การวิเคราะห์เปรียบเทียบชิ้นส่วนเทโลเมียร์ของบลาสโตซิสมนุษย์ จากกลุ่มมารดาอายุต่างกันโดยเทคนิคการหาลำดับเบส แบบเน็กซต์เจนเนอเรชั่น

นางสาวกฤชกร เสวกวงศ์พระ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ระดับโมเลกุลทางจุลชีววิทยาทางการแพทย์และวิทยาภูมิคุ้มกัน ภาควิชาเวชศาสตร์การธนาคารเลือดและจุลชีววิทยาคลินิก
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

Comparative analysis of telomere regions in human blastocysts between maternal age groups by next-generation sequencing

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Molecular Science of Medical Microbiology and Immunology
Department of Transfusion Medicine and Clincal Microbiology
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กฤชกร เสวกวงศ์พระ : การวิเคราะห์เปรียบเทียบชิ้นส่วนเทโลเมียร์ของบลาสโตซิสมนุษย์จากกลุ่มมารดาอายุต่างกันโดยเทคนิคการหาลำดับเบส แบบเน็กซต์เจนเนอเรชั่น (Comparative analysis of telomere regions in human blastocysts between maternal age groups by next-generation sequencing) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.เทวิน เทนคำเนาว์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.สัญชัย พยุงภร, วิวัฒน์ กว้างคณานุรักษ์, 82 หน้า.

การศึกษานี้ เป็นการเก็บข้อมูลย้อนหลัง โดยมีวัตถุประสงค์เพื่อ เปรียบเทียบความยาวของเทโลเมียร์ ในตัวอ่อน มนุษย์ระยะบลาสโตซิสต์ ระหว่างกลุ่มมารดาที่มีอายุต่างกัน โดยใช้การตรวจวิเคราะห์ ด้วยการหาลำดับเบส แบบเน็กซต์เจนเนเรชั่น โดย บลาสโตซิสต์จากกลุ่มแม่อายุน้อย(น้อยกว่า หรือเท่ากับ 35ปี จำนวน 47 ตัว) และ บลาสโตซิสต์จากกลุ่มแม่อายุมาก (มากกว่า 35ปี จำนวน 44 ตัว) โดยผู้ที่มารับการรักษาภาวะมีบุตรยาก ทำการตรวจคัดกรอง โครโมโซมในตัวอ่อนเนื่องมาจาก เป็นกลุ่มมารดาที่มีอายุมาก (มากกว่า 35ปี) และหรือ มีประวัติการแท้งซ้ำซาก โดยข้อมูล (BAM files) จะถูกคัดเลือกจากตัวอ่อนที่มีการตรวจคัดกรองโครโมโซม ด้วยวิธีการหาลำดับเบส แบบเน็กซต์เจนเนเรชั่น จากผลการตรวจวิเคราะห์พบว่า ความยาวเฉลี่ยของเทโลเมียร์ของแขนแต่ละข้างของโครโมโซม จากบลาสโตซิสต์ที่ได้จาก แม่อายุมาก และแม่อายุน้อย ส่วนใหญ่ พบว่า ไม่มีความแตกต่างกันอย่างมีนัยสำคัญ พบ ความยาวเฉลี่ยของเทโลเมียร์ ที่ยาวกว่าในกลุ่มแม่อายุมาก ในแขนของโครโมโซม 1P, 2Q, 3P, 5P, 9Q, 11P, และ 15Q ($P < 0.05$, 15%) การวิเคราะห์การซ้ำกันของลำดับเบส TTAGGG พบว่าส่วนใหญ่ ไม่มีความแตกต่างกันอย่างมีนัยสำคัญ ที่แขนแต่ละข้างของโครโมโซม แต่มี แขน 1Q, 2Q, 9Q, 12Q, 13P, และ 20P ที่มีความแตกต่างอย่างมีนัยสำคัญ($P < 0.05$, 13%) การศึกษานี้ประสบความสำเร็จในการพัฒนาวิธีชีวสารสนเทศน์ศาสตร์ ในการประเมินความยาวของโลเมียร์ในตัวอ่อน โดยใช้ข้อมูลการหาลำดับเบส แบบเน็กซต์เจนเนเรชั่น และความแตกต่างที่พบในการศึกษานี้ ไม่มีความสัมพันธ์ต่อผลของการรักษาภาวะมีบุตรยากในทางคลินิก

ภาควิชา	เวชศาสตร์การธนาคารเลือดและจุลชีววิทยาคลินิก	ลายมือชื่อนิสิต	.....
		ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
สาขาวิชา	วิทยาศาสตร์ระดับโมเลกุลทางจุลชีววิทยาทางการแพทย์และวิทยาภูมิคุ้มกัน	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....
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KRITCHAKORN SAWAKWONGPRA: Comparative analysis of telomere regions in human blastocysts between maternal age groups by next-generation sequencing. ADVISOR: ASST. PROF. TEWIN TENCOMNAO, Ph.D., CO-ADVISOR: ASST. PROF. SUNCHAI PAYUNGPORN, Ph.D., WIWAT QUANGKANANURUG, M.D., 82 pp.

The retrospective investigation was carried out to compare the telomere regions using next-generation sequencing (NGS) technique in human blastocysts derived from infertile Asian couples classified into two different maternal age groups. Bioinformatics methods were used to compare the average telomere length and telomeric repeated sequences (TTAGGG) of 94 human blastocysts derived from two maternal age groups, young mothers (≤35 years, N=47), old mothers (> 35 years, N=44). Patients subjected to chromosome screening were due to advanced maternal age (older than 35 years) and / or mothers with recurrence miscarriage or implantation failure. BAM files were selected from all patients undergoing preimplantation blastocyst biopsied on day 5 or day 6 after insemination with chromosome screening by NGS technique. We found that the majority of chromosome arms were not significantly different between the two maternal groups. The longer telomere length was found in older mother group's chromosome arms 1P, 2Q, 3P, 5P, 9Q, 11P and 15Q ($P < 0.05$, 15%), while the majority of chromosome arms was not different. Most chromosome arms were not different in telomeric repeated sequences between the two maternal groups, whereas the chromosome arms 1Q, 2Q, 9Q, 12Q, 13P and 20P were significantly distinct ($P < 0.05$, 13%). Therefore, we successfully applied the bioinformatics approach for determination of telomere length and telomeric repeated sequences based on NGS data, and these differences detected might be correlated with reproductive outcome clinically.

Department:	Transfusion Medicine and Clincal Microbiology	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Molecular Science of Medical Microbiology and Immunology	Co-Advisor's Signature .....
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Chapter 1

Introduction

Telomeres are a region of repetitive nucleotide sequences at each end of chromosomes which participate in process of chromosome repair, prevent nonspecific chromosomal recombination and help the chromosome binding to nuclear matrix.[1, 2] Human telomere consist of tandem repeats of TTAGGG.[3] Telomeres length decreased by aging both in dividing and non-dividing cells, which cause by signaling cell cycle arrest, senescence, and cell death. In mitotic cell division process, DNA is duplicated by the action of a DNA polymerase. This enzyme does not duplicate the ends of chromosome so telomeres get shorter after each cell division.[4] The telomere theory of reproductive senility supports that telomere will be shortening after fetal development and the long period of time before ovulation in adults lead to reproductive aging in woman.[5] Moreover, the mechanism of telomere shortening causes misalignment and defective paring of homologous chromosome in early meiosis cycles.[6] Telomere DNA deficiency is correlated with genomic instability and has a role in development aneuploidy in female germ cells and human embryos.[7] Comparing of the telomere length in oocytes, they were shorter in woman who did not get pregnant than pregnancy individuals.[7, 8]

There are many tests developed for telomere length detection. Standard methods for telomeres length are classified in three groups: Southern blot analysis of the terminal restriction fragment measures length in sample of cells.[9] The detection of variation between chromosome and cells ,for example, fluorescence *in situ* hybridization (FISH) technique, including of Quantitative Fluorescence in-situ Hybridization (Q-FISH) and Flow-FISH.[10] Quantitative polymerase chain reaction (qPCR) measure telomeres DNA content.[11]

Next-generation sequencing (NGS) is an advance technology in the molecular science. It provides an opportunity to access whole genomic information and adaptable methods of copy number detection by comparing the short sequence between patients and reference database.[12] NGS use the ability to massively parallel sequence of millions of DNA templates. The genomic DNA was cut into short fragments and tagged with barcode sequence. The short products were amplified and size selected before sequencing. The advantage of this method is a rapid sequencing of

large genomes. NGS specificity was 99.98% with the sensitivity 100% for aneuploidy call in human embryos compared with the gold standard for genetic diagnosis of copy number, the array comparative genomic hybridization (array CGH).[13] The applications of NGS are available in genetic diagnosis and research purpose such as point mutation, chromosome abnormalities and preimplantation genetic screening (PGS). There are less information about the low copy number pattern of human blastocyst form next-generation sequencing result. This study was interested in application of next-generation sequencing in preimplantation genetic screening to investigation the pattern of low copy number of the telomeric regions in human embryos.

1. Review of related literature

1.1. Telomere structure and Telomere Function

"Telomere" is derived from Greek words "Telo" means end and "mero" means part. Telomeres are nucleoprotein structure present at the both end of chromosomes. Elizabeth Blackburn, Carol Greider and Jack Szostak discovered the telomere structure and function and they won the Nobel Prize in Medicine in 2009.

1.1.1. Telomere structure

Telomeres are at the both ends of chromosomes containing of hundreds of TTAGGG tandem repeats. Telomeres binding with proteins cap the chromosome ends to protect the ends of chromosome break during cell division.[14] In humans, there are between 9-15 kb in length. In eukaryotic cell, telomeres are formed of double strand tandem repeat sequences which have G rich and contemporary C rich strand. The numbers of telomeric repeats are difference between species. The double strands of tandem repeats consist of G rich strand in 5' to 3' end and C rich in complementary strand. The most of telomeric DNA structure is double-stranded. At 3' G rich the single-stranded overhangs of several repeats approximately 150-200 bp. The 3' overhang turns back into invading the previous double-stranded. As the result, loop formation called "T-loop" In human G-strand overhang is approximately 100-280 nucleotides binding with proteins. This formation is known as the shelterin complex.[15-17] The shelterin complex consists of six proteins that are binding with telomeric sequences; telomere repeat factor 1 (TRF1), telomere repeat factor 2 (TRF2). Telomere interact protein 2 (TIN2), protection of telomere 1 (POT1), TIN2-POT1 interacting protein (TPP1) and repressor/activator protein 1 (RAP1). The binding of proteins with the TTAGGG sequence forms the telosome structure and responsible for telomere

maintenance by controlling the action of telomerase. TRF, TRF2 and POT1 directly interact with the TTAGGG sequence of mammalian telomeres. RAP1 is joined with TRF2 and regulating telomere length. Moreover, TIN2 is associates with TRF1, TRF2, TTP1 and POT1. TIN2 is a chain of TTP1 and POT1 to TRF1 and TRF2. In addition, over expression of TRF2 results in prevention of chromosome aberration but the loss expression has been shown to activate the p53 pathway and senescence.[18, 19]

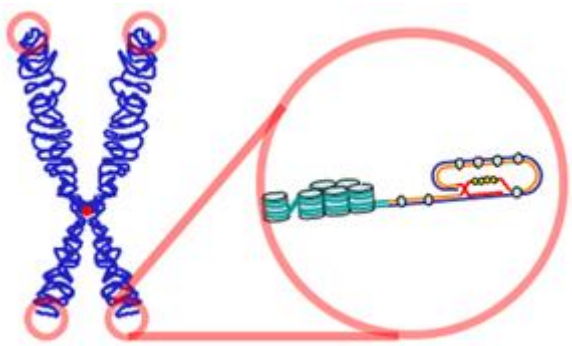


Figure 1: Telomere structure

Telomeres are the end of chromosome and made up of repetitive sequences of DNA associated with proteins.[20]

1.1.2. Telomere Function

Telomeres play essential roles in biological mechanisms. The mainly function of telomere is to protect gene near the end of chromosome from DNA replication process and maintain genomic stability by preventing chromosome fusion. Moreover, telomeres have function in nuclear organization during mitosis and meiosis.

1.1.2.1. Telomere and DNA replication

During DNA replication process, the starting point of replication called “origin of replication double-stranded DNA” is denatured into single-stranded DNA by helicase and topoisomerase. Then DNA polymerase binds to single-strand DNA and begin making up the new strand in 5’ to 3’ direction. This results in the 5’ to 3’ direction continuous new DNA known as “leading strand”, but in 3’ to 5’ direction the discontinuous DNA strand.[21-23] It requires RNA primers to elongated into short fragment called Okazaki fragments. For this reason, RNA primer is lost at the 5’ end of the lagging strand when the RNA primer is removed from replication later.[24-26]

Since telomeres contains no vital genetic information or non-coding DNA sequence. In this way, telomeres can protect the genes near the end of

chromosome from degradation. The loss of telomere sequences after replication, eventually the length is reduced to critical telomere length, the cell leaves the cell cycle G1 and undergoes senescence.[27]

1.1.2.2. Senescence

Senescence is a process which the cell stop dividing and leaves the cell cycle.[28]It is defined as several characteristics as follows: morphological changes (increased cell size), expression profiles changed (inhibition of cell cycles and DNA damages), signaling molecules secreted, presence of heterochromatin and absence of proliferation markers. Senescence is an important cellular response to cell damage because it prevents the increasing of damaged cells by immune cells and this is vital in the prevention of tumorigenesis. In addition, senescence has an important role in tissue renewal and embryogenesis by limiting amount of cell that being produced. The loss of telomere repeats leads to DNA damage response by ATM or ATR pathways. This leads to the phosphorylation of p53, expression of p21, and inhibition of cyclin dependent kinase.[29]

1.1.2.3. Telomeres and genome stability

In normal situation, a broken DNA can be found in the action of DNA repair mechanism. In 1939, Barbara McClintock found that fusion event happened in broken chromosome only.[30] Blasto et al., in 1997 demonstrated that the shortened telomere cannot support the telosome as a result of chromosome fusion events in mouse model.[4] So, maintaining telomere length is important in vital life for preventing the chromosome fusion that can cause harmful mutation and genetic disorders.

1.1.3. Telomere homeostasis and ageing

In the living eukaryote cell, telomere shortening after the end of replication is a part of homeostasis and preserving genome stability. Normal telomere homeostasis is controlled by the balance between mechanism of lengthening and shortening. Telomerase activity is regulated during development and inactive in normal somatic cells but expressed only in male germ cells, stem cells and cancer cells. Telomere length is decreased with aging and normal cell division. Telomere length used as biological marker for aging. Telomerase is an enzyme functioning in maintaining telomere length.[31] Many studies show a relation between cellular aging and telomere length *in vivo*. In 1990, Calvin Harley et al., show a decrease of telomere

length in fibroblast after the initial passage. In 1992, Allsopp et al., show a similar result that telomere length of fibroblast decreased with age. Moreover, there were many *in vivo* studies show that telomere length was shortened by aging in skin, blood sample and colorectal.[32, 33] In normal cells having telomere shortened to a critical length result in senescence by cell damage pathways.[34, 35]

1.2. Gametogenesis

To begin with the sexual reproduction, the reproductive organ produces haploid gametes in the gametogenesis process. The precursor cells undergo meiosis cycles. Starting with the precursor cells undergo a pre-meiotic division in DNA replication. After the completion of pre-meiotic division, meiosis may then ensure.

1.2.1. Meiosis

Meiosis consists of two cellular divisions such that four haploid cell for one diploid cell.[36] The first division resulting in a product containing half genetic of the mother cell (meiosis I). The second meiosis division produces four haploid daughter cells (meiosis II). The offspring has a random combination between maternal and paternal genome. This is completed by the random segregation of chromosome into daughter cells, DNA recombination and crossing over.[37] which happens in prophase stage during the first meiotic division which is sub-divided into five stages: Leptotene, zyotene, pachytene, diplotene and finally diakinesis as in the picture[36, 38].

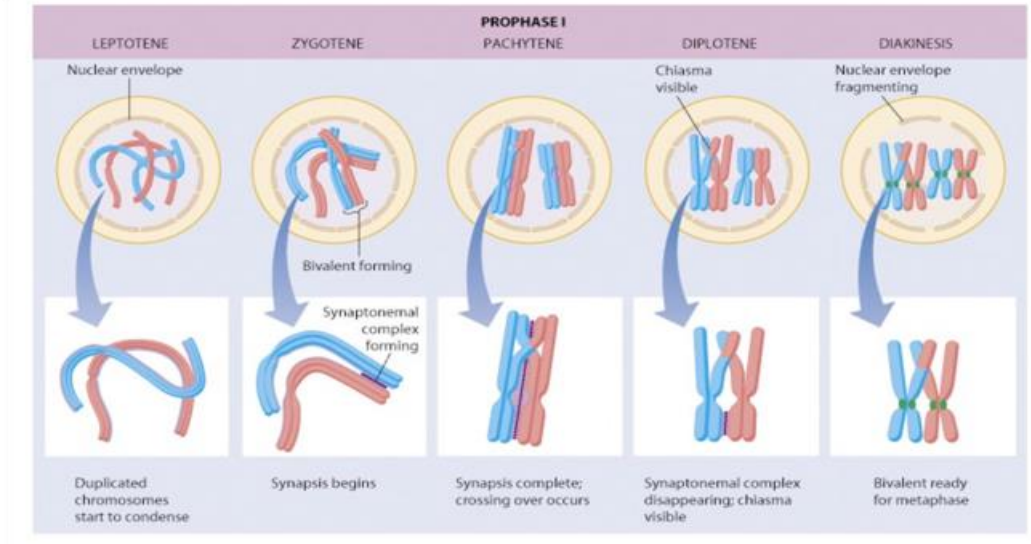


Figure 2: Prophase I of meiosis.

Chromosomes begin to condense in lepotene followed by chromosome pairing and synnaptonemal complex formaltion in zygotene. Next, in pachytene crossing over happens between sisters homologous chromosomes. Chiasma continue through the following stage of diplotene, when the chromosomes begin to align along the equator and synaptonamal complec begin to disappear. Finally in diakinesis chromosomes are fully aligned at the equator, the synaptonemal complex disappear and the nuclear envelop begins fragmentation.[39]

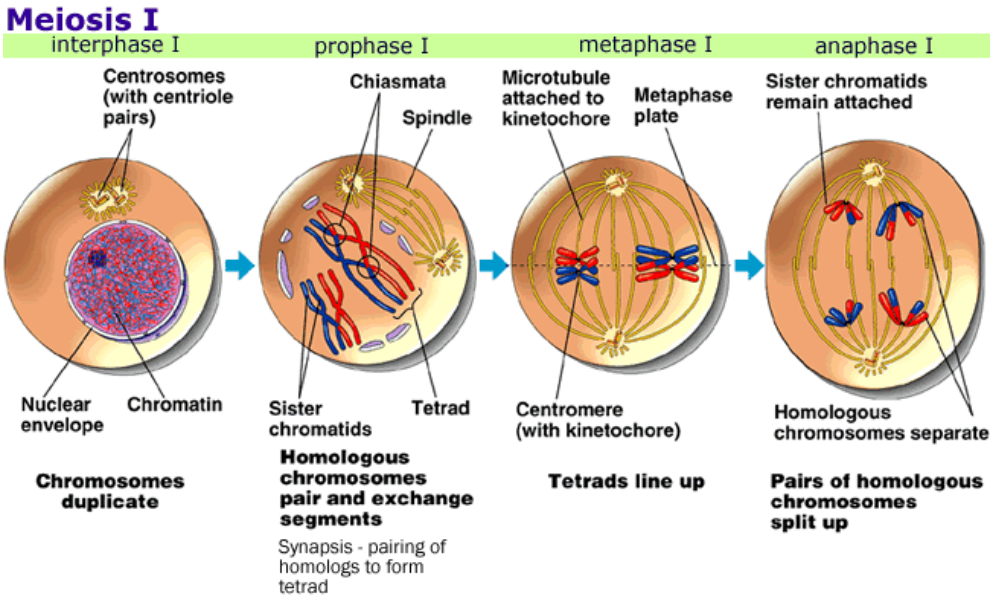


Figure 3: Meiosis 1

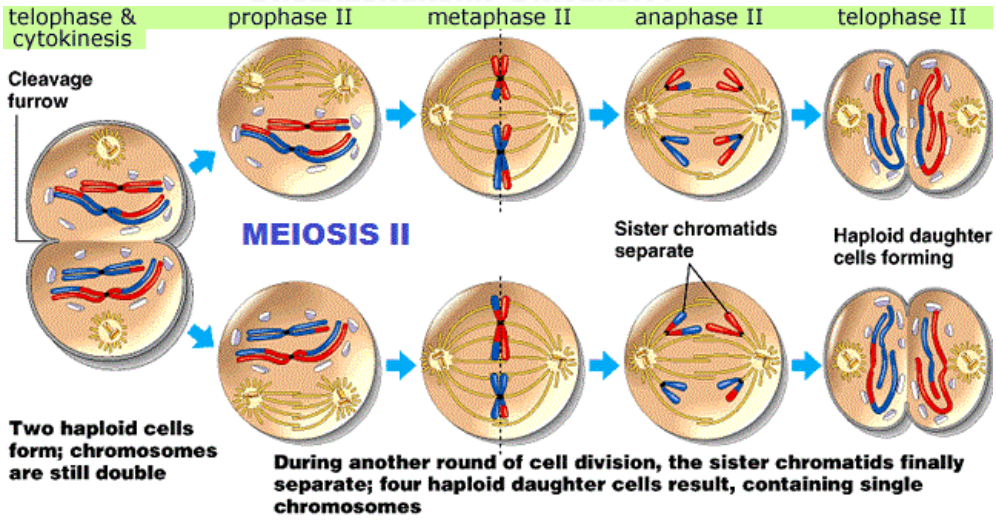


Figure 4: Meiosis 2

Meiosis 1 and 2 During prophase chromosomes pair and decondence and the meiotic spindle begins to form. This is followed by metaphase, in which chromosomes align along the equator of the nucleus and attach to spindle fibers. Chromosomes are then pulled apart in anaphase. During telophase, chromosome arrives at opposite poles in the cell. Meiosis completes with cytokinesis, when the nuclear envelope pinches between the newly formed nuclei to produce two daughter cells. [20]

1.2.2. Spermatogenesis

There are three unique cell types within the testes. Germ cells or spermatogonia are the cells that divide and develop into mature sperm. Sertoli cells are important to spermatogenesis. Sertoli cells are joined together to form a protective blood-testis barrier, preventing any contract between the developing and fragile sperm and the blood stream with all of its potential toxins. Sertoli cells nourish developing sperm and are able to eliminate defective germ cells. Sertoli cells also secrete seminiferous tubules fluid, protein and hormones. Outside of the seminiferous tubule and in the interstitial space is the leydig cell, which produces and secretes testosterone. Beginning at puberty, spermatogenesis happens continuously within the seminiferous tubules. Passing the cell production and division in mitosis and meiosis. The spermatogonia develop into spermatocyte and spermatid. Spermatids are haploid, containing only one copy of each chromosomes. Spermatogenesis takes 72-74 days, with several hundred million sperms reaching maturity daily. Spermiogenesis is the maturation process in which the round spermatids are transformed into elongated spermatozoa with tail. The spermatid nucleus condenses, an acrosome forms that surrounds the head, the cells organize into the midpiece and tail. Spermiation is the process in which fully developed but nonmotiles spermatozoa are released from the sertoli cells and propelled out of the tubules into the collecting tubules, rete testis, and then epididymis. The spermatozoa are immotile at this stage. The epididymis is where sperm mature, concentrate, and stored. When sperms initially enter the epididymis, they are immotile and do not have the capacity to fertilize with oocyte. As sperm pass through the segments of the epididymis. They become motile and capable of fertilization.[40-42]

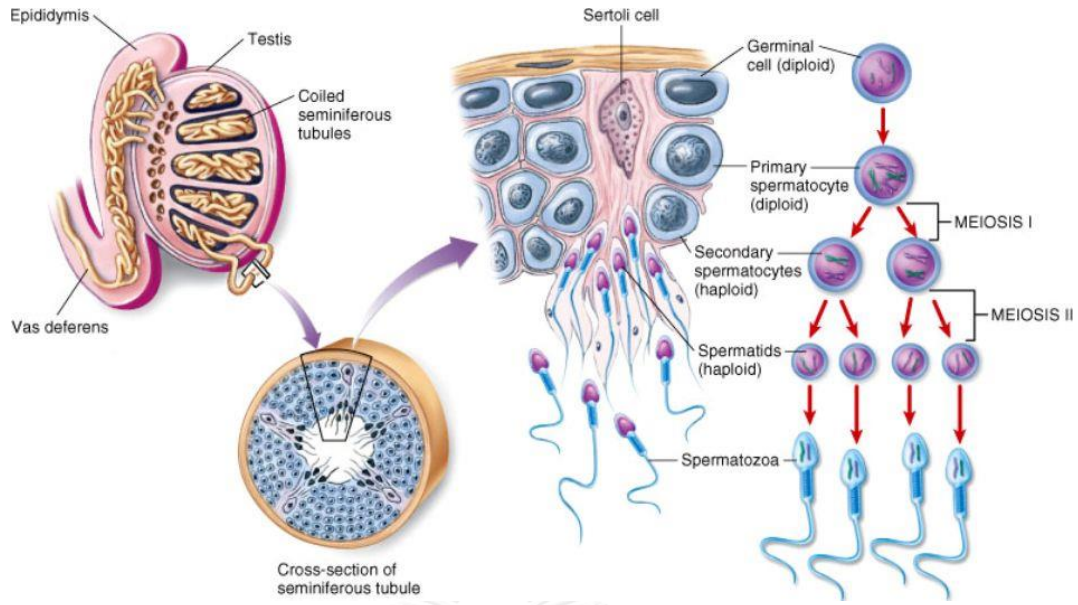


Figure 5: Spermatogenesis

The process of spermatogenesis. In the seminiferous tubules spermatogonia undergo mitotic division to produce haploid primary spermatocytes, which in turn undergo the first meiotic division to produce secondary spermatocytes. Following meiosis II, round spermatid are produced which differentiate first into elongation spermatids and the elongated spermatid before finally differentiating into mature spermatozoa.

1.2.3. Oogenesis

A woman's ovaries and germ cells form during the first few weeks of her embryonic life. These germ cells contain 46 chromosomes and divide by a process called mitosis, so that the new daughter cell contains the same number of chromosomes as the parent cell. During the first trimester of embryonic growth, a pre-oocyte cell is called an oogonium. During the second trimester of female fetal development, the 46 chromosomes in the oogonia start to replicate and separate through the process of meiosis. Meiosis certifies that human have the same number of chromosome in each generation. It contains two processes that reduce the chromosomes by half (from 46 to 23). The primary oocyte was developed from the oogonia during fetal life. The chromosomes of the primary oocyte remain in prophase I until maturation is reduced by the LH surge. Additional chromosome separation and oocyte development are arrested until after a girl reaches puberty. Under the control of luteinizing hormone (LH), the primary oocyte in the most dominant follicle resumes meiosis. Normally, several oocytes are recruited during each menstrual cycle, with just one continuing to mature and be released with ovulation. In response to LH just prior to ovulation, the primary oocyte completes the first meiotic division, resulting in the

formation of a polar body and a secondary oocyte. These remain paused in this phase until after fertilization. Following fertilization, both the secondary oocyte and the first polar body each produce meiotic division. The remaining polar bodies degenerate. If there is no fertilization, the ovulated mature ovum also degenerates.[43]

1.3. Telomere length in gametogenesis

During gameogenesis, telomere has a functiona in making the "bouquet formation" in meiosis. This is characterized by the clustering of telomere at one pole under the nuclear envelop and the dispersion of centromeres at the opposite pole as look like a bouquet of flowers.[44] There are three stages in telomere appears bouquet formation: Nuclear attachment, clustering of telomere and finally movement of telomeres.[44] Telomere led bouquet formation is essential for chromosome paring, recombination and synapsis.[45, 46] A synapsis brings to a collection of double strand break (DSB) repair protein, which are recognised at cell checkpoints, leading to apoptosis. In addition, meiotic silencing of unsynapsed chromatin (MSCU) leads to silencing of autosomal gene essential for survival.[47-49] In addition to its essential during gametogenesis, several studies show evidence that the length of telomere play an important role in meiosis. Telomere length of homologous chromosomes functions in synapsis, homologous recombination, and segregation. Several resulting performing telomerease knockout in mice have shown telomere shortening result in meiotic arrest, segregation error, aneuploidies, and apoptosis. Moreover, telomere loss brings to senescence and apoptosis in spermatocytes and meiotic arrest in oocytes.

1.3.1 Telomere length in spermatogenesis

Telomere length in sperm is variably diverse but not in somatic cells. Moreover, sperm telomere length varies with ages. Several studies have suggested that telomere length in the offspring is paternal inherited. So, telomere length in male gamete is a vital for life. The heritability of telomere length was shown to increase in a study investigating in offspring from older fathers.[50] Telomerase expresses in early germ cell precursor and increase levels of expression from spermatogonia to primary spermatocyte and decreases in secondary spermatocytes. Spermatids and mature spermatozoa do not show the expression of telomerease.[14, 51, 52] Although mature spermatozoa has longer telomeres than spermatogonia, the relationship between telomerase activity and sperm maturity is opposite.[40, 51, 53]

1.3.2. Telomere length in oogenesis

In contrast to spermatogenesis, telomere length reduces in oogenesis.[54-56] This may be explained by the fact that the mitochondria load is altered and ATP is increased during oocyte maturation.[57] This may lead to oxidative stress induced telomere length decrease in the maturing oocyte. Unlike spermatogenesis, telomere is expressed in all stages of oogenesis. In 2001, Wright et al research showed that telomerase expression was highest in immature oocytes but reduce in the maturation and fertilization process.[58] This result was confirmed in later study in cattle and rat by Turner et al in 2013. It is supposed that the highly expressed of telomerase in immature oocytes is important for maintaining telomere length in the cells because telomere leads to synapsis and recombination during meiosis[54]. The studies of telomere lengths and telomerase activity in oogenesis are few. There are some studies in animal models such as bovine and porcine.[51, 59, 60] Meerdo et al., found that telomere length of cumulus cells in the cattle are shorter than oocytes. Moreover, several studies showed that telomerase activity is absent in pre-granulosa cells of primordial follicles, but returns to active when follicles start to grow.[61] It expresses at the highest level in pre-antral and small antral follicles and reduces in later stage of follicular development. The study investigated in pigs, confirmed that telomerase expression is appear in granulosa cells and immature oocytes. During antral differentiation, telomerase expression in cumulus cells and somatic cells in concurrence to the antrum. This result correlated with the lengthen of telomeres only in those cells expressing telomerase in folliculogenesis.[62] Expression of telomerase reverse transcriptase (TERT), which is RNA subunit of telomerase, appeared to rearrange the cytoplasm of oocyte during preantral to antral differentiation of folliculogenesis. This rearrangement of TERT in the ooplasm remains evident in MII oocytes, which is thought to prepare a stock of telomerase protein for the zygote after fertilization.[60, 62]

1.4. Telomere function in fertilization and embryogenesis

After prenetration of spem through cumulus cells, fertilization begins when a spermatozoa attaches with the zona pellucida of oocyte. This attachment results in acrosome reaction which leads to a release hyaluronidase and acrosin, permitting the zona pellucida to be digested and fusion of sperm and oocytes occurs. This action protects other sperm from prenetrating into the oocyte. Then maternal and paternal pronuclei can be appeared and the zygote is activated from a calcium influx.[63] This results in a signaling cascade and completion of meiosis II. Finally the two haploid

nuclei fuse to a diploid zygote.[64] After fertilization, the zygote move through the fallopian tube toward the uterus. During this time it is in mitotic division and produces two daughter cells and continues to divide to four and eight cells in days three after fertilization known as the cleavage stage and each cell in cleavage called blastomere. At the fourth day, blasomeres begin fuses called a morula. In fifth day, the embryo has a cavity and divide into two cell types are trophectoderm and inner cell mass. This stage called blastocyst. Trophectoderm will develop to placenta and inner cell mass is growing to the fetus. During the blastocyst stage, the embryos will hatch from zona pellucida to prepare the process of implantation in the uterus wall.

1.4.1. Telomere homeostasis during embryogenesis

During embryogenesis, the genomes from sperm and ovum combined during fertilization process and replication and cell division and under embryogenesis. Telomere length is 'reset' in order to produce a viable offspring and certify that the telomeric reserve is sufficient to develop a normal and healthy life. After fertilization, telomere length in cleavage stage is shorten comparing to the oocyte.[56] Telomere length more shorthen at the morula stage then increases at the blastocyst stage.[65] Interestingly, Telomere length shows highly degree of variation in single blastomeres in cleavage stage, morula and blastocyst stage.[56] Wright et al in 2001, showed a highly degree variation of telomerase actively in single blastomeres.[66] This may be a result of chromosomal mosaicism or ability and polarity to activate the embryonic genome. There are some studies concerning to telomere in human embryo as the following; Anastasia Mania, et al [67] studied the telomeric length in human embryos at day 4 after fertilization and investigated the correlation between chromosome ploidy, embryo development and patient age. The study show that the embryos in developmentally slow group have abnormal chromosome associated with shorter telomere comparing to normal chromosomes groups (p= 0.0003). Telomere length changed during embryogenesis is shown in Figure 6.

Telomeres in early embryo development and pregnancy

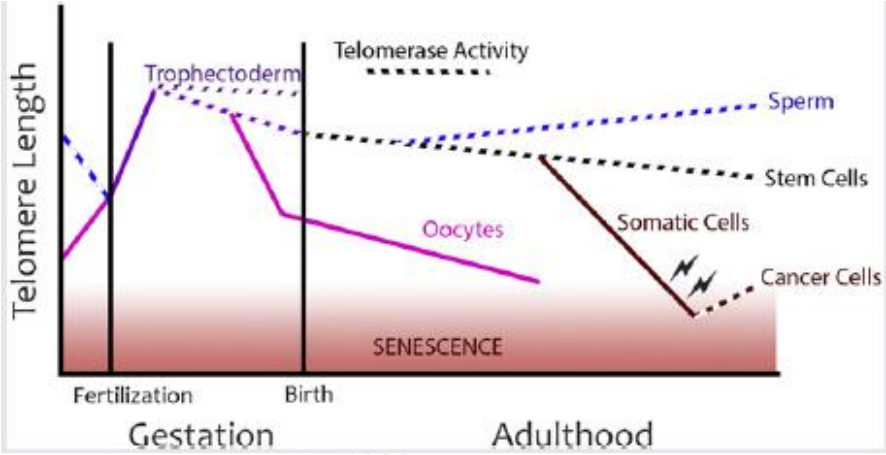


Figure 6: Telomere length in mammalian development

In the earliest stages of preimplantation embryo development, telomeres increase lengthening as shown in the picture.[65]

1.5. Infertility

Infertility is described as a failure to archieve and maintain pregnancy following one year of unprotected sexual intercourse. According to WHO, infertility incidence is around one in six couples, with 38% increasing from maternal causes., 27% increasing from both parent, and 20% increasing from paternal causes. The remaining 15 to 20% are unknown causes.[68] So, the main objective of infertile research is to try to reduce this number. However, the reports of male and female infertility prevalence are variable between literatures. Several studies show that male prevalence infertility is over 50%.[68], other report around 40%.[69] The cause of infertility is difficult to differentiate between true infertility (couple which never achieve pregnancy) and sub-fertility (couple which fertilization is possible but difficulties are experienced). Because of the cause of infertility are complex, It is difficult to identify a true cause from either partner. Infertility occur due to many reasons, for example, physiological cause, hormone dysfunction, infection, lifestyle factors and age.[70-75] Moreover, the cause may due to genetic causes, including of chromosome aberrations, reciprocal translocation, micro-deletion, and DNA damage.[76-79] In addition, a growing level of study in the role of telomeres function in infertility couples continues. Given more importance details of telomeres in organism and genome stability during gametogenesis and embryogenesis discussed in previous section, it confirms to reasons that telomere

length could be involved in fertility. For this reason, it is possible that telomere length may be useful in assisted reproductive technique.

1.6. Assisted reproductive technique

The first baby from in-vitro fertilization technique and embryo transferred was born in 1978. Last decade, ART was developed to help patients that have infertile problem such as endometeosis in female and azoospermia in male. ART is the last choice of treatment because it is expensive and complicated. ART includes techniques such as intrauterine insemination (IUI), *in vitro* fertilization (IVF), Intracytoplasmic sperm injection (ICSI), cryopreservation, preimplantation gnetic diagnosis (PGD) and preimplantation genetic screening (PGS).[80] In case of infertility causing by male origins or in case using sperm donors, IUI offers for this problem. Semen is inserted directly into the recipient and fertilization occurs *in vivo*. In case which the cause of infertility is much more complex. IVF may be help. Briefly, a woman is hyperstimulated by injecton of gonadrotrophins in order to achieve many oocyte are ovalted in one cycle. The assessement by ultra-sonography is used to determine the follicle size then oocytes were retrieved by ultra-sonography transvaginal aspiration of follicular fluid. The mature oocytes are isolated and co-incubated with sperm fertilization occurs *in vitro*. The zygote is cultured to blastocyst before transfer into the uterus.[81] Alternately, in case which sperm motility and concentration is low. The sperm cannot penetrate the oocyte. ICSI is suitable for this solution. Briefly, oocyte retrieval, sperm is injected into the oocyte. The fertilization begining *in vitro*. The zygote is cultured to blastocyst stage before transfer into uterus.[80] Furthermore, genetic analysis of sperm or embryo may be performed. PGD provides for couples with a risk of transmitting genetic disorders. Moreover, genetic analysis may offer the couples who have recurrent miscarriage, implantation failure or woman of advance maternal age (AMA) because of improving the chances of having healthy baby. The well-known of preimplantation genetic screening is the detection of aneuploidy screening.[82], [83] The role of telomeres in genetic stability is to prevent aneuploidy and chromosome pairing and synapsis in meiosis cycle. It is possible that telomere length assessment might be useful in PGS.

1.6.1. Embryonic development and morphological assessment

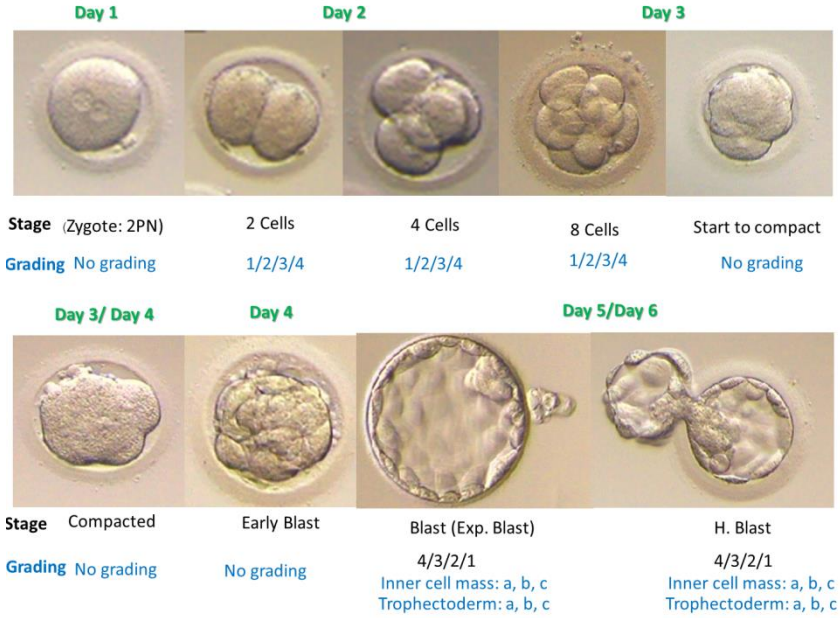


Figure 7: Human embryo development

After the fertilization process, 16-18 hours later 2 pronucleis and 2 polar body are appeared and progressed to normal or abnormal fertilization. The 2PN zygote develops to cleavage stage. It has 4 cells and 6-8 cells on day 2 and 3, respectively. The embryologist assesses non-invasively by using steromicroscope. The assessment of morphological at cleavage stage depends on blastomere size, cytoplasmic fragmentation and multinucleation.

Table 1. Normal embryonic growth[84]

Day	Embryonic stage	Appearance
D1	Fertilized ovum	2 PN(12 hours) and Syngamy(18-24 hours)
D2	Cleaving embryo	2-6 cells: rounded blastomeres
D3	Cleaving embryo Compacting embryo	8-10 cells : rounded blastomeres Blastomeres show evidence of adhesion
D4	Compacted morula Early cavitating	Blastomeres show increased adhesion Beginning of blastocoele formation
D5	Early blastocyst Expanding blastocyst	Blastocoele formed Trophectoderm expanding, Zona thining out, Embryo growing, Blastocoele much increased
D6/7	Late blastocyst Hatching blastocyst Fully hatched blastocyst	Expanded about 150-200 cells Trophectoderm hatching out of zona Trophectoderm and ICM hatched out of empty zona

Table 2: Consensus scoring system for cleavage-stage embryos (In addition to cell number)[85]

Grade	Rating	Description
1	Good	• <10% fragmentation • Stage-specific cell size • No multinucleation
2	Fair	• 10–25% fragmentation • Stage-specific cell size for majority of cells • No evidence of multinucleation
3	Poor	• Severe fragmentation (>25%) • Cell size not stage specific • Evidence of multinucleation

Table 3: Consensus scoring system for blastocyst-stage embryos

	Grade	Rating	Description
Stage of development	1		Early
	2		Blastocyst
	3		Expanded
	4		Hatched/hatching
ICM	1	Good	Prominent, easily discernible, with many cells that are compacted and tightly adhered together
	2	Fair	Easily discernible, with many cells that are loosely grouped together
	3	Poor	Difficult to discern, with few cells
TE	1	Good	Many cells forming a cohesive epithelium
	2	Fair	Few cells forming a loose epithelium
	3	Poor	Very few cells

The scoring system for blastocysts is a combination of the development stage and the grade of Inner cell mass (ICM) and Trophectoderm (TE). It can use alphabet A-C instead of number 1-3 for example expanded blastocyst 3AA

1.7. Preimplantation genetic screening

PGS provides a selection of normal embryos for transfer following IVF and ICSI procedures, to improve the chances of pregnancy in infertile couples. PGS can use genetic sample materials from several sources but there are some limitations and reflective of the embryo development. Genetic sample material for PGS testing include the first and/or second polar bodies derived from mature oocyte, a single blastomere derived from cleavage stage and trophectoderm biopsy from blastocyst stage embryo.

The advantages of polar bodies analysis are to avoid removing the embryonic cells. This technique assesses information on the chromosome number of the oocyte which detects aneuploidy embryo from non-disjunction chromosome number during meiosis 1 of oogenesis. The maternal meiosis error is largely thought to be aneuploidy.

Use of the second polar body in PGS is for determining of inferences of missing maternal meiosis II error. However, the affected oocytes remain have a chance of aneuploidy rescue and the potential in successful pregnancy. Moreover, biopsy of either the first or second polar body cannot detects potential aneuploidy from paternal origins.

The blastomere biopsy on day three after fertilization, provides the investigation of both maternal and paternal components of the developing embryo. Althought the data has been reported with consideration as the safety of embryo biopsy, the evidence shows the removal of one blastomere has no hamful effects on the potential development of embryo. But the blstomere of cleavage stage may be highly mosaic. This means that one of blastomere shows aneuploidy. However, this may not be predicted of the whole embryo.[86]

The trophectoderm biopsy is usually performed on day five or six after fertilization. The embryos have more time to rescue for chromosomal errors derived from maternal or paternal genome. Furthermore, there are more genetic material provided for analysis than previous stage. Trophectoderm develop into placenta without any reflects of inner cell mass which develop into the fetus.

1.7.1 PGS technique

The embryo selection for transfers is an important process in the success of assisted reproductive technique. The reduction of multiple gestations, neonatal morbidity and mortality, the embryologist works to reduce the number of embryos transferred and selected the competent embryos to increase of the successful outcome. Since the early time of ART till the present, the embryos assessment based on embryos selection in the routine has increased the pregnancy rates.[87] In contrast, some studies stated that there are no correlation with the ploidy status and morphology of embryo selection without chromosome screening would transferred an incompetent embryo.[88] The embryo selection by morphology criteria alone cannot show the chromosomal error. Its like “normal looking” an embryo carries abnormality risks.[89] The embryos derived from ART have the most common aneuploidy risk. This problem lead to poor implantation outcomes.[90] Preimplantation genetic diagnosis (PGD) is a method to identify genetic abnormality in embryos before transfer to the uterus. After PGD it is only normal embryo for transfer to get a healthy baby. There three are indications for application of PGD. Firstly, PGD is used for aneuploid screening and chromosome abnormality. Secondly, the structural change or abnormality of chromosome such as translocation and inversion were identifying.

Thirdly, Finding of human leukocyte antigens compatibility for stem cell transplantation in a child. The chromosome in human somatic cells contains 46 chromosomes. (22 pairs of autosome and 1 pair of sex chromosome, n=23). Any gain or loss from this chromosome set is called abnormality or aneuploidy. The human preimplantation embryos contains aneuploidy have a major risk in implantation failure, pregnancy loss and abnormal live birth.[91] Although the aneuploidy rate correlated with the increase maternal age, PGD result showed the high incident of aneuploidy and mosaicsm chromosome in both young and advance maternal age groups.[92] There are several methods being used for the screening of aneuloidy in human embryos such as FISH (Fluoresence *in-situ* Hybrization), aCGH (array Comparative Genomic Hybridization), SNP-arrays, Real-time PCR, and Next-Generation Sequencing.

1.7.1.1 Fluoresence In-situ Hybridization (FISH)

In the FISH technique, DNA probes were labeled and hybridized with fluorescence tags that specific for chromosomal regions. FISH consists of the following steps: fixation of biopsied cell, denature, hybridization, washing, staining step and analysis the result under fluorescence microscope. The advantage of FISH technique is a conventional and rapid because it does not required cultured metaphase chromosome. The limitation of FISH inability to detect all 24 chromosomes and mainly technical specially. Moreover FISH panel not efficient in all trisomy chromosome that cause spontaneous abortion. FISH was first used for PGD in 1994, then PGS quickly developed. Many studies showed that FISH technique in PGS is not beneficial. Since then, several techniques have been developed.

1.7.1.2. Comparative Genomic Hybridization

CGH has an ability to detect all 24 chromosomes from embryo biopsy. CGH is a method used to compare the fluorescence intensity between DNA samples and reference DNA. The DNA was hybridized on probes that imprinted on glass slide. CGH is highly accurate. However, it has clinical limitation in the fields of PGD. For example, it cannot distingquish the error maternal and paternal or discrimination between meiotic and mitotic error of chromosome segregation. This method predicted on high-quality DNA and successful hybridization. The result possibility showing borderline could be miscalled. More recently, other techniques have been developed with the increasing of accuracy for PGD, including single

nucleotide polymorephism (SNP) microarray, multiplex qRT-PCR, and next-generation sequencing.

1.7.1.3. Next-generation sequencing (NGS)

Next-generation sequencing (NGS) is an advance technology in the molecular science of the last 30 years. The second generation sequencing platforms can sequence in a single instrument run in two weeks for whole genome sequencing. The NGS can sequence the whole genome in 6 hours. The principle of NGS is similar to capillary electrophoresis (CE). NGS platforms use a feature massive parallel sequencing. The genomic DNA was cut in short fragment and tagged with barcode sequence. The short products were amplified and size selected before sequencing. The advantage is rapid sequencing of large genome. It is suitable for small to medium size laboratory. In this research use MiSeq (illumia).

Principles of MiSeq are Tamplate DNA is fragmented into shot length of hundred base pair and tagged with oliginucleotide adapter. The adapter nucleotides are complementary to the flow cells anchors. The single-stranded DNA is added to the flow cell and immobilized by hybridization to the anchors used as DNA template. Templates are amplified in the flow cell by “bridge” amplification as show in the picture 2. Multiples amplification cycles convert the single-stranded DNA template to a clonally amplified arching “cluster” with each cluster containing approximate 1000 clonal molecules. For sequencing, the cluster are denature and a subsequent chemical cleavage reaction and wash leave forward strands for single-end sequencing. Sequencing of the forward strands is initiated by hybridizing a primer complaementary to the adapter sequencs, which followed by addition for polymerase and a mixture of 4 differently colored fluorescent reversible dye terminators. The terminators are incorporated according to sequence complementarily in each strand in a clonal cluster. After incorporation, the clusters are interrogated and the fluorescence is recorded.

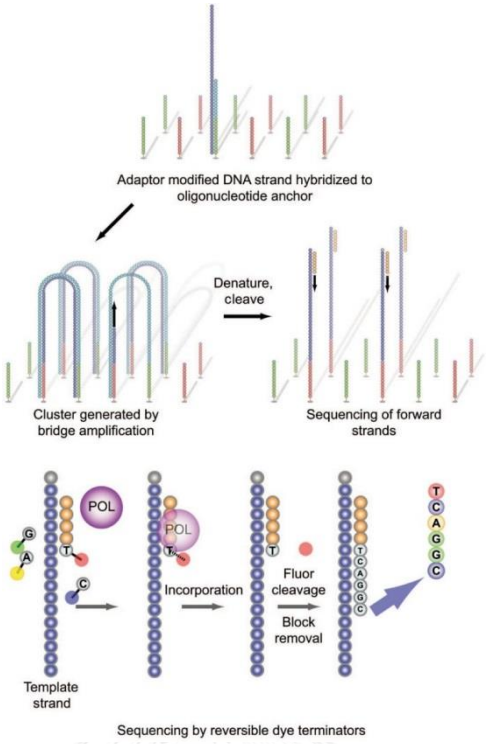


Figure 8: Bridge amplification

single-strand DNA is added in flow cell and immobilized by hybridization with anchors. Bridge amplification generates clonally amplified cluster. Clusters are denatured and cleaved. Then sequencing is initiated with addition of primer and 4 reversible dye terminator.

PGS in ART has been shown to effectively improve the pregnancy rate. Several studies reported that PGS does improve the success rate of pregnancy, other also reported the negative effects of PGS. The debate of using PGS in ART remains inconclusive the result seeking for new tools and techniques to assist in ART continues. The author then suggested that telomere functional analysis provide a novel PGS tool in human ART.

1.8. Telomere and reproductive

Many reports made consideration telomere homeostasis in normal reproduction. Several studies determined telomere function in infertility couples. Telomere dysfunction in infertile patients has been detailed. These support the useful of telomere length evaluation in PGS.

1.8.1. Telomere length and female reproductive aging

The loss of reproductive capacity according to aging is only found in women. Fertility in woman begins to decrease the middle age. This leads to the risk of pregnancy loss, miscarriage, and chromosome abnormalities. Theoretically of reproductive aging that effected to oocyte dysfunction in woman is mostly a result of telomere defects. Telomere shortening in egg promotes genomic instability, apoptosis and cell cycle arrest.[97] Telomere function is necessary for meiosis. During the early prophase of meiosis, telomere helps homologous chromosomes pairing and begins the synapsis and chiasmata. Normal segregation of chromosome in adult is important to fetal life. Liu L, et al reported that the telomere shortening reduced the synapsis and recombination process in mice.[52] Several studies reported telomere length in human oocytes and embryos, for instant, development as follows; Keefe DL, et al compared telomere length in human oocytes form pregnant and non-pregnant women after IVF treatment. They found telomere length from failed pregnancy was shorter than success pregnancy ($p< 0.005$).[8]

Nathan Treff, et al [7] reported the correlation of the telomeric DNA content andembryo development and aneuploidy chromosome status. The study showed that aneuploid polar body displayed significant lower quantities of telomeric DNA than euploid polar body ($p = 0.016$). Aneuploid blastomeres exhibited a significant reduction of telomeric DNA comparing with euploid blastocyst ($p = 0.002$). Aneuploid and euploid blastocyst trophectoderm displayed similar quantity of telomeric DNA ($p = 0.340$). David L. Keefe, et al [98] studied the effect of telomere length on cytoplasmic fragmentationand morphologic appearance of preimplantation embryo. The study showed that telomere length was not significantly related to cyotplasmic fragmentation in day 2 embryos. They also found a decrease in telomere length the embryos. Ghosh S., et al studied the length of telomere in leukocyte from older mother who gave birth to Down

syndrome children. They found that telomere length in the Down syndrome mothers was significantly shorter than mothers who have normal children at the same age mother groups.[99] Moreover, women who have unexplained recurrence pregnancy loss havealso had a shorter leukocyte telomere length comparing to women who have success in pregnancy at the same age groups.[100] Further studies have also reported that the older mothers who gave who Down's syndrome children have significantly shorter telomeres than the matched age control groups.[101], [99]Very few studies have detailed the role of telomere distribution in oocyte of infertile woman due to the difficulty of access to oocytes The study in mice has shown that SUN1 mutations in sperms and oocytes have similar outcome by showing the absence of mature oocyte and impaired chromosomal alignment because of the impaire meiotic division.

Many studies have shown the association of maternal age and the shorten of telomeres of oocytes , generally, they are shorter than somatic cells even more shorten with age [69], [102], [103], [71]. These lead to the hypothesis that oocytes of advance maternal age with a shorter telomeres are unable to support fertilization and embryogenesis. It is also thought that the advanced maternal age involve a high risks associated pregnancy and live birth due to implantation failure or abnormal chromosome development in embryo.[71]The maternal age affects reproductive potential in older age (above 35 years). It brings to some factors including a dwindling in the number of oocytes and a reduction in oocyte quality.[95, 104-106] Moreover, oocytes from older woman are more prone to have meiosis errors, leading to aneuploidy embryos. This is known as a major cause of the first trimester pregnancy loss in advance maternal age.[107] Current finding have described two mechanisms involving causes of this defect. Firstly, the oocyte appear to be produced during fetal oogenesis and produced from primary cells that have undergone many mitotic divisions which also lost a number of telomere repeats due to the end replication problem [108-110]. Secondly, female gametes exposed to a long time collective oxidative stress during oogenesis and ovulation. Since telomeres are rich in guanine (G) and oxidative stress induce telomere shortening, this bring to a reduction of reproductive potential.[110-114] It has also been shown that telomere depletion caused an abnormal chromosome fusion and apoptosis, mostly, associated with mitochondrial dysfunction [115]. Additionally, the shortening of telomere length and low telomerease activity in the meiosis II oocytes in older mice have been shown to related with a higher level of reactive oxygen species (ROS) comparingto younger mice [116].

1.8.2. Telomeres and male reproduction

Telomere length of sperm increase by the action of telomerase which is continued action of telomerase. The expression of telomerase is high in spermatogonia in order to maintain the longer average telomere length. The variation of telomere length in sperm varies in individual men and individual spermatozoa.[117, 118] The offspring from advanced paternal ages have the increase telomere length [119]. The effect of paternal age in telomere length can be found across multiple generations [53]. The study in mouse model showed chemically induced obstruction to the chromosome organization in sperm nucleus failed the embryogenesis [120]. Moreover, mutation of SUN1, a gene accountable for building telomere led bouquet formation of chromosome during meiosis, results in impaired synapsis, crossing over, and finally apoptosis.[121] Some indirect evidence support that male infertility associated with nuclear organization that showed the loss of telomere-telomere interaction which lead to telomere led bouquet formation.[57, 122, 123] In mouse model, shortened telomeres in sperm and telomerease deficiency are associated with male infertility.[52, 124, 125] Moreover, a reduction of sperm motility and sperm concentration in mice are associated with shorten telomere length as a result of telomerease deficiency.[125] DNA sperm fragmentation in male mice is correlated with the shorten telomere length [126].

Correlative studies of telomere length and male fertility are very few. Some studies reported a positive correlation of sperm competence and telomere length and telomere length, others have not. For example, Santiso et al reported that sperm selected by swim-up technique had longer telomeres and less DNA damage.[117] However, some studies found no correlation between telomere length and DNA fragment in any semen parameters.[57, 127]

1.8.3. Telomere and ART outcome

Many studies have assessed telomere length in human embryo. The result showed that telomeres are shorter in cleavage stage of aneuploid embryos than euploid embryos. However, there is no difference in euploid blastocyst when comparing to aneuploid blastocyst. This suggests that telomerase mediated telomere lengthening is due to genome activation in blastocyst stage.[7] As the same result from Mania et al. (2014) and Treff et al. (2011b) reported the indifference of telomere length

in blastocyst embryos and arrested embryo. It is suggested that telomere length dose not have an important role in developmentally competent embryos .[7, 70] The study of Mania et al in 2014 also noted that telomere length in embryo from woman with advance maternal age and have a history of recurrent miscarriage had trended shorter telomeres. Similarly, the result from Hanna et al.(2009) showed the association of with the shorter telomere length from leukocyte telomere length in parent with recurrent miscarriage in comparison with control cases.[100] Studies determining correlation between embryo quality and telomerase activity found that telomerase activity associated with the poor development of cleavage and blastocyst stage embryos. However, the overall level of telomerase expression did not changed. The present of some variations may effects telomere function.[128] This result was confirmed by the study of Wright et al, (2001) which showed the lower telomerase activity in multinucleated zygote comparing to normal pronuclear zygotes and arrested embryos in cleavage stage. There was no significant change of telomerase activity in comparison with developing blastocysts. These finding started that the level of telomerase activity is not correlated with the potential of embryo development.[61]

1.9. Measuring telomere length

Telomeres are complex structure. Measurement of telomeres is challenging. Moreover, it is also complicated because of the limited in samples size for telomere length analysis such as in biopsied cell from embryos. Several methods are deployed for measuring the length of telomere. Each has its own pros and cons in which may affect the interpreting results.

1.9.1 Telomere restriction fragment analysis

Telomere restriction fragment analysis (TRF) was developed by Calvin Harley in 1990s to measure telomere restriction fragment and calculate the average of telomere length. The process uses restriction digestive enzyme for digesting non-telomeric DNA and leaving only telomere sequence. DNA fragments containing telomere sequence are separated by gel electrophoresis and following by standard southern blotting technique.[129, 130] The advantages of this technique are the simplicity of technique, the minimal equipments required and a cheaper cost. Additionally, several optimized commercial kits are now available. However, there are some disadvantages of TRF. The separation of telomere fragments using agarose gel

electrophoresis causes two problems. Firstly, complete DNA digestion is necessary in term of avoiding over measuring telomere length. Secondly, the inclusion of non-telomeric sequence close to telomere can cause of a variation in telomere length.

1.9.2 Slot blot analysis and hybridization protection assay

The slot blot base technique developed in 1997 is used to replace some difficulties of TRF analysis. This technique does not required restriction enzyme digestioin and agarose gel electrophoresis. Technically, the telomere specific probe is applied on the membrane for exposure step, following exposure, telomere probe is washed off and recorded the signal intensity. Centromere specific probe is also applied, exposed, and recorded signal intensity in the same way and blotted on a nylon membrane. A telomere to centromere (T/C) ratio can be calculated from the intensity of two signal detected.[131] Similar to the hybridization protection assay (HPA), this technique uses alu sequence instead of telomere sequence to calculate a ration of telomere to alu sequence.[132] The advantages of this technique is the ability of avoiding any artefacts that may caused from non-telomeric DNA fragment. Moreover, calculation of T/C ratios (telomere/alu ratio) avoids any inaccuracies in DNA volume before loading or incomplete transfers of DNA on nylon membrane. The DNA quality does not affect the accurate quantitation of telomere length by slot blot or HPA. This technique is also suitable a small DNA volume. It uses only 10-20 nanograms of DNA volume to generate effective signals. However, it has been reported the lack sensitivity and reproducibility in repeat experiment is the disadvantage of this technique.[133]

1.9.3 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) is used for quantitation the copy numbers of a target sequence. The cycle threshold (Ct) is directly equal to the number of copies of target sequence in the sample. In 2002, qRT-PCR was used for telomere length analysis by calculating T/S ratio which determine a reference DNA sample in ratio of telomere sequence copy number (T) and a single copy gene copy number (S). The advantage of using qRT-PCR is the clear and accuracy of T/S ratio interpretation. [11] Additionally, non-telomeric sequences are not required to estimate relative telomere length because the primers are designed for specific sequence of interest. The qRT-PCR is highly sensitive and only five nanograms of DNA required for the experiment. This technique is less time consuming, generally can be

completed in three hours. The disadvantage of this method is the limited detail references to telomere lengths of individual chromosomes.

1.9.4. Single telomere length analysis

Single Telomere Length Analysis (STELA) is a ligation polymerase chain reaction (PCR) technique, developed specifically to determine the telomere length of individual chromosome. Briefly, this process involves designed linker at G rich 3' overhang of the telomere, which consists of 7 base complementary with the telomere hexameric repeat following by a 20 non-telomeric base. The linker is bond to C rich complementary 5' strand and creating a tag on the telomere. The PCR reaction is including of a 'teltail' primer and primer specific to sub-telomere upstream. After PCR amplification, the products are run on the agarose gel and quantified by southern blotting. [93, 94] Several studies used STELA for measuring of telomere of several chromatids such as Xp, Yp, 2p, 11p, 12p, and 17p. [94-96] The advantage of this method is able to measure a range of telomere length of interested chromatids. Furthermore, less DNA required (only 10 nanograms) is also beneficial. However, it has a limitation in detecting long telomeric DNA sequences. Less than 20 kbps of telomeric DNA is allowed to give thebest result due to limitations of the amplification step. [94]

1.9.5. Quantitative fluorescence in situ hybridization

Quantitative fluorescence in situ hybridization (Q-FISH) modified from standard Fluorescence in situ hybridization (FISH). This process is DNA labelled with specific probes that complimentary to sequence of interest and detected by visualizing where sequence is in the genome. Q-FISH is advantage technique for telomere length analysis because it can analysis telomere length from each chromosome in the cell population. Probe is specifically to the telomere sequence only, so sub- telomeric regions are exclude. The disadvantage if Q-FISH such as it is required expensive equipment. Telomeres of individual chromosomes can be detected only metaphase chromosome so it is required good quality metaphase preparation.

Research Question

1. Is the telomere length different in human blastocysts different in maternal age groups?
2. Is the number of teromeric repeats different in human blastocyst different in maternal age groups?

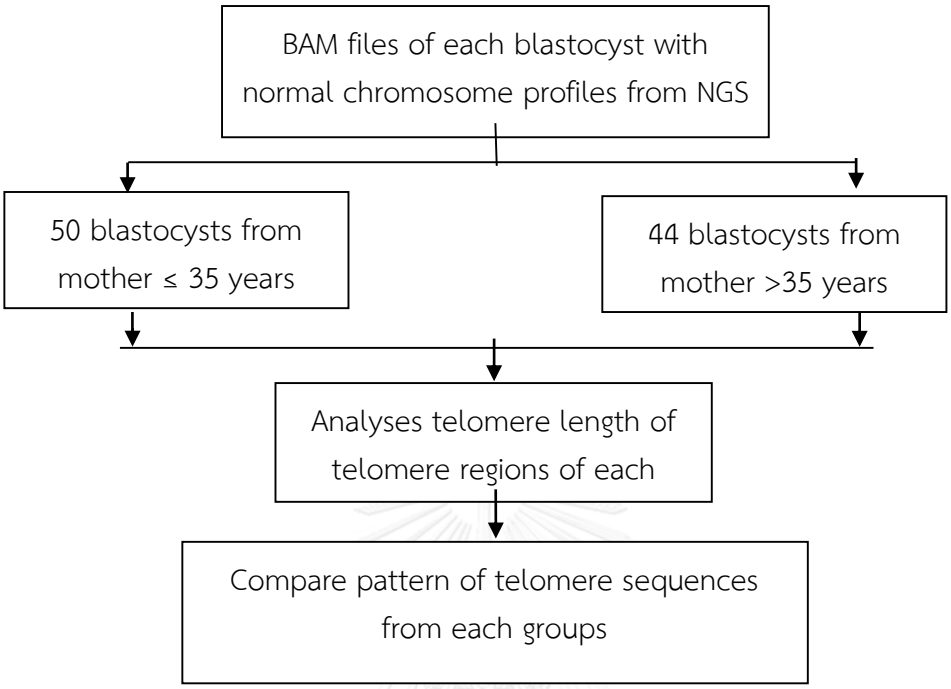
Objective

To compare the telomere length and telomeric repeates in human blastocyst between maternal age groups in infertile couples by next-generation sequencing.

Hypotesis

The telomere length and the number of telomeric repeats telomeric regions in human blastocysts are different between maternal age groups in infertile couples.

Conceptual Framework



Abbreviations/Operational definitions[84]

Assisted reproductive technology (ART): All treatments or procedures that include the *in vitro* handing of both human oocytes and sperm of embryos for the purpose of establishing a pregnancy. This includes, but is not limited to, *in vitro* fertilization and embryo transfer, gamete intrafallopian transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy. ART does not include assisted insemination (artificial insemination) using sperm from either a woman's partner or a sperm donor.

Blastocyst: an embryo, 5 or 6 days after fertilization, with an inner cell mass, outer layer of trophectoderm, and a fluid-filled blastocele cavity.

Cryopreservation: the freezing or vitrification and storage of gametes, zygotes, embryos, or gonadal tissue.

Copy number variation (CNV): A copy number variations were the number of copies of a particular gene varies from one individual to the next. Following the completion of the Human Genome Project, it became apparent that the genome experiences gains and loss of genetic material. The extent to which copy number variation contributes to human disease is not yet known. It has long been recognized that some cancers are associated with elevated copy number of particular gene.

Delivery: the expulsion or extraction of one or more fetuses from the mother after 20 completed weeks of gestational age.

Embryo: the product of the division of the zygote to the end of the embryonic stage, 8 week after fertilization.

Embryo transfer: the procedure in which one or more embryos and placed in the uterus or fallopian tube.

Fertilization: the penetration of the ovum by the spermatozoon and combination of their genetic material resulting in the formation of a zygote.

Hatching: The process of an embryo at the blastocyst stage separates from the zona pellucid.

Implantation: the attached and subsequent penetration by the zona-free blastocyst (usually in the endometrium) that starts 5 to 7 days after fertilization.

***In vitro* fertilization (IVF):** an ART procedure that involves extracorporeal fertilization.

Infertility (clinical definition): a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse.

Intracytoplasmic sperm injection (ICSI): a procedure in which a single spermatozoon is injected into the oocyte cytoplasm.

Live birth: the complete expulsion or extraction from its mother of a product of fertilization, irrespective of the duration of the pregnancy, which, after such separation, breathes or show any other evidence of life such as heart beat, umbilical cord pulsation, or definite movement of voluntary muscles, irrespective of whether the umbilical cord has been cut or the placenta is attached.

Micromanipulation: a technology that allows micro-operative procedure to be performed on the spermatozoon, oocyte, zygote, or embryo.

Preimplantation genetic diagnosis (PGD): analysis of polar bodies, blastomeres or trophectoderm from oocytes, zygotes or embryos for the detection of specific genetic, structural, and/or chromosomal alteration.

Preimplantation genetic screening (PGS): analysis of polar bodies, blastomeres or trophectoderm from oocytes, zygotes or embryos for the detection of aneuploidy, mutation, and /or DNA rearrangement.

PESA: percutaneous epididymal sperm aspiration.

Recurrent spontaneous abortion/ miscarriage: the spontaneous loss of two or more clinical pregnancies.

Spontaneous abortion/ miscarriage: the spontaneous loss of a clinical pregnancy before 20 completed weeks of gestational age (18 weeks after fertilization) or, if gestational age is unknown, the loss of an embryo/fetus of less than 400 g.

Telomere: a region of repetitive nucleotide sequences at each end of a chromatid, which protects the end of the chromosome from deterioration or from fusion with neighboring chromosomes.

TESE: testicular sperm extraction

Zygote: a diploid cell resulting from the fertilization of an oocyte by a spermatozoon, which subsequently divides to form an embryo.

Research Design

Retrospective Cohort study

Chapter II

Materials and Methods

Sample collection

This study was performed in subjects who selected in assisted reproductive technique and preimplantation genetic screening program at Safe Fertility & PGD Center during November, 2014 to January, 2015. BAM files were selected based on inclusion criterias. Biopsies of blastocysts on day 5 or day 6 after insemination were collected for chromosomal screening by next-generation sequencing technique. Patients underwent chromosome screening were based on reasons in accordance with advanced maternal age (> 35 years) or couple with recurrence miscarriage or implantation failure. This study was approved by the Reasearch Ethic Review Committee for Research Involving Human Research Participant, Health Science Group, Chulalongkorn University (RECCU) Thailand (COA 162/2015)

Inclusion criteria lists;

- Patients with ICSI and NGS.
- Patient's race is Asian.
- Blastocysts with no aneuploid chromosomes from NGS result and biopsied on day 5 or 6.
- BAM files were selected from NGS runs and samples with QC value in acceptance criteria as follow; Data files uses from old result only.

Table 1: Summary of VeriSeq PGS-MiSeq Acceptance Criteria

	Parameter	Optimum Value	Acceptance Value	Source of Values
Amplification Test	Gel Electrophoresis	Positive	Positive	Laboratory tests
	Concentration dsDNA ng/µl (single cells)	25 – 35	> 10	
Flow Cell-based Parameter	Density (K/mm²)	1200 –1400	1100 – 1600	"Run summary" page on BaseSpace or "Summary" in SAV
	% of clusters passing filter (PF)	85	75	
	Total number of reads	30,000,000	25,000,000	
	Total number of reads (PF)	25,000,000	19,000,000	
	% of reads reaching Q30	95	90	
	Maximum phasing/pre-phasing	0.5 / 0.05	0.65 / 0.1	
Sample-based Parameter	% of reads identified (PF)	95	85	"Indexing QC" page on BaseSpace or "Indexing" in SAV
	% of reads identified (PF) per sample	5	2.5 – 8	
	% of reads identified (PF) for empty wells	< 0.025	< 0.05	
	Number of total reads	1,000,000	700,000	Downstream analysis from BlueFuse Multi
	Number of reads after filtering	500,000	250,000	
	% of total reads after filtering	> 50	> 35	
	Average quality score (Q-score)	> 35	> 30	
	Average alignment score	> 35	> 30	
	Sample noise score (DLR)	0.2	< 0.4	
Chromosome-based scores	Region Confidence (value in CNV Table)	1.0	> 0.7	Downstream copy-number analysis from BlueFuse Multi

Figure 9: Summary of VeriSeq PGS-MiSeq Acceptance Criteria

Please see detail in appendix

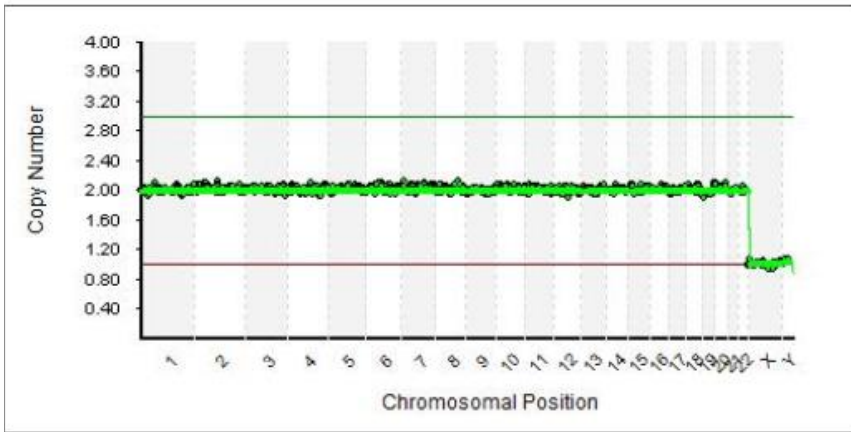


Figure 10: Normal male embryo from NGS results.

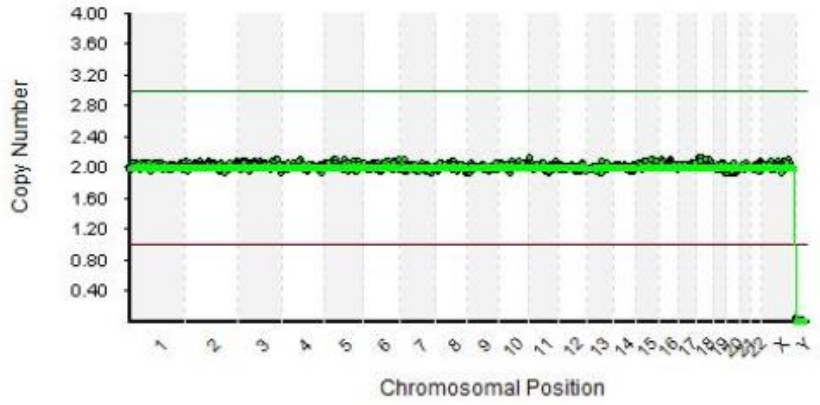


Figure 11: Normal female embryo from NGS results.

Exclusion Criteria

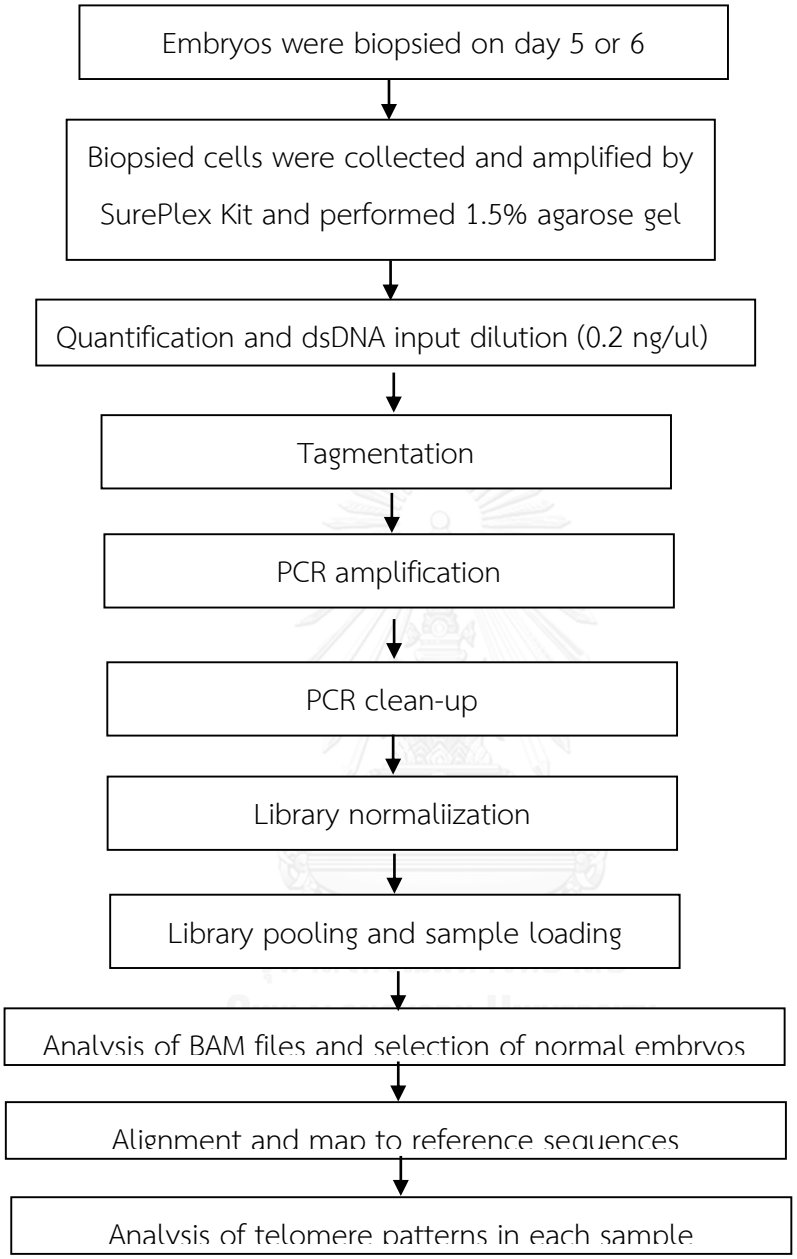
- NGS profiles show structural chromosome abnormality.
- Patient were performed TESE, PESA, IUI and IVF
- NGS runs and samples are not in acceptable range.

1. Sample size

1. 50 blastocysts from mother ≤ 35 years old.
2. 44 blastocysts from mother > 35 years old.

The reference of sample size is based on the previous study of Zhihao Ding et al. 2014. [97]

Research Methodology



Data were extracted from the results of routine work.

Embryo culture and Biopsy procedure

After intracytoplasmic sperm injection (ICSI), embryos were cultured in microdrops of medium under culture oil at 37 °C in 5% CO_2 incubator. On day 3, all embryos were assisted hatching and separated to culture individually in a micro drop containing blastocyst medium until day 5. On day 5 and/or day 6, the hatching blastocysts were selected by embryologist for biopsy by vitrification technique following by the freezing step for further application.

Cell lysis and whole genome amplification

The biopsied cells were washed and placed in to PCR tube for a NGS testing. All biopsied cells and negative control were lysed and genomic randomly amplified using SurePlex DNA Amplification System (illumina). These were processed in PCR room. The amplification master mixed were added (SurePlex amplification kit, illumina) and amplified by using Thermal cycle machine. Briefly, biopsies collected in 2.5 ul of 1x PBS were lysed by SurePlex cell extraction buffer and 5 ul of SurePlex extraction master mix added and incubated at 75°C for 10 minutes and 95 °C for 4 minutes. The pre-amplification step, the 5 ul of master mixes were added in to biopsied samples, genomic control and negative control and incubated by the following protocol: one cycle of 95 °C for 2 min, 12 cycles of 95 °C for 15 sec, 15 °C 50 sec, 25 °C for 40 sec, 35 °C for 30 sec, 65°C for 40 sec, 75 °C for 40 sec, followed by a hold at 4°C. After that, the 60 ul of master mix (SurePlex amplification cocktail) was added to each tube. The mixtures were amplified according to the program: one cycle of 95 °C for 2 min, cycles of 95 °C for 15 sec, 65 °C for 1 minute and 75 °C for 1 minute, hold at 4 °C. The PCR products were blotted on 1.5% agarose gel electrophoresis to confirm products size and verify the positive and negative controls Images were captured and analyzed by Gel Doc machine.

Preimplantation Genetic Screening

96 blastocysts from 93 Asian couples were biopsied to collect TE cells in PCR strips and further processed whole genome amplification (WGA) using Sureplex amplification kit (illumina). WGA product (1-2 ug) was used for library preparation according to the protocol (illumina) after measuring the single strand concentration. All samples were processed for high-throughput sequencing using Miseq Illumina.

Next-generation sequencing analysis

1. Quantification of Unpurified SurePlex Products

This step used the dsDNA High-Sensitivity (HS) Assay Kits to quantify the dsDNA content of unpurified PCR products. By entering the calculated dsDNA concentration of the 1/10 diluted PCR product into the "VersiSeq PGS Assay Plate" of the BlueFuse Workflow manager to help calculate the dilution of PCR product with water to achieve diluted DNA at final concentration of0.2 ng of dsDNA per microliter.

2. Tagmentation of Input DNA

In this step, the PCR product is tagmented and fragmented by transposome. The transposomes contemporary fragments the PCR product and add adapter sequences to the ends. The quantified dsDNA at 0.2 ng/ul was added to 5 ul of Amplicon Tagmentation Mixture (ATM) and 10 ul of Tagmentation DNA buffer (TD). The tangmentation step was incubate at 55°C for 5 min and hold at 10 °C The tagmented mixture was neutralized by adding 5 ul of Neutralization buffer (NT) and leave on room temperature at 5 min.

3. PCR Amplification

The tagmented PCR product is amplified by a limited-cycles PCR program. The PCR step is also included index 1 and index 2 and sequence for cluster formation. The tagmentation product was further added of 5 ul of index 1 (i7), 5 ul of index 2 (i5) and 15 ul of Nextara PCR Master Mix (NPM) to each tube. The mixture was amplified by PCR program: one cycle of 72 °C for 3 min, 12 cycles of 95 °C for 10 sec, 55 °C for 30 sec and 72 °C for 30 sec, one cycle at 72°C for 30 sec and followed by a hold at 4°C

4. PCR Clean-up

After the PCR process, PCR products were purified by AMPure XP beads and provide a size selection step to remove shorts library fragments and primers from the population. Briefly, add 45 ul of PCR product into deep well plate containing 45 ul of AMPure XP beads Mixed, sealed plate and put on plate shaker at 1,800 rpm for 2 min. Then incubate at room temperature on magnetic strand without shaking for 5 min until supernatant cleared. Kept plate on magnetic strand and wash twice with 80% alcohol freshly prepared. The purified libraries were eluted from magnetic beads with 50 ul of Nextara XT Resuspension Buffer.

5. Library Normalization

Clean-up dsDNA was normalized to achieve the quantity of each library ensuring more equal library representation in pooled samples. The normalized library consisted of single-stranded DNA (ssDNA). The dsDNA purified libraries were denatured to give ssDNA by addition of 0.1N NaOH. In the first step, preparation of the LNA1 (Library Normalization Additive 1) / LNB1 (Library Normalization Beards 1) by addition of LNA1 1.1 ml and LNB1 200 ul in 5 ml eppendrop tubes and mixed well by inverting the tube 10-15 times. Using the multichannel pipette 45 ul in deep well plate and added PCR clean-up product 20 ul sealed and shake on micoplate shaker 1,800 rpm 30 min. Put plate on the magnetic strand for 2 min or until the supernatant cleared. Discarded the supernatant. Added LNWI (Library Normalization Wash 1) 45 ul and put the plate on the microplate shaker at 1,800 rpm for 5 min. Put plate on the magnetic stand for 2 min removed the supernatant and repeat this step once time. At the last time after discarded supernatant remove the plate form magnetic strand and added 30 ul of fresh prepare 0.1 N NaOH put plat on microplate shaker at 1,800 rpm for 5 min and put on the magnetic strand for 2 min. Then use multichannel pipette to transfer 25 ul of supernate into the plate containing of 25 ul of LNS1 (Library Normalization Storage Buffer 1) and mixed. The product of this step contained single-strand DNA.

6. Library Pooling and Miseq Sample Loading

In this step, the preparation of cluster generation and sequencing was performed. The equal volumes of normalized library were combined, diluted in Hybridization Buffer, and heat denatured before sequencing. This step began with the transfer 5 ul of each samples to pool into a low-binding DNA tube following by the transfer of 20 ul of pool library into the clean PCR tube. Addition of 80 ul of HT1 (Hybridization buffer 1) and placing in to the Thermal cycler and run the following program: 96°C for 3 min, 4 °C for 5 min and hold at 4°C. Then transfer the 100 ul of cold and denature pool/ HT1 mixed to 600 ul of HT1. Using 1 ml pipette transfer pool library to cartridge.

7. Data collection

Cycle characteristic from each couples including the number of oocytres retrieved, MII oocytes, fertilization rate, and chromosomal screening results were collected and recorded by the author.

8. Bioinformatics analysis of telomere length

Primary analysis of the NGS data was performed by MiSeq reporter software version 2.6 (Illumina) to obtain FASTQ and BAM files. The secondary analysis was performed by using BlueFuse multi software version 2.2 (Illumina) to provide molecular cytogenetic and *in vitro* fertilization (IVF) data analysis. This study selected BAM files from euploid blastocyst only.

The BAM files were used to align to Human reference genome (Hg19: NCBI Build36) by CLC genomic workbench 8.5.1 (QAIGEN). Sequencing reads were trimmed by the cutting-off at the Q30 for accuracy of the data (>99.9%) and mapped with telomere regions within each chromosome of the human genome. The improvement of sequence quality of mapped sequences was done by extraction of concensus sequences. Then consensus sequences were exported to Fasta files and the repeated sequences (TTAGGG) were analyzed by BioEdit program. Finally, the telomeric length was compared between two maternal age groups (>35 years, ≤ 35 years).

9. Statistical analysis

Independent t-test was used to analyse the statistical differences between telomere length and mathernal age groups and the numbers of telomeric repeats sequence (TTAGGG). Microsoft Excel and graph pad program were used to achieve the *p-value*. *P-value* less than 0.05 was considered as statistical significance.

Sequencing data analysis

Table 4: Selection of telomere reference from Human reference genome (Hg19: NCBI Build36)

RegionB4:	Chr.	Start bp	End bp	Band	NCBI Reference Sequence	No. of repeated
Telomere	chr1	1	100125	1p36.33	NT_077912.2	17
Telomere	chr1	22614012	22828024	1q44	NT_032977.10	33
Telomere	chr10	194401	344400	10p15.3	NT_008705.17	20
Telomere	chr10	91900000	92093901	10q26.3	NT_030059.14	56
Telomere	chr11	136968	286967	11p15.5	NT_009237.19	29
Telomere	chr11	49940000	50701348	11q25	NT_009237.19	31
Telomere	chr12	231058	381057	12p13.33	NT_009759.17	19
Telomere	chr12	57564748	57752858	12q24.33	NT_029419.13	30
Telomere	chr13	49191894	49386767	13q34	NT_024524.15	37
Telomere	chr14	5700000	5876477	14q11.2	NT_026437.13	27
Telomere	chr14	70300000	70436672	14q32.33	NT_026437.13	24
Telomere	chr15	55200000	55427441	15q26.3	NT_010194.18	33
Telomere	chr16	93688	389999	16p13.3	NT_010393.17	36
Telomere	chr16	43619318	43724708	16q24.3	NT_010498.16	17
Telomere	chr17	1	150000	17p13.3	NT_024972.9	33
Telomere	chr17	54600000	54806562	17q25.3	NT_010783.16	24
Telomere	chr18	136938	287039	18p11.32	NT_010859.15	21
Telomere	chr18	56957959	57104975	18q23	NT_010966.15	23
Telomere	chr19	199397	349396	19p13.3	NT_011295.12	18
Telomere	chr19	31100000	31366742	19q13.43	NT_011109.17	74
Telomere	chr2	3336	203335	2p25.3	NT_005334.17	44
Telomere	chr2	145303985	145503985	2q37.3	NT_005403.18	25
Telomere	chr20	1	396719	20p13	NT_113914.2	51
Telomere	chr20	33100000	33282659	20q13.33	NT_011362.11	>100
Telomere	chr21	1	156246	21p13	NT_187300.1	23
Telomere	chr21	47940318	48090317	21q22.3	NT_011512.12	65
Telomere	chr22	31000000	31264301	22q13.33	NT_011520.13	34
Telomere	chr3	165238	315237	3p26.3	NT_022517.19	23
Telomere	chr3	103913846	104063845	3q29	NT_005612.17	12

RegionB4:	Chr.	Start bp	End bp	Band	NCBI Reference Sequence	Number of repeated
Telomere	chr4	26788	176787	4p16.3	NT_006051.19	29
Telomere	chr4	131105810	131283174	4q35.2	NT_016354.20	100
Telomere	chr5	57834	223585	5p15.33	NT_006576.17	38
Telomere	chr5	24785483	24935482	5q35.3	NT_023133.14	17
Telomere	chr6	100644	250643	6p25.3	NT_007592.16	28
Telomere	chr6	50170066	50286111	6q27	NT_025741.16	21
Telomere	chr7	83133	222484	7p22.3	NT_007819.18	31
Telomere	chr7	96471885	96621884	7q36.3	NT_007933.16	16
Telomere	chr8	341356	491355	8p23.3	NT_023736.18	22
Telomere	chr8	13172735	13432735	8q24.3	NT_008183.20	54
Telomere	chr9	194195	344194	9p24.3	NT_008413.19	30
Telomere	chr9	70000000	70114165	9q34.3	NT_008470.20	93
Telomere	chrX	283056	402499	Xp22.33	NT_187358.1	14
Telomere	chrX	27600000	27830040	Xq28	NT_011786.17	27
Telomere	chrY	293529	512250	Yp11.32	NT_167201.2	24
Telomere	chrY	10000000	10146379	Yq12	NT_011875.13	23

Chapter III

Results

3.1.General characteristic of patients who performed PGS and BAM files selected for analysis of young mother (≤ 35 years) and old mother (>35 years)

This study was a retrospective analysis. The characteristics of all patients were summarized in Table 1. Data included of maternal age, paternal age, total retrieved oocytes, number of mature oocytes (MII), fertilization rate (2PN), and blastocyst rate. We found that there was no significant difference in most parameters except maternal and paternal ages (P<0.05).

	young mother (n=47)	old mother (n=44)	*P*-value
Maternal age (years)	32.1(31.1-33.0)	38.7(38.1-39.3)	0.0001*
Paternal age (years)	36.1 (33.9-38.2)	43.3 (41.0-45.6)	0.0001*
Total oocytes (n)	16.9(15.0-18.8)	17.6 (15.0-20.2)	0.6573
MII (n)	14.3(12.0-16.6)	13.4 (11.6-15.1)	0.5336
2PN (n)	11.7(9.8-13.6)	10.8(9.5-12.1)	0.4195
Blastocyst rate (n)	7.56 (6.48-8.64)	7.37(5.90-8.84)	0.8314

Table 5: General characteristics of patients who performed PGD using next-generation sequencing (n=94).The results showed none of the parameters had significant differences between the two groups except mother age (P <0.05).

3.2. Distribution of patient ethnicities from total 91 couples.

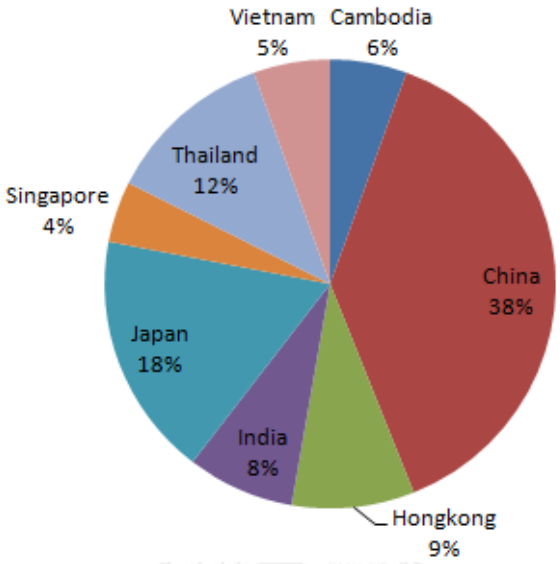


Figure 12 : Distribution of patient ethnicities from total 91 couples.
This pie graph shows the summary of nationality of sample size. Chinese patients are the majority of sample size and Cambodia patients are the minority.

3.3. Determination the length of telomere in human blastocyst from difference maternal age groups

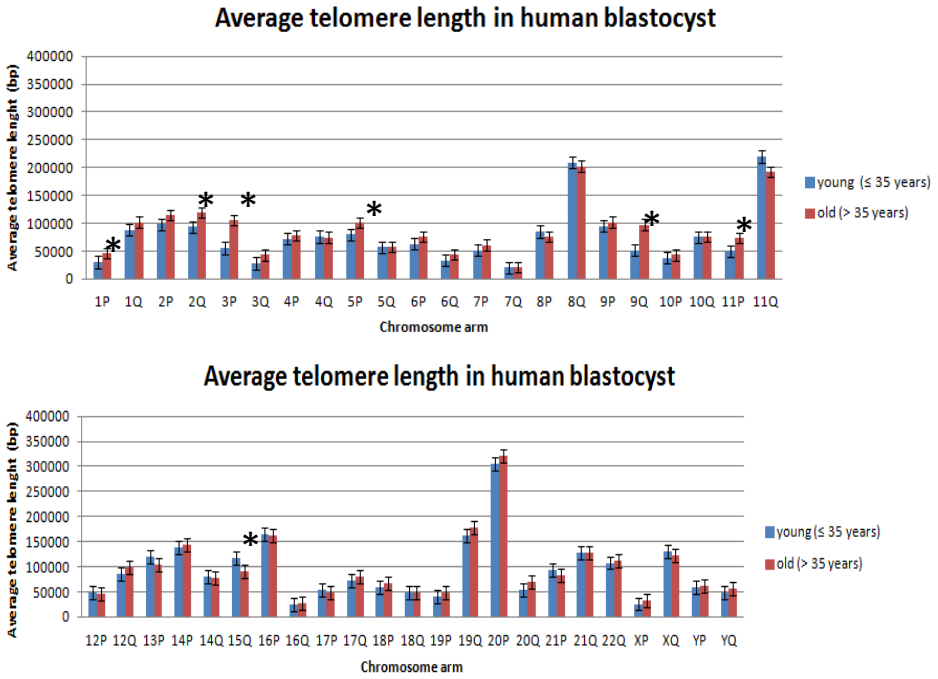


Figure 13 : Average telomere lengths of individual P and Q arms form each chromosome in blastocyst from young mother age (blue) and blastocyst from old mother age (red).

This result showed that the most of chromosome arms are similar between mother groups. Some of chromosome arms had statistically different such as 1P, 2Q, 3P, 5P, 9Q, 11P, 15Q (P <0.05, 15%). In the significant groups, we found that the telomere length of blastocyst derived from older age was longer than that of young mother. *indicates significant of values compared between to maternal age groups.

3.4. Average number of telomeric repeated sequence in human blastocyst

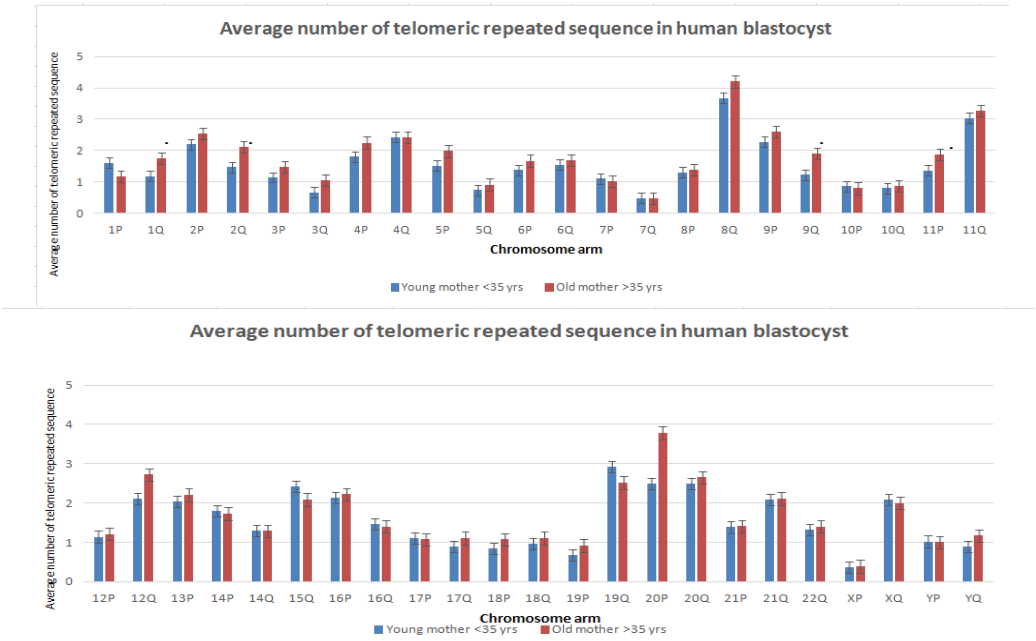


Figure 14 : Average the number of telomeric repeated sequences individual P and Q arms form each chromosome in blastocyst from young mother age (blue) and blastocyst from old mother age (red).

This result showed that the most of chromosome arms were non-significant between mother groups. Some chromosome arms were significantly differed such as 1Q, 2Q, 9Q, 12Q, 13P, 20P ($P < 0.05$, 13%). We found the telomeric repeated sequences of blastocysts derived from older age were longer than young mother. *indicates significant of values compared between to maternal age groups.

3.5 Determination the length of telomere in human blastocyst from difference pregnancy results (excluded young mother with old father)

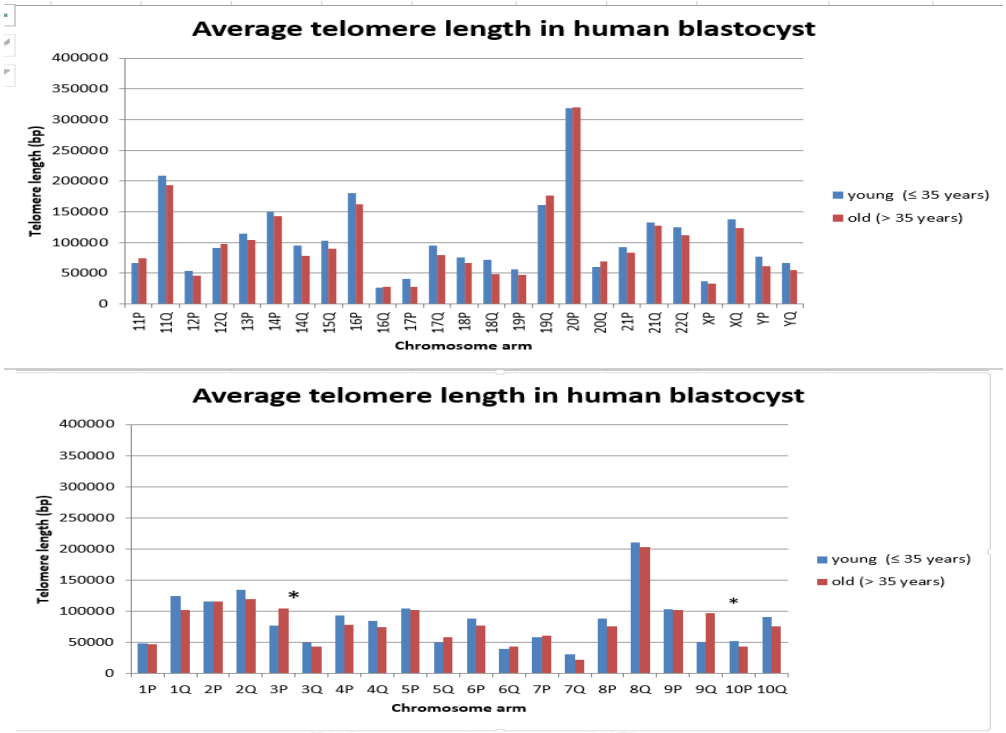


Figure 15: Average telomere lengths of individual P and Q arms form each chromosome in blastocyst from maternal age groups with excluded young mother with old father

This result shows the most of chromosome arms non-significant from maternal age groups with excluded young mother with old father and some chromosome arm had significantly such as 3P, 10Q (P <0.05) .

3.6 Average number of telomeric repeated sequence in human blastocyst difference pregnancy results (excluded young mother with old father)

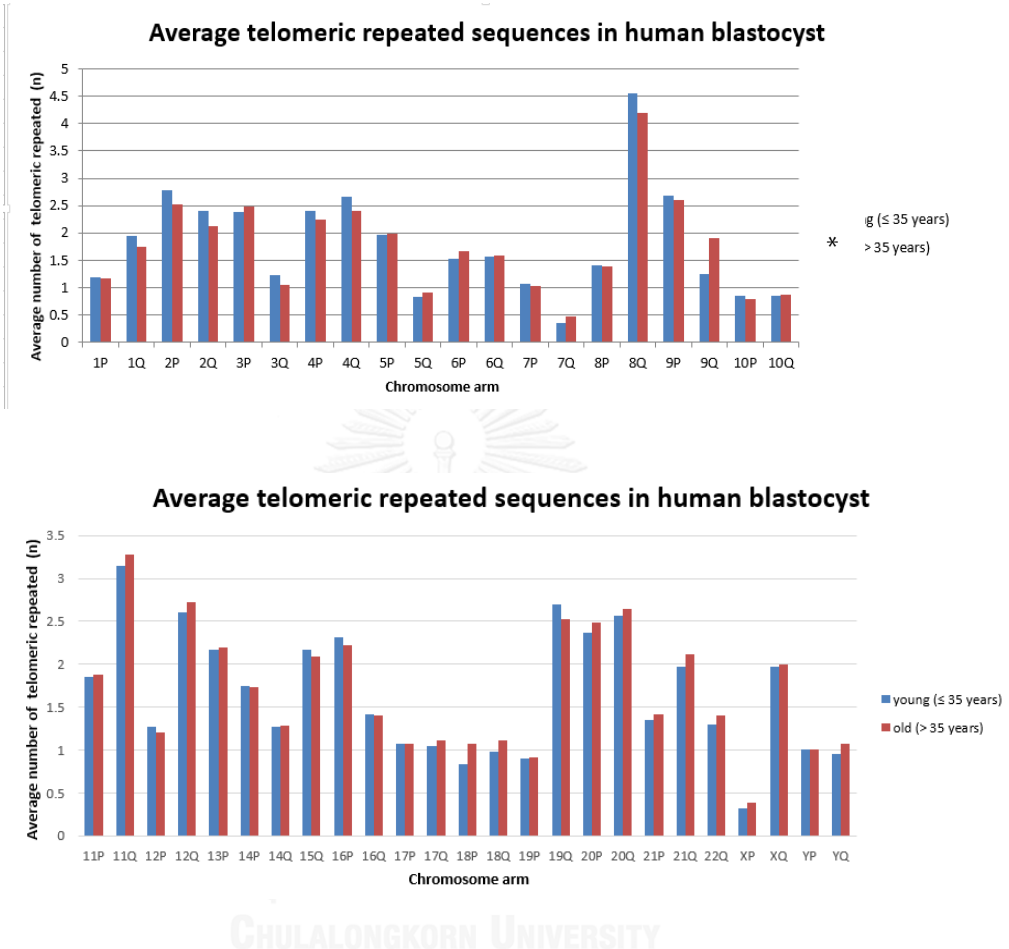


Figure 16 : Average the number of telomeric repeated sequences individual P and Q arms form each chromosome in blastocyst from maternal age groups with excluded young mother with old father.

This result shows the most of chromosome arms non-significant from maternal age groups with excluded young mother with old father and some chromosome arm had significantly such as 9Q (P <0.05)

3.7 Determination the length of telomere in human blastocyst between Japanese and non- Japanese ethnicities in young mother groups (≤ 35 years)

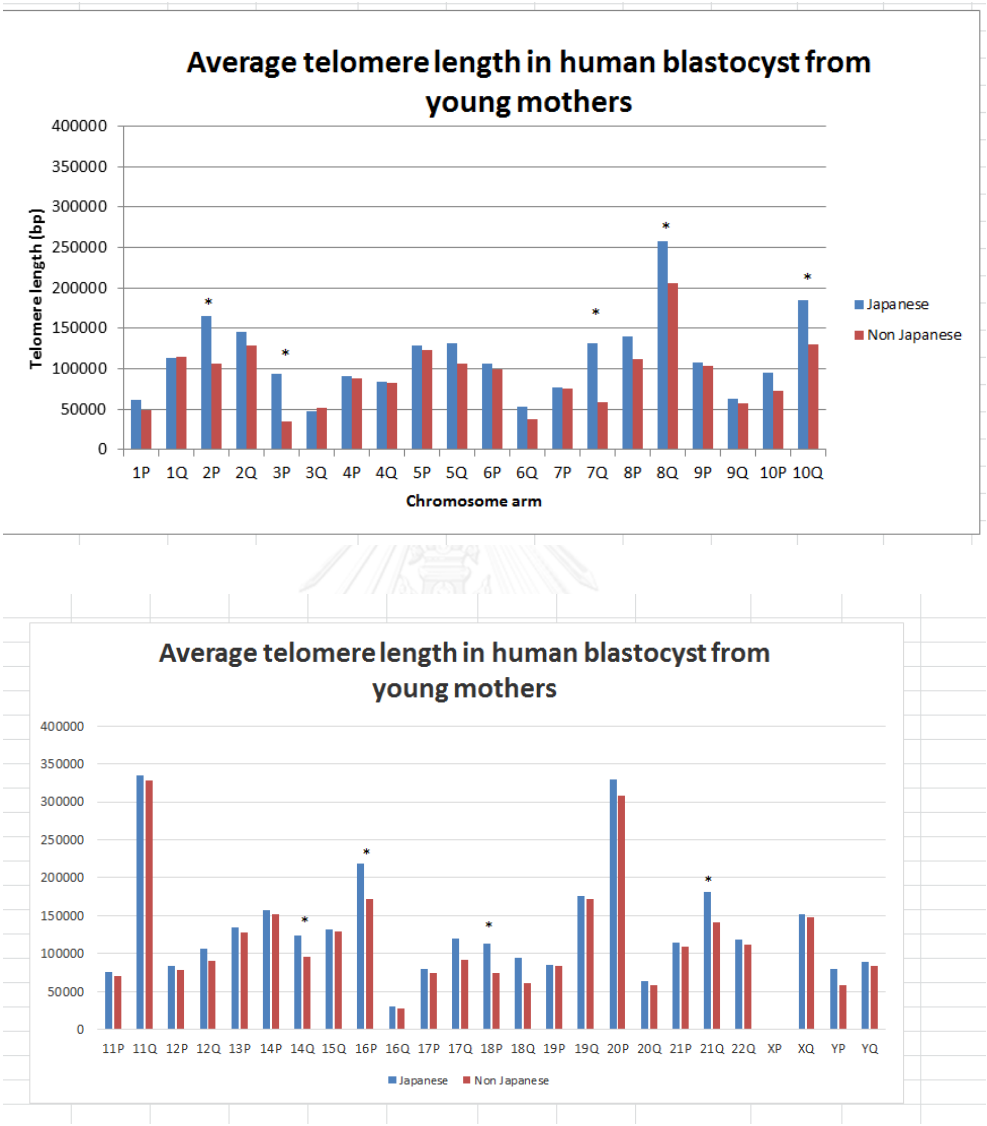
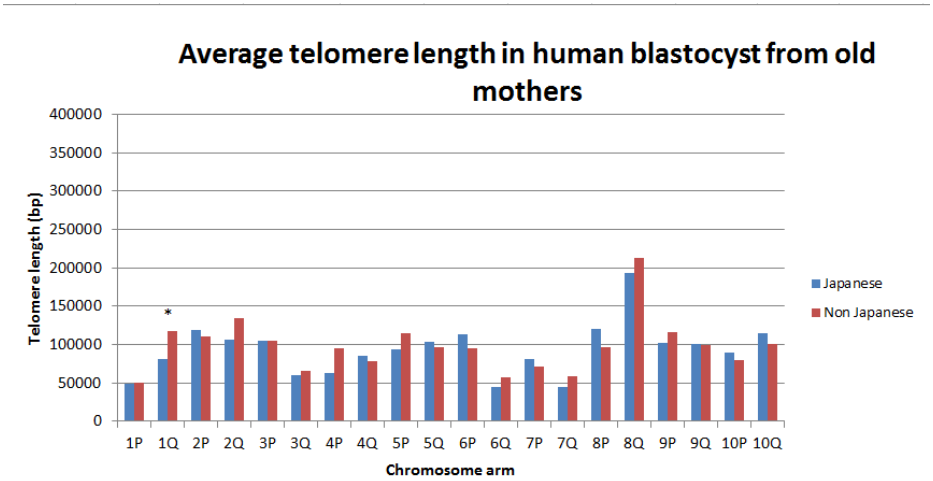


Figure 17 : Average telomere lengths of individual P and Q arms form each chromosome arm in human blastocyst from young mother groups between Japanese and non- Japanese mothers

This result shows the most of chromosome arms non-significant from young mother groups. Some chromosome arms were significantly differed such as 2P, 3P, 7Q, 8Q, 10Q, 14Q, 16P, 18P, 21Q (P <0.05, 20%). (*P* <0.05)

3.8 Determination the length of telomere in human blastocyst between Japanese and non- Japanese ethnicities in old mother groups (> 35 years)



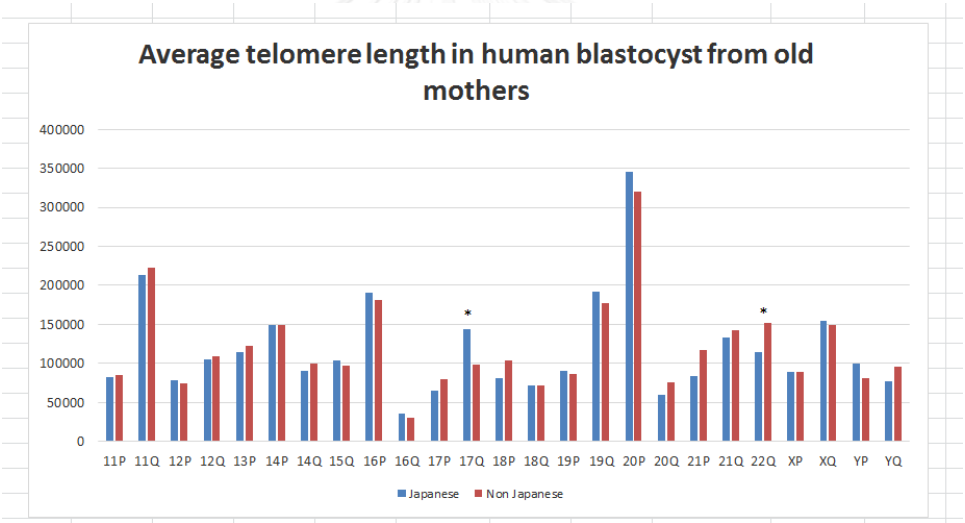


Figure 18 : Average telomere lengths of individual P and Q arms form each chromosome arm in human blastocyst from old mother groups between Japanese and non- Japanese mothers

This result shows the most of chromosome arms non-significant from old mother groups. Some chromosome arms were significantly differed such as 1Q, 17Q, 22Q ($P < 0.05$, 20%). ($P < 0.05$)

Chapter IV

Discussion

The aim of this study was to compare the telomere length and telomeric repeated sequences derived from human blastocysts of two maternal age groups. We used the NGS technique to determine the telomere length of 94 human blastocysts derived from 91 couples undergoing ART and PGS program. We employed Sureplex amplification (Illimina) for whole genome amplification since previous reports have demonstrated this particular technique and platform suitable for detecting the telomere sequences. Treff et al, (2011) analyzed telomere length by quantitative real-time PCR used primer specific with telomere regions and used embryo biopsy WGA by Genomeplex amplification kit (Sigma Aldrich, St. Louis, MO).[98] Primer specific telomere regions are confidential data of producer. K.J.Turner et al, (2014) verified of the faithful representation of telomere length using Sureplex kit the result indicated acceptable representation of telomeres in WGA process [7, 54, 99].

Our present study showed that the telomere length and telomic repeated sequences of almost chromosome arms were not different between the two maternal age groups. These findings are in line with previous reports showing that telomere at the morula-blastocyst could reset their lengths in this stage. [60, 100] Our result also suggested that telomere length in blastocyst stage of embryos derived from young mother group was similar to that of the old mother group.

Attempting to investigate the telomere length of chromosome arms, the blastocyst biopsies (day 5 or 6 after fertilization) were subjected to comparison of telomere length between young and old mother groups. Mania *et al* (2014) showed the reduction of telomere length in day 5 embryos derived from advanced maternal age. In their study, all embryos included were aneuploid embryos from different development stages. They also included the blastocyst, morulae and arrested embryos. But our study selected only the euploid blastocysts to assess the telomere patterns. As the previous study included only 35 embryos from 7 couples, our study included 94 embryos from 91 couples, thus being considered as a larger sample size.

Our result found the significant between maternal age groups is chromosome 3p and 10q. Chromosome 3p is correlated with chromosome 3p deletion syndrome

disease. The characteristic of disease, including, low birth weight, autism. 10q deletion syndrome has the most common features, such as, growth delay, kidney and urinary tract abnormality, genital abnormality.[101, 102]

The average telomere length between Japanese and non-Japanese groups shown that in the young mother groups the most chromosome of Japanese blastocysts are longer than non-Japanese. But the old mother groups found non-singnificant in the most of chromosomes. Maybe explain that Japanese and non-japanese mother are difference life style factors, hereditary, and food intake.

Regarding of our limitation in the present work, all patients were Asian. It would be of value if a larger sample size with more ethnicities can be grouped and studied in the future. In addition, analysis of the telomere length in sperms and follow up the pregnancy of the couples should be further performed to get a relevant outcome. This first study analyzes telomere repeated by bioinformatics methods. The previous studies are modified and compared telomeric amplification methods.

This study selected Human Hg 19 bilt36 for reference database because this reference used in MiSeq program analyzer for generate BAM files and based on clinical application and this the latest version.

NGS is the advantage technology and recently used in many routine lab. There many advantages such as access to the whole genome, high coverage in target regions, reduced for cost large genomes, reduced turnaround time. For disadvantage, NGS requires minimum sample in run such as 24 samples per run, and required skills for sample preparation process[39].

We found the differences in average numbers of telomere length (15%) and telomeric repeated sequences (13%) of all chromosome arms studied between the two maternal age groups. With respect to the finding, certain chromosome arms were significant different in the telomere length in blastocyst derived from old mother age. The telomere length was found to be longer than that of young mother age groups. As far as our explanation was concerned, the increase of telomere length in blastocysts derived from older women would be the effect of paternal age as suggested by previous reports. [54, 103, 104] In other word, the older mothers coupling with the older husbands would have paternal effect on the telomere length from the father too. It is

of great interest to study the variation of telomere length and use this for detecting any correlation with other clinical parameters such as embryo development and disease in the future.

REFERENCES

1. Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. The EMBO journal. 1992;11(5):1921-9.
2. de Lange T. Human telomeres are attached to the nuclear matrix. The EMBO journal. 1992;11(2):717-24.
3. Blackburn EH. Telomere states and cell fates. Nature. 2000;408(6808):53-6.
4. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, et al. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell. 1997;91(1):25-34.
5. Blasco MA, Gasser SM, Lingner J. Telomeres and telomerase. Genes Dev. 1999;13(18):2353-9.
6. Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, et al. Longevity, stress response, and cancer in aging telomerase-deficient mice. Cell. 1999;96(5):701-12.
7. Treff NR, Su J, Taylor D, Scott RT, Jr. Telomere DNA deficiency is associated with development of human embryonic aneuploidy. PLoS Genet. 2011;7(6):e1002161.
8. Keefe DL, Liu L, Marquard K. Telomeres and aging-related meiotic dysfunction in women. Cell Mol Life Sci. 2007;64(2):139-43.
9. Kimura M, Stone RC, Hunt SC, Skurnick J, Lu X, Cao X, et al. Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths. Nat Protoc. 2010;5(9):1596-607.
10. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, et al. Extension of life-span by introduction of telomerase into normal human cells. Science. 1998;279(5349):349-52.
11. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res. 2009;37(3):e21.

12. Hayes JL, Tzika A, Thygesen H, Berri S, Wood HM, Hewitt S, et al. Diagnosis of copy number variation by Illumina next generation sequencing is comparable in performance to oligonucleotide array comparative genomic hybridisation. Genomics. 2013;102(3):174-81.
13. Fiorentino F, Biricik A, Bono S, Spizzichino L, Cotroneo E, Cottone G, et al. Development and validation of a next-generation sequencing-based protocol for 24-chromosome aneuploidy screening of embryos. Fertil Steril. 2014;101(5):1375-82.
14. Bekaert S, Derradji H, Baatout S. Telomere biology in mammalian germ cells and during development. Dev Biol. 2004;274(1):15-30.
15. de Lange T. T-loops and the origin of telomeres. Nat Rev Mol Cell Biol. 2004;5(4):323-9.
16. Makarov VL, Hirose Y, Langmore JP. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell. 1997;88(5):657-66.
17. Wright WE, Tesmer VM, Huffman KE, Levene SD, Shay JW. Normal human chromosomes have long G-rich telomeric overhangs at one end. Genes Dev. 1997;11(21):2801-9.
18. Karlseder J, Kachatrian L, Takai H, Mercer K, Hingorani S, Jacks T, et al. Targeted deletion reveals an essential function for the telomere length regulator Trf1. Mol Cell Biol. 2003;23(18):6533-41.
19. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev. 2005;19(18):2100-10.
20. Meiosis 1, 2 Available http://apocketmerlin.tumblr.com/post/21587555401/cell-division-meiosis.
21. Meselson M, Stahl FW. The Replication of DNA in Escherichia Coli. Proc Natl Acad Sci U S A. 1958;44(7):671-82.
22. Ohki R, Tsurimoto T, Ishikawa F. In vitro reconstitution of the end replication problem. Mol Cell Biol. 2001;21(17):5753-66.
23. Waga S, Stillman B. The DNA replication fork in eukaryotic cells. Annu Rev Biochem. 1998;67:721-51.

24. Ogawa T, Okazaki T. Discontinuous DNA replication. Annu Rev Biochem. 1980;49:421-57.

25. Okazaki R, Okazaki T, Sakabe K, Sugimoto K, Sugino A. Mechanism of DNA chain growth. I. Possible discontinuity and unusual secondary structure of newly synthesized chains. Proc Natl Acad Sci U S A. 1968;59(2):598-605.

26. Sakabe K, Okazaki R. A unique property of the replicating region of chromosomal DNA. Biochim Biophys Acta. 1966;129(3):651-4.

27. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol. 1973;41(1):181-90.

28. Hayflick L. The cell biology of human aging. N Engl J Med. 1976;295(23):1302-8.

29. Munoz-Espin D, Serrano M. Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol. 2014;15(7):482-96.

30. McClintock B. The Behavior in Successive Nuclear Divisions of a Chromosome Broken at Meiosis. Proc Natl Acad Sci U S A. 1939;25(8):405-16.

31. Greider CW, Blackburn EH. The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell. 1987;51(6):887-98.

32. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. Telomere reduction in human colorectal carcinoma and with ageing. Nature. 1990;346(6287):866-8.

33. Lindsey J, McGill NI, Lindsey LA, Green DK, Cooke HJ. In vivo loss of telomeric repeats with age in humans. Mutat Res. 1991;256(1):45-8.

34. Herbig U, Jobling WA, Chen BP, Chen DJ, Sedivy JM. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). Mol Cell. 2004;14(4):501-13.

35. Shay JW. Telomerase therapeutics: telomeres recognized as a DNA damage signal: commentary re: K. Kraemer et al., antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. Clin. Cancer Res., 9: 3794-3800, 2003. Clin Cancer Res. 2003;9(10 Pt 1):3521-5.

36. Kleckner N. Meiosis: how could it work? Proc Natl Acad Sci U S A. 1996;93(16):8167-74.

37. Terasawa M, Ogawa H, Tsukamoto Y, Shinohara M, Shirahige K, Kleckner N, et al. Meiotic recombination-related DNA synthesis and its implications for cross-over and non-cross-over recombinant formation. Proc Natl Acad Sci U S A. 2007;104(14):5965-70.

38. Roeder GS. Meiotic chromosomes: it takes two to tango. Genes Dev. 1997;11(20):2600-21.

39. Schneeberger K. Using next-generation sequencing to isolate mutant genes from forward genetic screens. Nat Rev Genet. 2014;15(10):662-76.

40. Achi MV, Ravindranath N, Dym M. Telomere length in male germ cells is inversely correlated with telomerase activity. Biol Reprod. 2000;63(2):591-8.

41. Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev. 1972;52(1):198-236.

42. Tanemura K, Ogura A, Cheong C, Gotoh H, Matsumoto K, Sato E, et al. Dynamic rearrangement of telomeres during spermatogenesis in mice. Dev Biol. 2005;281(2):196-207.

43. Ohno S, Klinger HP, Atkin NB. Human oogenesis. Cytogenetics. 1962;1:42-51.

44. Bhalla N, Dernburg AF. Prelude to a division. Annu Rev Cell Dev Biol. 2008;24:397-424.

45. Cooper JP, Watanabe Y, Nurse P. Fission yeast Taz1 protein is required for meiotic telomere clustering and recombination. Nature. 1998;392(6678):828-31.

46. Nimmo ER, Pidoux AL, Perry PE, Allshire RC. Defective meiosis in telomere-silencing mutants of Schizosaccharomyces pombe. Nature. 1998;392(6678):825-8.

47. Hemann MT, Rudolph KL, Strong MA, DePinho RA, Chin L, Greider CW. Telomere dysfunction triggers developmentally regulated germ cell apoptosis. Mol Biol Cell. 2001;12(7):2023-30.

48. Lee HW, Blasco MA, Gottlieb GJ, Horner JW, 2nd, Greider CW, DePinho RA. Essential role of mouse telomerase in highly proliferative organs. Nature. 1998;392(6676):569-74.

49. Liu L, Franco S, Spyropoulos B, Moens PB, Blasco MA, Keefe DL. Irregular telomeres impair meiotic synapsis and recombination in mice. Proc Natl Acad Sci U S A. 2004;101(17):6496-501.

50. Eisenberg DT, Hayes MG, Kuzawa CW. Delayed paternal age of reproduction in humans is associated with longer telomeres across two generations of descendants. Proc Natl Acad Sci U S A. 2012;109(26):10251-6.

51. Eisenhauer KM, Gerstein RM, Chiu CP, Conti M, Hsueh AJ. Telomerase activity in female and male rat germ cells undergoing meiosis and in early embryos. Biol Reprod. 1997;56(5):1120-5.

52. Yashima K, Maitra A, Rogers BB, Timmons CF, Rathi A, Pinar H, et al. Expression of the RNA component of telomerase during human development and differentiation. Cell Growth Differ. 1998;9(9):805-13.

53. Fujisawa M, Tanaka H, Tatsumi N, Okada H, Arakawa S, Kamidono S. Telomerase activity in the testis of infertile patients with selected causes. Hum Reprod. 1998;13(6):1476-9.

54. Turner S, Hartshorne GM. Telomere lengths in human pronuclei, oocytes and spermatozoa. Mol Hum Reprod. 2013;19(8):510-8.

55. Hardarson T, Hanson C, Lundin K, Hillensjo T, Nilsson L, Stevic J, et al. Preimplantation genetic screening in women of advanced maternal age caused a decrease in clinical pregnancy rate: a randomized controlled trial. Hum Reprod. 2008;23(12):2806-12.

56. Turner S, Wong HP, Rai J, Hartshorne GM. Telomere lengths in human oocytes, cleavage stage embryos and blastocysts. Mol Hum Reprod. 2010;16(9):685-94.

57. Duran HE, Simsek-Duran F, Oehninger SC, Jones HW, Jr., Castora FJ. The association of reproductive senescence with mitochondrial quantity, function, and DNA integrity in human oocytes at different stages of maturation. Fertil Steril. 2011;96(2):384-8.

58. Wright DL, Jones EL, Mayer JF, Oehninger S, Gibbons WE, Lanzendorf SE. Characterization of telomerase activity in the human oocyte and preimplantation embryo. Mol Hum Reprod. 2001;7(10):947-55.

59. Betts DH, King WA. Telomerase activity and telomere detection during early bovine development. Dev Genet. 1999;25(4):397-403.

60. Xu J, Yang X. Telomerase activity in bovine embryos during early development. Biol Reprod. 2000;63(4):1124-8.

61. Meerdo LN, Reed WA, White KL. Telomere-to-centromere ratio of bovine clones, embryos, gametes, fetal cells, and adult cells. Cloning Stem Cells. 2005;7(1):62-73.

62. Russo V, Berardinelli P, Martelli A, Di Giacinto O, Nardinocchi D, Fantasia D, et al. Expression of telomerase reverse transcriptase subunit (TERT) and telomere sizing in pig ovarian follicles. J Histochem Cytochem. 2006;54(4):443-55.

63. Mengerink KJ, Vacquier VD. Glycobiology of sperm-egg interactions in deuterostomes. Glycobiology. 2001;11(4):37R-43R.

64. Dupont G. Link between fertilization-induced Ca2+ oscillations and relief from metaphase II arrest in mammalian eggs: a model based on calmodulin-dependent kinase II activation. Biophys Chem. 1998;72(1-2):153-67.

65. Passos JF, Saretzki G, von Zglinicki T. DNA damage in telomeres and mitochondria during cellular senescence: is there a connection? Nucleic Acids Res. 2007;35(22):7505-13.

66. Liu L, Bailey SM, Okuka M, Munoz P, Li C, Zhou L, et al. Telomere lengthening early in development. Nat Cell Biol. 2007;9(12):1436-41.

67. Mania A, Mantzouratou A, Delhanty JD, Baio G, Serhal P, Sengupta SB. Telomere length in human blastocysts. Reprod Biomed Online. 2014;28(5):624-37.

68. Seli E, Sakkas D. Spermatozoal nuclear determinants of reproductive outcome: implications for ART. Hum Reprod Update. 2005;11(4):337-49.

69. Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ, Jr., Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod. 1999;14(11):2801-7.

70. Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, et al. Population study of causes, treatment, and outcome of infertility. Br Med J (Clin Res Ed). 1985;291(6510):1693-7.

71. Paavonen J, Eggert-Kruse W. Chlamydia trachomatis: impact on human reproduction. Hum Reprod Update. 1999;5(5):433-47.

72. Homan GF, Davies M, Norman R. The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatment: a review. Hum Reprod Update. 2007;13(3):209-23.

73. Practice Committee of the American Society for Reproductive M. Smoking and infertility. Fertil Steril. 2004;81(4):1181-6.

74. Pasqualotto FF, Lucon AM, Sobreiro BP, Pasqualotto EB, Arap S. Effects of medical therapy, alcohol, smoking, and endocrine disruptors on male infertility. Rev Hosp Clin Fac Med Sao Paulo. 2004;59(6):375-82.

75. Baird D, Benagiano, G., Cohen, J., et al Physiopathological determinants of human infertility. Hum Reprod Update. 2002;8(5):435-47.

76. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl. 2002;23(1):25-43.

77. O'Flynn O'Brien KL, Varghese AC, Agarwal A. The genetic causes of male factor infertility: a review. Fertil Steril. 2010;93(1):1-12.

78. Shah K, Sivapalan G, Gibbons N, Tempest H, Griffin DK. The genetic basis of infertility. Reproduction. 2003;126(1):13-25.

79. Zini A, Fischer MA, Sharir S, Shayegan B, Phang D, Jarvi K. Prevalence of abnormal sperm DNA denaturation in fertile and infertile men. Urology. 2002;60(6):1069-72.

80. Cooke S, and Felming, S.D. Textbook of Assisted Reproduction for Scientist in Reproductive Technology. Vivid Publishing2009.

81. Van Voorhis BJ. Clinical practice. In vitro fertilization. N Engl J Med. 2007;356(4):379-86.

82. Keltz MD, Vega M, Sirota I, Lederman M, Moshier EL, Gonzales E, et al. Preimplantation genetic screening (PGS) with Comparative genomic hybridization (CGH) following day 3 single cell blastomere biopsy markedly improves IVF outcomes while lowering multiple pregnancies and miscarriages. J Assist Reprod Genet. 2013;30(10):1333-9.

83. Rubio C, Bellver J, Rodrigo L, Bosch E, Mercader A, Vidal C, et al. Preimplantation genetic screening using fluorescence in situ hybridization in patients with repetitive implantation failure and advanced maternal age: two randomized trials. Fertil Steril. 2013;99(5):1400-7.

84. S G. The A-Z Encyclopedia on Male and Female Infertility. New Delhi: Jaypee; 2005. 146 p.

85. Alpha Scientists in Reproductive M, Embryology ESIGo. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Hum Reprod. 2011;26(6):1270-83.

86. Scott RT, Jr., Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. Fertil Steril. 2013;100(3):624-30.

87. Sakkas D, Gardner DK. Noninvasive methods to assess embryo quality. Curr Opin Obstet Gynecol. 2005;17(3):283-8.

88. Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, et al. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study. Mol Cytogenet. 2012;5(1):24.

89. Alfarawati S, Fragouli E, Colls P, Stevens J, Gutierrez-Mateo C, Schoolcraft WB, et al. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. Fertil Steril. 2011;95(2):520-4.

90. Wilton L. Preimplantation genetic diagnosis and chromosome analysis of blastomeres using comparative genomic hybridization. Hum Reprod Update. 2005;11(1):33-41.

91. Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet. 2001;2(4):280-91.

92. Baart EB, Martini E, van den Berg I, Macklon NS, Galjaard RJ, Fauser BC, et al. Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. Hum Reprod. 2006;21(1):223-33.

93. Baird DM, Jeffreys AJ, Royle NJ. Mechanisms underlying telomere repeat turnover, revealed by hypervariable variant repeat distribution patterns in the human Xp/Yp telomere. EMBO J. 1995;14(21):5433-43.

94. Baird DM, Rowson J, Wynford-Thomas D, Kipling D. Extensive allelic variation and ultrashort telomeres in senescent human cells. Nat Genet. 2003;33(2):203-7.

95. Britt-Compton B, Rowson J, Locke M, Mackenzie I, Kipling D, Baird DM. Structural stability and chromosome-specific telomere length is governed by cis-acting determinants in humans. Hum Mol Genet. 2006;15(5):725-33.

96. Xing J, Ajani JA, Chen M, Izzo J, Lin J, Chen Z, et al. Constitutive short telomere length of chromosome 17p and 12q but not 11q and 2p is associated with an increased risk for esophageal cancer. Cancer Prev Res (Phila). 2009;2(5):459-65.

97. Ding Z, Mangino M, Aviv A, Spector T, Durbin R, Consortium UK. Estimating telomere length from whole genome sequence data. Nucleic Acids Res. 2014;42(9):e75.

98. Treff NR, Su J, Tao X, Miller KA, Levy B, Scott RT, Jr. A novel single-cell DNA fingerprinting method successfully distinguishes sibling human embryos. Fertil Steril. 2010;94(2):477-84.

99. Turner KJ. Telomere Length and Distribution im Three Developmental Stages ;https://kar.kent.ac.uk/47463/. 2014.

100. Schaetzlein S, Lucas-Hahn A, Lemme E, Kues WA, Dorsch M, Manns MP, et al. Telomere length is reset during early mammalian embryogenesis. Proc Natl Acad Sci U S A. 2004;101(21):8034-8.

101. 10q25 and 10q26 deletions ;http://www.rarechromo.org/information/Chromosome%2010/10q25%20and%2010q26%20deletions%20FTNW.pdf.

102. 3p25 deletions; http://www.rarechromo.org/information/Chromosome%20%203/3p25%20deletions%20FTNW.pdf.

103. Kimura M, Cherkas LF, Kato BS, Demissie S, Hjelmborg JB, Brimacombe M, et al. Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm. PLoS Genet. 2008;4(2):e37.

104. Baird DM, Britt-Compton B, Rowson J, Amso NN, Gregory L, Kipling D. Telomere instability in the male germline. Hum Mol Genet. 2006;15(1):45-51.

APPENDIX

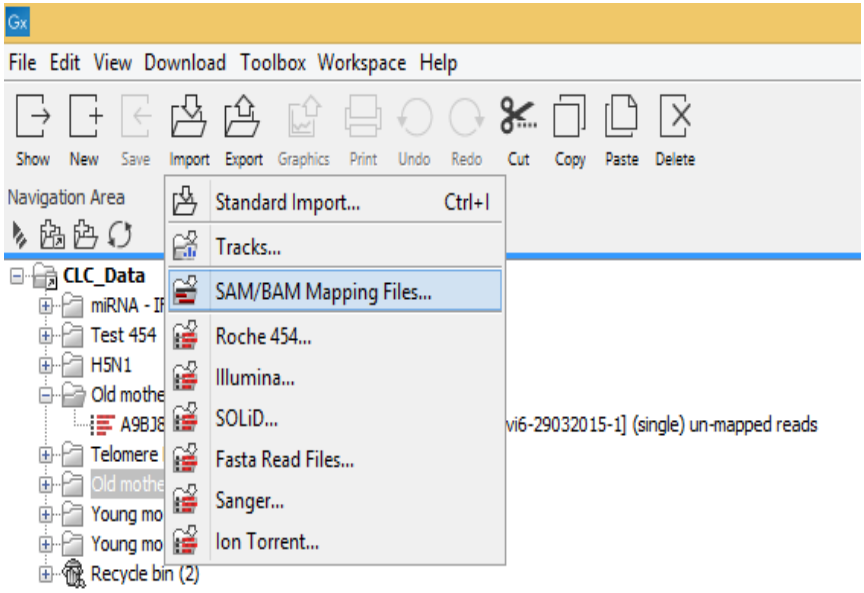
- Sequencing Run: The total Cluster Number corresponds to how many position with distinct DNA molecule detected on the flow cell surface (raw cluster). The number is ideally close to 30 Million (M), and reaches at 25 M raw clusters. Less than 25 M or more than 30 M clusters indicate that the amount of DNA added to the flow cell requires optimization for subsequence sequencing runs. While lower numbers do not provide enough output for reliable analysis, higher numbers result in misreading of the sequence. The SAV can provide more detailed information about the cluster density and should be used after every run. Optimal density is between 1,200k and 1,400k/mm2.

  From all the raw clusters that formed in the flow cell, only a subset is considered to be reliable enough to be used in downstream processing. Ideally this subset, "passing filter" (PF) clusters, reaches at least 85% of the total number of raw cluster. If lower values of PF clusters are reached (i.e. 75%), it indicates that the amount of denature library used for seeding the flow cell was suboptimal. In absolute numbers, this result would relate to minimum of around 19 M clusters reported as Cluster Number Passing Filter.
- Average Quality Score: The base quality score (Q-score) is a measure of confidence in the base that was called at a given position. The Q-score is calculated as mean for all read per sample. A Q-score of 30 estimates that there are 0.1 % wrong bases in the result. For the report, this score is recalculated after filtering and is ideally around 35 with a minimum score of 30.
- Average Alignment Score: The alignment software calculates this score and takes into account the Q-score, read length, and the number of alignments for every read. The average alignment score is ideally around 35 with a minimum score of 30.
- Number of Total Reads: For a well-balanced library within 24 samples. There is ideally around 1M reads reported for each sample, with a minimum of 700,000 reads.
- Number of Reads after Filtering: Aligned reads are further filtered. The number of filtered reads usable for copy-number calling is ideally around 60% of the reads, i.e. 600,000 reads. The minimum value is 250,000 reads, but examples with this number of reads can result in noisy profiles that are difficult to interpret.

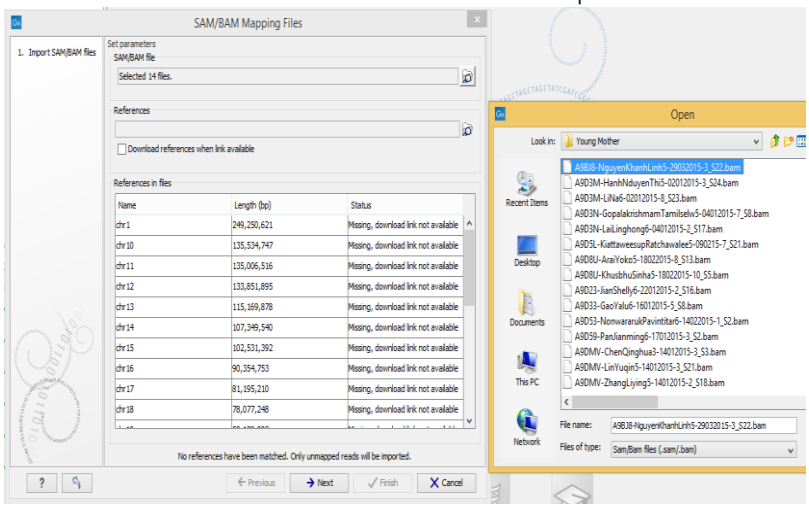
- Overall Noise (DLR-Derivative Log Ratio): The overall sample noise measures the spread of the difference in copy number values between all bins of a chromosome. Values are ideally less than 0.3. Values above 0.4 indicate low quality sample DNA or problems during the amplification steps.

CLC Software manual

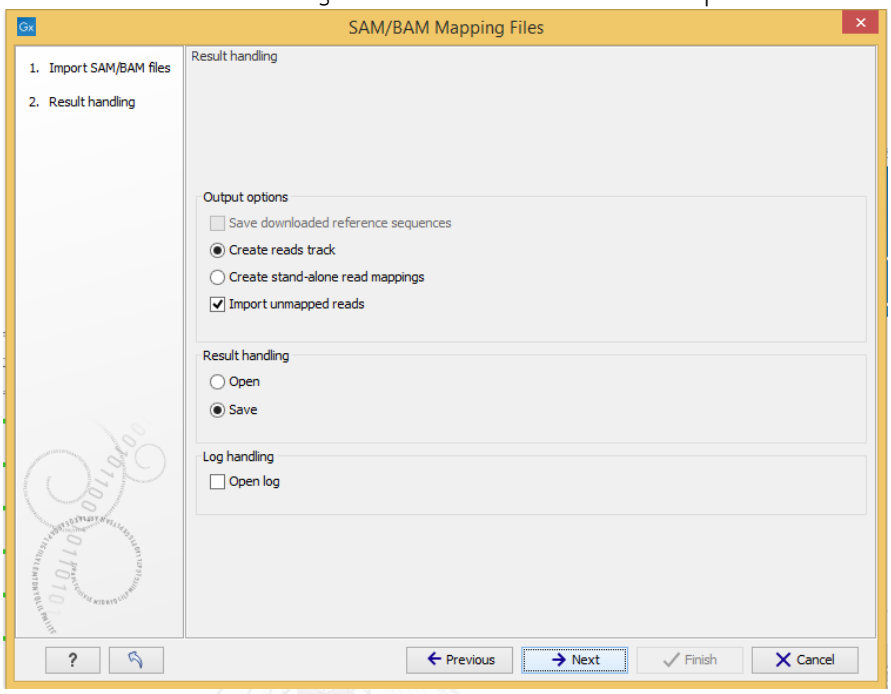
1. BAM files were imported to align with Hg 19 NCBI Build 36 Reference of each chromosome arm by CLC genomic workbench 8.5.1 (QAIGEN) Selected Import Menu and SAM/BAM Mapping files.



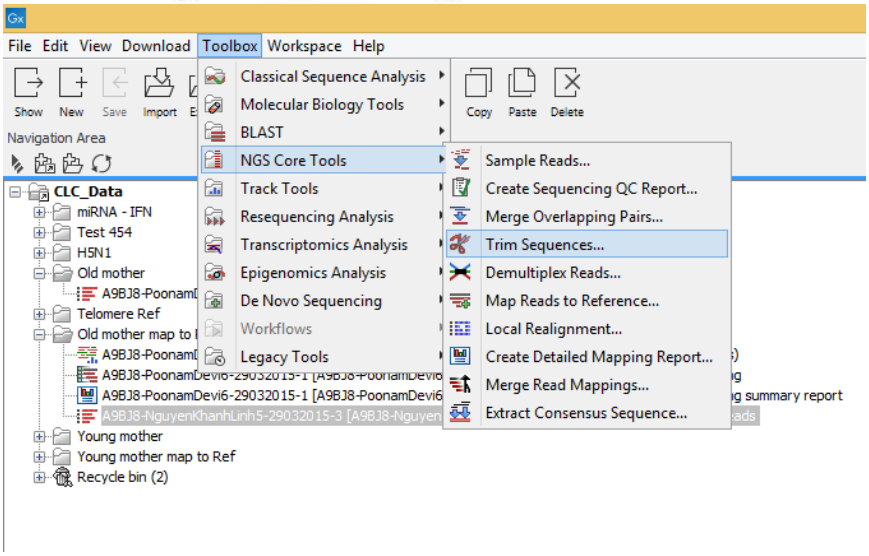
2. Select BAM files and reference from folders. Then pressed next.



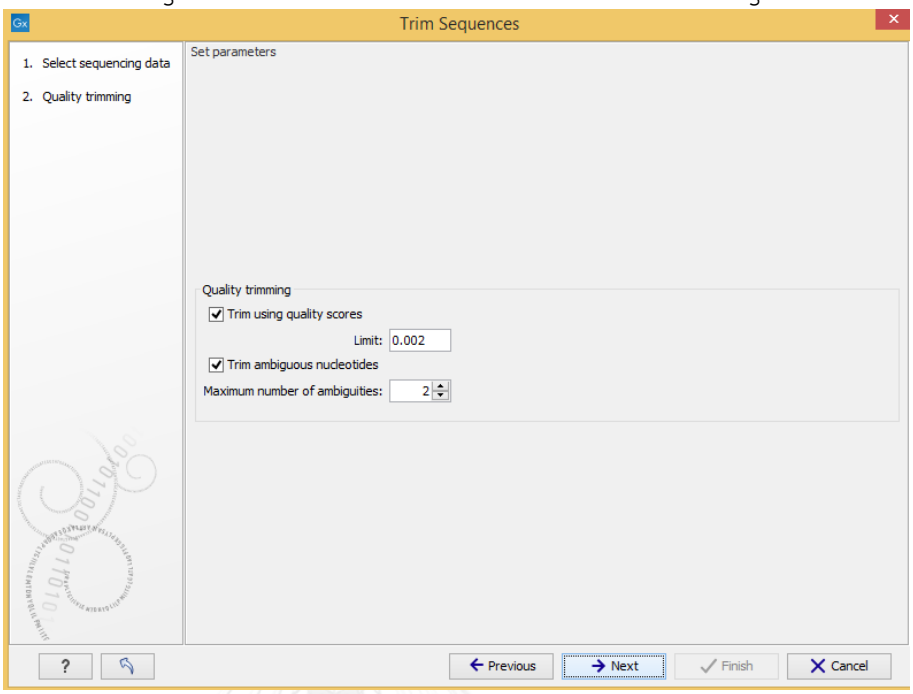
3. On the output option selected Created reads tract and Import unmapped reads. On the result handing menu. Selected Save. Then pressed next.



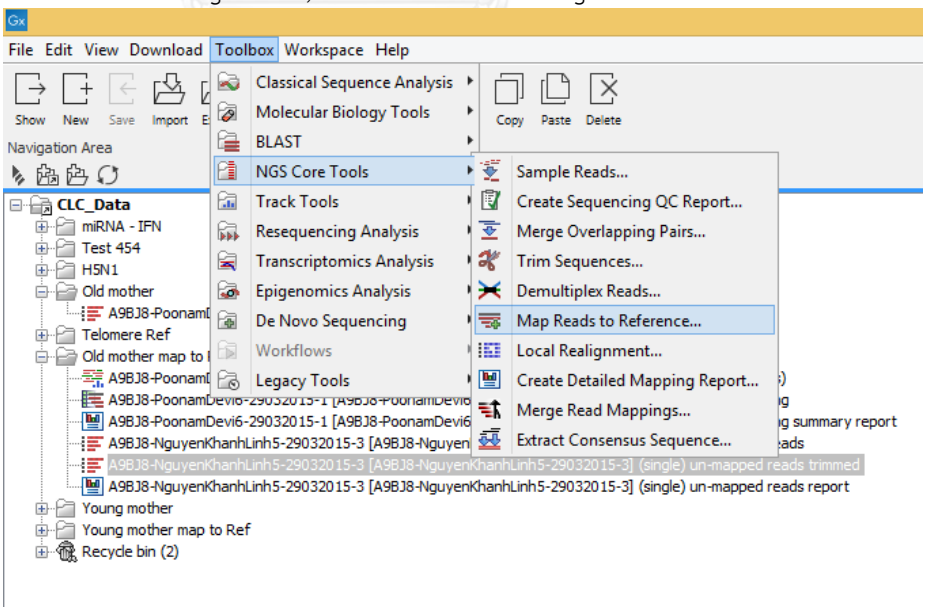
4. Sequencing reads were trimmed by the cut-off at Q30 for accuracy of data (>99.9%) and remove adapter from reads to avoid interfering of the result interpretation. Selected menu Toolbox, NGS Core Tools and Trim Sequence.

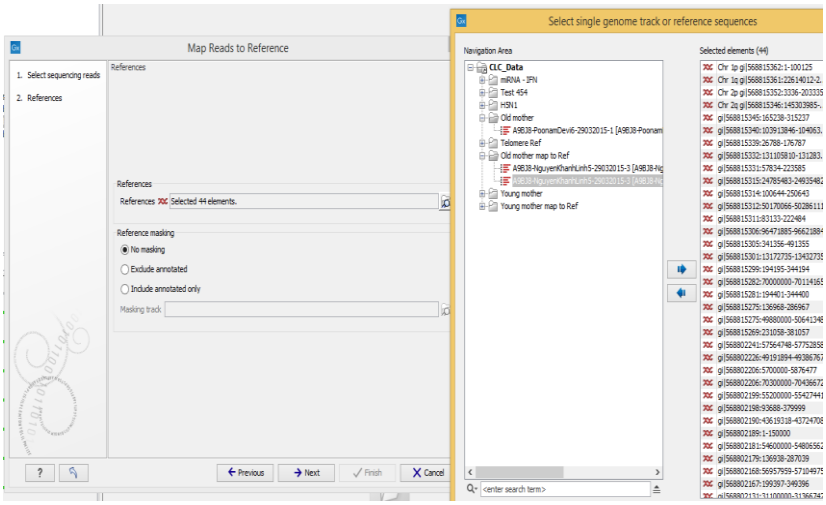


5. On quality trimming menu, selected Trim using quality score Limits 0.002 and Trim ambiguous nucleotides. Maximun number of ambiguities :2.

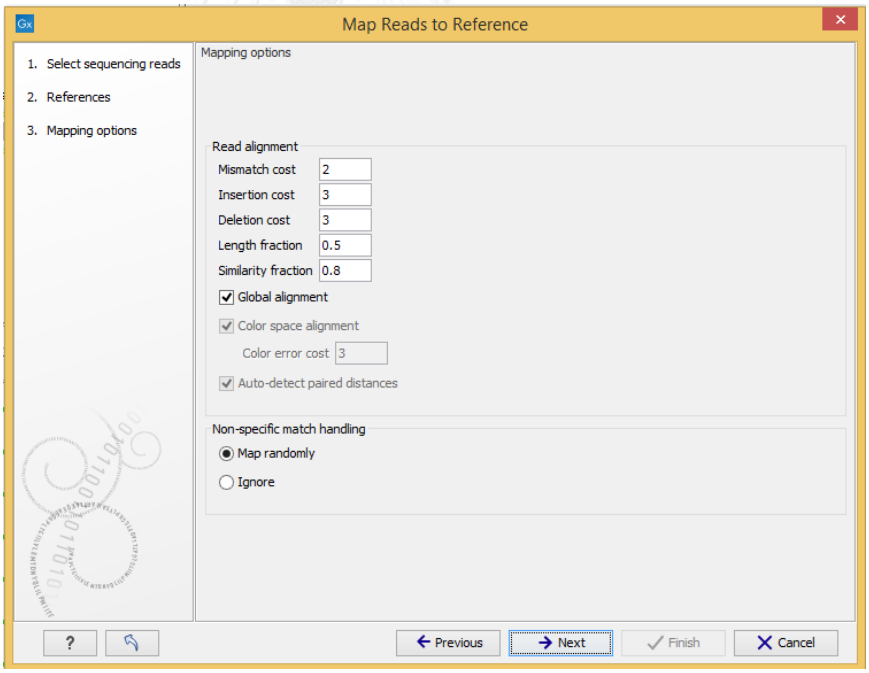


6. Then mapped reads with reference. Selected Toolbox, NGS Core Tools and Map Reads to Reference. On Reference menu, selected references. Reference masking menu, selected no masking.

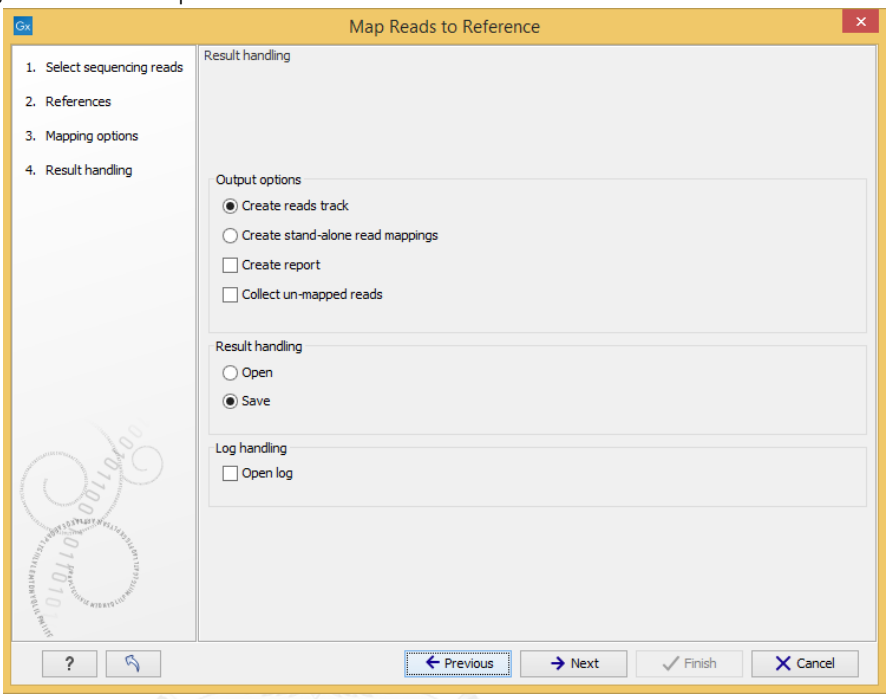




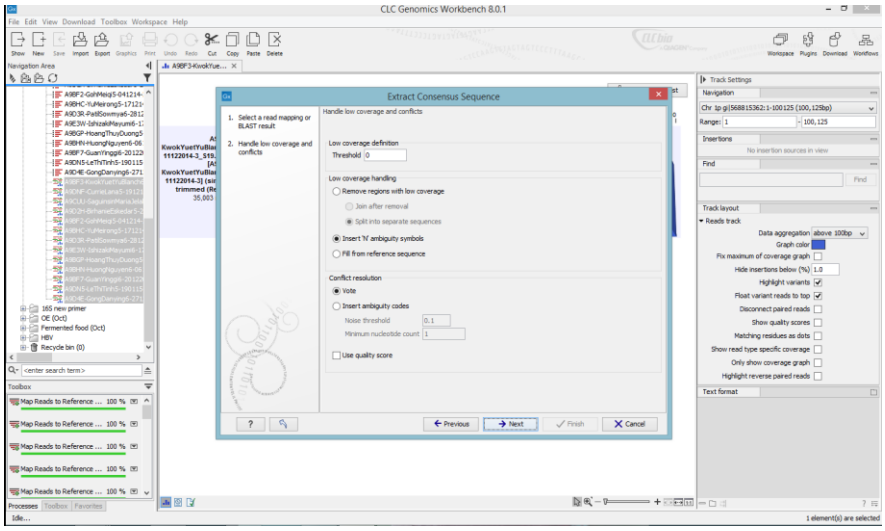
7. Mapping option. Read alignment menu. Mismatch cost =2, insertion cost=3, deletion cost =3, length fraction = 0.5, similarly fraction = 0.8. Selected Global alignment. On non-specific match handing menu, selected map randomly.



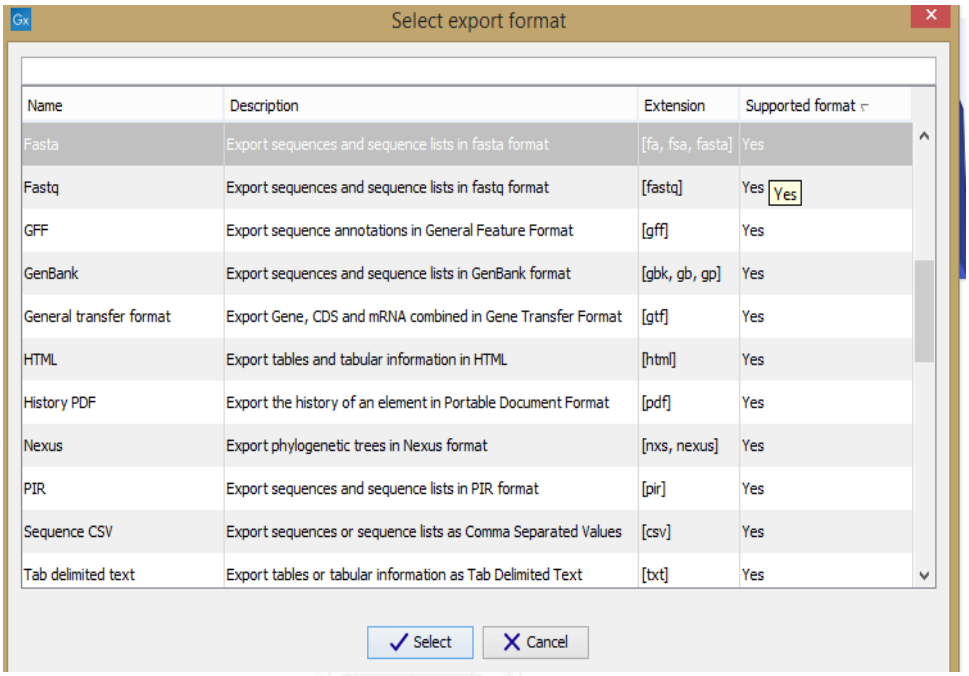
8. Out put option menu, selected created reads tract. Result handing menu, selected save. After that we got mapped sequences files. This files were short multiple fragment reads mapped with reference. Then we used extract consensus sequences for improving sequence quality to change in single strands sequences.



9. Selected Toolbox, NGS Core Tools, Extract Consensus Sequence. Low coverage definition menu. Thershold =0. Low coverage handing. Selected insert “ N” ambiguity symbol. Confrict resolution. Selected vote.



10. Then exported to Fasta files and analysed the repeated sequence (TTAGGG) by Bioedit Bioedit Version 7.2.5.



11. Finally, the telomere lengths were compared between maternal age groups and pregnancy result.

VITA

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