervening sequence 2. Specificity and sensitivity of amplification were

achieved by two sequential reactions with 2 sets of primers, amplifying first a 725 base pair (bp) sequence and secondly a 680 bp sequence from with in the first amplified fragment. A restriction enzyme digestion of the DNA amplified from a single oocyte with the endonuclease *Dde* I conform the identity of the amplified beta-haemoglobin fragment. This technique provides a diagnostic test for the genetic defect responsible for sickle cell anemia. Analysis of the DNA from the first polar body may enable detection of such defects in unfertilized eggs from carrier woman. Selection of eggs without the detection for fertilization may therefore obviate the need for diagnostic procedure on embryo (Monk and Holding, 1990).

In 1991, Varewalla et al. used PCR to amplify a single copy fragment of the beta globin gene from 2-32 human embryonic cells obtain from arrested preimplantation embryo. Successful amplification was obtained in 14 out of the 15 embryonic sample analysed. Amplification Refractory Mutation System (ARMS) have used to detect beta thalassemia mutations [IVS-I nt1(G-T), IVS-I nt5(G-C), codon 8/9 (+G), codon 41/42 (-CTTT) and codon15 (G-A)] with account for 77% of the beta thalassemia mutations present in an Asian Indian population selected for study]. As it was not possible to obtain embryonic cell sample form couple carrying beta thalassemia mutations, diluted DNA solutions (approximately 10 pg of DNA) from various individual whose beta thalassemia mutations had already been characterized were used as experimental model (Varawalla et al., 1991).

In 1992, human preimplantation embryos at various stage of development have been analysed using PCR to amplify 680 bp fragment of beta globin gene. Successful amplification was achieved more frequently with DNA form intact embryos containing between 1-11 cells (100%), single cumulus cells (83%), unfertilized oocyte (71-100%) and polar bodies (100%) than from single blastomere (45%). The distribution of nuclei demonstrated of nuclear chromophore diamino-phenyl–indole showed considerable inter-blastomere variation; however, no clear correlation between staining pattern and successful amplification was observed (Peckering et al., 1992).

In 1996, Ray et al. examine the feasibility of PGD in an Indian couple each carrying a point mutation common in that population at different nucleotide position IVS-I nt1(G-T) and IVS-I nt5(G-C), they analysed single lymphocytes from their affected compound heterozygous son , from a normal individual, and single cells from cleavage stage human embryos. Nested PCR was used to amplify 208 bp fragment encompassing both mutation site (from –29 to IVS-I nt31). The mutations in the amplified fragment were detected by restriction enzyme digestion (a *Bsl* I/*Bsi* Y1 and *Kpn* I detect IVS-I nt1 (G-T) and IVS-I nt5 (G-C) respectively). The single lymphocyte and single blastomere were amplified 88% and 83% success (Ray et al., 1996).

In 1997, El-Hashemile et al. detected of selected beta thalassemia mutations in intron I [nt1 (G-A), nt1 (G-T), nt5 (G-C), nt6 (T-C) and nt110 (G-A)] at the single level by the amplification of nested PCR and Single Strand Conformation Polymorphism (SSCP) analysis. A total of 294 single somatic cells of different type (buccal cell, lymphocytes, fibroblasts) was cell amplified with 96% success and all tested mutations in homologous and heterozygous from were identified correctly. To assess the efficiency of nested PCR on single blastomere prior to clinical application, 10 single blastomeres were amplified and gave the expected normal pattern when analysed by SSCP (El-Hashemile et al., 1996).

In 1998, Kuliev et al. performed PGD for thalassemia in clinical cycle (IVS-I nt110 and IVS-II nt745 mutations) using biopsy of the first and second polar bodies (PBs) extruded from oocytes during maturation and fertilization, couple with nested PCR analysis and restriction digestion. A total of 118 oocytes were obtained, of which 78 has results for both the first and the second PBs. This resulted in the selection and transfer of 30 unaffected embryos. The application of PB analysis in six patients resulted in 2 ongoing pregnancies with a thalassemia free fetus already confirmed in both of by prenatal diagnosis (Kuliev et al., 1998).

In 1999, Vrettou et al. reported a strategy which includes a first round of PCR allowing subsequent nested PCR and Denaturing Gradient Gel Electrophoresis (DGGE) analysis for genotyping the wide spectrum of beta thalassemia mutations in Greek population in single cell sample. Optimization, accuracy and reliability of the method were investigated by genotyping single blastomere, amnioctyes and lymphocytes. Results confirmed that PCR efficiency and occurrence of allelic drop out (ADO) are improved by higher denaturation temperature in the first cycle of the first round PCR, influenced by the size of the fragment amplified in the first round of PCR and additionally by the quality and type of cells being genotyped (Vrettou et al., 1999).

Another report described the application of the DGGE-base diagnostic strategy in 11 clinical IVF/PGD cycle, in 10 couples at risk for transmitting beta thalassemia major, the transfer of at least 1 embryo diagnosed as unaffected for beta thalassemia majoring 9 couples has resulted in the initiation of 6 pregnancies. Four pregnancies have so far been confirmed as unaffected for beta thalassemia major by first or second –trimester prenatal diagnosis, 2 of which have resulted in the birth of 2 healthy babies. Three singleton pregnancies are still on-going and one ectopic pregnancy was terminated (Kanavaskis et al., 1999).

In 2001, the study two PGD protocol for deleting beta thalassemia mutation (codon 41/42 and IVS-I nt110) and one for alpha thalassemia (SEA mutation) have been designed and tested. These methods contain fail-safe mechanisms to reduce the risk of misdiagnosis due to ADO or contamination and utilize multiplex fluorescent PCR (F-PCR). Interestingly, amplification efficiency and ADO were significantly affected by the choice of DNA polymerase and the freshness of the single cells used. The close similarity between the DNA sequence of beta globin and delta globin was also found to be an important issue that necessitated careful design of primers for beta globin gene (Piyamongkol et al., 2001).

In 2002, Palmer et al. present the results from 15 PGD cycle (15 couples) in which at risk of transmitting beta thalassemia major mutations [IVS-I nt110 (G-A), codon 39 (C-T), IVS-I nt6 (T-C), Frameshift mutation codon 6 (-A),

IVS-II nt745 (C-G), IVS-I nt5 (G-A) and Sicilian delta-beta thalassemia deletion. They evaluate a strategy involving embryo biopsy on day 3 post insemination (p.i) genetic analysis by PCR following the nested PCR and analysed by DGGE on day 4, following culture in blastomere sequential media, transfer of unaffected embryos on day 5 p.i. Eight pregnancies resulted, providing support for the view that, this strategy has the advantage of extending the time allowed for embryo biopsy and genetic analysis for clinical PGD cycle without compromising pregnancy outcome (Palmer et al., 2002).

The first successful application of PGD for beta thalassemia and sickle cell anemia reported by Chamayou et al. PDG performed on seven Sicilian couples and carrier of beta globin gene mutations (codon 39,HbS, IVS-I nt1, IVS-I nt6 and IVS-I nt110). The mutation diagnosis was performed by restriction enzyme digestion and reverse dot blot. The amplification efficiency was 97.2%. four pregnancies obtained, three resulted in live births and miscarried at 11 weeks. Prenatal diagnosis at the 11^{th} week and miscarriage material analysis confirmed the PDG results (Chamayou et al., 2002).

Beta thalassemia is a heterogeneous disorder due to mutations that reduce or abolish synthesis of the beta globin chains. More than 180 different mutations are known to produce the beta thalassemia phenotype, most of which are single-base change in an around the beta globin gene (Weatherall and Proven.,2000). However, mutations found in each population usually consist of a few common mutations and variable number of rare mutation. In Thailand there are about 25 beta thalassemia mutations but only 4 mutations [-28(A+G), codon 17(A-T), codon 41/42 (-CTTT) and IVS-II nt654 (C-T)] account for about 80% of the cases (Winichagoon et al., 1990).

Practically, detection for the high risk couples of beta thalassemia can be carried out by conventional haemoglobin electrophoresis or chromatographic technique. However, detection for beta thalassemia mutations is required when the couple plans for or is having a baby. Many techniques based on PCR have been developed for the detection. In allele specific oligonucleotide (ASO) hybridization technique the normal and mutant alleles can be distinguished from

one another by filter hybridization with enzymatically-labelled allele specific oligonucleotide probes (Saiki et al.,1988; Cai et al., 1989; Randall et al., 1988).

Reverse dot blot hybridization have been developed from ASO, this method offers a means of screening for several mutations with a single hybridization reaction. The probe are filter bound and the non-radioactively labeled PCR product is hybridized to the immobilized ASOs. The strength of this method lies in its ease, reliability and mostly in rapid identification, usually within a single working day, of the mutation underlying beta thalassemia. Therefore, it is the method of choice for carrier screening (Saiki et al.,1989; Sutcharitchan et al., 1995; Chan et al., 1999) and prenatal diagnosis of beta thalassemia in both clinical and research laboratory setting (Maggio et al.,1993; Winichagoon et al., 1999). Recently, there is a report represent the first successful application of PGD for beta thalassemia using reverse dot blot method (Chamayou et al., 2002).

Regardless of the strategy employed, the major problems with single cell PCR remain the omnipresent threat of contamination and ADO, a dphenomenon whereby only one of two alleles present in a single heterozygous cell is amplified successfully. Previous investigations of affected of ADO have considered the effects of denaturation temperature in the primary PCR (Ray and Handyside., 1996b), the use or not of a lysis buffer (Sermon et al., 1995; Gitlin et al., 1996) and the choice of that buffer (EI-Hashemite and Delhanty., 1997; Thornhill et al., 2001).

In this study, furthermore optimize PCR condition for beta globin gene amplification of single blastomere , also compare 5 lysis method in an effort to maximize PCR amplification of beta globin target sequence efficiency. Genetic analysis would be then followed by Reverse dot blot hybridization.

CHAPTER III

MATERIALS AND METHODS

A. Materials

1. instrument for separate cell (Embryo biopsy, zona pelluzida digestion)

1.	CO_2 Incubator	Forma Scientific
2.	Inverted microscope With hoffman lens system	Nikon, Japan
3.	Laminar flow hood	Lab service,Thailand
4.	Micromanipulator	Narishige,Japan
5.	Pasteur pipette	Corning, New York
6.	Holding pipette	COOK®, Australia
7.	Aspiration pipette	COOK®, Australia
8.	Zona drilling pipette	COOK®, Australia
9.	Sterilized plastic petridish	Falcon,France

2. DNA preparation and analysis

1.	Thermal cycle	Perkin-Elmer,USA
2.	Gel electrophoresis apparatus	Hoefer, USA
3.	Electrophoresis power supply	Hoefer, USA
4.	High speed centrifuge	Denver instrument
5.	UV Transluminator	Ultralum, Ultra-lum Inc., USA
6.	Variable micropipette	Eppendorf, Germany
7.	Vortex mixer	Fisher Scientific Industries, USA
8.	Waterbath	Stuart scientific, Great Britain
9.	PCR tube	Eppendorf, Germany
10.	Pipette tip	Eppendorf, Germany
11.	Thermometer	
12.	Disposable glove, parafilm	

3. Reverse dot blot hybridization

1.	Hybridization Oven	Hybaid, UK
2.	Spotting manifold	The Convertible™ GibcoBRLGaitherburg,MD.
3.	UV Crosslinker	Hoefer Scientific Instrument, San Francisco
4.	Nylon membrane	Biodyne®-B; Glen Cove, N.Y.)
5.	Aluminium foil	Reynolds®, USA
6.	Cylinder	Pyrex®, USA
7.	Timer	Canon, China
8.	Microcentrifuge tube	Eppendorf, Germany

B. Chemicals and reagents

1.	Amplitaq DNA polymerase	Perkin-elmer,Connecticus
2.	Agarose	Sigma, USA
3.	Bovine serum albumin, Fraction V	Sigma, USA
4.	Bromophenol blue	Sigma, USA
5.	Calcium chloride dihydrate	Sigma, USA
6.	Citric acid	Sigma, USA
7.	dNTPs	Promega, Wisconsin
8.	dTTP	Promega, Wisconsin
9.	Dithiothreitol	Sigma, USA
10.	Ethidium bromide	Sigma, USA
11.	Ethylene diaminetetraacetic acid disodium salt dehydrate (EDTA)	Sigma, USA
12.	Glucose	Sigma, USA
13.	Glycerol	Sigma, USA
14.	Horseradish peroxidase streptavidin	Burlingame, CA
15.	Hydrochloric acid	Sigma, USA
16.	Hydrogen peroxide	Sigma, USA
17.	Lactic acid	Sigma, USA

18. 25mM $MgCl_2$ — Perkin-elmer,Connecticus
19. Magnesium chloride hexahydrate — Sigma, USA
20. Oligonucleotide Primer — Bio-Synthesis
21. Oligonucleotide Probe — Bio-Synthesis
22. 10X PCR buffer — Perkin-elmer,Connecticus
23. Paraffin oil — GRP™,BDH, England
24. PhiX 174DNA / *Hae* III Markers — Promega, Wisconsin
25. Potassium chloride — Sigma, USA
26. Polyvinylpyrolidone: PVP — Sigma, USA
27. Potassium phosphate monobasic — Sigma, USA
28. Proteinase K — Gibco BRL Life Technologies
29. Protease — Sigma, USA
30. Pyruvic acid — Sigma, USA
31. Sodium chloride — Sigma, USA
32. Sodium dodecyl sulfate — Sigma, USA
33. Sodium phosphate dibasic anhydrous — Sigma, USA
34. Sodium hydroxide — Sigma, USA
35. Sucrose — Sigma, USA
36. Terminal deoxynucleotidyl transferase — Promega, Wisconsin
37. Tetrametylbenzidine (TMT) — Boehringer Mannheim

C. Sample

- **Single blastomere**

Single blastomere samples were separated from human embryos donated by couple undergoing IVF treatment for infertility at the Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University. We only used IVF embryos that were not required for transferration or cryopreservation, after informed and signed the consent from the couples donating the embryos, approved by the ethical committees of Faculty of Medicine, Chulalongkorn University.

- **Genomic DNA (Whole blood)**

The blood samples used in this study were obtained from blood stock of patient whose beta thalassemia mutation had already been characterized from beta thalassemia screening program at Hematology unit, Department of Medicine, Faculty of Medicine, Chulalongkorn University.

D. Methods

❖ ***DNA extraction from Whole blood***

1) Pipette 500 ml of blood into labeled centrifuge tube
2) Add 1 ml TE buffer (pH 8.0)
3) Centrifuge 12,000 rpm for 2 minutes (min)
4) Do step 2) and 3) for several times until there is no red blood cell in the tube
5) Decant the supernatant
6) Add 90 μl of sterilized deionized water (SDW)
7) Add 10 μl of 10Xduplex buffer (see Appendix C)
8) Add 2 μl of10 mg/ml proteinase K
9) Centrifuge 12,000 rpm for 2 min
10) Incubate in waterbath at 65°C for 45 min, following 100°C for 15 min.
11) Measurement of DNA concentration and store at 4°C until use.

❖ ***Collection of blastomere***

a) <u>Embryo bipsy</u>

1. Place 6-8 cell stage embryos individually in 10 μl drops of biopsy medium under sterilized paraffin oil for at least 30 min in CO_2 Incubator.
2. Attach the holding and zona drilling pipette to the left and the right micromanipulator respectively. The micro tools are placed to lie parallel at the bottom of the dish.
3. Load the acid tyrode's solution into the zona drilling pipette.
4. Fix embryo by suction with a holding pipette.
5. Create a hole at zona pelluzida by gentle blowing acid tyrode's solution using a zona drilling pipette.

6. Replace zona drilling pipette with the aspiration pipette.
7. Aspirate single blastomere by gentle suction through the hole made in the zona.
8. Pick up the biopsied cell/s using a siliconined drown out pipette and wash 3 times in biopy medium, following placed in the PCR tube contaning SDW or lysis buffer (1 single blastomere/tube), then keep at -20°C until use.

b) Zona pelluzida removal (adapted from Avner et al.,1994)

1. Incubate arrested embryo for 30 min in biopsy medium
2. Remove the zona pelluzida (ZP) of embryo by incubate for 2 min. in 10 IU pronase (follow the disappearance of ZP under the stereomicroscope).
3. Wash each single blastomere 3 times in biopsy medium.
4. Place a single blastomere in PCR tube containing SDW or lysis buffer, then keep at -20°C until use.

Note: For each collection of blastomere, an aliquot of the last washing droplet was added to separate PCR tube to serve as a blank (negative control).

❖ **Lysis of the blastomere**

Each individual blastomere was transferred to PCR tube containing 3 μl of TE buffer and was kept at -20°C until future processing.

❖ Two-step (nested) PCR method

Protocol I :
Protocol II : } Reaction mixture and PCR cycling program described in appendix A
Protocol III :

❖ **Agarose gel electrophoresis**

1. Weigh 0.6 gram. Of agarose and heat to dissolve in 30 ml 1X TAE buffer to make 2% agarose gel.
2. Pour melted agarose into a gel mould after it cool down.
3. Insert a comb to make wells.
4. Remove a comb gently after agarose wassolidified.
5. Pour 1XTAE buffer covering the gel to depth about 1-2 cm.
6. Mix 0.7 μl PCR product with 3 μl loading dyed and slowly apply into the well. (Use PhiX 174 DNA / *Hae* III Markers serve as a DNA maker).
7. Run the gel at 100 volts until bromophenol blue migrate to the other edge of the gel.
8. Stained the gel in 1XTAE buffer + 0.25 μl/ml ethidium bromide for 20 min (Shaking)
9. Destain the gel with deionized H_2O for 10-15 min to leach out unbound ethidium bromide.
10. Place the gel on a transluminator and visualize DNA band under ultraviolet light.
11. Take a photograph of the gel.

Amplification of beta globin gene

Determine the minimum genomic DNA concentration

Determine the minimum genomic DNA concentration that can amplify the beta globin sequence, using PCR protocol I (which is well modified and be accepted by the Haematology laboratory, Department of Medicine, Faculty of Medicine, Chulalongkorn University). PCR success was indicated by the presence of a correctly sized PCR product on agarose gel electrophoresis.

Method :

1. Dilute genomic DNA from 100 ng/μl to 100,50,25,12.5 and 6.25 pg/μl .
2. Perform two-step (nested) PCR according to PCR protocol I
3. Analyse PCR product by gel electrophoresis.

Single blastomere PCR testing

For DNA analysis, a multiplex two-step (nested) PCR system was used.

Method :

1. Heat sample (collected in TE buffer 3 μl) at 95 ^{o}C for 10 min.
2. Perform two-step (nested) PCR of single blastomere using PCR protocol I
3. Analyse PCR product by gel electrophoresis.

Note: Positive and negative controls (including 1 μl of 100 ng/μl Genomic DNA and 10 μl of last wash drop of washing medium, respectively were also subjected to the multiplex two-step PCR procedure together with the embryo samples.

PCR optimization of annealing temperature

For most purpose annealing temperature (T_A) has to be optimized empirically. PCR reactions for the optimization of T_A investigated five temperature level; 54°C, 56°C, 58°C, 60°C and 62°C.

Method :

1. Heat sample (lysed-single blastomere in TE buffer at 95 ^{o}C for 10 min.
2. Perform two-step (nested) PCR using PCR protocol I. But in the nested PCR step, thermal cycling profile at annealing temperature were optimized. (54°C,56°C, 58°C, 60°C and 62°C.)
3. Analyse PCR product by gel electrophoresis.

Note: Positive and negative controls were 100 ng/μl genomic DNA and SDW, respectively

The effect of Primer

In the most PCR applications, it is the sequence and the concentration of the primer that determine the overall assay success, this part was separated in to 2 sections following this:-

Section A : Changing location of nested primer.

According to Winicahgoon et al.(1999) claimed the successful of amplification beta glogin sequence containing the most Thai common mutations same as this study. In contrast they used different primer set to perform PCR. So that is primer set, primer set D&E (Table A.1, Appendix A) were choosen to use as a nested primer in this study.

Method :

1. Heat sample (collected in TE buffer 3 μl) at 95 ^{o}C for 10 min.
2. Perform two-step (nested) PCR of single blastomere using PCR protocol II
3. Analyse PCR product by gel electrophoresis.

Note: Positive and negative controls as same as the method of single blastomere PCR testing.

Section B: Primer concentration modification

The choice of primer concentration has a significant influence on PCR. A high primer concentration increases the probability of spurious priming and leads to the generation of nonspecific product. At the same time, it enhances the generation of primer-dimer. A substantial surplus of primer can therefore even result in a reduction of the amplification yield.

Method :

1. Perform two-step (nested) PCR of various dilutions of DNA (100, 10, 1ng/μl,100, 50,10pg/μl) using PCR protocol III (see appendix A) which vary primer concentration from 0.1μM to 1.0 μM
2. Analyse PCR product by gel electrophoresis.

Note: Primer for first round PCR : primer set G and polymorphism primer (Set F) (see Appendix A) and primer set B&C used for nested PCR

Effects of lysis cell method to the amplification efficiency

In single cell PCR, the requirement for high quality template DNA is important as it is for any PCR. The lysis method is involved in the quality of DNA template, so that amplification efficiency of single cell PCR using 5 different lysis method was compare.

Method :

1. Lysis single blastomere according to Table 3.1
2. Perform two-step (nested) PCR using PCR protocol III (primer concentration was 0.1 μM) . The positive and negative controls were 10 pg/μl genomic DNA and 10 μl of last wash drop respectively.
3. Analyse PCR product by gel electrophoresis.

Table 3.1 Lysis method

Lysis solution	Lysis condition
1) SDW 5 μl	94°C 15 min
2) SDW 5 μl	94°C 30 min
3) Alkaline lysis buffer 5 μl	94°C 30 min
4) Alkaline lysis buffer 5 μl	65°C 10 min
5) TE buffer 3 μl	95°C 10 min

Note ; Samples which lysis in alkaline lysis buffer have to add 5 μl of neutralization buffer before doing PCR (Cui et al., 1989).

Detection of beta globin gene mutation

Reverse dot blot hybridization was used for detection of beta globin gene mutation of successfully amplify a region of the beta globin which is known to be the site of many of beta thalassemia mutation from 10 pg of DNA which individuals known to carry these mutation and from single embryonic cell. This method consists of 3 major steps as follow :-

Step1 : Probe preparation by Poly-T tailing of oligonucleotide probe

1. Pipette 20 μl of 10 pmol oligonucleotide probe into microcentrifuge tube.
2. Add 80 μl of reactions mixture containing 100 mM Potassium cacodylate, 25mM Tris-HCl, 1mM $CoCl_2$, 0.2 mM dithiothreitol, pH 7.6 with 160 nmol dTTP, and 60 Units of TdT.
3. Incubate the reaction at 37^{o}C overnight (in water bath)
4. Add 100 μl 10 mM EDTA to stop reaction
5. Keep at 4 ^{o}C until use

Step2 : Membrane preparation and fixation of ASO probe

Since only 10 beta thalassemia mutations are commonly found in Thai population (see Table 2,Appendix A), the membrane strips were accordingly prepared to sets of ASO probe;

Membrane A: containing probe for the 10 most common Thai beta thalassemia mutations, the beta thalassemia haemoglobinopathies Hb Malaya and HbE. (Figure A.2, Appendix A)

Membrane B: containing probe for 5 less common beta thalassemia mutations, the beta thalassemia haemoglobinopathies Hb khon kaen and HbE. (Figure A.2, Appendix A)

Note : On each filter strip the normal probe (N) are at the top and the mutant probe are at the bottom. Mutants with close proximity may use the same oligonucletiode as the normal probe.

Protocol as following :-

1. Cut Nylon membrane in 11cm x 7.5 cm. (for 4 strips).
2. Soak membrane in TE buffer (pH 8.0) for 1 min.
3. Place membrane on spotting manifold.
4. Dilute tailed oligonucleotide probe (probe for membrane A&B) into 100 μl TE to optimum concentration [The quantities of probe pairs (mutant and normal alleles) range between 1.0-8.0 pmol; tested and adjusted by trial and error by hybridizing with control DNA sample]
5. Apply oligonucleotide probe 50μl /dot on a nylon membrane in each well and suction to fix probe on membrane.
6. Add 250 μl TE pH 8.0 (0.5M EDTA+ 1M Tris-HCl) in each well and suction.
7. Place the dotted membrane into UV Crosslinker and irradiated at 245 nm, 600 mJ/cm^2.
8. Soak the dotted membrane in 5XSSPE + 0.5%SDS (warm at 64^oC before use) with shaking for 30 min.
9. Wash with dH_2O 2 times.
10. Place dotted membrane on filter paper for drying.
11. Store at room temperature until use.

Step3 : Hybridization with PCR product and detection

** All buffer should be pre-warmed before use**

1. Label number of DNA on Hybridization tube (HD tube).
2. Cut dotted membrane and label DNA's number.
3. Put dotted membrane (membrane A & B) into HD tube.
4. Pipette 20 μl Denature solution(10M NaOH+0.5 M EDTA) into each 0.5 ml microcentrifuge tube.
5. Add 20 μl DNA sample (the nested PCR product) into microcentrifuge tube containing denature solution and mix
6. Pipette 3 ml Hybridization buffer (4XSSPE+0.5%SDS, warm at 57^oC before use) into HD tube.
7. Pipette all of denatured DNA (from 5.) into HD tube.
8. Incubate HD tube in Hybridization oven 57^oC 30 min with rotating.

9. Wash hybridized membrane in tray containing washing buffer (1XSSPE+0.2%SDS) 1 times.
10. Soak hybridized membrane in washing buffer for 10 min in HD oven to remove excess DNA.
11. Wash hybridized membrane with washing buffer 1 times
12. Transfer hybridized membrane to a tray containing 1XSSPE and Steptavidin (3ml 1XSSPE, 0.3 μl Streptavidin /sample) for conjugation DNA 10 min and shake
13. Wash hybridized membrane with washing buffer 3 times.
14. Wash hybridized membrane with 100mM Sodium citrate 3 times.
15. Mix 100mM Sodium citrate, 0.1mg/ml TMB and 0.015 % H_2O_2 together in flash covered with aluminium foil to prevent the light (in dark room)
16. Incubate hybridized membrane with solution from 16. (in dark room) with shaking for 30 min.
17. Color on hybridized membrane will occur, and able to be visualized with naked eyes.
18. Take a photograph and store membrane in dH_2O at room temperature.

❖ **Statistical analysis**

The results of comparison of lysis methods were analysed by a χ^2 two-by-two table test. Significance was considered as $P< 0.05$.

CHAPTER IV

RESULTS

Amplification of beta globin gene

Determine the minimum genomic DNA concentration

The minimum genomic DNA concentration was determined, a multiplex two step PCR system, protocol I was used.

Initially primer set A (table A.1, Appendix A) was designed for the amplification of 2062 bp fragment, subsequently two sets of primers (set B and set C) was designed to amplify two separate smaller products, 774 and 378 bp product (which together encompassed all the known Thai beta thalassemia mutations) of the first round of PCR.

Ten experiments were performed to amplify 774 bp and 378 bp fragment of the beta globin gene from various dilutions of genomic DNA (100, 50, 25,12.5,6.25 pg/μl). The result of these experiments were listed in Table 4.1.

Figure 4.1 shown an ethidium bromide-stained agarose gel of amplified fragment of dilution genemic DNA.

The dilution DNA at 100, 50, 25,12.5 pg/μl was successful amplify (show both 774 and 378 bp products) in all experiment, but dilutions at 6.25 pg/μl could not amplify the target product except in experiment 5, only 378 bp product (poor band) was amplified. So that , The minimum genomic DNA concentration was able to amplify beta globin sequence using PCR protocol I was 12.5 pg/μl.

Table 4.1 Amplification of beta globin gene of various dilutions of genomic DNA

Experiment No.	Diluted genomic DNA (pg/μl)					Negative control (SDW)
	100	50	25	12.5	6.25	
1	1	1	1	1	0	0
2	1	1	1	1	0	0
3	1	1	1	1	0	0
4	1	1	1	1	0	0
5	1	1	1	1	0*	0
6	1	1	1	1	0	0
7	1	1	1	1	0	0
8	1	1	1	1	0	0
9	1	1	1	1	0	0
10	1	1	1	1	0	0
Total	10	10	10	10	0	0

1 Denotes the presence of amplification and 0 denotes its absence.

***** see 378 bp product

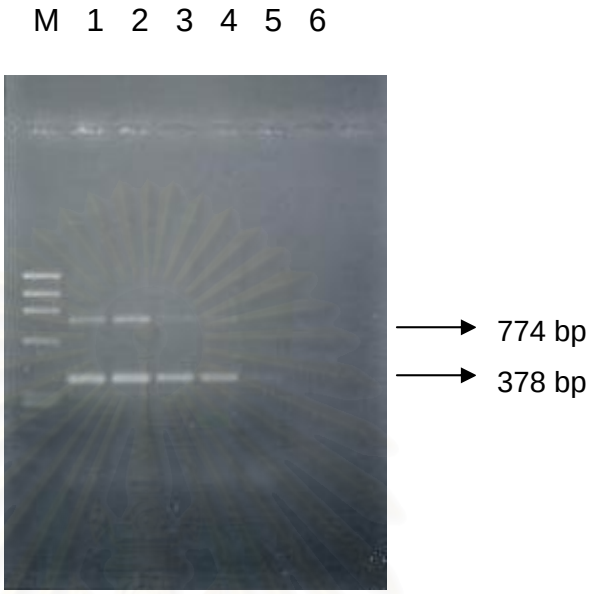

Figure 4.1 Ethidium bromide stainning of dilution genomic DNA (100-6.25 pg/μl) of experiment 5. The DNA were run on 2% agarose gel at 100 volts for hour.

Lane M : DNA Marker (phi X 174 DNA / *Hae* III)
Lane 1 : 100 pg/μl Genomic DNA
Lane 2 : 50 pg/μl Genomic DNA
Lane 3 : 25 pg/μl Genomic DNA
Lane 4 : 12.5 pg/μl Genomic DNA
Lane 5 : 6.25 pg/μl Genomic DNA
Lane 6 : Negative Control

Single blastomere PCR testing

To determine whether the multiplex two-step PCR method using PCR protocol I could detect the target sequence from single human blastomere, successful amplification was indicated by the presence of a correctly sized PCR product on agarose gel electrophoresis.

Twenty experiments were performed to amplify 774 bp and 378 bp fragment of the beta globin gene from single blastomere. The result of these experiments were listed in Table 4.2, genotyping of 106 single blastomere samples using first round primer set A and nested primers with primer set B&C resulted in 26.4% (28/106).

Figure 4.2 revealed an ethedium bromide-stained agarose gel showing the 774 bp and 378 bp of beta globin amplified fragment obtained from the embryonic cell sample. A band was observed in the lane with the embryonic cell sample, identical to that seen in the lane with the positive control, confirm that the PCR product is a fragment of the beta globin gene. The absence of any band in the negative control (last wash drop) lane indicated the absence of contamination.

Table 4.2 Amplification of beta globin gene by PCR from single blastomere

Experiment No.	Embryonic cell sample		Negative control (last wash drop)		Positive Control (100ng/μl Genomic DNA)	
	+	-	+	-	+	-
1	1	4	0	1	1	0
2	0	4	0	1	1	0
3	2	3	0	1	1	0
4	1	4	0	1	1	0
5	2	5	0	1	1	0
6	2	3	0	1	1	0
7	1	4	0	1	1	0
8	0	5	0	1	1	0
9	1	3	0	1	1	0
10	2	4	0	1	1	0
11	1	4	0	1	1	0
12	2	5	0	1	1	0
13	1	4	0	1	1	0
14	1	4	0	1	1	0
15	3	4	0	1	1	0
16	2	4	0	1	1	0
17	1	4	0	1	1	0
18	2	4	0	1	1	0
19	2	3	0	1	1	0
20	1	4	0	1	1	0
Total	28	78	0	20	20	0

+ Denotes the presence of amplification and - denotes its absence.

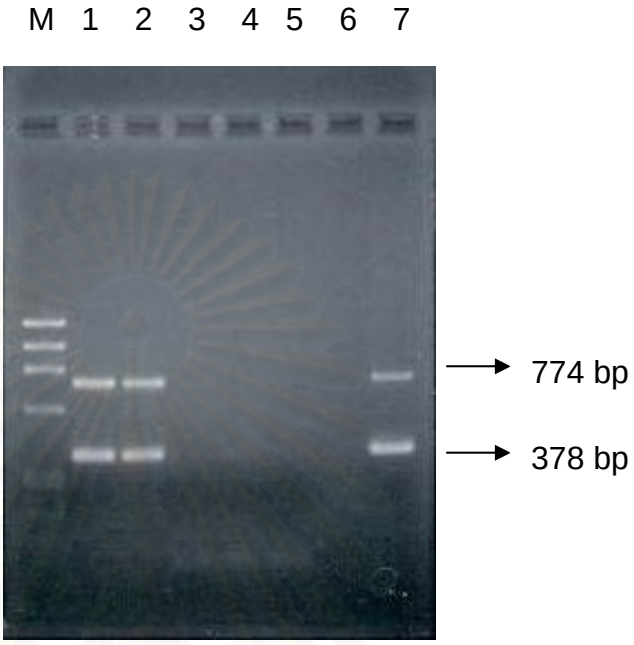

Figure 4.2 Amplification of the beta globin from single blastomere of experiment 6. Ethedium bromide-stained agarose gel showing the presence of 774 bp and 378 bp product.

Lane M : DNA marker (phi X 174 DNA / *Hae* III)
Lane 1 : Single blastomere no.1
Lane 2 : Single blastomere no.2
Lane 3 : Single blastomere no.3
Lane 4 : Single blastomere no.4
Lane 5 : Single blastomere no.5
Lane 6 : Negative Control (last wash drop)
Lane 7 : Positive Control (100 pg/μl genomic DNA)

PCR optimization of annealing temperature

To optimization of annealing temperature (T_A) of nested PCR, PCR amplifications of beta globin gene in single blastomere (25 single blastomeres) were performed the annealing temperature at 54°C, 56°C, 58°C, 60°C and 62°C, under PCR protocol I.

The result in Figure 4.3 revealed intense band of 774 and 378 bp product (Lane 5) when performed T_A at 58°C, while perform other T_A, show no amplification of target sequence of single blastomere (Figure not shown).

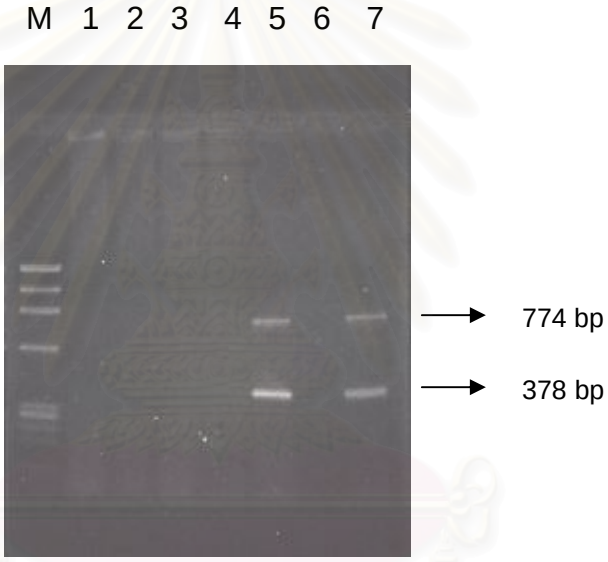

Figure 4.3 Amplification of the beta globin gene of single blastomere performed nested PCR using 58°C as an annealing temperature.

Lane M : DNA marker (phi X 174 DNA / *Hae* III)
Lane 1 : Single blastomere no.1
Lane 2 : Single blastomere no.2
Lane 3 : Single blastomere no.3
Lane 4 : Single blastomere no.4
Lane 5 : Single blastomere no.5
Lane 6 : Negative Control (SDW)
Lane 7 : Positive Control (100 pg/μl genomic DNA)

The effect of Primer

Section A: Changing location of nested primer.

Ten experiments were performed to amplify 602 bp and 423 bp fragment of beta globin gene form single blastomere

The amplification efficiency of beta globin gene of single blastomere using first round primer set A and Nested PCR with primer set D&E and performing two-step PCR protocol II is 22.0% (11/50). As shown in Table 4.3. The location of nested primer with respect to the beta globin sequence is shown in Figure 4.4.

Table 4.3 Amplification of beta globin gene of single blastomere using nested primer set D&E

Experiment No.	Embryonic cell sample		Negative control (last wash drop)		Positive Control (100ng/μl Genomic DNA)	
	+	-	+	-	+	-
1	1	5	0	1	1	0
2	0	5	0	1	1	0
3	1	4	0	1	1	0
4	2	3	0	1	1	0
5	1	4	0	1	1	0
6	0	5	0	1	1	0
7	2	4	0	1	1	0
8	2	4	0	1	1	0
9	1	4	0	1	1	0
10	1	5	0	1	1	0
Total	11	39	0	10	10	0

+ Denotes the presence of amplification and - denotes its absence.

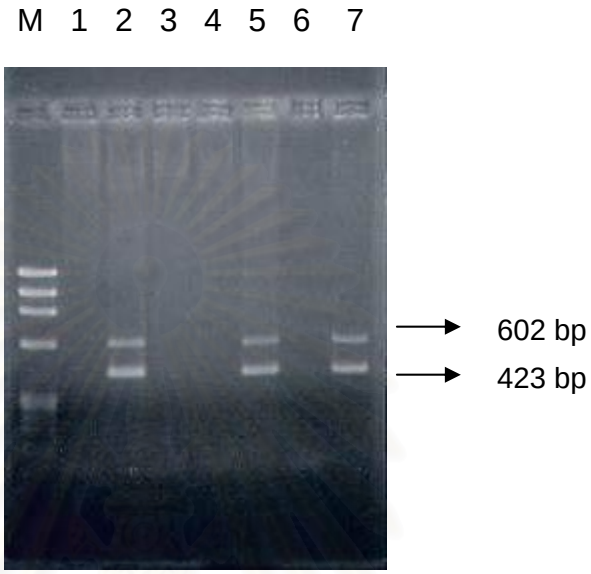

Figure 4.4 Amplification of the β-globin gene from single blastomere of experiment 4. Ethedium bromide-stained agarose gel showing the presence of 602 bp and 423 bp product

Lane M : DNA marker (phi X 174 DNA / *Hae* III)
Lane 1 : Single blastomere no.1
Lane 2 : Single blastomere no.2
Lane 3 : Single blastomere no.3
Lane 4 : Single blastomere no.4
Lane 5 : Single blastomere no.5
Lane 6 : Negative Control (last wash drop)
Lane 7 : Positive Control (100 pg/μl genomic DNA)

Section B : Primer concentration modification

The primer concentration was titrated in range of 0.1–1.0 μM. The result shown that primer concentration at 0.1 μM gave the best result, it gave both 774 bp and 378 bp products of beta globin gene. The more primer concentration, the less of PCR product of target sequence as shown in Figure 4.5 and Figure 4.6. The HUMTH01 PCR products (279 bp, using polymorphic linked marker, primer set F), presented in the first round PCR which act as a internal control (Figure not shown).

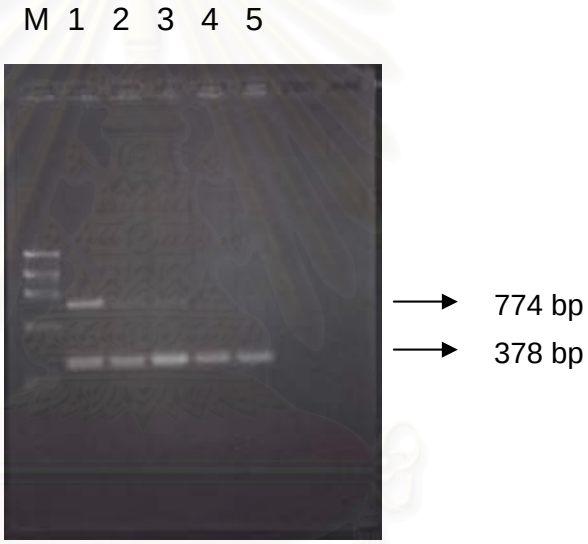

Figure 4.5 Titration of primer concentrations which range from 0.1-0.5 μM using 10 pg/μl Genomic DNA as a DNA template.

Lane M : DNA Marker (phi X 174 DNA / *Hae* III)
Lane 1 : Primer concentration = 0.1 μM
Lane 2 : Primer concentration = 0.2 μM
Lane 3 : Primer concentration = 0.3 μM
Lane 4 : Primer concentration = 0.4 μM
Lane 5 : Primer concentration = 0.5 μM

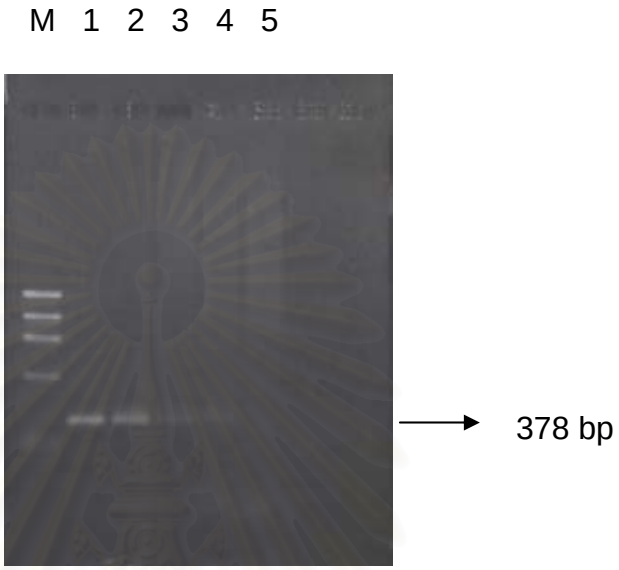

Figure 4.6 Titration of primer concentrations which range from 0.6-1.0 μM using 10 pg/μl Genomic DNA as a DNA template.

Lane M : DNA Marker (phi X 174 DNA / *Hae* III)

Lane 1 : Primer concentration = 0.6 μM

Lane 2 : Primer concentration = 0.7 μM

Lane 3 : Primer concentration = 0.8 μM

Lane 4 : Primer concentration = 0.9 μM

Lane 5 : Primer concentration = 1.0 μM

Effects of lysis cell method to the amplification efficiency

To study effects of lysis cell method to the amplification efficiency of single cell, five lysis methods were compared.

There was a difference in amplification efficiency according to the lysis procedure: boiling in water for 15 min gave a signal in 29 out of 50 samples (58.0%) where as boiling for 30 min decreased the efficiency to 10.8% (5 out of 46 samples amplified). As an alternative to boiling in water, alkaline lysis treatment gave a worse result : when boiled at 94° C for 30 min and 65°C for 10 min, these samples shown no amplification at all. But another lysis method, TE buffer (95°C 10 min) treatment gave a same result, it gave a signal in 36 out of 65 samples (55.4%). When comparing the first lysis method to the second, the third and the fourth one, there was always a statistical difference ($P<0.05$), but when comparing the first lysis method to the fifth one, there was not a statistical difference ($P>0.05$). These results, together with the number of experiments performed and the numbers of embryos used, were mentioned in Table 4.4.

Table 4.4 Number of blastomeres used, number of experiments perform and amplification efficiencies per lysis method.

Lysis method	In water		ALB		TE buffer
	94°C 15′	94°C 30′	94°C 30′	65°C 10′	95°C 10′
No. of experiment	7	6	6	7	10
No. of blastomere	50	46	45	50	65
Amplification efficiency (%)	29/50 (58.0%)[a,b,c]	5/46 (10.8%)[a]	0/45 (0%)[b]	0/50 (0%)[c]	36/65 (55.4%)

ALB = Alkaline lysis buffer

“ ′ “ = minute

[a,b,c] Values with same superscript were statistically different with χ^2–test ($P<0.05$)

(for the detail see appendix A)

Detection of beta globin gene mutation

The beta globin DNA products of two-steps PCR (Shown 774 bp and 378 bp product) were performed hybridization with filter-bound probes to detect beta globin mutation.

Reverse dot blot testing could detect beta thalassemia mutation by allele specific hybridization of PCR-amplified target DNA to oligonucleotide probes fixed to membrane. The presence of a particular sequence could be detected by the appearance of a blue-color dot on the membrane.

Normal DNA hybridized only to probe complementary to normal sequences. The positive hybridization with the mutation oligonucletide probe was interpreted as having the particular mutation in the beta globin gene. Therefore, a positive signal with both normal and mutant probes would be indicated the heterozygous state. DNA homologous for a particular mutation hybridized to the probe complementary to that mutation but not its normal analogue. Compound heterozygous DNA containing one copy each of two different mutations hybridized to both mutations and their normal analogues and to all other normal probe but not their mutant analogue. Mutations with close proximity may use the same oligonucleotide as the normal probe, there would be no hybridization to the normal probe either with homozygosity for one of the mutations or with compound heterozygosity for both.

On each filter strip the normal probes (N) were at the top and the mutant probe (M) were at the bottom. The presence of a color signal at each position indicated the presence in amplified target DNA of sequence complementary to that specific probe.

Each of 16 allele specific oligonucleotide probe set tested was able to correctly detect the beta thalassemia mutations using 10 pg of DNA from individuals known to be homozygous, heterozygous, and normal for the particular mutation.

The DNA sample homozygous for codon 17(A-T), heterozygous codon 19 (A-G) and heterozygous codon 41 (-C) mutation showed the presence of a color signal at codon 17(A-T), codon 19 (A-G) and codon 41 (-C) mutation as shown in Figure 4.7.

As shown in Figure 4.8, the DNA sample homozygous for Hb E, heterozygous codon 27/28 (+C), heterozygous of Hb C and heterozygous codon 43(G-T) mutations showed the presence of a color signal at Hb E, codon 27/28 (+C), Hb C [codon 19 (A-G)] and codon 43(G-T) mutations.

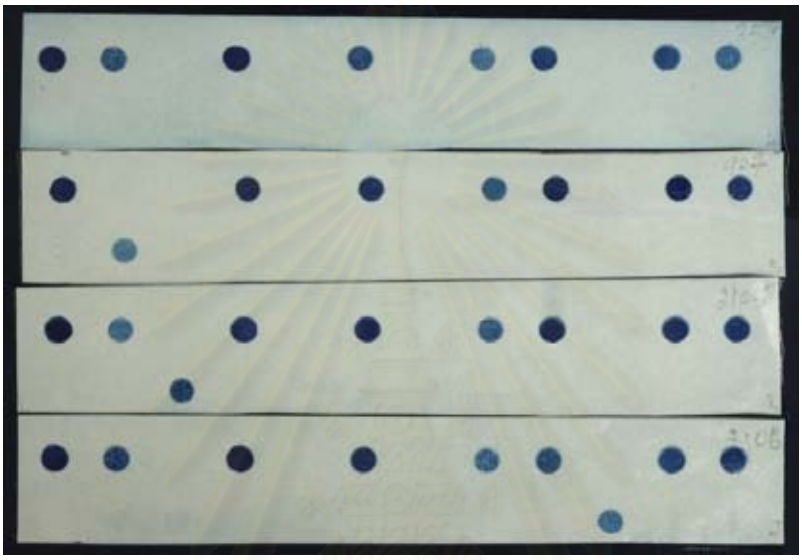

Figure 4.7 Reverse dot blot strip (membrane A) after hybridization and color development of 10 pg of genomic DNA (known mutation)

Strip no.1 : Normal control
Strip no.2 : Homozygous codon 17(A-T)
Strip no.3 : Heterozygous codon 19 (A-G)
Strip no.4 : Heterozygous codon 41(-C)

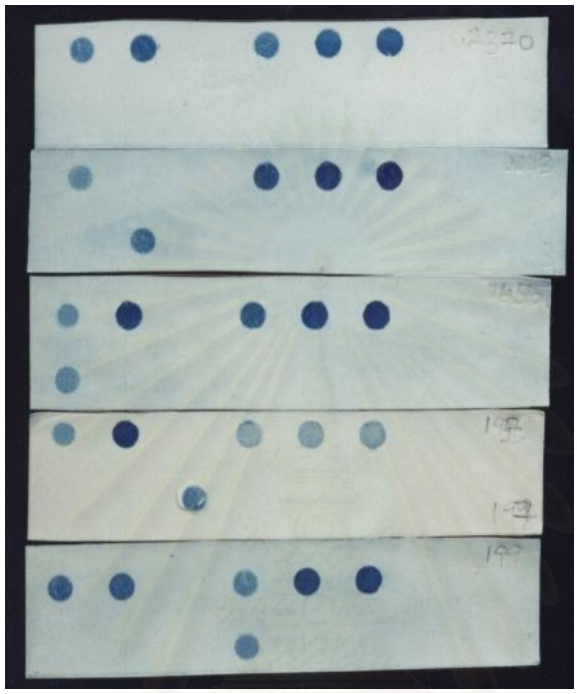

Figure 4.8 Reverse dot blot strip (membrane B) after hybridization and color development of 10 pg of genomic DNA (known mutation)

Strip no.1 : Normal control
Strip no.2 : Homozygous Hb E
Strip no.3 : Heterozygous Hb C
Strip no.4 : Heterozygous codon 27/28 (+C)
Strip no.5 : Heterozygous codon 43 (G-T)

Each single blastomere which could amplify beta globin gene (shown 774 bp and 378 bp products of beta blobin on agrose gel electrophoresis) was performed reverse dot blot hybridization. Single blastomere from normal patient shown normal beta globin gene, the appearance of a blue-colored dots only shown at the top row in both membrane A and B, as shown in Figure 4.9.

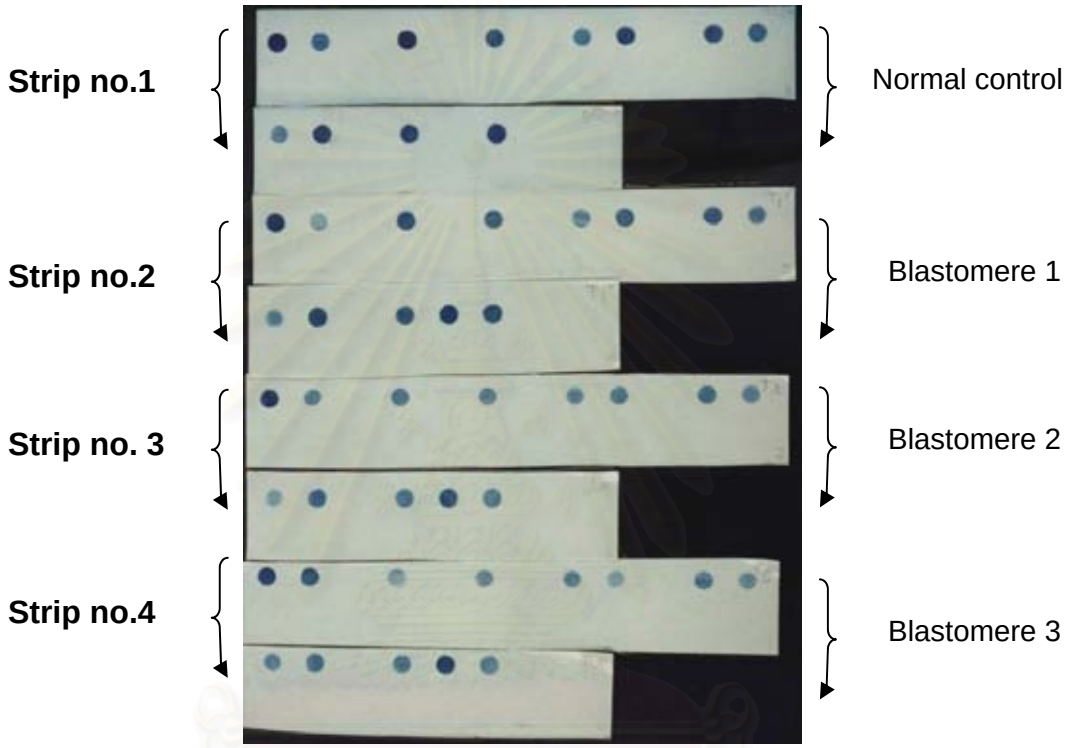

Figure 4.9 Reverse dot blot strips after hybridization and color development of individually normal single blastomere.

Strip no. 1 : Normal control
Strip no. 2 : Normal beta blobin gene
Strip no. 3 : Normal beta blobin gene
Strip no. 2 : Normal beta blobin gene

CHAPTER V

DISCUSSION AND CONCLUSIONS

A couple of thalassemia carrier has a 25% probability of having an affected baby with each pregnancy. Current therapies for thalassemic patients are blood transfusion, iron chelation and allogenic bone marrow transplantation while choronic villus sampling (CVS), amniocentesis and genetic analysis by the polymerase chain reaction (PCR) (Saiki et al.,1985) have made first- and second- trimester prenatal diagnosis possible by DNA analysis.

Although the number of beta thalassemia births has been reduced by the treatments and the methods as above, pregnancy termination is still unacceptable for many families and couple at risk are looking for an alternative way of producing non-affected offspring.

Successful pregnancies following the transfer of human embryos which had been sexed by amplification of Y-specific repeat sequence have been reported (Handyside et al., 1990). Amplification of single copy beta globin gene from mouse blastomere (Holding and Monk, 1989) and from human oocytes and polar bodies (Monk and Holding, 1990) had also been described. Thus, it should be possible to perform preimplantation diagnosis for hereditary disorders of the beta globin gene such as beta thalassemia by PCR analysis of the genetic defect using embryonic cell. A technique for obtaining a single blastomere biopsy from the cleavage stage embryo has been described which would provide an embryonic cell sample for genetic analysis.

This study aimed to amplify the beta globin gene of single blastomere in order to detect the beta globin gene mutation by reverse dot blot hybridization. In the first part, The determination of the minimum genomic DNA concentration could be amplify beta globin sequence was performed, the result shown that PCR condition I can successful amplify beta globin gene from dilute DNA to 12.5 pg/μl, making it suitable for analysis of 2 or more blastomeres , the amplification of 6.25 pg/μl of genomic DNA, only 378 bp product of beta globin gene was amplified, so that this PCR condition may be not suitable for amplify

DNA of single blastomere (the quantity of DNA present in a 10-30 cell trophectoderm biopsy would be 60-180 pg, considering that 6 pg of DNA is contained in a single diploid cell; Varawalla et al.,1991).

The next part, single blastomere was amplified beta globin sequence. The result demonstrated that a fragment of the beta globin gene (774 bp and 378 bp product) could be amplified from a single blastomere using two rounds of amplification with nested primers. But the successful amplification was poor, only 28 out of 106 blastomeres could be amplified.

Several explanations for this low amplification can be considered. Firstly, it is unlikely that the blastomere did not enter the reaction tube as the collection process was monitored throughout very carefully. Second, blastomere may have been anucleated thus having no DNA available for amplification. Third, the DNA containing the sequence to be amplified could have been destroyed. With the method of collection the sample was boiled either prior to or just after freezing, which should prevent the possibility of DNA degradation of the DNA in the sample by nucleases during the PCR reaction. However, should degradation have occurred, it would be necessary to propose that DNA of blastomere origin was susceptible to degradation (Pickering et al., 1992). The remaining possibility is that the sequence itself is missing and therefore unavailable for amplification. There is evidence of chromosomal abnormality and nuclear heterogeneity in embryos of IVF origin (Winston et al., 1991) and it is possible that sequence loss might occur if all or part of the chromosome carrying the sequence was absent from the blastomere under test. Fourth , embryo quality decreases PCR reliability such as the study of Findlay an colleague, they compare reliability rate of amplification of blastomere from good quality and poor quality. They achieved a reliability rate of 91% with blastomere from grade 1 and 2 embryo (good quality) but only 27% with blastomere from grade 3,4 or fragmenting embryo. This may reflect that our embryos were “spare” and only become available after the “best” embryos were selected for embryo transfer or freezing (Findlay et al., 1995). From the reason described above, the conclusion is that even though the embryo appeared morphologically normal, there may have been some unknown mechanism which the embryo itself interfering with the PCR process. This may be due to unavailability of DNA or an impairment of normal cellular process.

Furthermore, the reason of amplification failure describe above, the PCR condition also have some parameter that effects the amplification efficiency. The choice of primer annealing temperature is probably the most critical factor in designing a high specific PCR. If the temperature is too high, no amplification occur, but if it is too low, non-specific annealing would be increase dramatically. Primer-dimer would perform if the primers have one or more complementary based so that base pairing between the 3′ ends could occur.

Optimization of annealing temperature (T_A) of nested PCR shown that amplification efficiency of T_A at 54°C, 56°C, 60°C and 62°C were 0 % because no amplification of beta globin sequence from single blastomere. The T_A at 58°C gave the better result, 1 out of 5 single blastomere revealed successful amplification. From this study, T_A at 58°C was an optimal annealing temperature

Another interesting parameter affected PCR amplification efficiency was primer, because the primers determine the overall assay success. So that the effect of primer was be studied.

According to the report of Winichagoon and colleague claimed the successful amplification of beta globin sequence containing the most Thai common mutation same as this study, primer set from this report (primer set D&E) was chosen to use as a nested primer set in stead of primer set B&C and followed both PCR reaction mixture and PCR cycling program of that report. The beta globin gene amplification efficiencies of single blastomere of this part was only 22% (11/50). Although insufficient for statistical analysis, this data show that the amplification efficiency of single blastomere using this nested primer is lower than amplification efficiency of single blastomere using old nested primer set. So it was necessary to find out what was the major parameter affected PCR efficiency in this study.

The size of the first round product may affect PCR efficiency, the study of Vrettou and colleague proved this idea, they compared PCR success rate of using 3 first round primer set; first round primer set I gave 1158 bp product and first round primer set II and III were designed for the amplification of two shorter fragment of 689 bp and 637 bp respectively. The result show, with all first round PCR conditions tested on blastomere with primer set I , they could not achieve

higher than 67% PCR success, but using first round primer set I and II , they achieves 80% PCR success.

Based on the above report, first round primer set G ware designed for the amplification of shorter fragments of 1784 bp of beta globin gene. (The old first round primer set A gave 2062 bp product), and used in section B of the fourth part. To modify primer concentration in both first round and nested PCR, the primer concentration was varied from 0.1 μM to 1.0 μM. The beta globin gene amplification condition (PCR cycling program and reaction mixture) was all change from PCR protocol I to protocol III. The dilution genomic DNA (100,100,1 ng/μl, 100,50,10 pg/μl) as a DNA template, and HUMTH01 polymorphic primer (set F) used as a internal control primer. The result of this part shown that all reactions containing 0.1 μM of primer could successful amplify both 774 bp and 378 bp beta globin gene product (our target product). The result of using the highest genomic DNA concentration (100 ng/μl) and the lowest DNA concentration (10 pg/μl) gave the same result, when primer concentrator was increase, amplification efficiency was decrease.

To improve PCR success rate of amplifying beta globin gene in single blastomere five lysis method were compared (using optimized PCR protocol from previous part); first , boiling at 94°C in water for different time periods; 15 and 30 min. Boling for 30 min gave an efficiency of 10.8% but boiling for 15 min gave better result (58.0% amplification). Other reports have also used boiling for 15 min in water as a lysis method for blastomeres with very variable results; 45% for beta globin gene (Pickering et al.,1992) 67 and 77% for two different CF mutations (ΔF508 and WI282X) (Avner et al.,1994) 94 -100% of cystic fibrosis (CF) (Liu et al.,1995) respectively. Boiling in alkali buffer was chosen as a second alternative, the method was first reported to improve amplification efficiencies in spermatozoa from 24 to 85% (Cui et al., 1989) but our experiment gave an opposite result, lysis method using alkali buffer boiling at 94°C for 30 min and at 65°C for 10 min have an efficiency of 0% in both method. It is possible that this method would be too stringent for blastomere, a DNA from blastomeres appeared to be more readily released than from spermatozoa (Xu et al.,1993). Comparing PCR success rate using the first lysis method (water, 94°C 15 min) with the fifth lysis method (TE buffer,

95°C 10min) found that no different between two lysis methods (58.0% and 55.4% respectively). From this result, TE buffer can maintain DNA quality of single blastomere as same as water, and lysis methods are effect to amplify efficiency of single blastomere.

Most of beta thalassemia is point mutation, the technique that can detect beta globin gene mutation rapidly, high sensitivity and simple approach to type a large number of alleles is reverse dot blot hybridization. This technique can detect beta thalassemia mutation by allele–specific hybridization of PCR-amplified target DNA to oligonucleotide probes fixed to membrane. So in the last part, applications of this technique to detect beta globin gene mutation of single blastomere was performed. As it was not possible to obtain embryonic cell sample from couple caring beta thalassemia mutation, diluted DNA solution (10 pg/μl) from various individual whose beta thalassemia mutation had already been characterized were used as experimental model to test the reliability of this technique before applying this technique for preimplantation genetic diagnosis .

The result of this experiment show that each of 16 allele specific oligonucleotide probe set tested was able to correctly detect the beta thalassemia mutation using 10 pg of DNA from individuals known to be homozygous, heterozygous and normal for the particular mutation, an example shown in Figure 4.7 and Figure 4.8. Similar results were obtained from detect from of single blastomere from normal patient, normal DNA hybridizes only to probes complementary to normal sequence (see Figure 4.9). As the PCR amplification efficiency affected the detections of beta globin gene mutations by reverse dot blot hybridization. The study achievement of amplified beta globin gene from single blastomere was 58%. The failure to detect an amplified product would waste the embryo that could have been transferred. This may be considered an inappropriate method.

Conclusion, application of preimplantation genetic diagnosis for beta globin gene mutation using this two step PCR protocol followed by reverse dot blot hybridization was not appropriated at the present time.

In further study, the technique which provide greater reliability and accuracy, less allelic drop out (ADO) and less time consuming for genetic

analysis such as fluorescence PCR or real time may be better choice for single blastomere diagnosis for beta thalassemia.

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APPENDICES

APPENDIX A

Primer and Probe

Table A.1 Primers designed for multiple two-step PCR

Primers	Sequence of primer	Product size (bp)
First round PCR primers		
Set A		
f	5′ CAT CTA CAT ATC CCA AAG 3′	2062
r	5′ GAC CTC CCA CAT TCC CTT TT 3′	
Set F (HUMTH01: polymorphism primer)		
f	5'-AGG GTA TCT GGG CTC TGG-3'	279
r	5'-CTT CCG AGT GCA GGT CAC-3'	
Set G		
f	5'-TGT ACT GAT GGT ATG GGG CC-3'	1784
r	5'-GGC CCT TCA TAA TAT CCC CCA G-3'	
Nested PCR primers		
Set B (X = biotin labeled)		
f	5′ XAAC TCC TAA GCC AGT GCC AGA AGA 3′	774
r	5′ XTCA TTC GTC TGT TTC CCA TTC TTA 3′	
Set C		
f	5′ XTAT CAT GCC TCT TTG CAC CAT TCT 3′	378
r	5′ XTGA TAC TTG TGG GCC AGG GCA TTA 3′	
Set D		
f	5′ GTA CGG CTG TCA TCA CTT AGA CCT CA 3′	602
r	5′ TGC AGC TTG TCA CAG TGC AGA TCA CT 3′	
Set E		
f	5′ GTG TAC ACA TAT TGA CCA AA 3′	423
r	5′ AGC ACA CAG ACC AGC ACG TT 3′	

f : Forword primer.

r : Reverse primer.

PCR PROTOCOL

Protocol I

Reagent mixture (Final volume 100 μl)

First round PCR	Nested PCR
1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl),	Same as first round PCR
2.5mM $MgCl_2$	"
0.2 μM of each primer (set A)	0.2 μM of each primer (set B&C)
200 μM each of dNTPs	400μM each of dNTPs
2.5 units of taq polymerase	4.0 units of taq polymerase
SDW	Same as first round PCR

PCR cycling program

First round PCR

	Temperature (^oC)	Time	Number Of cycles
Initial denaturation	95	1 min	1(first)
Denaturation	94	1 min	
Annealing	40	45 sec	35
Elongation	72	1 min	
Final elongation	-	-	-
Hold at	4	∞	

Nested PCR

	Temperature (^oC)	Time	Number Of cycles
Initial denaturation	-	-	-
Denaturation	95	30 sec	
Annealing	58	3 sec	35
Elongation	72	3 sec	
Final elongation	72	5 min	-
Hold at	4	∞	

Protocol II

Reagent mixture (Final volume 50 μl)

First round PCR	Nested PCR
1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl)	1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 0.01% gelatin)
2.5mM $MgCl_2$	1.5mM $MgCl_2$
0.2 μM of each primer (set A)	12.5 μM of each primer (set D&E)
200 μM each of dNTPs	100μM each of dNTPs
2.5 units of taq polymerase	1.25 units of taq polymerase
SDW	Same as first round PCR

PCR cycling program

First round PCR

	Temperature (^oC)	Time	Number Of cycles
Initial denaturation	95	1 min	1(first)
Denaturation	94	1 min	
Annealing	40	45 sec	40
Elongation	72	1 min	
Final elongation	-	-	-
Hold at	4	∞	

Nested PCR

	Temperature (^oC)	Time	Number Of cycles
Initial denaturation	94	5 min	1 (first)
Denaturation	94	1 min	
Annealing	55	30 sec	35
Elongation	72	30 sec	
Final elongation	72	10 min	-
Hold at	4	∞	

Protocol III

Reagent mixture (Final volume 50 μl)

First round PCR	Nested PCR
1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl)	Same as first round PCR
1.5mM $MgCl_2$	"
0.1 μM of each primer (set G)	0.1 μM of each primer (set B&C)
200 μM each of dNTPs	"
3.0 units of Amplitaq Gold polymerase	"
SDW	"

Note: Section B: Vary primer concentration, vary primer concentration from 0.1-1.0 μM and used polymorphic primers (set F) as an internal control.

PCR cycling program

First round PCR and Nested PCR

	Temperature (^{o}C)	Time	Number Of cycles
Initial denaturation	95	15 min	1 (first)
Denaturation	95	45 sec	
Annealing	60	45 sec	35
Elongation	72	2 min	
Final elongation	72	5 min	
Hold at	4	∞	

Table A.2 Point mutation that caused beta thalassemia among Thai.

Position	Mutation	Phenotype	Probe	Sequence
−28	A → G	β^+	Normal	5′ GGCTGGGCATAAAAGTCAGG 3′
			Mutant	5′ TGCCCTGACTTCTATGCCCAG 3′
Codon 17	A → T	β°	Normal	5′ GGGCAAGGTGAACGTGGAC 3′
			Mutant	5′ CTGTGGGGCTAGGTGAAC 3′
Codon 19	A → G	β^+	Normal	Same as for Codon 17
			Mutant	5′ AAGGTGAGCGTGGATGAA 3′
Codon 26 (Hb E)	G → A	β^+	Normal	5′ AGGGCCTCACCACCAACCC 3′
			Mutant	5′ GTTGGTGGTAAGGCCCTG 3′
Codon 26	G → T	β°	Normal	Same as for Hb E
			Mutant	5′ GTTGGTGGTTAGGCCCTG 3′
Codon 27–28	+C	β°	Normal	Same as for Hb E
			Mutant	5′ TGAGGCCCCTGGGCAG 3′
IVS I-1	G → T	β°	Normal	5′ CCTTGATACCAACCTGCCCAG 3′
			Mutant	5′ GGGCAGTTTGGTATCAAGG 3′
IVS I-5	G → C	β^+	Normal	Same as for IVS I-1
			Mutant	5′ CCCTGGGCAGGTTGCTATCAA 3′
Codon 35	C → A	β°	Normal	5′ GGTGGTCTACCCTTGGACCC 3′
			Mutant	5′ GGTGGTCTAACCTTGGACC 3′
Codon 41	−C	β°	Normal	5′ CAAAGGACTCAAAGAACCTCTG 3′
			Mutant	5′ CCAGAGGTTTTTGAGTCC 3′
Codon 41–42	−TTCT	β°	Normal	Same as for codon 41
			Mutant	5′ GGACTCAACCTCTGGGTCC 3′
Codon 43	G → T	β°	Normal	Same as for codon 41
			Mutant	5′ CCCAGAGGTTCTTTTAGTC 3′
Codon 71–72	+A	β°	Normal	5′ GTGCCTTTAGTGATGGCCTG 3′
			Mutant	5′ CCATCACTTAAAGGCACCG 3′
Codon 95	+A	β°	Normal	5′ ACAAGCTGCACGTGGATC 3′
			Mutant	5′ TGCAGCTTTGTCACAGTG 3′
IVS II-654	C → T	β°	Normal	5′ CTGGGTTAAGGCAATAGCAATCC 3′
			Mutant	5′ TTGCTATTACCTTAACCCAGAAATCC 3′
Codons 123–125	−ACCCCACC	β°	Normal	5′ GAATTCACCCCACCAGTGC 3′
			Mutant	5′ AGAATTCAGTGCAGGCTGC 3′

Figure A.1 Location of nested oligonucleotide primers

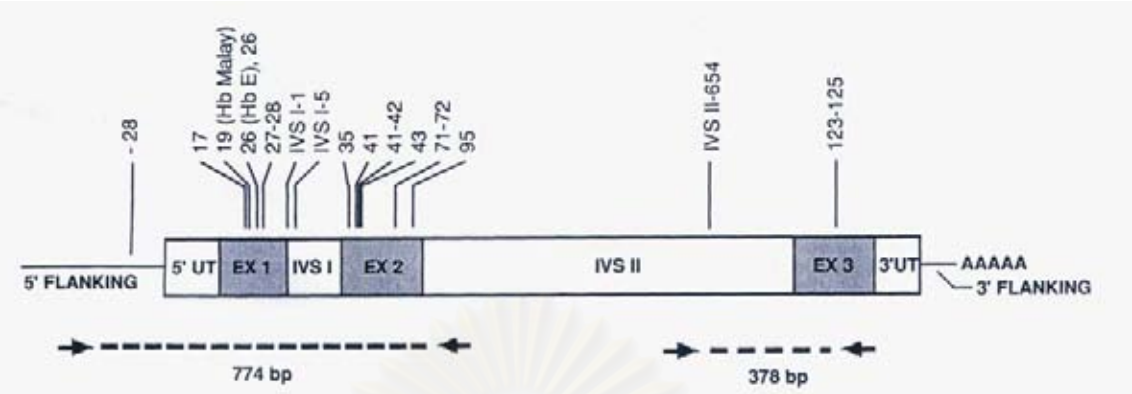
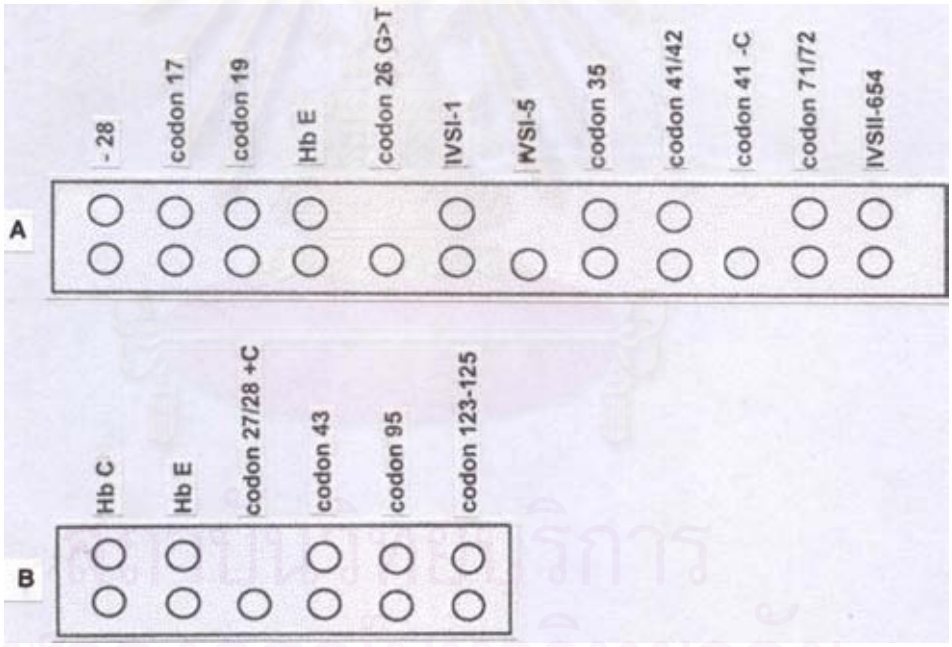


Figure A.2 Reverse dot blot strips of membrane A and B



Membrene A (long strip) : 10 most common Thai beta Thalassemia mutation including Hb Malay (codon 19) and Hb E

Membrene B (short strip) : 5 less common Thai beta Thalassemia mutation including Hb Khonkaen (codon 123-125) and Hb E

Comparision of Lysis method

Lysis I	: SDW 5 μl	94°C	15 min
Lysis II	: SDW 5 μl	94°C	30 min
Lysis III	: Alkaline lysis buffer 5 μl	94°C	30 min
Lysis IV	: Alkaline lysis buffer 5 μl	65°C	10 min
Lysis V	: TE buffer 3 μl	95°C	10 min

	Lysis I	Lysis II	Total
	29	5	34
	21	41	62
Total	50	46	96

X^2 = 23.27, *P* values < 0.05

	Lysis I	Lysis III	Total
	29	0	29
	21	45	66
Total	50	45	95

X^2 = 37.57, *P* values < 0.05

	Lysis I	Lysis IV	Total
	29	0	29
	21	50	71
Total	50	50	100

X^2 = 40.85, *P* values < 0.05

	Lysis I	Lysis V	Total
	29	36	65
	21	29	50
Total	50	65	115

X^2 = 0.08, *P* values = 0.7791 (NS)

APPENDIX B

Stock solution for preparation of single blastomere

Acid tyrode's solution

Nacl	8.0	g/l
KCl	0.2	g/l
Glucose	1.0	g/l
PVP	4.0	g/l
$MgCl_2.6H_2O$	0.1	g/l
$CaCl_2.2H_2O$	0.24	g/l

Dissolve all component in dH_2O and adjust pH to 2.5 with 0.1 N HCl.

Filter sterilization and store at 0°C.

Biopsy medium (Gordon & Gang, Repro 1990)

106 mM NaCl	6.195	g/l
2.7 mM KCl	0.201	g/l
1.5 mM KH_2PO_4	0.204	g/l
8.1 mM Na_2HPO_4	1.150	g/l
56 mM Glucose	1.009	g/l
2 mM EDTA	0.672	g/l
100 mM Sucrose	34.300	g/l
0.33 mM Sodium pyruvate	0.036	g/l
25 mM Sodium lactate (60%)	3.65	ml/l
4mg/ml BSA fraction V	4.0	g/l

Dissolve all component in dH_2O and adjust pH to 7.3-7.4 with 0.1 N NaOH.

Filter sterilization and store at 4°C.

10 IU Pronase

Protease (3.9 units/mg solid)	0.0128	g

Dissolve protease in dH_2O 5 ml.

Filter, aliqout and store at -20°C.

Alkaline lysis buffer (ALB) (200 mM KOH, 51 mM DTT)

KOH (FW. 56)	1.12	gm
DTT (FW. 154.2)	0.771	gm

Dissolve KOH and DTT in dH_2O 100 ml.

Filter, aliqout and store at -20°C.

Neurtralization buffer (900 mM Tris-HCl (pH 8.3), 300 mM KCl, 200 mM HCl)

1M Tris-HCl (pH 8.3)	45	ml
KCl (FW. 74.5)	1.1182	gm
2N HCl	5	ml

Add dH_2O to 100 ml.

Filter, aliqout and store at -20°C.

APPENDIX C

Stock solution for PCR and Reverse dot blot hybridization

TE buffer

1 M Tris-HCl (pH 8.0)l	10	ml
0.25 M EDTA	0.4	ml

Dissolve Tris-HCl and EDTA in dH_2O and adjust pH to 8.0

Sterilize by autoclaving and store at room temperature.

TE buffer

50X TAE	242	g/l
Glacial acetic acid	57.1	ml
0.5 M EDTA (pH 8.0)	100	ml/l

Dissolve Tris-base, Glacial acetic acid and EDTA in dH_2O and adjust pH to 8.05 with HAc.

Sterilize by autoclaving and store at room temperature.

20X SSPE

3.6 M NaCl	210.4	g/l
0.2 M Na_2HPO_4	27.6	g/l
20mM EDTA	0	ml/l

Dissolve NaCl, Sodium phosphate and EDTA in dH_2O and adjust pH to 7.4 with NaOH.

Dispense into aliquots, sterilize by autoclaving and store at room temperature.

Denature solution

10 M NaOH	10	μl
0.5 M EDTA	0.4	μl

Add dH_2O to 1000 μl.

****** Prepare freshly before use.

10% SDS

SDS	10	g/100 ml

Dissolve SDS in dH_2O and store at 4°C.

Buffer for dot probe (5XSSPE+ 0.5%SDS)

20XSSPE	50	ml
10% SDS	10	ml

Add dH_2O to 200 ml.

****** Prepare freshly before use and warm at 64°C.

Hybridization buffer (4X SSPE+ 0.5 % SDS)

20XSSPE	20	ml
10% SDS	5	ml

Add dH_2O to 100 ml.

****** Prepare freshly before use and warm at 57°C.

Washing buffer (1X SSPE+ 0.2 %SDS)

20XSSPE	5	ml
10% SDS	2	ml

Add dH_2O to 100 ml.

****** Prepare freshly before use and warm at 57°C.

Citrate buffer (100mM)

Na citrate (FW. 294.1)	29.41	g/l

Dissolve Na citrate in dH_2O and adjust pH to 5.0 with Citric acid (FW 192.1).
Sterilize by autoclaving and store at room temperature.

TMB (2 mg/ml)

TMB	20	mg
100% Ethanol	10	ml

Mix TMB with Ethanol. The solution can store for 2 months at 4°C.

****** Keep in dark place.

3% H_2O_2

30% H_2O_2	200	µl

Add dH_2O to 2 ml. Store at room temperature.

Loading dye

Loading dye	0.005	g
Bromphemol blue	600	µl

30 % Glycerol

Add dH_2O to 2 ml. Store at room temperature.

10X duplex buffer

1 M (NH_4SO_2)	1.245	ml
1 M Tris-HCl (pH 8.0)	5.025	ml
1 M $MgCl_2$	0.503	ml
14.4 M βME	0.026	ml
0.5 M Na_2 EDTA	1.02	µl
dH_2O	8.02	ml

Mix, store at 4 °C.

BIOGRAPHY

Miss Jiraporn Pansatha was born on January 2, 1972 in Nakornsawan, Thailand. She graduated with the degree of Bachelor of Biochemical & Biochemical technology of Science in 1994 from the Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. She has enrolled at Chulalongkorn University in graduate program for the degree of Master of Science in Medicine Science in 2003. At present, She works as an embryologist in the Division of Reproductive Medicine, Department of Obstetrics and Gynaecology, Faculty of Medicine, Chulalongkorn University.