Determination of source and quantity of DNA in spent embryo culture medium

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Alkiodiagnostiikka hedelmöityshoidoissa on enenemässä määrin tarpeellinen erityisesti ensisynnyttäjien keski-iän noustessa, jolloin alkioiden kromosomipoikkeavuudet lisääntyvät ja raskaaksi tuleminen hankaloituu. Tällä hetkellä kromosomien seulontatutkimukset perustuvat alkiosta otettuun biopsiaan. Tuoreissa tutkimuksissa alkioiden viljelyliuoksesta on löydetty alkioiden DNA:ta ja näin ollen viljelyliuoksen käyttöä alkiodiagnostiikassa on tarpeen tutkia. Tutkimushypoteesina oli oletus, että alkioperäistä DNA:ta löydetään viljelyliuoksesta ja siitä voidaan tehdä päätelmiä alkion laadusta. Tämän tutkimuksen tarkoituksena oli optimoida DNA:n eristysmenetelmä viljelyliuoksesta, ja osoittaa, että DNA on peräisin alkiosta käyttäen Y-kromosomaalisen TSPY-geenin määritystä liuoksesta sekä kromosomiseulontaa verraten tuloksia jo analysoituihin alkiodiagnostiikan tuloksiin. Tutkimuksessa analysoitiin lisäksi alkioista saatavaa kuvamateriaalia kontaminaatiolähteistä ja alkion kehitysominaisuuksista EmbryoScope®-inkubaattorista, verraten sitä viljelyliuoksesta eristetyn DNA:n määrään.

Menetelmät: Suolasaostusta ja NucleoSpin plasma XS -kittiä verrattiin DNA:n eristyksessä käyttäen qPCR-menetelmää, elektroforeesia sekä absorbanssi- ja pitoisuusmittauksia NanoDropilla. Viljelyliuokset kerättiin alkion viljelyn päättyessä, DNA eristettiin ja sen kokonaismäärää arvioitiin käyttäen Alu4-aluketta sekä TSPY-alukkeita osoittamaan Y-kromosomaalinen DNA liuoksista qPCR:llä. Kuva-analyysissä käytettiin lisäksi IBM SPSS -tilasto-ohjelmaa. Kromosomiseulontaan käytettiin aCGH-menetelmää ja sen tuloksia verrattiin aiemmin analysoituihin NGS-tuloksiin.

Tutkimustulokset: NucleoSpin plasma XS -kitti oli herkempi menetelmä sekä DNA:n saannin, puhtauden, että toistettavuuden osalta. Kumulussolujen ($p<0,001$), kuten myös kuolleiden solujen määrä ($p<0,019$) nosti DNA:n määrää viljelyliuoksessa. Havaittiin myös, että mitä pidempään alkiota viljeltiin (4, 5 tai 6 päivää), sitä suurempi oli DNA:n määrä liuoksessa ($p<0,001$). Muiden tekijöiden, kuten siittiöiden, ei havaittu merkitsevästi vaikuttavan DNA:n määrään. Kromosomiseulonnan tulokset viljelyliuoksesta aCGH-tekniikalla eivät vastanneet NGS-tekniikan tuloksia biopsioista.

Johtopäätökset: NucleoSpin plasma XS -kitti toimi suolasaostusta paremmin DNA:n eristyksessä saannin ja puhtauden sekä toistettavuuden osalta. Kumulussolujen, alkion viimeisen viljelypäivän sekä viljelyssä havaittujen kuolleiden solujen määrän havaittiin lisäävän DNA:n määrää liuoksessa. Muiden tekijöiden ei havaittu merkitsevästi vaikuttavan DNA:n määrään liuoksessa. Lisää tutkimuksia on tarpeen tehdä suuremmilla näytteiden määrillä. Kromosomiseulonnan tulokset eivät vastanneet NGS-tuloksia, joten menetelmän optimointi on edelleen tarpeen.

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Abstract

Background and aims: Infertility is more common phenomenon especially due to increase in maternal age. Embryo diagnostics is necessary within the couple with repetitive miscarriages and disruption of fertilization. Current procedures for embryo chromosomal screening require still an invasive biopsy of the embryo. There have been promising results of embryo´s spent medium use in analyzing embryo´s DNA and that field is necessary to be investigated more. The hypothesis of this study was to expect embryonic DNA to be found in spent medium and be demonstrated to correlate with the quality of the embryo. The purpose of this study was to optimize DNA extraction method from embryo culture medium and indicate that DNA in spent medium originated from embryo determining Y-chromosomal TSPY gene as marker and comparing aCGH results of spent media to NGS results of chromosomal screening. The aim was also to investigate if the evaluated events from time-lapse EmbryoScope® device correlate with amount of DNA. Especially, feasible DNA contamination sources and factors as well as embryo development properties were analyzed to explore their impact to released DNA amount in spent media.

Methods: Salt precipitation method and NucleoSpin plasma XS kit (Macherey-Nagel) for DNA extraction were compared using electrophoresis, qPCR and NanoDrop measurements. Embryo´s spent media from D4 to D6 embryo cultures were collected. DNA from spent media was determined using Alu4 as target to evaluate DNA amount and TSPY to determine Y-chromosomal DNA. Statistical analyses of time-lapse incubator images were used to investigate relation between contamination sources and embryo properties with DNA amount in spent media. IBM SPSS was used for statistical analyses. To compare chromosomal screening of spent medium with NGS results, aCGH (Agilent) was used.

Results: Yield and purity of the extracted DNA as well as repeatability of the method were better using NucleoSpin plasma XS kit than using salt precipitation method. Cumulus cells as contaminant DNA source ($p<0,001$), as well as lysed cells ($p<0,019$) were observed to increase the DNA amount in spent medium. Culture time was demonstrated to increase the DNA amount among D4, D5 and D6 embryo culture medium samples. Other evaluated factors had no impact on DNA amount in spent medium. Results of spent medium chromosomal screening using aCGH were not consistent with chromosomal screening results of biopsy by NGS.

Conclusions: DNA extraction using NucleoSpin plasma XS kit was more accurate. Cumulus cells and lysed cells, were demonstrated to increase DNA amount in spent medium. The culture time had also impact on DNA amount in culture medium. Other factors, such as sperm cells, had no impact on DNA amount, but more studies must be done because the number of samples was low. Also, the procedure of aCGH must be optimized because or re-evaluated the results were confusing.

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Abbreviations

aCGH	array comparative genomic hybridization
Alu	Alu element
ART	assisted reproductive technique
Bp	base pair
CGH	comparative genomic hybridization
Ct	cycle threshold
FET	frozen embryo transfer
FISH	fluorescence in-situ hybridization
ICSI	intracellular sperm injection
ICM	inner cell mass
IVF	in vitro fertilization
NGS	next-generation sequencing
NS	NucleoSpin plasma XS kit
PCR	polymerase chain reaction
PGD	preimplantation genetic diagnosis
PGS	preimplantation genetic screening
qPCR	quantitative polymerase chain reaction
SM	spent medium
SNP	single nucleotide polymorphism
SP	salt precipitation
TE	trophectoderm
TSPY	testis specific protein Y linked

1. Introduction

Across the Western countries, childbearing rates have been dramatically decreased over the past decades. Life style factors such as smoking and obesity cause risk of reduced fertility among both females and males. The rhythm of life has changed and there has been an increasing trend of age of primiparas. Due to these factors, quantity and quality of oocytes and sperm cells has decreased thereby impeding conceiving. For these reasons, the demand on treatments using assisted reproductive techniques (ART) is growing. (Kovacs 2014; Hart 2016)

The most common ART methods are in vitro fertilization (IVF) and intracytoplasmic injection (ICSI). In both of these methods, oocytes and sperm cells are collected from the couple or donor. In the IVF method, the sperm cells are washed, after which the sperm cells are transferred into the same dish with the oocyte. One of the most vital sperm cells penetrates the oocyte membrane and hence fertilizes the oocyte. In ICSI method, one of the washed and diluted sperm cells is selected and injected into the oocyte. (Tiitinen and Unkila-Kallio 2011)

Risk of spontaneous miscarriage is elevated the higher the maternal age and it is highly associated with chromosomal aneuploidy of the oocyte and therefore of the embryo. Aneuploidy usually leads to disruption of embryonic development at early days of in vitro culture, during the implantation or development after implantation to womb. (Hart 2016) Babies born with chromosomal abnormalities have usually severe injuries (Sariola 2015).

Nowadays, aneuploidy of embryos can be minimized in ART performing preimplantation genetic screening (PGS) for all chromosomes of the embryo. There are many PGS methods to detect a chromosomally normal embryo: array comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) arrays, multiplex quantitative polymerase chain reaction (qPCR) and next-generation sequencing (NGS). However, these methodologies do not work with full accuracy and precision. (Alan and Handyside 2013) Currently, PGS methods require invasive biopsy of cleavage-stage embryo or trophectodermal (TE) cells of blastocyst-stage embryo. Taking a biopsy from a developing embryo may decrease the quality of the embryo. (Cimadomo et al. 2016) Long-term biosafety of biopsy has not yet been evaluated and this technique also demands high experience of practice from the embryologists creating substantially high costs (Xu et al. 2016).

Recently, there have been studies and reports on embryonic genomic DNA (Galluzzi et al. 2015; Mousa et al. 2016; Shamonki et al. 2016; Xu et al. 2016; Feichtinger et al. 2017; Hammond et al. 2017; Liu et al. 2017) and mitochondrial DNA (mtDNA) (Stigliani et al. 2013 and 2014) in embryo culture medium, which are at least partly correlated with the embryo quality or PGS result. Use of embryo spent medium in PGS, instead of invasive biopsy, would be revolutionary in infertility treatments.

The procedure, how the DNA is released from an embryo to the culture medium is still unknown. Apoptotic and necrotic pathways are suggested to be possible DNA releasing pathways to transfer genomic DNA from cytosol to extracellular space. (Chi et al. 2011; Gianaroli et al. 2014; Herrera et al. 2015; Magli et al. 2016; Xu et al. 2016; Liu et al. 2017) Moreover, the degree to which this DNA in spent medium is representative of the developing embryo is currently unclear although correlating results of extracted DNA in spent medium and embryo quality have been obtained. In these studies, the amount of DNA in spent medium has been characterized and the normality of the chromosomes has been evaluated. Nevertheless, more accurate studies of spent medium use in embryo diagnostic are needed. For instance, possible contamination sources, such as maternal cumulus cells, paternal sperm cells and media containing traces of DNA must be scouted precisely to avoid foreign DNA in diagnostics of embryonic genomic DNA. (Feichtinger et al. 2017)

In this master's thesis, spent medium DNA was investigated by evaluating the amount of genomic DNA in spent medium with qPCR and studying the possible DNA origins by comparing amount of observed DNA to different parameters obtained from time-lapse incubator image analysis. In this image analysis, embryos, which were cultured in time-lapse incubator, EmbryoScope®, were analyzed by counting numbers of visible sperm cells, cumulus cells, and dead cells during development, fragmentation level, feasible hatching vesicles and vacuoles. Time frames from the fertilization to further cell number stages were also noticed and the impact of all these factors on observed DNA amount were analyzed. Hence, the correlation of contaminating foreign DNA (from cumulus or sperm cells) was inspected by comparing the DNA amount with evaluated cumulus or sperm cell numbers. The aCGH assay of spent medium DNA was also tested with 3 various embryonic spent medium samples. Results were compared to the corresponding embryos, which had been analyzed with traditional biopsy PGS.

2. Literature review

2.1 Germ cells and fertilization

Germ cell production is called spermatogenesis in males and oogenesis in females. Spermatogenesis differs from oogenesis by its property to produce new spermatogonium cells for the whole life whereas primordial female germ cells are produced up to its total number during fetal development. Approximately 300 million sperm cells are produced daily when fewer than 500 oocytes are ovulated during the lifetime of a woman. (Sainio and Sariola 2015)

2.1.1 Primary germ cell division

Meiosis is a cell division, which takes place in germ cells producing male and female gametes. Male and female germ cell division varies from somatic cell division by its amount of divisions to reduce number of chromosomes from diploid 46 to haploid number of 23. Meiosis requires 2 cell divisions, when mitosis in somatic cells requires only one. In meiosis, 4 haploid male sperm cells are generated. In the female meiosis only one haploid gamete is produced while the remaining genetic material is removed in the first (diploid) and second (haploid) polar body. Steps are shown in figure 1. (Sainio and Sariola 2015)

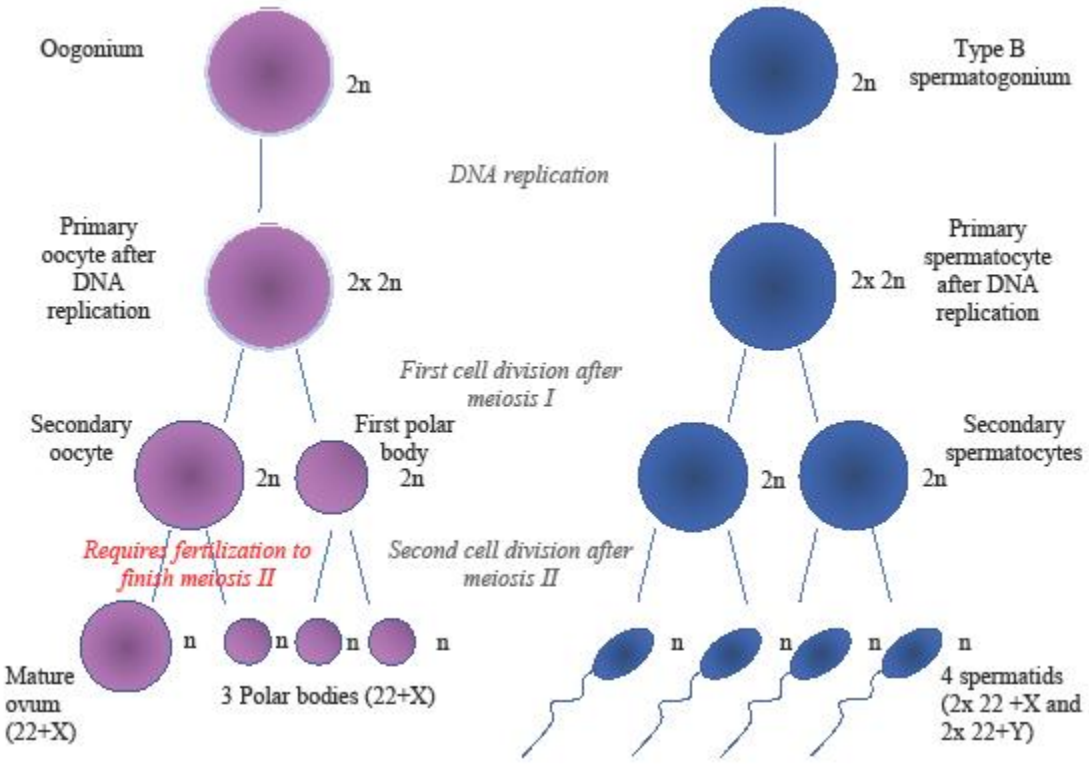

Figure 1: Male and female germ cells division. (Figure modified from Sainio and Sariola 2015)

At meiosis I, DNA is replicated to generate a double set of chromosomal material and thereafter the cell is divided to form 2 daughter cells each having 46 chromosomes. At this stage, genetic variability is enhanced through recombination (crossing overs) and random distributions. Crossing overs occur, when duplicated maternal and paternal chromosomes are pairing with homologous chromosomes and a part of chromosomes change places with these chromosomes. (Sainio and Sariola 2015)

2.1.2 Oogenesis

The total maximum number of female germ cells is reached by the 5th month of prenatal development of fetus. These primordial germ cells are localized in ovaries and after mitotic divisions they differentiate into oogonia. Their amount is estimated to be about 7 million even though estimated number of primary oocytes varies from 600 000 to 800 000 at the birth of a baby. During oogenesis, oogonia differentiate into mature oocytes, which undergo DNA replication to duplicate chromosomes and start meiosis I, arresting at prophase already during fetal development. At puberty, primary oocytes complete meiosis I and after meiotic I cell division, the secondary oocyte (and the first polar body) start meiosis II, which is arrested in oocyte at metaphase approximately 3 hours before ovulation as shown in figure 1. First polar body completes the first meiotic division. After the ovulation, meiosis II is completed in the secondary oocyte only if it is fertilized. The approximate diameter of an oocyte is 100 ㎜ but sizes vary substantially. (Sainio and Sariola 2015)

Oogenesis is localized in both of the ovaries of a woman. The primary oocytes mature inside the follicles, which undergo maturation from a primordial follicle to a preovulatory one synchronously with oogenesis. A matured follicle, graafian follicle, consists of primary oocyte, its surrounding zona pellicuda and corona radiata that is the inner layer of the cumulus cells. The antrum separates oocyte-cumulus cell complex from mural granulosa cells inside the edge of follicle and granulosa membrane (Khamsi and Roberge 2001). Theca cells surround the granulosa membrane outside the follicle. (Sainio and Sariola 2015)

After a hormone stimulated follicular maturation, during ovulation, the secondary oocyte is released from a follicle. Releasing secondary oocyte is still surrounded by zona pellucida and cumulus cells, which support the oocyte and supply the vital proteins for the oocyte. (Sainio and Sariola 2015)

2.1.3 Spermatogenesis

Spermatogenesis is initiated in seminiferous tubules in testis at the beginning of puberty when the gonadal cords, which are solid up till then in the juvenile testis, develop a lumen. They then gradually transform themselves into spermatic canals. Seminiferous tubules are lined by germinal epithelium and it consists of primary germ cells and large Sertoli cells. Male germ cells mature from type A spermatogonia, which undergo mitotic divisions to form daughter spermatogonium cells, of which another becomes same type A and other becomes type B spermatogonia. Type B spermatogonia are transformed into spermatozoa through meiotic cell division according figure 1. Sertoli cells feed these maturing spermatogonia and spermatocytes but they also form a blood-testis-barrier to protect developing spermatozoa cells from immune attack because immune cells do not recognize them as own cells of the human body. (Sainio and Sariola 2015)

The spermatids are carried to the lumen of the tubule after meiosis II cell division. They differentiate into sperm cells (spermiogenesis) on their way from testis through epididymis in order to complete their morphology. In this process, the nuclear condensation, the acrosome formation and the flagellum formation reach the final morphology and functionality. During spermatids´ maturation into spermatozoa, histones in nucleosomes are widely replaced by highly basic proteins: at first by transition proteins and finally by protamines. About 8 % of matured sperm´s genome is packaged by histones and 92 % is packaged by protamines, which bind to DNA condensing the spermatid genome into a genetically inactive state compacting it at 10-fold. (Castillo et al. 2015) Spermatogenesis takes around 74 days at total. Semen consists of sperm cells and plasma produced by the seminal vesicle, the prostate and the Bulbourethral gland. Plasma consists of necessary amino acids and carbohydrates for vitality, enzymes, Zinc ions, C-vitamin and citric acid. There are also prostaglandins against female immune attack, and prostate specific antigen to release single sperm cells from coagulate, which is stabilized by proteoglycans in semen. In ejaculate, there may also be for example some epithelial cells from the glands and urethra. The amount of sperm cells per ejaculate is normally from 200 million to 500 million. (Sainio and Sariola 2015)

2.1.4 Fertilization and early development of embryo

During ovulation, the secondary oocyte, surrounded by zona pellucida and corona radiata (cumulus cells and extracellular matrix), is released from a follicle in the ovary to the fimbriae

of the fallopian tube. The oocyte is fertilized in the ampulla of the distal end of the fallopian tube according to the figure 2. The fertilization requires a vital sperm cell that has undergone capacitation in the female reproductive tract. The capacitation enables the sperm penetration into the oocyte passing through the corona radiata cell layer with hyaluronidase, after which it undergoes an acrosome reaction to fuse with the oocyte membrane penetrating the zona pellucida after binding its receptors. When the sperm binds to the zona pellucida, it enhances a hardening of the zona pellucida proteins preventing polyspermic fertilization of the oocyte (the zona reaction) and commence of intracellular calcium oscillations. Fusion of sperm acrosome and oocyte cell membrane allows sperm nuclei passage into oocyte cytoplasm completing meiosis II in the secondary oocyte. The second polar body is transported to the edge of the ovum. (Okabe 2013)

Male and female haploid pronuclei approach each other and nuclear membranes break down. The pronuclei can be seen clearly in vitro with microscope and they disappear approximately after 12 hours from the fertilization when chromosomal pairing, DNA replication and the first mitosis occur and the embryo (zygote) begins to divide. The dividing cells inside the zygote are

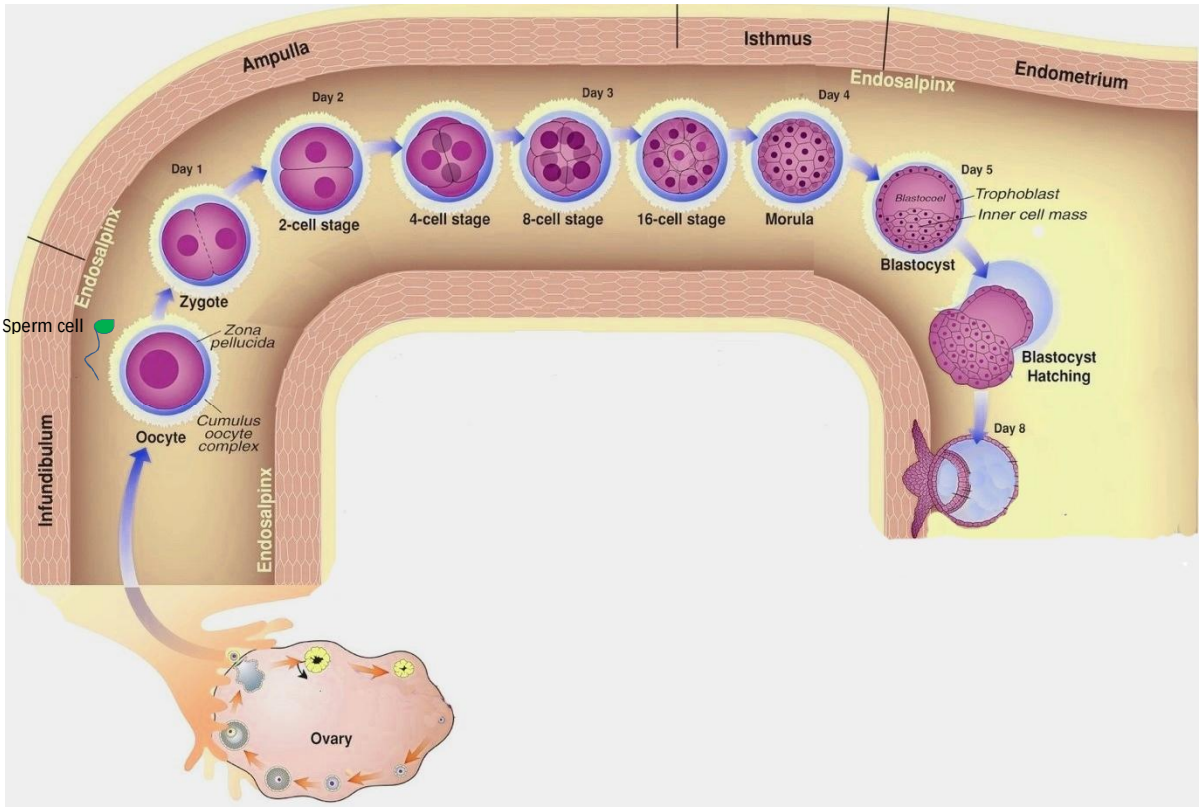

Figure 2: Fertilization and early development of embryo in female reproductive organs. (Figure modified from Atwood and Vadakkadath 2016)

called blastomeres. During embryo development (figure 2), blastomeres cleavage continues up to an approximately 16-cell stage when the embryo is called morula. By the morula stage, blastomeres are very sensitive to the environmental factors because the cells work singly. After the compaction of morula, when the blastomeres get polarized, the embryo is expanded to form two distinct groups in a structure called blastocyst and it is not as sensitive to outer conditions as in cleavage-stage. There are two different cell types in blastocyst: inner cell mass (ICM) cells and TE cells. Blastocoel fluid fills the hollow structure of the blastocyst. ICM cells form later the fetus, while the outer TE cells give rise to extraembryonic tissues such as the placenta. (Wolpert et al. 2007; Magli et al. 2016) The blastocyst is carried to the uterus by day 5. Differentiated cell types keep on dividing and the blastocyst is implanted to the womb with TE cells after embryo hatching out from zona pellucida, which has become thinner during the blastocyst development and expansion. Implantation occurs usually by day of 8. (Partanen et al. 2015)

2.1.5 Chromosomal abnormalities of embryo

Embryo's chromosomal abnormalities are a result from nondisjunction of meiosis I or II in gametes or first DNA replication and mitotic cell divisions of the zygote. An abnormal number of chromosomes is called aneuploidy and it is the most common cause of reproduction failure throughout the nature. (Hassold et al. 1980; Kalousek et al. 1993). Aneuploidy as result from oocyte is usually the most common cause for embryo's aneuploidy (Dailey et al. 1996). Maternal age is highly associated with nondisjunction increasing the risk of meiosis failures (Munné et al. 1995). Majority of aneuploidies are originated from nondisjunction at meiosis I in primary oocyte (Hassold et al. 1987 and 1995; May et al. 1990; Antonorakis et al. 1991 and 1993; Zaragoza et al. 1994), except trisomy 18, which mostly occurs at maternal meiosis II (Fisher et al. 1995). Trisomies 47XXY and 47XYY are 50 % and 100 % paternally derived, respectively (Hassold et al. 1987; McDonald et al. 1994). Trisomy 8, 9, 13, 18, 21, monosomy X (Turner syndrome) or trisomy XXY (Kleinefelt syndrome) may lead to live child birth but aneuploidies in other chromosomes lead to miscarriage during the pregnancy. (Munné and Cohen 1998)

Mosaicism is a chromosomal abnormality form that is not present in all cells, only some as results of the occurrence of two genetically distinct populations of cells within an individual, derived from a postzygotic mutation (Freed et al. 2014).

2.2 View on assisted reproduction techniques

After the birth of the world's first IVF baby in the United Kingdom 1978, the use, development and demand on assisted fertility treatments have increased (Kovacs 2014). Across the Western countries, childbearing rates have dramatically decreased over the past decades. It is estimated, that every 6th man or woman suffers from infertility at some point during the life. In Finland, 14 100 assisted fertility treatments were performed in 2015, of which 18,2 % resulted in ongoing pregnancy and approximately 2570 babies were born by these treatments. It was 5,6 % of all childbirth that year. (https://www.thl.fi/fi/tilastot/tilastot-aiheittain/seksuaali-ja-lisaantymisterveys/hedelmoityshoidot, 30.10.2017) In 2014, it was estimated that over 5 million children have been conceived in vitro worldwide and nowadays the multiple pregnancy rates have been decreased after single fresh and frozen embryo transfer has been observed to be safer for mother and the fetus (Kovacs 2014).

2.2.1 Causes of infertility and the use of ART methods

Infertility is defined as not conceiving a pregnancy after at least 12 months of unprotected coition regardless of whether or not a pregnancy ultimately occurs. There are two types of infertility: primary and secondary. Primary infertility means, that no child has been born or the pregnancies have been failed but discussing about secondary infertility, the couple has already a child but after 12 months coitus, there is no suggested pregnancy. Causes of infertility can be physical, genetic or based on life style factors among males and females. Usually, the primary reason for infertility is unknown. (Tiitinen and Unkila-Kallio 2011)

Beside the physiological and anatomical influences among women, such as disruptions in ovaries, fallopian tubes and uterus as well as endometriosis (Tiitinen and Unkila-Kallio 2011), the most powerful influence relating to chance of conceiving is her age. As the age of the woman increases, ovarian follicular pool reduces, as well as probability of ovulation perturbations and meiotic failures in oocyte meiosis increases. (Oktem and Urman 2010) Women´s childbearing is increasingly postponed into 30s (instead of previous 20s), resulting to the increased need of recourse for assisted fertility treatments. Besides of the increasing maternal age, life style factors, such as obesity cause risk of reduced fertility among both females and males. However, also impacts of low peripheral body fat and hard exercise have been reported having negative impacts on fertility. Prevalence of sexually transmitted diseases and smoking have significant negative effects on female fecundity at a population level (Sharara

et al. 1994). Also, viruses, such as mumps, have been found to increase the risk for infertility. Physiological factors, such as myomas and greater prevalence of polycystic ovary syndrome have also been reported to influence negatively to conceiving. (Tiitinen and Unkila-Kallio 2015, Hart 2016)

Among men, there are several reasons for poor quality and quantity of semen. Azoospermia is a medical condition, when semen contains no sperm. It can result from many disturbances: hormonal, testicular or incomprehensive of ejaculation even though semen is normally produced. Oligozoospermia, when concentration of sperm in semen is low, is the most common medical condition to cause infertility in males. (Tiitinen and Unkila-Kallio 2011)

Nowadays, excess weight and obesity have become a serious problem among adult men of reproductive age throughout the world and obesity has been observed to be highly associated with azoospermia and oligozoospermia (Wang et al. 2017; Sallmén et al. 2006). Life style factors, such as smoking, are also cause for weak quantity and/or quality of semen or disorders of ejaculation. Physiological disorders in male reproductive organs, hormonal regulation disruptions or immunological factors, such as Ig A and Ig G, in semen may cause infertility as well. Usually the primary reason for male infertility is not found even though semen analysis is done. (Tiitinen and Unkila-Kallio 2011) According recent studies, alterations in epigenetic factors, distribution of histones and the additional sperm chromatin-associated proteins, like protamine isoforms, are also being detected in infertile patients and suggested to have greater role in fertilization and genetic regulation than was thought previously (Castillo et al. 2015).

Genetic influences, such as Turner's syndrome or Fragile X permutation carrier status and numerous genes, are involved in infertility, for example caused by ovarian failures (Ledig et al. 2010). A common single-nucleotide polymorphism of BRCA2 is also associated with severe oligozoospermia (Zhoucun et al. 2006). Cancers in reproductive organs, metastasis and treatments (chemotherapy, radiotherapy and surgery) for a wide of range cancer types are also related for infertility among female and male (Anderson et al. 2006; Howell and Shalet 2005; Huddart et al. 2005). In addition, autoimmune causes have been reported of being associated with infertility (Wheatcroft et al. 1997).

2.2.2 ART methods

The most common ART methods are IVF and ICSI. Treatments are carried out in a scrupulously clean laboratory. IVF is performed by placing the harvested oocytes, after cumulus cell denuding, from the woman and the sperm separated from the man's semen sample onto the same common culture dish to fertilize. Before oocyte harvesting from the follicles in the ovaries under ultrasound, the woman receives hormonal treatment that helps more than one follicle to grow and the oocytes contained in them to mature for fertilization. (Tiitinen and Unkila-Kallio 2011)

ICSI is used as a treatment especially for infertility due to the man if the sperm count in the semen sample is very low or if the motility of the sperm is particularly poor. The reference values for semen according WHO (2010) are: semen volume ≥ 1.5 ml, sperm concentration ≥ 15 million per ml, progressive motility 32 % and morphologically normal forms ≥ 4 %. (Cooper et al. 2010) ICSI is also used when normal IVF has not led to fertilization. Before the ICSI treatment, the woman receives hormonal treatment and oocytes are collected just as in IVF. Motile sperm are separated from the man's semen sample for intracytoplasmic injection. Fertilization is aided by injecting one sperm into a ripe oocyte with a thin glass needle. (Joris et al. 1998) Special form of ICSI, called physiological intracytoplasmic injection (PICSI), has been evolved to select a sperm that is capable of binding to hyaluronan such as in natural fertilization when sperm must penetrate the corona radiate with hyaluronidase. PICSI is a technique that stimulates the natural selection of mature sperm. The principle of this method is the cultivation of mature sperm in a hyaluronan gel containing dish. The selected sperm cells are then used in intracytoplasmic injection. (Witt et al. 2016)

Insemination is also labeled as one of the ART methods. In this procedure, sperm cells are inserted into female reproductive tracts to fertilize the oocyte. This method requires the hormonal or antagonist stimulation of female follicles to mature like in other in vitro fertilization methods as well as semen ejaculation sample processing before the insemination. (Tiitinen, Unkila-Kallio 2011)

2.2.3 Embryo culture

In both methods, IVF and ICSI, the fertilized oocytes are cultivated in incubator in which the temperature is +37 °C and humidity and gas content (O_2 5 % and CO_2 5 %) are carefully

controlled to mimic the conditions in the female reproductive organs. Embryos are cultured either as group culture or singly in embryo culture medium droplets. Droplets are covered with mineral oil that protects the embryo in a droplet from feasible harm of excessive O_2 or other compounds and prevents the medium evaporation and due to that, osmotic and pH changes. The oocyte fertilization and development of the embryos are monitored during each phase and scored according to the morphology requirements. The treatment results of ICSI are comparable to those of IVF. Usually 70 % of the oocytes become fertilized in the culture dish and about 30–50 % of the fertilized oocytes develop into good-quality embryos. (https://www.ovumia.fi/koeputkihedelmoityshoidot-ivf-icsi/, 26.10.2017) The best of these is selected for fresh embryo transfer, during which the embryo is placed into the uterus 2–5 days after in vitro fertilization. 30–40 % of fresh embryo transfers lead to a clinical pregnancy. Usually the remaining good-quality graded embryos are frozen by vitrification technique and stored in liquid N_2 to wait for possible frozen embryo transfers (FET) in the future. FET has become more common when the vitrification methods have been developed. FET is also used when the embryo has undergone some diagnostics procedure. In these cases, biopsy is taken from the blastomeres or TE cells and then the embryo is vitrified to wait for the biopsy diagnostics results. (Tiitinen, Unkila-Kallio 2011)

2.2.4 Embryo grading

Accurate selection of embryos for transfer and prediction of implantation success is important topic in fertility treatments (Depa-Martynov et al. 2007). In general, the quality and the rate of development in embryos that are fertilized in vitro may vary widely and these differences may indicate the inherent diversity in the potential of gametes as well as in details of the in vitro fertilization method (Cummins et al. 1986). Embryo selection is normally based on the embryo morphology grading. Current embryo grading systems differ with regards to selection of embryo stage and criteria for evaluation of embryo quality. In grading, development stage according morphology of cleavage stage embryo or expansion of blastocyst are evaluated but also cell morphology of ICM and TE cells are evaluated in blastocysts to form the score of the embryo. (Nahid and Poopak 2015) In Ovumia Fertinova, cleavage-stage embryos are graded using a modified scoring system (Fridström et al. 1999; Mohr et al. 1985). In this scoring system, an embryo with the expected developmental stage receives a starting score of 3,5, whereas fast or slowly cleaving embryos receive a starting score of 3,0. Then, every observed reducing factor reduce the score with increment of 0,5: an unequal size of blastomeres, the

presence of more than 10 % fragmentation, a large perivitelline space or cytoplasmic abnormalities. The presence of more than 25 % fragmentation or the presence of multinucleated blastomeres reduce the score with increment of 1,0. (Fridström et al. 1999; Mohr et al. 1985) In grading blastocysts, scoring system of Gardner et al. (2000) is in use in Ovumia Fertinova. The expansion grade is evaluated from 1 to 6 (1: blastocyst development and stage status; 2: blastocoel cavity more than half of the volume of the embryo; 3: full blastocyst, cavity completely filling the embryo; 4: expanded blastocyst, cavity larger than the embryo, with thinning the zone pellucida; 5: hatching out of the zone pellucida and 6: hatched out of the zone pellucida).

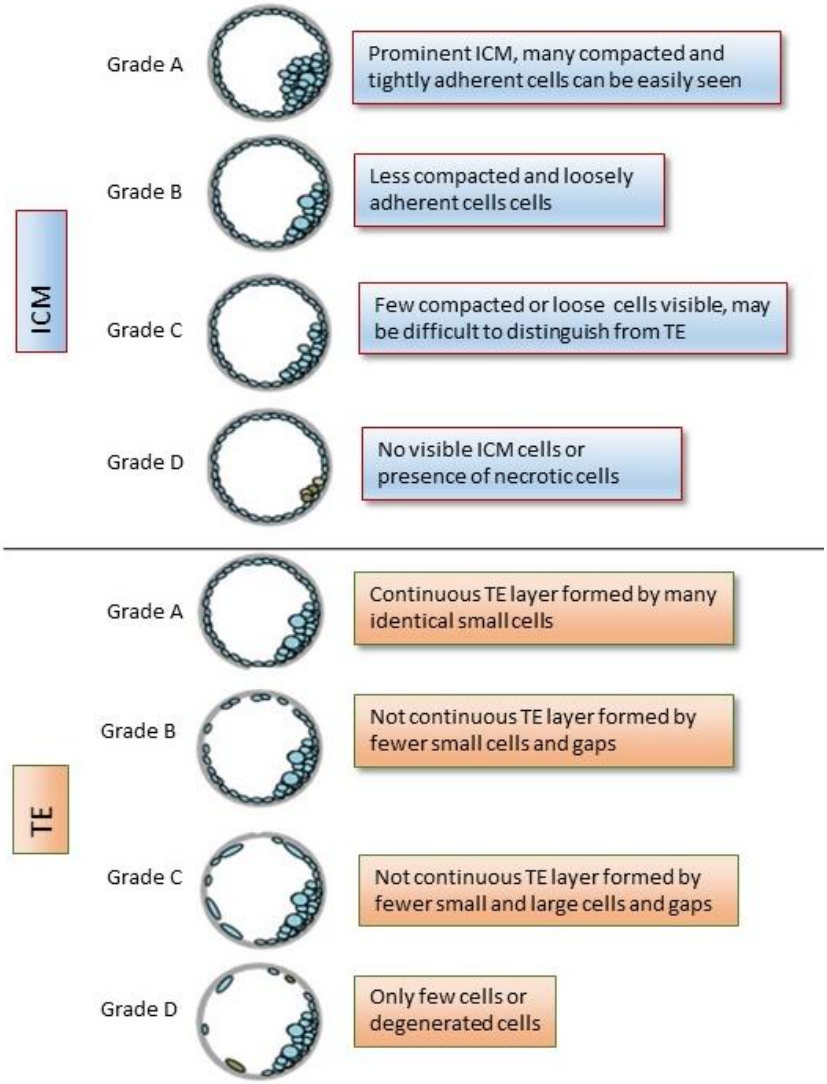

Figure 3: Inner cell mass (ICM) and trophectoderm (TE) grading in blastocysts according to Gardner et al. 2000. (Image modified from https://gamete-expert.com/news/article-32.html, 31.10.2017)

Additionally, ICM and TE of blastocyst are also scored by Gardner from A to C according the figure 3 (ICM: A: many cells, tightly packed; B: several cells, loosely packed and C: very few cells, TE: A: many cells, forming a cohesive layer; B: few cells, forming a loose epithelium and C: very few cells). (Gardner et al. 2000)

2.2.5 Time-lapse monitoring of embryos

The traditional procedure in embryo culture by embryologists is removing the embryo from the incubator once per day to assess morphology and cleavage-stage. In time-lapse incubator, the embryos can be monitored by the inside build microscope camera and scored without removing them from the optimal culturing conditions in the incubator. The camera images the embryos at preset intervals of about 5-20 min. It has been previously reported, that earlier cleaving embryos have a better chance to develop into blastocysts and implant but also the blastocyst reached embryos are less likely to be aneuploid (van Montfoort et al. 2004). Thus, with time-lapse monitoring software, video represents embryo development and contains much information of embryos on time points of the cleavages and the dynamics of morphologic changes, such as fragmentation. Hence, this automated time-lapse systems that identify the embryos to be transferred with the help of a software program also may ease the embryologist's work. (Kovacs 2014)

EmbryoScope® and Primo Vision (Vitrolife) are the most widely used time-lapse technologies according to review of time-lapse techniques by Kovacs (2014). In this thesis, EmbryoScope® time-lapse incubator was used. In EmbryoScope®, the embryos are individually cultured in microwells on special culture dishes called Embryoslides (Vitrolife), which allows the monitoring of up to 12 individually cultured embryos per slide. The maximum number of slides in EmbryoScope® is 6 at the same time. The camera system uses low intensity red LED illumination (635 nm) with <0.5 secundum per image light exposure and it can evaluate the embryos in 7 focal planes. (http://www.vitrolife.com/en/Products/EmbryoScope-Time-Lapse-System/, 19.10.2017)

2.3 Embryo diagnostics

Despite numerous advances in the field of reproductive medicine, the likelihood of achieving a live birth in some couples undergoing ART remains still low. For this reason, modern molecular diagnostic methods make it possible to obtain a genetic profile of an embryo to enhance live

births by ART treatments. With these methods, it can be avoided transferring embryos with chromosome set defects or chromosome rearrangements leading to congenital pathology, and even to prevent a number of genetically determined diseases. (Munne et al. 2010; Alfarawati et al. 2011)

Infertile couples, which were required for ART (IVF or ICSI), appear to be affected by higher frequency of chromosomal rearrangements than the general population (Clementini et al. 2005). Thus, PGS has become a more common tool to screen in vitro embryos for the possible aneuploidy and to select the euploid embryos with normal number of chromosomes to be implanted. In PGS, all chromosomes are screened and it has been studied and reported to improve treatment results reducing failures of embryo´s development and implantation, decreasing miscarriage and reducing the need for prenatal trisomy screening. PGS is especially relevant in cases of recurrent miscarriage (Munné et al. 2005; Shahine and Lathi 2014), repeated implantation failures (Blockeel et al. 2008), advanced maternal age (Munné et al.1995) and male infertility (Harper and Sengupta 2012). More than 26 000 PGS treatments had been cycled worldwide by year 2015. (De Rycke et al. 2015; Lu et al. 2016) Preimplantation genetic diagnosis (PGD), instead, is a procedure whereby oocytes or in vitro fertilized (IVF or ICSI) embryos are examined in cases the mother and/or the father is a carrier for special gene mutation, translocation or inversion. After PGD, the healthy embryo can be transferred. (Harper 2009) More than 6000 cycles of PGD have been reported in 2015 being performed in the past 13 years (De Rycke et al. 2015).

There are few techniques for PGS: Digital PCR, Real-time PCR, comparative genomic hybridization (CGH), aCGH, SNP microarray and NGS (Alan and Handyside 2013). In PGD, Fluorescence in-situ hybridization (FISH) was first used for translocation carriers (De Ugarte et al. 2008) and it was usually used to examine from five to nine chromosomes in single blastomeres at day 3 of embryo culture. Fluorescent labelled probes of target gene sequence are used in FISH. The probes hybridize the template DNA and the fluorescence can be localized visualizing with microscope. DNA probes are specific to chromosomes 13, 18, 21, X, and Y to detect the most common chromosome failures found at birth. (O´Connels 2008)

Afterwards, it was demonstrated in many studies that the aneuploidies may occur in any of the 24 chromosomes in preimplantation embryos (Wells et al. 2008; Schoolcraft et al. 2010) thus aCGH has been employed in PGS in the majority of the cases. This microarray-based CGH

compares amplified DNA content from two different fluorophore labelled genomes. The two genomes, a test sample and reference are cohybridized onto a solid support, which is usually a glass microscope slide, on which the DNA fragments have been immobilized. It allows for high-resolution evaluation of DNA copy number alterations associated with chromosomal abnormalities. (Bejjani and Shaffer 2006) SNP array is based on the array technique as well, where the immobilized allele-specific oligonucleotide probes are chosen to determine the specific polymorphism and used to detect the both alleles. In this method, hundreds of thousands of SNPs can be genotyped simultaneously. (Alan and Handyside 2013)

Digital PCR analysis is used mainly in countries, like Germany, in which legal restrictions prevents PGS on embryos after pronuclear stage of embryo (Brezina et al. 2016). In this method, polar bodies are usually used. After polar body lysis, lysate is pipetted into separate wells and multiplex PCR is performed with several multicopy chromosome specific targets per chromosome. With real-time qPCR, at least two sequences of each arm of each chromosome are amplified. (Alan and Handyside 2013) The protocol of qPCR is discussed in section 2.6.1.

NGS, also known as high-throughput sequencing, has been used for PGS. NGS is based on high number of sequencing reactions, which are run in parallel, leading to large output in terms of obtained. Various NGS techniques are available. Possibility to customize the scope of testing, for example changing the needed numbers of reads per sample, examining several tens of samples simultaneously and reducing the testing costs are important advantages of NGS. (Fiorentino et al. 2014; Aleksandrova et al. 2017)

Conventional PGD/PGS procedures require mainly the invasive removal of the blastomere or TE cells from embryo, which may intervene with embryonic development (Kirkegaard et al. 2012; Cimadomo et al. 2016). It also requires technical expertise with training and experience from the embryologists to perform the biopsy that raises the costs of PGS or PGD for the clinic and patient. Therefore, less invasive and easier methods are needed. Less invasive techniques, such as use of the blastocoel fluid and polar body biopsy have been under study. PGS analysis from blastocentesis sample has been reported (Gianaroli et al. 2014) to contain embryonic DNA, but sample collection, aspiration of blastocoel fluid after penetrating the trophectodermal layer with a sharp pipette, may lead to developmental damage of TE cells. This technique also requires micromanipulation, as does taking a biopsy. Even though polar body biopsy is mildly invasive for the embryo by removing the polar body from the zygote, it has been suggested to

improve live birth rates, but as polar body, it only mirrors the maternal oocyte chromosome status. Due to that, paternally derived or mitotic defects are not analyzed. (Capalbo et al. 2013) In addition, the DNA in spent embryo culture media is investigated for its embryonic origin. Spent medium use in embryo diagnostics is discussed in section 2.4.1.

2.4 Embryo culture medium

In vitro developing embryos are cultured in specific embryo culture media. There are various embryo culture media in use. Sequential media are purposed to be changed during the culture and whereas some are planned for the whole culture period. Culture medium that has been used in this thesis is "1-step" so it is not meant to be changed during the culture. The purpose of culture media is to ensure the optimal cell growth and development circumstances for the embryo. The medium droplet, where the embryo grows, mimics the conditions of the woman´s fallopian tube and uterus. Media contain mainly carbohydrates and amino acids for nutriment, buffers to equal the pH changes and human serum albumin. There are also vitamins, and antibiotics in the media. (Lane and Gardner 2007) The human serum albumin is a protein, that has a good binding capacity for H_2O, ions, fatty acids, hormones, bilirubin and drugs and it is the main zinc transporter through the plasma to the cells. In addition, albumin has an ability to bind to DNA. (http://www.uniprot.org/uniprot/P02768, 18.10.2017)

2.4.1 Earlier studies of spent medium in embryo evaluation

According previous studies (Galluzzi et al. 2015; Mousa et al. 2016; Shamonki et al. 2016; Xu et al. 2016; Feichtinger et al. 2017; Hammond et al. 2017; Liu et al.2017), embryonic DNA has been reported to be found in spent embryo culture medium. In these studies, the amount of extracted embryonic DNA has varied a lot. At first, Stigliani et al. (2013 and 2014) observed mitochondrial and genomic DNA in spent medium, which correlated with embryo quality. Galluzi et al., reported the DNA amounts being among day 3 spent medium samples 58 pg (median) and among day 5 spent medium samples 67 pg (median), which corresponds the DNA amount of about 10 cells (Galluzzi et al. 2015). Used methods for evaluating the DNA amount and origin, have been qPCR, aCGH and NGS. qPCR has been used to evaluate DNA amount using multicopy gene targets and sex chromosome linked gene targets to prove the embryonic origin of the DNA in spent medium. For patients with known gene mutation, spent medium has also been analyzed using specific mutated gene targets as proof of concept for embryonic origin of DNA in spent medium (Galluzzi et al. 2015; Liu et al. 2017) with partly corresponding

results. (Galluzzi et al. 2015). Chromosomal screening results using aCGH of spent media have correlated quite well with trophectodermal biopsy in proof of concept study (Shamonki et al. 2016) and polar body biopsy PGS (Feichtinger et al. 2017) as mirror image. In IVF and ICSI procedures, cumulus cells are denuded but there might still exist some foreign cells contaminating the medium. Additionally, sperm cells and fresh culture medium may act as DNA contamination sources. Hence, contamination sources should be investigated when the embryo culture procedure is to cultivate the embryo in the same medium during the whole culture.

2.4.2 Possible origins of embryonic DNA in spent medium

Today, it is not clear how the DNA is released from the embryo into the medium (Liu et al. 2017). The main hypothesis is that the dead cells within the embryo would be responsible of the DNA found in the medium, but exosomes as DNA releasing transporters are also under investigations. In addition to passive release of DNA from the apoptotic or necrotic cells, there have been studies among cell culture experiments, such as frog heart, lymphocytes and chicken embryo fibroblasts of active DNA deliver to the culture medium. This phenomenon has been demonstrated to occur without notable amount of apoptotic or necrotic cells. In these studies, the released DNA concentration in the medium after incubation was the same every time after medium change. Thus, it is assumed to be some kind of homeostatic method because the DNA seemed to be released by the living cells indicated by the absence of cell death markers. (Gahan and Swaminathan 2008) However, there is not researched significant connections with this kind of DNA release method among human embryos but after own genome activation at the end of cleavage stage, this type of DNA releasing method should be investigated.

Apoptosis is the most suggested theory of DNA release (Gianaroli et al. 2014; Herrera et al. 2015; Magli et al. 2016; Xu et al. 2016; Liu et al. 2017). Based on apoptosis theory, DNA is cut into nucleosome-sized fragments of approximately 180 base pairs (bp) and transported inside the vesicles. Among embryos, the unnecessary and chromosomally abnormal cells are demonstrated to undergo apoptosis. (Hardy 1997) According Wu et al. and Tobler et al., number of mosaic cells in cleavage-stage embryos might be even 60 % but the mosaicism is observed to decrease among blastocysts (Wu et al. 2015; Tobler et al. 2015). Apoptosis has been observed in mosaic mouse blastocyst cells (Feichtinger et al. 2017), which supports the theory of apoptosis-mediated DNA release. Cleavage-stage embryos are not demonstrated to undergo

apoptosis, whereas especially 5-6 days old blastocyst-stage embryos are observed of having enhanced apoptosis (Hardy et al. 1989). This phenomenon is suggested to be due to the genome inactivation during early cleavage stage of the embryos (Bakri et al. 2016). In addition, DNA is observed to be fragmented in the spent medium according Hammond et al. (2016). This supports the apoptotic pathway hypothesis because in apoptosis, the DNA is cut into 180 bp fragments and this length and its multiply fragments are demonstrated to exist in blastocoel fluid and culture medium samples (Stigliani et al. 2013; Liu et al. 2017).

Apoptotic components are usually removed by phagocytes or phagocytosis particles in cell membrane. These cell membrane molecules appear onto the cell membrane not until during blastocyst stage. (Hardy 1997) However, Galluzzi et al. (2015) observed the median of DNA amount in day 3 spent medium samples to be 58 pg, which corresponds to the DNA amount of more than about 10 cells. Three days old embryo contains about 6-10 cells (Ottolini et al. 2015), thus apoptotic pathway for DNA release cannot be, at least, the only secretion method.

Another DNA releasing method is necrosis-mediated pathway. Necrosis has been also observed to occur among embryos. In necrosis, the DNA is cut into random sized fragments and it is released as free, unlike apoptotic DNA fragments are transported in the vesicles. (Chi et al. 2011). Phagocytosis is also common for necrotic cell components, but in different and weaker way than in apoptosis using pinocytosis enabling a release of larger DNA amount into the medium (Krysko et al. 2008). In general, necrosis is demonstrated to act as a response to environmental signal unlike apoptosis, which is a response to cell regulation, for example if mutation in genome has been observed. Thus, if the pathway is necrosis, the DNA may represent better the embryo than apoptotic cut DNA fragments. (Chi et al. 2011)

Fragmentation of the embryo at day 3 has been associated to both apoptosis and necrosis as well as increased aneuploidy, polyploidy and weakened developing potential (Hardy et al. 2003; Stigliani et al. 2013; Chi et al. 2011). The elevated DNA quantity in spent media has been reported to associate with the increased fragmentation level in embryos (Stigliani et al. 2014; Liu et al. 2016). As results from fragmentation, the cells lose cytoplasm and cell organelles, such as mitochondria and mtDNA. The role of mtDNA rate has been under investigations. At first, Stigliani et al. (2013) reported the increased mtDNA amount in spent medium of fragmented embryos but later they (2014) demonstrated the mtDNA increase also among embryos with good morphology. (Stigliani et al. 2013 and 2014)

There have also been suggestions of exosome pathway as DNA transporter. Exosomes are small vesicles with an average size from 30 to 150 nm and they are believed to originate from endosomes. They contain a variety range of components, such as cytoskeletal and chaperon proteins, messenger RNA, micro RNA, proteins such as metabolism enzymes. Their major function and role of exosomes are not well understood but it is suggested that they are involved in protein turnover, immune system and RNA carriers but also the somatic cell properties are suggested to be transferred for other cells through the exosome-mediated pathway. (Aucamp et al. 2016; Cresticelli et al. 2013) It is suggested that exosomes play a critical role in embryo development. According Qu et al., embryo development was observed to weaken after medium change but when the exosomes were transferred into new embryo culture medium, the embryo development improved notably (Qu et al. 2017). In the recent study, Pallinger et al. observed the lower level of exosomes and nucleic acids in the embryo culture media among the embryos, of which were determined as "confirmed competent". Due to that, it was suggested, that the low level of exosome and nucleic acid amount would identify the competent embryos. (Pallinger et al. 2017)

2.5 Analyzing DNA amount with qPCR

To determine DNA amounts or gene pool, target for PCR detection has to be selected. Autosomal gene target represents the DNA overall, depending on the copy number of that target in genome. Chromosomes 1-22 are autosomes and there are 2 copies of each. Instead, sex chromosomes XX in females and XY in males are not autosomes. Autosomal multicopy gene targets are useful in PCR based analyses for increase the chances of amplification, moreover sex chromosome linked gene targets are used to evaluate the reliability of PCR results. (Galluzzi et al. 2015)

In spent media analysis, for example mitochondrial targets have been used (Stigliani et al. 2013 and 2014), as well as multicopy gene target TBC1D3 and Y-linked TSPY target (Galluzzi et al. 2015). Targets of mutated genes have also been used to determine reliability of spent medium analysis if the parent´s gene mutation is known (Liu et al. 2017; Wu et al. 2015). In this thesis, discussion of different DNA targets is focused in used targets, Alu and TSPY.

2.5.1 Quantitative polymerase chain reaction

qPCR is a method based on the principle of the traditional PCR. PCR was invented in 1985 by Kary Mullis et al. revolutionizing the possibility to amplify specific DNA sequences. In real-time qPCR the amplification is monitored at the same time as the reaction proceeds. This property enables to better determine the starting amount of DNA in the sample. It was demonstrated first in early 1990´s, when Roche Molecular Systems and Chiron published their versions of real-time PCR: they added ethidium bromide (EtBr), a common fluorescent dye, in the PCR run and videotaped the PCR reactions under the ultraviolet light enabling to visualize the DNA accumulation during the run. With nowadays qPCR, it is possible to accurately and quickly determine the changes in gene expression by experimental stimuli, physiological effect or individual properties in DNA even in very low levels of genetic material. (Ishmael and Stellato 2008)

The basic protocol of qPCR is the same as in conventional PCR: it is both a thermodynamic and an enzymatic process. A template of the DNA that is to be copied, two sequence-specific, single stranded oligonucleotides that act as primers to start the amplification are required. The primers each hybridize to their complementary target DNA sequences at the separated template strands of the DNA forward and reverse primer and during each cycle of the PCR, amount of the target DNA is doubled in the sample. Nucleotide triphosphates (dNTPs) are required to form the new fragments according the DNA template and a heat-stable DNA polymerase enzyme, that catalyzes that DNA extension from 3´end of the primers, is needed as well. In addition, the reaction components require magnesium ion containing buffer to stabilize the PCR conditions. As a baseline in the PCR reaction, a passive 6-carboxy-X-rhodamine (ROX) reference dye was used in this thesis. Depending on the template properties, some PCR additives can be used to stabilize the reaction conditions. (Valasek and Repa 2005; Ishmail and Stellato 2008)

PCR is based on the temperature changes in reaction: denaturation, annealing and extension. First, at denaturation, the temperature is raised to about 95 °C, when the DNA double strands separate. The temperature is then lowered to allow for primer annealing, and finally the temperature is raised around 72 °C, which is optimal for the polymerase enzyme to extend the primers adding dNTPs to the DNA according to the template. This cycle is repeated several times, usually about 35-45 cycles, depending on the application. Sometimes the annealing and extension steps can be combined. (Valasek and Repa 2005; Ishmael and Stellato 2008)

During the PCR run, the number of amplified PCR products is doubled every cycle thus the reaction will eventually reach a plateau. At the first cycles in the PCR, the fluorescence signal is weak but when the product accumulates exponentially, the signal of fluorescence also amplifies. In a typical qPCR run all response curves reach plateau at the same level thus the difference between curves of the samples is separated in the exponential phase, which reflects the difference in the initial amount of the template. The difference of curves is quantified and measured in this thesis by using the cycle threshold (Ct) values, which reflects the cycle amount to reach the threshold fluorescence signal level. Setting the threshold level is selected by the software by varying algorithms or the threshold level can be also set manually by users. In these analyses, melting curve of each sample can be detected as well. It is based on double-stranded DNA dissociation during heating, leading to a change in the fluorescence intensity, hyperchromicity. In the temperature, when 50 % of DNA is denatured (Tm) is known as the melting point in this consensus. If there are multiple melting points, it can be result from some unspecific PCR products. This step is important when unspecific fluorescent dyes are used to determine the accuracy and reliability of reaction. (Valasek and Repa 2005)

There are two types of fluorescence techniques: non-sequence specific dyes and sequence specific probes. In dye-based qPCR method, the dyes have not the fluorescence property when they are free in the solution but when they bind to negatively charged double stranded DNA minor groove, it becomes highly fluorescent. To note, the dyes bind to all kind of double strands, also to unspecific targets. Because of this, the melting curve must be done. In contrast, the probes are specific for DNA sequence, which is usually somewhere between the primer binding sites. Probes contain 2 special sites: fluorescent label and its quencher. During the polymerase reaction, the polymerase enzyme removes the quencher at the other site of probe. Thus, the fluorescent property of the label activates. (Heid et al 1996)

There are two basic ways to quantify the nucleic acid amounts in the samples: the absolute and relative quantification. In absolute quantification, the absolute amount of DNA or RNA molecules is determined. To achieve this, it is necessary to construct a standard curve in which the absolute DNA amount is known. In relative quantification, the amount of DNA in the target sample is determined in relation to reference sample, which is chosen to be the control sample. Quantity of target DNA/RNA in all target samples is then expressed relating to the control sample. In this thesis, Ct values of all samples are compared. (http://dyes.gene-quantification.info, 31.10.2017; Valasek and Repa 2005)

2.5.2 Alu elements as target

In this study, Alu family member genes are used as multicopy targets in DNA amount determination. These genes are short interspersed repetitive DNA elements in human genome, of which a diploid genome has over a million copies. They emerged 65 million years ago by a fusion of RNA gene and amplified throughout the human genome by retrotransposition to reach the present amount of 11 % of the human genome. They have a wide range of influences for gene expression regulation at the post-transcriptional level, such as alternative splicing, translation regulation and RNA editing. (Deininger 2011; Häsler and Strub 2006) Alu-based qPCR method for the detection of circulating cell-free DNA was developed by Lou et al. to validate rapidly and sensitively the DNA amount. Alu targets (primers or probes) are short fragments (in this study the lengths of primers were from 76 bp to 200 bp). (Lou et al. 2015) They were selected to determine (hypothetically) low level DNA amounts due to their multicopy property and the short primer property - if the DNA outcome method from embryo to medium occurs via apoptosis, DNA fragments are cut into 180 bp long or shorter fragments thus short Alu genes might be found despite apoptotic fragment cutting.

2.5.3 TSPY as target

"Testis specific protein, Y-linked", TSPY, is a protein that belongs to the nucleosome assembly protein (NAP) family. It is found being expressed only in testis. TSPY Isoform 1 and isoform 2 are suggested to be expressed in spermatogonia and spermatocytes and many functional paralogs and pseudogenes of this gene are present in a cluster in humans. TSPY has alternative splicing results in multiple transcript variants. (http://www.genecards.org/cgi-bin/carddisp.pl?gene=TSPY1, 19.10.2017). In these studies, used isoform 1, TSPY1, is tandemly-repeated gene forming an array of approximately 21-35 copies. The copy number variation is investigated to be involved in spermatogenesis and suggesting that low copy number of TSPY is associated with high risk of poor spermatogenesis and infertility. (Giachini et al. 2009) However, TSPY gene is located in Y-chromosome thus it is found only in males, and the existence of that gene in spent medium analysis might demonstrate the embryonic origin of DNA in spent medium.

3. Objectives

This study was part of Fertility clinic Ovumia research and development project, which aims to develop in-house embryo diagnostic methods. Hypothesis of this study was to expect embryonic DNA to be found in spent medium and it was supposed to correlate with the quality of the embryo. Purpose of this study was to optimize DNA extraction method from embryo culture medium and indicate that DNA in spent medium was originated from embryo determining Y-chromosomal TSPY gene as a marker and comparing aCGH results of spent media to NGS results of chromosomal screening. In this study, aim was also to investigate, how evaluated images by time-lapse EmbryoScope® correlated with the amount of DNA. Especially, feasible DNA contamination sources and factors due to embryo development properties were analyzed to explore their impact to released DNA amount in spent media.

4. Materials and methods

4.1 Embryo culture

This study was part of Fertility clinic Ovumia Oy research and development project. Blastocysts from various maternal age patients (25-40 years, average 34) for this method development were selected during culture depending on the score and age. For time-lapse (EmbryoScope®) analysis, culture media only from either transferred or vitrified blastocysts were analyzed. All patients, whose spent embryo culture medium was used, had signed a research agreement for method development. Spent media samples from embryos of patients, who had not given the agreement, were collected and combined for pooled samples.

Oocytes were denuded and then fertilized according to the standard ICSI or IVF procedure in embryo laboratory by embryologists. Embryos were cultured according embryo laboratory protocols in 25 µl SAGE 1-step medium (Origio, Målov, Denmark) in single droplets, which were overlaid with paraffin oil (Liquid Paraffin, Origio, Målov, Denmark) on culture dishes or in EmbryoScope® slide wells under oil at +37°C in MIRI® incubator (Esco Medical, Singapore) or EmbryoScope® (Vitrolife, Göteborg, Sweden). O_2 gas concentration level was 5 % and CO_2 gas concentration level was 5 %. Embryos were cultured up to 4-6 days, depending on patient´s treatment and time schedule. Blastocysts were scored according the grading system (Gardner et al. 2000).

4.2 Samples

4.2.1 Spent medium collection

For all studies, culture medium of embryos was collected after finishing the culture. After embryos were transferred out of the 25 µl culture droplets or wells, dishes or EmbryoScope® slides were put into a refrigerator (+4 °C) to wait for the collection. Single culture droplets or EmbryoScope® slide wells had been overlaid with oil, thus pipetting the medium had to be made avoiding taking the oil. Embryo spent media were collected with gel pipette tips. At first 16 µl of spent medium was collected from EmbryoScope® slide wells or droplets, next the wells or droplets were washed with 8 µl of fresh SAGE 1-step medium and finally the rest 8 µl of spent medium was collected. Media were collected into 0,2 ml PCR tubes. Some media droplets were combined taking 20 µl of spent medium from each droplet of one patient into same 0,2 ml PCR tube. Single embryo spent medium tubes were stored in liquid N_2 and pooled

media tubes were stored at -20 °C. Pooled spent media were used for optimizations of the methods. As negative controls, the same amount of embryo culture medium, but without its being used for embryo culture, were collected from the same slides or dishes, from which the embryo spent media samples were collected.

4.2.2 Spiked medium

To optimize DNA extraction method and to compare salt precipitation method and NucleoSpin plasma XS DNA extraction kit (Macherey-Nagel, Düren, Germany) DNA mastermix consisting of different sized DNA fragments was prepared. DNA mastermix consisted of 10 µl 50 bp DNA ladder (NEB, Ipswich, Massachusetts, USA), 2 µl NoLimits 3000 bp DNA fragment (ThermoFisher Scientific, Waltham, Massachusetts, USA), 2 µl NoLimits 10000 bp DNA fragment (ThermoFisher Scientific, Waltham, Massachusetts, USA) and 36 µl H_2O. Ladder amounts per sample were 1 µg, 100 ng and 100 ng, respectively. DNA mastermix was aliquoted to 8 tubes, 5 µl in each. 19 µl of fresh SAGE 1-step medium was added into each tube to get total solution amount of 24 µl. Salt precipitation method was made for 4 of these samples and extraction with NucleoSpin plasma XS kit was made for 4 of the samples.

4.2.3 Reference DNA

Male reference DNA (SureTag Complete DNA Labeling Kit, Agilent, Santa Clara, California, USA) was used as standard in qPCR experiments. Female reference DNA (SureTag Complete DNA labeling kit, Agilent, Santa Clara, California, USA) was also used as negative control in TSPY optimizations. Both male and female reference DNA (concentration 200 ng/µl) was diluted into TE buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA) to concentration of 10 ng/µl. Further dilutions were made into H_2O. Concentrations from 0,54 ng/µl to 0,052 pg/µl were used.

4.3 Sample preparation and DNA extraction

4.3.1 Sample preparation

Spent medium samples were thawed and each volume was added to total amount of 24, 25 or 32 µl with fresh SAGE 1-step medium depending on the experiment. Used volumes are summarized in table 1.

For DNA extraction optimization, pooled SM samples were centrifuged (Labnet Prism™, Edison, New Jersey, USA) at 750 g for 10 min to pellet possible cumulus cells to the bottom of the tube. Supernatant from these was transferred into new tubes avoiding touching the bottom of the tube. Other preparations are presented later but they are summarized in table 1.

Table 1: Used volumes, preparation treatments, DNA extraction method and number of samples in experiments. SP= salt precipitation, NS=NucleoSpin plasma XS DNA extraction kit, SM=spent medium, MM=mastermix.

Experiment	Sample preparation	Sample volume for DNA extrac-tion	DNA extraction method	Final volume	No of samples	Notices
Salt precipitation optimization	- Proteinase K treatment	25 µl	SP	Pellet diluted straight into Luna MM	2+2 pooled SM	
Detection of TSPY in SM samples	- Proteinase K treatment	25 µl	SP	3 µl	4 SM	Incubating at +37°C for 25 min before adding the primer MM
Comparing DNA extraction methods	- Centrifugation - Proteinase K treatment	24 µl	SP or NS	5 µl	4+4 pooled SM	
Sample volume optimization	- Dividing sample into different volumes	32 µl	NS	5 µl	3SM+ 3Pooled SM+ 3Ctrl SM	
Repeata-bility testing	- Dividing sample for TSPY and Alu4	32 µl	NS	5 µl	2 SM	
Alu4 and TSPY determi-nation	- Dividing sample for TSPY and Alu4	32 µl	NS	5µl	45 SM+ 15 Ctrl SM	

4.3.2 Salt precipitation

Proteinase K treatment was done for the samples by adding 0,5 µl of proteinase K (ThermoFisher Scientific, Waltham, Massachusetts, USA) to each sample and incubating in incubator (RS Biotech, Irvine, United Kingdom) at +37 °C for 30 min. Proteinase K was inactivated at 95 °C (Grant, Cambridgeshire, United Kingdom) for 15 min with glycogen (ThermoFisher Scientific, Waltham, Massachusetts, USA), $MgCl_2$ (ThermoFisher Scientific, Waltham, Massachusetts, USA) and Natrium acetate (NaAc) (ThermoFisher Scientific, Waltham, Massachusetts, USA). Before inactivation, mastermix of glycogen, $MgCl_2$ and NaAc was made to final concentration of 0,13 ng/µl, 10 mM and 0,27 M, respectively. After inactivation, ethanol (70 %) was added and the solution was incubated in -20 °C for 1 hour. The solution was centrifuged (Labnet Prism™, Edison, New Jersey, USA) at 14 000 g for 30 min, supernatant was discarded and ethanol (80 %) was added. Solution was centrifuged at 14 000 g for 15 min and the supernatant was discarded bewaring of touching the pellet. Pellet was diluted into needed volume of H_2O in TSPY detecting or elution buffer (Macherey-Nagel Nucleo Spin plasma XS kit) when comparing the methods or straight into the Luna mastermix (NEB, Ipswich, Massachusetts, USA) in method optimizations.

4.3.3 NucleoSpin plasma XS kit

DNA extraction was performed according to protocol of manufacturer (Macherey-Nagel, Düren, Germany), using the volume of sample 24 µl or 32 µl. All solutions which were used at the extraction protocol were proportioned with the initial volume of samples. After elution, samples were incubated (Grant, Cambridgeshire, United Kingdom) at 60 °C for 15 min to evaporate the residual traces of ethanol which could have inhibited the following PCR reaction. After evaporation samples were adjusted to the final volume with elution buffer or H_2O.

4.4 NanoDrop measurements

DNA concentration and yield were measured with NanoDrop (ThermoFisher Scientific, Waltham, Massachusetts, USA). Concentration measurement with NanoDrop was performed according to NanoDrop manufacturer instructions pipetting 1,5 µl of sample onto device. Samples were measured twice to ensure the quality of measurements. Elution buffer from DNA extraction kit (Macherey-Nagel) was used as blank.

4.5 Electrophoresis

Comparing extraction methods, extraction products were separated by agarose gel electrophoresis (Flashgel, Lonza, Verviers, Belgium). 50 bp DNA ladder (NEB, Ipswich, Massachusetts, USA) and GeneRuler 1 kb DNA ladder (ThermoFisher Scientific, Waltham, Massachusetts, USA) were used as standards. Samples were treated with Loading dye (ThermoFisher Scientific, Waltham, Massachusetts, USA). Standards and samples were run in 2,2 % agarose gel cassette in 180 V in the Flashgel system device (Lonza, Verviers, Belgium) for 15 min. The bands were visualized with Lonza Flashgel camera during the run.

4.6 qPCR

Real-time qPCR experiments were carried out using 7500 Real-Time PCR Detection System (Applied Biosystems®, ThermoFisher Scientific, Waltham, Massachusetts, USA). Each qPCR reaction consisted of Luna universal qPCR mastermix (NEB, Ipswich, Massachusetts, USA), primers and cod UNG (ArticZymes, Tromsø, Norway). Cod UNG enzyme was used to hydrolyze uracil-glycosidic bonds in DNA containing dUTP. Hence, potential contamination from previous qPCR reactions could be minimized. Reaction conditions were optimized for each primer pair. The used primers are summarized in the table 2. In addition, sample volumes varied in different qPCR studies, thus used qPCR programs are shown in table 3 and the mastermix volumes are summarized in table 4.

Table 2: Used primer sequences

Target	**Primer name**	**Sequence**	**Product size**
Alu element	Alu4 Sense	5’- TAC AAA AAA TTA GCC GGG CG -3’	113 bp
	Alu4 Antisense	5’- GAT CTC GGC TCA CTG CAA G -3’	
TSPY	Dys14 Forward	5'-GGG CCA ATG TTG TAT CCT TCT C -3'	84 bp
	Dys14 Reverse	5'- GCC CAT CGG TCA CTT ACA CTT C -3'	

Table 3: qPCR programs

Target	Analysis	Initial denaturation	Cycles	Denaturation	Extension	Melt curve
Alu4 qPCR	Experiments	90°C for 30 s	35	95°C for 5 s	60°C for 34 s	Melt curve
TSPY qPCR	Experiments	95°C for 1min 30 s	35	95°C for 10 s	60°C for 45 s	Melt curve
	Optimization	95°C for 1min 30 s	35	95°C for 10 s	62°C for 45 s	Melt curve

Table 4: Used primers and primer mastermix volumes in qPCR analysis

Experiment	qPCR primer	Primer mastermix volume
Salt precipitation optimization	TSPY	9 µl
Detection of TSPY in SM samples	TSPY	7 µl
Comparing extraction methods	Alu4	5 µl
Sample volume optimization	Alu4	5 µl
Repeatability test and Alu4 determination	Alu4	8 µl
Repeatability test and TSPY determination	TSPY	4 µl
TSPY qPCR optimization	TSPY	9 µl

4.6.1 Alu

Alu1, 4 and 5 primers (Lou et al. 2015) (Eurogenetec, Seraing, Belgium) were optimized and compared with each other under different conditions of qPCR annealing temperature, cycle amount and concentration of primers. For all Alu determination studies, qPCR mastermix was combined with the samples. 10 µl of Luna qPCR mastermix was mixed with Alu primer mastermix. Volume of primer mastermix varied depending on the study, which are shown in table 4. Total qPCR reaction was 20 µl in every study. Concentrations of Alu4 forward and reverse primers were 0,4 µM. Volume of COD UNG was 0,25 µl and the rest of primer mastermix volume were added with H_2O.

To test the needed volume of spent medium for Alu4 determination in experiments, various volumes of extracted DNA samples were tested: ≤10 %, 20 %, 30 % vs. 40 % of 10 µl of eluted volume. The elution was made using volume of 8 µl and then the samples were evaporated at 60 °C for 15 min to evaporate the sample to volume of 5 µl. The dilution was added to 10 µl of elution buffer by NucleoSpin plasma XS extraction kit.

In Alu4 and TPY determination from the same sample, in DNA extraction with NucleoSpin plasma XS kit, the used volume for final elution was 10 µl and evaporation was performed at 60 °C for 15 min to evaporate the sample to volume of 6 µl. H_2O was added to each sample to adjust volume to 8 µl after the yield was measured with pipette and with the same pipette tip the whole volume of sample (8 µl) was transferred into new fresh qPCR tube. 2 µl of that sample (25 %) was further transferred into new fresh qPCR tube for Alu4 qPCR determinations. Residual volume (6 µl) was for analysis of TSPY qPCR. For both samples Luna mastermix was

added first and then the primer mastermix. All the samples were handled in the same way. Control male reference DNA (0,01 ng/µl) was handled in the same pipetting procedure.

4.6.2 TSPY

10 µl of Luna qPCR mastermix was mixed with TSPY primer mastermix. Volume of primer mastermix varied depending on the study, which are shown in table 4. Total qPCR reaction volume was 20 µl in every study. Concentration of TSPY forward and reverse primers were 0,25 mM and Cod UNG volume was 0,25 µl in every study. Optimization of DMSO (ThermoFisher Scientific, Waltham, Massachusetts, USA) concentration was tested with 3 % (0,6 µl in primer mastermix) and 0 % DMSO concentration. For the experiments after optimization, 3 % DMSO concentration was used. The rest of the primer mastermix needed volume was added with H_2O.

Pipetting procedure of TSPY and Alu4 determinations from the same sample is described at 4.6.1. Processing spent media for TSPY qPCR, for 10 spent medium samples and 2 control spent medium samples were added Alu4 mastermix inadvertently instead of TSPY mastermix. Hence, they were not analyzed further after qPCR.

4.7 aCGH

aCGH analyses were done for 3 samples of spent media 45-9, 65-1 and 80-3 and their embryo biopsies. Their chromosomal screening results were known (by biopsy NGS, Reprogenetics, UK) and these results were compared with each other. TE biopsy was performed by experienced embryologist. Biopsy was taken from 3-10 cells, which had been herniated through the zona pellicuda from 3 embryos.

DNA extraction from spent media was performed according NucleoSpin plasma XS kit modified instructions (initial medium volume 32 µl and added reagents were proportioned to that volume) and final elution was 8 µl and it was evaporated at 60 °C to volume of 2,5 µl.

GenetiSure Pre-Implantation Array-Based CGH for Aneuploidy Screening kit (Agilent Technologies, Santa Clara, California, USA) was used. Whole genome amplification and sample labeling was done according to the instructions of manufacturer. During purification of

labelled samples, sample concentration to dryness was done at +37 °C in incubator (Termaks, Bergen, Norway) for 3 hours instead of vacuum concentration. Hybridization, microarray washing and scanning were done according to the instructions of manufacturer.

4.8 Image analysis of EmbryoScopeâ time-lapse incubator images

45 embryos were cultured until 4-6 days after fertilization in a time-lapse incubator (EmbryoScope®). Images of the embryos were acquired every 10 minutes with EmbryoViewer®, time-points of key embryonic events were registered by embryologists, and possible contamination factors, which could release DNA into spent media, were analyzed. To analyze contamination impact to diagnostic results, amount of cumulus cells and sperm cells were counted. During the embryonic development, number of lysed cells within the embryo was counted to investigate impact of dead cells to DNA amount of the embryo culture medium. Fragmentation level before embryo compaction was evaluated to investigate its impact on spent medium DNA amount. Number of perivitelline space cells, hatching vesicles and vacuoles were also counted to investigate their impact on spent medium DNA amount. Time of each cell number stages (8C, 9+C, M=morula stage, SB=small blastocyst stage, B=blastocyst stage, HB=hatching blastocyst stage and EB=expanded blastocyst) were evaluated by embryologists. Diameter of the blastocyst at the most expanded stage was measured with measurement tool of EmbryoViewer® software. Measurements were performed from edge of TE cells to edge of opposite TE cells at 2 sites according to figure 4.

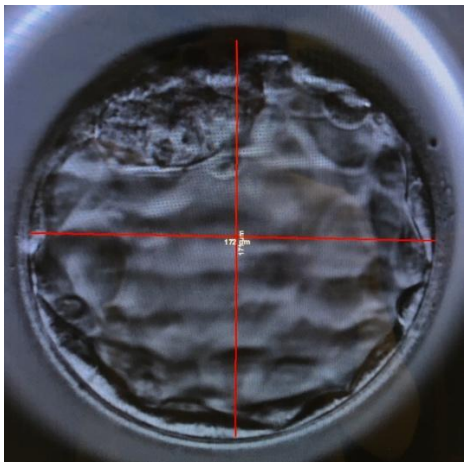

Figure 4: Blastocyst diameter measurement of image of embryo in EmbryoScope®.

4.9 Statistical analysis

Statistical analyses were made with IBM SPSS software using Independent t-test or ANOVA (p-value <0,05) and correlation tests with Pearson's and Spearman's test ($p<0,05$) or Mann-Whitney U test ($p<0,05$).

5. Results

5.1 Optimization of DNA extraction method for spent medium samples

At first, ethanol-salt precipitation was thought as primary method for DNA extraction. In this method, the whole sample is in the same tube during the extraction protocol, thus sample loss resulting from pipetting or column use is avoided. The demand of using proteinase K in salt precipitation method was analyzed by comparing pooled spent media samples, which were either processed with proteinase K or not processed. It is still unknown, if the cell free DNA in spent medium is bound to some protein or transferred outside the cell in vesicles. If the DNA is for example bound to some protein it could be necessary to digest the protein to release the DNA for following PCR reaction. Requirement of proteinase K was tested with TSPY determination in qPCR, which results are shown in the figure 5.

Standard DNA samples were analyzed as triplicates and averages of them are shown in figure 5. All standard samples, except one (0,18 ng; 62,13 °C and 80,15 °C) had only one melting point in the melt curve –analysis. Additionally, NTC and both proteinase K treated samples had two melting points: 62,13 °C and 79,37 °C; 62,13 °C and 78,80 °C; 62,13 °C and 76,47 °C, respectively.

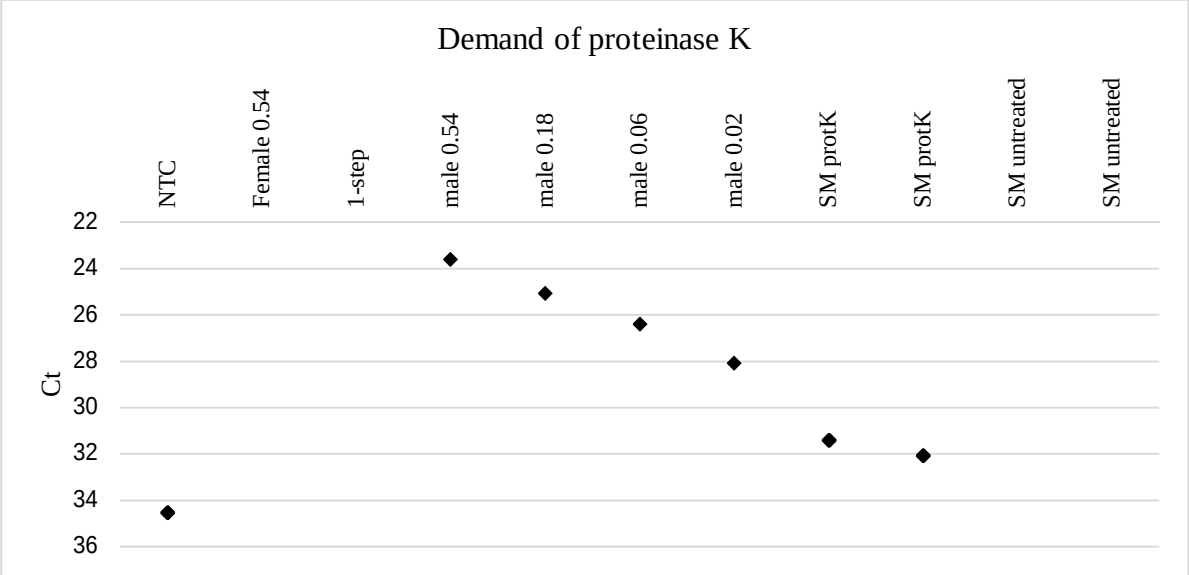

Figure 5: Demand of proteinase K in salt precipitation optimization. TSPY qPCR cycle threshold (Ct) values of NTC (H_2O), female DNA sample, fresh SAGE 1-step, standards (ng) and 2 pooled spent medium samples with proteinase K treatment and 2 pooled spent medium samples without proteinase K treatment. Ct values of female DNA, fresh 1-step medium and untreated spent medium samples were undetermined.

Overall, Ct values were determined for proteinase K treated pooled spent medium samples and undetermined for untreated samples. For further experiments, proteinase K treatment was used. H_2O was used as no template control (NTC).

5.1.1 Comparing extraction methods

DNA extraction method for further experiments was selected by comparing DNA salt precipitation method and NucleoSpin plasma XS kit. Extraction was made using 1-step medium spiked with DNA and with pooled spent medium samples. Gel electrophoresis was used to compare DNA extraction methods with spiked DNA and their concentration and absorbance were measured with NanoDrop. Pooled spent medium samples were used for comparing Alu4 qPCR determination results between extraction methods.

Concentrations of samples extracted from 1-step medium spiked with DNA were measured with NanoDrop. NanoDrop was used, because properties of DNA, such as single- or double strand form, were not known. Measurements with NanoDrop cannot be selected by single or double strand property. Concentrations were measured twice per each sample and averages of absorbance ratios and DNA yields are shown in figure 6. A260/280 ratio was measured to indicate DNA purity and for pure DNA it is 1,8. Salt precipitation method samples had lower

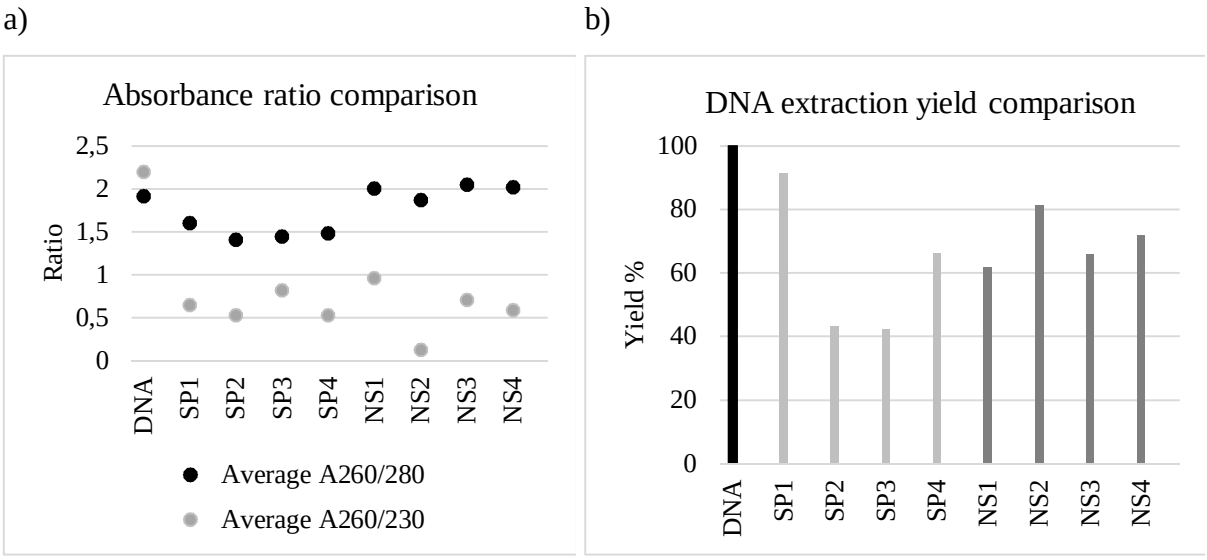

Figure 6: Absorbance ratio and yield. Salt precipitation spiked DNA samples (SP1-4) and NucleoSpin plasma XS spiked DNA samples (NS1-4) were compared. Each sample was measured twice and the average of them is shown in the diagrams. a) Absorbance 260/280 (black) and 260/230 (grey) ratios and b) yield percentage were measured using NanoDrop. Yield percentage was calculated based on the used DNA (not extracted) yield value (100 %).

ratio of A260/280 (scale from 1,4 to 1,6) than NucleoSpin samples (scale from 1,87 to 2,05). A260/230 ratio was measured to indicate contamination sources and optimal ratio is 2,0-2,2. Absorbance ratios 260/230 were low in both methods. According to figure 6b yields were more equal among NucleoSpin method samples and the absorbance ratios (figure 6a) were better in NucleoSpin method samples overall.

According to the figure 7 of electrophoresis, the bands are brighter among NucleoSpin method samples. Repeatability is good and all the samples look quite similar among NucleoSpin samples.

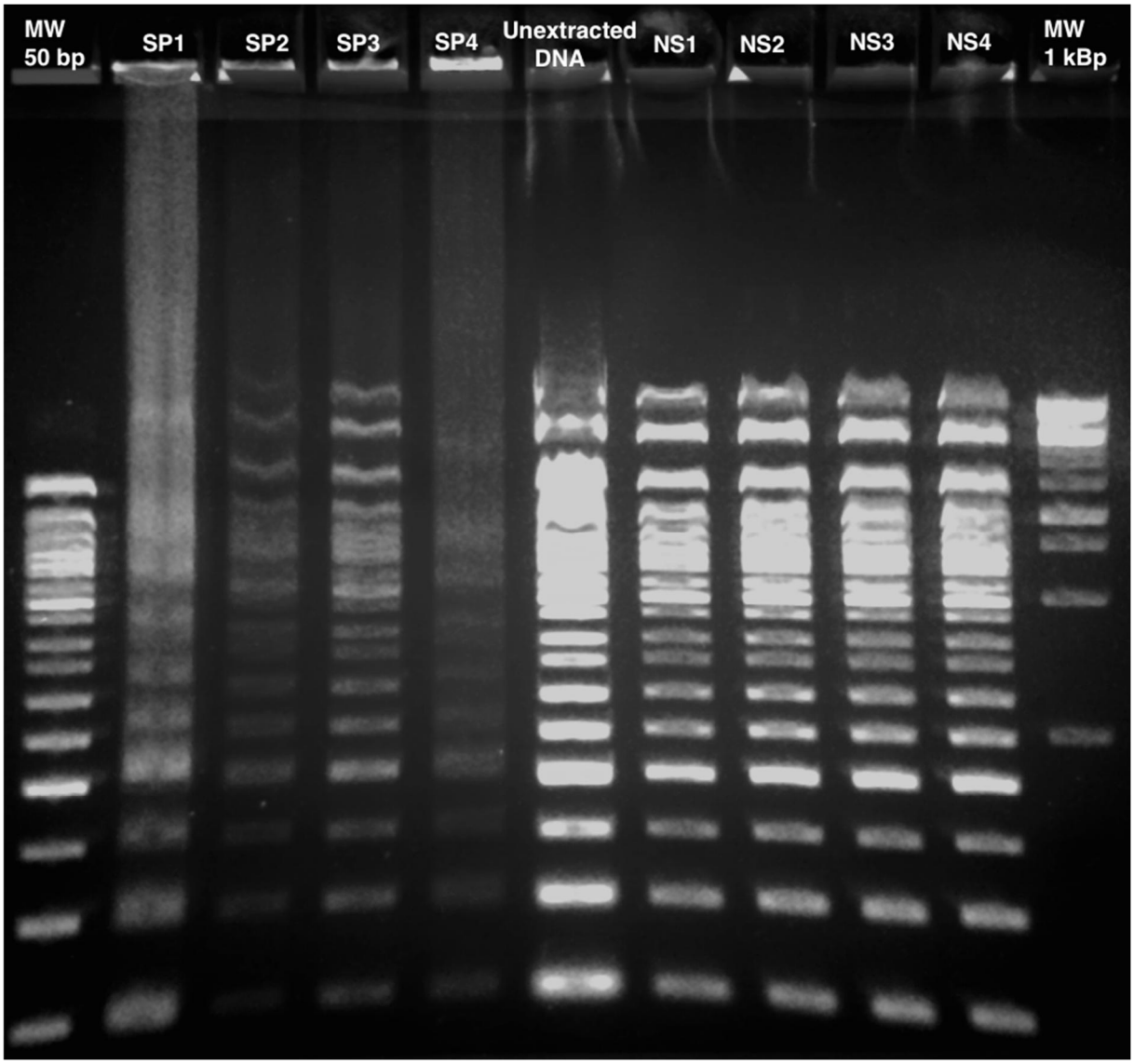

Figure 7: Electrophoresis of comparing DNA extraction methods with spiked DNA. Electrophoresis run of spiked DNA samples, which have been extracted using salt precipitation (SP1-4) and NucleoSpin plasma XS kit (NS1-4). Ladders (MW) 1000 bp and 50 bp and pure DNA can be seen in the image as well to perceive DNA fragment sizes.

Among salt precipitation method samples, there is a lot of difference between the samples and there might be also some single strand DNA fragments, which ran slower than double strand fragments.

In addition, DNA extraction methods were compared with each other using pooled spent media sample with qPCR, Alu4 as target. Comparison of Ct values between salt precipitation method and NucleoSpin plasma XS kit can be seen in figure 8. Standard samples of 6,6 pg and 1,6 pg DNA were analyzed as duplicates and averages of them are shown in figure 8. Scale of Ct values among samples extracted with salt precipitation was from Ct value of 26,66 to 26,89 which corresponds the evaluated DNA amount from 0,19 pg to 0,23 pg. One of salt precipitation samples had undetermined Ct value. Average of Ct value among samples with salt precipitation method was 26,81 (SPd not included) corresponding the evaluated DNA amount of 0,2 pg. Scale of Ct values among NucleoSpin plasma XS kit extracted samples was from 24,14 to 30,98. Sample 4 of NucleoSpin plasma XS kit (NSd in the figure 8) was not evaporated completely (yield ~8 ml when others had ~3-4 ml). Average of the NucleoSpin samples (NSd not included) of the quantity amount of DNA was 1,52 pg. Fresh 1-step control sample had lower Ct value with NucleoSpin plasma XS kit extraction (26,49) than with salt precipitation

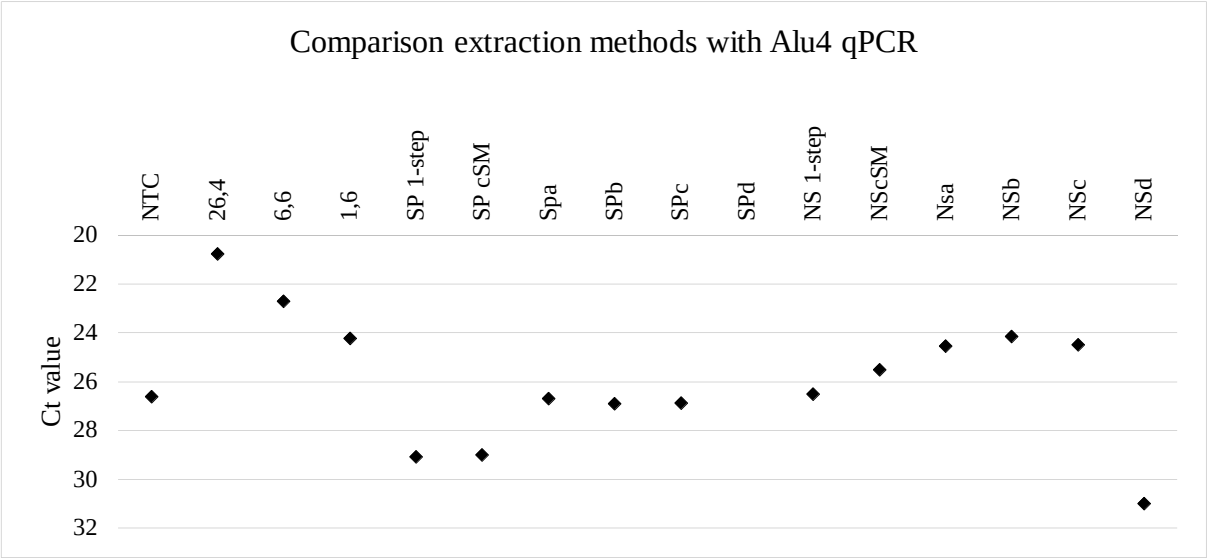

Figure 8: DNA extraction comparison with Alu4 qPCR using pooled spent medium samples. Alu4 qPCR cycle threshold (Ct) values of NTC (H_2O), standards (pg), salt precipitation method samples (SP) and NucleoSpin plasma XS kit method samples (NS). Extraction was made using pooled spent media (SP/NS a-d), pooled control spent media (SP/NS cSM) and fresh SAGE 1-step medium (SP/NS 1-step). Ct values among NS samples can be seen being lower than SP samples despite NS4 which was not evaporated correctly. Also, Ct values among control samples are lower in NS method.

(29,06). Spent medium control had lower Ct with NucleoSpin plasma XS kit extraction (25,50) than with salt precipitation (28,97). Tm values for all samples, NTC included, were 86-87 °C except NSd (84,3 °C) and SPd (65,44 °C). Based on these comparison results, NucleoSpin plasma XS kit was selected for further experiments.

5.2 Optimizing qPCR protocols for detecting embryonic DNA in spent medium samples

TSPY was used to detect the Y-chromosomal DNA in spent media that was assumed to originate from the embryo. As a hypothesis of DNA properties, we thought that DNA would be more or less fragmented in spent medium. Due to that, primers used in analyzing spent medium should have been quite short. To determine DNA amount in spent media, target Alu primers were selected and Alu1, 4 and 5 were compared. Lengths of these primers were 200 bp, 113 bp and 76 bp, respectively. In preliminary analysis, the smallest DNA concentrations could not be determined with Alu5 and Alu1 (data not shown). Due to that, the next shortest primer, Alu4 (113bp) was selected as target to determine DNA amount in spent media.

5.2.1 Optimizations of qPCR conditions for TSPY detection in spent medium samples

Optimal DMSO concentration in TSPY qPCR was determined comparing concentrations of 0 % with 3 % DMSO in qPCR reaction.

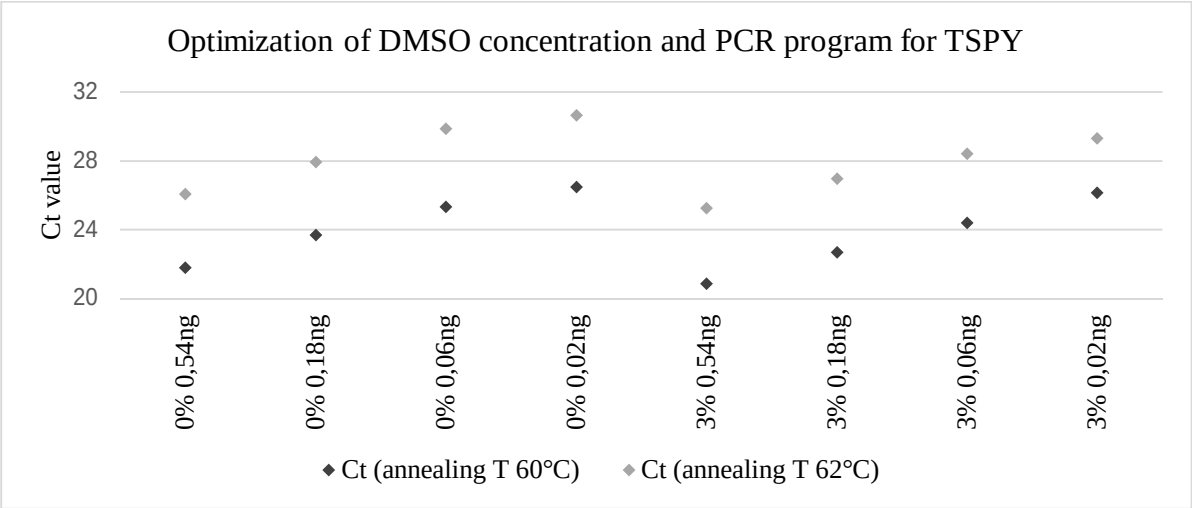

Figure 9: DMSO concentration and qPCR annealing temperature impact on TSPY Ct values. TSPY qPCR cycle threshold (Ct) values of each standard concentrations. In this optimization 0 % (4 samples from the left) or 3 % (4 samples from the right) DMSO in qPCR reaction, as well as qPCR program annealing temperature 60 °C (black) and 62 °C (grey) were compared.

Annealing temperature in qPCR was also optimized comparing temperatures of 60 °C and 62 °C. Annealing time (45 sec) and other conditions of the qPCR program were the same. PCR programs can be seen in table 3. Male reference DNA was used as standards and female DNA and H_2O were used as negative controls. According to qPCR results (figure 9), sensitivity of 3 % DMSO samples was better with lower concentrations of DNA. Ct values of 3 % DMSO samples increased equally while Ct values of 0 % DMSO samples did not. Ct values with annealing temperature of 60 °C were lower than with 62 °C. Control Ct values were undetermined (not shown in figure 9). Based on these results, further TSPY experiments were processed using 3 % DMSO concentration and annealing temperature of 60 °C.

5.2.2 Detecting TSPY gene in spent medium samples

TSPY was detected from various male DNA samples to determine the copy number variance. Additionally, TSPY was used to determine the sex of embryo from spent medium samples. Karyomapping was performed earlier for these embryos thus the sex of them was known. Salt precipitation was used as DNA extraction protocol. Fresh SAGE 1-step medium and H_2O were used as controls. PCR program can be seen in table 3.

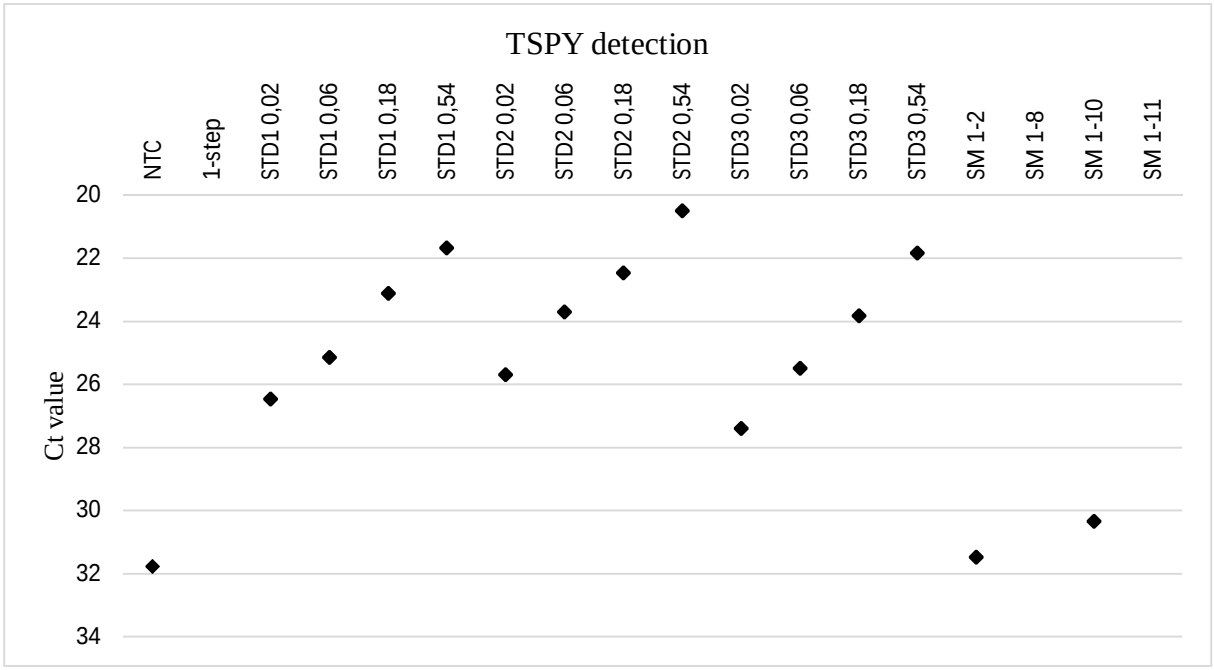

Figure 10: TSPY detection from DNA of various individuals and spent medium samples. TSPY qPCR cycle threshold (Ct) values of negative controls NTC (H_2O) and fresh 1-step medium, different standard DNAs (STD1-3) and concentrations (ng) and spent medium samples (SM1-2, -8, -10 and -11).

There was different amount of TSPY gene in standard male DNA samples (STD1-3) depending on the individual (figure 10). According to the TSPY qPCR results (figure 10), embryos from SM wells 1-2 and 1-10 seemed to be male embryos, because the Y-chromosomal signal was detected from these media.

According to karyomapping done earlier for these embryos, embryos 1-2 and 1-10 were males, while embryos 1-8 and 1-11 were females. Spent media results corresponded with karyomapping. Based on this preliminary study, TSPY could be used to detect embryonic DNA in spent media. In fresh 1-step control sample, TSPY Ct-value was undetermined but NTC (H_2O) control gave Ct signal.

5.2.3 Optimization of needed sample volume for Alu4 qPCR detection

For the determination of Alu4 and TSPY from the same spent medium sample, the needed sample volume had to be optimized. Tested volumes were 4 µl (40 %), 3 µl (30 %), 2 µl (20 %) and the rest <1 µl (<10 %) (the greater amounts were pipetted first from the total volume of 10 µl). Determination was made using three SM samples (105-1, 105-8 and 105-11) and H_2O, elution buffer and control spent media as controls.

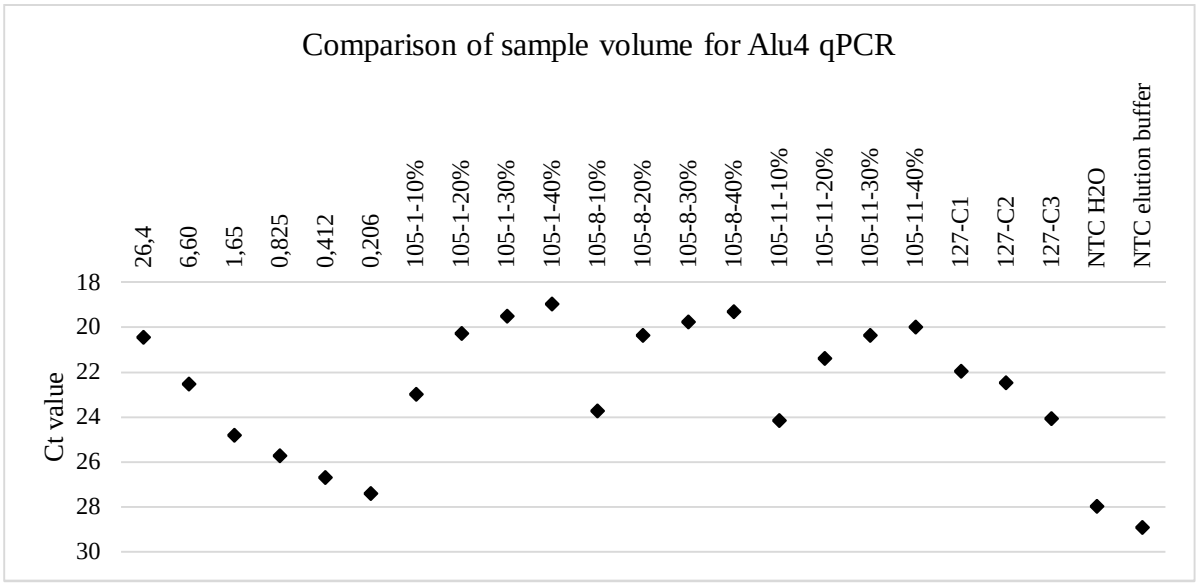

Figure 11: Sample volume determination for Alu4 qPCR experiments. Alu4 qPCR cycle threshold values of each standard samples (pg), control samples (NTC H_2O and NTC elution buffer) and different volumes of each spent medium samples (105-1, 105-8 and 105-11) and control spent medium samples (127-C1, -C2 and -C3). Percentiles are portions from the original 10µl sample volume.

It can be noticed from the figure 11 that Ct values are quite low overall and small volumes of extracted DNA solution get signal as well as the greater amounts. Due to that, 25 % of sample volume was selected for further Alu4 analyses and the rest 75 % for TSPY determination.

5.2.4 Repeatability test of pipetting procedure for Alu4 and TSPY detection

Aim of this determination was to test the repeatability of pipetting protocol of processing the same DNA sample for Alu4 and TSPY analysis. In the pipetting procedure, the idea was to pipette 25 % of sample volume for Alu4 (2 µl) and leave the rest 75 % (6 µl) of the sample for TSPY determination. Repeatability was done for spent medium samples 105-16 and 105-18 as well as DNA spiked medium samples. Results of the repeatability test can be seen in figures 12 and 13. Average of Alu4 Ct value among spiked DNA samples was 21,58, when the lowest Ct was 21,38 and the highest Ct was 21,81. Average TSPY Ct values among spiked DNA samples was 27,81, when the lowest Ct is 27,30 and the highest Ct is 28,50. Alu4 Ct values of spent medium samples were 21,49 and 20,92 when TSPY Ct values of spent medium samples were 30,12 and 28,62. Variances of determinations of Alu4 and TSPY of DNA samples were low: 0,16 and 0,40, respectively. Standard samples of small amounts of DNA (<1,65 pg) seemed to be failed because there were not PCR product in the reactions. Standard sample of 6,6 pg is out of the expected line as well even though the PCR product exists. Based on these studies and used pipetting procedure, spent medium experiments were determined to work.

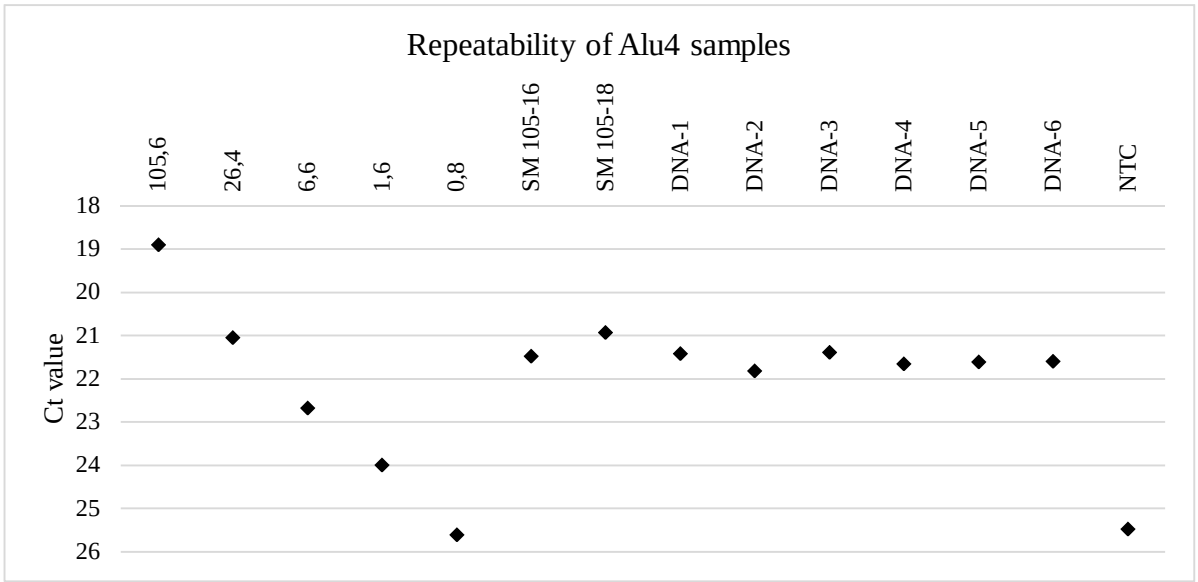

Figure 12: Repeatability test of Alu4 determination. Alu4 qPCR cycle threshold (Ct) values of standards (pg), spent medium samples 105-16 and 105-18, DNA spiked medium samples (DNA1-6) and NTC (H_2O).

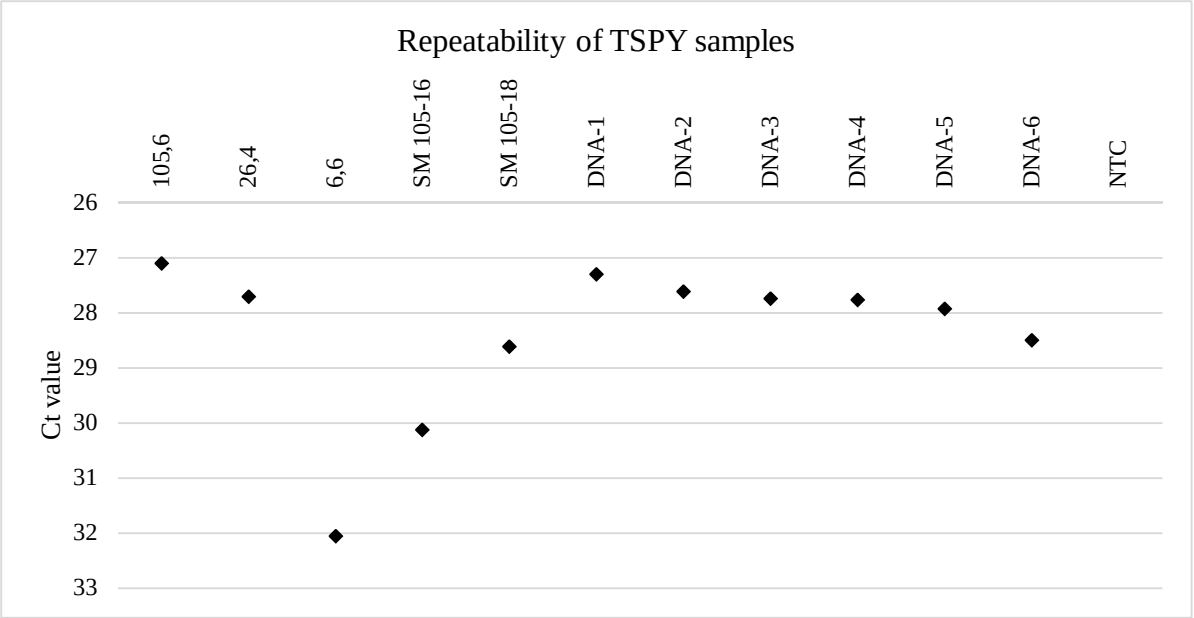

Figure 13: Repeatability test for TSPY determination. TSPY qPCR cycle threshold (Ct) values of standards (pg), spent medium samples 105-16 and 105-18, spiked DNA samples (DNA1-6) and NTC (H_2O).

5.3 Comparing the amount of spent medium DNA to EmbryoScope® image data

45 embryos were cultured until 4-6 days after fertilization in a time-lapse incubator (EmbryoScope®). Images of embryos were acquired every 10 min. With EmbryoViewer®, time-points of key embryonic events were registered by embryologists, and possible contamination factors, which could release DNA into SM, were analyzed.

Blastocysts were also scored (according Gardner et al. 2000) and the age of embryos was registered for analyses. DNA of spent media were extracted according to NucleoSpin plasma kit instructions (Macherey-Nagel) and Alu4 was used as qPCR target to detect DNA amount in spent media. Image and statistical analyses were done for 44 embryos, because one spent medium sample extraction failed during evaporation. TSPY determination was done for 35 samples to determine the embryonic origin of DNA in spent medium. Statistical analyses were processed with IBM SPSS software.

5.3.1 TSPY analysis as proof of concept for Alu4 determination

TSPY was used to determine embryonic DNA from embryo culture medium. TSPY determination was done for 35 samples, of which 16 (46 %) samples had TSPY Ct signal and 19 (54 %) Ct values were undetermined. TSPY is located in Y-chromosome and thus the undetermined Ct refers to female sex of the embryo. According to table 5, where the samples

of TSPY determination are set in order according to the TSPY amount, the impact of embryo´s age can be seen clearly. All day 6 samples had the lowest TSPY Ct values while day 4 samples had the highest Ct values. There was no impact of sperm cells or treatment method (IVF or ICSI) on TSPY Ct value but correlation between TSPY and Alu4 Ct values can be seen in the table 5 and in the figure 14. Pearson correlation of TSPY and Alu4 Ct values was 0,869 (n=16, p<0,001): the smaller the Alu4 Ct, the smaller the TSPY Ct value. There were 13 control spent media used in TSPY determination, of which TSPY Ct value was undetermined for 11 and determined for 2 control samples: 30,4 and 31,7. Alu4 Ct values for these 2 control samples were 27,5 and 25,4, respectively. Based on these results, Alu4 determinations were thought to be proofed.

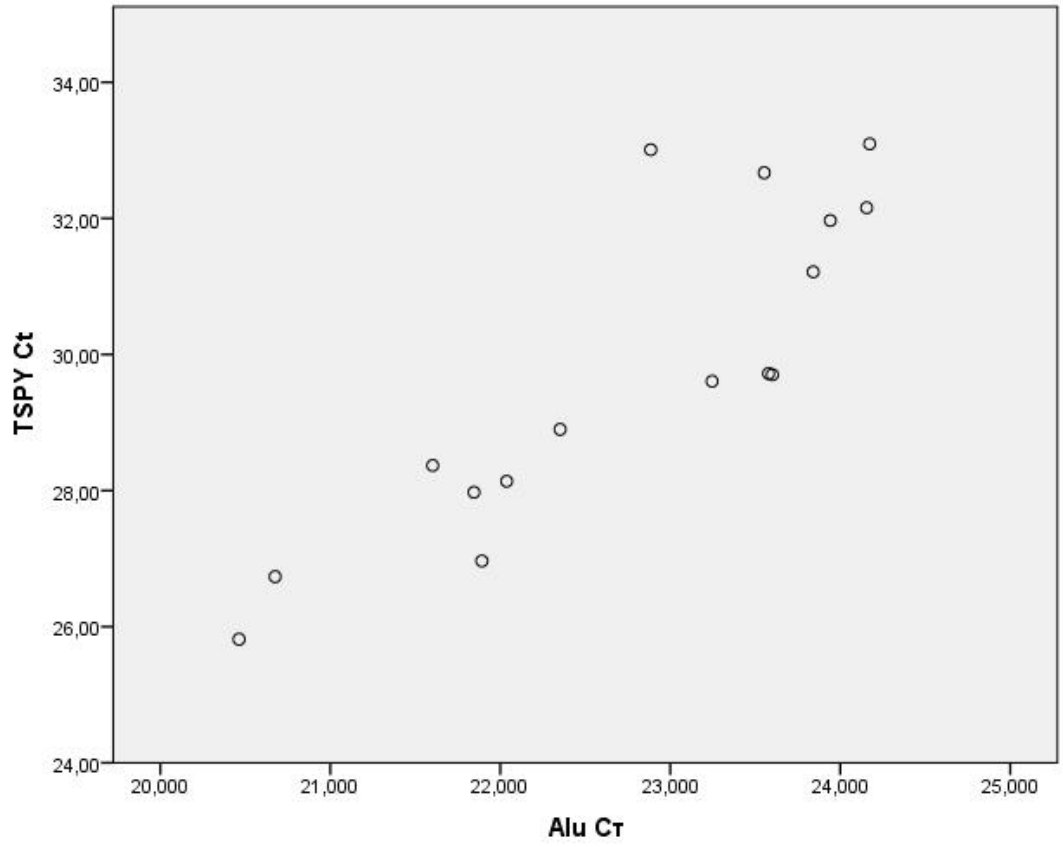

Figure 14: Correlation between Alu4 and TSPY threshold cycle (Ct) values. Pearson correlation r=0,869 (n=16, p<0,001).

Table 5: Table of samples with TSPY analysis. Samples are put in order according to the increasing threshold cycle (Ct) value of TSPY. TSPY Ct was determined for 16 samples out of 35. Last culture day of the embryo, observed sperm cell number and treatment (IVF/ICSI) are presented in the table. At the right, Ct values of Alu4 qPCR are shown. Among samples with determined TSPY Ct, all day 6 spent media had the lowest Ct values and day 4 spent media samples were quite downstream of the order. There was no significant correlation between sperm cell number and TSPY Ct or difference in TSPY Ct values between treatments.

Sample Name	Day	Treatment	Sperm cells	TSPY Cт	Alu Cт
71-5	6	ICSI	0	25,82	20,46
118-8	6	IVF	25	26,73	20,68
118-3	6	IVF	25	26,97	21,89
63-4	6	ICSI	0	27,98	21,85
35-3	6	ICSI	0	28,14	22,04
118-6	6	IVF	25	28,37	21,60
91-5	5	IVF	50	28,90	22,35
70-2	5	IVF	60	29,61	23,25
43-6	5	IVF	5	29,70	23,60
91-2	5	IVF	40	29,72	23,58
36-11	5	ICSI	0	31,21	23,84
100-2	5	ICSI	0	31,97	23,94
74-9	4	IVF	10	32,16	24,16
74-7	4	IVF	15	32,67	23,55
43-7	5	IVF	60	33,01	22,88
63-3	5	ICSI	0	33,09	24,17
43-3	5	IVF	5	Undetermined	19,74
71-9	6	ICSI	0	Undetermined	20,97
30-5	6	ICSI	0	Undetermined	21,01
22-16	6	IVF	10	Undetermined	21,50
71-8	6	ICSI	0	Undetermined	21,54
22-19	5	IVF	10	Undetermined	21,55
36-3	5	ICSI	0	Undetermined	22,67
35-11	5	ICSI	0	Undetermined	22,68
71-15	6	IVF	5	Undetermined	22,96
43-1	5	IVF	0	Undetermined	22,99
36-10	5	ICSI	0	Undetermined	23,75
63-1	5	ICSI	0	Undetermined	23,78
22-13	5	IVF	20	Undetermined	23,84
22-15	5	IVF	6	Undetermined	23,88
43-4	5	IVF	2	Undetermined	24,07
36-1	5	ICSI	0	Undetermined	24,24
63-5	5	ICSI	0	Undetermined	24,60
22-14	5	IVF	3	Undetermined	25,05
91-3	5	IVF	70	Undetermined	25,35

5.3.2 Impact of embryo properties and contamination factors to detected Alu4 amount

Alu4 gene has a large copy number in human genome, over a million in a human diploid genome. Alu4 amplicon was selected to evaluate the amount of DNA in culture medium. In this section, impact of contamination sources and impact of embryo properties for Alu4 Ct values, are presented. Based on earlier studies, impact of embryonic age (day 4, day 5 or day 6) was hypothesized to correlate with DNA amount in spent medium. In addition, contamination sources, cumulus cells and sperm cells, were analyzed because hypothetically they were thought to release DNA into culture medium and thus contaminate the spent medium. Impact of observed dead cells in the embryo was also investigated. According to standard curve, the DNA amount as the lowest was 2,23 pg (Ct 25,35) and as the highest 73,57 pg (Ct 19,74) among all the samples (n=44). These values correspond to DNA amount of approximately 1/3 cell and 12 cells. Average of all DNA amounts was 14,04 pg, which corresponds the DNA amount of about 2 cells. In this study, the Ct values were compared instead of DNA amount because the DNA amounts are only evaluations and they depend on the used standard DNA.

Impact of embryo age (day 4, day 5 or day 6) was analyzed with ANOVA test (figure 15a). Difference between embryonic age was significant ($p<0,001$). Averages of Alu4 Ct values among day 4 (n=3), day 5 (n=29) and day 6 (n=12) embryos were 24,03 (std. dev. 0,43); 23,59 (std. dev 1,16) and 21,52 (std. dev. 0,68), respectively. Due to the significant impact of embryo age to DNA amount in spent medium, further analyses were made only for day 5 samples (n=29).

Cumulus cell impact was analyzed using Independent t-test comparing 0-1 cumulus cell group (n=22) with >1 cumulus cells (n=7): t(27): 3,850 when ($p<0,001$). In this analysis, difference between these groups was significant ($p<0,001$). Average of Alu4 Ct among group of 0-1 cumulus cells was 23,98 (std. dev. 0,78) and among group of >1 cumulus cells 22,40 (std. dev. 1,38). Boxplot of cumulus cell impact to Alu4 Ct can be seen in figure 15c. Because of contaminating impact of cumulus cells, samples with >1 cumulus cells were omitted in further analyses. Impact of embryonic age was determined again without samples with cumulus cell contamination, resulting the sample amount of 22 (figure 15b). There was still a significant difference in Alu4 Ct values depending the culture day when using ANOVA analysis ($p<0,001$). Dead cell impact (figure 15d) was analyzed using Mann-Whitney U test comparing dead cells groups 0 (n=17) or >0 (n=5) dead cells. The difference between these groups was statistically significant ($p<0,019$). Medians of Alu4 Ct in groups of 0 dead cells and >0 dead cells were

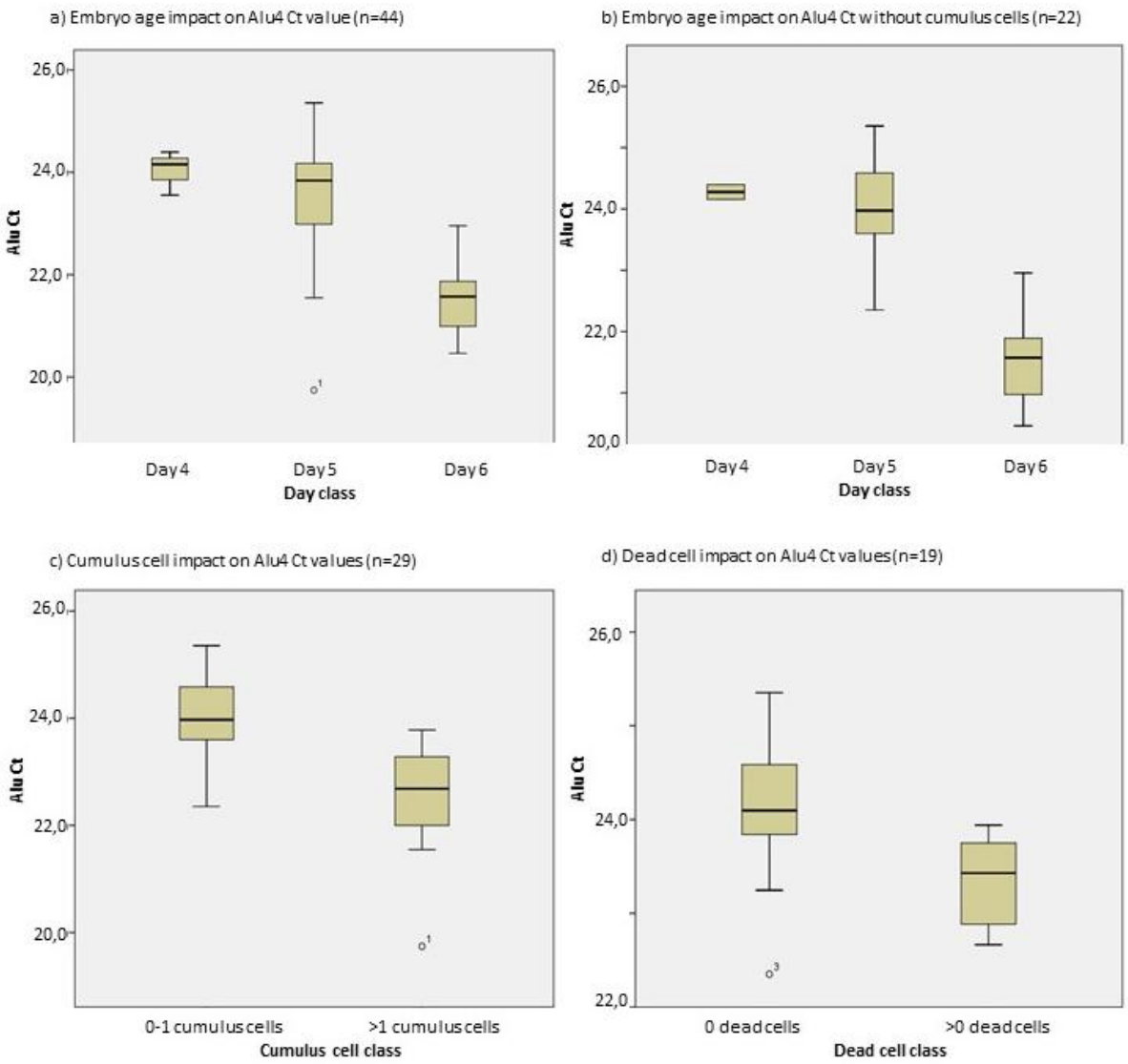

Figure 15: Age, cumulus cell and dead cell impact on Alu4 Ct. a) Embryo age impact when comparing D4, D5 and D6 samples, b) Embryo age impact when comparing D4, D5 and D6 samples without samples of >1 cumulus cells, c) Cumulus cell impact, d) Impact of dead cells. The middle line in the boxes is the median, the bottom of the box is the lower quartile and top of the box is the higher quartile. The lowest and uppermost lines in each box reflect the lowest and highest values.

24,00 and 23,16, respectively. Based on these results, also samples with dead cells were omitted from further analyses. Among fragmented embryo samples, there was no difference in Ct values ($p<0,837$). Boxplot images of age, cumulus cell and dead cell impacts to Alu4 Ct values can be seen in figure 15a-d. When analyzing sperm cell impact, there was no significant difference ($p<0,178$) when using independent T-test ($t(15):1,412$) in Alu4 Ct values between classes of 0 sperm cells (n=8) or >0 sperm cells (n=9). Averages were 24,43 (std. dev. 0,44) and 23,93 (std. dev. 0,89), respectively. Amount of DNA in spent medium was not either dependent on used

treatment method IVF or ICSI. In addition, against expectations, there was no correlation between Alu4 Ct and diameter of the most expanded stage of each blastocyst. Furthermore, hatching vesicles, PVS cells or vacuoles did not impact on the Alu4 Ct values. Development times from 2-cell to 8-cell stage, morula stages or various blastocyst stages were analyzed too but there was no correlation with periods and Alu4 Ct values.

Alu4 determination for 15 spent medium control samples were done as well. Median of the control samples was 28,8, when the lowest Ct value was 25,4 and the highest Ct value was 29,8.

5.4 Preliminary study of non-invasive PGS

aCGH was performed for three spent medium samples and biopsies from these embryos. The purpose of this experiment was to test the in-house aCGH protocol because the used embryos had been previously determined as aneuploid in NGS analyses (Reprogenetics, UK). The embryos were thawed and 1-2 trophectodermal biopsies from each embryo were taken by embryologists. Also, the spent media were thawed for the analyses. Comparisons are shown in figures 16, 17 and 18.

In the sample 65-1 (figure 16), aCGH result of spent medium suggested trisomies in the chromosomes 15 and 19. Instead, in the aCGH result of the biopsy showed trisomies in chromosomes 1, 2, 3, 7, 15, 16, 18 and X. According to NGS results, trisomies were found in chromosomes 1, 2, 3, 7, 15, 16 and 18, and monosomies in chromosomes 21 and X/Y. Hence, the trisomy 15 was the only coherent aneuploidy that was found in both biopsies and spent medium.

Sample 80-3 (figure 17) biopsy was analyzed by NGS having partial trisomy in chromosome 1, trisomy in chromosome 15 and monosomy in chromosome 21. There were differences between two biopsies obtained from the same embryo studied with aCGH method. In the other biopsy (biopsy 4), there was trisomy in chromosomes 1, 2, 5, 6, 11 and monosomy in chromosomes 10 and X while the other biopsy (biopsy 5) was analyzed only having trisomy 15. This biopsy was analyzed again using mosaic filter in the Agilent software. When analyzed with mosaic filter, biopsy had partial trisomy 1 and 2, trisomy 11, 13, 15, and monosomy 21 and X. Using mosaic filter, the result corresponded better with NGS result. However, the spent medium sample result had only trisomy X.

Sample 49-5 (figure 18) was determined as euploid embryo according to spent medium sample. Instead, according to the NGS results, in this embryo, there was trisomy in chromosomes 6, 8, 13, and 22, and monosomy in chromosomes 7 and 18. In contrast, trisomy 18 was observed using aCGH in the biopsy 4 in addition to trisomy 13 and monosomy X. There was a lot of difference between biopsies and spent medium samples in aCGH, because another biopsy was analyzed having trisomy 3 and 14 and partial monosomy in 19 and monosomy in X chromosome.

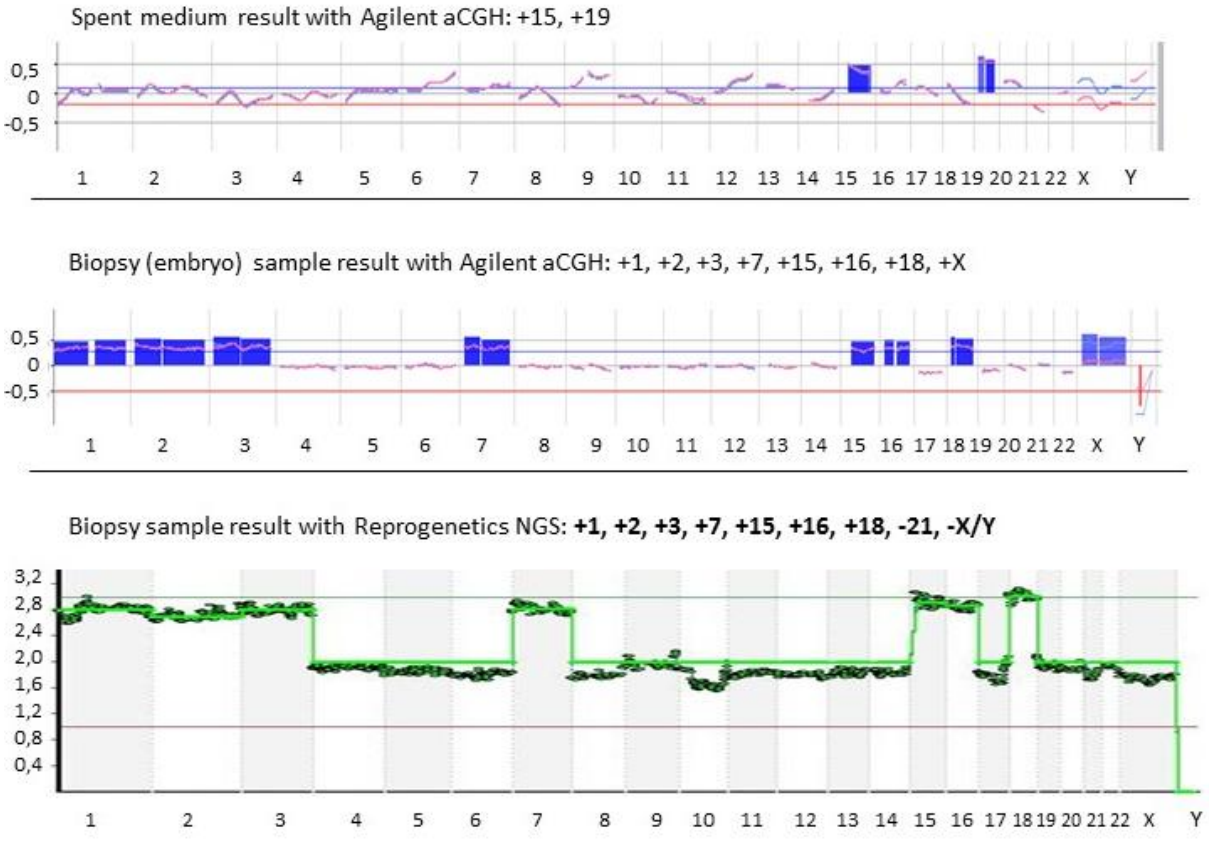

Figure 16: Chromosomal screening results of embryo 65-1 (score 2BB). Ploidy results in each analysis are shown in images and texts. Copy numbers are shown in Y-axis and chromosomal positions are shown in X-axis.

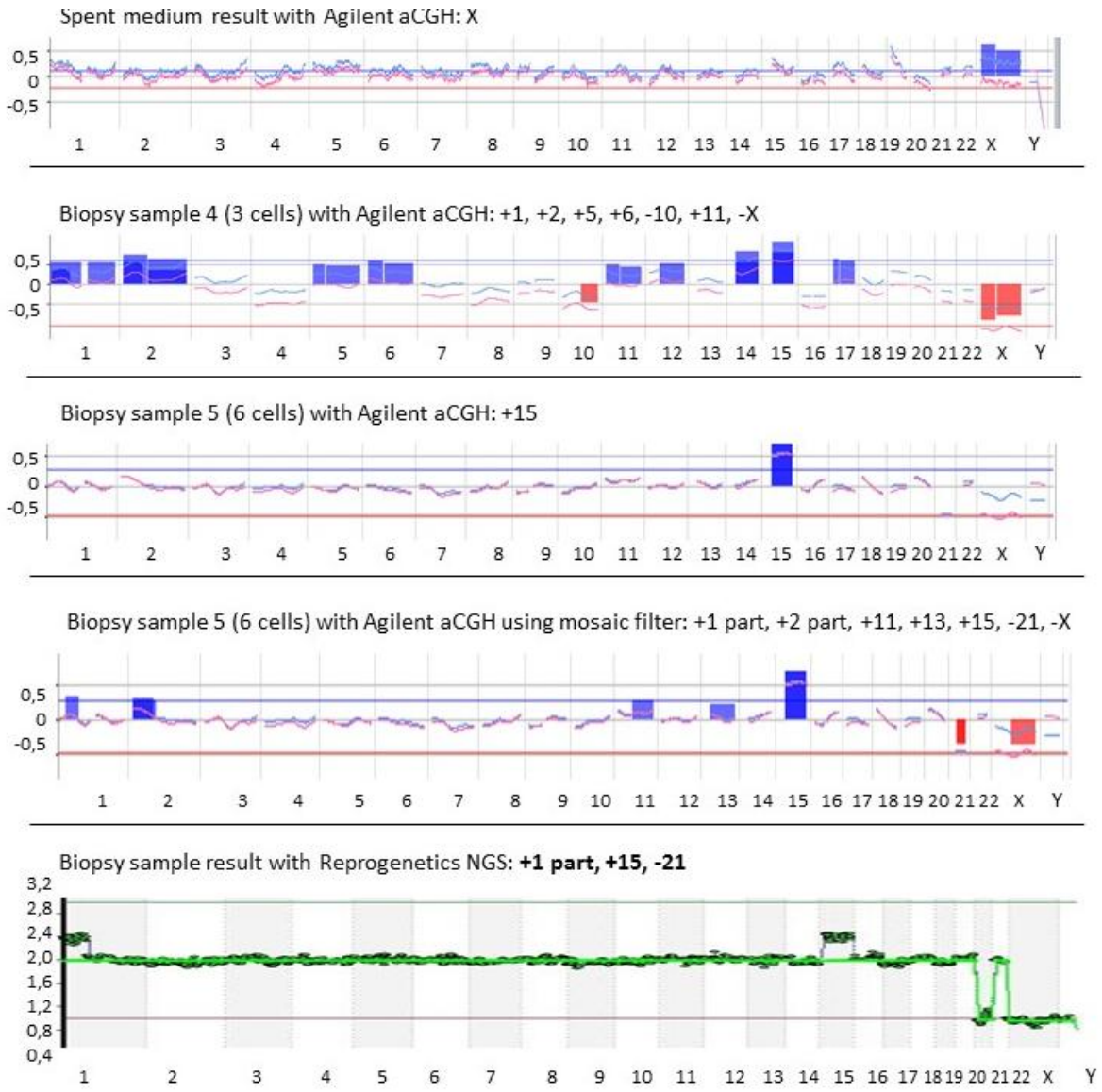

Figure 17: Chromosomal screening results of embryo 80-3 (score 4AB). Ploidy results in each analysis are shown in images and texts. Copy numbers are shown in Y-axis and chromosomal positions are shown in X-axis.

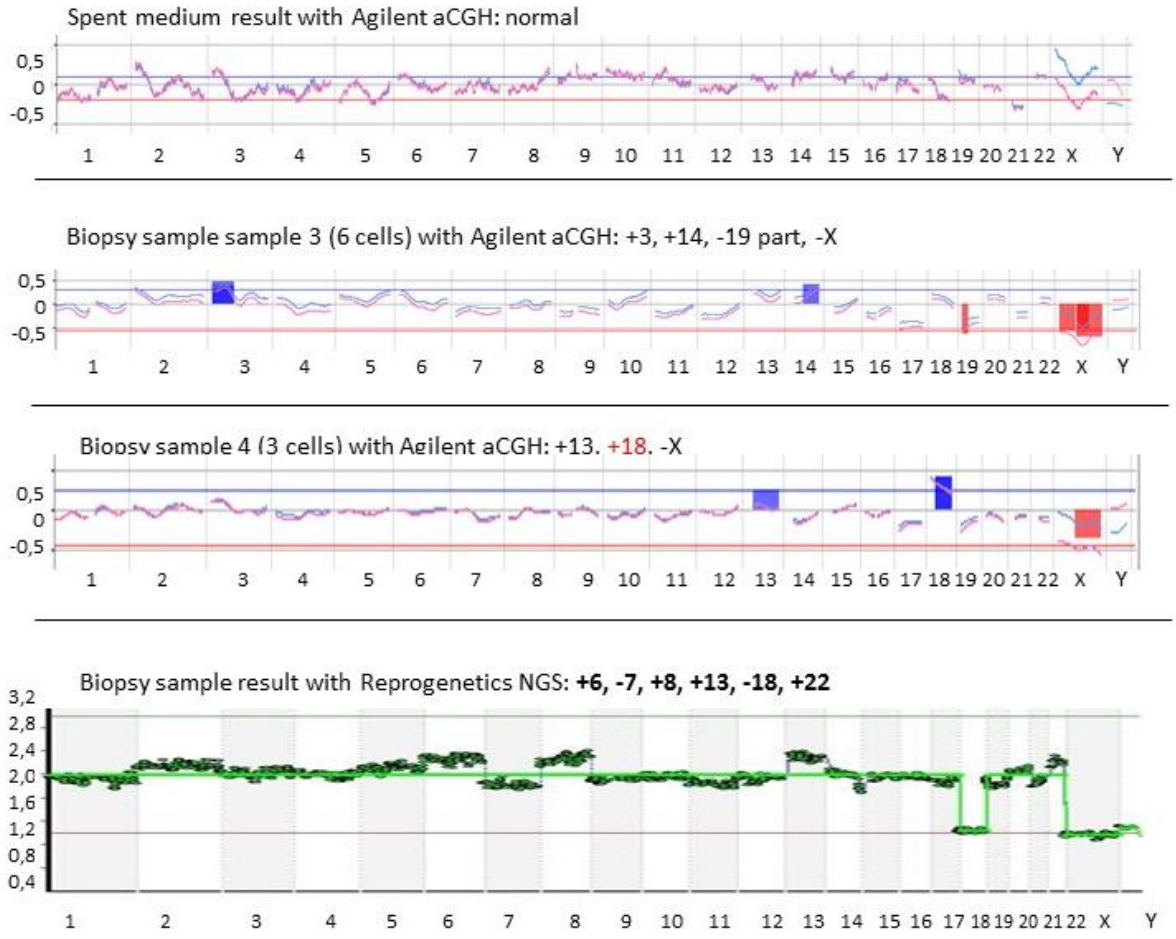

Figure 18: Chromosomal screening results of embryo 49-5 (score 4BB). Ploidy results in each analysis are shown in images and texts. Copy numbers are shown in Y-axis and chromosomal positions are shown in X-axis.

6. Discussion

6.1 Optimizing DNA extraction method for spent medium samples

For starters, DNA extraction method for extracting DNA from spent medium samples was optimized. Two different DNA extraction methods were compared: salt precipitation and NucleoSpin plasma DNA XS kit (Macherey-Nagel). Salt precipitation was attempted to use at first, because it is a traditional technique to extract nucleic acids, DNA or RNA. Furthermore, in this method, the whole sample is in the same tube during the whole extraction process and thus sample loss from pipetting or column use is avoided.

6.1.1 Salt precipitation optimization

Proteinase K is used to digest possible proteins in the samples, for example histones, which act as spools around which DNA winds. Demand of proteinase K use before extracting DNA from pooled spent medium samples with salt precipitation method was tested using TSPY qPCR. Results were clear, proteinase K treated samples had Ct values (31,4 and 32,0) but TSPY Ct values were undetermined for untreated samples. Hence, DNA in spent medium can be assumed to bind in some protein. Two melting points were observed among four samples, which suggests the unspecific PCR product existence but it may be result from unoptimized TSPY qPCR determination, too. For some reason, threshold of fluorescence rate was reached at cycle 34,5 also in NTC (H_2O) sample and its Ct was quite high, thus some unspecific primer dimers might have disturbed fluorescence measurement or the TSPY qPCR conditions were not optimal enough.

Salt precipitation method was optimized with glycogen concentration (0,13 ng/ml vs. 0,5 ng/ml), $MgCl_2$ concentration (0 mM vs. 10 mM) and ethanol concentration (70 % vs. 75 %) ending up using the concentrations of 0,13 ng/ml, 10 mM and 70 %, respectively. Results from these optimizations were not presented in this thesis. Natrium acetate concentration was recommended to be 0,3 M, but we used 0,27 M, because of the existence of salts in SAGE 1-step medium. Natrium acetate was used to make nucleic acids less hydrophilic by interacting the PO_3^- with Na^+ ion and positively charged sugar backbone to be neutralized less hydrophilic with acetate (CH_3COO^-). Magnesium chloride was used to increase the yield of precipitation product when it was expected to be in low rate and glycogen was used to make the precipitation pellet more visible after centrifugation. After inactivation, ethanol (70 %) was added and the

solution was incubated at -20 °C for 1 hour. Ethanol is less polar than water and adding ethanol to solution disrupts the screening of charges by water. Also, the electrical attraction between phosphate groups and any positive ions (Na^+) present in solution becomes strong enough to form stable ionic bonds thus the DNA will be precipitated. This is found to happen, when ethanol composes over 64 % of the solution. In this reaction, portion of ethanol was 74 %. (https://bitesizebio.com/253/the-basics-how-ethanol-precipitation-of-dna-and-rna-works/, 30.8.2017)

6.1.2 Comparing extraction methods

When comparing DNA extraction methods, the NucleoSpin plasma XS kit was more sensitive and accurate method according to the results of electrophoresis, absorbance ratio comparison, yield and Alu4 Ct value comparison. In electrophoresis, among salt precipitation samples, one of the four samples was considerably better (SP3) than the others but the reason is unclear. Although one of the samples was better, the repeatability of the samples with salt precipitation was patchy and brightness of the bands was much poorer than among NucleoSpin samples. In addition, among NucleoSpin samples, all different-sized fragments in DNA spiked medium samples can be seen. Among salt precipitation samples “smear” on the passages can be seen, more (SP1 and SP4) or less (SP2 and SP3). This could be a result of failed extraction when the single strand DNA fragments cause this unspecific smear.

NanoDrop absorbance and yield measurements were used, because properties of DNA in spent media, such as single- or double strand form, were not known. Measurements with NanoDrop cannot be selected by single or double strand property. In NanoDrop measurements, absorbance 260 nm is optimal for nucleic acids, absorbance 280 nm for proteins (especially with aromatic group) and absorbance 230 nm for organic compounds. Optimal A260/280 ratio for pure DNA is 1,8. Among salt precipitation method, samples had notably lower ratio of A260/280 (scale from 1,4 to 1,6) than NucleoSpin samples (scale from 1,87 to 2,05). Among NucleoSpin samples, high A260/280 ratio (average about 2) may indicate the presence of RNA. A230/260 was measured to indicate contamination sources. According to NanoDrop instructions, for pure DNA samples, ratio of A260/230 should be bigger than ratio of A260/280, about 2,0-2,2. Absorbance ratios 260/230 were low in both methods, which might indicate the presence of contaminants. EDTA, carbohydrates and phenol all have absorbance near 230 nm. Hence,

Phenol red and EDTA• Gentamicin in SAGE 1-step medium and glycogen in salt precipitation method might have disturbed absorbance measurements.

Yield measurements were discordant with electrophoresis results among salt precipitation. The best samples (SP2 and SP3) according to electrophoresis had the lowest yield. The highest yield samples (SP1 and SP4) had smear in electrophoresis. On the other hand, maybe the single strand DNA amount was greater in the samples, which had the highest DNA concentrations. In addition, the yield was the lowest in the SP3 sample, which electrophoresis result was the best of the salt precipitation samples. This result supports the previous suggestion about single strand DNA existence based on the smear on the electrophoresis. All the NucleoSpin samples had over 60 % yield thus the yield was better and more equal overall than among salt precipitation samples.

In Alu4 target qPCR determination of pooled spent medium samples, the Ct values of three salt precipitation samples SPa-c (scale from 26,66 to 26,89) corresponded to the DNA amount, according to the standard curve, of from 0,19 pg to 0,23 pg. This scale means the DNA amount of about 1/33 cell. However, the Ct values (scale from 24,14 to 24,53) among NucleoSpin samples NSa-c (without the unsuccessful extraction sample NSd, whose Ct was 30,98), match up with the average DNA amount of about ¼ cell. The difference is 8-fold, which is a lot of DNA in cleavage stage and blastocyst embryos. Based on this comparison, DNA yield after extraction was found to be considerably better when using NucleoSpin plasma kit. According to the standard curve, DNA amount of the salt precipitated fresh 1-step medium (medium was taken straight from the bottle to the extraction) was about 9 times lower than the sample with NucleoSpin treatment. Difference between control spent medium samples was 20-fold. According to these control sample findings, the DNA transfer during culture or contamination possibility during pipetting the embryos to the wells or drops and/or collecting spent media, must be considered. In addition, NTC sample was contaminated at some point of the procedure due to same Tm value as template samples besides the low Ct value.

All qPCR experiments were performed using double-stranded DNA binding fluorescence dyes thus the possibility of unspecific sequence binding by dyes, elevating the fluorescent signal, exists. Additionally, primer dimer effect may interfere the signal monitoring and reliability.

The salt precipitation protocol, as a whole, took at least 4 hours with small sample amount while the time for NucleoSpin extraction took about 1,5 hours. The visibility of the pellets after centrifugation was poor and dissolving the pellet after final centrifugation was troublesome in salt precipitation. Based on all these results, which corroborate the further use of NucleoSpin kit, the practical laboratory work and working hours were considered when selecting the NucleoSpin plasma kit as DNA extraction method for further experiments. However, salt precipitation was still used in some experiments (TSPY detecting from spent medium) because these experiments were performed before the NucleoSpin plasma XS kit was tested.

6.2 Optimizing qPCR

6.2.1 Optimization of DMSO concentration and annealing temperature in TSPY qPCR reaction

According to New England Biolabs' instructions for optimal PCR conditions, 3 % DMSO, as a PCR additive, is recommended for GC-rich templates. DMSO aids in denaturing templates with high GC contents. Thus, if high DMSO concentration is used, annealing temperature must be lowered from the recommended temperature, as DMSO decreases the melting point of the primers. Because it has been reported that 10 % DMSO decreases the annealing temperature by 5,5-6,0 °C, we tried 2 °C temperature decrease. When comparing the annealing temperatures of 62 °C and 60 °C among both DMSO concentrations, Ct values were lower among 60 °C annealing temperature. The difference in each standard DNA concentrations was about 4 cycles of PCR. When comparing DMSO concentrations, the difference was about 1 cycle more with 0 % DMSO concentration. Based on these findings, we deduced positive impact of DMSO use and lower annealing temperature in PCR conditions.

6.2.2 Detecting TSPY in spent medium samples

TSPY, as Y-chromosomal DNA target in spent medium DNA determinations, was used by Galluzzi et al. thus this target was expected to work (Galluzzi et al. 2015). As a preliminary proof that the DNA in spent medium at least partly is embryonic in origin, we studied the existence of Y-chromosomal TSPY. These samples were the first collected spent media samples and thus the collection protocol had not been optimized to its final form. There was quite much oil in the samples, which had to be removed before the salt precipitation. The yield of the spent media after removing the oil was about half of the expected 32 ml. Salt precipitation was used because we did not have NucleoSpin plasma XS kit at that time. Nevertheless, the results of

this detection were expected and corresponded with the known sex of the embryos. Different DNA standards were used to determine the different TSPY copy number variation among men. The copy number is assumed to vary from 21 to 35 (Giachini et al. 2009). According to the figure 10, there were lower Ct values in standard 2 male DNA samples corresponding to the higher copy number of TSPY gene. Correspondingly, Ct values of standard 3 male DNA were higher. This comparison reflects the normal copy-number variation among men. Doubtless, there might be pipetting inaccuracies in DNA dilutions, which distort the results as well. However, the standard curves among each standard were equal. NTC (H_2O) control gave Ct signal thus there was something contaminant.

6.2.3 Determining the needed volume of spent medium DNA sample for Alu4 determination

As a hypothesis of DNA properties, we thought that DNA would be more or less fragmented. Due to that, we thought that the amplicons used in DNA detection from spent medium samples should be quite short. Furthermore, use of multicopy gene targets are preferable to use than single-copy targets, if the single certain gene cannot be found in the sample. On the other hand, there is high contamination risk when using multicopy gene targets because the sensitivity of this kind of primer or probe is wide. To determine DNA amount in spent media, target Alu primers were selected and compared because the copy number of Alu element genes in genome is wide, approximately million copies (Häsler and Strub 2006), and the level of DNA amount can be evaluated with that target (Lou et al. 2015). Preliminary optimizations among Alu1, 4 and 5 primers resulted in the use of Alu4 primers for determination of DNA amount in spent medium. The amplicon lengths for these primers were 200 bp, 113 bp and 76 bp, respectively. In these preliminary analyses, the smallest DNA concentrations could not be determined with Alu5 and Alu1. Due to that, the next shortest amplicon using Alu4 (113 bp) primers was selected as target to determine DNA amount in spent media.

Based on the Alu4 determination in comparing extraction methods with pooled spent media using NucleoSpin plasma XS kit, it was expected to get the DNA amount of about 1,52 pg but the analysis was still capable to determine lower DNA amounts. Thus, we wanted to determine Alu4 and TSPY from the same sample to detect Y-chromosome and the total DNA amount. Ct values in TSPY detection among spent medium samples and pooled spent medium samples were demonstrated to be quite high with the whole sample volume. Hence, the needed sample volume for TSPY determination was assumed to require at least more than half of the sample

volume. The adequate sample volume for Alu4 determination had to be specified. Tested volumes for Alu4 determination were 4 ml (40 %), 3 ml (30 %), 2 ml (20 %) and the rest < 1 ml (<10 %) (after these greater amounts were pipetted from the total volume of 10 ml). The volume of the rest <1 ml must have varied among the samples. The greater volumes were pipetted first in order of magnitude (4 ml first and so on) thus there might be some sample loss during this pipetting procedure. According to the results, where the Ct values of <10 % volume samples were such high, the accuracy of qPCR was not sensitive or the sample volume was very small, or both. Among samples in which the sample volume was doubled from 20 % to 40 %, the Ct value differences were 1,32; 1,05 and 1,39, corresponding to the DNA quantity difference of 45,7 pg; 31,0 pg and 23,2 pg. This kind of quantity difference cannot be seen when the volume is doubled from <10 % to 20 %. The Ct value differences among each spent medium samples were quite equal. The Ct values in 20 % and 30 % were low and equal enough and thus 25 % was decided to use for further analyses. Among pooled control samples 127-C1-3, there was the same initial volume of extracted samples but the difference in Ct values between control samples was wide but also surprisingly low. Also, negative controls H_2O and elution buffer had Alu4 Ct values 27,99 and 28,93, respectively. Tm values of controls were as high as the template samples Tm values were (about 86-87 °C). These observations refer to DNA contamination among these samples because they had also as high Tm values as the spent medium samples did.

6.2.4 Repeatability test of pipetting protocol

The method of pipetting first the lower volume (2 ml) from the total volume of 8 ml for Alu4 determination was thought to minimize the relative sample loss during pipetting. For example, 10 % loss of pipetting 2 ml is much less than the 10 % loss of 6 ml if the larger amount would have been transferred. The pipetting method was tested in repeatability experiments. According to the results of Alu4 determination (figure 12), among spiked DNA samples the variation of Ct values was quite low. The average of spiked medium samples´ Ct values was 21,58 while the highest Ct was 21,82 and lowest 21,38. Still, observations of determined Ct value for NTC and it´s same Tm value as template samples had, refer to DNA contamination from PCR reagents or from the pipetting process.

When comparing spent medium Ct values of Alu4 to TSPY, the amount of DNA was similar in both determinations; high TSPY Ct and high Alu4 Ct in sample 105-16, as well as low TSPY

and low Alu4 Ct in sample 105-18. These spent medium samples were selected to this optimization because the embryos from these media were male. Overall, the variability was found to be low enough for further experiments among spiked DNA samples. The variances among DNA samples may have been results, for example, from primer dimer formation. However, the repeatability was found as good enough for further analyses.

6.3 Statistical analyses of spent medium analyses compared to EmbryoScope® image data

6.3.1 TSPY determination as a proof of concept

Ratio of determined and undetermined TSPY Ct values were 54 % versus 46 %. It was quite near to the natural sex ratio or sperm cell X and Y chromosome containing 50 % -50 % ratio, however sample size was quite small (35). As expected according to previous studies (Galluzzi et al. 2015), the period the embryos had been cultured in the spent medium had an increasing impact on the TSPY amount. D6 embryos, with determined TSPY signal, had the lowest Ct values corresponding to the highest TSPY amounts. Instead, sperm cells from IVF embryo samples had no significant impact on TSPY determination, the reasons are discussed later. However, there was still TSPY Ct values in two samples among 13 control spent medium samples. They had high Ct values, but the contamination possibility must be taken into account: these 2 samples had 2 or 3 Tm values, which one of them was the same as the template samples had. The contamination may have come during the embryo culture or spent medium collection when the samples were pipetted. In addition, the fresh 1-step medium contain serum albumin, which binds DNA but it is not known if there actually is foreign DNA bound to albumin. However, I doubt that, because the TSPY signal was determined only in 2 control of 11 samples (18 %) and in previous studies, there had been a TSPY signal in H_2O as well.

According to figure 14, TSPY and Alu4 Ct values (to note, threshold states were both set by the software) have quite strong correlation. This observation supports the theory of DNA of embryo origin and Alu4 as a marker evaluating the whole amount of DNA.

6.3.2 Influences of embryo properties and contamination sources to Alu4 Ct values

According to previous studies, the DNA amount increases during the embryo culture time in the medium when comparing D3 and D5/6 media (Galluzzi et al. 2015; Wu et al. 2015;

Hammond et al. 2017). In this study, there were 3 D4 embryos, 29 D5 embryos and 12 D6 embryos. Among these variables, the average of evaluated DNA quantity differences between age groups (D4, D5 or D6) according to the Ct values were: 5,0 pg (Ct 24,03); 6,7 pg (Ct 23,59) and 24 pg (Ct 21,53), respectively. These evaluated DNA amounts refer to whole diploid DNA amounts of about 5/6 cell, 1 cell and 4 cells, respectively. We had only 3 D4 samples, and the number should be larger for deeper conclusions of that group. The average quantity of D6 samples is found to be almost 4-fold comparing to D5 quantity average. That difference means the DNA amount of about 3 cells. In the previous studies, the yield of DNA in spent media has been reported at a higher level. For example, according to Wu et al., they average yield of DNA was 14,24 pg among D4 samples, 48,78 pg among D5 samples and 54,35 pg among D6 samples (Wu et al. 2015). Additionally, the median of DNA according Galluzzi et al. was 58 pg among D3 samples and 67 pg among D5 samples (Galluzzi et al. 2015). The measurement method influences to DNA amount evaluation, such as used standard DNA and target gene used in qPCR.

Observations of feasible contamination DNA sources, such as maternal cumulus cells and paternal sperm cells in spent media are reported and discussed in the previous studies (Xu et al. 2016; Hammond et al. 2016 and 2017; Feichtinger et al. 2017). Even though the cumulus cells that are surrounding the oocytes are denuded after oocyte collection, some cumulus cells may remain in the culture. Additionally, we tried to centrifuge the contaminating cumulus cell with different speeds (3000 g, 10 000 g and 17 000 g) before spent medium collection (data not shown) but the results of DNA amount in these studies were confusing and quite random. Additionally, Crescitelli et al. demonstrated the centrifugation-based protocol to distinguish microvesicles and exosomes from the cell culture medium (Crescitelli et al. 2013). Because the DNA releasing method from embryo into the culture medium is not clear, there is the possibility of exosome-mediated releasing pathway (Pallinger et al. 2017). Even though the centrifugation protocol of Crescitelli et al. (2013) was different than ours, we could not have been sure, that the embryonic DNA was not pelleted inside the exosomes during centrifugation. Thus, we decided not to centrifuge the samples in the further studies. The cumulus cell impact was determined comparing 0-1 cumulus cell to group of more than 1 cumulus cells, which were seen on the EmbryoViewer®. The strong difference between 0-1 cumulus cell group and group of >1 cumulus cells was detected, even though the number of samples was quite low.

It was a little bit surprising, that there was not even weak impact of sperm cells to DNA amount in the IVF spent medium samples. Sperm cell chromosomes are known to be packed tightly by protamines, which have some property to protect the genome against digestion (Tobler et al. 2015). Hence, maybe DNA is not extracted as the DNA extraction method used was originally meant for DNA extraction from blood plasma. Although the proteinase K treatment was included to the protocol, maybe it was not capable to release the DNA from protamines. However, even the large sperm cell amounts (60-70 sperm cells) had no impact on DNA amount. Furthermore, there was not significant difference in Alu Ct values between IVF or ICSI although in IVF treatment, usually sperm cells bound to zona pellucida and may act as contamination source in PGS or PGD. Thus ICSI treatment is recommended usually for PGD and PGS patients. I think that further studies with larger sample amounts need to be done to reliably clarify this area. The impact of sperm cells could be investigated by changing the spent medium at day 3 and comparing the DNA determinations between D1-D3 media to D4-D5/6 media like Xu et al. (2016) has suggested. This kind of procedure could be used to determine the influence of other factors as well, by changing the media every day to demonstrate the DNA amount changes.

Lysed cell impact on increasing DNA amount was expected too and this investigation supports the previous suggestions of apoptosis and/or necrosis mediated DNA releasing pathway. In this part, it must be taken into account that the analysis was done “semi-quantitatively”, just counting the visible cells which were observed to be clearly lysed. There might have been much more necrotic/apoptotic cells, which were not observed. We decided to investigate the impact of fragmentation, since the supposition was that the DNA in the culture media originates from dying cells and embryo fragmentation is known to be associated with apoptosis (Stigliani et al. 2013; Liu et al. 2017). However, only good-quality embryos, which had been selected for transfer or vitrification, were used in this study. Thus, it is actually not surprising, that there was not much fragmentation among these embryos or that we could not find statistical influence of fragmented embryos to the DNA amount.

Vacuoles, hatching vesicles and perivitelline cells were counted because it was expected that they could have impact as possible DNA carrier on DNA amount in the medium. However, the number of samples was low and the number of observations of these factors was minor. Studies, focusing on these factors with much larger number of samples, would be necessary to perform

to make deeper conclusions about PVS cell, vacuole and vesicle influence to DNA amount in spent medium.

The diameter of the most expanded stage of the blastocyst was measured to investigate, if the larger (more expanded) blastocyst would release more DNA because the releasing area is wider and zona pellucida is thinner. Surprisingly, there was no correlation between the diameter and DNA amount. In addition, there was expectation to observe correlations between the development time periods and Alu Ct values. Especially it was expected, that the time period from morula stage to early blastocyst phase would correlate with DNA amount: the longer the time the higher the amount of DNA. Embryo is labeled as early blastocyst when the blastocoel cavity can be seen. Though, there was no correlation between these time periods and Alu4 Ct values. The development stages were evaluated by embryologists thus in must be taken into account, that they might have evaluated the embryos differently.

To be mentioned, the number of samples in the statistical analyses was very low after omitting the D4 and D6 samples and samples containing either cumulus cells or dead cells. To summarize, the cumulus cells and culture day have effects on the DNA amount in spent media but the impact of dead cells, which was analyzed as significant using Mann Whitney U test ($p<0,019$) should be performed again using larger number of samples.

6.4 Preliminary study of non-invasive PGS

The main future goal of research and development project in Ovumia is to develop in-house procedures in embryo diagnostics of PGS and PGD. PGS by aCGH has been reported in many studies to increase the implantation and pregnancy rates after embryo transfer (Harton et al. 2013; Aleksandrova et al. 2017). In the most optimal set, the DNA in the spent media would represent the chromosomal status of the whole embryo, while the biopsy reflection is still under deeper discussion. In general, misleading results of traditional PGS may occur as a results of mosaicism of the cells in biopsy, which does not present the whole embryo quality. Hypothetically, based on earlier studies of chromosomal screening results of spent media, the released DNA from the embryo to the medium could present the embryo better than singly biopsies because it presents several cells. (Gleicher et al. 2016). Both methods, aCGH and NGS, have been used in spent medium PGS. There was very promising study by Xu et al. using NGS as method. Nonetheless, in their study, embryos were vitrified and thawed at age of 3 days and

cultured up to 5 days in single droplets. Thus, the embryo had not been in the same culture media from the beginning of fertilization. Due to that, feasible contamination sources, such as cumulus cells, were minimized. (Xu et al. 2016)

In this thesis, the spent medium results obtained from aCGH did not correspond with NGS results. The aCGH results of the biopsies were shown in this thesis to demonstrate how the spent media results are compared with them. aCGH of biopsies were performed simultaneously with spent medium samples using the same procedure. Among embryo 65-1, the spent medium screening result suggested the trisomy in chromosomes 15 and 19 but in NGS as well as in aCGH analyzed biopsy, that was actually the whole embryo, there was a lot more aneuploidy. The embryo biopsy aCGH and NGS results corresponded well within this embryo. According to this case, the reflection of the chromosomal composition of the whole embryo in that NGS biopsy was really good. In contrast, aCGH biopsy results and NGS result of embryo 80-3 did not correspond that well. According to NGS result, trisomy 1 and 15 and monosomy 21 were observed. The differences between biopsy results were quite confusing. In the other biopsy, there were 3 cells and the screening suggested a lot of aneuploidies while the biopsy of 5 cells suggested only trisomy 15 but when the mosaic filter was used (in the software), the number of aneuploidies increased notably. In spent medium analyses of this particular embryo, only trisomy X was observed. All in all, the embryo would be graded as aneuploid according to any of these samples but the reliability of this aCGH is questionable in these circumstances.

As intriguing detail, I think, is the NGS result of monosomy 18 in biopsy sample of 49-5 while the biopsy sample using aCGH suggested it as trisomy. Due to that, mosaicism might be significant among that embryo. Mosaicism is associated to accumulated apoptosis in mice (Feichtinger et al. 2017) and mosaicism is decreased in blastocyst cells (Wu et al. 2015; Tobler et al. 2015). In this case, that theory as DNA releasing method is contradictory, because aneuploidy was not detected in the spent medium sample.

Differences in aCGH and NGS results may be attributed to higher resolution of NGS, because the technique of NGS is based on to “the sequence building” while the array based method is using pre-defined probes to detect genes in chromosome´s arms. In addition, the used DNA extraction methods have been different, as well as different kits have been used to perform whole genome-amplification. Currently, aCGH on is employed in PGS in the majority of cases. Beside all these discussion of the confusing aCGH results compared to NGS results, it must be

mentioned, that other users besides us, had also background errors with the scanner (Agilent) that we used. It was later observed that the ozone level in the laboratory building was so high that the ozone-protocol and ozone barrier lid should have been used. However, be whatever the reason for different results, the protocol should be repeated with larger amount of spent medium samples and reference samples and maybe with another the ozone-protocol by the manufacturer.

There are a lot of advantages in aCGH technique. The capability of detecting both microscopic chromosomal disorders, such as aneuploidies, including mosaic ones and unbalanced translocations are major points of using it in PGS. Also, the marker chromosomes and submicroscopic defects, like microdeletion and -duplication syndromes can be determined in the total genome at the same time. Using NGS, multiple single gene disorders from the same biopsy can be simultaneously be done. (Fiorentino et al. 2014; Aleksandrova et al. 2017) Even though advantages of NGS are wide and I believe this technique is overtaking the scene in PGS area step by step, at the moment the development of clinical aCGH is needed to optimize further.

7. Conclusions

NucleoSpin plasma XS kit was better and more accurate than salt precipitation method for DNA extraction from spent medium. Alu4 and TSPY target primers in evaluating of DNA quality and quantity in spent medium were demonstrated as workable using qPCR but further studies are needed to resolve the source of TSPY signal in control samples during the PCR run with this PCR device. Sex of the embryos were determined successfully from the spent medium samples using TSPY qPCR.

There were significant differences when comparing D4, D5 and D6 embryo culture medium samples ($p<0,001$). Amount of DNA was increased in D6 spent medium samples ($n=12$) compared to D5 ($n=29$) and D4 samples ($n=3$). DNA amount was also bigger among D5 samples compared to D4 samples. Contaminant DNA factors in spent medium were evaluated by time-lapse EmbryoScope® incubator and their impact on determined DNA amount was analyzed. Impact of cumulus cells (>1 cumulus cell) was significant ($p<0,001$) in increasing the DNA amount in embryo culture media. Dead cells were also demonstrated to influence the DNA amount increasingly ($p<0,019$) but the number of samples was quite low ($n=19$) and thus this determination should be repeated using more samples. Sperm cells, treatment (IVF/ICSI), evaluated fragmentation level before compaction, perivitelline cells, vacuoles or hatching vesicles were not observed to impact on the DNA amount in spent medium. Embryo development time periods (h) from lower cell-stage to higher-stage/blastocyst stage or the measured diameter of the most expanded stage of each blastocysts did not influence to the DNA amount either. More studies are anyway needed with larger number of samples to determinate better these factors' influence on DNA amount in spent medium.

Chromosomal screening results of three spent medium samples using aCGH were not consistent with NGS results. Further optimizations with this procedure must be done.

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